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Signaling pathways activated by extracellular nucleotides through the $P2Y_2$ receptor: an *in vitro* ischemia model

A dissertation submitted in partial fulfillment for the degree of Doctor of Philosophy

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Abstract

Extracellular nucleotides through the $P2Y_2$ receptor activate signal transduction that involve the activation of protein kinases such as the MAPK family and Akt/PKB and transcription factors regulating gene expression to induce cellular responses such as proliferation and survival. The amino acid sequence of the human $P2Y_2$ receptor have indicated the presence of an arginine-glycine-aspartic acid (RGD) integrin binding motif that is localized at the first extracellular loop of this receptor. The $P2Y_2$ receptor also posses a proline rich sequence at the intracellular region (C-terminal) which encompasses an SH3 binding domain that may regulate the signal transduction that induce the receptor. Receptors for adenosine and ATP are expressed in astrocytes. Larger amounts of nucleotides and nucleosides are released following brain trauma and ischemia, which can activate immediately P2 receptors such as $P2Y_2$ receptors. Therefore, this study investigated the role and molecular mechanisms that extracellular nucleotides through the $P2Y_2$ receptor activate during and after a cerebral ischemia episode and the molecular determinants (extracellular and intracellular domains) of the $P2Y_2$ receptor by which extracellular nucleotides mediate astrocytic activation. The activation of MAPK and Akt/PKB is partially dependent of the interaction of the $P2Y_2$ receptor with the $\alpha_v\beta_3$ integrin through the RGD sequence and partially dependent of the proline rich sequence. The activation of these protein kinases during ischemia is mediated principally by the nucleotides released and the activation of $P2Y_2$ receptor but also by glutamate receptors. The activation of these protein kinases by $P2Y_2$ receptor induces a survival signal during an episode of ischemia.

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Biographical Sketch

Laura I. Santiago Pérez was born in Humacao, P.R. on January 17, 1971. Laura is the elder daughter of two children born to Laura E. Pérez and Elizardo Santiago, and Elizardo Santiago Pérez is her younger brother. As a child she enjoyed science so much that she dreamed of being a great doctor, pharmacist, medical technologist and/or scientist. She also had other interests such as drawing. During her years in the Rufino Vigo Elementary School she demonstrated drawing abilities inherited from her father. She joints the Art Club taking classes with her teacher Mrs. Delia Brito during her 6^{th} grade. She participated in a Painting Competition sponsored by Jayce of P.R. and Banco Popular de P.R., where children from Puerto Rico and the rest of the Caribbean participated, and the theme was Peace for the World. She won the first place in this competition and was recognized by her school during the Student Day and by her town during Religious Town Festivities ("Fistas Patronales"). She continued her art studies, in spite of her passion for science. During her years at Ana Roqué High School, she approved more credits in science than those required by the school and she also approved advanced mathematics exam with high scores, which lead her to obtain advanced credits at the university that she close to attend.

Laura enrolled in the Premedical Program at the University of Puerto Rico Río Piedras Campus, at Río Piedras, Puerto Rico in August 1989. There she was selected to be part of the "Group of One Hundred" of the Natural Science Faculty which allowed her to take sophomore level science courses (Chemistry, Biology, Calculus) while a freshman. Her chemistry teacher, Prof. Nadia Cordero inspired in her a great interest in chemistry, and she decided to reclassify to the Chemistry program. During her senior year, she took chemistry research with Dr. Osvaldo Rosario, where she learned more about analytical techniques to study and develop methods to study pollutants that affect the environment. During the same year, she also worked as a practical student in the Alcon Pharmaceuticals, where she recognized that she really wanted to do research. Follow her interests, she graduated in 1993 with a Bachelor degree in science (Chemistry mayor) and continue graduate studies in the Graduate Chemistry Program at the U.P.R.

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During the first year in Graduate School, she attended the Advanced Biochemistry course with Dr. Fernando A. González, who expanded her vision of science and gave her a better perspective of what she really wants, that is to study biochemistry and its implications in health. After 6 years of hard work she finished her Master degree. Then, she continued graduate studies in UPR at Río Piedras, to obtain the PhD. During this time, she also did extracurricular work in the UPR Humacao to share and help students to understand chemistry concepts.

During the past 6 years to obtain her Master degree and the five years to obtain her PhD, she contributed to the scientific community and presented her research work in Scientific Meetings such as: Annual Meeting Society for Neuroscience, Purines 2000, Annual P.R. Neuroscience Conference, ACS Technical Meeting, as well as in symposiums of Recent Progress in P2 Receptor Research and Recent Developments in $P2Y_2$ receptor Research among others. She also published her work in several scientific journals that include:

Publications:

1. Weisman, G.A., Erb, L., Garrad, R.C., Theiss, P.M., **Santiago-Pérez, L.I.,** Flores, R.V., Santos-Berrios, C., Méndez, Y., and González, F.A., (1998) "P2Y nucleotide receptors in the immune system: signaling by a P2Y2 receptor in U937 monocytes" *Drug Develop. Res.* **45**, 222-228.
2. González, F.A., **Santiago-Pérez, L.I.,** Flores, R.V., Santos-Berrios, C., Chorna, N., Garrad, R.C., Erb, L., Weisman, G.A., (2000) "$P2Y_2$ nucleotide receptor signaling in U937 cells: activation, desensitization and coupling to mitogen-activated protein kinases" *Drug Develop. Res.***50**, 1, 51.
3. Erb, L., Liu, J., Ockerhausen, J., Garrad, R.C., Griffin, K., Neal, C., Krugh, B., **Santiago-Pérez, L.I.,** González, F.A., Gresham, H.D., Turner, J.T., Weisman, G.A., (2001) "An RDG Sequence in the $P2Y_2$ Receptor binds to $\alpha v\beta 3$ Integrins and is responsible for coupling to G0-meditaed calcium signal transduction" *J. Cell. Biology.* 153, 491-501.
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7. Chorna, N.E., **Santiago-Pérez, L.I.,** Erb, L., Neary, J.T., Sun, G.Y., Weisman, G.A., González, F.A. "$P2Y_2$ receptors activate neuroprotective mechanisms in astrocytic cells" *J. Neurochem.*, submitted.

After 5 years of hard work, disappointments, frustrations and also happy moments, she completed this thesis.

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This thesis is dedicated to:
My father Elizardo Santiago,
to my brother Elizardo (Eli)
and specially to
my mother Laura E. Pérez for all
her patience, comprehension and love.

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List of Abbreviations

ADP	adenosine 5'- diphosphate
AFX	acute-lymphorytic-leukemia-1 fused gene from chromosome x
AMP	adenosine 5'- monophosphate
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APAF-1	apoptotic protease-activating factor 1
ATF-2	atriad transcriptional factor 2
ATP	Adenosine 5'-triphosphate
BDGF	basic fibroblast growth factor
BDNF	neurotrophin brain-derived neurotrophic factor
BMK1	Big MAP kinase 1
BSA	bovine serum albumin
CaMK	Ca^{2+}/calmodulin protein kinase
CHOP/GADD153	growth arrest and DNA-inducible gen 153
CNS	central nervous system
CREB	cAMP response element binding protein
CSBP	Cytokine Suppression anti-inflammatory drug-binding protein
CYTD	cytochalasin D
DAG	diacylglycerol
DMEM	Dubelcco's Modified Eagle's culture medium
DTT	DL-dithiothreitol
EAA	excitatory amino acids
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	Extracellular Signal-Regulated Kinase
FAK	focal adhesion kinase
FDA	Food Drug Administration
FKHR	forkhead in rhabdomyosarcoma
GABA	γ-aminobutyric acid A
GFAP	glial fibrillary acidic protein

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GPCR	G protein coupled receptors
GSK-3	glycogen synthase kinase-3
HI	hypoxic-ischemic
HRP	horseradish peroxidase
IGF-1	insulin like growth factor
IKK	IκB kinase
IL-1beta	interleukin-1- beta
ILK-1	integrin linked kinase-1
IP_3	inositol-1,4, 5-triphosphate
JNK	c-Jun-N-terminal Kinase
LDH	Lactate dehydrogenase
LPS	lipopolysaccharide
MAP-2	microtubule-associated protein 2 kinas
MAPK	Mitogen-Activated Protein Kinase
MAPKK	MAP Kinase Kinase
MAPKKK	MAP Kinase Kinase Kinase
MAPKAP-K	MAP kinase activated protein kinase
MBP	myelin basic protein
MEF2C	myocyte-enhance factor 2
MEKK	MEK Kinase-1
mGluR	metabotropic glutamate receptor
MKK	MAP Kinase Kinase
NAD^+	nicotinamida adenine dinucleotide
NDPK	nucleoside diphosphokinase
NMDA	N-methyl-D-aspartate
NGF	nerve growth factor
OGD	oxygen glucose deprivation
PARP	Poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDK1	3-phosphoinositide-dependent protein kinase

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PH	pleckstrin homology domain
PI3K	phosphatidylinositol 3-kinase
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PLCβ	phospholipase Cβ
PMSF	phenylmethylsulfonyl fluoride
PNS	peripheral nervous system
PtdIns(3,4)P_2	phosphatidylinositol 3,4-biphosphate
PtdIns(3,4,5)P_3	phosphatidylinositol 3,4,5-triphosphate
Pyk2	proline rich tyrosine kinase 2
RGD	arginine-glycine-aspartic acid
RGE	arginine-glycine-glutamic acid
RK	Reactivating Kinase
ROS	reactive oxygen species
RTK	tyrosine kinase receptors
SAPK	Stress activated protein kinase
SEK1	SAPK/ERK kinase-1
SFK	src family kinase
SH3	Src Homology 3
SKK	Stress Kinase Kinase
TCF	ternary complex factors
TNFalpha	tumor necrosis factor alpha
t-PA	tissue plasminogen activator
UDP	uridine -5'-diphosphate
UMP	uridine 5'-monophosphate
UTP	uridine 5'-triphosphate
4-VO	four-vessel occlusion
2-VO	two-vessel occlusion

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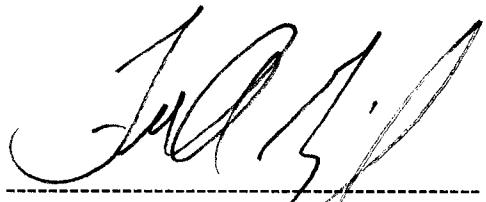

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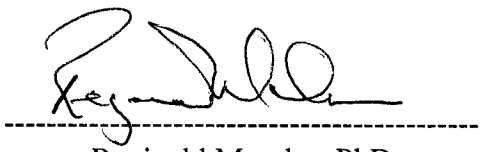

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CHAPTER I
INTRODUCTION

I. Central nervous system

The central nervous system (CNS) is responsible for the control of many cellular responses. It is composed of neurons and glial cells. Neurons have fiberlike extensions composed of axon and dendrites that conduct information (chemical and electrical signals) and communicate with other cells through the synapsis. Glial cells maintain neuronal homeostasis, synaptic plasticity and repair [1]. Glial cells are responsible of the neuron's integrity. They are classified in two subgroups: macroglial cells, astrocytes and oligodendrocytes which are of ectodermal origin and microglial cells that are thought to stem from the mesoderm [2]. Microglial cells are the principal immune cells in the CNS and have a critical role in host defense against invading microorganisms and neoplastic cells. They play a dual role, amplifying the effects of inflammation and mediating cellular degeneration as well as protecting the CNS [3] , [2].The Shwam cells in the peripheral nervous system (PNS) are the equivalent of oligodendrocytes called in the CNS. Oligodendrocytes are close to nerve cell bodies and form myelin, the substance that facilitates action potentials propagation in the CNS. They synthesize an array of trophic factors and appear to be sensitive to neurotrauma [4]. Astrocytes can be subdivided into three major populations: radial astrocytes, fibrous astrocytes and protoplasmic astrocytes [2]. Astrocytes have a starlike shape and are found mainly in regions of axons and dendrites (neurons) contributing in synaptic transmission. Astrocytes are electrically unexcitable cells that traditionally are regarded as the brain's support cells. Astrocytes communicate with one another by calcium signaling and these calcium signals are transmited to neurons [5]. Astrocytes contribute to brain homeostasis, regulating the local concentrations of ions and neurotransmitters [5]. Neurons and astrocytes are in communication through the release of neurotransmiters such as glutamate and the activation of the glutamate receptors [6]. Astrocytes play critical roles in the development and physiology of the CNS, being involved in key aspects of neuronal function such as trophic support, neuronal survival, and differentiation, neuronal guidance, neurite outgrowth and synaptic efficacy [7]. They tend to surround and to be in close contact

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with the advential surface of blood vessels where they elicit the expression of proteins in cerebral endothelial cells that constitute the blood brain barrier that restricts the passage of most substances into the brain [8]. While most of the past studies have been performed in neurons, the physiology and function of astrocytes *in vivo* remain largely undefined and are currently under study [9]. In processes such as stroke or ischemia, the CNS is far more vulnerable than other organs. The death mechanisms by which neurons die after ischemic brain injury are more or less identified. However, less is known about what is happening in astrocytes during these processes and how astrocytes modulate the responses in neurons associated to death or survival.

II. Cerebral ischemia

Cerebral ischemia is the third cause of death in the US and the most common cause of adult disability [10], [11]. Cerebral ischemia is a type of stress that involves global or focal loss of blood flow to the brain after a stroke. The decrease of the supply of energy substrates, primarily oxygen and glucose, in the brain causes neuronal injury indicative of its vulnerability to ischemia and its high metabolic rate (25% of basal metabolism from the 2.5% of body weight in humans) [12]. Cerebral ischemia may be either transcient and followed by reperfusion, or essentially global or permanent. In focal ischemia, a region of the brain may be affected, as occurs during an arterial or venous stroke in which a thrombi or emboli commonly cause ischemic arterial obstruction due to atherosclerosis or other disorders (eg, arteritis, rheumatic heart disease, arterial stenosis, platelet adherence), or the entire brain may become globally ischemic, as occurs during a cardiac arrest and cardiopulmonary bypass surgery [12], [13], [11]. Ischemia may also contribute secondarily to the brain damage in the settings of mass lesions, blood vessel rupture leading to hemorrhage, or trauma. Two general therapeutic approaches have emerged: 1) enhancing blood flow by the lysis of the arterial thrombus with tissue plasminogen activator (tPA) approved by the US Food and Drug Administration (FDA) in 1996, and 2) neuroprotection that aims to reduce the intrinsic vulnerability of brain tissue to ischemia on blocking excitotoxicity triggered by the excitatory transmitter glutamate [10], [11].

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During cerebral ischemia, complete interruption of blood flow to the brain for only five minutes can trigger the death of vulnerable neurons in several brain regions. It causes a decrease in intracellular ATP levels, a massive shutdown of neuronal activity (induced by K^+ efflux from neurons, mediated initially by the opening of voltage-dependent K^+ channels and later by ATP-dependent K^+ channels), and transient plasma membrane hyperpolarization. Then, despite this energy sparing response, an abrupt and dramatic redistribution of ions occurs across the plasma membrane, associated with membrane depolarization (efflux of K^+ and influx of Na^+, Cl^-, and Ca^{2+}). With the depolarization, larger amounts of neurotransmitters such as: glutamate, dopamine and nucleotides are released [14]. The same response is observed in astrocytes with an increase of intracellular calcium as a result of glutamate released that modulates a synaptic transmission [9]. There has been thought that the main cause of brain injury and cells death caused by cerebral ischemia is by the excitotoxicity of glutamate that are released in greater amounts (initially mediated by vesicular release from nerve terminals, and later by reverse transport from astrocytes responsible of the recycling of the neurotransmitter that do not respond), increasing the concentration to neurotoxic levels (mM) during this process [15] The Zn^{2+} ion is also implicated with the death of neurons in ischemia, and some reports suggest that Zn^{2+} could be co-released with glutamate at the extracellular medium [16, 17].

During focal or transcient cerebral ischemia, the brain cells suffer damage which ends in apoptosis, necrosis or neuronal death [18]. Apoptotic death is rapid with a time course of a few hours. In contrast, necrosis does not involve a death signal, it is a passive form of cell death which is characterized by cell and organelles swelling and rupture [19]. Mammalian apoptosis is known as a programmed death signal which consist of three main pathways: the death receptors, apoptotic cue toward mitochondrial pathway and endoplasmic reticulum stress [20]. Biochemical mechanisms of apoptosis which include cytochroma c release, activation of caspases cascades and activation of apoptotic proteins could be observed [21]. A series of biochemical processes that include Bcl-2 family proteins, the adaptor protein Apaf-1 (for apoptotic protease-activating factor 1) and the cysteine-dependent aspartate-specific protease caspase family (caspase-3 and caspase-9) are the main factors that regulate apoptosis resulting in DNA cleavage by the

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mitochondria pathway as a result of withdrawal of growth factors [19]. Biochemical studies of tissues and cells during the treatment of ischemia, preconditioning ischemia and ischemia/reperfusion show that protein kinases such as MAPK family; ERK1/2, p38 and JNK, are differentially activated [22],[23],[24] [25]. But these differential activations are also implicated in survival signals together with the inhibition of the protein kinases that phosphorylate them as well as the activation of their phosphatases [26], [27]. In ischemia, it is also observed that Ca^{2+}/calmodulin protein kinase (CaMKII) activity decreases [28]. Inflammatory responses are also implied with synthesis and the release of proinflammatory cytokines such as: IL-1β and TNF-α [29]. Up- and down- regulation of proteins and genes are also observed [30]. The production of the glial fibrillary acidic protein (GFAP) is used as a marker in ischemic astrocytes [31]. Although much of the present work on neuroprotection has focused on the survival on neurons, a concomitant effect of cerebral ischemia is the death of astrocytes. Identification of molecular mechanisms responsible of astrocyte death after ischemia is highly desirable, but also the development of novel strategies aimed specifically at enhancing astrocyte survival.

Glutamate arrive in greater amounts (at toxicity levels) at the extracellular medium activating its receptors and inducing a cascade of events that can induce survival as well as apoptosis signals depending of the receptor activated and the duration of this activation. Glutamate has two types of receptors, ionotropic and metabotropic receptors in glia (astrocytes) promoting Na^+ influx and K^+ efflux, inducing an increase of intracellular calcium [14], [32], modulating transmitter uptake and the production and the release of neuroactive substances such as neurotrophin brain-derived neurotrophic factor (BDNF), which could mediate subsequently cell survival signal [33]. The ionotropic glutamate receptors are classified in three subtypes: N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate. NMDA receptor regulate or display high Ca^{2+} permeability with significantly lower Na^+ influx, AMPA and kainate receptors predominantly conduct the influx of Na^+ and K^+ ions (although in some circumstances they might allow Ca^{2+} entry) [34]. The metabotropic glutamate receptors are classified in 8 subtypes (mGluR1-mGluR8) and are classified into three major groups on the basis of sequence homology, pharmacological profile, and coupling to G-proteins and effector systems. Group I of GluRs (mGluR1 and mGluR5) is

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coupled to the $G_{q/11}$ family of G-proteins and activation of phospholipase $C_{\beta 1}$ and phosphoinositide hydrolysis. Group II of mGluRs (mGluR2 and mGluR3) and group III (mGluRs4,6,7 and 8) couple to $G_{i/o}$ and associated effectors such as ion channels and inhibition of adenylate cyclase [35], [34], [36].

As mentioned before, glutamate induces a survival or apoptosis signal depending of the receptor and the duration of the stimulus that is activated. Some reports have suggested that the ionotropic receptor NMDA is responsible of the damage caused by the exocitotoxicity of glutamate [32]. However, blockade of NMDA receptors for only few hours during letal fetal or early neonatal life triggered widespread apoptotic neurodegeneration in the development rat brain suggesting that glutamate acting through the NMDA receptor control neuronal survival [37]. Activation of the group II mGlu receptor may contribute to neuroprotective mechanisms attenuating the NMDA excitotoxicity and staurosporine induced neuronal death and against transient global ischemia in gerbils [38]. Pharmacological blockade of mGlu1 or mGlu5 receptors or pharmacological activation of mGlu2/3 or mGlu4/7/8 receptor produces neuroprotection in a variety of *in vitro* or *in vivo* models [39]. Inhibition of mGlu1 receptor attenuated necrosis but activation exacerbated such injury induced by oxygen glucose deprivation (OGD) [40]. Inhibition of both mGlu1 and mGlu5 receptors play a role in attenuation of neuronal apoptosis [40]. Some mGlu5 receptor antagonists and agonist are neuroprotective when administered after focal cerebral ischemia [41]. Activation of mGlu2/3 receptor by agonists inhibits glutamate release, but also promotes the synthesis and release of neurotrophic factors in astrocytes [39]. The activation of mGlu4/7/8 receptors by agonists potentially inhibit also glutamate release and may play roles in seizure disorders [39].

In most cases, the underlying goal of stroke treatment has been the reduction of glutamate release, glutamate receptor activation or associated cellular Ca^{2+} overload to reduce the excitotoxicity caused by glutamate. The development and use of drugs that act as antagonist of NMDA, AMPA and Kainate receptors have demonstrated their involvement in neurotoxicity and how these antagonists can play a role in neuroprotection [10]. However, a major limitation in clinical trials of these antagonists

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has been dose ceilings imposed by drug side effects altering the brain's major excitatory transmitter system, pharmacokinetics problems, psychotomimetic side-effects and peripheral toxicities or lack of efficacy [42], [43]. Although it is thought that NMDA-receptor overactivation leading to neuronal calcium overload contributes importantly to neuronal cell death after focal brain ischemia, this may not be the predominant mechanism. It is possible that other forms of injury are present that are masked by the NMDA response. Oxidative stress is thought to be the cause of several injuries and glutamate causes an increase in reactive oxygen species (ROS) and intracellular calcium.

Several models of study cerebral ischemia are currently practiced such as *in vivo* by surgical occlusion of brain arteries and *in vitro* where tissues of certain regions of the brain or culture cells are exposed to an oxygen glucose deprived environment. These models will be described further.

Nucleotides such as ATP and adenosine are released during cerebral ischemia and the signals that they regulate are mediated through their receptors that are widely expressed in astrocytes [44], [45], [14]. Some reports have demonstrated that glutamate is released in response to extracellular ATP [46], but also ATP is released from astrocytes by glutamate receptor activation [47]. Therefore, there is a potential feedback loop between the release of glutamate and nucleotides in astrocytes [46]. It is possible that both neurotransmitters are regulated to modulate a specific response during an ischemia episode. Other reports have demonstrated a role of adenosine, product of ATP degradation, during ischemia. Adenosine is produced in the extracellular space where it is observed to act as a neuroprotective agent [48]. In neurons, adenosine activates K^+ and Cl^- conductances, which limit synaptically evoked depolarization, thus counteracting the Ca^{2+} influx though voltage-dependent and NMDA receptor-operated ion channels. In glial cells, adenosine regulates the Ca^{2+} influx and cAMP-dependent molecular signaling that determines the cellular proliferation rate, the differentiation state and related functions [49], [50]. Its neuroprotective role may be due to blocking of Ca^{2+} influx, which results in the inhibition of glutamate release via presynaptic receptors and reduction of its excitatory effects at postsynaptic level through the A_1 receptors, contrary to the A_2 receptor which mediate depolarization and enhanced transmitter release [51],

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[52], [53], [54]. Its neuroprotective effects have been seen after hours or days after the ischemia or reperfusion treatment. An increase of ecto-nucleotidases activity is also observed during ischemia which is responsible to hydrolize triphosphates of nucleotides such as ATP to nucleosides as adenosine [55]. Nucleotides, before their hydrolysis, could readily activate P2 receptors such as the $P2Y_2$ receptor which could modulate apoptosis, death or survival signal. It is our hypothesis that extracellular nucleotides, through the $P2Y_2$ receptor, can induce neuroprotective or survival signals before being hidrolyzed to the nucleoside form.

III. Purines and Pyrimidines Nucleotides

Adenosine 5'-triphosphate (ATP) is known as an intracellular energy source involved in many metabolic cycles of the cell. Like ATP, other nucleotides such as uridine 5'-triphosphate (UTP) participate in several events inside the cell such as: nucleic acids synthesis and RNA synthesis, biomembranes synthesis, sugar and lipid metabolism and enzyme regulation [56]. Nucleotides were thought to be confined to those functions until Drury and Szent-Gyorgyi reported in 1929 the potent action of extracellular nucleotides (adenylic acid and adenosine) on the cardiac rhythm of the heart of the dog and in other physiological roles [57].

After that report, other investigators have become interested in knowing more about the actions of extracellular nucleotides in several systems. Therefore, ATP was found to act as a neurotransmitter causing vasodilatation when released during an antidromic stimulation of sensory nerves which are non-cholinergic and non-adrenergic nerves [58]. Such nerves were denominated 'purinergic' by Burnstock [59] and 'purinergic transmission' to the signal exerted by these nerves [60]. Uridine and its nucleotides were found to act also as neurotransmitters modulating peripheral and central nervous system. Uridine, UDP, UTP and UDP-glucose have been shown to depolarize or hyperpolarize amphibian ganglia at submicromolar concentrations and similar actions is also observed in rat superior cervical ganglia [56, 61, 62]. UDP and UTP were found to modulate neurotransmitter release (noradrenaline) from cultured rat superior cervical ganglia [56].

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Purines such as ATP and pyrimidines such as UTP are released to the extracellular medium by four routes: lysis of cells, transmembrane transport, mechanical stimulation and exocytosis [63], [64-66]). ATP is also released to the extracellular medium during cell injury caused by cerebral ischemia [14]. The storage of ATP in synaptic vesicles and its secretion from stimulated neurons have been seen in the central and peripheral nervous system [67]. Acetylcholine and ATP are jointly released in synaptic vesicles by nerve stimulation [68], and is also co-released with other neurotransmitters such as norepinephrine, glutamate, GABA and neuropeptide Y [69].

In addition to the nervous system, extracellular ATP can modulate the cardiovascular, immune, pulmonary and inflammatory systems as well as the functions of several organs [70]. ATP can modulate the inflammatory responses of the immune system through the release of cytokines and the release of arachidonic acid which can be transformed to prostaglandins and prostacyclins by the action of enzymes such as cyclooxygenase [71]. Pyrimidines, as UTP, were shown to induce endothelium-dependent relaxation and stimulate prostacyclin production in various vascular beads, to enhance exocytosis and O_2^- production in neutrophiles; and to mobilize intracellular Ca^{2+} in various cell types [72]. ATP has been involved in autocrine and paracrine regulation of the release of hormones in pituitary cells [73]. ATP has been linked with the regulation of the cardiovascular system through the modulation of many functions of the heart [74] and also during ischemia [75]. Extracellular nucleotides have been implicated in the stimulation of mitogenesis [76, 77], in the regulation of proliferation and differentiation of several cell lines [78], [79] and can also promote cell cycle progression [80, 81]. Studies have been proposed the use of extracellular nucleotides as therapeutic drugs for the treatment of cystic fibrosis [82, 83].

A. Classification of receptors

The nucleotide receptors were initially classified by Burnstock in 1978 in two mayor types: P1-purinoceptors, which are selective for adenosine and act through adenylyl cyclase (and are antagonized by low concentrations of methylxanthines); and

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P2-purinoceptors, which were defined as being activated preferentially by ATP and ADP ([84]). Through the years, this classification has been updated and modified due the diversity in effects and the variable responses exerted by nucleotides reported in the literature. The P1-purinoceptors were subdivided into A1 and A2 subtypes [85] and P2-purinoceptors into P_{2X} and P_{2Y} subtype [86]. Then, P1-purinoceptors were subdivided in 4 subtypes A_1, A_{2A}, A_{2B}, and A_3 after new discoveries [87]. The P_{2X} subtype refers to ligand gated ion channels permeable to Na^+, K^+ and Ca^{2+} [88], whereas the P_{2Y} subtype is a G-protein coupled receptor that modulates membrane phosphoinositide metabolism and, hence, inositol-1,4, 5-triphosphate (IP_3) and diacylglycerol (DAG) generation [89]. Additional transduction mechanism seen to be utilized in some tissues, such as modulation of cAMP generation [90] and arachidonic acid mobilization [91].

Gordon proposed in 1986 [92] another subdivision of P2 purinoceptors. The designation of P_{2T} to the receptor for ADP on blood platelets, thrombocytes and megakaryocytes, and a designation of P_{2z} to the receptor that mediates responses to ATP^{4-} in mast cells and macrophages. Another designation for a specific receptor for diadenosine polyphosphate, which are released from platelets, was termed P_{2D} (Ap4A, Ap5A, and Ap6A) [93] and the receptor that showed equipotency for UTP and ATP was termed P_{2u} purinoceptor [94]).

However, Fredholm proposed the receptors to be termed "P2 receptors" because some receptors were activated by both purines and pyrimidines nucleotides and dinucleotides [87]. Therefore, Abbracchio and Burnstock in 1994 proposed a new nomenclature in which divided P2 receptors in two main families: P2X consisting of ligand gated cation (ionotropic ATP receptors) and P2Y consisting of G-protein coupled receptors (metabotropic nucleotides receptors) [95]. This new nomenclature is based in numbering the receptor subtypes and was approved by the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR). In the present, the P2X and P2Y receptor family has 7 subclasses and 8 subtypes respectively, that are readily studied and pharmacologically defined, in which for P2X are: $P2X_1$, $P2X_2$, $P2X_3$...$P2X_7$; and for P2Y are: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ [96, 97], [66], [98, 99]. The potency order of each P2Y

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receptor is shown in Table 1.1. The potency profile of several of these receptors has been changed by new discoveries and may depend on the experimental conditions, the cell type and species.

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Table 1.1 Pharmacological profile of agonist potency of P2Y receptors

P2Y receptors	Pharmacological profile/Agonist potency
$P2Y_1$	ADP> > ATP >> UTP, UDP *
$P2Y_2$	UTP=ATP> ADP, UDP *
$P2Y_4$	UTP>>ATP, UDP, ADP (human) *
$P2Y_6$	UDP> UTP> ADP>>ATP *
$P2Y_{11}$	ADP> ATP>> UDP, UTP *
$P2Y_{12}$	ADP> ATP>>UDP, UTP *
$P2Y_{13}$	ADP> ATP>>UDP, UTP **
$P2Y_{14}$	UDP-glucose>> UTP, ATP, ADP, UDP ***

Taken form [96]

* Taken from [97].

** Taken from [100], [101], [102]

*** Taken from [103]

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1. Structural determinants of $P2Y_2$ receptor

A $P2Y_2$ receptor was cloned from a NG108-15 neuroblastoma x glioma cell cDNA library by expression cloning in *Xenopus laevis* oocytes [104] and functionally expressed in mammalian cells [105]. To identify the $P2Y_2$ receptor protein, cDNA for the receptor was modified to incorporate a hexahistidine epitope tag at the C-terminus which enabled purification of the expressed receptor from membrane extracts of K562 cell transfectants and this modification did not affect $P2Y_2$ receptor signaling as compared to the wild type receptor [105]. The apparent molecular weight of 53 kDa determined for the $P2Y_2$ receptor suggested that ~20% of its apparent mass is due to N-linked glycosylation at sites in its extracellular domain. Figure 1.1 shows a two dimensional structure of the $P2Y_2$ receptor determined from the nucleotide sequence of the $P2Y_2$ receptor cDNA and hydropathicity analysis of the predicted amino acid sequence of the encoded protein [104], [106], [82]. The $P2Y_2$ receptor is activated equipotentially by ATP and UTP. To learn more about the ligand binding site of the cloned NG108-15 cell $P2Y_2$ receptor, a series of mutant receptors were produced by site-directed mutagenesis of the $P2Y_2$ receptor cDNA and expressed in a clonal line of human 1321N1 astrocytoma cells that are devoid of endogenous P2Y receptor activity [107]. It was postulated that positive charged amino acids in the $P2Y_2$ receptor could served as conterions for binding the negatively charged receptor agonists. Substitution of the amino acids, ^{262}His, ^{265}Arg, and ^{292}Arg, with the neutral amino acids leucine or isoleucine diminished ATP and UTP potencies and substitution of ^{289}Lys with Arg conserved the positive charge but increased the potencies of ADP and UDP and decreased the potencies of ATP and UTP. The location of these four basic amino acids is depicted in Figure 1.1. The positively charged amino acids in the 6^{th} and 7^{th} transmembrane domains appear to be conserved among all of the established P2Y receptors subtypes [108].

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Figure 1.1 Structure and amino acid sequence of the $P2Y_2$ receptor

The $P2Y_2$ receptor presents the N-linked glycosylation at sites in the extracellular domain, the arginine-glycine-aspartic acid (RGD) integrin binding motif that is localized at the first extracellular loop of this receptor, positive charged amino acids as UTP and ATP binding sites, consensus phosphorylation sites for PKC, MAPK and GRKs and the proline rich sequence at the intracellular (C-terminal) domain

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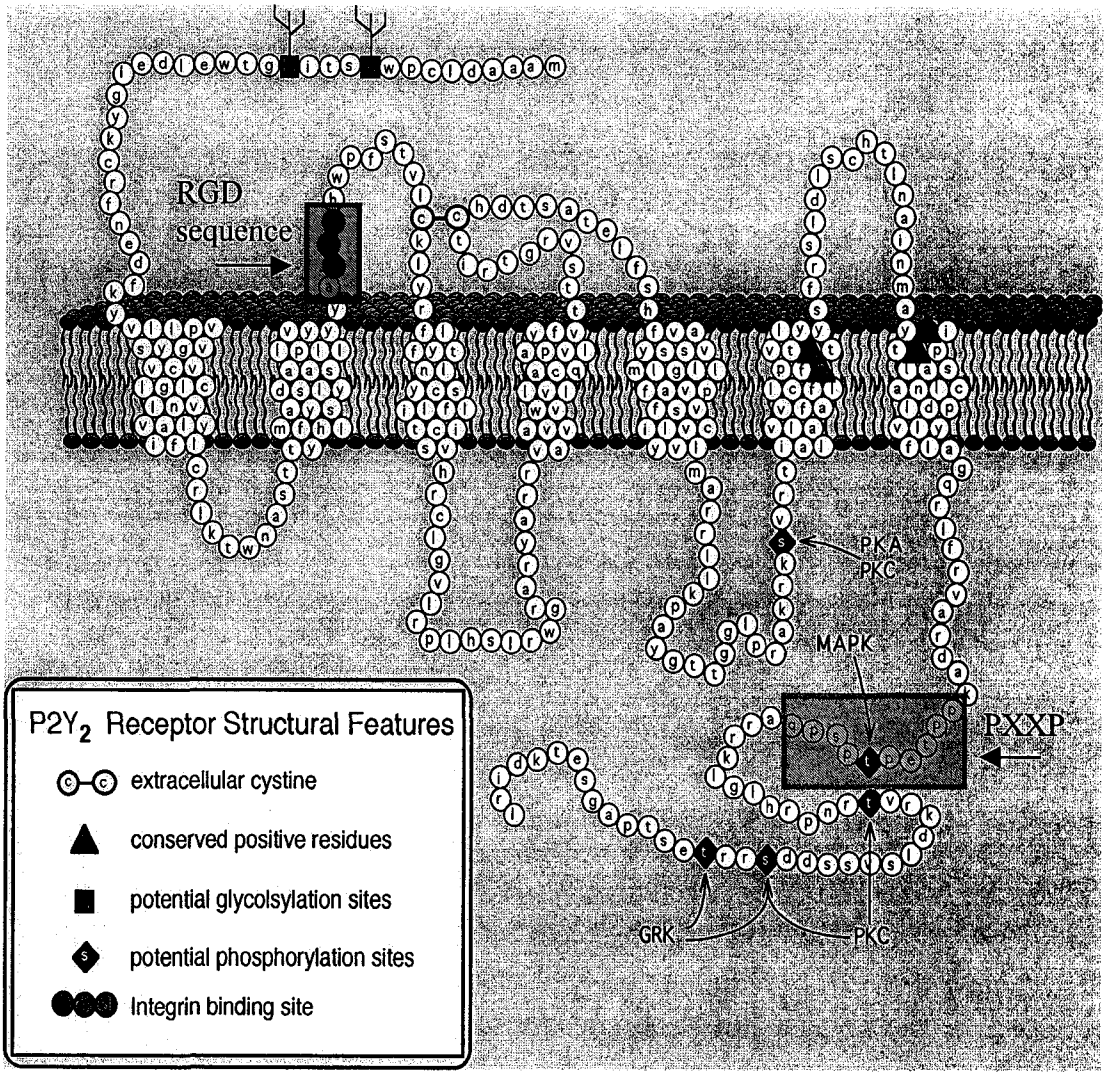

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The P2Y receptors undergo agonist induced desensitization [109] similar to other members of the G protein coupled receptors (GPCRs) superfamily. The C-terminus of the $P2Y_2$ receptor contains two consensus phosphorylation sites for PKC (another site is present in the third intracellular loop) and potential phosphorylation sites for GRK (see figure 1.1). A series of five truncation sites of the NG-108-15 cell $P2Y_2$ receptor cDNA was constructed using polymerase chain reaction (PCR) to study the role of the C-terminal [110]. An N-terminal hemagglutinin (HA) epitope-tag was also incorporated into the cDNA [110]. The potency and efficacy of UTP-induced calcium mobilization were relatively unaffected in the truncated mutant receptors, but a deletion of 18 or more amino acids from the C-terminus increased by approximately 30-fold the concentration of UTP necessary for desensitize the receptor as well as the rate and extent of UTP-induced sequestration [110]. The time of recovery from sequestration was markedly increased with the longest truncation mutant as compared to the wild type receptor [110].

The amino acid sequence of the human $P2Y_2$ receptor have indicated the presence of an arginine-glycine-aspartic acid (RGD) integrin binding motif that is localized at the first extracellular loop of this receptor [106], [104], [82] See figure 1.1. This RGD sequence is rare among cloned GPCR where is found also at the first extracellular loop of the $P2Y_6$ receptor and at the third extracellular loop of the H_2 histamine receptor (GPCR database: http://www.gpcr.org). However, the homologue rat $P2Y_2$ receptor contains a QGD instead of RGD sequence [111] that is suggested to maintain integrin binding.

The RGD motif is a sequence present in integrins ligands such as vitronectin, fibrinogen, collagen, fibronectin, osteopontin, von Willebrand factor, thrombospondin, laminin, entactin, tenascin, bone sialoprotein and other extracellular matrix proteins that bind the integrins to modulate cell adhesion and cell shape changes involved in cell spreading and locomotion [112, 113]. Integrins are transmembrane proteins that are composed of noncovalently associated alpha and beta subunits which form heterodimeric receptor complexes. The α and β subunits contain a large extracellular domain, a short transmembrane domain and a cytoplasmic carboxy terminal domain of variable length. The mammalian system has 18 α and 8 β subunits, which are known to combine to

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produce 24 distinct different receptors. Although each integrin has its own binding specificity, many bind to the same ligand or to partially overlapping sets of ligands. The major adhesive components of matrix, such as fibronectin and laminin, are recognized by multiple integrins [114], [113]. The integrin that binds ligands with RGD sequence are $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_5$, $\alpha_v\beta_3$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, and $\alpha_7\beta_1$, [115].

The binding of ligands to integrin receptors leads to a cross-linking or clustering of integrins. Integrins binding undergo a conformational change in their cytoplasmic domains which promotes the formation of structures at the cell membrane known as focal adhesions where integrins link the outside matrix to intracellular cytoskeletal complexes [116]. These protein assemblies play an important role in modulating cell adhesion and induce cell shape changes involved in cell spreading and locomotion. Actin binding proteins that colocolize with integrins at focal adhesion plaques include: α-actinin, talin, tensin, paxillin and vinculin. Protein kinases that colocolize with these structures include: focal adhesion kinase (FAK), c-Src, protein kinase C (PKC), integrin linked kinase (ILK) [113].

Integrins regulate early angiogenic responses including the endothelial cells adhesion, migration and proliferation [117], [118]. These angiogenic responses in the endothelium respond to physical injury or disease [117]. The endothelium regulates blood flow and platelet aggregation through the release of prostacyclin and nitric oxide by GPCR activation [119-121]. The expression and release of nitric oxide were seen in ischemic process [122]. Also focal cerebral ischemia induces the expression of $\alpha_v\beta_3$ integrin and its ligand fibrinogen in a subpopulation of brain microvessels [123]. Thus, integrins could be modulating processes related to neurological diseases, specifically during protection. The signals from integrins receptors facilitate growth factor-mediated activation of MAPK [113] and extracellular nucleotides activate a signal transduction that includes MAPKs by the $P2Y_2$ receptor. Integrin signaling activates ERK through two major mechanisms: the SFK (src family kinase)/FAK pathway, which is activated by most, perhaps all, integrins through the cytoplasmic portion of their β subunit, and SFK/Shc pathway, which is activated by a subset of integrins through the transmembrane segment of their α subunit [124].

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Some studies have demonstrated cross-talk between the intracellular signaling pathways of tyrosine kinase receptors (RTK) and G protein coupled receptors (GPCR) [125]. In fact, there have been reports suggesting intracellular cross talk between P2Y receptors and integrins [126] and P2Y and EGF receptors [127, 128]. Interactions between integrins and other types of receptors such as the urokinase-type plasminogen-activated receptor and PDGFb receptor have also been reported [129]. However, no one has demonstrated a direct physical interaction between a GPCR and an RGD-binding integrin receptor. Recent studies from our laboratory in collaboration with Weisman et al. of University of Missouri, have suggested that the $P2Y_2$ receptor could be associated or interacting with other receptors, specifically integrins [130]. Thus, it is possible that the $P2Y_2$ receptor associates with the $\alpha_v\beta_3/\beta_5$ integrins through the binding of its RGD motif to the $\alpha_v\beta_3/\beta_5$ integrins, and both receptors coupling elicit a protection or survival signal during ischemia regulating the blood flow to the brain.

The signal transduction exerted by the $P2Y_2$ receptor may be regulated by specific sequences of amino acids at the intracellular domain. As mentioned before, previous studies from our laboratory in collaboration with Dr. Gary A. Weisman of the University of Missouri have demonstrated that the deletion of segments of the C-terminal domain of the $P2Y_2$ receptor are important in the regulation of $P2Y_2$ receptor signaling [110]. However, C-terminal domains may also play other roles in addition to regulate desensitization or sequestration. Murine and human $P2Y_2$ receptor posses a proline rich sequence at the intracellular domain (C-terminal) which may interact with the SH3 (Src Homology 3) domain of other proteins such as the c-Src protein kinase [131]. (See figure 1.1) The SH3 domains regulate protein localization, enzymatic activity and often participate in the assembly of multicomponent signaling complexes [132]. Some of the proteins that contains SH3 domains such as Grb2, src and PI3K, also contain SH2 domains that act as enzymes and adaptors [131]. The minimal sequence requirement for the SH3 domain ligands is the PXXP motif [133]. The general consensus sequence for SH3 domain ligands is ψ PX ψP (where ψ is a hydrophobic residue) [132]. This SH3 binding motif in other receptors such as β_2-adrenergic and β_3-adrenergic receptor was shown to be critical for the formation of the intracellular signaling complexes that

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mediate the activation of MAPK [134]. Src has an SH2 domain that has been shown to mediate the activation of FAK (focal adhesion kinase) which is implicated in the assembly of focal adhesions in the intracellular actin cytoskeleton of the cell and in the activation of MAPK [135]. Changes in the endothelial actin cytoskeleton which regulate the activation of protein kinases are important for neuroprotection after ischemic stroke [136]. It was also shown that stimulation of β_2-adrenergic receptor induces an increase of NGF expression in rat brain after transient forebrain ischemia thereby protecting the neurons against apoptosis [137]. Thus, it would be important to investigate the potential role of this sequence (SH3 binding motif) in the signal transduction exerted by the $P2Y_2$ receptor related to neuroprotection and the possible intracellular signaling protein complex responsible for this effect (protein kinases activation).

IV. Animal and cell culture models used to study ischemia

Many experimental models are used to study ischemia damage which includes the *in vivo* and *in vitro* models. The three principal classes on *in vivo* rodent models are global ischemia, focal ischemia, and hypoxia/ischemia. The latter consist in vessel occlusion combined with breathing an hypoxic mixture. The *in vitro* models include exposure of neuronal, neuronal/glial, organotypic slice cultures, or the freshly isolated hippocampal brain slice, to anoxia or to anoxia in the absence of glucose (*in vitro* ischemia) [12] .

A. The *in vivo* models

The basis for the systematic study of the brain cell damage in rodents were determined by the Levine model, in which hypoxia is combined with unilateral carotid occlusion [138], and then adapted in the mid-1960s in landmark studies by Brown, Brierly, and co-workers [139]. Most of the *in vivo* models now rely on vessel occlusion that predominantly affects the forebrain, developed between the late 1970s and early 1980s [140], [141]. They are generally divided in two categories: global and focal ischemia, but a modified Levine preparation (hypoxia/ischemia) has some properties of both models.

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Global ischemia insults are most commonly produced by vessel occlusion, and less commonly by complete brain circulatory arrest. The former are not actually global, a large portion of the fore brain is quite uniformly affected. The three most widely used global ischemia models are four-vessel occlusion (4-VO), two-vessel occlusion (2-VO) combined with hypotension in the rat, and two-vessel occlusion in the gerbil. The 4-VO in rat involves permanent coagulation of the vertebral arteries (which has no deleterious effects) and temporary ligation of the two common carotids. The 2-VO in rat involves ligation of the common carotid arteries only, along with a blood pressure reduction to ~ 50 mmHg and has slightly more profound effects than 4-VO. The 2-VO in gerbil is induced by temporarily ligating the carotid arteries, with no reduction in blood pressure [12]. Because there are no posterior communicating arteries in gerbils, this produces profound forebrain ischemia. And finally, there is a complete global ischemia, generally achieved by neck cuff [142], cardiac arrest [143], or by ligating or compressing all arteries stemming from the heart [144]. Blood flow in the whole brain is zero or <1% in these models [145]. Vessel occlusion is often termed "incomplete ischemia" because of the residual blood flow, and there is a controversy as to whether complete or incomplete ischemia is more damaging, with the thought that the larger fall in pH or increase in free radicals during incomplete ischemia might enhance damage. Overall, gerbil vessell occlusion is the most studied model because the operation is simpler than the rat [12].

Focal ischemia involves occlusion of one middle cerebral artery and in some cases the carotid artery is also ligated [141]. The insult is differentiated from the global ischemia in two important ways. First, even at the core of the lesion, the blood flow is almost always higher than during global ischemia so that longer insults are required to get damage. Secondly, there is a significant gradation of ischemia from the core of the lesion to its outermost boundary, and hence there are different metabolic conditions within the affected site. Because of its duration, and heterogeneity, the insult is much more complex than global ischemia, but it is an invaluable model for stroke and is thus widely studied. In permanent focal ischemia, the arterial blockage is maintained throughout an experiment, usually for 1 to several days, whereas in temporaray focal models, vessels are blocked for up to 3 hrs, followed by prolonged reperfusion. Unlike global ischemia,

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which leads to neuronal cell death in isolated regions, focal ischemia produces a contiguous mass damaged brain tissue termed the infarct [12].

B. The *in vitro* models

In this type of model, the bathing solution over the cultured cells is rapidly changed from O_2/CO_2 equilibrated to N_2/CO_2 equilibrated. When glucose is maintained in the anoxic buffer, the insult is termed anoxia (or hypoxia), and when glucose is omitted, the insult is termed *in vitro* ischemia or oxygen/glucose deprivation (OGD) [12]. Intracellular ATP falls less completely during *in vitro* ischemia than it does during global ischemia and falls more slowly in the presence of glucose. Generally 5-7 min *in vitro* ischemia at 36-37°C, or a period of ischemia extending 2-3 min beyond the anoxic depolarization, leads to a rapid damage to various properties of CA1 pyramidal cells which lasts for the 8- to 14-hrs life of the slice. Somewhat longer exposures are necessary for damage to dentate granule cells so that the slices do show the same ranking of selective vulnerability as *in vivo* tissue. However, differences are not nearly as dramatic because acute damage, rather than prolonged damage, is being measured, and acute damage are very similar in different hyppocampal regions *in vivo* [12].

1. Brain slices

Brain slices, particulary the hyppocampal slice, have become widely used models for studying anoxic or ischemic damage [146]. Properties that are damaged include synaptic transmission [146], protein synthesis [147], maintenance of ATP levels [148], cytoskeletal integrity [149], and neuronal morphology [150]. Changes in protein synthesis are similar to those observed *in vivo*, but the profound loss in synaptic transmission and the very intense morphological damage and disruption of the cytoskeleton are generally not observed as early or as strongly *in vivo,* indicating that slices are more sensitive to ischemic damage than are cell *in situ*. Slices fulfill a very important role by facilitating analysis of mechanisms of early changes. Although there are some differences, rates of change of ions, metabolites, and protein synthesis

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measurement are quite similar to those measured *in vivo,* suggesting that the mechanisms are the same. Shorter insults, which are stopped just when the anoxic depolarization occurs, lead to a more slowly developing damage, requiring ~ 12 h to be manifested which is closer to the delayed neuronal death seen *in vivo* [12].

2. Cell Culture

Primary neuronal/glial cultures from cortex [151], hippocampus [152], cerebellum and hypothalamus [153] of embryo or perinatal rats and mice have been used extensively to study anoxic or ischemic damage since 1983 [154]. Several differences in vulnerability between regions as occurs in CA1 versus CA3 versus dentate gyrus if the *in vitro* ischemia is kept relatively short (30 min), but twice as much cell death in CA1 as CA3 [155]. Longer durations (60 min) produce larger and equal amounts of cell death in all hyppocampal regions [155]. Damage is usually developed 8-24 h after 30- to 60-min *in vitro* ischemia and is generally monitored as a gross increase in membrane permeability to dyes or protein, in particular leak of LDH [153], or nonexclusion of trypan blue or propidium iodide [156].

A major difference from damage *in vivo* is the very long duration of OGD that is generally required to induce cell death particularly for both primary cultures and organotypic cultures [157], [155]. The possible reasons for this difference is that ATP does not fall nearly as rapid as it does *in vivo* and the release of glutamate is very delayed [157]. The cell in culture could adopt a protective mechanism that reduce ATP turnover during hypoxia [158]. Another difference between cell cultures and *in vivo* tissue is that damage in primary culture following 30-35 min exposures is due or dependent of NMDA receptors activation [157], but longer exposure (90 min) produce an independent NMDA receptor manner of damage that appears to be apoptotic [159]. Astrocytes are more resistent to this type of insult and are more dealyed in *in vitro* studies.

The use of an *in vitro* model facilitates the interpretation of the results in absence of several factors that confound *in vivo* studies such as blood flow and blood vessels in culture, brain temperature and trauma due to survival surgery. An another advantage is

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that the *in vitro* model can allow five to ten uniform cultures to be isolated from a single animal, therefore fewer animals are needed whereas animal models require sacrifice one animal per one unit data [12], http//www.rfsunny.org). In our model, the absence of neurons and other cell type helps us to understand the astrocyte function, astrocytic interrelation and the astrocytic response to ischemia especially in terms of the role, activation and regulation of the $P2Y_2$ receptor. Astrocytes express several types of P2 ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2X_7$ subtypes) and P1 (A_1, A_{2B}, and perhaps A_3 subtypes) receptors [44], [45] as well as several glutamate receptors types that could be activating and modulating different responses after an episode of ischemia [160]. To know the role of extracellular nucleotides in ischemia specifically the role of the $P2Y_2$ receptor, we employed a cell line of astrocyte lineage, which does not express functional P2Y receptor and was transfected with HA-tagged human $P2Y_2$ receptor cDNA to determine the role of the $P2Y_2$ receptor in ischemia [161], [82], [107]. With this cellular model, we eliminate other factors such activation of other P2 receptor such as $P2X_7$ that mediate apoptosis which interfere with the interpretation of data regarding the possibility of the $P2Y_2$ receptor activation signaling pathway that activates and regulates a particular cellular response. Although may be this model does not represent the complete response of this type of cell to this type of stress (ischemia), it may help us understand the mechanism that astrocytes carry to a particular response and the role of $P2Y_2$ receptor in this process.

This type of *in vitro* model tries to ressemble as much as possible to the *in vivo* model and the information obtained in the *in vitro* model can be extrapolate or used to understand the responses observed in the *in vivo* model. This type of *in vitro* model has been amply used by many investigators to determine activation of signaling molecules, effectiveness of glutamate antagonists, neuroprotection responses, gene expression, among others ([162], [163], [164]). Moreover, the information that will be obtained, will provide a molecular and cellular framework that helps us to understand the development and function of astrocytes network as well as brain function.

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V. Mitogen-Activated Protein Kinase

Mitogen-Activated Protein Kinase (MAPK) is a family of serine-threonine protein kinases that mediate signal transduction from the cell surface to the nucleus and participate in many cellular responses such as: proliferation, differentiation, development, apoptosis, inflammation, regulation of genes expression, activation of transcription factors, control of metabolism, and regulation of the immune system (protein kinase receptors, hormones, neurotransmitters, nucleotides, among others through specific membrane receptors such as tyrosine protein kinase receptors and G-protein coupled receptors [125], [165], [166], [167]. Other activators of MAPK not mediated by membrane receptors, are phorbol esters, stress, UV light, osmolarity [168]. Because MAPKs regulate a large number of responses, they are attractive targets for drugs in therapeutic studies as cancer [169] and inflammation [170].

In mammals, MAPKs have been identified and classified in 3 subfamilies: ERK (Extracellular Signal-Regulated Kinase), p38/CSBP/RK (CSAID binding protein/Reactivating Kinase and is the homologue of the *Saccharomyces cerevisiae* (HOG1) gene) and JNK/SAPK (c-jun-N-terminal Kinase or Stress-Activated Protein Kinase). MAPKs are activated by the phosphorylation of both threonine and tyrosine residues found in the regulatory TXY motif present in all the MAPKs [171]. This phosphorylation is carried by dual upstream threonine-tyrosine protein kinases known as MAPK Kinase or MAPKK which vary between the three subfamilies.

The differences between the 3 subfamilies may be summarized as follows:

a. They differ in their activators and the receptors whose signal is carried to MAPKs
b. the consensus of phosphorylation (TXY) in which X vary in each subfamily
c. their substrates or effectors
d. the cellular response that are exerted
e. structural features
f. the signal transduction that activate them
g. their dual upstream protein kinase MAPKK

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These differences permit us to elucidate which MAPK are involved in specific cellular response. The next few sections will summarize our current knowledge of this family of protein kinases.

A. ERK

Extracellular Signal-Regulated Kinase (ERK) was discovered by Sturgill and coworkers and was initially known as MAP-2 kinase (microtubule-associated protein 2 kinase) [172], [173]. The acronym MAP-2 was changed to MAPK (Mitogen-Activated Protein Kinase) because it is activated by diverse mitogens and growth factors [174]. Then, the name of MAPK was proposed to be changed to ERK (Extracellular Signal-Regulated protein Kinase), because a wide variety of extracellular signals activated it [175].

The ERK subfamily is composed of at least 8 isoforms. ERK1 (p44), ERK2 (p42), ERK3 (p62)/(p63) and ERK4 (p45) [175], [176]. Other smaller isoforms, p40 and p41, were identified (cloned) as a result of an alternative splicing process [177]. ERK5/BMK1 (Big MAP Kinase) is considered as a member of the ERK subfamily by some investigators, but also is considered as a fourth member of the MAPK family [178], [179]. Other members are ERK 6, considered as a p38 member [180], ERK7 (61kDa) [181] and ERK8 which has 69% amino acid sequence identity with ERK7 [182].

ERK activation depends of the dual phosphorylation occurred within the sequential motif Thr-Glu-Tyr [183]. The activation of ERK1/2 may be mediated by autophosphorylation in addition of the dual upstream threonine-tyrosine protein kinase known as MAPKK or MEK1 and MEK2 [184], [185], [186]. The activation of these kinases, MEK1 and MEK2 were regulated by another group of protein kinases known as MAPKKK and MAPK inclusive [187]. Among the MAPKKK known to phosphorylate and activate MEK1/2, the family of Raf (Raf-1, A-Raf, B-Raf, c-Raf, v-Raf) is the best characterized [188]. The most common, Raf-1, is known to phosphorylate MEK1/2 and MEK Kinase [189]. To determine the dependency of MEK1/2 or MEKK in the activation of ERK1/2 and to probe its involvement in a specific cellular response, some specific

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inhibitors of MEK1/2 or MEKK heve been designed and frecuently used such as PD 098059, and U0124, U0125, and U0126 [190, 191], [192].

The family of ERKs are proline-directed protein kinases that phosphorylate a consensus sequence Pro-Xaa_n-Ser/Thr-Pro, where Xaa is any amino acid and n=1 or 2 [193]. Some of the substrates that have been demonstrated to posses this consensus of phosphorylation are proteins such as $cPLA_2$ and transcription factors such as c-Myc, c-Fos, Elk-1, Sap-1, c-Jun [194], [195] that once the protein kinase is translocated to the nucleus, regulate gene expression [196].

B. JNK/SAPK

JNK (c-jun-N-Terminal Kinase) or SAPK (Stress-Activated Protein Kinase) was discovered regulating apoptosis and the responses that exert stress [197]. They are activated by stress, UV radiation, osmolarity changes, TNFα, IL1β, protein synthesis inhibitors (anisomycin and cycloheximide), hypoxia, etc. [168]. Although JNK is a Pro directed kinase similar to the other MAPK, its substrate specificity is different from that of ERK1/2 [194], [193]. This kinase was discovered by the phosphorylation of one of its principal substrates, c-Jun [198]. c-Jun was phosphorylated in the NH_2-terminal activation domain on Ser 63 and Ser 73, causing increased c-Jun transcriptional activity [198] [199]. The phosphorylation sequence motif Leu/Ala-Ser*-Pro-Asp/Glu was determined in c-Jun to be the phosphorylation site for JNK [199].

JNK was cloned in human (JNK) [199] and in rat (SAPK) [200]. Three principal isoforms: JNK1 (p46), JNK2 (p55) and JNK3 (p49) have been identified to be encoded by three distinct genes: Jnk1, Jnk2 and Jnk3 [200],[201]. At least 10 different spliced isoforms exist in mammalian cells [202]. Four spliced variants of the mouse JNK/SAPK α-isoform have also been identified [203]. Each isoform binds to the substrate with different affinities suggesting a regulation of signalling pathways based on substrate specificity of different JNKs *in vivo* [202], [201]. This idea was demonstrated by the difference in binding of JNK1 and JNK2 to c-Jun [204].

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JNK1 and JNK2 are activated by dual upstream threonine-tyrosine protein kinases, which are structurally related to the MAP Kinase Kinase (MEK) known as MKK7 [205], SEK1(SAPK/ERK kinase-1) [206], JNKK1 [207], JNKK2 [208] and MKK4 [209]. The phosphorylation of Thr^{183} and Tyr^{185} was required for JNK activation (JNK1 and JNK2) [199] and for JNK3 is Thr ^{221} and Tyr ^{223} [210]. An analogue of Raf-1 which phosphorylates MEK1/2 was identified and denominated MEKK (MEK Kinase-1) which phosphorylates SEK1 in Thr ^{223} and in turn regulates the activation of JNK/SAPK [189, 206]. A number of MKKK/MEKK-like kinases have been postulated to regulate JNK activation. Another MKKK has been demonstrated to regulate JNK activation and was denominated MEKK4 which is regulated by Cdc42/Rac [209].

JNK as well as ERK and p38, is translocated to the nucleus from the cytosol once stimulated. However, the activation of JNK may vary depending of the stimulus and the cell type. JNK1 may be translocated to the nucleus without being activated, and may be activated in the nucleus by SEK1 during ischemia and reperfusion process in the heart [211]. SEK1 different from MEK1, is distribuited in the cytosol and the nucleus under control conditions and translocated to the nucleus by ischemia and reperfusion [211]. Once in the nucleus, JNK phosphorylates transcription factors such as: c-Jun, ATF2, Elk-1, TCF, etc [195].

B. p38

The p38 MAP kinase was first identified as an intracellular protein rapidly phosphorylated on tyrosine upon treatment with lipopolysaccharide (LPS) from gram-negative bacteria in murine monocytes and macrophages transfected with the LPS-complex receptor or CD14 receptor, which is the known receptor for LPS [212]. This protein has an apparent molecular weight of 38 kDa [212], but it was found that its cDNA encoded a 360 amino acid protein with a molecular weight of 41 kDa. Thr^{180} and Tyr^{182} are the phosphorylated residues in p38 [213] found in the TXY motif [187] characterized in all the MAPKs [214]. The X amino acid is G which is different to the other MAPKs. The sequence Thr^{180}-Gly^{181}-Tyr^{182} in p38 must be phosphorylated for p38 activation, and experiments performed to change one or both amino acids confirmed this requirement

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[213]. The dual phosphorylation of the regulatory sequence of p38 was achieved by the upstream dual specificity threonine/tyrosine kinase known as MAP Kinase Kinase (MKK) or Stress Kinase Kinase (SKK). The p38 MAPK was phosphorylated mainly by MKK6 and MKK3 [215] and SAPKK2 and SAPKK3 (which corresponds to the mammalian MKK3 and MKK6) [216], although it is also phosphorylated by MKK4/SEK1 which phosphorylates JNK [217].

The p38 MAPK, like JNK, is activated by stress-induced signal transduction pathways which include: enviromental stress (UV radiation, osmotic shock), proinflammatory cytokines (IL-1 and TNF), endotoxic LPS, high osmolarity, H_2O_2, anisomycin, sodium arsenite, NaCl, Na_3VO_4 [218]. With strong activators of ERK1/2 like EGF and phorbol esters, a modest increase in p38 activity was observed, but it depended of the cell type [219]. With respect to its activity, p38 has been shown to modulate the synthesis of proteins and/or cytokines at the translational and transcriptional level [220]. Some of the substrates that are phosphorylated by ERK1/2, are phosphorylated by p38 including: the EGF- receptor, MBP [213], and $cPLA_2$ [221]. The principal substrate of p38 is ATF2 (atriad transcriptional factor 2) which is phosphorylated at the N-terminal activation domain [213]. Other substrates of p38 include: transcription factors such as CHOP/GADD153 (growth arrest and DNA-inducible gen 153) [218]; MEF2C (myocyte-enhance factor 2) [222], TCF (ternary complex factors) such as: Elk-1, Sap-1 [195]; CREB (cAMP response element binding protein) and ATF1 [223], [224] among others.

At least six isoforms of p38 (α1, α2, β1, β2, γ, δ) have been identified [225], [226], [227], [228], [214]. Some of p38 isoforms could be the result of alternatively spliced variants [229]. Recently, it was found that different p38 isoforms regulate different cellular events such as is the case of p38α and p38β [230], [231]. Compounds of pyridinyl imidazoles have been designed with potential anti-inflammatory function [170] and are specific in inhibiting p38. These inhibitors do not cross react with other members of the MAPK. Not all the inhibitors of p38 inhibit all the p38 isoforms. SB203580 is one that specifically inhibits p38 activity and the downstream cascade that activate p38 [232]. This inhibitor and other such as SB202190, have been used to identify the presence and the regulatory function of p38 isoforms in cell function [233] .

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VI. PKB/Akt Protein Kinase

PKB/Akt is a serine/threonine protein kinase of 57 kDa involved in regulation of cell proliferation, migration, transcription, glucose metabolism, angiogenesis, apoptosis and survival [234]. PKB/Akt was discovered in 1977, by Staal and co-workers, of a transforming murine leukemia virus termed AKT8 from mice with a high incidence of spontaneous lymphoma [235]. By its homology with other protein kinases such as protein kinase A, cGMP-dependent protein kinase and protein kinase C, PKB/Akt was designed to belong to a family termed AGC family [236]). Until now, there are three isoforms of PKB/Akt identified in mammalian cells: PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 [234]. PKBβ and PKBγ show 81% and 83% amino acid identity with PKBα respectively and show a wide tissue distribution [237].

The activation of PKB/Akt has been shown to be induced by insulin, serum and growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and insulin like growth factor (IGF-1), through their respective tyrosine kinase receptors [234], [238]. The activation has also been seen by integrins such as $\alpha_5\beta_1$ and by G protein coupled receptors such as adrenergic and muscarinic receptors [239]. The activation of PKB/Akt is regulated by their phosphorylation in Ser 473 in the regulatory C-terminal hydrophobic motif and Thr 308 in the A-loop of the kinase domain and by protein-protein interactions that are mediated by the association with membrane phospholipids generated by an enzyme known as phosphatidylinositol 3-kinase (PI3K) [234].

The mechanism of activation of PKB/Akt is conducted by its recruitment to the plasma membrane by phospholipids such as phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3) and phosphatidylinositol 3,4-biphosphate (PtdIns(3,4)P_2), products of phosphatidylinositol 3-kinase (PI3K) [240]. The recruitment is mediated by a pleckstrin homology domain (PH) that is present in the N-terminal region of the PKB/Akt [241]. Another protein that contains a PH domain which is also associated to the plasma membrane is 3-phosphoinositide-dependent protein kinase, PDK1 [242], [243]. PDK1 is

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the protein kinase responsible of the Thr308 phosphorylation in PKB/Akt [242]. Although the protein kinase responsible for the phosphorylation of Ser 473 is not yet identidied, studies *in vitro* suggest that integrin-linked kinase-1 (ILK-1) another lipid-activated kinase may be responsible of this phosphorylation [244]. The product of PI3K, PtdIns(3,4,5)P_3, is responsible for the translocation of both kinases to the plasma membrane and subsequently the activation of PKB/Akt by PDK1 [243], [241]. The use of PI3K inhibitors such as wortmannin and LY 294002 has demonstrated the role of PI3K in the regulation of PKB/Akt activation and MAPK [245], [246].

After the PKB/Akt activation, there have been demonstrated to regulate multiple biological process including apoptosis, cell cycle, gene expression, metabolism, cytoskeleton, and cellular proliferation [234]. This regulation is mediated by the phosphorylation of a serie of substrates. The first substrate discovered was glycogen synthase kinase-3 (GSK-3) that exists in two isoforms α and β [247]. Using synthetic peptides based on the phosphorylation site of GSK-3 have accelerated the identification of other substrates of PKB/Akt [238]. The Akt phosphorylation consensus was identified as RXRYaaZaaS/THyd where X is any amino acid, Yaa and Zaa are preferably small residues other than glycine and Hyd is a bulky hydrophobic residue (phenylalanine (F) or leucine (L)) [238]. The phosphorylation of GSK3β in the Ser 9 residue by PKB/Akt has been shown to inactivate it and inhibit apoptosis [247]. Other PKB/Akt substrates identified that regulate apoptosis are BAD [248], procaspase-9 [249], FKHR [250], AFX, [251], CREB [252] and ASK1 [253]. Each one of these proteins, once are phosphorylated, inhibits apoptosis, but unphosphorylated induce apoptosis.

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VII. Statement of the problem:

Receptors for adenosine and ATP are expressed in astrocytes. Larger amounts of nucleotides and nucleosides are released following brain trauma and ischemia, which can activate immediately P2 receptors such as $P2Y_2$ receptors. Therefore, the study presented in this thesis was aimed at determining the role and molecular mechanisms that extracellular nucleotides through the $P2Y_2$ receptor activate during or after a cerebral ischemia episode and to elucidate the molecular determinants of the $P2Y_2$ receptor by which extracellular nucleotides mediate astrocytoma activation. This information could help to elucidate novel therapeutic strategies to treat cerebral ischemia.

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CHAPTER II

Methods

I. Cell Culture

Human 1321N1 astrocytoma cells are adherent cells that grow in monolayers. Human 1321N1 astrocytoma cells do not express functional P2Y receptors, making it a suitable model for study the roles of P2Y receptors after transfection with specific nucleotide receptor genes. Therefore, human 1321N1 astrocytoma cells were transfected with HA-tagged human $P2Y_2$ receptor cDNA ($P2Y_2$-1321N1 cells) using the pLXSN retroviral vector, as described previously [82], [107]. The $P2Y_2$-1321N1 cells were cultured in Dubelcco's Modified Eagle's culture medium (DMEM) supplemented with 5% fetal clone bovine serum (Clonetech), 1% kanamycin antibiotic (FISHER), 1% antimycotic antibiotic (SIGMA), 5mM HEPES (SIGMA) and 500μg/ml Geneticin (G418, Life Technologies, Inc.) at 37°C under a humidified 5% CO_2, 95% air atmosphere.

II. An *in vitro* model of Cerebral Ischemia

An *in vitro* model of cerebral ischemia was utilized in our studies as described in reported procedures [254], using 1321N1 astrocytoma cell culture. The $P2Y_2$-1321N1 astrocytoma cells in the exponential phase of growth were seeded at a concentration of 1.0×10^6 cells/ml in a 35 x 10 mm sterile dish plates (Nunclon) in growth medium (Dubelcco's Modified Eagle's culture medium (DMEM) supplemented with 5% fetal clone serum (Cloneteck), 1% kanamycin antibiotic (FISHER), 1% antimycotic antibiotic (SIGMA), 5mM HEPES (SIGMA) and 500μg/ml Geneticin (G418, Life Technologies, Inc.) and incubated for 24 hrs. The next day, the medium was changed to 1:1 DMEM:Waymouth medium without serum and incubated 18 to 24 hrs before the experiment. On the day of the experiment, the cells were exposed to a glucose-oxygen free DMEM medium and placed to an anaerobic chamber without oxygen and gassed with N_2:CO_2 (19:1 or 95%:5%) for the indicated time. After the time of incubation, the

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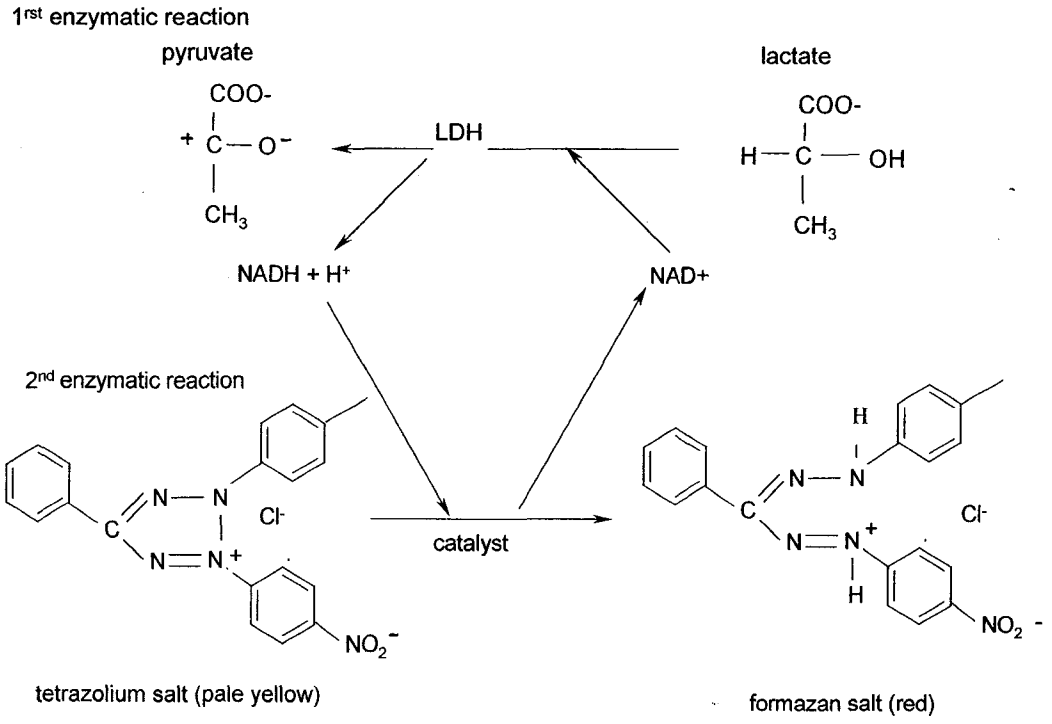
cells were incubated with UTP and test agents at specific concentration and time of incubation.

III. Lactate dehydrogenase (LDH) Assay



The $P2Y_2$-1321N1 astrocytoma cells in the exponential phase of growth were seeded at a concentration of 1.0×10^5 cells/ml in a 96 well sterile plate (Nunclon) in the growth medium Dubelcco's Modified Eagle's culture medium (DMEM) supplemented with 5% fetal clone serum (Cloneteck), 1% kanamycin antibiotic (FISHER), 1% antimycotic antibiotic (SIGMA), 5mM HEPES (SIGMA) and 500μg/ml Geneticin (G418, Life Technologies, Inc.) and incubated for 24 hrs. The next day, the medium was changed to 1:1 DMEM:Waymouth medium without serum and incubated 18 to 24 hrs before the experiment. On the day of the experiment, the medium was change to Dubelcco's Modified Eagle's culture medium (DMEM) without glucose and phenol red. The cells were exposed to a glucose-oxygen free DMEM medium and placed to an anaerobic chamber without oxygen and gassed with N_2:CO_2 (19:1) for the indicated times. After complete the time, the cells were incubated with UTP at specific concentration and time

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of incubation. After the treatment of ischemia, the supernatant (100µl) was collected and LDH assay was performed as manufacturer's instructions. (Roche Molecular-BoehringerMannheim). The absorbance of the samples was performed in a Microplate Reader at a maximal wavelength λ of 490 nm. The results were reported as % of citotoxicity using the low control (cells without OGD treatment) and high control (cells disrupted with triton x-100 representing total LDH in the cells) and using the following question.

$$\% \text{ of cytotoxicity} = \frac{\text{experimental value} - \text{low control}}{\text{high control} - \text{low control}}$$

IV. Protein Kinases Phosphorylation Assays

Protein Kinases activation assays in $P2Y_2$-1321N1 astrocytoma cells were performed following methods in our published article in Santiago-Pérez, L.I. et.al. [167]. The $P2Y_2$-1321N1 astrocytoma cells in the exponential phase of growth were seeded at a concentration of 1.0 x 10^6 cells/ml in a 35 x 10 mm sterile dish plates (Nunclon) in growth medium Dubelcco's Modified Eagle's culture medium (DMEM) supplemented with 5% fetal clone serum (Cloneteck), 1% kanamycin antibiotic (FISHER), 1% antimycotic antibiotic (SIGMA), 5mM HEPES (SIGMA) and 500µg/ml Geneticin (G418, Life Technologies, Inc.) and incubated for 24 hrs. The next day, the medium was changed to 1:1 DMEM:Waymouth medium without serum and incubated 18 to 24 hrs before the experiment. On the day of the experiment, the cells were incubated with test agents and treatment (ischemia or OGD) for the indicated times. The cells were placed on ice and the medium was suctioned and the cells were rinsed with ice-cold PBS. Then, the cells were lysed with 250 µl of ice-cold lysis buffer (25 mM Tris HCl pH 7.4, 25 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 10 mM $Na_4(P_2O_7)$, 25 mM β-glycerophosphate, 25 mM p-nitro phenylphosphate, 0.5 mM EGTA, 0.5 %(w/v) Triton x-100, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 nM okadaic acid) and centrifuged (8,243 x g 10 min, 4 °C) to remove insoluble material. Then, 100 µl of 3x SDS loading buffer (187.5 mM Tris HCl (pH 6.8 at 25°C), 6% (w/v) SDS, 150 mM DTT, (DL-dithiothreitol)

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0.3% (w/v) bromophenol blue) was added to the supernatant followed by a 5 min 96°C incubation to denature the proteins. Aliquots were subjected to 12% SDS-PAGE and after the run, the gels were incubated for 30 min in transfer buffer (48 mM Tris base pH 9.0, 39 mM glycine, 20% methanol, 0.038 % (w/v) SDS). Then, the samples in the gel were transferred to a nitrocellulose membrane using a semi-dry electroblotting transfer device (Bio-Rad). Transfer was performed at 5.5 mA/cm^2, 15 V and 1.5 hr at room temperature. Then, the membranes were incubated with 5% (w/v) blocking reagent (low fat milk) in TBS-T solution (20 mM Tris base pH 7.6, 137 mM NaCl, 1 mM HCl, 0.1% (w/v) Tween 20) for 1 hr at room temperature with gentle agitation on an orbital shaker. Successively, the membranes were incubated with one of the following specific primary antibodies in blocking buffer (5% blocking reagent in TBS-T or 5% BSA in TBS-T (bovine serum albumin) overnight at 4°C at the 1:1000 dilution:

a. rabbit anti phospho p44/42 (Thr202/Tyr204) IgG
b. rabbit anti p44/42 IgG
c. rabbit anti phospho p38 (Thr180/Tyr182) IgG
d. rabbit anti p38 IgG
e. rabbit anti phospho SAPK/JNK (Thr183/Tyr185) IgG
f. rabbit anti SAPK/JNK IgG
g. rabbit anti phospho Akt (Thr 308) IgG
h. rabbit anti phospho Akt (Ser 243) IgG
i. rabbit anti Akt IgG
j. rabbit anti phospho GSK3 (αβ) (Ser21/9)IgG
k. rabbit anti phospho IκBα (Ser32) IgG
l. rabbit anti IκBα IgG
m. rabbit anti phospho BAD (Ser112) IgG
n. rabbit anti phospho BAD (Ser136) IgG
o. rabbit anti phospho BAD IgG
p. rabbit anti phospho p70S6k (Thr389) IgG
q. rabbit anti phospho p70S6k (Thr421/Ser424) IgG
r. rabbit anti p70S6k IgG

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Then, the membranes were rinsed with TBS-T three times (1 x 15 min and 2 x 5 min) and incubated with a goat anti rabbit horseradish peroxidase (HRP) conjugated IgG as secondary antibody (at a dilution of 1:5000) and biotin conjugated to HRP (1:2000) (for biotinylated protein markers) in blocking buffer for 1 hr at room temperature. After the incubation, the membranes were rinsed with TBS-T as mentioned above. The proteins were detected using Super Signal ® West Dura Extended Duration Substrate Kit [134] following the manufacturers instructions. The chemiluminiscence in the blots was measured after direct quantitation by exposing them to an imaging screen-CH and analized using a Molecular Imager GS-525 (Bio-Rad) and the Multi Analyst software (Image Analysis Systems Version 1.0, Bio-Rad) in a Machintosh Workstation.

For reprobing of the membranes with different antibody, the membranes were treated with 50 ml stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris HCl, pH 6.7) for 30 min at 60°C. The antibodies were also stripped with a solution of Restore ™ Western Blot Stripping Buffer [134] and/or Re-Blot Plus- Strong (10x) (Chemicon International) for 10 min at room temperature (@ 25°C). Then, the membranes were washed twice with TBS-T and incubated with blocking buffer. After stripping the membranes of antibody, the probing protocol was followed without further modifications as described above.

V.Transcription factors Phosphorylation Assays

Determination of the phosphorylation of transcription factors was done by Western blot by the methodology described previously in the determination of the phosphorylation of protein kinases. The specific primary antibodies employed to detect transcription factors at a 1:1000 dilution were:

a. rabbit anti phospho Elk-1 (Ser383) IgG
b. rabbit anti Elk-1 IgG
c. rabbit anti phospho ATF-2 (Thr 69/71) IgG
d. rabbit anti ATF-2 IgG
e. rabbit anti phospho c-Jun (Ser63) IgG
f. rabbit anti phospho c-Jun (Ser73) IgG

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g. rabbit anti c-Jun IgG
h. rabbit anti phospho CREB (Ser133) IgG
i. rabbit anti CREB IgG
j. rabbit anti phospho c-Myc (Thr58/Ser52) IgG
k. rabbit anti c-Myc IgG
l. rabbit anti phospho FKHR (Ser256) IgG
m. rabbit anti FKHR IgG
n. rabbit anti phospho NF-κB p65 (Ser 536) IgG
o. rabbit anti NF-κB IgG

VI. Determination of cleaved PARP (apoptosis)

Determination of the PARP cleavage was done by Western blot by the methodology previously described in the determination of the phosphorylation of protein kinases and transcription factors. The determination was performed determining the cleaved protein and the total protein. The specific primary antibodies employed to detect PARP at a 1:1000 dilution were:

a. rabbit cleaved PARP (Asp 214) IgG
b. rabbit PARP IgG

VII.Blocking possible association of $P2Y_2$ receptor with integrins

To block the possible association of integrins with $P2Y_2$ receptor, the following procedure with some modifications was performed [130]. The $P2Y_2$-1321N1 astrocytoma cells in the exponential phase of growth were seeded at a concentration of 1.0×10^6 cells/ml in a 6 well sterile plates (Nunclon) in growth medium Dubelcco's Modified Eagle's culture medium (DMEM) supplemented with 5% fetal clone serum (Cloneteck), 1% kanamycin antibiotic (FISHER), 1% antimycotic antibiotic (SIGMA), 5mM HEPES (SIGMA) and 500 μg/ml Geneticin (G418, Life Technologies, Inc.) and incubated for 2 hrs. Then, after the cells were adherent, the medium was removed and changed carefully to DMEM with 0.5% BSA, 1% kanamicin and 1% antimycotic antibiotic overnight (18

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hrs) and incubated with the following integrins antibodies (Santa Cruz Biotechnology) at a 10 μg/ml concentration:

a. anti mouse monoclonal integrin αvβ3 (23C6) IgG_1 Ab 10μg/ml
b. anti goat polyclonal integrin αv (N-19) IgG Ab 10μg/ml

Then, the Protein Kinase Phosphorylation Assay was performed.

VIII.Materials

The nucleotides were obtained from SIGMA. Primary antibodies were obtained from Cell Signaling and secondary antibodies from Pierce for western blotting and other antibodies were obtained from Santa Cruz Biothechnology. Inhibitors, antagonists and ecto-ATPase were obtained from SIGMA, Calbiochem and Chemicon International. Other reagents and biochemicals were obtained from Fisher and SIGMA.

IX. Statistical Analysis

All data are presented as the mean ± SEM using Graph Pad Prism (version 3.03) software. Statistical analysis was calculated by One-way ANOVA followed by Tukey (compare all pairs of columns) post test using Graph Pad InSTAT (version 3.0) software. Values of $P<0.05$, were considered statistically significant.

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CHAPTER III

RESULTS

To better understand the role of extracellular nucleotides and $P2Y_2$ receptor in a cerebral ischemia episode, 1321N1 human astrocytoma cells expressing the human $P2Y_2$ receptor were used as a study model. This cellular model was also used to understand the functional role of two structural motifs (amino acid sequences) at the extracellular and intracellular domain in the human $P2Y_2$ receptor in mediating the signal transduction exerted by this receptor. Several reports have demonstrated that extracellular nucleotides induce phenotypic differentiation, proliferation as well as inflammatory responses through the activation of the $P2Y_2$ receptor [255], [256], [257]. Some of these responses modulated by extracellular nucleotides are through a signal transduction which include calcium mobilization as well as ERK1/2 activation [167]. Other members of the MAPK family such as p38 and JNK are activated by the $P2Y_2$ receptor to regulate proliferation [258], [259]. Another protein kinase which has been activated by extracellular nucleotides through the $P2Y_2$ receptor is Akt [260]. Akt as well as MAPKs regulate survival and proliferation signals [261]. Knowing that the family of MAPK and Akt/PKB are involved in survival signals, we decided to determine if extracellular nucleotides through the $P2Y_2$ receptor mediate a survival signal by the activation of these protein kinases and downstream substrates during a cerebral ischemia episode in 1321N1 astrocytoma $hP2Y_2$-transfected cells.

I. Characterization of the human $P2Y_2$ receptor in 1321N1 transfected cells

A. Kinetics of protein kinases

To study the activation of MAPK family and Akt that are related to survival signals, Western blot assays were performed as described in CHAPTER II. To determine if the MAPKs are activated by extracellular nucleotides in the human 1321N1 astrocytoma cells transfected with the human $P2Y_2$ receptor, time course experiments of UTP-stimulated phosphorylation or activation of ERK1/2, p38 and JNK were performed. For some assays, 100 μM UTP was used as a maximal dose as previously reported in the

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literature by us [167] and others [255]. Figure 3.1 shows the autoradiography of the blotting membrane detecting the phosphorylation of ERK1/2 in 1321N1-hP2Y_2 astrocytoma cells. Figure 3.1A shows the detection of ERK1/2 in its phosphorylated (activated) form and shows the detection of total (phosphorylated and non-phosphorylated form) ERK1/2. The detection of the phosphorylated form was performed by the use of phospho-specific ERK1/2 antibody and the total kinase was detected by reprobing the same membrane with anti-ERK1/2 antibody. As shown in Figure 3.1A, ERK1/2 display maximal phosphorylation at 5 min after addition of UTP and shows that each sample (each well) contains similar amount of kinase (detecting with antibody against ERK1/2). The autoradiographies were digitized and analyzed as described in CHAPTER II. A graphical representation shown in figure 3.2A presents the time course of ERK1/2 phosphorylation by UTP through the activation of the P2Y_2 receptor and demonstrates its transient activation that reaches maximal values at 5 min.

To determine if UTP through the P2Y_2 receptor activates other members of the MAPK family, Western blots assays were performed using specific antibodies for p38 and JNK. Figure 3.1B shows the detection of p38 activation by 100 μM UTP (figure 3.1B shows the p38 phosphorylated form and p38 total kinase). The time of maximal phosphorylation of p38 was 5 min. Normalization of the phospho p38 by the total p38 content revealed that the activation of p38 is also transient as observed for ERK1/2 with a maximal activation at 5 min (see figure 3.2B). Figure 3.1C shows the detection of p54 JNK and p46JNK activation by 100 μUTP (figure 3.1C shows the JNK phosphorylated and JNK total kinase respectively). Although the activation of p54JNK and p46JNK is transcient, the maximal activation was more delayed, at 25 min as shown in figure 3.2C.

Recently, it was demonstrated that PKB/Akt is activated by extracellular nucleotides through the P2Y_2 receptor in renal messangial cells [260]. Knowing this information and that PKB/Akt modulates protection or survival signals, a time course with 100μM UTP was performed detecting the phosphorylated and total PKB/Akt protein in hP2Y_2-1321N1 transfected astrocytoma cells. Figure 3.3 shows that PKB/Akt

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Figure 3.1 Time course of UTP-stimulated protein kinase phosphorylation in 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected were treated with 100 μM UTP for the indicated period of time. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using (A) anti phospho specific ERK1/2 antibody and blots were stripped and reprobed with anti ERK1/2 antibody to confirm uniform loading, (B) anti phospho specific p38 antibody and blots were stripped and reprobed with anti p38 antibody and (C) anti phospho specific JNK (p54/p46) antibody and blots were stripped and reprobed with anti JNK antibody (p54/p46). Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 4 independent experiments.

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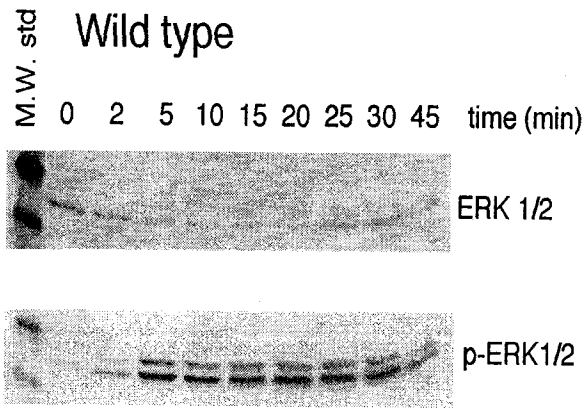
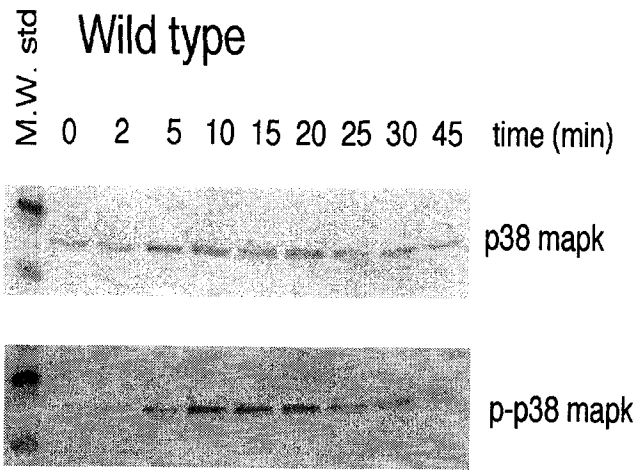
(A)



(B)



(C)

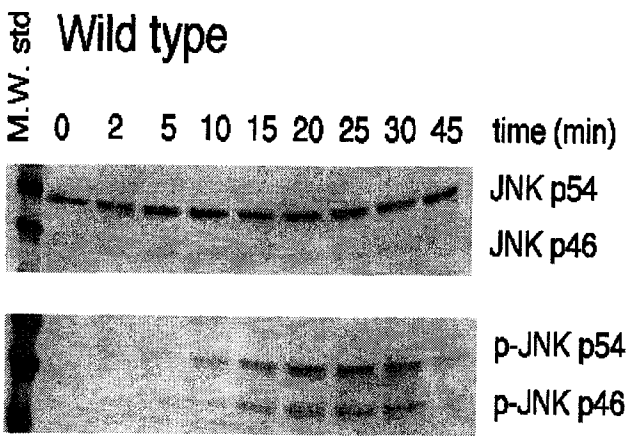


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Figure 3.2. Activation of ERK1/2, p38 and JNK by UTP in 1321N1-hP2Y_2 transfected cells. Data from figure 3.1 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of ERK1/2, p38 and JNK were calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 4 independent experiments.

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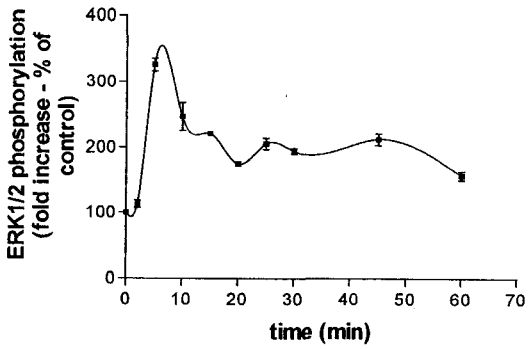
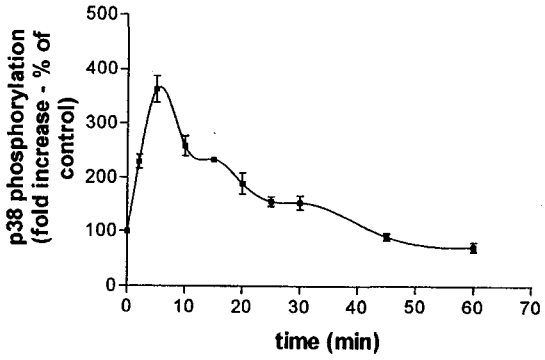
(A)



(B)



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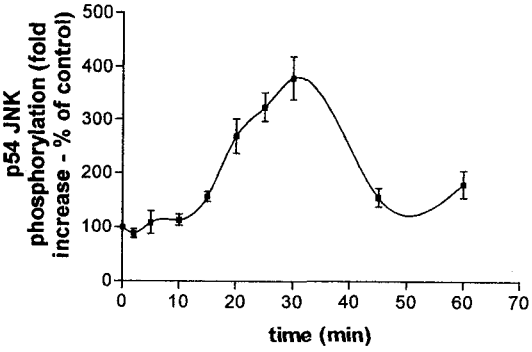

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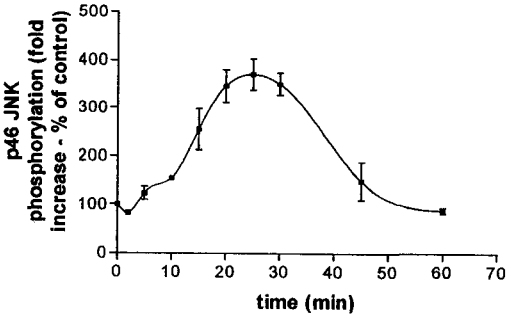

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Figure 3.3 Time course of UTP-stimulated Akt phosphorylation in 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected were treated with 100 μM UTP for the indicated period of time. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western blot analysis was performed using anti phospho specific Akt antibody and blots were stripped and reprobed with anti Akt antibody to confirm uniform loading. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 4 independent experiments.

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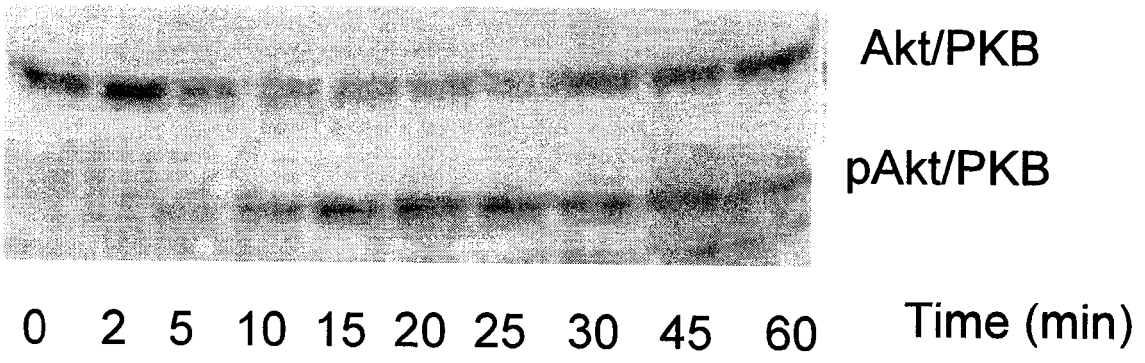

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Figure 3.4. Activation of Akt by UTP in 1321N1-hP2Y_2 transfected cells. Data from figure 3.3 were digitized and quantitated using Molecular Imager (Bio Rad). The phosphorylation of Akt was calculated by normalizing the contents of phosphorylated Akt with the content of total (phosphorylated and nonphosphorylated) Akt. Data are representative as the % of control untreated cells and are the mean +/- SEM from at least 4 independent experiments.

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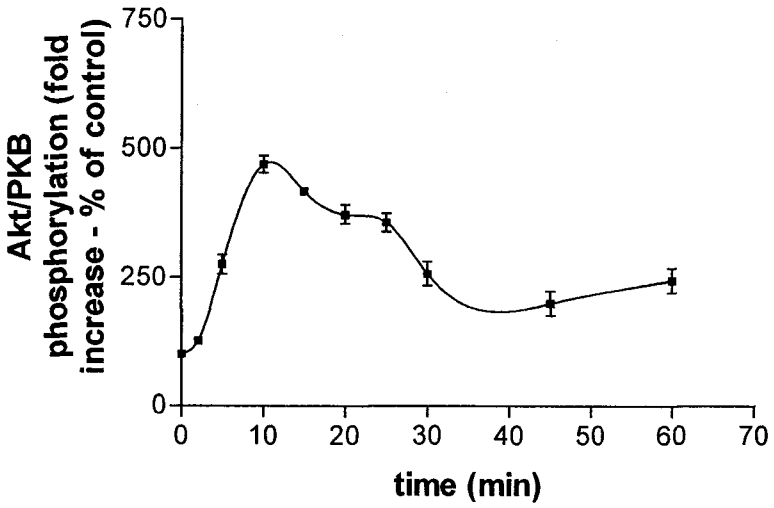

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phosphorylation is also transcient but more prolonged than that for the MAPK family. Figure 3.4 shows the graphical representation of PKB/Akt activation with a maximal time at 20 min. This information suggests the $P2Y_2$ receptor induce a survival signal through a route which includes Akt/PKB and MAPK family in $hP2Y_2$-1321N1 astrocytoma transfected cells.

B. Kinetics of transcription factors

Cellular proliferation, survival and anti-apoptotic as well as death signals are known to be regulated by the phosphorylation of transcription factors. To determine which transcription factors are phosphorylated by the signal transduction that UTP activates, a series of time couse experiments of UTP-stimulated phosphorylation of transcription factors in $hP2Y_2$-1321N1 transfected cells were performed. Figure 3.5A shows the phosphorylated ATF-2 protein activated by 100 μM UTP. Figure 3.5A and 3.6A show that ATF-2 is phosphorylated transciently with a maximal time of activation at 15 min. Figure 3.5B and 3.5C show the phosphorylation of c-Jun at Ser 63 and Ser 73 induced by 100 μM UTP. Figure 3.6B and 3.6C show that the phosphorylation of c-Jun is also transcient with a maximal peak after 15 min. Figure 3.5D and 3.6D show the phosphorylation of c-Myc which is also show a maximal time of activation at the same of time of ATF-2 and c-Jun, 15 min. With respect to CREB and ATF1, both are activated by UTP with a maximal value of 15 min as shown in figure 3.5E and 3.6E. The FKHR transcription factor whose phosphorylation is mediated by Akt/PKB, is also phosphorylated after $P2Y_2$ receptor activation, although with a different time course. FKHR as shown in figure 3.5F and 3.6F has two peaks of phosphorylation, one at 15 min and the other time at 1hr. The phosphorylation induced by the signal transduction activated by the $P2Y_2$ receptor suggest that the $P2Y_2$ receptor could be modulating signals related to death, anti-apoptotic or survival by the phosphorylation of these transcription factors.

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Figure 3.5. Time course of UTP-stimulated transcription factors phosphorylation in 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected cells were treated for the indicated period of time. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using (A) anti phospho specific ATF-2 Thr69/71 antibody, (B) anti phospho specific c-Jun Ser63 and anti phospho specific c-Jun Ser73 antibodies, (C) anti phospho specific c-Myc Thr58/Ser62 antibody, (D) anti phospho specific CREB Ser 133 antibody and (E) anti phospho specific FKHR Ser 256 antibody. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 3 independent experiments.

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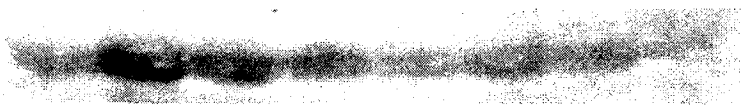
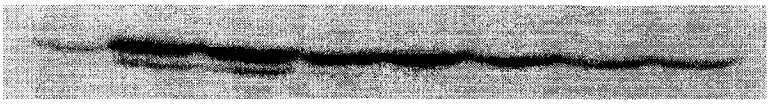
(A)



pATF2 (Thr69/71)

(B)



pcJun (Ser63)

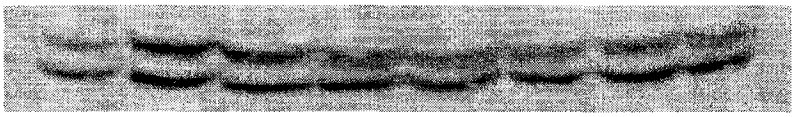
(C)

pcJun (Ser73)

(D)

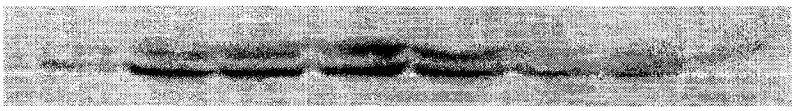
pcMyc(Thr58/Ser62)

(E)



pCREB(Ser133)
pATF1

(F)



pFKHR (Ser 256)

0 0.25 0.5 1 2 3 4 5 Time (hrs)

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Figure 3.6. Activation of transcription factors by UTP in 1321N1-hP2Y_2 transfected cells. Data from figure 3.5 were digitized using Molecular Imager Model GS-525) (Bio Rad). The phosphorylation of each transcription factor was calculated by (using the phospho) normalizing the contents of phosphorylated transcription factor with the content of the total (phosphorylated and nonphosphorylated) transcription factor. Data are expressed of the % of control untreated cells and are the mean +/- SEM from at least 3 independent experiments.

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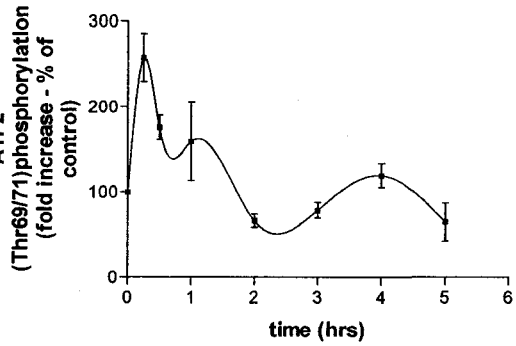

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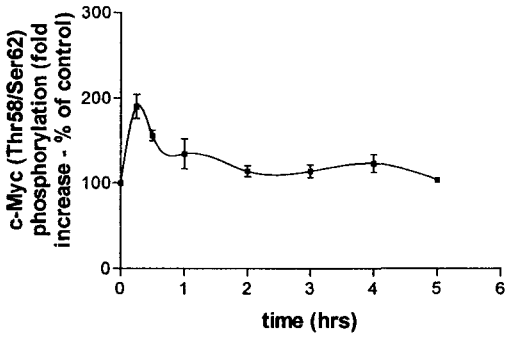

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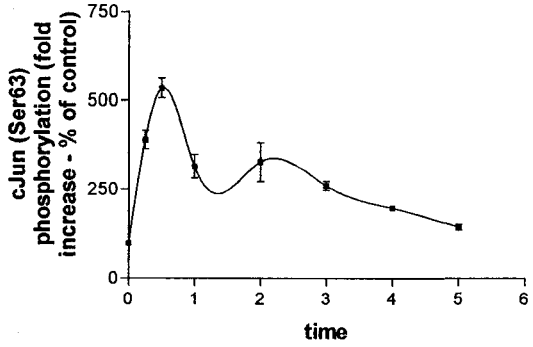

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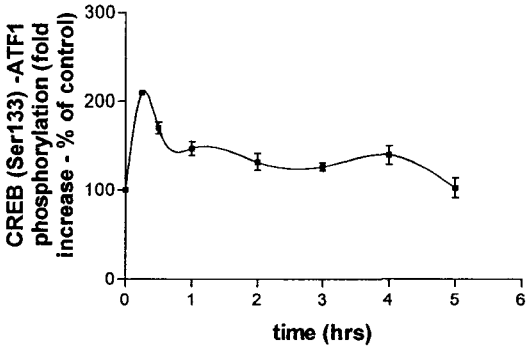

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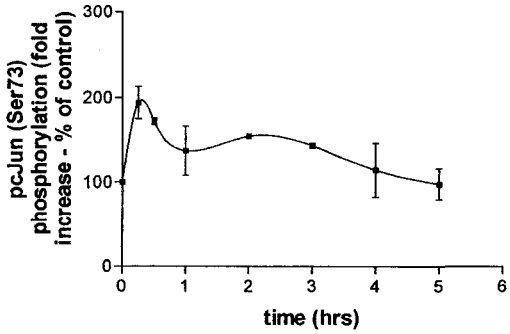

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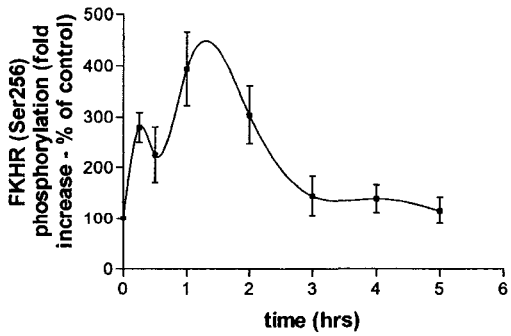

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C. Protein kinase responsible for the phosphorylation of transcription factors

Because the transcription factors studied are substrates of some of the protein kinases studied, and one protein kinase can phosphorylate more than one transcription factor as well as one transcription factor can be phosphorylated by more than one protein kinase, we decided to use specific inhibitors of these protein kinases to determine which protein kinase is responsible for their phosphorylation. According of our results, the maximal time of phosphorylation of the transcription factors studied was 15 min, and we employed this time of incubation of UTP 100μM. The cells were preincubated with 30μM PD98059 [191]; MEK1 inhibitor (ERK1/2 inhibitor pathway), 10μM SB203580; p38 inhibitor [262], 25μM JNK inhibitor II [263], 100nM wortmannin; PI3K inhibitor [246], [245], for 30 min before the stimulus with 100μM UTP for 15 min. Figure 3.7A and 3.8A show that ATF-2 was phosphorylated by the incubation with UTP and its phosphorylation was inhibited by the JNK inhibitor. SB 203580 partially inhibited the phosphorylation of ATF-2. However, the phosphorylation was not significantly inhibited by wortmannin as demonstrated by the ANOVA analysis. These results suggest that JNK is the principal kinase responsible for the phosphorylation of ATF-2 but p38 could also be modulating this phosphorylation during this period of time of incubation with UTP. Figure 3.7B, 3.7C, 3.8B and 3.8C show the phosphorylation of c-Jun at Ser 63 and Ser 73 by the incubation with UTP. The phosphorylation of c-Jun was inhibited principally by the JNK inhibitor. None of the other protein kinase inhibitors affected the phosphorylation of c-Jun induced by $P2Y_2$ receptor activation.

Another transcription factor studied was c-Myc. However, the phosphorylation of c-Myc was inhibited by PD98059 indicating that ERK1/2 is the protein kinase responsible of its phosphorylation and confirming that c-Myc is a main substrate of ERK1/2 [194] (figure 3.7D and 3.8D). The other protein kinase inhibitors did not inhibit the phosphorylaltion of c-Myc induced by the $P2Y_2$ receptor. Other transcription factor that could be modulated by ERK1/2 is CREB. UTP induced the phosphorylation of CREB and ATF1. However, none of the protein kinase inhibitors employed completely inhibited their phosphorylation induced by the $P2Y_2$ receptor as shown in figure 3.7E,

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3.8E and 3.8F. These results suggest that other protein kinases modulate the phosphorylation of these transcription factors or a combination of several of these protein kinases are responsible of the regulation of CREB and ATF1 phosphorylation induced by UTP. Contrary to the response observed with the other transcription factors, FKHR and AFX remains phosphorylated and with the incubation of UTP for 15 min a little increase in their phosphorylation was observed. This result is indicative that the cells have a survival response, because if the cells were in apoptosis, the protein would not be phosphorylated and the phosphorylated protein acts as an anti-apoptotic signal [264]. As expected, the phosphorylation of FKHR was only inhibited by wortmannin which is indicative of the PI3K-PKB-FKHR pathway [236] (figure 3.7F, 3.8G and 3.8H).

II. Structural determinants of the human $P2Y_2$ receptor

A. RGD sequence

The amino acid sequence of the $P2Y_2$ receptor indicates the presence of an arginine-glycine-aspartic acid (RGD) integrin binding motif that is localized at the first extracellular loop of this G protein coupled receptor (see figure 1.1). The RGD motif is a sequence present in integrins ligands such as vitronectin, fibrinogen, collagen, fibronectin, osteopontin, von Willebrand factor, thrombospondin, laminin, entactin, tenascin, bone sialoprotein and other extracellular matrix proteins that bind the integrins to modulate cell adhesion and cell shape changes involved in cell spreading and locomotion [112, 113]. To investigate the role of the RGD motif in the $P2Y_2$ receptor and its influence in the signal transduction that activates UTP through $P2Y_2$ receptor, a dose-dependency of UTP-induce MAPK phosphorylation were performed using wild type $P2Y_2$ and RGE-$P2Y_2$ receptor mutant where the aspartic acid was changed by a glutamic acid (see figure 1.1). The change of the aspartic acid by glutamic acid reduce the activity of the ligand in cell attachment 100-fold or more or the association or binding of the ligand (the receptor $P2Y_2$) with the integrin that recognize this type of ligands [265]. The aspartic acid may be important because its potential to contribute to divalent cation binding [266]. Studies performed with our collaborators indicate the presence of several integrins that recognize the RGD sequence and that colocolize with the $P2Y_2$ receptor [130].

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Figure 3.7. Effect of specific protein kinase inhibitors on transcription factor phosphorylation by UTP in 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected cells were pretreated with 30μM PD98059 (PD), 10 μM SB203580 (SB), 25 μM JNK inhibitor II (JI), and 100 nM wortmannin (W), for 30 min. Then, the cells were stimulated with 100 μM UTP for 15 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using (A) anti phospho specific ATF-2 Thr69/71 antibody, (B) anti phospho specific c-Jun Ser63 antibody and (C) anti phospho specific Ser73 antibody, (D) anti phospho specific c-Myc Thr58/Ser62 antibody, (E) anti phospho specific CREB Ser133 antibody and (F) anti phospho specific FKHR Ser256 antibody. Blots were stripped and reprobed with anti each protein antibody. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 3 independent experiments.

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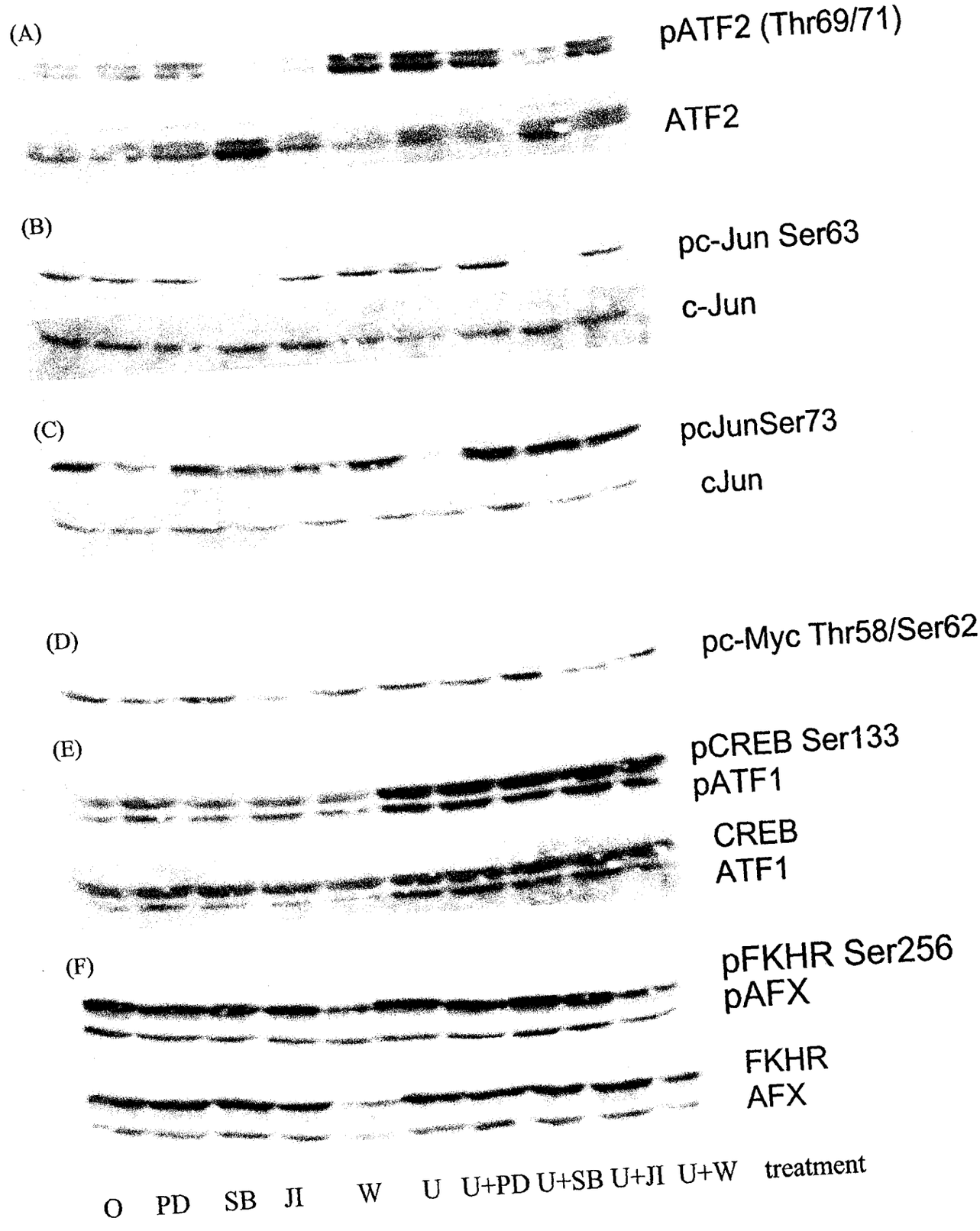

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Figure 3.8. Effects of various specific protein kinase inhibitors on the UTP-induced phosphorylation of transcription factors. 1321N1-hP2Y_2 transfected cells were pretreated with 30μM PD98059 (PD), 10 μM SB203580 (SB), 25 μM JNK inhibitor II (JI), and 100 nM wortmannin (W), for 30 min. Then, the cells were stimulated with 100 μM UTP for 15 min. Data from figure 3.7 were digitized and quantitated Molecular Imager Model GS-525) (Bio Rad). The phosphorylation of each transcription factor was calculated by (using the phospho) normalizing the contents of phosphorylated transcription factor with the content of the total (phosphorylated and nonphosphorylated) transcription factor. Data are expressed of the % of control untreated cells and are the mean +/- SEM from at least 3 independent experiments. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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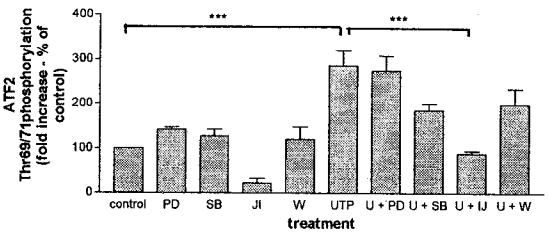

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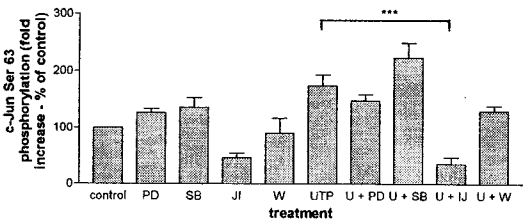

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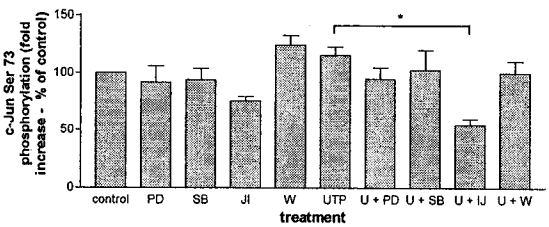

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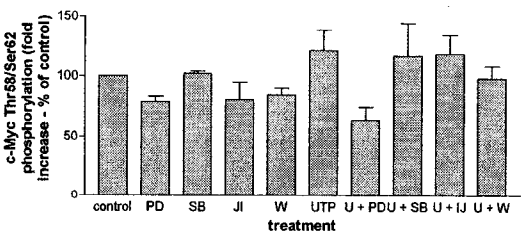

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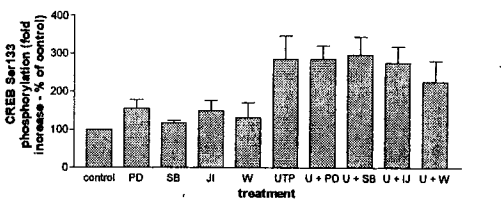

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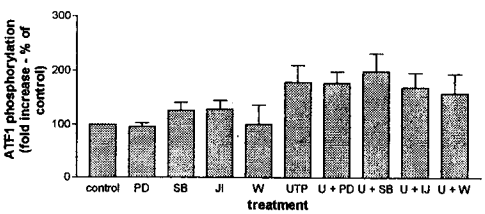

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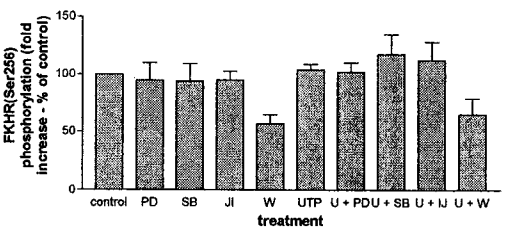

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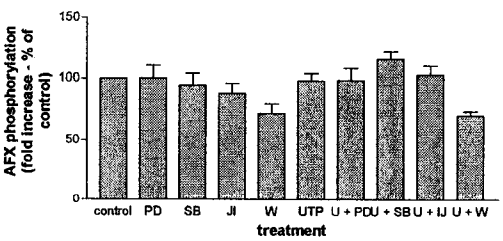

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1. Dose response

To determine the role of the RGD motif in the $P2Y_2$ receptor and its influence in the signal transduction that activates UTP through its receptor, a dose-dependency of UTP-induced MAPK phosphorylation were performed using wild type $P2Y_2$ and RGE-$P2Y_2$ receptor mutant. As we can see, a partial decrease in the phosphorylation of ERK1/2 was observed in the RGE mutant receptor although a similar dose dependency remained. As shown in figure 3.9 and 3.10, ERK1/2 phosphorylation/activation by wild type $P2Y_2$ receptor was dependent on the UTP concentration with an EC_{50} of 0.21 to 1.9μM {(log EC_{50}) = -6.678 to -5.712}. ERK1/2 activation by the RGE-$P2Y_2$ mutant was also dependent of UTP concentration, but with an EC_{50} of 0.37 to 17μM {(log EC_{50}) = -6.432 to -4.766}. These results suggest that $P2Y_2$ receptor signaling transduction induced by UTP is partially dependent on the interaction or coupling with the integrins by the RGD motif.

Because ERK1/2 activation is partially dependent of the RGD motif of the $P2Y_2$ receptor, the same experiment was performed to determine the dependency of the other protein kinase activation. The same dose dependence or response is observed for p38 activation with an EC_{50} of 0.45μM {(logEC_{50}) = -6.349} by the wild type $P2Y_2$ and an EC_{50} of 393.6μM {(logEC_{50}) = -3.405} by the the RGE-$P2Y_2$ mutant (figure 3.11 and 3.12). This result suggests that p38 activation is also dependent on the RGD motif of the $P2Y_2$ receptor and its coupling with the integrins. With respect of JNK activation, the response was similar, which is dependent of the coupling of the integrins by the RGD motif. The activation of JNK isoforms by the wild type $P2Y_2$ receptor has an EC_{50} of 0.367μM of p54JNK {(log EC_{50}) = -6.435} and 247.8 μM of p46JNK {(log EC_{50}) = -3.606}. For the RGE-$P2Y_2$ mutant, p54JNK activation decreased and the EC_{50} of 0.158 μM of p54JNK {(log EC_{50}) = -6.799}, but for the p46JNK was 54.0 μM and {(log EC_{50}) = -4.267} (figure 3.13 and 3.14).

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Figure 3.9. Dose response of UTP-stimulated ERK1/2 phosphorylated in wild type 1321N1-hP2Y_2 and RGE-mutant 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected cells were treated with the indicated concentrations of UTP for 5 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using anti phospho specific ERK1/2 antibody and blots were stripped and reprobed with anti ERK1/2 antibody to confirm uniform loading. (A) wild type 1321N1-hP2Y_2 and (B) RGE-mutant 1321N1-hP2Y_2. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 4 independent experiments.

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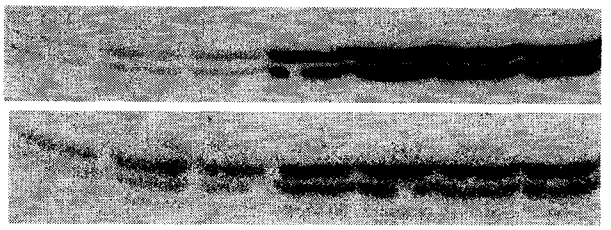
(A)

Wild type



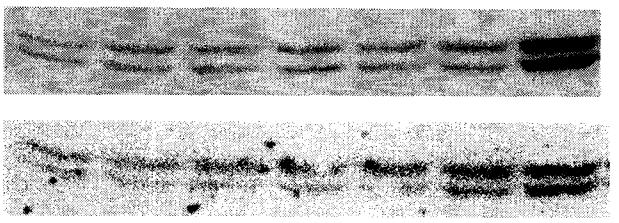
pERK 1/2

ERK1/2

0 0.001 0.01 0.1 1 10 100 [UTP] (μM)

(B)

RGE-mutant



pERK 1/2

ERK1/2

0 0.001 0.01 0.1 1 10 100 [UTP] (μM)

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Figure 3.10. Dose dependency of UTP-mediated ERK1/2 activation in wild type 1321N1-hP2Y_2 and RGE-mutant 1321N1-hP2Y_2 transfected cells. Data from figure 3.9 were digitized and quantitated using Molecular Imager Model GS-525) (Bio Rad). The phosphorylation of ERK1/2 was calculated by normalizing the contents of phosphorylated ERK1/2 with the content of total (phosphorylated and nonphosphorylated) ERK1/2. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 4 independent experiments, EC_{50} of 0.21 to 1.9μM {(log EC_{50}) = -6.678 to -5.712} for the wild type and EC_{50} of 0.37 to 17μM {(log EC_{50}) = -6.432 to -4.766} for the RGE mutant receptor.

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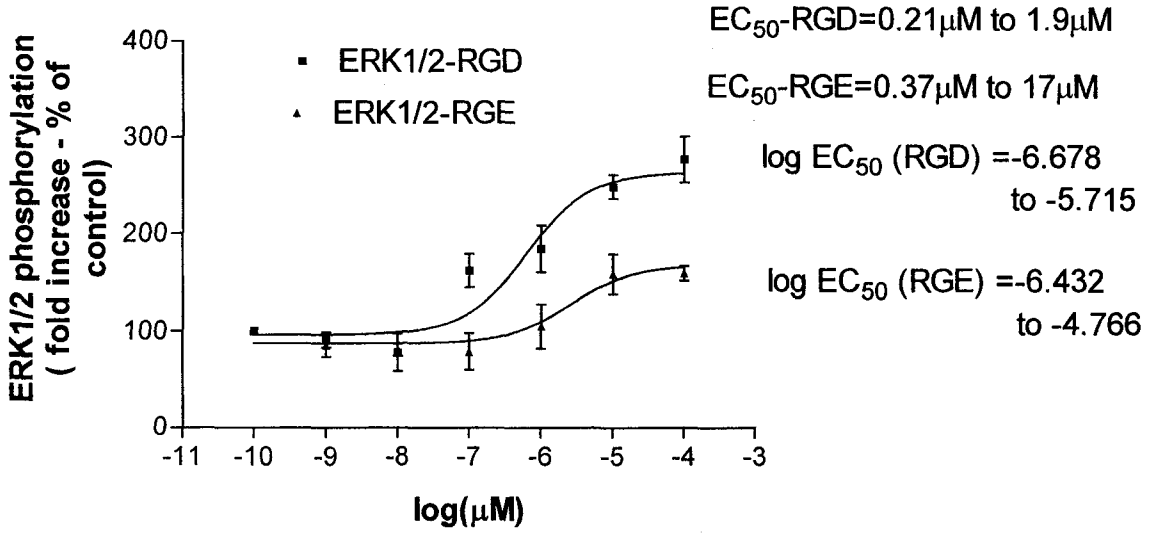

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Figure 3.11. Dose response of UTP-stimulated p38 phosphorylated in wild type 1321N1-hP2Y_2 and RGE-mutant 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected cells were treated with the indicated concentrations of UTP for 5 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using anti phospho specific p38 antibody and blots were stripped and reprobed with anti p38 antibody to confirm uniform loading. (A) wild type 1321N1-hP2Y_2 and (B) RGE-mutant 1321N1-hP2Y_2. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 4 independent experiments.

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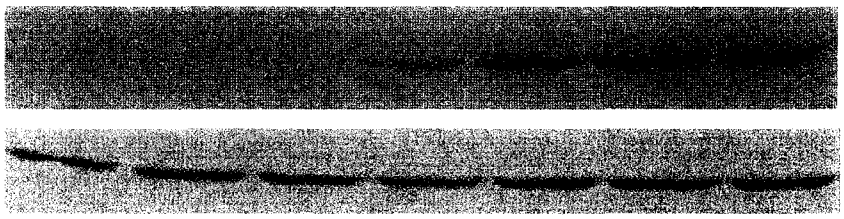
(A)

Wild type



(B)

RGE-mutant

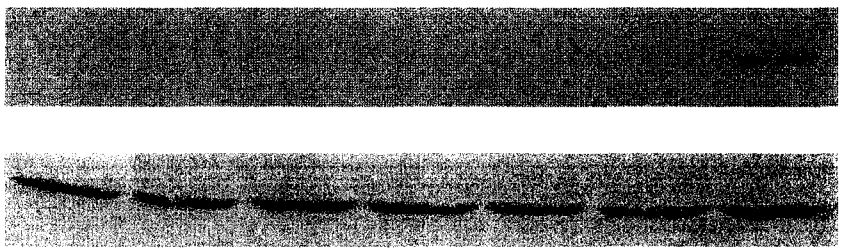


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Figure 3.12. Dose dependency of UTP-mediated p38 activation in wild type 1321N1-hP2Y_2 and RGE-mutant 1321N1-hP2Y_2 transfected cells. Data from figure 3.11 were digitized and quantitated using Molecular Imager Model GS-525) (Bio Rad). The phosphorylation of p38 was calculated by normalizing the contents of phosphorylated p38 with the content of total (phosphorylated and nonphosphorylated) p38. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 4 independent experiments, EC_{50} of 0.45 μM {(log EC_{50}) = -6.349} for the wild type and EC_{50}= 393.6 μM of {(log EC_{50}) = -3.405} for the RGE mutant receptor.

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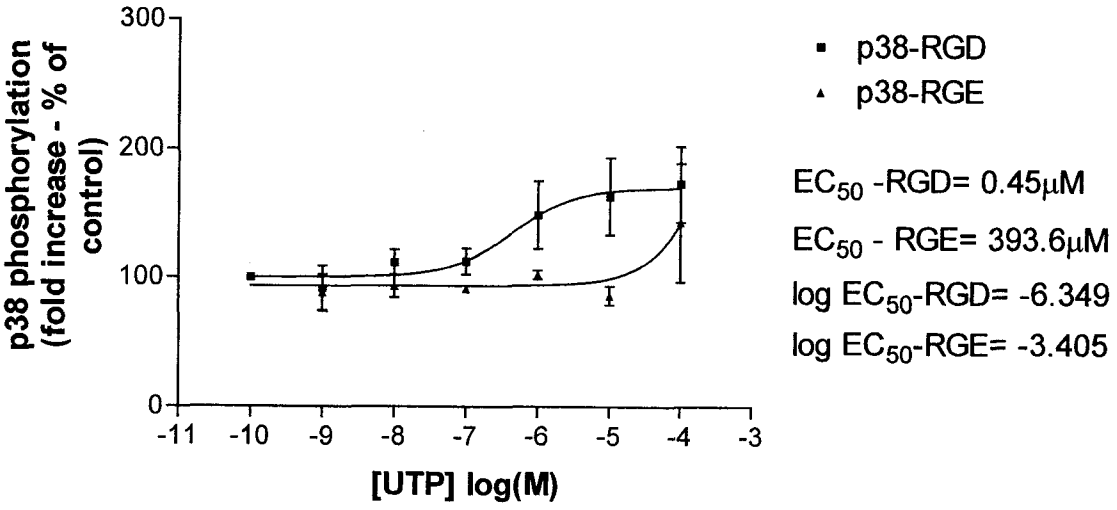

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Figure 3.13. Dose response of UTP-stimulated JNK phosphorylated in wild type 1321N1-hP2Y_2 and RGE-mutant 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected cells were treated with the indicated concentrations of UTP for 25 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using anti phospho specific JNK antibody and blots were stripped and reprobed with anti JNK antibody to confirm uniform loading. (A) wild type 1321N1-hP2Y_2 and (B) RGE-mutant 1321N1-hP2Y_2. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 4 independent experiments.

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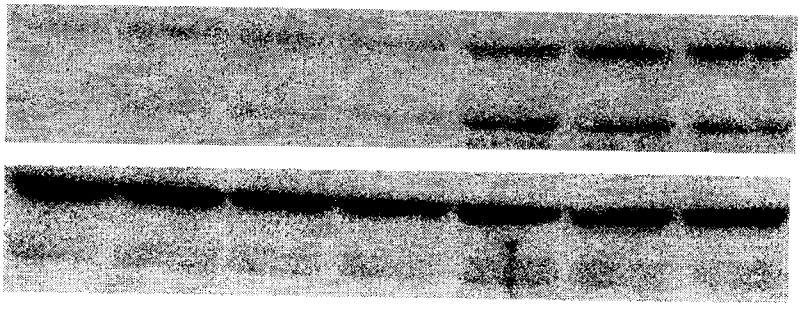
(A)

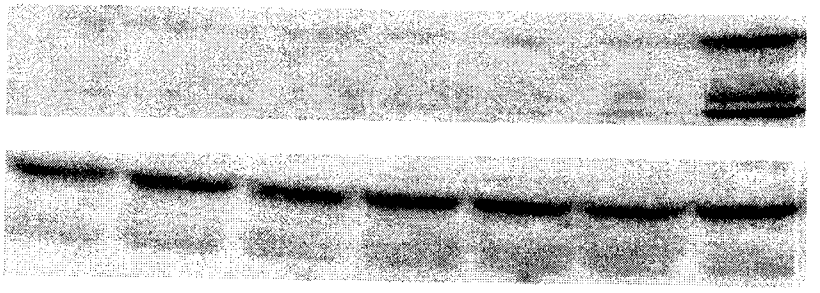
Wild type



0 0.001 0.01 0.1 1 10 100 [UTP] (μM)

(B)

RGE-mutant



0 0.001 0.01 0.1 1 10 100 [UTP] (μM)

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Figure 3.14 Dose dependency of UTP-mediated JNK activation in wild type 1321N1-hP2Y_2 and RGE-mutant 1321N1-hP2Y_2 transfected cells. Data from figure 3.13 were digitized and quantitated using Molecular Imager Model GS-525) (Bio Rad). The phosphorylation of JNK was calculated by normalizing the contents of phosphorylated JNK with the content of total (phosphorylated and nonphosphorylated) JNK. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 4 independent experiments, EC_{50} of 0.367μM of p54JNK {(log EC_{50}) = -6.435} and 247.8 μM of p46JNK {(log EC_{50}) = -3.606} for the wild type and EC_{50} of 0.158 μM of p54JNK {(log EC_{50}) = -6.799}, but for the p46JNK was 54.0 μM and {(log EC_{50}) = -4.267} for the RGE mutant receptor. (A) p54JNK and (B) p46JNK.

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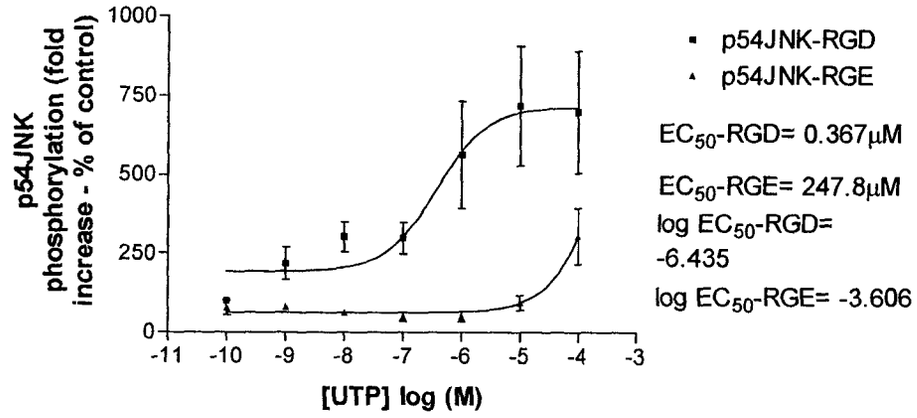
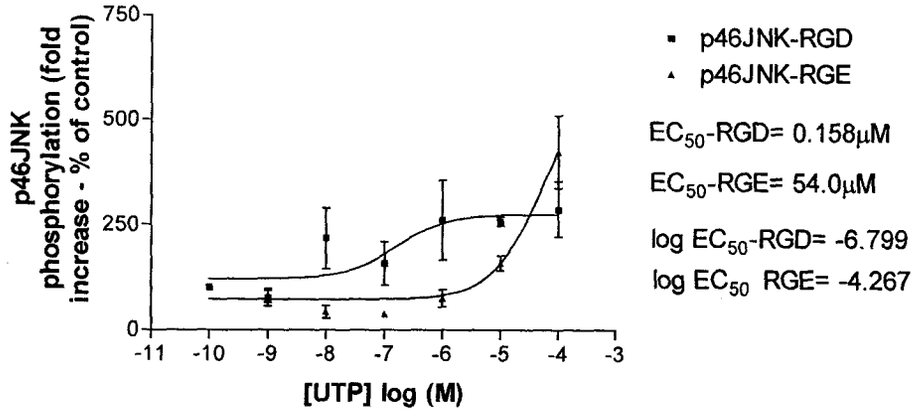
(A)



(B)



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There are several reports that demonstrate that Akt is activated by integrins to induce a survival signal in neurons [267]. As shown in figure 3.15 and 3.16. Akt/PKB activation was dose dependent with an EC_{50} = 0.405 μM {(log EC_{50}) =-6.392} for the wild type receptor and for the RGE-mutant an EC_{50}=6030 μM {(log EC_{50}) = -2.219, which indicates also that Akt/PKB activation was dependent on the RGD motif of the $P2Y_2$ receptor and its coupling with integrins.

2. Blocking interaction of $P2Y_2$ receptor with integrins using with anti integrins Ab

To investigate the possibility of an interaction of the $P2Y_2$ receptor with integrins receptors which recognize ligands with RGD sequence such as: $\alpha_5\beta1$, $\alpha_v\beta_5$, $\alpha_v\beta_3$ and $\alpha_v\beta_6$ among others, incubations with some integrins antibodies to block this possible interaction between the $P2Y_2$ receptor and the integrin were performed. Our collaborators in Missouri and us demonstrated the colocalization of $\alpha_v\beta_3$ intergrin with the $P2Y_2$ receptor [130]. Knowing this information, we decided to use α_v and $\alpha_v\beta_3$ integrins antibodies to determine its influence in the protein kinase phosphorylation mediated by the $P2Y_2$ receptor. Figures 3.17 and 3.18 show that incubation with α_v and $\alpha_v\beta_3$ antibodies inhibited almost totally the phosphorylation of ERK1/2, p38 and Akt induced by UTP through the $P2Y_2$ receptor. This inhibition was very significant as demonstrated by the ANOVA analysis. However, JNK phosphorylation induced by UTP was slightly decreased, but not totally with the presence of anti-integrins antibodies. The inhibition was significant, but not as observed with the other protein kinases. This information suggests that the binding of integrins antibodies block $P2Y_2$ signaling.

B. Proline rich sequence

Knowing that the human $P2Y_2$ receptor contains a proline rich sequence characteristic of SH3 binding sequence, we decided to study if this intracellular sequence may be important for the signal transduction that activates MAPKs and Akt by UTP. To investigate the functional role of this sequence we used cells expressing wild type human

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Figure 3.15. Dose response of UTP-stimulated Akt phosphorylated in wild type 1321N1-hP2Y_2 and RGE-mutant 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y2 transfected cells were treated with the indicated concentrations of UTP for 25 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using anti phospho specific Akt antibody and blots were stripped and reprobed with anti Akt antibody to confirm uniform loading. (A) wild type 1321N1-hP2Y_2 and (B) RGE-mutant 1321N1-hP2Y_2. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 4 independent experiments.

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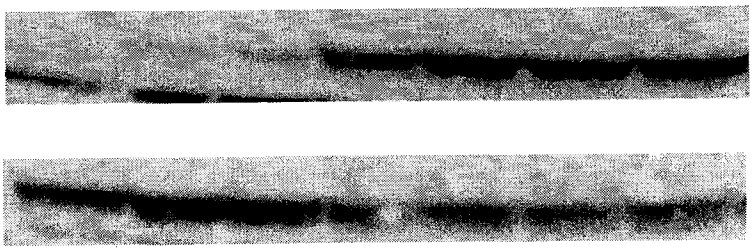
(A)

Wild type



(B)

RGE-mutant

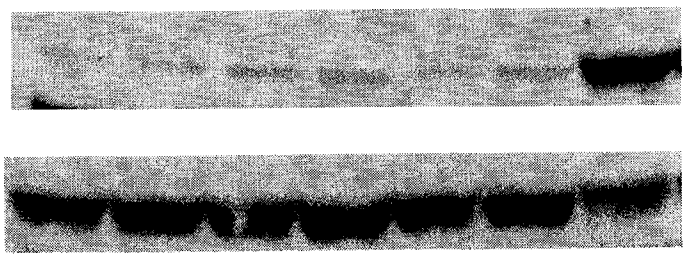


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Figure 3.16. Dose dependency of UTP-mediated Akt activation in wild type 1321N1-hP2Y_2 and RGE-mutant 1321N1-hP2Y_2 transfected cells. Data from figure 3.15 were digitized and quantitated using Molecular Imager Model GS-525) (Bio Rad). The phosphorylation of Akt was calculated by normalizing the contents of phosphorylated Akt with the content of total (phosphorylated and nonphosphorylated) Akt. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 4 independent experiments, EC_{50} = 0.405μM {(log EC_{50}) = -6.392} for the wild type receptor and for the RGE-mutant an EC_{50}=6030 μM {(log EC_{50}) = -2.219.

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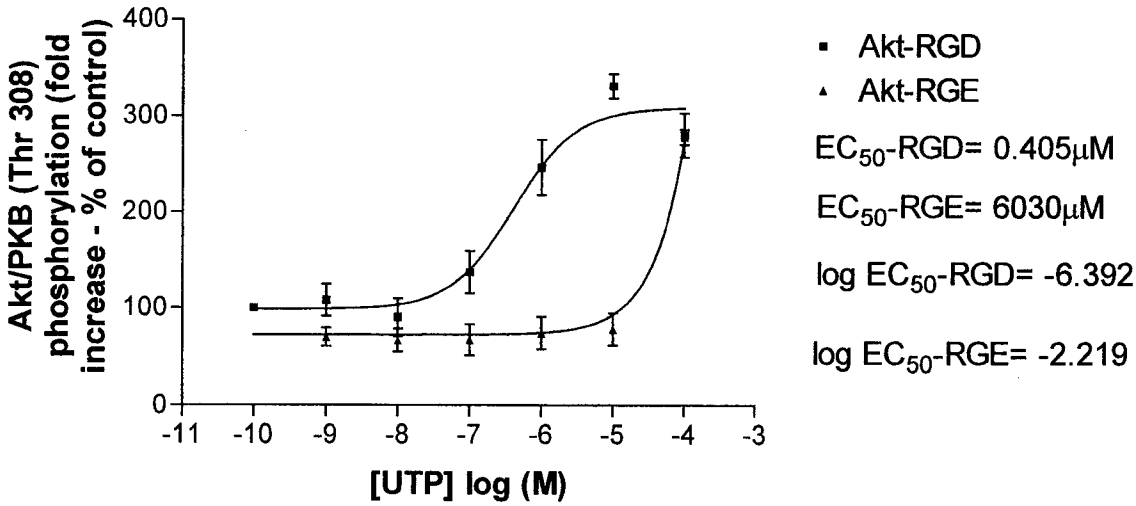

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Figure 3.17. Effect of specific anti integrins antibodies on protein kinase phosphorylation in 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected cells were pretreated with 10 μg/ml anti integrins $\alpha_v\beta_3$ and α_v for 18 hrs. Then, the cells were stimulated with 100 μM UTP for 5 and 25 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using (A) anti phospho specific ERK1/2 antibody and blots were stripped and reprobed with anti ERK1/2 antibody to confirm uniform loading, (B) anti phospho specific p38 antibody and blots were stripped and reprobed with anti p38 antibody, (C) anti phospho specific JNK (p54/p46) antibody and blots were stripped and reprobed with anti JNK antibody (p54/p46) and (D) anti phospho Akt antibody and blots were stripped with anti Akt antibody. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 4 independent experiments.

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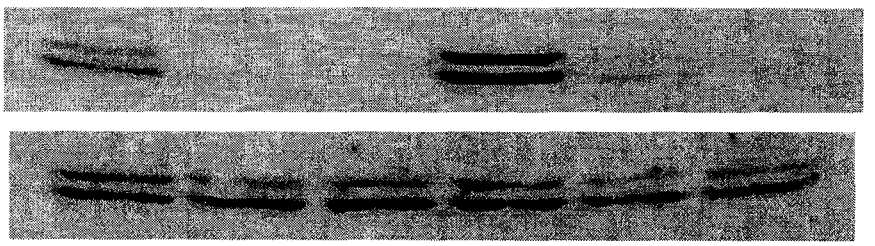
(A)



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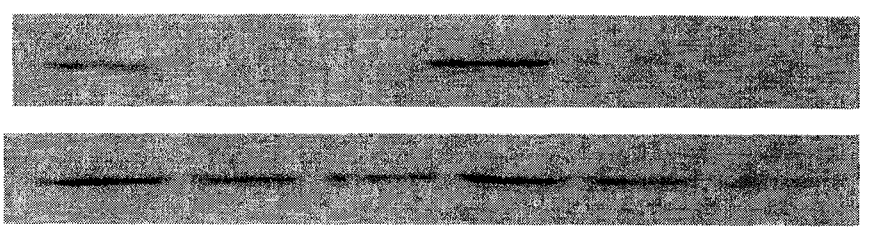

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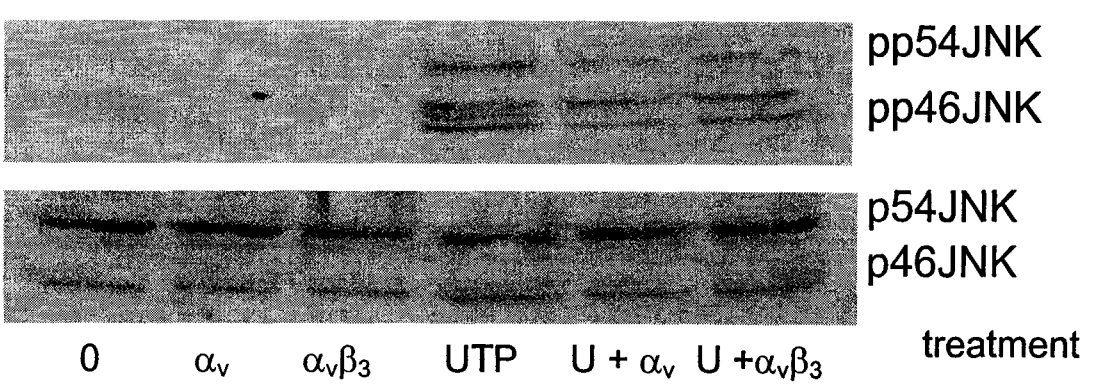

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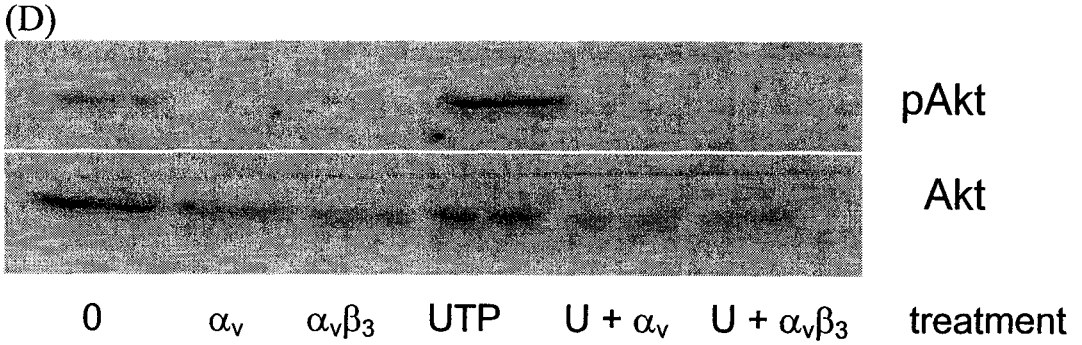

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Figure 3.18. Effects of various specific anti integrins antibodies on the UTP-induced phosphorylation of protein kinase inhibitors in 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected cells were pretreated with 10 μg/ml anti integrins $\alpha_v\beta_3$ and α_v antibodies for 18 hrs. Then, the cells were stimulated with 100 μM UTP for 5 and 25 min. Data from figure 3.17 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of ERK1/2, p38, JNK and Akt were calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 4 independent experiments. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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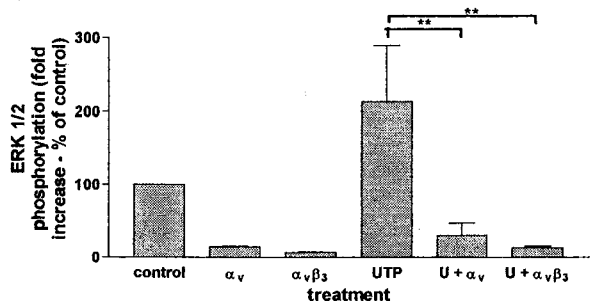
(A)



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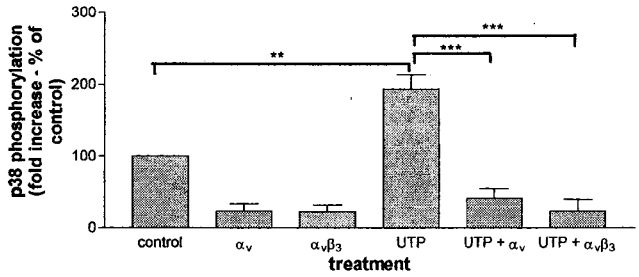

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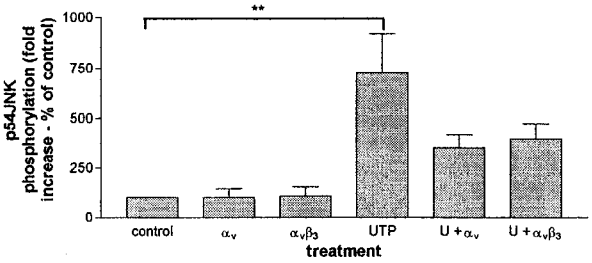

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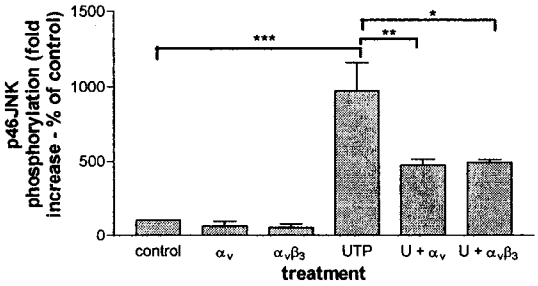

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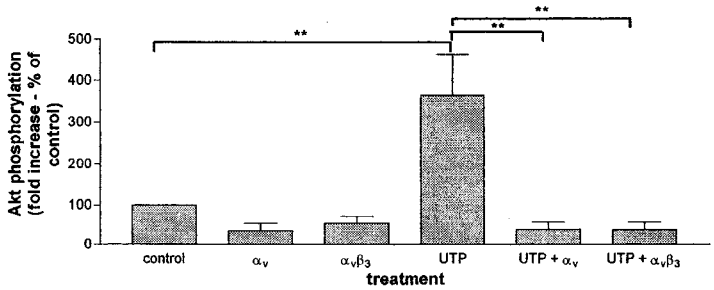

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$P2Y_2$ receptor or mutants, eliminating part of the cytoplasmic domain of the receptor but leaving the proline rich sequence (Pro+) or eliminating the cytoplasmic domain and the proline-rich sequence as well (Pro-) (see figure 1.1 and 3.19). We also used specific protein kinase inhibitors to determine the possible role of certain proteins in the activation of these protein kinases induced by UTP that may be dependent of this proline rich sequence.

1. Activation of the receptor and treatment with PP2, CYTD, AG1478

One of the proteins that regulate ERK1/2 activation is src [268]. Src is composed of six distinct functional regions in which one of them is a SH3 domain which bind short contiguous amino acid sequence rich in proline residues [269]. Since src protein kinase has a SH3 binding domain that binds to proline rich sequence, the cells were preincubated with PP2 10 μM (4-amino-5-(4chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) [270], an inhibitor of src kinase activity, for 30min before the stimulus with 100μM for 5 min. Figure 3.20A and 3.21A show that the ERK1/2 phosphorylation by the wild type receptor was inhibited by PP2. In the Pro+ mutant receptor, which eliminate part of the cytoplasmic domain but retained the proline rich sequence, ERK1/2 phosphorylation was inhibited too (figure 3.20B and 3.21B). However, the Pro- mutant receptor, which eliminates the proline rich sequence, ERK1/2 was not inhibited by PP2 (figure 3.20C and 3.21C). These results suggest that ERK1/2 activation is not mediated only by the proline rich sequence in the C-terminus of the $P2Y_2$ receptor. But also these results suggest that ERK1/2 may be activated by several different pathways which include src-dependent and –independent pathways. Although the results suggest that ERK1/2 activation is src-dependent; once this sequence is eliminated the receptor assume a src-independent pathway to activate ERK1/2. Src by its SH2 domain has been shown to mediate the activation of FAK [271] which is implicated in the assembly of focal adhesions in the intracellular cytoskeleton of the cell and with the activation of MAPK [272]. To investigate if the cytoskeleton complexes, which are related with src and FAK, regulates the activation of ERK1/2 by UTP through the $P2Y_2$ receptor, the cells were preincubated with 10 μM cytochalasin D, (which bind to the plus end of the actin filament blocking their elongation, depolymerizing the actin cytoskeleton and brake the

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Figure 3.19. Amino acid sequence structure of the wild type and mutants $P2Y_2$ receptor. Structure and amino acid sequence of the (A) wild type, (B) Pro+ -mutant, eliminating the part of the cytoplasmic domain leaving the proline rich sequence and (C) Pro- -mutant eliminating the proline rich sequence in the $hP2Y_2$ receptor.

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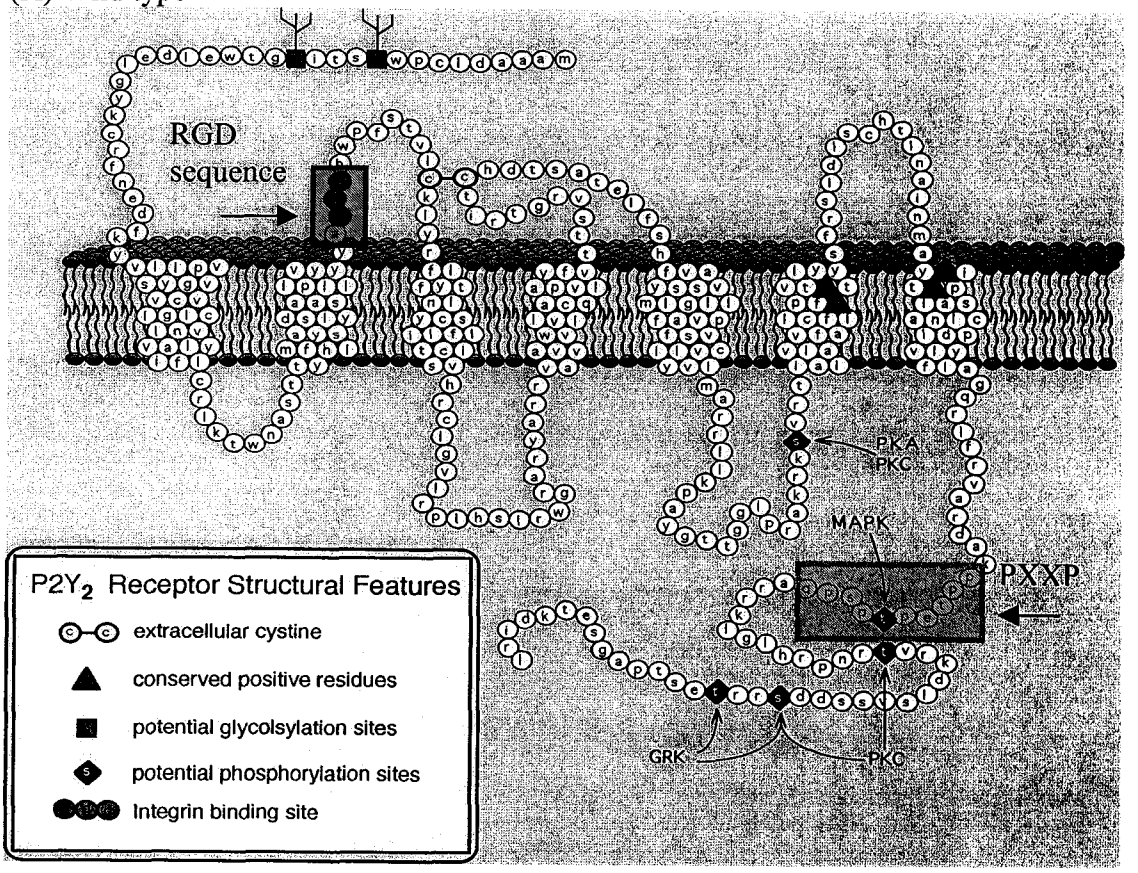
(A) Wild type



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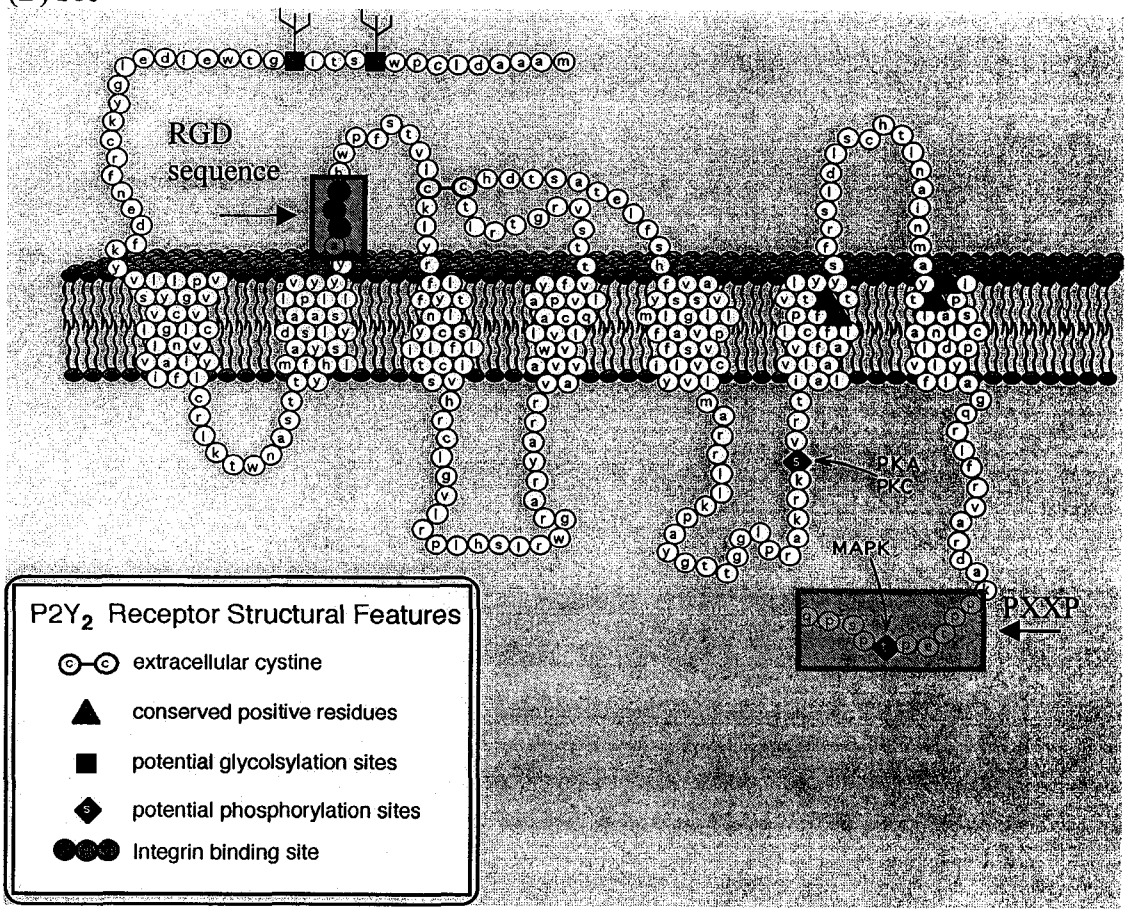
(B) Pro+



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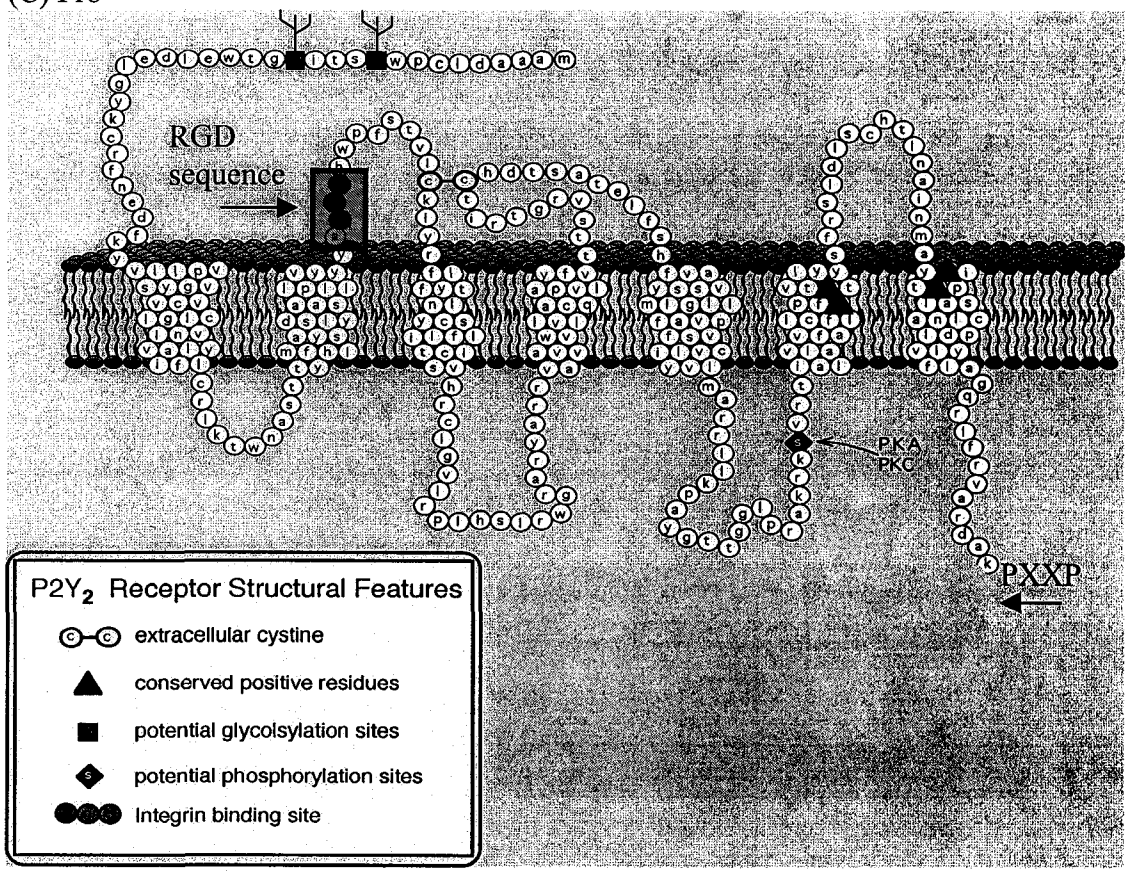
(C) Pro-



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assembly of focal adhesions [273], [274], [275] for 1 hr before the stimulus with 100 μM. Figure 3.20 and 3.21 show that ERK1/2 phosphorylation was partially inhibited by cytochalasin D in the wild type receptor as well as in the Pro+, but not in the Pro- mutant indicating that the ERK1/2 activation is regulated by the cytoskeleton complexes which may be modulated by the complex formed by src and FAK. However, once the proline rich sequence is eliminated, the protein complex of src-FAK is broken possibly due to the impediment of the formation of actin cytoskeleton which could modulate ERK1/2 activation by $P2Y_2$ receptor as observed. Once more the $P2Y_2$ receptor assumes a src-independent pathway to activate ERK1/2.

There are several reports suggesting that the activation of ERK1/2 could be mediated by the transactivation of the EGFR when GPCR are stimulated [276]. Knowing that the activation of the $P2Y_2$ receptor induce the phosphorylation of EGFR [277], incubation with 300 nM AG1478 (4-(3-chloroanilino)-6,7-dimethoxyquinazoline) ([127], an inhibitor of the EGFR, for 30 min before the stimulus with UTP 100 μM was used to determine if the phosphorylation of ERK1/2 induced by the $P2Y_2$ receptor is mediated by the transactivation of the EGFR. AG1478 did not inhibit the phosphorylation of ERK1/2 in the wild type, but decrease slightly the phosphorylation of ERK1/2 in the Pro+ and Pro- mutant receptors. These results suggest that ERK1/2 activated by the $P2Y_2$ receptor is modulated by two pathways, dependent and independent of the transactivation of the EGFR. (See figure 3.22 and 3.23)

With respect to p38, PP2 inhibited the phosphorylation of p38 induced by the wild type receptor (figure 3.24A and 3.25A). The same result was observed in the Pro- mutant receptor but not in the Pro+ mutant receptor suggesting that p38 can be activated by a src-dependent and –independent pathway (figure 3.24B, 3.24C, 3.25B and 3.25C). Contrary to ERK1/2, cytochalasin D did not inhibit the phosphorylation of p38 induced by UTP. Cytochalasin D increased its phosphorylation which could be due to a stress signal caused by the disruption of the actin cytoskeleton. These results suggest that p38 activation of the $P2Y_2$ receptor is modulated partially by src. The incubation with AG1478 which inhibits the activation of EGFR did not cause any effect in the phosphorylation of p38 induced by UTP except in the Pro- mutant suggesting that once

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Figure 3.20. Effect of specific inhibitors on ERK1/2 phosphorylation induced by UTP in wild type, Pro(+) and Pro(-) mutant 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected cells were pretreated with 10μM PP2 and 10μM CytD for 30 min and 1 hr respectively. Then, the cells were stimulated with 100 μM UTP for 5 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using anti phospho specific ERK1/2 antibody and blots were stripped and reprobed with anti ERK1/2 antibody to confirm uniform loading. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 4 independent experiments. (A) wild type, (B) Pro(+) mutant and (C) Pro(-) mutant.

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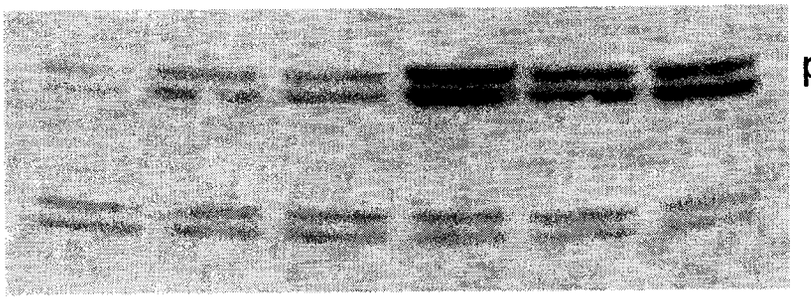
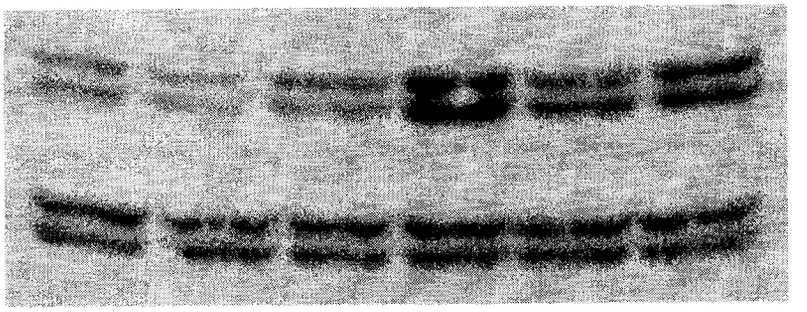
(A)Wild type



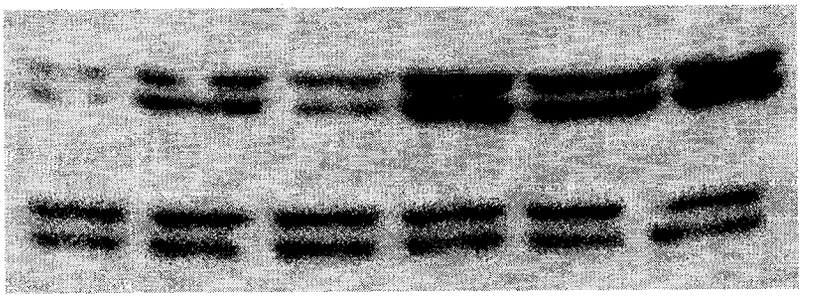
O PP2 CYTD U U+PP2 U+CYTD Treatment

(B) Pro+



O PP2 CYTD U U+PP2 U+CYTD Treatment

(C) Pro-



O PP2 CYTD U U+PP2 U+CYTD Treatment

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Figure 3.21. Effects of various specific inhibitors on UTP-induced ERK1/2 phosphorylation in wild type, Pro(+) and Pro(-) mutant 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected cells were pretreated with 10μM PP2 and 10μM CytD for 30 min and 1 hr respectively. Then, the cells were stimulated with 100 μM UTP for 5 min. Data from figure 3.20 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of ERK1/2 was calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 4 independent experiments. (A) wild type, (Pro(+) mutant and (C) Pro(-) mutant. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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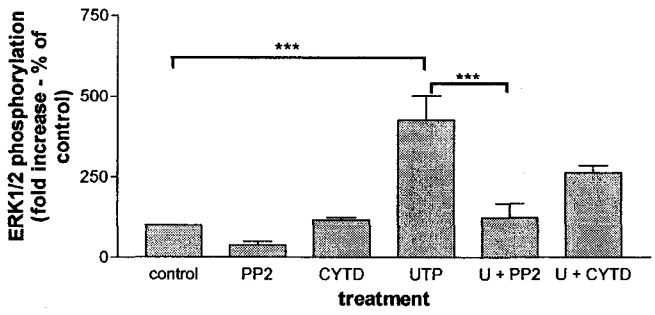
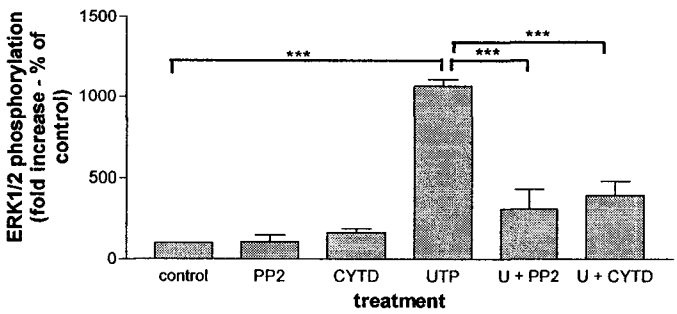
(A) Wild type



(B) Pro+



(C) Pro-

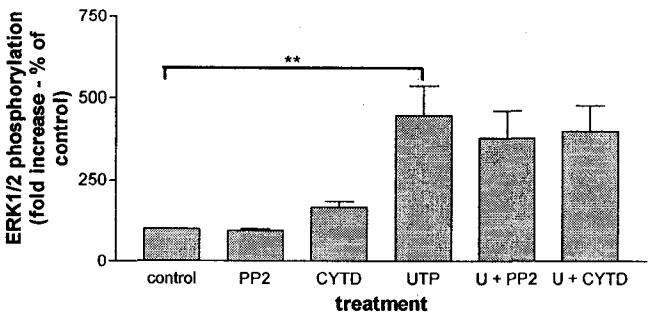


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Figure 3.22. Effect of an specific EGFR antagonist on ERK1/2 phosphorylation induced by UTP in wild type, Pro(+) and Pro(-) mutant 1321N1-hP2Y$_2$ transfected cells. 1321N1-hP2Y$_2$ transfected cells were pretreated with 300 nM AG1478 for 30 min. Then, the cells were stimulated with 100 μM UTP for 5 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using anti phospho specific ERK1/2 antibody and blots were stripped and reprobed with anti ERK1/2 antibody to confirm uniform loading. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 3 independent experiments. (A) wild type, (B) Pro(+) mutant and (C) Pro(-) mutant.

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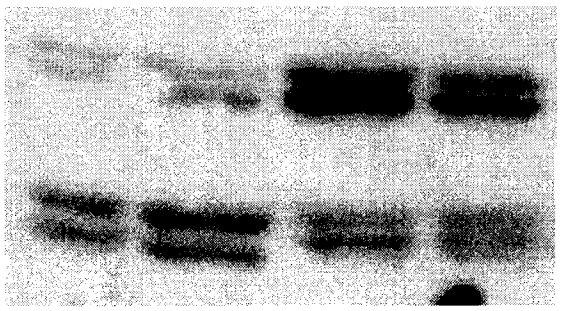
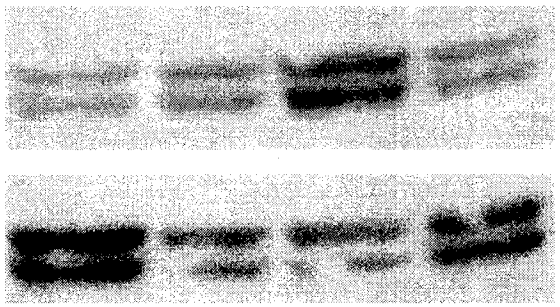
(A) Wild type



(B) Pro+



(C) Pro-

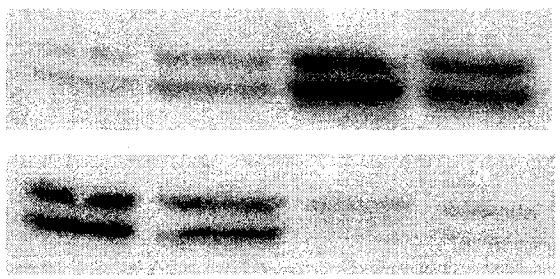


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Figure 3.23. Effects of AG1478, an EGFR antagonist on UTP-induced ERK1/2 phosphorylation in wild, Pro(+) and Pro(-) mutant 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected cells were pretreated with 300 nM AG1478 30 min. Then the cells were stimulated with 100 μM UTP for 5 min. Data from figure 3.22 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of ERK1/2 was calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 3 independent experiments. (A) wild type, Pro(+) mutant and (C) Pro(-) mutant. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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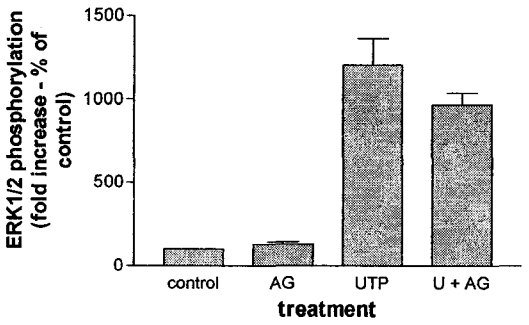
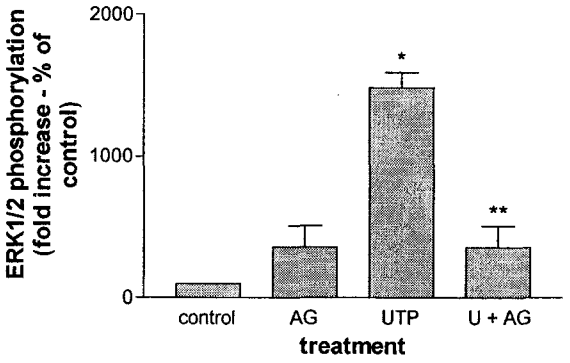
(A) Wild type



(B) Pro+



(C) Pro-

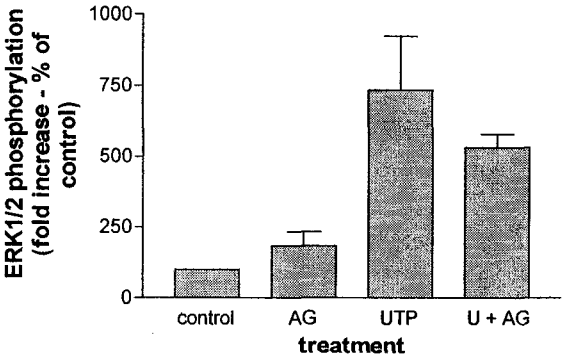


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Figure 3.24. Effect of specific inhibitors on p38 phosphorylation induced by UTP in wild, Pro(+) and Pro(-) mutant 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected cells were pretreated with 10μM PP2 (P), 300 nM AG1478 (AG) for 30 min and 10μM CytD (C) for 1 hr respectively. Then, the cells were stimulated with 100 μM UTP (U) for 5 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using anti phospho specific p38 antibody and blots were stripped and reprobed with anti p38 antibody to confirm uniform loading. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 5 independent experiments. (A) wild type, (B) Pro(+) mutant and (C) Pro(-) mutant.

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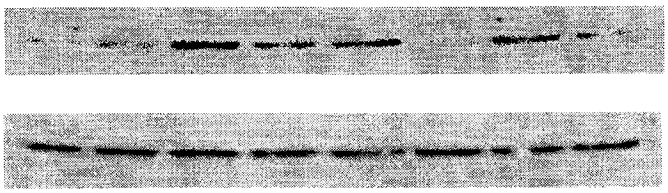
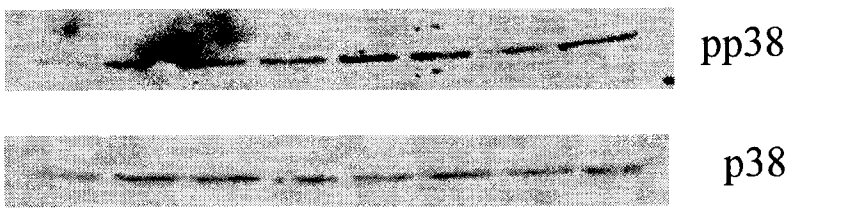
(A) Wild type



(B) Pro+



(C) Pro-

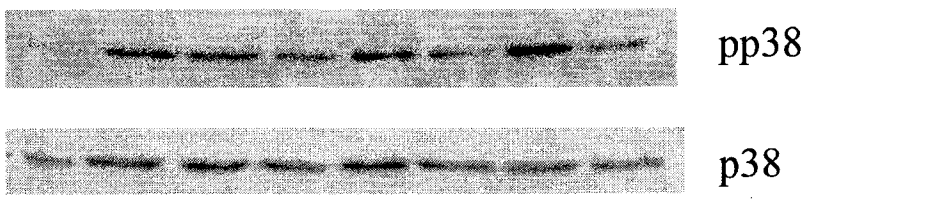


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Figure 3.25. Effects of various specific inhibitors on UTP-induced p38 phosphorylation in wild, Pro(+) and Pro(-) mutant 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected cells were pretreated with 10μM PP2 (P), 300nM AG1478 (AG) for 30 min and 10μM CytD (C) for 1 hr respectively. Then, the cells were stimulated with 100 μM UTP (U) for 5 min. Data from figure 3.24 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of p38 was calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 5 independent experiments. (A) wild type, Pro(+) mutant and (C) Pro(-) mutant. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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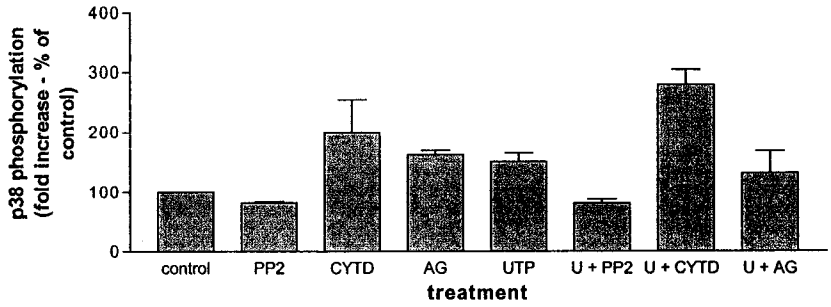
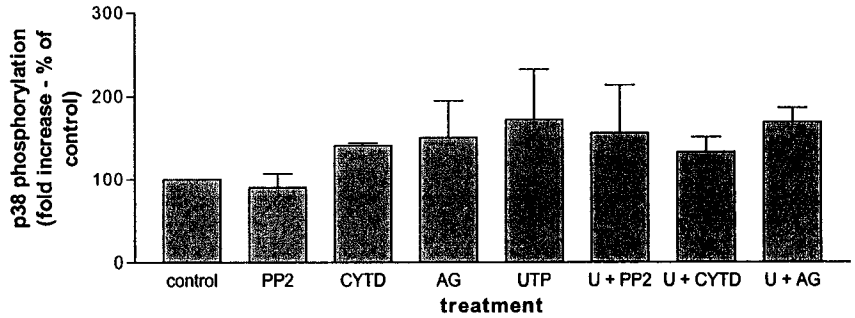
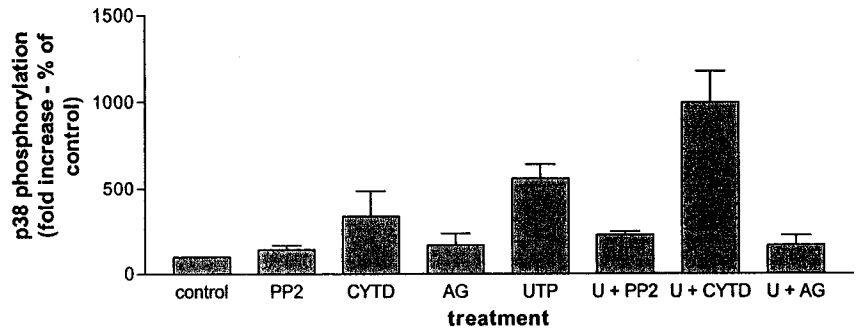
(A) Wild type



(B) Pro+



(C) Pro-



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the proline rich sequence is eliminated other pathway which include the transactivation of the EGFR is modulated the activation of p38 induced by UTP through the $P2Y_2$ receptor.

With respect to JNK, the two isoform behave somewhat different. The p54JNK phosphorylation was not inhibited by PP2 in the wild type and in the Pro+, but was slightly inhibited in the Pro- mutant. The p46JNK phosphorylation was not inhibited by PP2 in the wild type receptor, but was slightly inhibited by PP2 in the Pro+ and in the Pro- mutant. Similar to p38, cytochalasin D increased the phosphorylation of both isoforms of JNK in the wild type as well as in the mutants. AG1478 did not inhibit the response induced by UTP in the phosphorylation of JNK isoforms in the wild type, but inhibited slightly the phosphorylation of p46JNK in the Pro+ and in the Pro- mutant. These results suggest that both isoforms JNK are activated by different pathways which could involve the transactivation of the EGFR via proline rich sequence of the $P2Y_2$ receptor but not necessarily through a pathway which include src (Figure 3.26 and 3.27).

However, the activation of Akt is quite different. Akt phosphorylation was slightly inhibited by PP2 in the wild type receptor and in the mutants (figures 3.28 and 3.29). Treatments with AG1478 did not inhibit the Akt phosphorylation in any of the cells expressing the different receptor types. However, cytochalasin D strongly inhibited the Akt phosphorylation in the wild type as well as in the mutants. These results suggest that the pathway which activates Akt/PKB by the $P2Y_2$ depends on the formation of the actin cytoskeleton and partially on src, but did not depend on the transactivation of the EGFR.

III. *In vitro* cerebral ischemia (OGD)

A. Lactate dehydrogenase (LDH) released (necrosis/apoptosis) PARP cleaved

To investigate the role of extracellular nucleotides through the $P2Y_2$ receptor after an ischemia episode, an *in vitro* model of oxygen-glucose deprivation (OGD) was used to simulate an ischemia episode. During ischemia, activation of apoptosis or necrosis could mediate the death of cells. Apoptosis accounts for most of the physiological cell death characterized by the activation of a cascade of proteolytical enzymes known as caspases

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Figure 3.26. Effect of specific inhibitors on JNK phosphorylation induced by UTP in wild type, Pro(+) and Pro(-) mutant 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected cells were pretreated with 10μM PP2 (P), 300nM AG1478 (AG) for 30 min and 10μM CytD (C) for 1 hr respectively. Then, the cells were stimulated with 100 μM UTP (U) for 25 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using anti phospho specific JNK antibody and blots were stripped and reprobed with anti JNK antibody to confirm uniform loading. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 3 independent experiments. (A) wild type, (B) Pro(+) mutant and (C) Pro(-) mutant.

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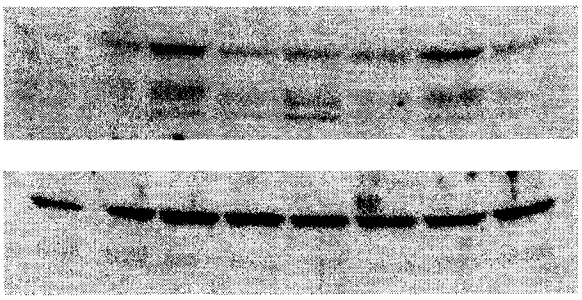
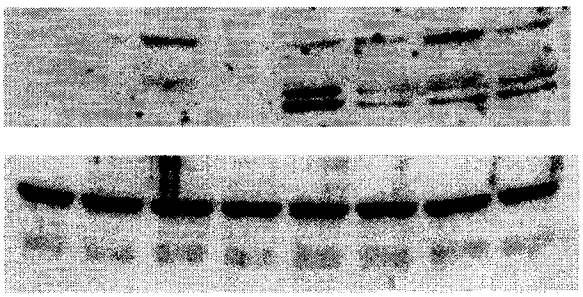
(A) Wild type



(B) Pro+



(C) Pro-

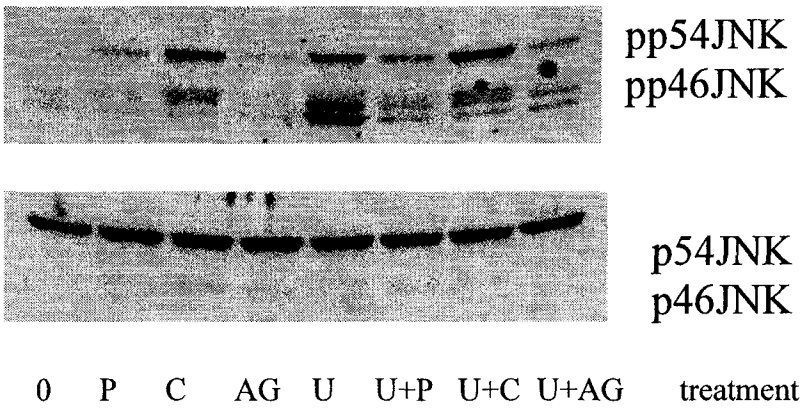


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Figure 3.27. Effects of various specific inhibitors on UTP-induced JNK phosphorylation in wild type, Pro(+) and Pro(-) mutant 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected cells were pretreated with 10μM PP2 (P), 300nM AG1478 (AG) for 30 min and 10μM CytD for 1 hr respectively. Then, the cells were stimulated with 100 μM UTP for 25 min. Data from figure 3.26 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of JNK was calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 3 independent experiments. (A) wild type, (B) Pro(+)-mutant and (C) Pro(-) mutant. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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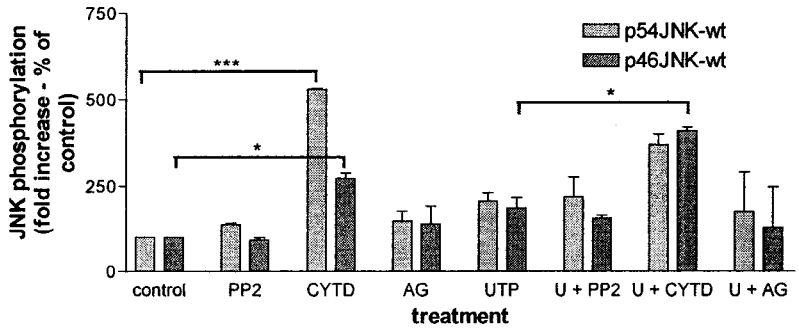
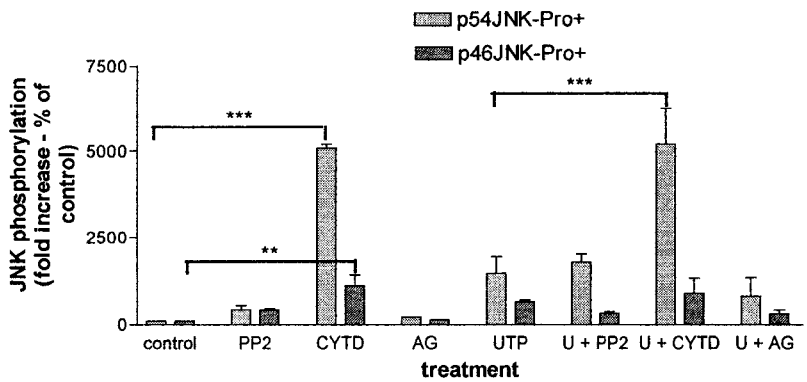
(A)



(B)



(C)

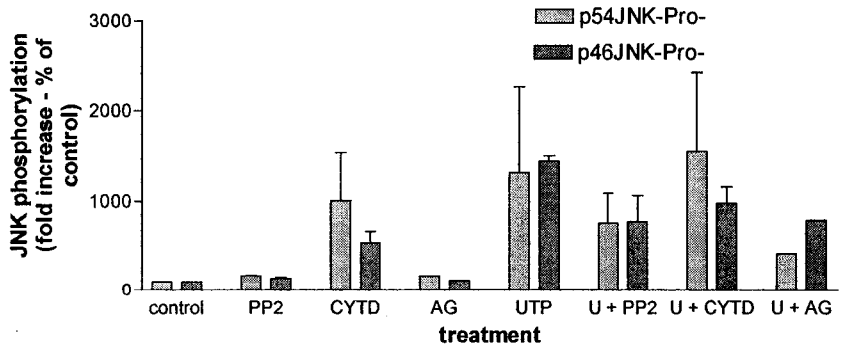


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Figure 3.28. Effect of specific inhibitors on Akt phosphorylation induced by UTP in wild type, Pro(+) and Pro(-) mutant 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected cells were pretreated with 10μM PP2 (P), 300nM AG1478 (AG) for 30 min and 10μM CytD (C) for 1 hr respectively. Then, the cells were stimulated with 100 μM UTP (U) for 25 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using anti phospho specific Akt antibody and blots were stripped and reprobed with anti Akt antibody to confirm uniform loading. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 4 independent experiments. (A) wild type, (B) Pro(+) mutant and (C) Pro(-) mutant.

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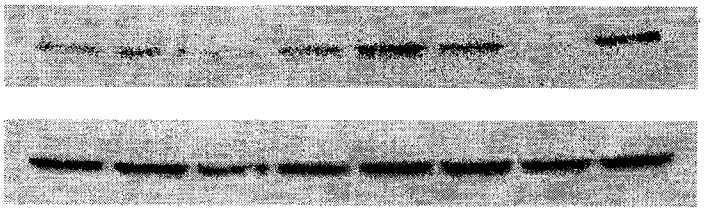
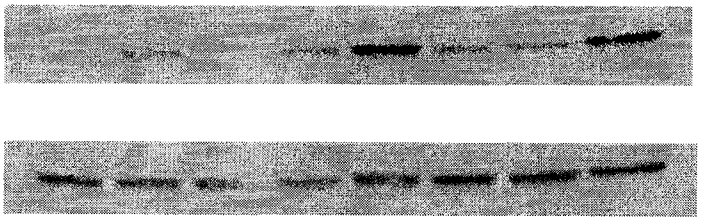
(A) Wild type



(B) Pro+



(C) Pro-

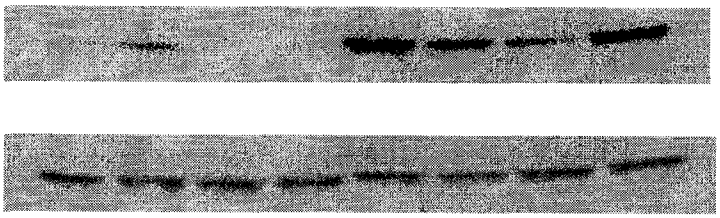


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Figure 3.29. Effects of various specific inhibitors on UTP-induced Akt phosphorylation in wild, Pro(+) and Pro(-) mutant 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected cells were pretreated with 10μM PP2 (P), 300nM AG1478 (AG) for 30 min and 10μM CytD (C) for 1 hr respectively. Then, the cells were stimulated with 100 μM UTP (U) for 25 min. Data from figure 3.28 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of Akt was calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 4 independent experiments. (A) wild type, (B) Pro(+)-mutant and (C) Pro(-) mutant. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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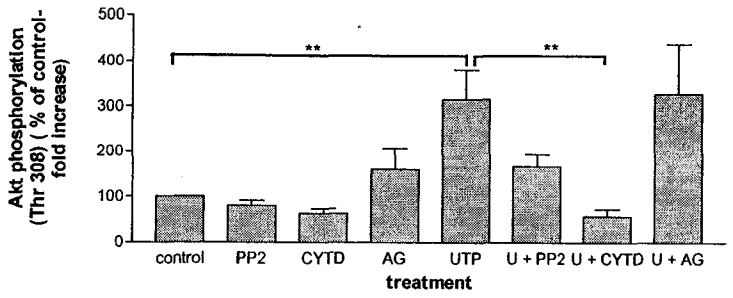
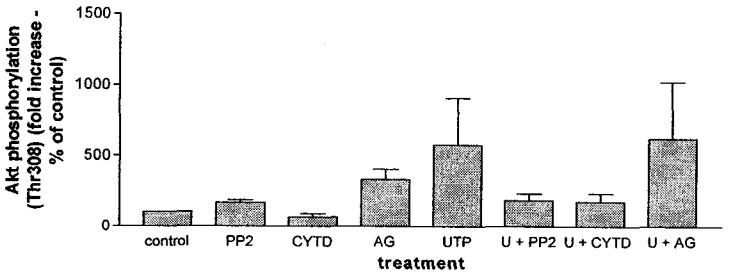
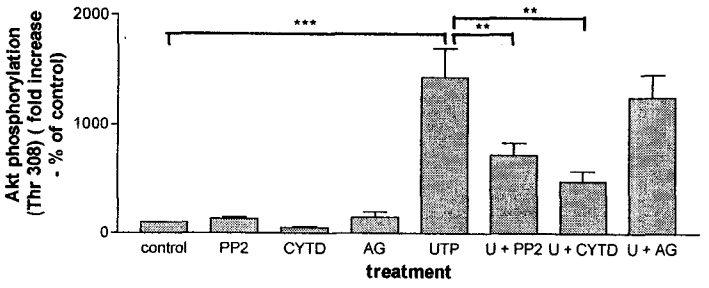
(A) Wild type



(B) Pro+



(C) Pro-



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[19]. Necrosis is usually induced in pathological situations by accidental and acute damage to cells characterized by cell swelling and disruption of the cell membrane, leading to the release of the cellular content [18]. As described in METHODS section, the cells were exposed OGD in an anaerobic chamber to determine the damage of the cells. To determine the damage of the cells, measurement of LDH release was performed as indicative of necrosis. LDH is a tetramer of 35 kDa cytosolic enzyme present in all mammalian cells [278]. The normal plasma membrane is impermeable to LDH, but damage to the cell membrane results in a change in the membrane permeability and subsequent leakage of LDH to the extracellular fluid. *In vitro* release of LDH from cells provides an accurate measure of cell membrane integrity and cell viability with reliable results. Figure 3.30A shows that during the first hours of OGD, (1-2 hrs) no release of LDH was observed, but then after 3 to 4 hrs (a longer exposition), the cells began to release LDH significantly. The cells exposed to OGD for longer time (until 18 hrs), died and released all of their LDH contents (figure 3.30B). These results suggest that the cells began to suffer cell damage and necrosis (as occur during ischemia) after the 4 hrs of OGD. The cells are alive or are surviving during the first hours of OGD exposition.

Poly (ADP-ribose) polymerase (PARP) is an abundant 116 kDa chromatin-associated enzyme involved in poly (ADP-ribosylation) of DNA binding proteins [279]. PARP enzyme is activated in response to DNA damage and is involved in DNA repair, DNA stability and transcriptional regulation [279]. Upon binding to DNA breaks, activated PARP catalyzes the covalent attachment of ADP-ribose units from NAD^+ to itself and to a limited number of nuclear DNA-binding proteins such as histones, adaptor factors and DNA repair effectors [279], [280]. When the DNA is repaired, the cell survives. PARP is implicated in necrosis as ocurr in ischemia by its overactivation, producing depletion of both NAD^+ and ATP stores [281]. However, it is also implicated in the final stage of apoptotic signals [282]. It is cleaved by several caspases specifically caspase -3 to generate the characteristic 89 and 24 kDa fragments [283]. Caspase cleavage at this site separates the DNA binding domain from the catalytic domain, resulting in the inactivation of PARP and the p89 and p24 inhibit the homoassociation and DNA binding of PARP [284]. A time course of OGD to detect cleaved PARP was performed to confirm that necrosis and not apoptosis is the possible predominant form of death of these cells

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Figure 3.30. Time course of LDH released by an *in vitro* ischemic treatment in human $P2Y_2$-1321N1 transfected cells. 1321N1-h$P2Y_2$ transfected cells were exposed to oxygen-glucose deprivation (OGD) for the indicated period of time. After the treatment of ischemia (OGD), the supernatant (100µl) was collected and LDH assay was performed as manufacturer's instructions (Roche Molecular-Boehringer Mannheim). The absorbance of the samples was performed in a Microplate Reader at a maximal wavelength λ of 490 nm. The results were reported as % of citotoxicity using the low control (cells without OGD treatment) and high control (cells disrupted with triton x-100 representing total LDH in the cells) using the equation as described in Chapter II. Results shown are representative of 6 independent experiments. (A) short kinetic, (B) long kinetic. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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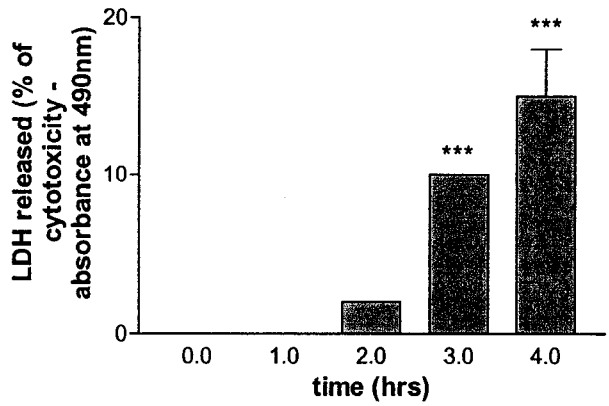
(A)



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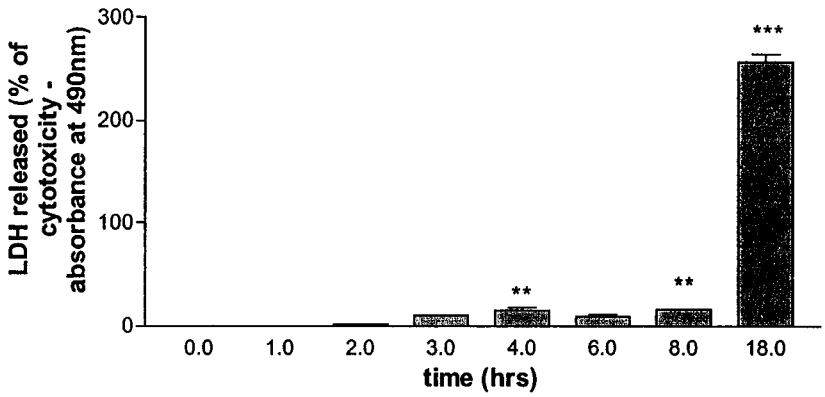

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exposed to these extreme conditions. As shown in figure 3.31, we can not detect cleaved PARP in the time course of OGD. However, cleaved PARP was detected in control cells (normal) showing the 89 and 24 kDa fragments. These cells were starved (without serum) which is implicated in apoptosis [285]. These results suggest that the predominant way of death during this OGD or ischemia treatment is necrosis and not apoptosis.

B. Protein kinase phosphorylation during OGD

Several reports have demonstrated that the MAPK superfamily of protein kinase cascades are activated differentially during ischemia [22, 23] [24] [25]. Knowing that MAPK is activated by the $P2Y_2$ receptor, we decided to investigate if MAPK activation by $P2Y_2$ receptor was altered after the OGD treatment. As shown in figures 3.32A and 3.33A, during the time course of OGD, a steady increase in ERK1/2 phosphorylation was observed until 4 hrs. After the OGD treatment, 100μM UTP was added for 15 min after each time point of the time course. A greater increase in ERK1/2 phosphorylation was observed during the time course of OGD after UTP addition. These results suggest that UTP, after an ischemia episode, increase the phosphorylation of ERK1/2 and through that activation could induce a survival signal.

An analogous experiment was performed to determine p38 and JNK phosphorylation. As shown in figure 3.32B and 3.33B a time-dependence of p38 phosphorylation is observed as the OGD time of exposure is increased. The addition of 100 μM UTP for 15min after this OGD exposure, a greater increase in p38 phosphorylation is observed; however after 4hrs of OGD incubation, no further change in p38 phosphorylation (after UTP addition) was observed, i.e. p38 remains the same as without UTP.

With respect of JNK isoform phosphorylation, the response is rather different. The p54JNK phosphorylation increases strongly at 1 hr and then decreases remaining phosphorylated almost the same until the 4 hrs of OGD. The increase in p46JNK phosphorylation remains the same until the 4 hrs of OGD. After the addition of UTP, an

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Figure 3.31. Time course of PARP cleaved by an *in vitro* ischemic treatment in human $P2Y_2$-1321N1 transfected cells. 1321N1-h$P2Y_2$ transfected were exposed to oxygen-glucose deprivation (OGD), for the indicated period of time. The, cell were stimulated with 100 μM UTP for 15 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using anti cleaved PARP antibody and PARP antibody. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 4 independent experiments.

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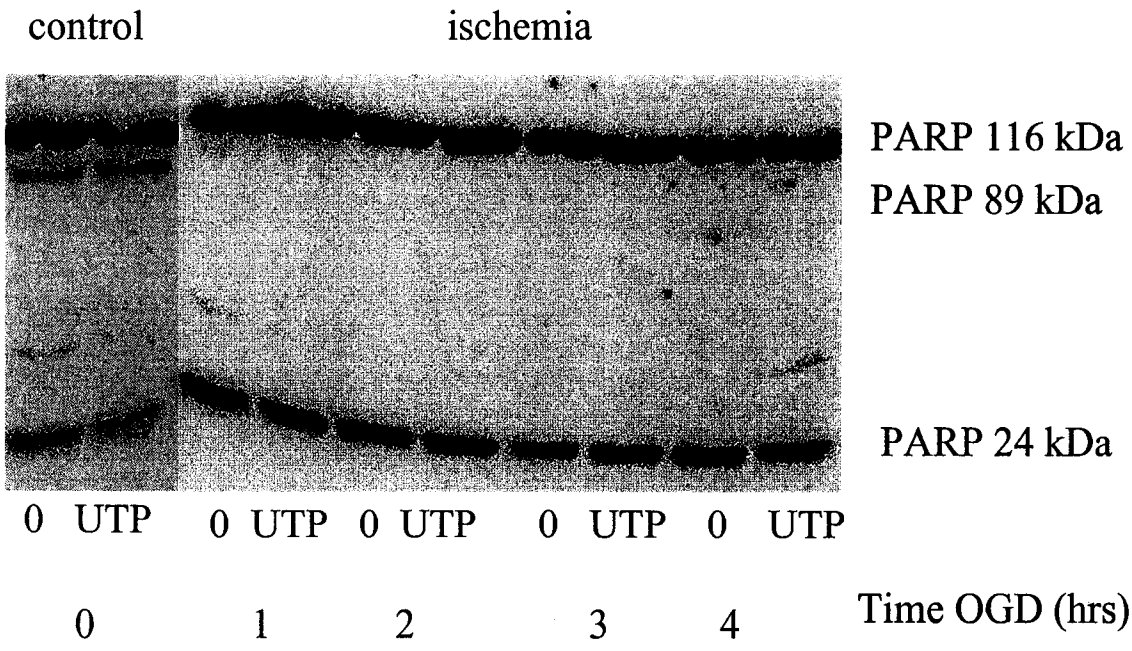

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increase in p54JNK and p46JNK phosphorylation was observed for each time point as shown in figure 3.32C, 3.33C and 3.33D.

For Akt, the response is different during the OGD treatment. As shown in figure 3.32D and 3.33E, Akt/PKB was not phosphorylated during the time course of OGD. But an increase in its phosphorylation was observed after the addition of UTP. The results are comparable with the control cells in which UTP incubation induced a strong phosphorylation of Akt/PKB. These results suggest the $P2Y_2$ receptor could be mediating a survival signal inducing the phosphorylation of ERK1/2, p38, JNK and AKT after an ischemia episode.

As observed with the kinetics of OGD in the LDH assay and protein kinase phosphorylation, we determined that 4 hrs of OGD is the precise time that the cells began to suffer severe damage and we want to determine which other proteins dowsteam of these protein kinases which are responsible to regulate apoptosis and survival signals are affected by this OGD treatment. The figures 3.34 and 3.35 summarize the results obtained of the protein kinases at 4 hrs of OGD and will permit a comparison with the other proteins determined at this time of OGD.

One more protein kinase that is also related to survival signal is p70S6k. This protein kinase is downstream of PI3K as PKB/Akt and may also be mediated by PKB/Akt [236]. The determination of p70S6k was performed by the detection of two principal phosphorylation sites: Thr389 which corresponds to the kinase activity domain and Thr421 and Ser 424 which corresponds to the initial step in p70S6k activation which appears to involve a phosphorylation-induced conformational change in the C terminus of the kinase domain to expose the site to be phosphorylated [234]. As shown in figure 3.36A, 3.36B, 3.37A and 3.37B, the two isoforms of p70S6k: p70 and p85 were phosphorylated in control conditions after UTP addition. In ischemic conditions and with UTP, no change in phosphorylation in Thr 421/424 and Thr 389 in p85 was observed. Although a slightly increase in p70 phosphorylation after UTP addition is observed, it is not very significantive.

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Figure 3.32. Time course of protein kinases phosphorylation by an *in vitro* ischemic treatment in human $P2Y_2$-1321N1 transfected cells. 1321N1-h$P2Y_2$ transfected were exposed to oxygen-glucose deprivation (OGD) for the indicated period of time. Then, the cells were stimulated with 100 μM UTP for 15 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using (A) anti phospho specific ERK1/2 antibody and blots were stripped and reprobed with anti ERK1/2 antibody to confirm uniform loading, (B) anti phospho specific p38 antibody and blots were stripped and reprobed with anti p38 antibody, (C) anti phospho specific JNK (p54/p46) antibody and blots were stripped and reprobed with anti JNK antibody (p54/p46) and (D) anti phospho specific Akt antibody and blots were stripped with anti Akt antibody. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 4 independent experiments.

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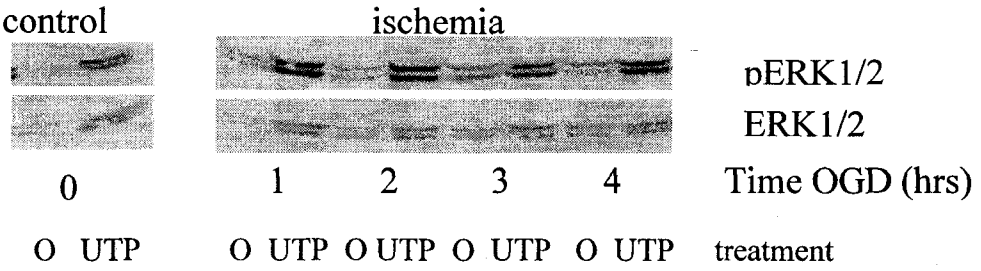

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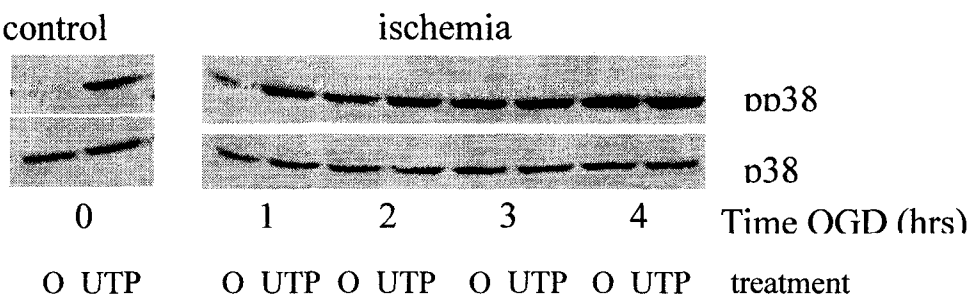

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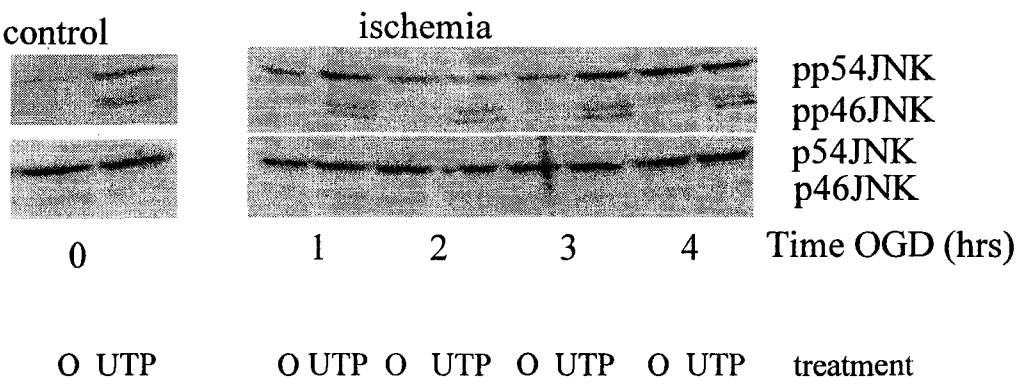

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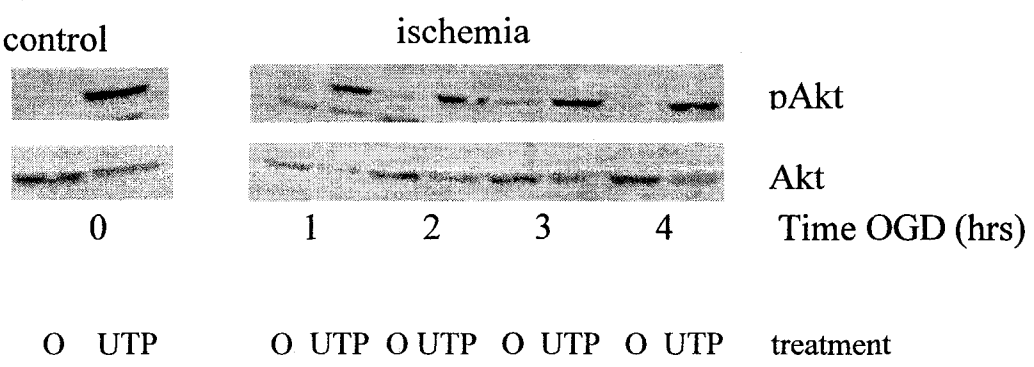

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Figure 3.33. Activation of ERK1/2, p38, JNK and Akt during an *in vitro* ischemic treatment and by UTP in 1321N1-hP2Y_2 transfected cells. Data from figure 3.32 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of (A) ERK1/2, (B) p38, (C-D) JNK and (E) Akt were calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 4 independent experiments. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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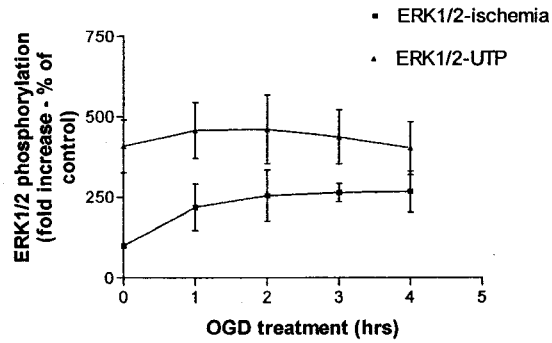

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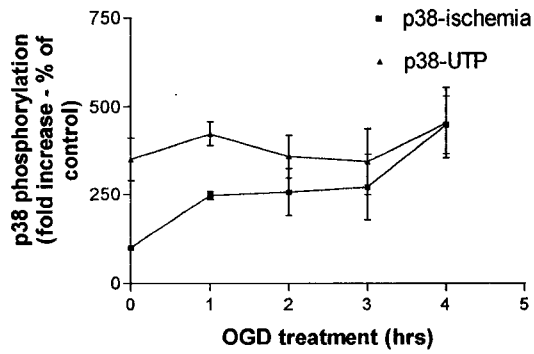

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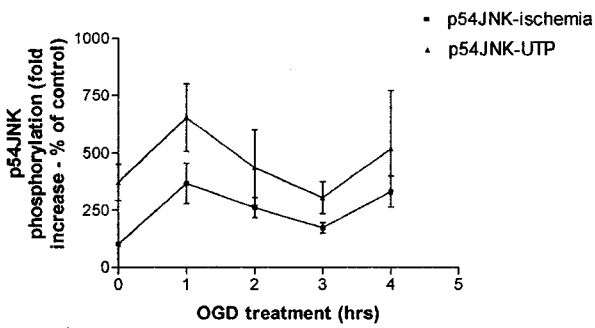

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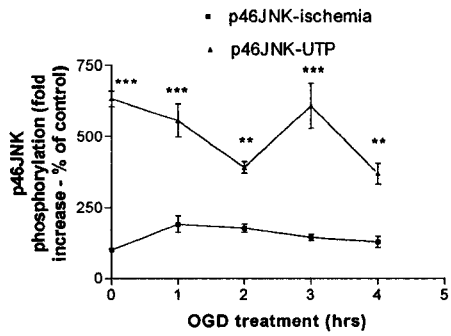

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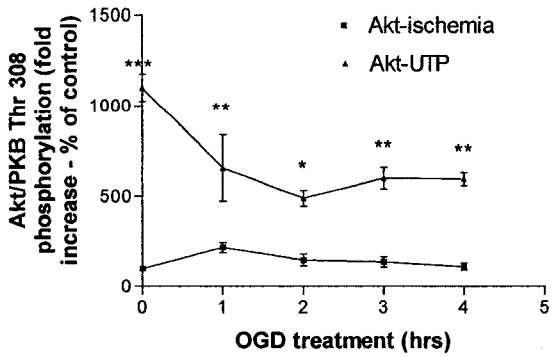

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Figure 3.34. Protein kinases phosphorylation during 4hrs of OGD, an *in vitro* ischemic treatment, and by UTP in human $P2Y_2$-1321N1 transfected cells. 1321N1-h$P2Y_2$ transfected were exposed to oxygen-glucose deprivation (OGD) for 4 hrs of OGD. Then, the cells were stimulated with 100 μM UTP for 15 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using (A) anti phospho specific ERK1/2 antibody and blots were stripped and reprobed with anti ERK1/2 antibody to confirm uniform loading, (B) anti phospho specific p38 antibody and blots were stripped and reprobed with anti p38 antibody, (C) anti phospho specific JNK (p54/p46) antibody and blots were stripped and reprobed with anti JNK antibody (p54/p46) and (D) anti phospho specific Akt antibody and blots were stripped with anti Akt antibody. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 7 independent experiments.

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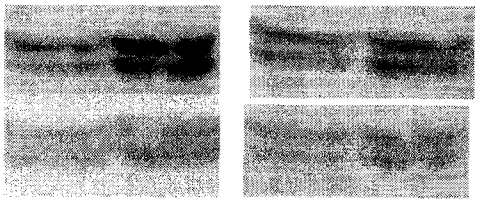
(A)



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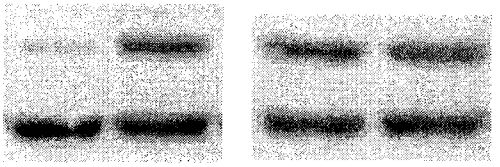

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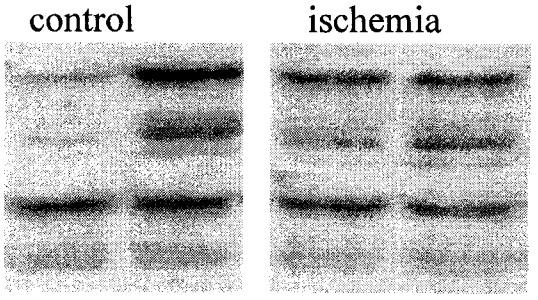

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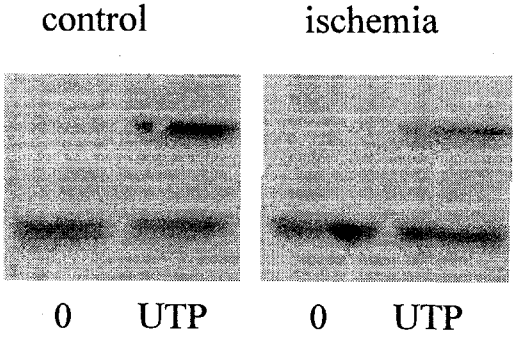

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Figure 3.35. Activation of protein kinases during 4hrs of OGD, an *in vitro* ischemic treatment, and by UTP in human $P2Y_2$-1321N1 transfected cells. 1321N1-h$P2Y_2$ transfected were exposed to oxygen-glucose deprivation (OGD) for 4 hrs of OGD. Then, the cells were stimulated with 100 μM UTP for 15 min. Data from figure 3.34 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of (A) ERK1/2, (B) p38, (C-D) JNK and (E) Akt were calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 7 independent experiments. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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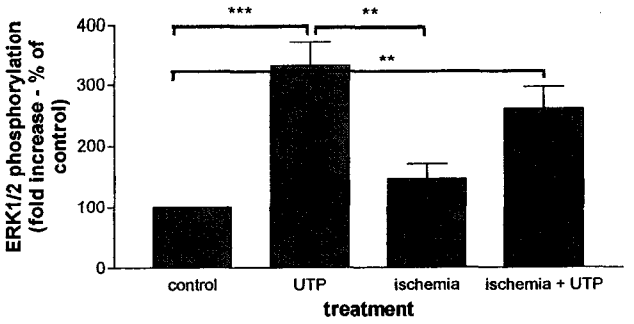

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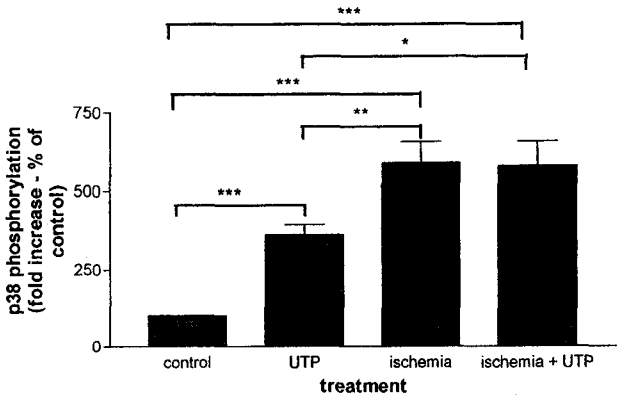

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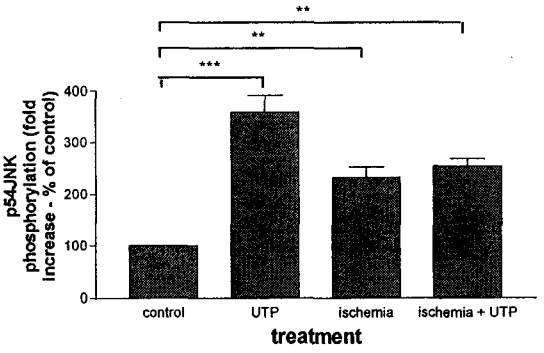

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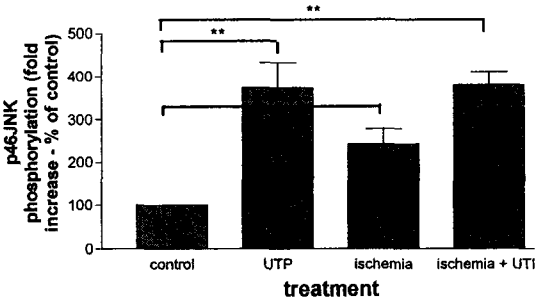

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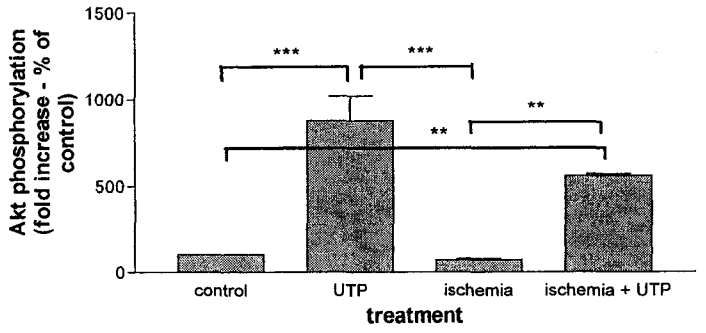

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Figure 3.36. The p70S6k phosphorylation during 4hrs of OGD, an *in vitro* ischemic treatment, and by UTP in human $P2Y_2$-1321N1 transfected cells. 1321N1-h$P2Y_2$ transfected were exposed to oxygen-glucose deprivation (OGD) for 4 hrs of OGD. Then, the cells were stimulated with 100 μM UTP for 15 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using (A) anti phospho specific p70S6k Thr 389 antibody and (B) anti phospho specific p70 S6k Thr 421/Ser424 and blots were stripped and reprobed with anti p70S6k antibody to confirm uniform loading. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 4 independent experiments.

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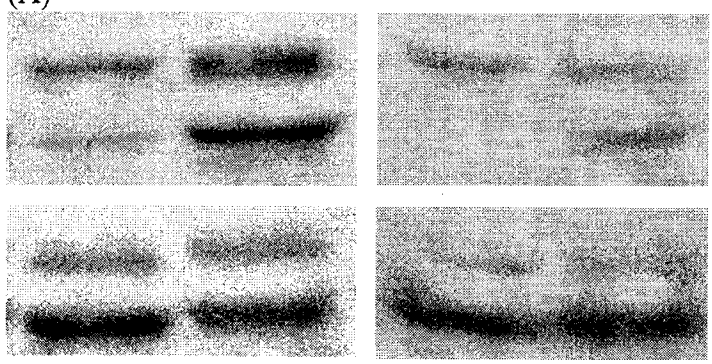
(A)



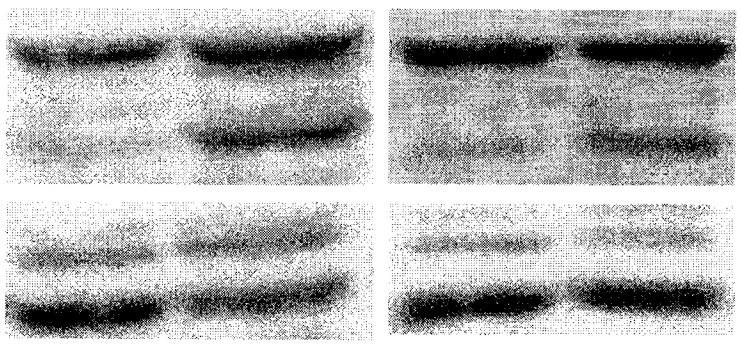
pp70S6kα/p85 (Thr389)

pp70S6kβ/p70 (Thr389)

p70S6kα/p85

p70S6kβ/p70

(B)



pp70S6kα/p85 (Thr421/S424)

pp70S6kβ/p70 (Thr421/S424)

p70S6kα/p85

p70S6kβ/p70

O UTP 0 UTP addition

Control Ischemia

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Figure 3.37. Activation of p70S6K during 4hrs of OGD, an *in vitro* ischemic treatment, and by UTP in human $P2Y_2$-1321N1 transfected cells. 1321N1-h$P2Y_2$ transfected were exposed to oxygen-glucose deprivation (OGD) for 4 hrs of OGD. Then, the cells were stimulated with 100 μM UTP for 15 min. Data from figure 3.36 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of p70S6k were calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 4 independent experiments. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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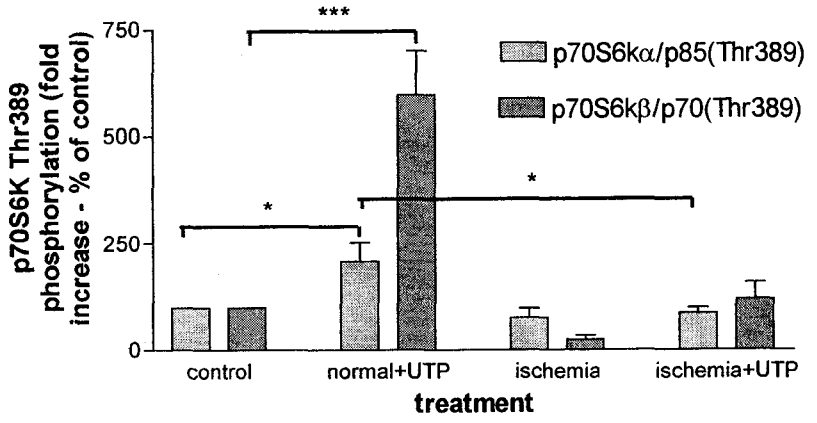

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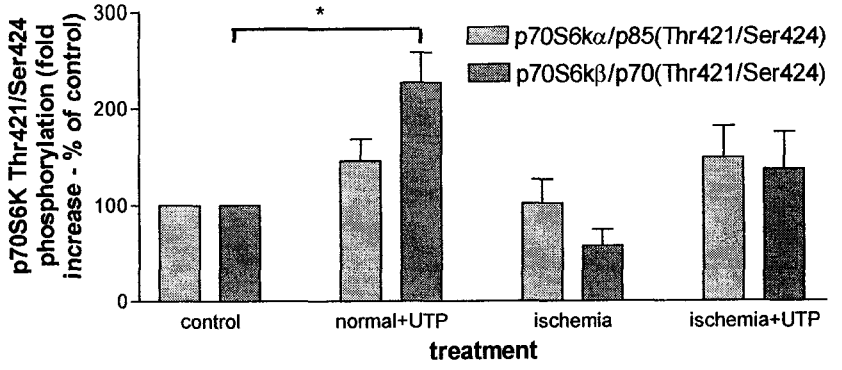

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Additional proteins related to survival signals are substrates of PKB/Akt. Therefore a determination of the phosphorylation of some of these proteins was performed. GSK3α and β phosphorylated by PKB/Akt mediates an anti-apoptotic or survival signal, while the non- phosphorylated forms mediate apoptosis. In figure 3.38 and 3.39A and 3.39B show that UTP induced an increase in the phosphorylation of both isoforms of GSK3 in control cells. During ischemia a decrease in their phosphorylation is observed which is comparable to basal levels. But after UTP induction, a slight increase in phosphorylation of both isoforms is observed, more in the alpha isoform than the beta isoform suggesting that UTP induced a survival signal. These results correlate with the results obtained of PKB/Akt indicating that PKB/Akt is the protein kinase responsible of the GSK3 phosphorylation.

Another substrate of PKB/Akt is BAD. BAD is phosphorylated in Ser 136 by PKB/Akt [248], but also is phosphorylated in Ser 112 by the ERK1/2 pathway [286]. Similar to GSK3α/β, when BAD is phosphorylated, a survival or antiapoptotic signal is induced. Figure 3.38B and 3.39C show the phosphorylation of BAD at Ser 112 induced by UTP through the $P2Y_2$ receptor in control conditions. However, in ischemic conditions there is no phosphorylation of BAD, suggesting that a death signal is present which could be mediated by BAD. After UTP addition, an increase in BAD phosphorylation at Ser 112 is observed. These results correlate with the results observed of PKB/Akt and ERK1/2, where UTP induce a survival signal through the $P2Y_2$ receptor.

Another protein kinase is IκBα that is related to survival signal which its activation is modulated by PKB/Akt through the IKK protein kinase [287]. As shown in figure 3.38C and 3.39D, UTP induce the phosphorylation of IκBα in control conditions, but not in ischemic or after UTP incubation for 15min.

C. Antagonist of glutamate receptors

During an ischemia episode large amounts of neurotransmitters such as glutamate are released [15]. Therefore glutamate antagonists were employed to determine if the

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Figure 3.38. PKB/Akt substrates phosphorylation during 4hrs of OGD, an *in vitro* ischemic treatment, and by UTP in human $P2Y_2$-1321N1 transfected cells. 1321N1-$hP2Y_2$ transfected were exposed to oxygen-glucose deprivation (OGD) for 4 hrs of OGD. Then, the cells were stimulated with 100 μM UTP for 15 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using (A) anti phospho specific GSK3 antibody, (B) anti phospho specific BAD antibody and blots were stripped and reprobed with anti BAD antibody and (C) anti phosphor specific IkBα antibody and blots were stripped and reprobed with anti IkBα antibody. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 4 independent experiments.

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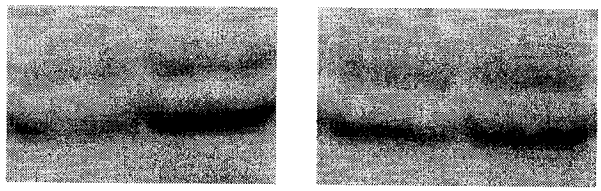
(A)



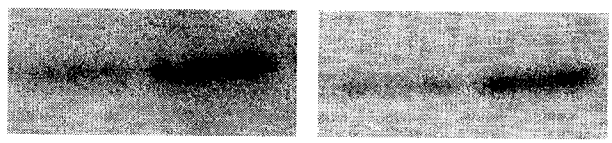
pGSK3α

pGSK3β

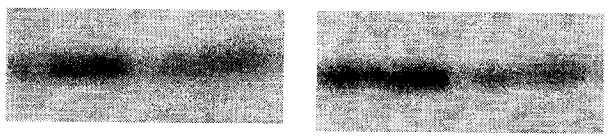
0 UTP 0 UTP addition

control ischemia

(B)



pBAD (Ser112)

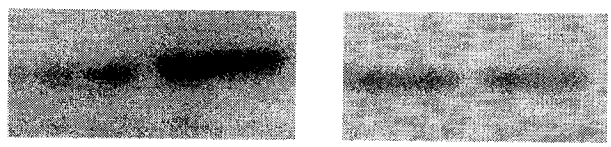


BAD

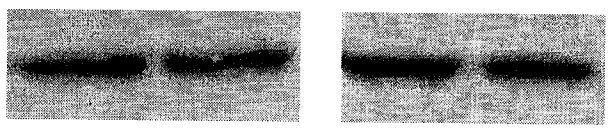
0 UTP 0 UTP addition

control ischemia

(C)



pIκBα



IκBα

0 UTP 0 UTP addition

control ischemia

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Figure 3.39. Inhibition of PKB/Akt substrates phosphorylation during 4hrs of OGD, an *in vitro* ischemic treatment, and by UTP in human $P2Y_2$-1321N1 transfected cells. 1321N1-h$P2Y_2$ transfected were exposed to oxygen-glucose deprivation (OGD) for 4 hrs of OGD. Then, the cells were stimulated with 100 μM UTP for 15 min. Data from figure 3.38 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation GSK3 α/β, BAD and IκBα were calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 4 independent experiments. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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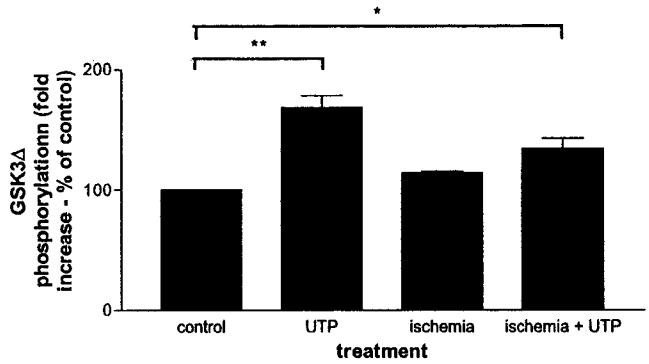

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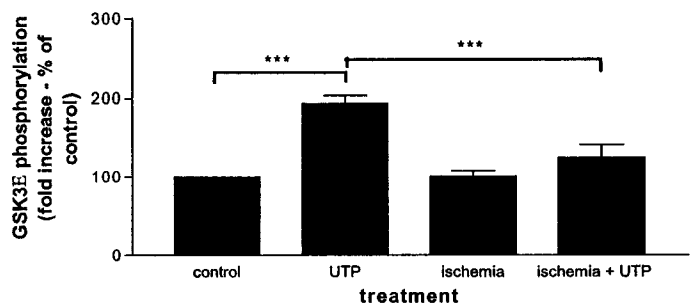

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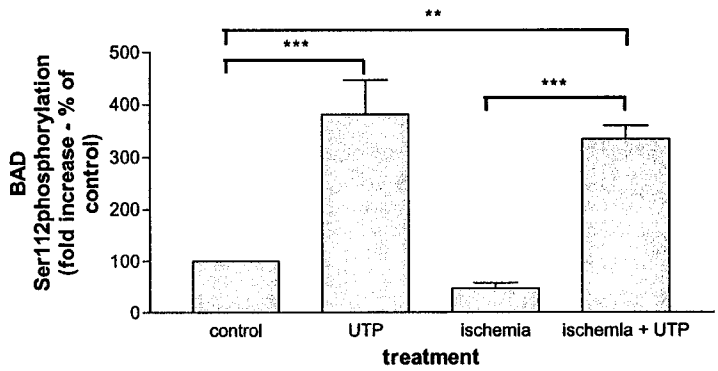

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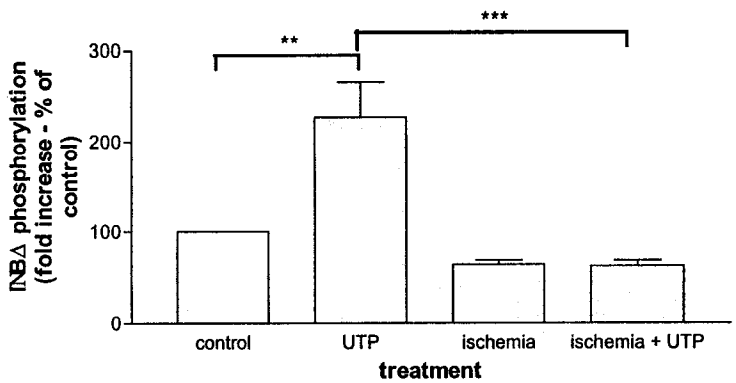

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response observed (phosphorylation of protein kinases) during ischemia with/without UTP was induced by the activation of glutamate receptors. First, we did control experiments to determine if glutamate could activate one of the protein kinases studied (MAPKs or Akt/PKB), time course experiments of glutamate-stimulated phosphorylation of MAPKs and/or Akt/PKB. For these assays, 100 μM glutamate was used as a maximal dose as it has been used in other reports [288]. As shown in figures 3.40 and 3.41, ERK1/2 and p38 were transciently phosphorylated with a maximal time of activation of 10 min. Contrary to the other protein kinases, Akt/PKB phosphorylation at Ser 243 (the site that could be phosphorylated by a signal transduction induced by glutamate) [289] is not as strong as the other protein kinases. However, addition of 100 μM of glutamate did not induce a phosphorylation of JNK (figure 3.40D). Addition of 1 mM of glutamate increases the phosphorylation of JNK transciently with a maximal time of activation between 5 and 10 min (figure 3.40E). This increase in phosphorylation is observed in p46JNK isoform, however there is no significant increase in phosphorylation of p54JNK isoform during this time course (figure 3.41D, 3.41E).

Knowing that glutamate can activate ERK1/2, p38, JNK and Akt, some antagonists of glutamate receptors were used at different doses to establish the effective concentration and if they antagonize the response elicited by glutamate as a further control. The cells were incubated with five types of glutamate antagonists, each representing a type of receptor antagonist that could be implicated in a signal. The cells were incubated with 100 μM DNQX (a potent competitive non-NMDA glutamate receptor) [290], [291], 300 μM trans(+/-) (1R,2R)-1—amino-1-carboxycyclopentane-2-acetic acid (competitive mGlu2 receptor antagonist) [292], 50 μM D(-)-2-amino-5-phosphonon pentanoic acid (competitive NMDA receptor antagonist) [293], and 100 μM L(+)-2-amino-(3)-phosphonopropionic acid (metabotropic glutamate receptor antagonist) [294] for 30 min and then stimulated with 100 μM glutamate for 10 min. As shown in figure 3.42 and 3.43, ERK1/2 phosphorylation induced by glutamate was decreased by all the glutamate receptor antagonists.

Knowing that the antagonists inhibited the response elicited by glutamate, the cells were incubated during the 4 hrs of OGD treatment with these antagonists: DNQX (a

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Figure 3.40 Time course of glutamate-stimulated protein kinase phosphorylation in 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 transfected were treated with 100μM and 1 mM glutamate for the indicated period of time. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using (A) anti phospho specific ERK1/2 antibody and blots were stripped and reprobed with anti ERK1/2 antibody to confirm uniform loading, (B) anti phospho specific p38 antibody and blots were stripped and reprobed with anti p38 antibody, (C) anti phospho Akt antibody and blots were stripped and reprobed with anti Akt antibody and (D-E) anti phospho specific JNK (p54/p46) antibody and blots were stripped and reprobed with anti JNK antibody. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 4 independent experiments.

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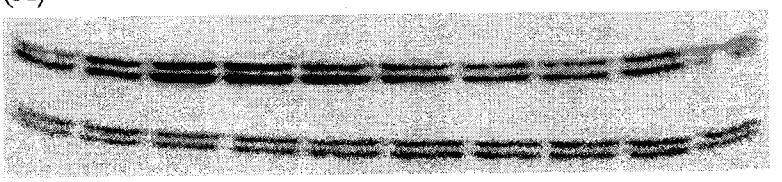
(A)

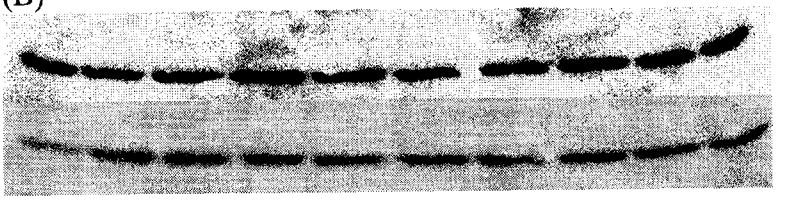


pERK1/2

ERK1/2

0 2 5 10 15 20 25 30 45 60 Time (min)

(B)

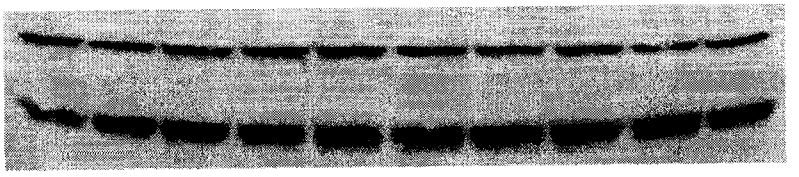


pp38

p38

0 2 5 10 15 20 25 30 45 60 Time (min)

(C)

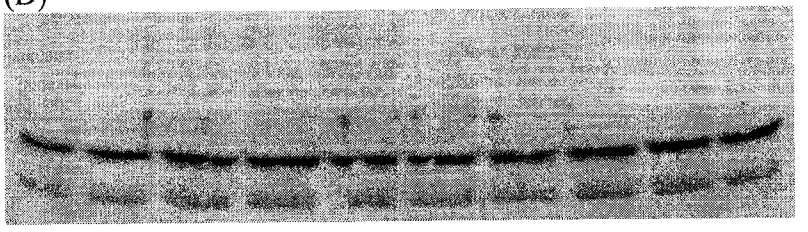


pAkt

Akt

0 2 5 10 15 20 25 30 45 60 Time (min)

(D)



pp54JNK

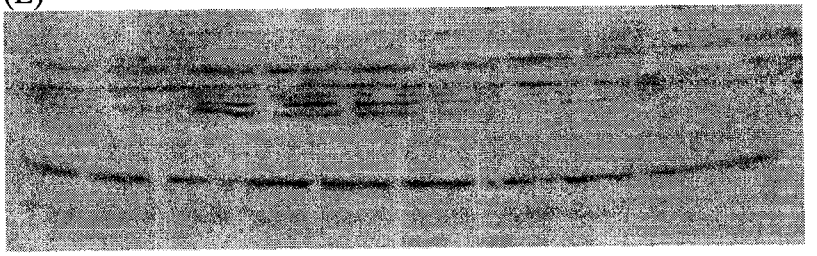
pp46JNK

p54JNK

p46JNK

0 2 5 10 15 20 25 30 45 60 Time (min)

(E)



pp54JNK

pp46JNK

p54JNK

p46JNK

0 2 5 10 15 20 25 30 45 60 Time (min)

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Figure 3.41 Activation of ERK1/2, p38, Akt and JNK by glutamate in 1321N1-hP2Y_2 transfected cells. Data from figure 3.40 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of ERK1/2, p38, Akt and JNK were calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 4 independent experiments. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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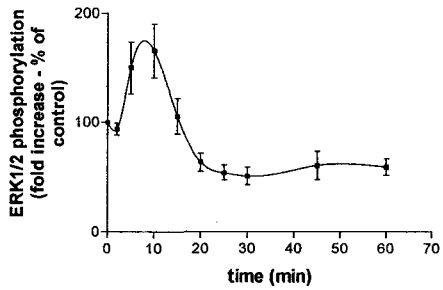

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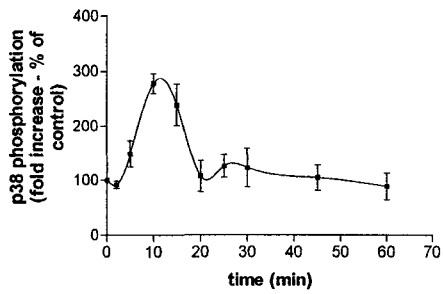

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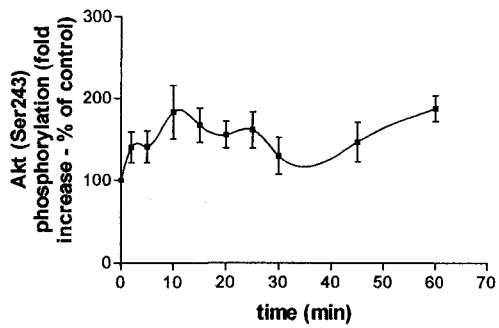

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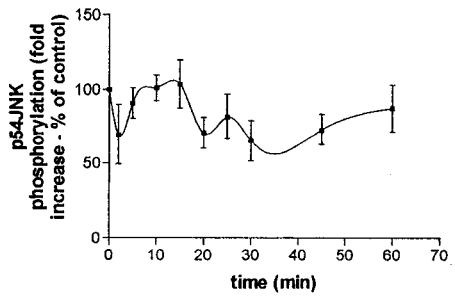

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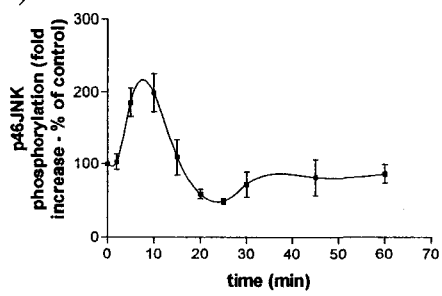

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Figure 3.42. Effect of glutamate receptor antagonists on ERK1/2 phosphorylation induced by glutamate in 1321N1-hP2Y_2 transfected cells. 1321N1-hP2Y_2 were pretreated with 100 μM DNQX (DN), 300 μM trans(+/-) (1R,2R)-1—amino-1-carboxycyclopentane-2-acetic acid (trans), 50 μM D(-)-2-amino-5-phosphonon pentanoic acid (D(-)), and 100 μM L(+)-2-amino-(3)-phosphonopropionic acid (L(+)) for 30 min and then stimulated with 100 μM glutamate (G) for 10 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using anti phospho specific ERK1/2 antibody and blot was stripped and reprobed with anti ERK1/2 antibody. Protein was detected by chemiluminiscence as described in Chapter II. Results shown are representative of 3 independent experiments.

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Figure 3.43. Effect of various specific glutamate receptor antagonists on the glutamate-induced phosphorylation of ERK1/2. 1321N1-hP2Y_2 were pretreated with 100 μM DNQX (DN), 300 μM trans(+/-) (1R,2R)-1—amino-1-carboxycyclopentane-2-acetic acid (trans), 50 μM D(-)-2-amino-5-phosphonon pentanoic acid (D(-)), and 100 μM L(+)-2-amino-(3)-phosphonopropionic acid (L(+)) for 30 min and then stimulated with 100 μM glutamate (G) for 10 min. Data from figure 3.42 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of ERK1/2 was calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM form at least 3 independent experiments. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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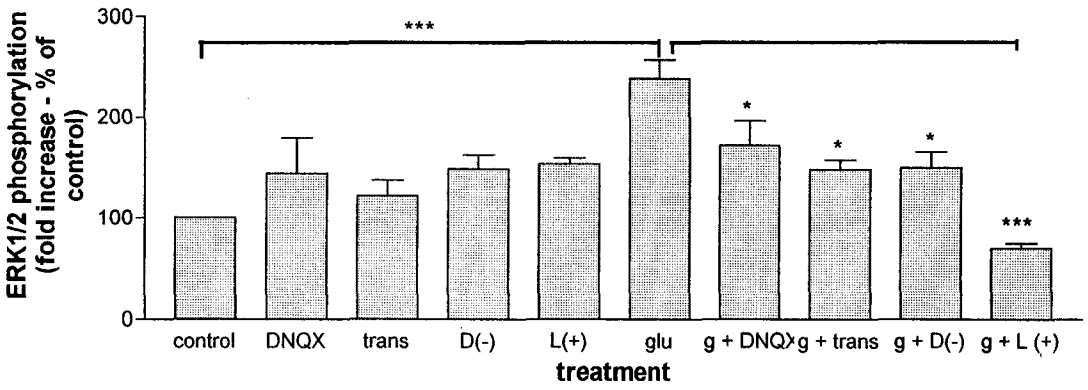

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potent competitive non-NMDA glutamate receptor used at 100μM [290], [291], trans(+/-) (1R,2R)-1—amino-1-carboxycyclopentane-2-acetic acid-competitive mGlu2 receptor antagonist used at 300μM [292], D(-)-2-amino-5-phosphonon pentanoic acid-competitive NMDA receptor antagonist used at 50μM [293] and L(+)-2-amino-(3)-phosphonopropionic acid- metabotropic glutamate receptor antagonist used ta 100μM [294]. As shown in figures 3.44 and 3.45, neither ERK1/2 nor Akt phosphorylation was affected by the incubation with the glutamate antagonists in the absence or presence of UTP incubation. The p38 phosphorylation was slightly affected by the glutamate antagonists during the OGD in the absence and presence of UTP. In the absence of UTP, p38 phosphorylation was affected by the glutamate antagonist L(+), but after UTP addition, p38 phosphorylation was affected by D(-) and L(+) suggesting that the NMDA and mGluR receptors may be regulating this phosphorylation. Similar results were obtained for JNK, in which p54JNK phosphorylation was decreased in OGD (absence of UTP) by D(-), however, after UTP addition it was not affected by any of the glutamate antagonists suggesting that NMDA receptor could be modulating the phosphorylation of p54JNK isoform during the OGD treatment. The p46JNK phosphorylation was affected by almost all the glutamate antagonists but the greatest effect was with L(+) according to the ANOVA analysis. None of the glutamate antagonists inhibited UTP-mediated p46JNK phosphorylation.

Because the phosphorylation of these protein kinases is not inhibited significantly or completly by the glutamate antagonists and we know that ATP is released during ischemia [14], the phosphorylation of these protein kinases during ischemia could be due to the released of ATP.

D. Apyrase treatment

Treatment with apyrase (which hydrolyse ATP to ADP and then to AMP) helps us to determine if the activation of protein kinases during ischemia is due to the release of nucleotides such as ATP before its hydrolysis and the activation of the $P2Y_2$ receptor. The cells were incubated with 20 u/ml apyrase, an ecto-ATPase [295], for the 4hrs of OGD. As shown in figure 3.46 and 3.47, no apyrase effect was seen in ERK1/2 and Akt

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Figure 3.44 Effect of glutamate receptor antagonists in protein kinase phosphorylation phosphorylation during 4hrs of OGD, an *in vitro* ischemic treatment, and by UTP in human $P2Y_2$-1321N1 transfected cells. 1321N1-h$P2Y_2$ transfected cells were pretreated with 100 μM DNQX (DN), 300 μM trans(+/-) (1R,2R)-1—amino-1-carboxycyclopentane-2-acetic acid (trans), 50 μM D(-)-2-amino-5-phosphonon pentanoic acid (D(-)), and 100 μM L(+)-2-amino-(3)-phosphonopropionic acid (L(+)), during the 4hrs of OGD. Then, the cells were stimulated with 100 μM UTP for 15 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using (A) anti phospho specific ERK1/2 antibody and blots were stripped and reprobed with anti ERK1/2 antibody to confirm uniform loading, (B) anti phospho specific p38 antibody and blots were stripped and reprobed with anti p38 antibody, (C) anti phospho specific JNK (p54/p46) antibody and blots were stripped and reprobed with anti JNK antibody (p54/p46) and (D) anti phospho specific Akt antibody and blots were stripped and reprobed with anti Akt antibody. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 5 independent experiments.

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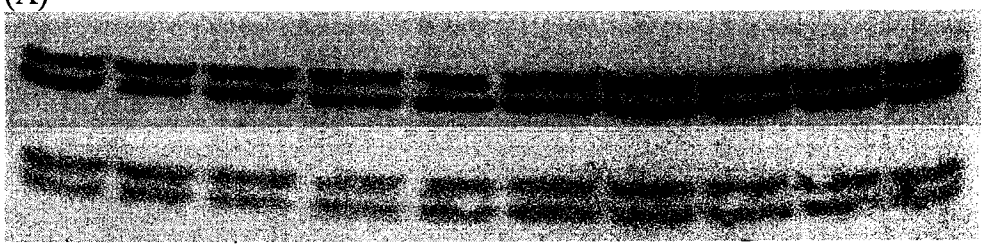
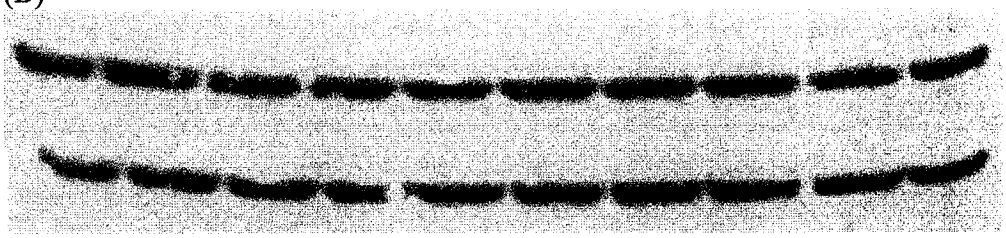
(A)



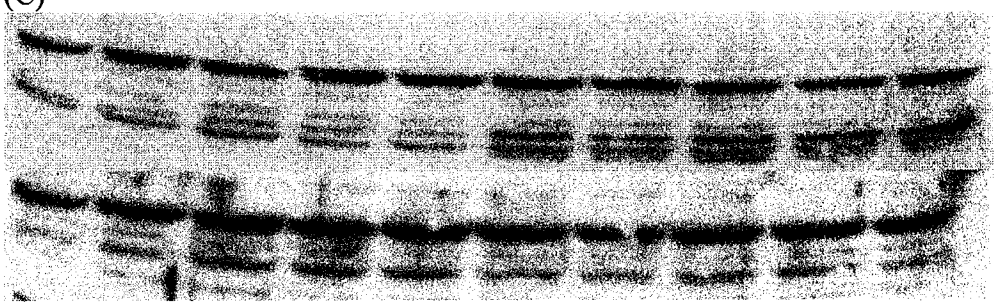
0 DN trans D(-) L(+) U U+DN U+trans U+D(-) U+L(+) treatment

(B)



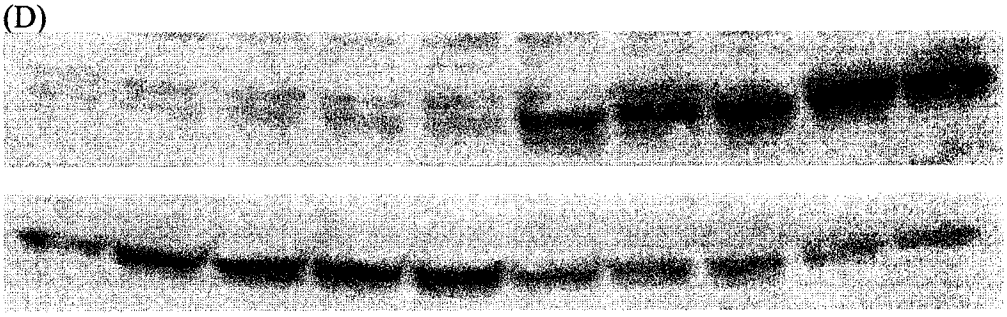
0 DN trans D(-) L(+) U U+DN U+trans U+D(-) U+L(+) treatment

(C)



0 DN trans D(-) L(+) U U+DN U+trans U+D(-) U+L(+) treatment

(D)



0 DN trans D(-) L(+) U U+DN U+trans U+D(-) U+L(+) treatment

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Figure 3.45. Effects of various specific glutamate receptor antagonists during the 4hrs of OGD on the UTP-induced phosphorylation of proten kinases. 1321N1-hP2Y2 transfected cells were pretreated with 100 μM DNQX (DN), 300 μM trans(+/-) (1R,2R)-1—amino-1-carboxycyclopentane-2-acetic acid (trans), 50 μM D(-)-2-amino-5-phosphonon pentanoic acid (D(-)), and 100 μM L(+)-2-amino-(3)-phosphonopropionic acid (L(+)), during the 4hrs of OGD. Then, the cells were stimulated with 100 μM UTP for 15 min. Data from figure 3.44 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of ERK1/2, p38, JNK and Akt were calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 5 independent experiments. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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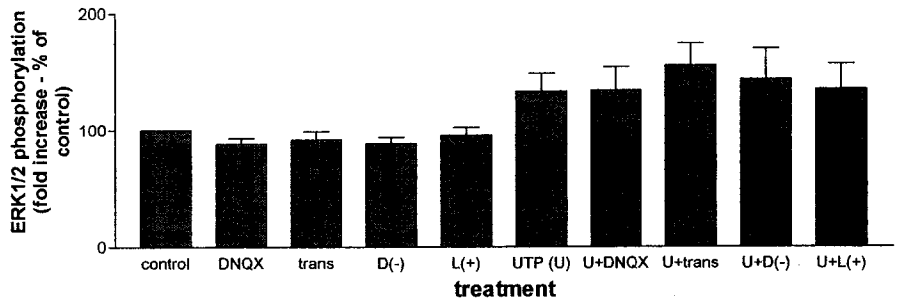

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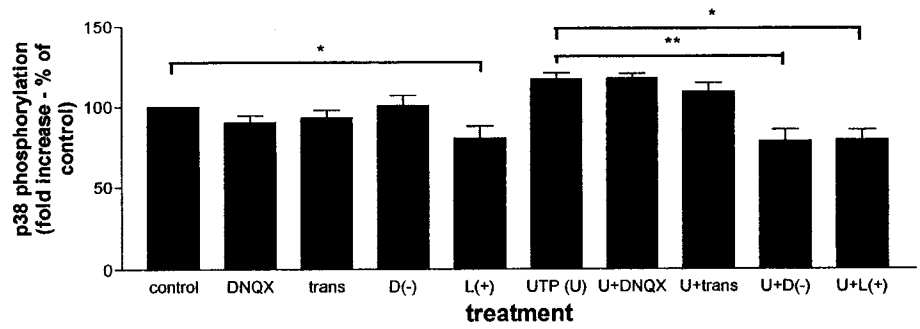

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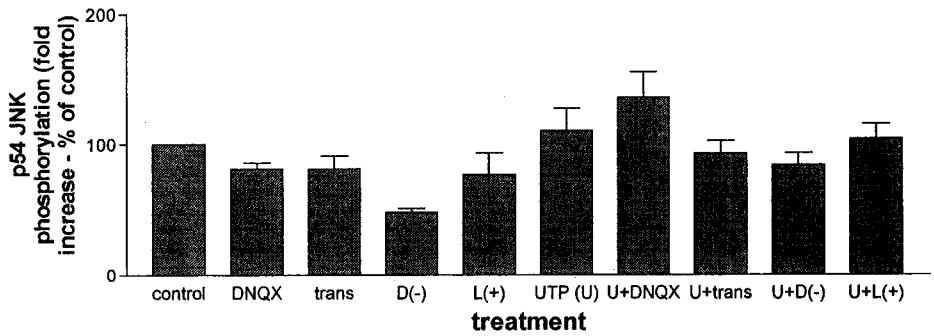

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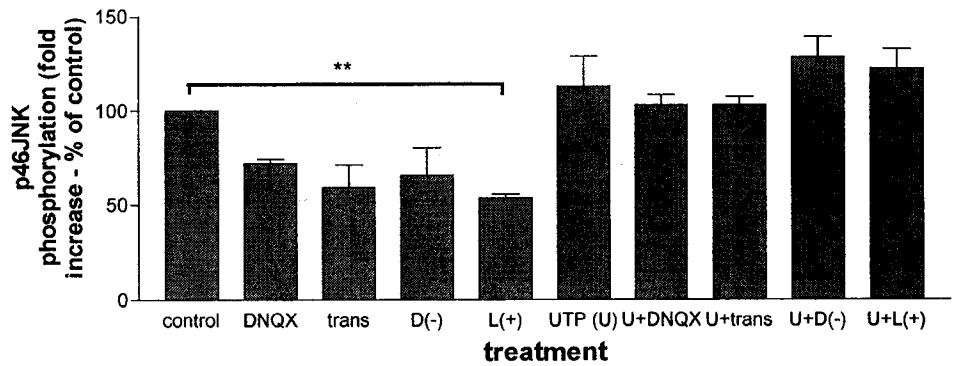

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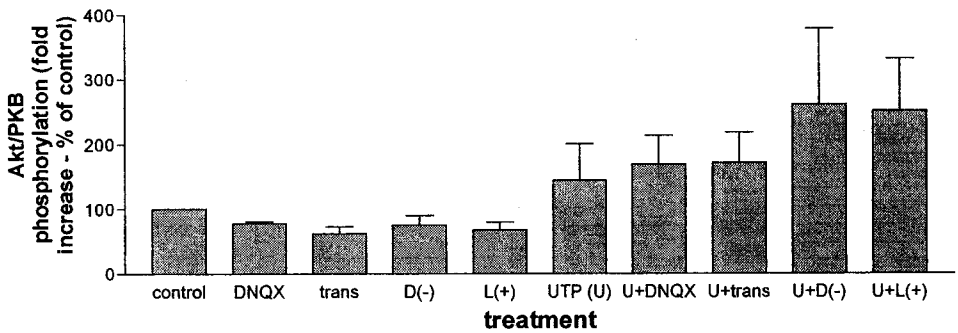

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phosphorylation, because at this point none of them are phosphorylated during the 4 hrs of OGD. However, p38 phosphorylation during ischemia decreased after treatment with apyrase suggesting that the activation of p38 during the 4 hrs of OGD were due to the release of ATP as a consequence of this type of stress and the activation of the $P2Y_2$ receptor. JNK phosphorylation during ischemia decreased slightly with apyrase treatment suggesting that this phosphorylation is due to the released of ATP.

E. Antagonist of P2 and A1 receptors

To confirm that the activation of ERK1/2, p38, JNK and Akt were due to the realease of ATP and the activation of $P2Y_2$ receptor during the OGD treatment, the cells were incubated with P2 antagonists during the 4 hrs OGD: 50 μM PPADS [296], 100 μM suramin [102] and 100 μM reactive blue [102]. As shown in figures 3.48 and 3.49, suramin and reactive blue attenuated the phosphorylation/activation of the ERK1/2 and p38. However, JNK phosphorylation was not affected by the P2 antagonists during the ischemia without UTP treatment. Akt was not phosphorylated during ischemia and is not affected by the antagonists. As expected, suramin and reactive blue decreased the phosphorylation of the protein kinases after the UTP was added, except p54JNK. PPADS decreased slightly the phosphorylation of ERK1/2 and p46JNK only after UTP addition, but not during OGD. These results are comparable with ones reported by Neary by the treatment of stress injury implicating P2 receptors in this response [296]. These results indicate that the response observed during the OGD is due to the ATP release and principally by its subsequent $P2Y_2$ receptor activation.

To verify that the results are due to antagonisms of the reagents, and not an artifact, the same experiment was performed in control or normal conditions, where a similar response was observed for ERK1/2 (figure 3.50 and 3.51). The antagonism by suramin and reactive blue was observed after the addition of UTP to the cells in each protein kinase, except for p54JNK. These results indicated and confirmed that the activation of these protein kinases is due to the activation of the $P2Y_2$ receptor.

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Figure 3.46. Effect of apyrase in the protein kinase phosphorylation during 4hrs of OGD, an *in vitro* ischemic treatment, and by UTP in human $P2Y_2$-1321N1 transfected cells. 1321N1-h$P2Y_2$ transfected cells were pretreated with 20U/ml apyrase during the 4 hrs of OGD. Then, the cells were stimulated with 100 μM UTP for 15 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using (A) anti phospho specific ERK1/2 antibody and blots were stripped and reprobed with anti ERK1/2 antibody to confirm uniform loading, (B) anti phospho specific p38 antibody and blots were stripped and reprobed with anti p38 antibody, (C) anti phospho specific JNK (p54/p46) antibody and blots were stripped and reprobed with anti JNK antibody (p54/p46) and (D) anti phosphor specific Akt antibody and blots were stripped and reprobed with anti Akt antibody. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 3 independent experiments. Apyrase (A), ischemia (I), UTP (U)

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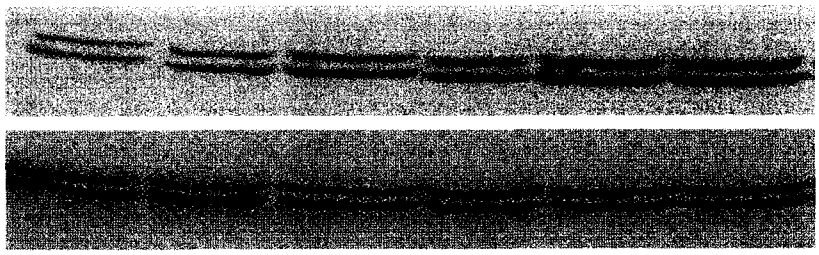
(A)



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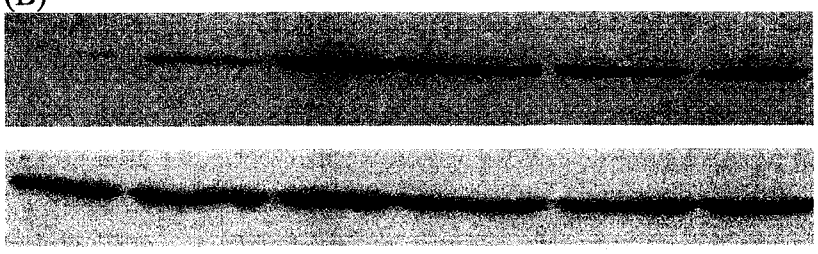

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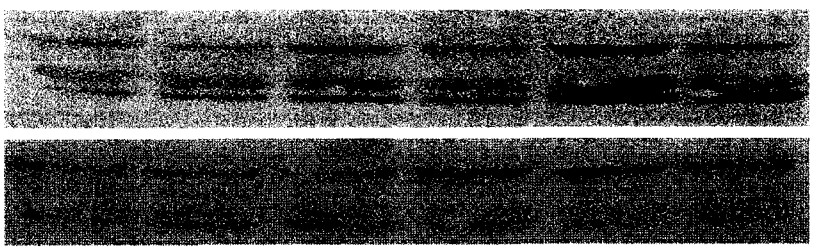

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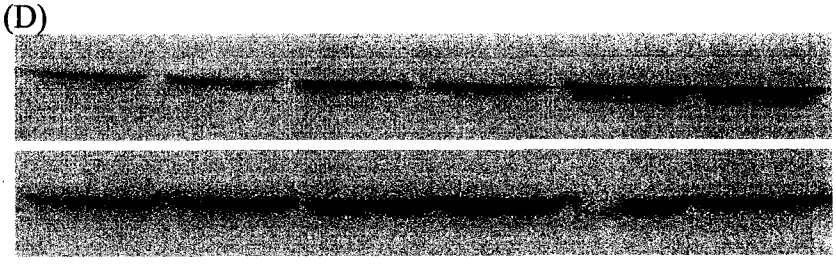

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Figure 3.47. Effects of apyrase during the 4hrs of OGD on the UTP-induced phosphorylation of proten kianses. 1321N1-hP2Y_2 transfected cells were pretreated with 20 U/ml apyrase during the 4hrs of OGD. Then, the cells were stimulated with 100 μM UTP for 15 min. Data from figure 3.46 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of ERK1/2, p38, JNK and Akt were calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 3 independent experiments. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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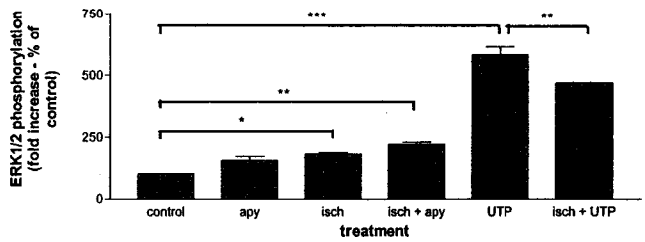
(A)



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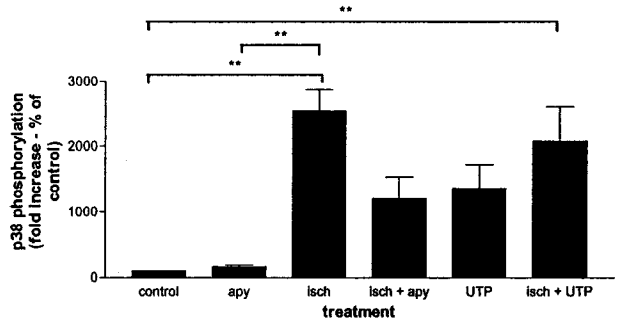

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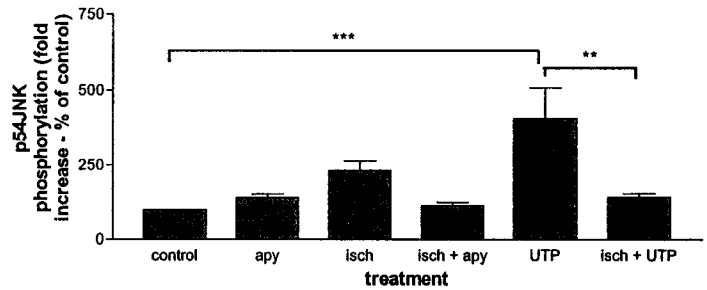

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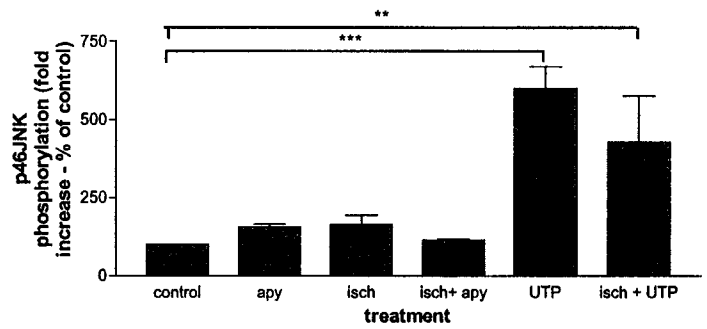

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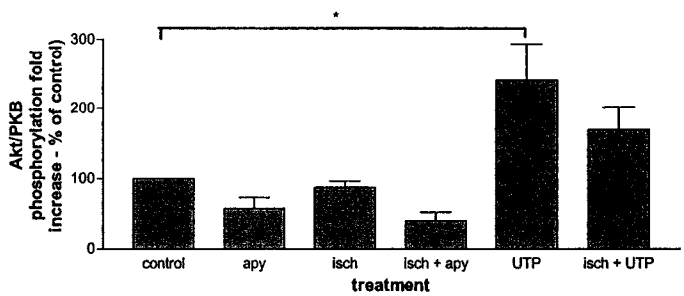

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Figure 3.48. Effect of P2 receptor antagonists in the protein kinases phosphorylation during 4hrs of OGD, an *in vitro* ischemic treatment, and by UTP in human $P2Y_2$-1321N1 transfected cells. 1321N1-h$P2Y_2$ transfected cells were pretreated with 50 μM PPADS (P), 100 μM suramin (S) and 100 μM reactive blue (RB) during the 4hrs of OGD. Then, the cells were stimulated with 100 μM UTP (U) for 15 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using (A) anti phospho specific ERK1/2 antibody and blots were stripped and reprobed with anti ERK1/2 antibody to confirm uniform loading, (B) anti phospho specific p38 antibody and blots were stripped and reprobed with anti p38 antibody, (C) anti phospho specific JNK (p54/p46) antibody and blots were stripped and reprobed with anti JNK antibody (p54/p46) and (D) anti phospho specific Akt antibody and blots were stripped and reprobed with anti Akt antibody. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 3 independent experiments.

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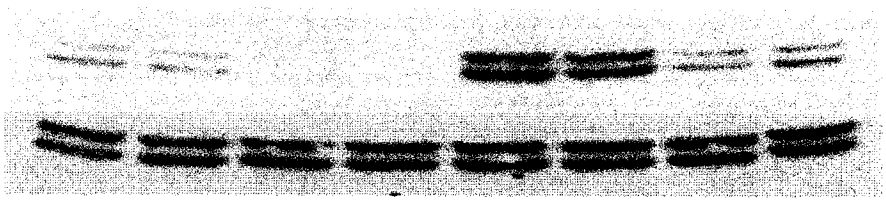
(A)



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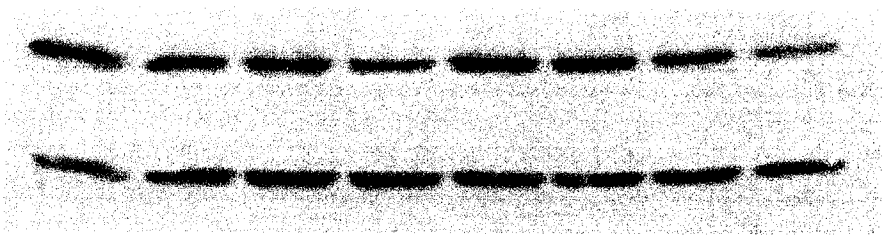

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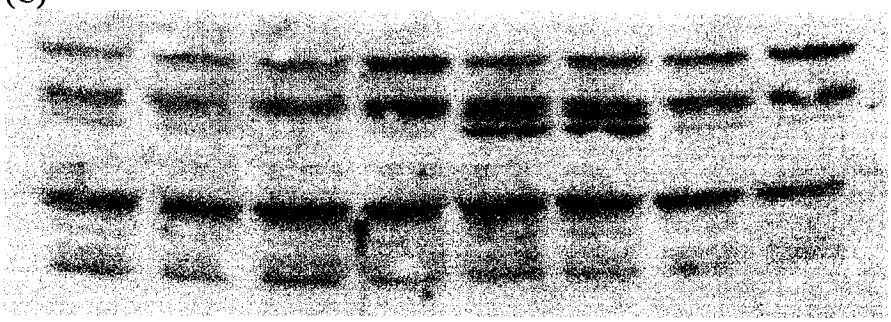

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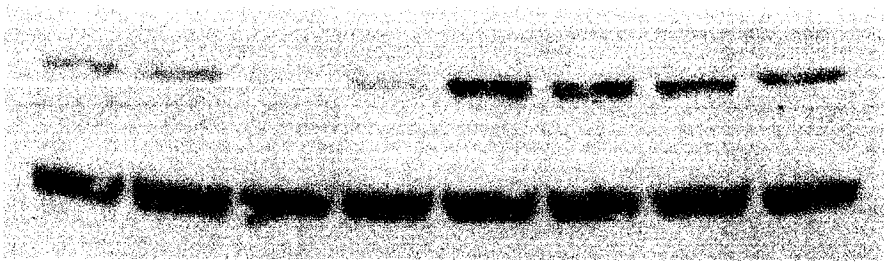

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Figure 3.49. Effects of various P2 receptor antagonists during 4hrs of OGD on the UTP-induced phosphorylation of proten kinases. 1321N1-hP2Y_2 transfected cells were pretreated with 50 μM PPADS (P), 100 μM suramin (S) and 100 μM reactive blue (RB) during the 4hrs of OGD. Then, the cells were stimulated with 100 μM UTP (U) for 15 min. Data from figure 3.48 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of ERK1/2, p38, JNK and Akt were calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 3 independent experiments. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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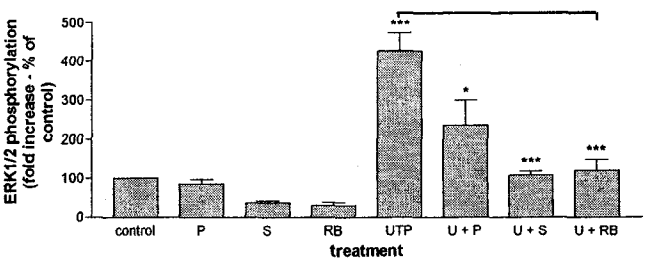

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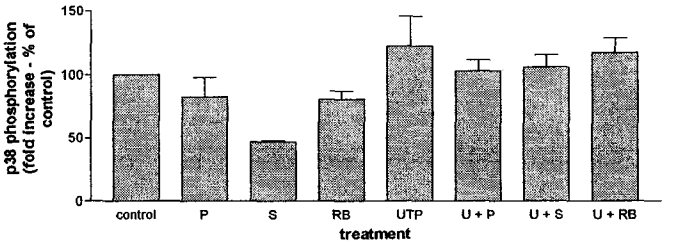

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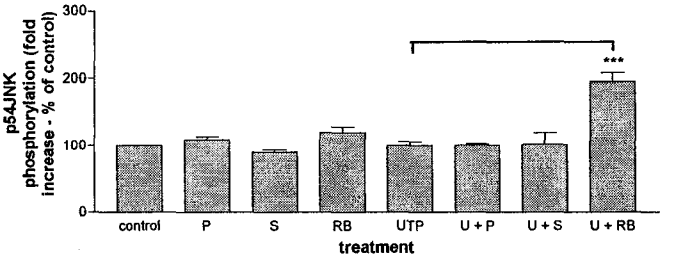

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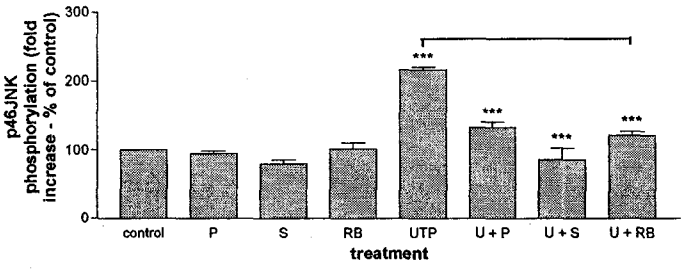

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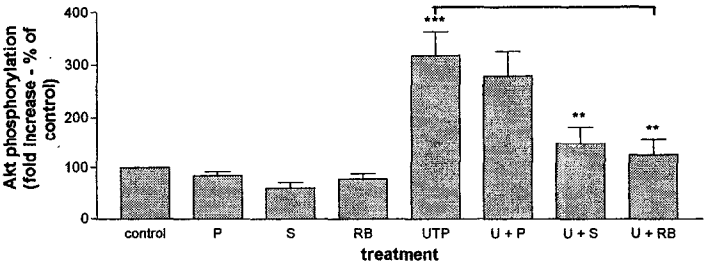

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Figure 3.50 Effect of P2 receptor antagonists in the phosphorylation of protein kinases in normal conditions. 1321N1-hP2Y_2 transfected cells were pretreated with 50 μM PPADS (P), 100 μM suramin (S) and 100 μM reactive blue (RB) during normal conditions (4 hrs). Then, the cells were stimulated with 100 μM UTP (U) for 15 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using (A) anti phospho specific ERK1/2 antibody and blots were stripped and reprobed with anti ERK1/2 antibody to confirm uniform loading, (B) anti phospho specific p38 antibody and blots were stripped and reprobed with anti p38 antibody, (C) anti phospho specific JNK (p54/p46) antibody and blots were stripped and reprobed with anti JNK antibody (p54/p46) and (D) anti phosphor specific Akt antibody and blots were stripped and reprobed with anti Akt antibody. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 3 independent experiments.

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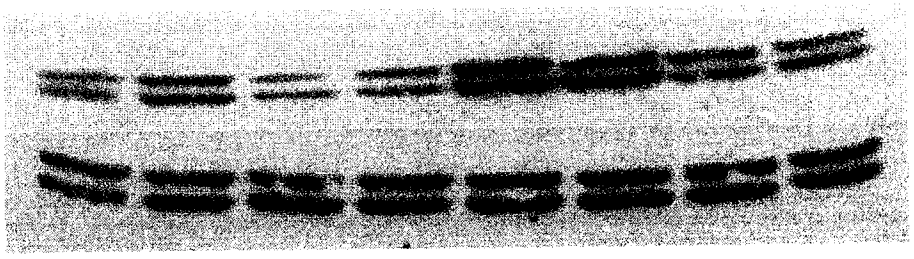
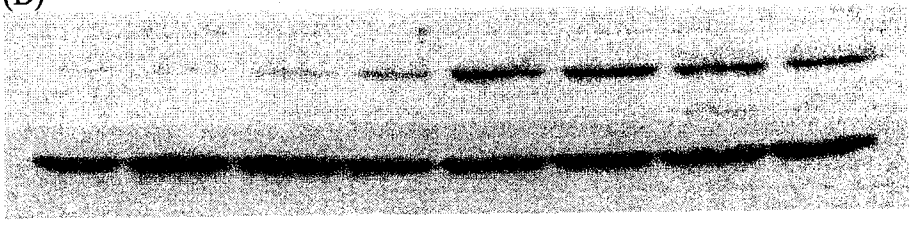
(A)



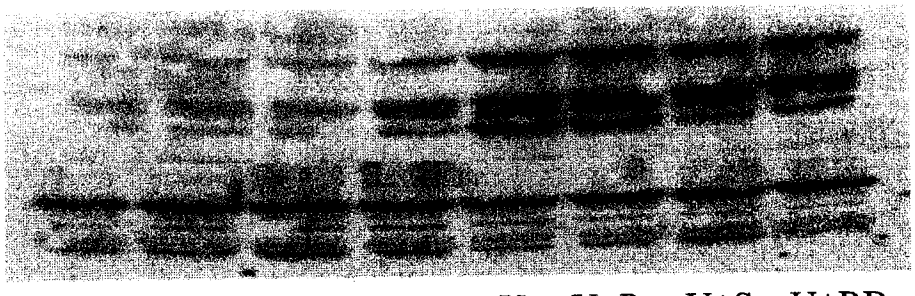
0 P S RB U U+P U+S U+RB treatment

(B)



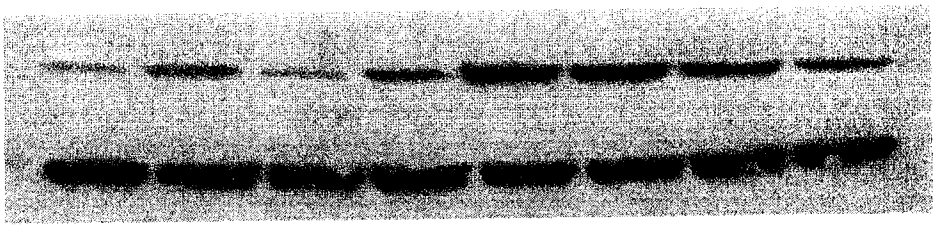
O P S RB U U+P U+S U+RB treatment

(C)



0 P S RB U U+P U+S U+RB treatment

(D)



0 P S RB U U+P U+S U+RB treatment

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Figure 3.51 Effects of various P2 receptor antagonists during normal conditions on the UTP-induced phosphorylation of proten kinases. 1321N1-hP2Y_2 transfected cells were pretreated with 50 μM PPADS (P), 100 μM suramin (S) and 100 μM reactive blue (RB) during normal conditions (4hrs). Then, the cells were stimulated with 100 μM UTP (U) for 15 min. Data from figure 3.50 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of ERK1/2, p38, JNK and Akt were calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 3 independent experiments. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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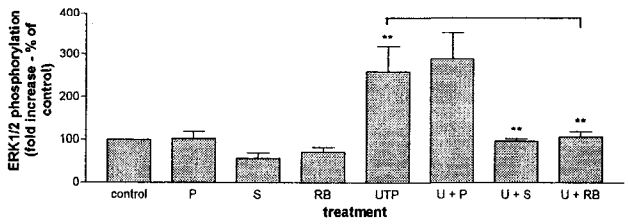
(A)



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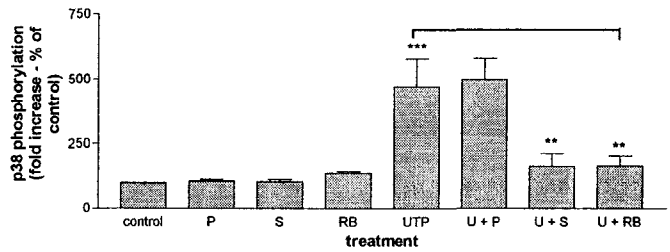

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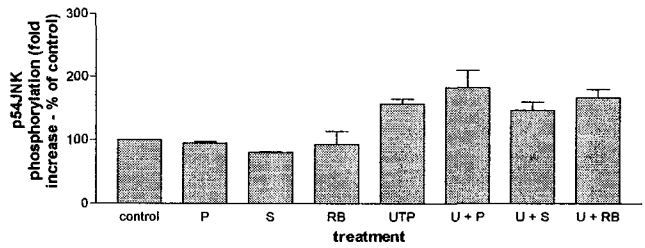

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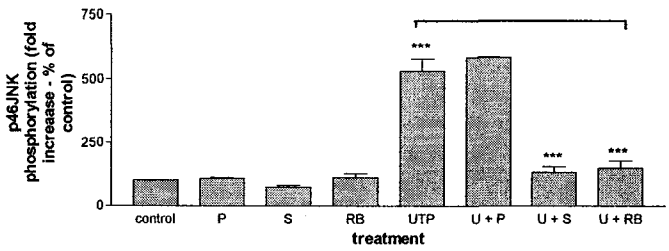

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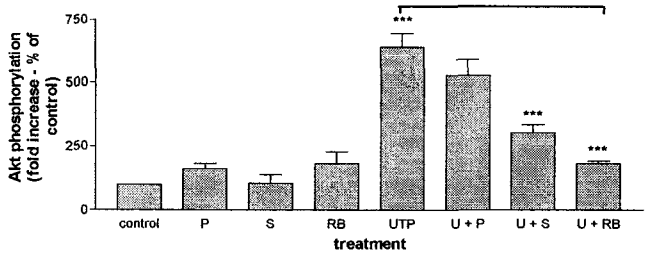

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Because the adenosine receptor A_1 is expressed in 1321N1 cells [297], and adenosine can arrive to the extracellular medium through its released directly from the cell or hydrolysis from ATP during ischemia, the cells were incubated during the 4 hrs of OGD with the A_1 antagonist, DPCPX (8-cyclopentyltheophylline) at 1μM [298], [299], to determine if adenosine could be influencing the response or the phosphorylation of these protein kinases during the ischemia. As shown in figure 3.52 and 3.53, DPCPX did not inhibit or affect the phosphorylation of ERK1/2, p38 or Akt during ischemia and after addition of UTP. However, and surprisingly, p54JNK phosphorylation decreased with DPCPX incubation suggesting that the activation of JNK during ischemia could be due to the adenosine released or hydrolyzed from the ATP released.

F. Transcription factors phosphorylation - 4 hrs OGD

Because these protein kinases studied regulate survival and apoptosis signal through the phosphorylation of transcription factors which in turn regulate gene expression, a determination of the phosphorylation of several transcription factors during ischemia were performed. The same conditions employed to study protein kinases phosphorylation (4 hrs of OGD) was used to study transcription factors phosphorylation. Incubation with 100 μM UTP for 15min after 4 hrs of OGD shows the following results. Figure 3.54A and 3.55A show a slightly increase in Elk1 phosphorylation during ischemia. After UTP addition, a more intense phosphorylation is observed which is comparable with the control cells after UTP addition. These results correlate with results observed of ERK1/2 during the 4hrs of OGD, and where Elk1 is one of the substrates of ERK1/2.

Another transcription factor is ATF-2. Figure 3.54B and 3.55B show that an increase in ATF-2 phosphorylation is observed in ischemic cells as well as with UTP incubation in control and after 4 hrs of OGD. These results correlate with ones obtained in ischemic conditions for p38 and JNK phosphorylation, but for p38 (figures 3.34 and 3.35) and as shown previously, these protein kinases are responsible of the phosphorylation of ATF2 (figures 3.7 and 3.8). One more transcription factor modulated

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Figure 3.52 Effect of A1 receptor antagonist in protein kinases phosphorylation during 4hrs of OGD, an *in vitro ischemic* treatment, and by UTP in human $P2Y_2$-1321N1 transfected cells. 1321N1-h$P2Y_2$ transfected cells were pretreated with 1μM DPCPX during the 4hrs of OGD. Then, the cells were stimulated with 100 μM UTP (U) for 15 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using (A) anti phospho specific ERK1/2 antibody and blots were stripped and reprobed with anti ERK1/2 antibody to confirm uniform loading, (B) anti phospho specific p38 antibody and blots were stripped and reprobed with anti p38 antibody, (C) anti phospho specific JNK (p54/p46) antibody and blots were stripped and reprobed with anti JNK antibody (p54/p46) and (D) anti phosphor specific Akt antibody and blots were stripped and reprobed with anti Akt antibody. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 4 independent experiments.

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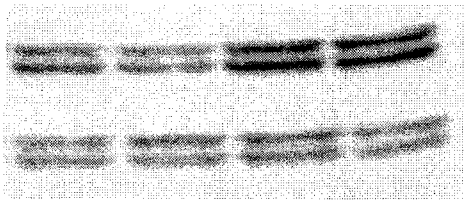
(A)

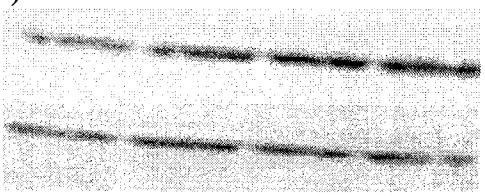


pERK1/2

ERK1/2

O DPCPX UTP U+DPCPX treatment

(B)

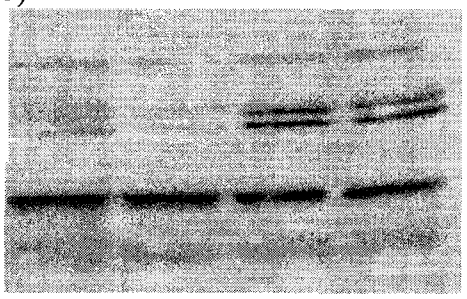


pp38

p38

O DPCPX UTP U+DPCPX treatment

(C)



pp54JNK

pp46JNK

p54JNK

p46JNK

O DPCPX UTP U+DPCPX treatment

(D)

pAkt

Akt

O DPCPX UTP U+DPCPX treatment

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Figure 3.53 Effects of A1 receptor antagonist during the 4hrs of OGD on the UTP-induced phosphorylation of proten kinases. 1321N1-hP2Y_2 transfected cells were pretreated with 1μM DPCPX during the 4hrs of OGD. Then, the cells were stimulated with 100 μM UTP for 15 min. Data from figure 3.42 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of ERK1/2, p38, JNK and Akt were calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 4 independent experiments. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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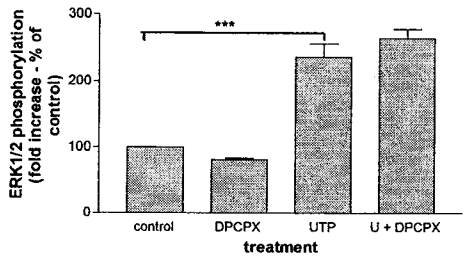

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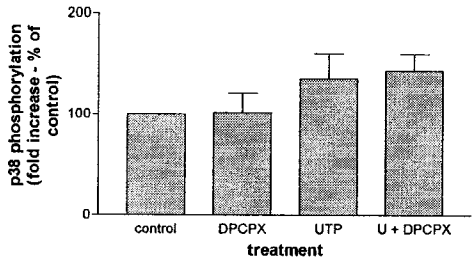

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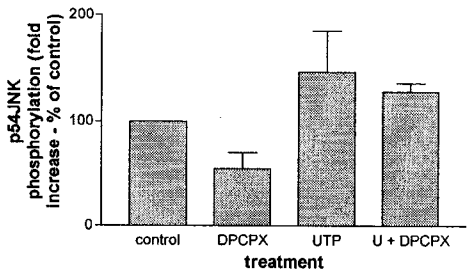

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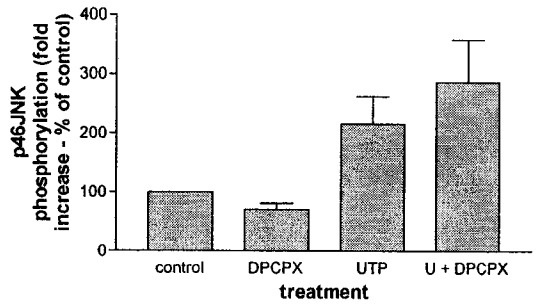

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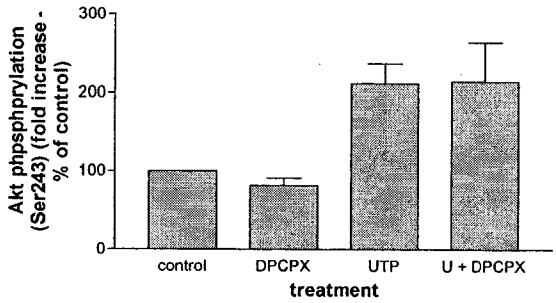

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by JNK is c-Jun at Ser 63/73. During ischemia, an increase in cJun Ser63/73 phosphorylation is observed during ischemia as shown in figure 3.54C, 3.55C and 3.55D. With UTP incubation after 4hrs of OGD, the phosphorylation was more magnified which correlate with the results obtained of JNK in ischemic conditions (figures 3.34 and 3.35).

The other transcription factor studied was c-Myc. Although c-Myc was not phosphorylated in ischemic conditions, a slightly increase in its phosphorylation is observed after UTP addition, which is more intense in control cells than in ischemic conditions (figure 3.54D and 3.55E). The response of c-Myc correlates with the one obtained of ERK1/2 (figure 3.34 and 3.35). Moreover, CREB and ATF1 were strongly phosphorylated after addition of UTP in control cells, and after 4 hrs of OGD, CREB and ATF1 phosphorylation were increased but CREB phosphorylation still more phosphorylated than ATF1 after addition of UTP in which ATF1 remains almost the same (figure 3.54E, 3.55F and 3.55G).

With respect of some substrates of PKB/Akt that are transcription factors, FKHR, AFX and NFκB were the transcription factors studied. As shown in figure 3.54F, 3.55H and 3.55I, FKHR and AFX were phosphorylated in control cells after addition of UTP. However, in ischemic cells, their phosphorylation decreased, although after addition of UTP a slightly increased was observed. These results are similar to the results observed of c-Myc and Akt. While for NFκB the response is somewhat different. NFκB was phosphorylated after addition of UTP in control cells, but in ischemic conditions, an increase in NFκB phosphorylation is observed. After UTP addition, NFκB phosphorylation remained the same as in ischemia treatment (figure 3.54G and 3.55J). This response is similar to the one observed by p38 in figure 3.34 and 3.35. These results suggest that UTP can modulate a survival signal and help the cells recover from the insult (ischemia) or can induce an apoptotic signal by the pathway that could include p38 and JNK.

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Figure 3.54. Transcription factors phosphorylation during 4hrs of OGD, an *in vitro* ischemic treatment, and by UTP in human $P2Y_2$-1321N1 transfected cells. 1321N1-h$P2Y_2$ transfected were exposed to oxygen-glucose deprivation (OGD) for 4 hrs of OGD. Then, the cells were stimulated with 100 μM UTP for 15 min. Cell extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western Blot analysis was performed using (A) anti phospho specific Elk1 antibody, (B) anti phospho specific ATF-2 antibody, (B) anti phospho specific c-Jun Ser63, (C) anti phospho specific c-Jun Ser73 antibody, (D) anti phospho specific c-Myc antibody, (E) anti phospho specific CREB antibody (F) anti phospho specific FKHR antibody and (G) anti phospho specific NFkB antibody. Blots were stripped and reprobed with each anti protein antibody. Proteins were detected by chemiluminiscence as described in Chapter II. Results shown are representative of 8 independent experiments.

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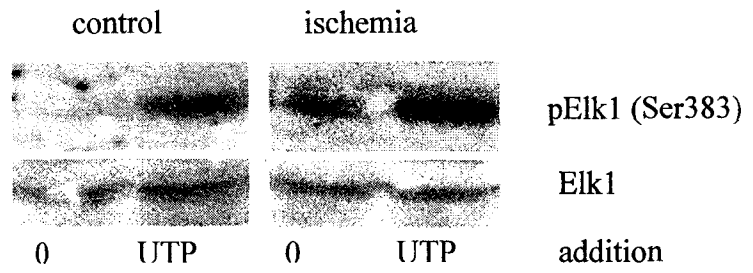
(A)



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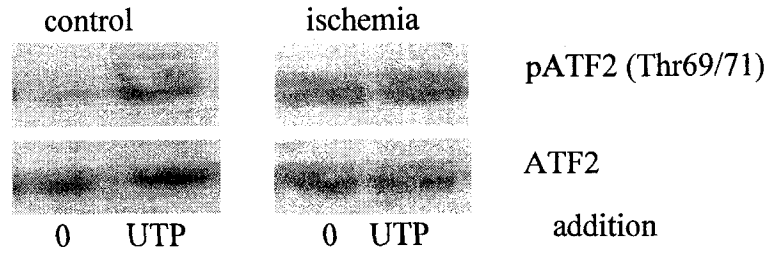

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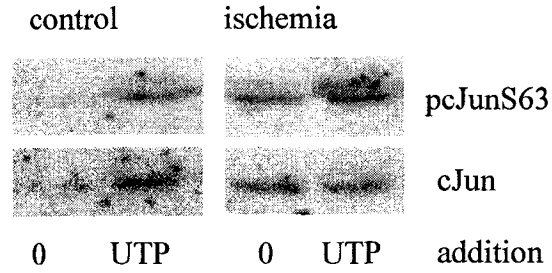

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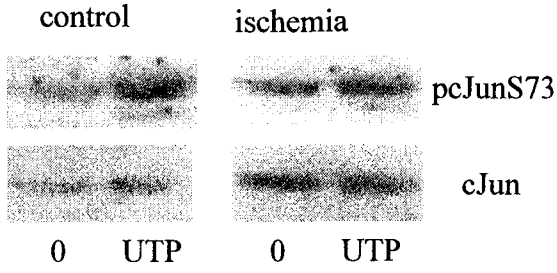

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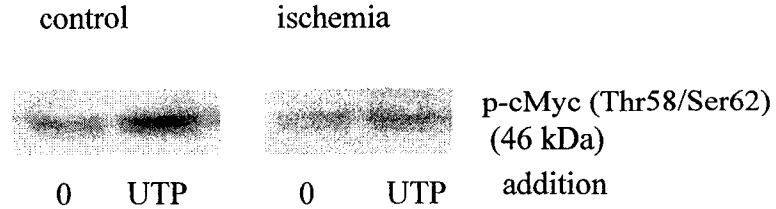

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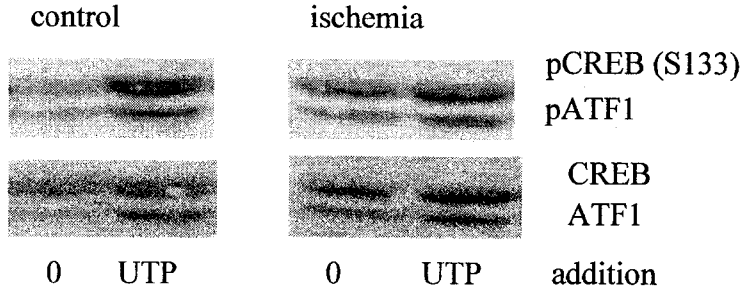
(F)



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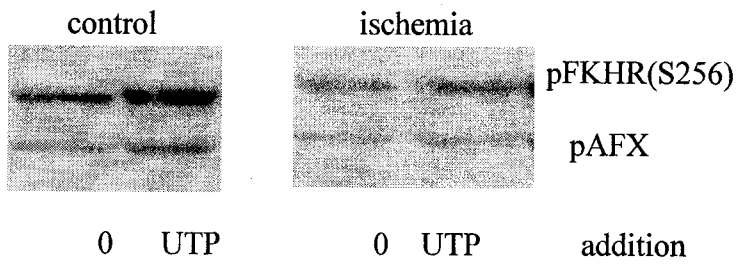

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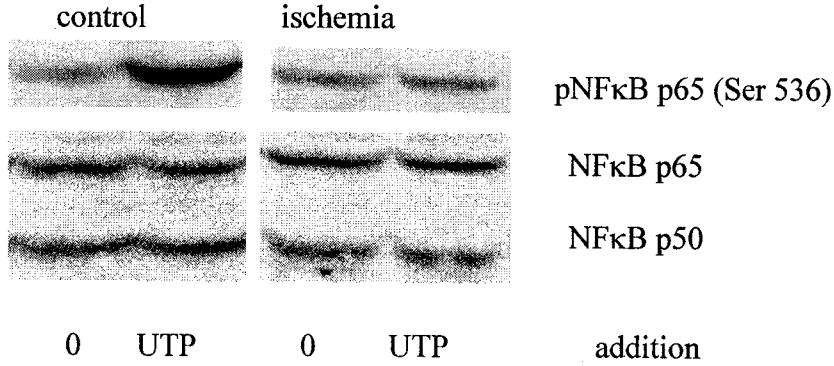

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Figure 3.55. Activation or inhibition of transcription factors in 4 hrs of OGD, an *in vitro* ischemic treatment, and by UTP in human $P2Y_2$-1321N1 transfected cells. 1321N1-h$P2Y_2$ transfected cells were exposed to oxygen-glucose deprivation (OGD) for 4 hrs of OGD. Then, the cells were stimulated with 100 μM UTP for 15 min. Data from figure 3.54 were digitized and quantitated using a Molecular Imager Model GS-525 (Bio Rad). The phosphorylation of Elk1, ATF2, c-Jun, c-Myc, CREB, FKHR and NFκB were calculated by normalizing the contents of phosphorylated protein kinase with the content of total (phosphorylated and nonphosphorylated) protein kinase. Data are expressed as the % of control untreated cells and are the mean +/- SEM from at least 8 independent experiments. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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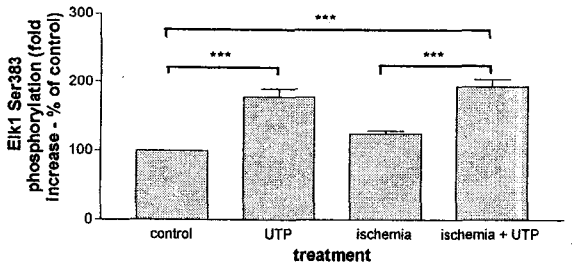
(A)



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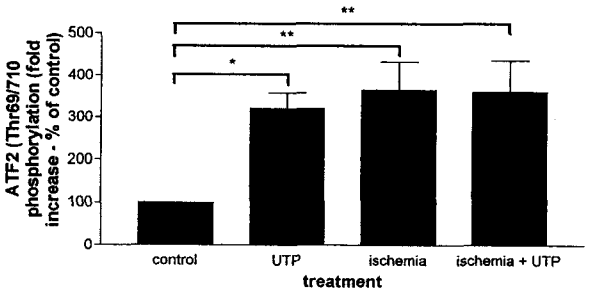

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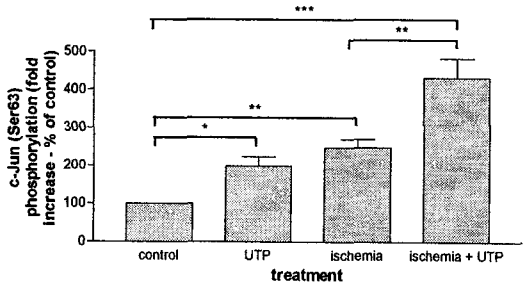

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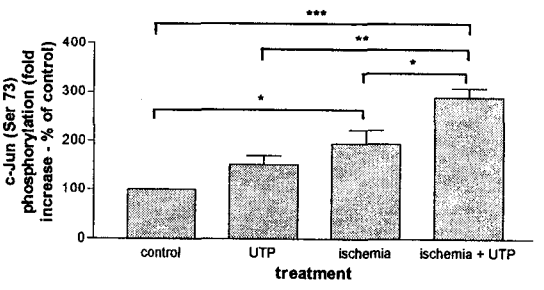

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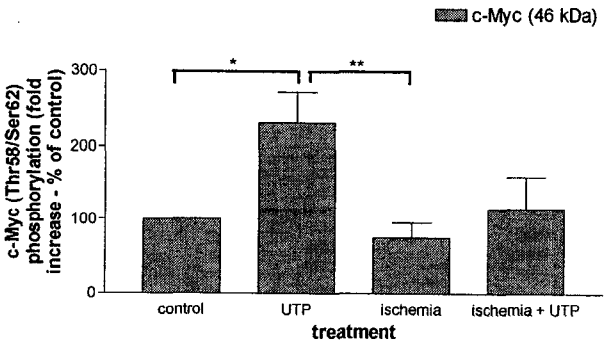

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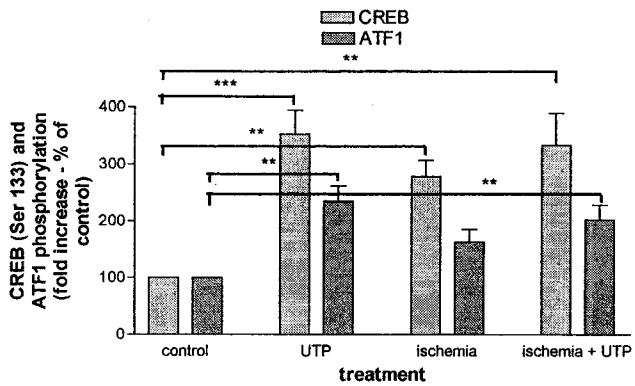

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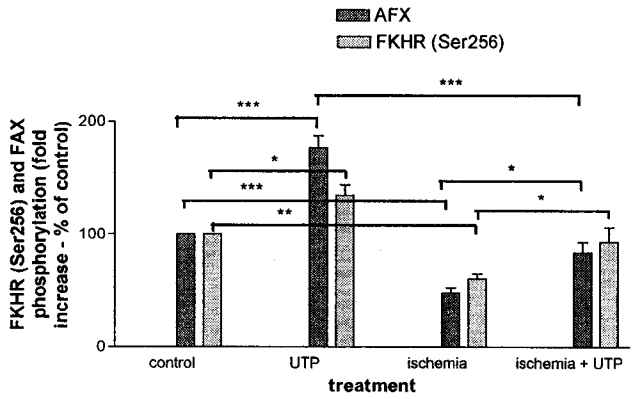

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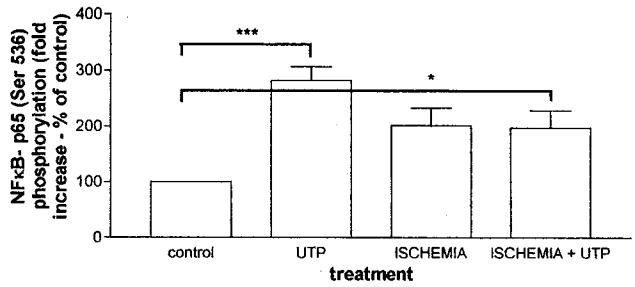

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G. Role of MAPK signaling pathway during OGD and glutamate and P2 receptors during OGD (cerebral ischemia) - Effetcs in LDH

The family of MAPK can modulate different cellular responses such as proliferation, apoptosis and survival signals. During the 4hrs OGD ERK1/2, p38, JNK but not Akt/PKB are activated. JNK and p38 has been implicated to apoptosis or death signals, but also to survival signals. To determine if these protein kinases mediate survival or death signals, incubation with 30 μM PD98059 [191], 10 μM SB203580 [262], 25 μM inhibitor of JNK II [263] and 100 nM wortmannin [246] during the 4 hrs of OGD was performed to see the effect of these protein kinase inhibitors on LDH released. As shown in figure 3.56, OGD caused LDH released as observed previously. The incubation of PD98059, SB203580 and inhibitor of JNK increased the amount of LDH released. However, wortmannin did not cause any change in LDH released. These results suggest that ERK1/2, p38 and JNK signaling pathways are mediating survival signals and their activation reduce or delay the death of the cells.

Knowing that glutamate and ATP are released during the OGD and they mediate the activation of these protein kinases, incubation of glutamate receptors antagonists and P2 receptors antagonists were performed during the 4 hrs OGD to see the effect of these antagonists in LDH released. As shown in figure 3.57, all the glutamate receptors antagonists decreased to control (basal) the LDH released caused by the 4hrs OGD. Reactive Blue and suramin also decreased the LDH released although the effect of suramin was incomplete. These results suggest that the LDH released during the 4hrs OGD is mediated by the release of glutamate and their subsequent receptors activation.

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Figure 3.56. Effect of protein kinase inhibitors in LDH released by an *in vitro* ischemic treatment in human $P2Y_2$-1321N1 transfected cells. 1321N1-h$P2Y_2$ transfected cells were exposed to 4 hrs oxygen glucose deprivation (OGD) and incubated with 30μM PD98059 (PD), 10 μM SB203580 (SB), 25 μM JNK inhibitor II (JI), and 100 nM wortmannin (W), during this period of time. After that treatment, the supernatant (100μl) was collected and LDH assay was performed as manufacturer's instructions (Roche Molecular-Boehringer Mannheim). The absorbance of the samples was performed in a Microplate Reader at a maximal wavelength λ of 490 nm. The results were reported as % of citotoxicity using the low control (cells without OGD treatment) and high control (cells disrupted with triton x-100 representing total LDH in the cells) using the equation as described in Chapter II. Results shown are representative of 6 independent experiments. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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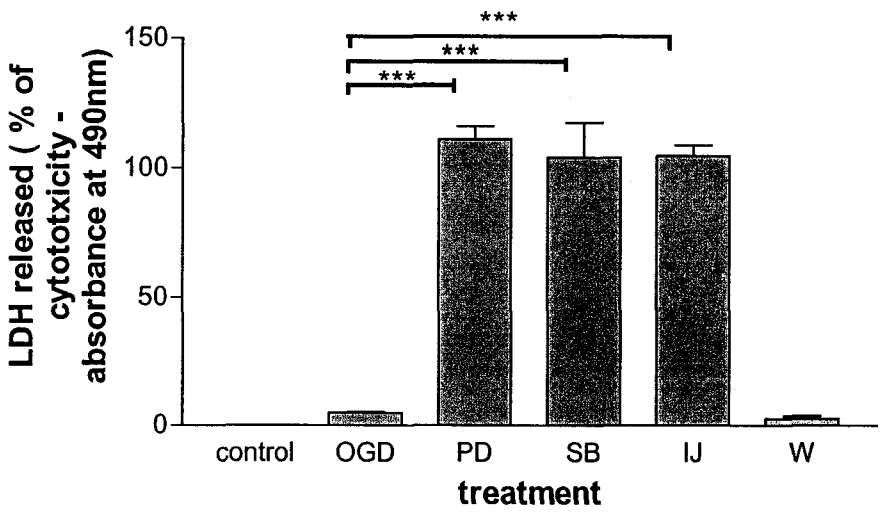

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Figure 3.57. Effect of glutamate and P2 receptor antagonists in LDH released by an *in vitro* ischemic treatment, in human $P2Y_2$-1321N1 transfected cells. 1321N1-h$P2Y_2$ transfected cells were exposed to 4 hrs oxygen glucose deprivation (OGD) and incubated with 100 μM DNQX (DN), 300 μM trans(+/-) (1R,2R)-1—amino-1-carboxycyclopentane-2-acetic acid (trans), 50 μM D(-)-2-amino-5-phosphonon pentanoic acid (D(-)), and 100 μM L(+)-2-amino-(3)-phosphonopropionic acid (L(+)), during this period of time. After that treatment, the supernatant (100μl) was collected and LDH assay was performed as manufacturer's instructions (Roche Molecular-Boehringer Mannheim). The absorbance of the samples was performed in a Microplate Reader at a maximal wavelength λ of 490 nm. The results were reported as % of citotoxicity using the low control (cells without OGD treatment) and high control (cells disrupted with triton x-100 representing total LDH in the cells) using the equation as described in Chapter II. Results shown are representative of 6 independent experiments. Statistical analysis was calculated by One-way ANOVA followed by Tukey post test. Values of $P<0.05$ (*), $P<0.001$ (**), and $P<0.0001$ (***) were considered statistically significant, very significant and extremely significant respectively.

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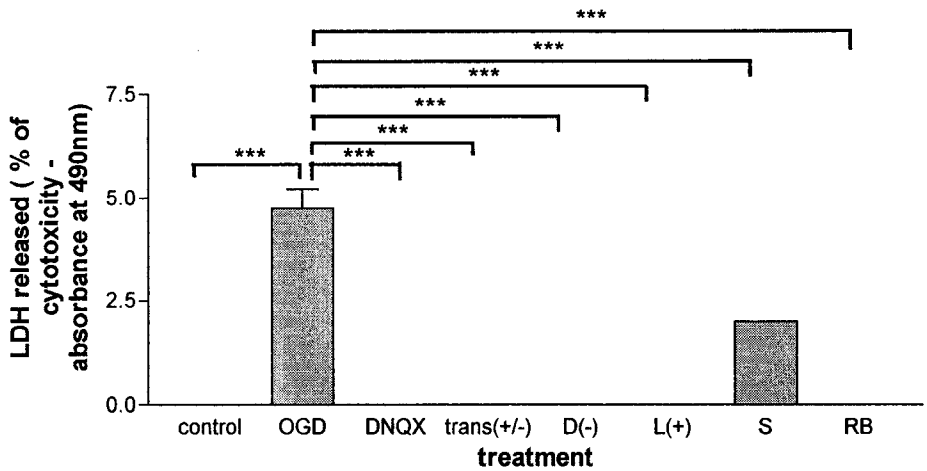

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CHAPTER IV
DISCUSSION

I. Characterization of the signal transduction of the $P2Y_2$ receptor in 1321N1-h$P2Y_2$ transfected cells

A. MAPK and Akt/PKB activation by the $P2Y_2$ receptor in 1321N1-h$P2Y_2$ transfected cells

The $P2Y_2$ receptor expressed in the 1321N1 cells mediate the activation of several protein kinases which include the MAPK family and Akt/PKB. Each one differs in its kinetics, its maximal activation time and the duration of the activation. ERK1/2 and p38 were activated transciently by UTP through the $P2Y_2$ receptor at concentration of 100 μM, reached a maximum at 5 min. The activation of JNK by UTP was more delayed and reached a maximum at 25 min, and the activation of Akt/PKB by UTP was also delayed, reaching a maximum at 20 min. The activation of ERK1/2 and p38 by the $P2Y_2$ receptor was reported with a similar kinetic and maximal time of activation of 5 min in rat renal messangial cells and glomerular mesangial cells respectively and U937 promonocytic cells [255], [258], [167]. The activation of JNK and Akt/PKB by the $P2Y_2$ receptor was also reported, however by a different kinetics that presented a maximal time of activation after 5 min [259], [260]. The difference in kinetics could be explained by differences in cell type. The activation of these protein kinases was relatively fast and their decrease in signal associated to the phospho kinase rapid deactivation could be explained by their translocation to the nucleus where they continue their function regulating the activation or inhibition of transcription factors and expression of several genes that are associated to survival or apoptosis.

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B. Transcription factors phosphorylation by the $P2Y_2$ receptor in 1321N1-h$P2Y_2$ transfected cells

Downstream of the protein kinases activated by the $P2Y_2$ receptor in 1321N1-h$P2Y_2$ transfected cells are transcription factors that regulate gene expression and modulates signals such as proliferation, differentiation, inflammation, survival and apoptosis. UTP through the $P2Y_2$ receptor induced the phosphorylation of several transcription factors that are substrates of these protein kinases. Almost all the transcription factors studied are phosphorylated transciently, they reached a maximum around 15 to 30 min. ATF-2, c-Myc, CREB, c-Jun and FKHR are substrates of the protein kinases activated and discussed above. It means that these protein kinases were translocated to the nucleus to exert a specific function which in its majority is regulating survival and apoptosis signal.

C. Determination of protein kinases that regulate transcription factors phosphorylation

Because several protein kinases can phosphorylate the same transcription factor or one protein kinase can phosphorylate several transcription factors, various protein kinases inhibitors were used to elucidate or to explain which protein kinases is responsible for the phosphorylation of a specific transcription factor. ATF-2 phosphorylation induced by UTP depends principally on JNK. Although p38 apparently regulate also its phosphorylation, the inhibition by SB203580 was not strong as with the JNK inhibitor. Several reports have shown that ATF-2 is a substrate of p38 and JNK [300]. A comparable result was demonstrated in human neuroblastoma cells where, ATF-2 phosphorylation was not inihibited by SB203580 but it was inhibited by a deficient MEKK1/JNK pathway ([301].

A similar result is obtained for the phosphorylation of c-Jun, except that SB203580 did not inhibit or decreased the phosphorylation induced by UTP. JNK is the principal protein kinase responsible of the phosphorylation of c-Jun as reported in the literature [200] and as demonstrated here.

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The other transcription factor studied was c-Myc that is modulated by ERK1/2 signaling pathway, where PD98059, inhibitor of MEK1/2, the protein kinase responsible of the activation of ERK1/2, inhibited the phosphorylation of c-Myc induced by UTP through the $P2Y_2$ receptor. The other inhibitors did not affect the phosphorylation of c-Myc indicating that only ERK1/2 is modulating its phosphorylation as demonstrated in several reports [194].

Contrary to the results obtained with the other transcription factors studied, CREB phosphorylation was not inhibited by any of the protein kinases inhibitors used suggesting that more than one protein kinase could be modulating its phosphorylation or other protein kinases not studied here could be modulating its phosphorylation. CREB phosphorylation can be mediated by several prorein kinases including PKA [302, 303], PKC [304], CaMK (I, II, IV) [305], p38 via MAPKAPK-2 [223] and ERK1/2 via $pp90^{RSK}$ or RSK [306], Akt [252], GSK3 ([307, 308] and possibly by JNK. ATF1 phosphorylation has the same response observed of CREB, and ATF1 forms homodimers and heterodimers with CREB [309]. CREB as well as ATF1 are regulated by increases in either cAMP or intracellular Ca^{2+} [302].

Other transcription factors studied were FKHR and AFX that are downstream substrates of Akt/PKB. The unique inhibitor that decreased the phosphorylation of both transcription factors was wortmannin, which is the inhibitor of PI3K, and PI3K is the protein kinase responsible or that modulate the activation of Akt/PKB. Although the phosphorylation of FKHR and AFX induced by UTP was not so strong as seen with the other transcription factors, we have to remember that this transcription factor is phosphorylated to maintain the cells alive, when it is not phosphorylated it modulate apoptosis. Therefore, the result suggests that the cells have entered into a survival response.

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D. Structural determinants of $P2Y_2$ receptor

1. RGD/RGE extracellular domain

The presence of the RGD sequence in the first extracellular loop of the $hP2Y_2$ receptor, could be acting as a ligand for the vitronectin receptor $\alpha_v\beta_3$ integrin activating a signal transduction that could be implicated in survival responses [130]. Dose responses experiments of UTP at maximal time of activation of each protein kinase determined that each protein kinase studied are RGD-dependent and UTP dose-dependent. The activation of these protein kinases depends on the presence of the RGD sequence in the $P2Y_2$ receptor and its coupling with integrins demonstrated by the decrease in their phosphorylation in the RGE-$hP2Y_2$ mutant receptor which needs a higher concentration to induce the activation of these protein kinases. However, the dependency of the RGD sequence in ERK1/2 activation was not much larger than the other protein kinases, suggesting that ERK1/2 activation is not totally dependent of the interaction of the $P2Y_2$ receptor with integrins or ERK1/2 activation is only partially modulated by the coupling with integrins. However, the RGE-mutant $P2Y_2$ receptor required approximately 1,000 times higher concentration of UTP or ATP to stimulate the mobilization of intracellular calcium as compared to the wild type $P2Y_2$ receptor [130]. Phosphorylation of FAK, (a protein known to be associated with integrins, focal adhesions, and cytoskeletal proteins [272]), by the RGE mutant $P2Y_2$ receptor requires also ~1,000-fold higher concentrations of the agonist UTP, as compared with the wild type $P2Y_2$ receptor [130]. FAK phosphorylation mediated by the wild type and RGE mutant $P2Y_2$ receptor was dependent on intracellular Ca^{2+} mobilization indicating the calcium-dependent and RGD-dependent signaling pathway of the $P2Y_2$ receptor [130]. One possible explanation for this result is that the RGD domain binds to the $\alpha_v\beta_3$ integrin in 1321N1 cells and this binding alters the conformation of the $P2Y_2$ receptor to increase ligand-binding affinity. Moreover, the integrin could interact with the RGD domain of the $P2Y_2$ receptor assisting in localizing it to focal adhesions to more efficiently activate intracellular signaling pathways. Studies with immunofluorescence demonstrated that vinculin, a cytoskeletal protein that is thought to associate exclusively with focal adhesions complexes colocalize in cells expressing the wild type receptor and not the RGE-mutant suggesting that the

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RGD domain of the $P2Y_2$ receptor is necessary for localizing the receptor to focal adhesions and that this localization occurs via a direct $\alpha_v\beta_3/P2Y_2$ receptor interaction [130].

The integrins antibodies are frequently used to determine which integrin is involved or interfere in a specific signal. Our collaboration with Dr. Gary A. Weisman at the University of Missouri and us demonstrated that the $\alpha_v\beta_3$ is one of the integrins that colocalize or associate with the $hP2Y_2$ receptor [130]. Colocalization of α_v and the wild type $P2Y_2$ receptor occurred whether or not the cells were treated with 100 μM UTP, indicating that association of the integrins is not dependent on $P2Y_2$ receptor activation [130]. Incubation with anti-$\alpha_v\beta_3$ and anti-α_v antibodies to block, interfere or impede the binding of the integrins with RGD-$hP2Y_2$ receptor demonstrated that ERK1/2, p38, Akt/PKB, and JNK activation were dependent of the interaction of the $P2Y_2$ receptor with the $\alpha_v\beta_3$ integrin specifically the α_v subunit. The incubation with anti-α_v antibody inhibited also FAK phosphorylation indicating the integrin dependence [130]. However, the activation of JNK is partially dependent of the coupling with integrins, because JNK activation was slightly decreased with the incubation of integrins antibodies. These results are consistent with other reports in which ERK activity was inhibited by the blocking of the α_v integrin ligation [310], [311]. Furthermore, a possible explanation for the inhibition of ERK1/2 and other protein kinases by the blockade of the integrins is suggested by a report where inhibition of integrins could affect the recruitment of focal contact and cytoskeletal components that then form a scalfold to allow efficient assembly of the various components of the signaling pathway to activates MAPK when a P2Y receptor is activated [126].

Studies have indicated that the use of peptide RGDS, which inhibits integrin dimerization and disrupts the formation of focal adhesions, can also inhibit GPCR-mediated ERK1/2 activation and FAK phosphorylation in certain cell types [312]. In collaboration with Dr. Gary A. Weisman in Missouri we demonstrated that overnight treatment with RGDS peptide inhibited FAK and ERK1/2 phosphorylation, whereas the control peptide, RGES, had no effect in the ability of the $P2Y_2$ receptor to induce phosphorylation of these protein kinases indicating one more time the RGD dependence

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on $P2Y_2$ receptor activation [130]. Incubation with a peptide derived from the human $P2Y_2$ receptor (YA**RGD**HWPFSTVLCKLVR ($P2Y_2$ $^{93-110}$)) binds to integrins but not the RGE mutant $P2Y_2$ receptor peptide (YA**RGE**HWPFSTVLCKLVR ($P2Y_2$ $^{93-110}$ [E^{97}])) [130].

However, it is important to mention that incubation with this type of integrins antibodies, $\alpha_v\beta_3$, promotes apoptosis-stimulating signals generated in response to disruption of the cell – matrix [313]. This type of apoptosis signal is denominated "anoikis" when occurred in epithelial and in endothelial cells [314]. Adhesion of many normal cell types to the extracellular matrix is essential to proceed through the cell cycle and maintain their survival, although transformed cells have abrogated the anchorage requirement [315]. Integrin-mediated adhesion not only activates ERK, but also JNK [316]. Cells attached to cell matrix by $\alpha_5\beta_1$ or $\alpha_v\beta3$ integrins express elevated levels of bcl-2 gene which modulates cell survival through Akt pathway [317]. Our laboratory has found an increase in bcl-2 gene expression induced by UTP in 1321N1-h$P2Y_2$ transfected cells inducing a survival signal [318]). It is possible that the combination of $P2Y_2$ and $\alpha_v\beta_3$ activation modulate survival. It is important to mention that MAPK (ERK) activation by the P2Y receptor, and possibly by $P2Y_2$ receptor requires integrin-mediated cell anchorage and does not involve transactivation of EGFR in HUCECs and ECV304 endothelial cells [319]. This type of result is similar to the one obtained here, where incubaton of integrins antibodies attenuate the phosphorylation/activation of ERK induced by UTP. Although the incubation of antibodies did not detach the cells from the cell culture dish plates, the morphology of the cells changed from the one stiffed to the one more contracted indicating that part of the cell adhesion is also affected and suggesting that the cells could be in anoikis, or in apoptosis with the incubation of anti-$\alpha_v\beta_3$ and anti-α_v antibodies. Although not all RGD-containing proteins mediate cell attachment, because the RGD sequence may not always be available at the surface of the protein, it could be influence the activation of these protein kinases. It is interesting that transformed cells (eg. astrocytes to astrocytoma) are more resistant to anoikis [320].

The activation of JNK induced by UTP observed in the presence of anti-$\alpha_v\beta_3$ and anti-α_v integrins antibodies indicate that JNK activation could partially activate or

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modulate an apoptosis signal. It can be due because the detachment from the cell matrix causes JNK activation and apotosis [321] and JNK activation in the absence of ERK1/2 activation induce apoptosis [197]. Although there are several reports demonstrating that JNK activation by integrins can regulate cell cycle progression [322]. The activation of JNK possibly mediated by integrins is abolished by the binding of integrins antibodies blocking the binding of the $P2Y_2$ receptor through its RGD motif to the integrin, but UTP induce a signal through the $P2Y_2$ receptor that is dependent of the RGD motif and the association with the $\alpha_v\beta_3$ integrin. It is important to mention that although p38 regulate apoptosis signals, here p38 is not phosphorylated after UTP addition in presence of anti-$\alpha_v\beta_3$ and anti-α_v antibodies suggesting that the response observed by the activation of p38 by UTP could be modulating a survival signal and not an apoptotic signal. If the cells are in anoikis, JNK phosphorylation should be observed with the incubation of anti integrins antibodies in the absence of UTP. However, JNK is not phosphorylated unless with the addition of UTP, suggesting that JNK is not related to detachment-induced apoptosis as seen in other reports [323]. JNK could be modulating an apoptosis signal induced by UTP through the activation of the $P2Y_2$ receptor in this type of condition. If the cells are in a condition that are suffering damage and are in an intermediate or in advanced stage of apoptosis, the cells could be in a point of no return (irreversible) of apoptosis towards death of the cells. However, if the cells are in a begining stage of apoptosis (reversible), the cells can recover from this condition and survival signals such as UTP through the $P2Y_2$ receptor can be initiated with the association of integrin $\alpha_v\beta_3$.

2. Proline rich motif intracellular domain

The signal transduction exerted by the $P2Y_2$ receptor may be regulated by specific sequences of amino acids at the intracellular domain. Murine and human $P2Y_2$ receptor posses a proline rich sequence at the intracellular domain (C-terminal) which may interact with the SH3 (Src Homology 3) domain of proteins such as the c-Src protein kinase [131]. (See figure 1.1) The SH3 domains regulate protein localization, enzymatic activity and often participate in the assembly of multicomponent signaling complexes [132]. To investigate the functional role of this sequence we used cells expressing wild type human $P2Y_2$ receptor or mutants, eliminating part of the cytoplasmic domain of the

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receptor but leaving the proline rich sequence (Pro+) or eliminating the cytoplasmic domain incluiding the proline-rich sequence (Pro-) (see figure 1.1 and 3.19). We also used specific protein kinase inhibitors to determine the possible role of certain proteins in the activation of these protein kinases induced by UTP that may be dependent of this proline rich sequence.

We found that the presence of the proline rich sequence in the intracellular C-terminal domain of the $P2Y_2$ receptor partially modulates the signal transduction that exerts UTP. This proline rich sequence binds proteins with SH3 domain such as Src. The use of PP2, an inhibitor of Src, demonstrated that the activation of ERK1/2 was dependent on Src. PP2 inhibited the ERK1/2 response exerted by UTP in the wild type and the Pro+ mutant receptor. However, in the Pro- mutant, ERK1/2 activation induced by UTP was not inhibited by the elimination of the proline rich motif not even with the treatment with PP2. These results suggest that the activation of ERK1/2 is mediated partially by Src and is not dependent completely of the proline rich sequence of the $P2Y_2$ receptor. ERK1/2 may be activated by several different pathways which include src-dependent and – independent pathways. Src kinases are activated by a variety of cell surface receptors such as integrins [270], GPCRs [268] and growth factors receptors such as EGFR and PDGR [324]. There are several reports demonstrating that the activation of ERK1/2 by GPCRs is dependent of a pathway mediated by src/Pyk2 complex and transactivation of the EGFR [325], [326]. The activation of ERK1/2 by the $P2Y_2$ receptor could be via src and its association with PYK2 and subsequently through the transctivation of the EGF receptor as suggested and reported by Soltoff [127]. Treatment with AG1478 did not inhibit the phosphorylation of ERK1/2 induced by UTP in the wild type, but decrease the phosphorylation of ERK1/2 in the Pro+ and decrease slightly in the Pro- mutant receptors. Our collaborators in Missouri and us demonstrated that, although the $P2Y_2$ receptor transactivates the EGFR, this transactivation did not influence the activation of ERK1/2 in the wild type receptor [277]. However, the phosphorylation in ERK1/2 in the Pro+ mutant receptor as well as in the Pro- mutant receptor could be due to the transactivation of the EGFR, because eliminating part of the cytoplasmic domain leaving the proline rich sequence as well as eliminating the proline rich sequence, the cell take another route to activates ERK1/2 and could be mediated by EGFR. The result obtained

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with the wild type receptor is similar to a report demonstrating that upon stimulation of GPCR, Pyk2 forms a complex with src, which in turn phosphorylates EGFR directly, but this activation protein complex was not required for linking GPCRs with ERK1/2 activation [327]. Additionally, src binds to the proline rich sequence of the $P2Y_2$ receptor modulating the activation of several tyrosine kinases including Pyk2 and growth factors but not intracellular calcium mobilization and ERK1/2 activation [277] similar to the results reported by Soltoff [328]. Src co-precipitates with the $P2Y_2$ receptor in 1321N1 astrocytoma cells stimulated with UTP [277]. It is also important to mention that dual immunofluorescence labeling of the $P2Y_2$ receptor and the EGFR indicated that UTP caused an increase in the colocalization of these receptors in the plasma membrane that was prevented by PP2 [277]. Studies have found that eliminating the proline rich sequence of the $P2Y_2$ receptor stimulate calcium mobilization and ERK1/2 phosphorylation but was defective in its ability to stimulate tyrosine phosphorylation of src and src-dependent tyrosine phosphorylation of the proline rich tyrosine kinase 2 (Pyk2), EGFR and PDGFR [277]. Thus, this information suggests that agonist-induced binding of src to the SH3 binding sites in the $P2Y_2$ receptor and allows src to efficiently phosphorylate the EGFR [277]. Thus, ERK1/2 activation by the $P2Y_2$ receptor is mediated by a src-dependent and –independent pathway and by transactvation of the EGFR-dependent and –independent pathway. If there is no phosphorylation of EGFR, neither src without the proline rich sequence, how is that AG1478 affects the ERK1/2 phosphorylation? Could be the cooperation with integrins may be through an unknown mechanism that is responsible of the transactivation of EGFR and then the activation of ERK1/2 pathway. However, integrins can activate ERK1/2 by two pathways that could be influencing the phosphorylation of ERK1/2 by the Pro+ and the Pro- mutant receptors: one through FAK and src which activates Cas-Crk-C3G-Rap1-B-Raf-MEK-ERK1/2 and the other through the activation of other src protein kinase family member such as Fyn or Yes and Shc which then subsequently activates the mSos-Grb2-Ras-Raf1-MEK-ERK1/2 pathway [329], [124]. Thus, eliminating the proline rich sequence may lead to a bypass or rerouting of the information flow via alternative pathways for coupling GPCR- activation with EGFR and integrins to activates ERK1/2 signaling cascade.

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The potent toxin cytochalasin D (CytD) is a specific reagent that causes disruption of actin filaments by binding to the barbed (plus) end of growing actin filaments, inhibiting polymerization [274], [275]. Actin filaments are implicated in transducing signals into cells by connection to focal adhesion molecules such as integrins, vinculin and talin [330], [331], [332]. Thus disruption of actin filaments leads to severe impairment in cell function, culminating in cell death [314]. Treatment with cytochalasin D inhibits slightly the activation of ERK1/2 in the wild type receptor and in the Pro+ mutant, but not in the Pro- mutant receptor. These results suggest that activation of ERK1/2 did not depend completely of the formation of protein complexes that depends of the actin filaments or cytoskeleton as occur in NIH3T3 cells induced by EGFR [329].

Migration and anti-apoptotic responses can be mediated by FAK when its is phosphorylated/activated by src in endothelial cells [271]. Integrin-dependent FAK signaling through focal adhesions is also thought to be critical for maintaining survival signals in endothelial cells and other adherent cells [333]. Studies have shown about the role of src in survival activating ERK1/2 and Akt through the PI3K pathway [261]. Data reported indicated the integrin-dependence of FAK phosphorylation induced by UTP [130]. Therefore, it is possible that activation of ERK1/2 induced by UTP can be modulated by the activation of Src and its association with FAK through the formation of cytoskeleton protein complex between the integrins and $P2Y_2$ receptor and also with the coupling of the $P2Y_2$ receptor with integrins through the RGD motif.

In addition of $P2Y_2$ receptor, other receptors have demonstrated that D3 and D4 dopamine receptors have in their amino acid sequence the proline rich motif that permit the formation of protein complexes that regulate the activation of protein kinases such as MAPK [334], [335]. The activation of MAPK (ERK) is not eliminated or affected by the deletion of the proline rich motif of the D4 receptor, although the incubation with PP2 decreases the activation of MAPK but not the activation of Akt [336]. These results are similar to the ones obtained here. Another example is the receptors β_2- and β_3-adrenergic receptors where the activation of ERK is mediated by src. However, the mechanism is different. For the β_2- adrenergic receptor, ERK activation depends on the delivery of the β-arrestin-bound c-src to the receptor through the interaction of the SH3

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domain of src with the proline rich motif of β-arrestin [337]. However, for the β_3-adrenergic receptors, ERK activation is dependent of src but independent of β-arrestin where src is bound directly to the proline rich motifs presents in the third intracellular loop and the carboxy terminus [134]. However, the interaction of src and β-arrestin did not depend completely on their interaction through the proline rich sequence present in the β-arrestin, the major interaction between them is through the catalytic or kinase domain of src [338]. Experiments showed that the lack of a sequence in the β_3-receptor abolished agonist induced activation of the ERK signal [134] which is not seen in the activation of ERK by $P2Y_2$ receptor. This result suggests that the ERK activation in addition of the signal of UTP is also modulated by the activation of other pathways, src-independent such as the coupling with integrins.

For the other protein kinases, the results are slightly different. The activation of p38 was dependent of Src, because in the wild type as well as the Pro- mutant the activation of p38 was inhibited with PP2. However, p38 activation was not inhibited in the Pro+ mutant treated with UTP. This is a curious result that suggests that part of the C-terminal of the $P2Y_2$ receptor could be involved in the activation of the signal transduction of p38 activation but not the proline rich sequence of the receptor. These results suggest that p38 activation of the $P2Y_2$ receptor is modulated partially by src. Some reports have demonstrated that p38 activation is mediated by EGFR transactivation as ocurr with thrombin [339]. The incubation with AG1478 which inhibits the activation of EGFR did not cause any effect in the phosphorylation of p38 induced by UTP except in the Pro- mutant suggesting that once the proline rich sequence is eliminated other pathways which include the transactivation of the EGFR is modulating the activation of p38. The addition of cytochalasin D increases its phosphorylation (p38) suggesting that disruption of the actin filament or cytoskeleton cause a stress that activates p38. Similar results were reported in human cancer cells and in 3T3 cells which disruption of filamentous actin cytoskeleton activate p38 and JNK [340], [341], [342]. These results suggest that the activation of p38 can be src-dependent and –independent. In addition, p38 activation is not dependent of the proline rich sequence of the $P2Y_2$ receptor because once this sequence is eliminated other pathways through the transactivation of the EGFR could be activated.

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Although the activation of JNK isoforms is affected slightly with PP2, it does not mean that their activation is dependent of src. The elimination of the proline rich sequence did not inhibit JNK phosphorylation, suggesting that JNK activation does not depend of this region. Similar to p38, cytochalasin D increased the phosphorylation of both isoforms of JNK in the wild type as well as in the mutants. Cytochalasin D-disruption of actin fibers activates JNK and stimulates apoptosis [343]. Treament with AG1478 did not inhibit the response induced by UTP in the phosphorylation of JNK isoforms in the wild type, but inhibited slightly the phosphorylation of p46JNK in the Pro+ and in the Pro- mutant. The activation of these isoforms could be modulated differently. These results suggest that JNK activation is not dependent on the proline rich sequence of the $P2Y_2$ receptor not src, but may be activated by other pathways that could include the transactivation of the EGFR.

With respect to Akt, its activation is quite different. The activation of Akt was slightly reduced with PP2 suggesting that src could modulate its activation. Activation of Akt by the EGFR is mediated by a complex that includes src/c-Cbl/PI3K [324]. Recently, it was discovered that src is required for Akt activation by its association of its SH3 domain with the proline-rich sequence present in the C-terminal of Akt [344]. It is important to mention that the SH3 domain of src can bind to the other protein that has the proline rich sequence such as FAK, PI3K etc [345]. It is possible that src could be interacting with several proteins that contain a proline rich sequence and did not have to necessarily modulate directly the activation of Akt. However, cytochalasin D strongly reduced Akt activation but not AG1478. This result is similar to one reported where Akt activation by α_2-adrenoceptor is mediated by src, but not by EGFR transactivation in porcine palmar lateral vein [346]. These results suggest that activationof Akt/PKB depends on Src and the formation of the actin filaments, but not on the proline rich sequence of the $P2Y_2$ receptor. An intact actin network is a crucial requirement for PI3K-mediated production of 3-phosphoinositide and therefore, for the activation of Akt/PKB [347]. The inhibition of Akt by cytochalasin D has been reported in capillary endothelial cells [348]. The disruption of actin filaments by cytochalasin D activates an apoptotic signal and inhibits survival signals in 3T3 cells [341] as observed in our results. This is

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why, cytochalasin D activated p38 and JNK but inhibits ERK1/2 and Akt. Activation of Akt/PKB mediated by Src has been observed in other systems such as in endothelial cells by estrogen [349]. The formation of the actin filaments recruit an adhesion complex of proteins which include catherins-catenin which is also associated with Src recruit PI3K and subsequently Akt to induce survival signals [350]. Thus it is possible that a pathway that includes these protein complexes could modulate Akt activation by UTP through the $P2Y_2$ receptor. Contrary to other reports that shown that Akt activation depends of the transactivation of the EGFR [351], these results suggest that the pathway which activates Akt/PKB by the $P2Y_2$ did not depend of the transactivation of the EGFR, but the formation of the actin cytoskeleton in association with src is important for its activation.

The partial inhibition of ERK1/2 phosphorylation by CytD could be explained in several ways. CytD disruption of the actin filament could impair the formation of protein complex possibly composed of src and FAK that mediate the activation of ERK1/2. The other pathway independent of the formation of src-FAK and actin filament formation complex could be activating ERK1/2 pathway. However, CytD in addition to induce a pro-apoptotic response, it could induce an anti-apoptotic response as observed in 3T3 cells in which it activated ERK1/2 [341]. The anti-apoptotic response of CytD has been suggested to be due by the activation of gelatinase A which is a member of the metalloproteinase family (a proteolytic enzyme that catalyses degradation of extracellular matrix components, Collagen IV) and has been implicated in the initiation of proliferation, differentiation and cell survival signals [352]. The survival signals induced by gelatinase A have been speculated to be due to the presence of the integrin $\alpha_v\beta_3$ that appears to be necessary for the functional activity of the enzyme [353]. Because the $P2Y_2$ receptor is coupled to the integrin $\alpha_v\beta_3$, their activation and the possibility of the activation of gelatinase A by CytD counteract the pro-apoptotic signal induced by itself (CytD) eliciting a survival signal mediated by ERK1/2 activation that override the pro-apoptotic activation of p38 and JNK pathways.

And it is also important to mention that the actin cytoskeleton and focal adhesion complex formation play an important role in integrin modulation of signaling such activation of ERK1/2 by tyrosine kinase receptors [329]. Moreover, treatment with

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cytochalasin D inhibits ERK1/2 and Akt activation suggesting the importance of the actin cytoskeleton in the activation of these protein kinases by $P2Y_2$ with a possibility that integrin-mediated recruitment of focal contact and cytoskeletal components that then form a scaffold to allow efficient assembly of the various components of the signaling pathways and to modulate the transactivation of the EGFR.

II. CEREBRAL ISCHEMIA- An *in vitro* model

A. LDH released and PARP cleaved determination

Cerebral ischemia cause irreversible and severe cell damage which finish in cell death by apoptosis or necrosis. Many experimental models are used to study ischemia damage which includes the *in vivo* and *in* vitro models. The three principal classes on *in vivo* rodent models are global ischemia, focal ischemia, and hypoxia/ischemia. The latter consist in vessel occlusion combined with breathing a hypoxic mixture. The *in vitro* models include exposure of neuronal, neuronal/glial, organotypic slice cultures, or the freshly isolated hippocampal brain slice, to anoxia or to anoxia in the absence of glucose (*in vitro* ischemia (OGD)) [12]. The in *vitro* model tries to ressemble as much as possible to the *in vivo* model. However, somewhat longer exposures are necessary for damage the slices, tissue or the cell culture so that they show the same ranking of selective vulnerability as *in vivo* tissue. Neurons are more suceptible to cell death than astrocytes, suggesting that astrocytes are able to deploy protective mechanisms that are absent in neurons. Studies have demonstrated that at 1hr of OGD, induced ATP depletion in neurons but not in astrocytes, an increase in necrotic but not apoptotic neurons was triggered [354]. Moreover, it has been recently reported that neurons reflect energy depletion and collapse of their plasma membrane potential after 1hr of OGD, whereas astrocytes tolerate an additional 2 or 3 hrs of OGD without cellular injury [355]. For this study, a time course of LDH released performed demonstrated that the *in vitro* model used to simulate an *in vivo* ischemia was useful. The release of LDH was an indicator of necrosis where the plasma membrane becomes permeable and releases all its content. Severe cell damage began once the cells were incubated to 3 to 4 hrs of OGD environment, but during the first hrs (1 to 2 hrs) of OGD, no release of LDH was observed. The cells exposed during the time course of OGD performed (1 to 4 hrs of

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OGD), are not death, they are surviving. And this result is in accordance with the previous study that the astrocytes, here astrocytomas, are more resistant to this type of stress of OGD than neurons. If the exposition of OGD is longer, i.e. until 18 hrs, the cells are disrupted totally and release all their LDH contents. These results correlate with a report where cultures of hyppocampal astrocytes exposed to oxygen-glucose deprivation observed a reduction in viability beginning at 2 hrs of OGD and reduction of cortical astrocytes viability was not observed until 4 hrs of OGD [356]. Our results are in accordance with a report which demonstrated that astrocytes in culture exposed to OGD remains unaltered after 2 hrs of OGD (ischemia), but were moderately or severely damaged after 4 or 6 to 8 hrs, respectively [357]. They suggested that astrocytes that survived various lengths of *in vitro* ischemia incubation retained their ability to produce ATP after ischemia and both ATP and ADP levels were increased in astrocytes that remained alive under *in vitro* ischemia for up to 6 hrs [357].

To confirm that the damage of the cells caused by the OGD treatment is mediated by necrosis and not by apoptosis, the same time course performed to LDH assay was performed to determine PARP cleaved which are downstream of caspases (caspase 3) and which is indicative of apoptosis. No PARP cleaved was detected during this period of time of OGD. Caspases activation (3, 7 and 9) (caspase cleaved), an early event in apoptosis, was not also detected (data not shown). However, cleaved PARP was detected in control cells showing the 89 and 24 kDa fragments. These cells were starved from serum. These starved cells were dying slowly by apoptosis and with DNA damage. No change in cleaved PARP with UTP addition was observed. Several reports have indicated that cells with growth factor withdrawal or serum deprivation die by a programmed cell death or apoptosis [285]. There has been reported that PARP is cleaved during apoptosis and not during necrosis in culture neuronal cells and in Swiss 3T3 fibroblasts [358], indicating or suggesting that in the 4hrs of OGD (ischemia) in our model, necrosis is predominant death signal and this is why PARP was not cleaved and no one of the caspases are activated. Moreover, UTP with OGD does not activate any of the caspases in study, suggesting that UTP is not inducing an apoptosis signal through them and is not activating a death signal. Another point is that the ATP levels indicate the type of cell death. High levels of intracellular ATP are needed to carry apoptosis but low ATP levels

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(ATP depletion) switch to necrosis and PARP activation [359], [281], [283]. These results indicate also that the *in vitro* model is like the *in vivo* model where ATP depletion ocurr and which implicate a death form of necrosis demonstrated by the amount of LDH released and PARP activation (not cleaved PARP detection). These results suggest that cells switch from apoptosis to necrosis once the cells are exposed to OGD. These results also suggest that once the ischemia is initiated and the plasma membrane suffer damage, UTP only can send a signal toward survival responses to counteract death by necrosis.

B. Activation of protein kinases

Several reports have demonstrated that MAPK family is activated differentially during ischemia. Focal cerebral ischemia in rat caused activation of ERK1/2 in neurons and cells with morphological appearance of oligodendrocytes in the penumbral-like regions where cells survive injury, while p38 activation occurs predominantly within cells with astrocyte-like morphology, but also within neurons in the 'penumbra-like' regions [25]. Long lasting phosphorylation and activation of JNK was observed in cortical and hyppocampal neurons after ischemia-reperfusion in rats [360]. Phosphorylation of p38, but not ERK1/2 or JNK are activated in microglia and not in CA1 neurons after ischemia [22]. ERK1/2, p38 and JNK were initially activated at 30, 10 and 5 minutes respectively, after focal cerebral ischemia in neurons and astrocytes of mouse brain [24]. Knowing this information, a similar time course of OGD was performed to determine the phosphorylation/activation of the MAPK family and Akt/PKB with or without UTP during this period of time. During the time course of OGD, a steady increase in ERK1/2 phosphorylation was observed until 4 hrs. And then, an increase in ERK1/2 phosphorylation was observed during the time course of OGD after UTP addition. These results suggest that UTP, after an ischemia episode, increase the phosphorylation of ERK1/2 and through that activation could induce a survival signal.

The same response is observed for p38 but it is more elevated than ERK1/2. A time-dependence of p38 phosphorylation is observed as the OGD time of exposure is increased and a greater increase in p38 phosphorylation is observed after addition of UTP; however

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after 4hrs of OGD incubation, no further change in p38 phosphorylation (after UTP addition) was observed, i.e. p38 remains the same as without UTP. With respect of JNK isoform phosphorylation, their phosphorylation increased during the OGD and after addition of UTP also increased. However, Akt was not phosphorylated or activated during the OGD time course which correlated with several reports that Akt is not activated during ischemia but is activated in the reperfusion process [361, 362].

These results are comparable to the one reported where astrocytes under ischemia in an anaerobic chamber, the level of phosphorylated ERK1/2 began to increase after 1 hr ischemia reached a maximum after 4hrs ischemia, then decreasing from 5 to 6 hr and Akt was activated later than ERK1/2 [361]. After addition of UTP, there is an increase in ERK, Akt, and JNK phosphorylation suggesting that UTP could be inducing a survival signal. Although p38 is also increased, its phosphorylation 4 hrs of OGD plus UTP remains at the same level of phosphorylation of ischemia alone suggesting that the enzyme is saturated. A report demonstrated that inactivation of a tyrosine phosphatase is responsible for the prolonged p38 activation during ischemia which causes cell death [27]. It is possible that during the 4 hrs of OGD, the tyrosine phosphatase was inactivated and this generated the increased in p38 activation. However, it is important to indicate that different isoforms of p38 has different functions. Inhibition of p38β by SB 202190 (p38 inhibitor) induced apoptosis by the activation of caspases through another p38, p38α [231]. The activation of p38β isoform protects cells from TNFα toxicity [363]. It is possible that the activation of p38 observed during ischemia can be due to the activation of p38β and induce a survival signal contrary to induce an apoptosis signal. But we can not discard the possibility of the activation of p38α can be activated which is the one that induce apoptosis.

The activation of p38 and JNK during ischemia suggests that a possible death signal is sent through their activation [364], [365]. The activation of JNK in hippocampal CA1 region was involved in cell death after cerebral ischemia [366]. However, JNK also is implicated in survival. Although JNK has been shown to function pro- and anti-apoptotic, it will depend on the cell type and the differentiation state of the cell [367]. A report demonstrated that early induction of JNK and p38 activity by TNF is independent

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of caspases and apoptosis (induce survival), whereas late phase-JNK and p38 correlate with caspase activation, are partially caspase dependent and occur only during apoptosis [368]. Because no caspase activation was determined during the OGD (ischemia) (data not shown), it is possible that the activation of p38 and JNK observed during the OGD (ischemia) is indicative that the cells are sending a message to survive to this type of stress through the release of nucleotides and activation of the $P2Y_2$ receptors. Some reports have been shown that ERK stimulation can inhibit the pro-apoptotic function of JNK [197]. It is possible that after UTP treatment during OGD, ERK is activated and suppress the pro-apoptotic function of JNK and inducing an anti-apoptotic function. However, Akt can inhibit the pro-apoptotic function of JNK also through the the phosphorylation of ASK1 and SEK1 which are protein kinases upstream of JNK and are responsible of its activation [253], [369]. Moreover, ERK, JNK and p38 in conjunction with AP-1 are activated coordinated and sustained manner to contribute to proliferation and cardiomyocyte differentiation of P19 cells [370]. Here, we obtained the concertated activation of the MAPK family in addition of Akt, therefore, all of them could influence or regulating a survival signal. The isoforms of JNK can modulate different responses as for example that JNK1 (p46) is protective during osteoclastogenesis [371]. In other report JNK2 play a key role in cell proliferation and cell cycle progression [372]. Additionally, JNK is implicated and required for survival in transformed cells such as transformed B lymphoblasts and the expression of Bcl-2 [373]. Because, the model used are the astrocytoma cells, JNK could be involved in survival signals induced by the nucleotides through the $P2Y_2$ receptor.

1. LDH released-protein kinase inhibitors incubation

To determine if the protein kinases studied mediate survival or death signal, incubation with inhibitors of these protein kinases during the 4 hrs OGD were used to test if ERK1/2, p38 and JNK mediate protective or survival signals. The inhibition of these protein kinases with PD98059, SB203580 and inhibitor of JNK increased the LDH released during the 4hrs OGD. However, wortmannin did not cause any change in LDH released suggesting that a pathway including PI3K and possibly Akt/PKB did not regulate the death or survival signals present during OGD, ischemia. The activation of

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ERK1/2 during OGD induces a protective or survival response due to the increased in LDH released by its inhibition with PD98059. Contrary to other studies where inhibition of ERK1/2 could modulate neuroprotective responses [26], is also comparable with other studies where inhibition of ERK1/2 induces death [26]. The activation of p38 could modulate survival and apoptosis signals which will depend of the isoform that is activated. Inhibition of p38 has been reported to be cardioprotective during ischemia [374]. The p38α modulates apoptosis and p38β modulates protection [231]. SB203580 inhibits both p38 isoforms [232]. The inhibition of p38 and increased in LDH released suggest that the isoform activated predominantly could be p38β, because it is the isoform that regulate protection, and not p38α. If p38α were activated, a decrease in LDH released were obtained as observed in rat ventricular cardiomyocytes after a 6hrs of simulated ischemia [375]. The activation of p38β in 1321N1-hP2Y$_2$ astrocytoma cells correlates with a report where demonstrated that p38β is induced in astrocytes in hippocampus after transient global ischemic insult, whereas p38α was concentrated in microglia in hippocampus as well as in frontal and parietal cortices of the brain [376]. The expression and activation of p38β was induced in reactive astrocytes after transient focal ischemia [377]. The protective role of p38 was demonstrated also during the activation of β-adrenergic-induced preconditioning when SB203580 were administrated and abolished cardioprotection [378].

C. Effects of glutamate, P2 and A1 receptors antagonists, and apyrase in the phosphorylation of protein kinases during the 4hrs OGD

Ischemia causes irreversible and fatal damage to the afected neurons and reperfusion of oxygen that follows may further be detrimental. Although the mechanism that give rise to ischemic brain damage have not been yet been definitely determined, it is believed that the release of excitotory amino acids (EAA) such as glutamate by its high concentration (initially mediated by vesicular release from nerve terminals, and later by reverse transport from astrocytes responsible of the recycling of the neurotransmitter that do not respond), neurotoxic levels (mM), that rise to cytotoxic levels as well as massive generation of free radicals during reperfusion are responsible of neuronal injury [379].

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Glutamate arrive in greater amounts (at toxicity levels) at the extracellular medium activating its receptors and inducing a cascade of events that can be induce survival as well as apoptosis depending of the receptor activated and the duration of this activation. The glutamate release has been divided in two phases to produce the neuronal death: 1) during ischemia or hypoxia, glutamate is released by reversed operation of glutamate uptake carriers and activates NMDA receptors, increases the intracellular concentration of Ca^{2+}, and triggers a long lasting potentiation of NMDA-receptor-gated currents; 2) after ischemia, glutamate released by (Ca^{2+})-dependent exocytosis activates an excessive influx of Ca^{2+} largely through potentiated NMDA-receptor-channels, which leads to neuronal death [380]. The activation of the protein kinase (MAPKs) observed during our *in vitro* ischemia could be due to the release of glutamate and the activation of several of its receptors.

Activation of ERK1/2, p38 and Akt by glutamate demonstrated that glutamate could be responsible of the phosphorylation/activation of these protein kinases during ischemia and the presence of some glutamate receptors in 1321N1 astrocytoma cells. The maximal phosphorylation time obtained for ERK1/2, p38 and Akt was 10 min. Their activation was transciently. Similar result was reported in cultured neurons exposed to glutamate where Akt was phosphorylated at Ser 243 transciently [289]. Another interesting point is that JNK phosphorylation induced by glutamate was not detected during the time course performed at 100 μM of glutamate. The activation of JNK was determined with a higher concentration of glutamate, 1 mM, suggesting that higher concentrations of glutamate as presented during ischemia to induce the activation of JNK.

To determine the reliance of the glutamate receptors antagonists, the cells were incubated with the glutamate receptors antagonists and then stimulated with glutamate. The glutamate receptors antagonists studied, decreased the phosphorylation of ERK1/2, suggesting that the activation of ERK1/2 by glutamate is mediated by NMDA, AMPA/Kainate and mGlu receptors and showed the reliable of these antagonists and the reliable of the concentrations.

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Knowing that ATP and glutamate are released during ischemia, we decided to determine if the protein kinases phosphorylation during OGD treatment was mediated by them or one of them activating their receptors. The principal protein kinases phosphorylated during the 4 hrs OGD are p38 and JNK. Treatment with apyrase demonstrated that extracellular nucleotides were involved in p38 and JNK isoforms activation after an OGD episode. The p38 phosphorylation was dependent of the activation of $P2Y_2$ receptor as well as metabotropic glutamate receptors during the 4 hrs OGD. After 4 hrs OGD and UTP addition, p38 phosphorylation was mediated by NMDA and mGlu receptors. With respect of JNK isoforms, during the 4 hrs OGD the phosphorylation/activation of p54JNK is mediated by NMDA and possibly by the adenosine A_1 receptor. The possible involvement of A_1 receptor in JNK activation during OGD can be understood because a report showed that JNK activation was mediated by A_1 and P2Y receptors in vascular smooth muscle cells through a mechanism involving autocrine stimulation of purinoceptors by ATP and its hydrolyzed product adenosine [299]. The p46JNK phosphorylation/activation was mediated by AMPA, Kainate, NMDA and some metabotropic receptors during the 4 hrs OGD. Addition of UTP after the 4 hrs OGD, the phosphorylation of p46JNK was not affected by the glutamate receptors antagonists, however the P2 antagonists decreased its phosphorylaytion indicating that the $P2Y_2$ receptor and may be another P2 receptor unknown that could be expressing during the OGD treatment. ERK1/2 and Akt/PKB activation with the addition of UTP after 4 hrs OGD is mediated by the $P2Y_2$ receptor activation. A curious effect is that ERK1/2 and p46JNK isoform phosphorylation could be mediated by $P2Y_1$ and/or P2X, because PPADS which decreased their phosphorylation during the 4 hrs OGD, is a P2 antagonist, but is more selective for P2X and $P2Y_1$ receptors [381]. These results suggest that may be these receptors are been expressed during the 4 hrs OGD (they are not currently expressed in 1321N1 cells), and modulate also the activation of these protein kinases. But the principal receptor that is expressed and modulates the phosphorylation of these protein kinases is $P2Y_2$ which is demonstrated by the results obtained with suramin and reactive blue which are also P2 antagonist, but they antagonize P2Y receptors [382].

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Another antagonist used very often in this type of studies is MK801, a noncompetitive NMDA receptor antagonist. The cells were incubated with MK801 during the 4 hrs of OGD at 1 μM [291]. JNK phosphorylation was slightly decreased with this antagonist during the OGD (absence of UTP) but not in the presence of UTP (data not shown) as seen with D(-) confirming the role of NMDA receptor in the activation of JNK during OGD. These results are in accord with the one reported during hypoxia, MK801 attenuated JNK-2 activation in the nucleus tractus solitarii indicating that JNK activation is by the NMDA activation [383]. It is important to mention that concentrations of MK801 higher than those used can induce the opposite effect [384]. Glutamate activates JNK and its substrates c-Jun, through the NMDA receptor in cortical striatal neurons [288]. Other reports have demonstrated that during ischemia and reperfusion, JNK is activated [385], [386].

Glutamate induce the release of ATP and ATP induce the release of glutamate resulting in a feedback loop [46]. This means that it is possible that both are regulating themselves during the OGD treatment. The mGlu receptors that are inhibited by L(+) and that is modulating the activation of p38 and JNK belongs to the group I (mGluR1 and mGluR5) which are coupled to $G_{q/11}$ family of G-proteins and activation of phospholipase $C_{\beta 1}$ and phosphoinositide hydrolysis [35], [34], [36]. One of these receptors or both may modulate the activation of NMDA which can explain the decreased in phosphorylation of p38 by D(-) after UTP addition after 4 hrs OGD not observed during the 4 hrs OGD alone [387], [388]. If there is some glutamate metabotropic receptor activation during our model of ischemia which can influence in the activation of these protein kinases, specially p38 and JNK, could be mGluR1 and mGluR5 which regulates apoptosis and necrosis during OGD [40]. The activation of mGlu1 receptors exarcebates neuronal necrosis whereas activation of both mGlu1 and mGlu5 receptors play a role in attenuation of neuronal apoptosis. This information suggests the possibility of the activation of mGluR1 in our model, because necrosis is implicated in our system. The results obtained on LDH with glutamate receptor antagonists confirmed that the role of glutamate and the activation of its receptors modulate necrosis, because incubation with these antagonists inhibited completely the LDH released caused by the OGD. However, incubation with suramin decreased slightly the LDH released suggesting that P2Y receptors are not

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modulating the necrosis response in OGD. Glutamate activates p38 in rat cerebellar granule cells inducing apoptosis [389]. However, the response that p38 modulates in our model is protection/survival by the results obtained where inhibition of p38 by SB203580 increased LDH released and subsequently the necrosis or death of the cells exposed to 4 hrs OGD. Thus, the activation of p38 modulating survival is mediated by ATP/UTP through the $P2Y_2$ receptor.

Although no change in phosphorylation was observed in Akt and ERK1/2 with the antagonists used during the 4hrs of OGD, other glutamate receptor could be activating during the first hrs of OGD, specially the mGluR2/3 and mGluR4/7/8 which regulate neuroprotection in glial cells [390] and during the time course of OGD ERK1/2 is activated. Glutamate activates ERK2 through the mGlu5 receptor and the transactivation of the EGFR in astrocytes [391]. It is possible also that glutamate could activate ERK1/2 through the activation of the mGlu5 receptor during the first hr of OGD. However, ERK1/2 phosphorylation here mediates neuroprotection due to the LDH results with PD98059. On the other hand, we can speculate that the amount of glutamate that could be released or remain at the extracellular medium during our model of ischemia could be minimal to exert a notably effect. Therefore, we can not discard that ERK1/2 could be modulated by $P2Y_2$ receptors during the first hrs of OGD when ATP is released.

Several reports have demonstrated the neuroprotective effect of adenosine after ischemia in which this effect has attributed to the hydrolisis of ATP and the consequence release of itself [48, 49, 51]. The way that adenosine arrive to the extracellular medium and mediate its neuroprotective role is through by the direct release or by the hydrolysis of ATP, ADP and AMP by ecto-ATPase, ecto-ATP diphosphohydrolase (ectoapyrase) and ecto-5'-nucleotidase respectively [55]. Extracellular concentrations of nucleotides are precisely regulated by several groups of membrane-associated ectoenzymes [392], [393]. We have to remember also the presence of the activity of extracellular nucleoside diphosphokinase (NDPK) which catalyzes transphosphorylation reactions to generate nucleotide triphosphates such as ATP. It is important to mention that an upregulation of ectonucleotidases activity such as ecto-apyrase (capable to hydrolyzing nucleoside 5'-triphosphate and diphosphates) and ecto-5'-nucleotidase (capable of hydrolyzing

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nucleoside 5'-monophosphates) are determined during global forebrain ischemia [55]. This upregulation of ectonucleotidase activities implies increased nucleotide release from the damage tissue and could play a role in the postischemic control of nucleotide-mediated cellular responses. It is also important to mention that the P2 antagonist used: PPADS, suramin and reactive blue in addition to antagonize P2 receptors, they also inhibit ectonucleotidase activity in various tissues and do not modify the ATP effects [394]. Thus, the results observed with these antagonists represent the response observed of ATP without degradation, through the $P2Y_2$ receptor. These results are in accordance with the results of apyrase treatment where, the response observed is decreased due to the degradation of ATP to ADP.

Recently, a report demonstrated that ATPase but not ADPase activity was significantly activated by glutamate in rat hyppocampal slices suggesting the production of degradation of ATP such as ADP and adenosine (a neuroprotective compound) and suggesting the neuroprotection role against the neurotoxicity of glutamate induced inclusively by ATP [395]. Extracellular hydrolysis of ATP has been demonstrated in astrocytic cultures [396]. It is possible that after 4 hrs OGD began the formation of adenosine by the hydrolysis of ATP. The protective effect of adenosine is mediated by inosine in rat cultured astrocytes submitted to OGD [397]. In normal conditions, the levels of adenosine increased by the hydrolysis of ATP, because there is not necessary the activation of P2 receptors, but in pathological circumstances where the extracellular ATP concentration is high, the amount of adenosine is negligible during the first hour, but then increase about 20 times after 4 hrs and maintained elevated during at least 16 hrs [398]. This behaviour can be done in our model. It is also important to mention that 1321N1 cells express the A_1 receptor which is responsible of the neuroprotection responses observed in several cells [297]. Adenosine inhibits the released of excitatory neurotransmitters (aspartate and gluatamate) [54] suggesting that the activation of A_1 receptor could inhibiting the released of glutamate promoting protective response. Some studies have shown that adenosine inhibits glutamate released via presynaptic receptors or act as a free radical scavenger [51]. The A_1-mediated neuroprotective effects of adenosine is due by the blocking of the presynaptic influx of Ca^{2+} which results by the inhibition of the glutamate released and the reduction of the effects of excititotoxic in the

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postsynaptic level [399]. The activation of A_1 receptor in hippocampal CA1 region of rats inhibits synaptic transmission (glutamatergic transmission) [400]. The activation of A_1 receptors syimulates astrocytes to produce trophic factors such as nerve growth factor (NGF) , S100beta protein and transforming growth factor beta which contributes to protect neurons against injuries [401], [402]. If adenosine is released during the ischemia or the ATP released is hydrolyzed as far as adenosine, adenosine by the activation of A_1 receptor could inhibit the release of glutamate and attenuate the injury caused by glutamate. Thus, the activation of p54JNK by A_1 can decrease the glutamate release and induce a survival signal, although it is also modulated by glutamate through NMDA.

ATP triggers the release of excitatory amino acids (EAA) such as glutamate and aspartate, moderately, but increases synergistically with cell swelling in astrocytes [403]. With this information, we can suggest that ATP released during the OGD or ischemia can induce the release of glutamate that may be further increased with the subsequent activation of NMDA receptors by the glutamate itself which can induce cell disruption and cell swelling. However, ATP acting presynaptically, inhibits glutamate synaptic release or glutamate-mediated excitatory postsynaptic potentials by acting through P2Y receptors such as $P2Y_1$ and $P2Y_2$ in pyramidal neurons of hipocampal slices [404]. Not only the neuroprotection associated to adenosine is due to the activation of adenosine receptors and the consequently inhibition of glutamate release. We postulate that ATP before its hydrolysis acting through the $P2Y_2$ receptor can induce neuroprotection inhibiting the release of glutamate. Studies have reported that integrins signaling via a PI3K-Akt pathway regulates the survival response inhibiting the death induced by glutamate in neurons [267]. It can be possible that the association of the $P2Y_2$ receptor with integrins induce a survival response inhibiting the death or delaying the death induced by glutamate in neural cells.

As we confirmed, nucleotides such as ATP are released during an ischemia episode. Although some reports have demonstrated that adenosine is released and is neuroprotective, we speculating that the protection is initiated by ATP through the activation of P2Y receptors such as $P2Y_2$ receptors. For example, C6 glioma cells do not released adenosine during ATP-depleting events (such as hypoxia or ischemia). The

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authors suggested that during ATP depleting conditions, astrocytes may salvage extracellular adenosine derived from ATP released from astrocytes and neurons or from adenosine per se released from neurons [405].

Extracellular nucleotides play an important role in cellular responses during ischemia. The data with PPADS, suramin, reactive blue and apyrase on LDH as well as on protein kinases indicate that survival signals are activated by the $P2Y_2$ receptor mediated by p38 and JNK protein kinases. Although the cells are releasing nucleotides continuously, this concentration is minimal and determines the basal signal transduction observed [406]. ATP released could also be mediated presynaptically by itself through the activation of P2 receptors and by A_1 receptors as well as other receptors [407]. ATP induces ATP release from astrocytes through the activation of P2Y receptors [408]. With the results obtained here, the activation of these protein kinases are mainly modulated by the nucleotides through the $P2Y_2$ receptor, although a contribution of glutamate receptor in specific protein kinases is observed and the activation of A_1 receptor by the presence of adenosine at the extracellular medium as well. This information suggests that ATP released acts paracrine and autocrine inducing a signal transduction to survival through the $P2Y_2$ receptor.

The importance of the released of nucleotides in addition to induce a survival signal to the neuron and astrocytes, is that ATP released from neurons could activate receptors in adyacent endothelial cells regulating the blood flow which is one of the problems of ischemia or stroke [409, 410]. The blood-brain barrier formed by the brain endothelium exerts a strong diffutional restriction on exchange between blood and brain. The brain endothelium increases permeability in response to nanomolar to milimolar of agents such as purines [410]. There are increased in permeability (drop in transendothelial electrical resistance) of pial vessels (frog) in response to ATP, ADP, and AMP [410]. Purines and pyrimidines such as ATP, ADP, and UTP have recently been recognized as having major vasoactive properties in the cerebral circulation [411].

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D. Protein kinases substrates phosphorylation in the OGD episode

For the reason that UTP induce the phosphorylation of Akt/PKB and Akt induce survival signal, we decided to determine if some of its substrates are also phosphorylated by UTP. The survival signal induced by Akt is mediated by the phosphorylation of several proteins that inhibits apoptosis. During the 4 hrs of OGD, a differential phosphorylation of these downstream effectors or proteins: BAD, GSK3 α/β, IkBα and p70S6k, which are in the Akt/PKB signaling pathway, are observed. Almost all of them are not phosphorylated during the OGD. However, each one of the proteins except IkBα increased its phosphorylation after UTP addition, inmmediatelly after 4 hrs OGD.

BAD is a proapoptotic protein that belongs to a Bcl-2 family that can bind to anti-apoptotic proteins such as Bcl-2 and Bcl-x_L and promote cell death [412]. The antiapoptotic effect is carried when BAD is phosphorylated in several sites: Ser 136 by PKB/Akt via PI3K pathway [248], at Ser 155 by PKA [413] and at Ser 112 by MAPK [286],[414]. BAD phosphorylated is sequestred by 14-3-3γ which inhibits the heterodimerization with the members Bcl-2 and Bcl-xL and then they are translocated from the surface of the mitochondria to the cytosol [415]. If BAD is not phosphorylated, it heterodimerize with Bcl-2 or Bcl-X_L and is conducted to the outer membrane of the mitochondria to induce the release of cytochrome c, activation of caspases and subsequently apoptosis [415]. UTP induced the phosphorylation of BAD at Ser112 in normal cells and after ischemic treatment indicating its antiapoptotic or survival role. In ischemic treatment alone no phosphorylation of BAD was observed indicating that a death signal is activated possibly through the heterodimerization of BAD with Bcl-2 and Bcl-X_L. The phosphorylation of BAD at Ser112 induced by UTP can be modulated by the ERK1/2 pathway through the p90RSK which recently have been shown that p90RSK is responsible of the phosphorylation of BAD at Ser112 [416]. However, the phosphorylation of Ser136 was not determined and possibly the Akt/PKB pathway could not modulating the BAD phosphorylation. Moreover, during the OGD treatment no phosphorylation of Akt/PKB was observed, suggesting that this pathway could not be

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activating during the OGD, but after UTP addition, Akt/PKB could induce the phosphorylation of BAD at Ser 136 to modulate survival.

GSK3 is one of the protein kinases that unphosphorylated regulate apoptosis but once phosphorylated inhibits apoptosis through the pathway of PI3K-Akt [417]. The inactivation of GSK3β by Ser9 (the residue detected) phosphorylation is implicated in mechanisms of neuronal survival, but phosphorylation of Tyr216 on GSK3β is necessary for its activity and is implicated in degenerating cortical neurons induced by ischemia (PC12 cells) [418]. GSK3β is also involves in degenerative conditions such as alzeheimer disease, where once it is activated and it is inhibited by litium [419]. UTP induce the phosphorylation of GSK3α and β, but more the alpha than beta indicating that a survival signal was induced. During ischemia a slightly phosphorylation not significantly was observed in GSK3α and β but after addition of UTP an increased in their phosphorylation was observed similar to the response observed of Akt. This information suggests that UTP through the Akt pathway induce a survival signal.

Another mitogen-regulated serine-threonine protein kinase that is related to a similar signal which activates PKB/Akt is ribosomal p70S6k. The p70S6k is activated mainly by growth factors and mitogenic signals and modulate proliferation and survival signals [420]. Here, UTP through the $P2Y_2$ receptor activates the two isoforms of p70S6k (p70 and p85), principally p70 in starved cells (normal cells), however not in ischemic cells. The slightly increased in p70S6k induced by UTP after OGD suggest that UTP can induce a survival signal through a pathway that includes p70S6k. The signal transduction that activates p70S6k is similar to the one that activates PKB/Akt that is PI3K-dependent. Thus, these protein kinases are not activated during the OGD similar to PKB/Akt. However, it can modulate proliferation or survival signals by the activation of the $P2Y_2$ receptor.

Other substrate is IκB which modulate the activation of NFκB. IκB was not phosphorylated during OGD. In normal cells, UTP induce the phosphorylation of IκB, but not in ischemic cells which correlates with the response observed of Akt. Apparently

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IκBα pathway is not activated during the OGD or ischemia, and UTP did not modulate this pathway.

Regulation of gene expression is mediated by the phosphorylation of transcription factors that are subtrates of MAPK family and Akt and the phosphoprylation of these transcription factors were affected by OGD episode as well as UTP addition.

Elk1 and c-Myc showed a similar response during OGD and after UTP addition. Both are substrates of ERK1/2 [194]. However, during the 4hrs of OGD treatment, Elk1 showed a slightly increase in its phosphorylation. There is no phosphorylation of c-Myc during the 4 hrs of OGD, a similar result observed in ERK1/2 phosohorylation. Glutamate induced the phosphorylation of Elk1 via ERK1/2 in brain slices [421]. It is also possible that glutamate released could induce the phosphorylation of Elk1 via ERK1/2 activation in our model. Although the glutamate antagonists did not exert any affect in ERK1/2 phosphorylation during the 4hrs of OGD, ERK1/2 can be activated early by glutamate (during the first minutes until hrs) and induce the signal transduction that carry to the phosphorylation of ERK1/2 and subsequently to Elk1. However, Elk1 can also phosphoryalted by other member of the MAPK family such as JNK [422] and p38 [423]. It can be also possible that the activation of p38 and JNK by the activation of mGLU or/and NMDA receptors by glutamate and by the activation of the $P2Y_2$ receptor by the ATP released induces the phosphorylation of Elk1 which can explain the slightly phosphorylation of Elk1 and not c-Myc during OGD.

During the 4hrs of OGD and increase in ATF2 phosphorylation was observed and then with addition of UTP, no significant difference was observed. This result is comparable or similar to the results obtained of phosphorylation of p38 which can be one of the protein kinase that can phosphorylate ATF2 and NFκB. ATF2 can be phosphorylated also by JNK [300] and was demonstrated by the protein kinase inhibitors experiment where the inhibitor of JNK decreases the phosphorylation of ATF2 more than the inhibitor of p38 SB 203580. Walton et. al. reported that following unilateral hypoxic-ischemic (HI) insult in neurons, an increase in ATF2 phoshorylation was found within cells undergoing apoptosis [424]. An increase in ATF-2 phosphorylation is also observed

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in CAI pyramidal cells after ischemia [425]. However, the phosphorylation of ATF-2 in the dentate gyrus granule cells by the kainic acid suggest that ATF-2 is involved in cell survival [426]. Because p38 and JNK apparently regulate ATF2 phosphorylation induced by the $P2Y_2$ receptor, and we have to remember that inhibition of JNK and p38 induce an increase in LDH release suggesting that JNK and p38 could be modulating a protective or survival signal, this survival signal can be through the phosphorylation of ATF2.

The same explanation can be applied to c-Jun. The increase in phosphorylation of c-Jun in both sites Ser63 and Ser73 in ischemia as well as after addition of UTP suggests that c-Jun can modulate one of the pathways or both: apoptosis or survival. Therefore, an abundant literature demonstrated a death-promoting role for c-Jun [427]. Other reports suggested that activated c-Jun has a selective role in protecting human tumor cells such as glioblastoma cells, from apoptosis induced by DNA damage [428]. Also, cJun promotes neurite outgrowth and survival in PC12 cells [429]. A similar protective role was suggested for c-Jun during cerebral ischemia based on the finding of c-Jun induction in resistant dentate gyrus cells and CA3 pyramidal cells, which survive the hypoxic-ischemic insult [426]. The excitotoxicity of glutamate have caused the activation of JNK and p38 and its subsequent phosphorylation of c-Jun and p53 respectively in rat cerebellar granular cells which is associated and precede apoptotic death [430]. The possible involvement of glutamate in the activation of JNK/c-Jun signaling could be present. JNK phosphorylation was inhibited by some glutamate receptors antagonists indicating that glutamate could be influencing or inducing a death signal through a JNK pathway. However, JNK modulate a protective or survival signal as mentioned early possibly by the ATP released through the $P2Y_2$ receptor and adenosine through A_1 receptor.

During the 4hrs of OGD, CREB and ATF1 are phosphorylated. After OGD and after UTP treatment an increase in their phosphorylation (not significantly change in phosphorylation) was observed. An increase in CREB phosphorylation was observed in dentate granule cells after ischemia [425]. CREB phosphorylation during the 4hrs of OGD could be induced by the activation of the $P2Y_2$ receptor or/and by the activation of metabotropic mGlu receptors as wells as for NMDA receptor or adenosine A_1 receptor.

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These results correlates with the ones reported in hippocapal and striatl neurons where CREB is phosphorylated during ischemia through NMDA receptor activation by glutamate [431]. Glutamate induced CREB phosphorylation through the ERK1/2 pathway in brain slices [421]. CREB phosphorylation in neurons after ischemia and exposure to glutamate is induced by NMDA receptor-gated calcium influx and subsequent activation of CaMKII-IV and CREB phosphorylation after metabolic stress might show a neuroprotective response through CRE-mediated gene induction [432]. In auditory neurons, activation of AMPA receptors induce the phosphorylation of CREB and it was not attenuated by PD98059 [433] and it was modulated by Ca^{2+}. Some studies have suggested that Ca^{2+} influx through the L-type calcium channels and/or NMDA receptor channels in neurons is required for CREB phosphorylation [291]. Membrane depolarization can induce Ca^{2+} influx and activates CaMKII which can activates CREB and also can induce cAMP which activates PKA and in turn activates CREB [303]. Na^{+} influx through the NMDA receptor could be depolarizing the membrane and trigger the opening of the L-type Ca^{2+} channels [291]. Thus, it is also possible that the activation of NMDA during ischemia (4hrs of OGD) as the results suggest, regulate the activation of CREB inducing the entrance or influx of Ca^{2+} though the L-type calcium channels and also by itself.

However, CREB phosphorylation was found in neurons which survive ischemic injury (penumbra-like regions) and ERK1/2 phosphorylation suggesting that activation of this pathway within the cells may be involved in neuronal survival following focal cerebral ischemia in rat [25]. Moreover, CREB phosphorylation is associated with protection of hippocampal CA1 pyramidal neurons following focal ischemia in rat [434]. This phosphorylation is also associated to neuroprotective through CRE-mediated gene induction. Recently, the Bcl-2 gene was found to have a CRE in the 5' promoter region, and cell survival mediated by neurotrophin-induced CREB phosphorylation in sympathetic and cortical neurons was associated with increased Bcl-2 expression [435]. It was reported that NGF regulates Bcl-2 expression through a p42/p44 MAPK cascade in PC12 cells [435]. Some reports indicates that overexpression of Bcl-2 regulated by phosphorylation of CREB provides protection against apoptosis and ischemic neuronal death [304]. The $P2Y_2$ receptor induces CREB phosphorylation in sensory neurons

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mediated by calcium release from intracellular stores [436]. Recently, we demonstrated that UTP increased the expression of Bcl-2 through the $P2Y_2$ receptor in astrocytes [318]. Thus, the neuroprotective effect of CREB phosphorylation against glutamate or ischemia or addition of UTP after OGD may be attributable to increased expression of Bcl-2.

As observed in control cells, UTP induce the phosphorylation of FKHR and AFX inducing an anti-apoptotic signal through them. During ischemia, no phosphorylation is observed indicating that the cells are dying, but with addition with UTP, a slightly increase in their phosphorylation was observed. None of the FKHR family proteins is phosphorylated after ischemia/reperfusion in the rat kidney, only occurs later after 24 to 48 hrs postreperfusion which is in accordance the Akt activation in these cells [437]. Thus, UTP through the $P2Y_2$ receptor induce the phosphorylation of FKHR and AFX via the Akt pathway inducing a survival signal.

During ischemia NFκB is phosphorylated (activated) and after ischemia UTP for 15 min remains unchanged, activated. The response is similar to the ones observed in ATF2 and p38. The kinases responsible of its phosphorylation remain to be elucidated, because it was not determinated with the treatment of protein kinase inhibitors. However, PI3K-Akt-IKK-IκBα pathway, which predominantly is responsible of its activation, apparently does not mediate NFκB activation because Akt and IκBα are not phosphorylated during the 4 hrs of OGD. Because the response of NFκB is similar to the one obtained in p38 and ATF2, we can suggest that the p38 pathway or protein kinases downstream of p38 such as MAPKAP-K family [438] can be inducing the transactivation of NFκB. NFκB activation could be mediated by MAPKAP-K, because MAPKAP-K has been shown to be activated during ischemic preconditioning of isolated rat hearts which resulted in enhance protection [439]. Several reports have demonstrated that p38 can modulate the activation of NFκB because the incubation with p38 inhibitors such as SB203580 interfere with NFκB-dependent gene expression (NFκB activity) but not its DNA binding activity [440], [441]. Moreover, activation of a signal pathway which include MKK6, the protein kinase upstream of p38 and responsible of its phosphorylation, induced NFκB activation and this activation inhibits apoptosis in a p38-

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dependent manner in myocardial cells [262]. We have to remember that different isoforms of p38 can modulate apoptosis or survival. The NFκB activation regulates survival signals, so it is possible that if p38 is implicated in the activation of NFκB could be inducing a survival signal through its pathway, because MAPKAP-K through the p38 pathway confers protection in cardiac myocytes [439]. And, with the results of LDH, p38 inhibition increased LDH released suggesting that p38 activation is inducing a survival signal.

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CHAPTER V
Conclusion

Extracellular nucleotides through the $P2Y_2$ receptor activate signal transduction that involve the activation of protein kinases such as the MAPK family, Akt/PKB and their downstream substrates including transcription factors and other protein kinases that regulate gene expression and regulate survival as well as apoptosis responses. The activation of these protein kinases is partially dependent of the interaction of the $P2Y_2$ receptor with the $\alpha_v\beta_3$ integrin through the RGD sequence present in the first extracellular loop of the $P2Y_2$ receptor. The responses observed also entail src-dependent and –independent pathways, possibly through the formation of focal adhesions proteins complexes in the cytoskeleton involving an intracellular proline rich domain in the $P2Y_2$ receptor, and by the transactivation of the EGFR modulated also by integrins and may be by other unknown mechanisms.

As demonstrated here, during ischemia, released glutamate, nucleotides (such as ATP) and nucleoside (as adenosine) participate in a signal transduction of protective or survival signals. The receptors that could be involved in the signal transduction activated to modulate survival are A_1 and principally $P2Y_2$ receptor. However, we can not discard the possibility that the glutamate receptors such as the family group 1 (mGluR1 and mGluR2) could be modulating survival. The activation of NMDA or other glutamate receptors (AMPA, Kainate and mGlu receptors) could be modulating death. Astrocytes, which are more resistant than neurons to this type of stress, are the cells that bring support to neurons when they suffer severe damage during this type of stress (ischemia). Nucleotides through the $P2Y_2$ receptor in astrocytes can induce survival before its hydrolysis to adenosine that has been demonstrated to be neuroprotectant. Nucleotides through the $P2Y_2$ receptor can inhibit the release of glutamate that is responsible of the death of the cells during ischemia. It is important to metion that it will depend on the duration of the ischemia episode, because if it is transcient, the nucleotides can exert their function as survival agents helping the cells to overcome this type of stress and survive, but if the stress is prolonged, the cells can go to apoptosis or to necrosis where there is no possibility to survive.

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ATP given shortly after ischemia in kidney (kidney dysfunction) increased new DNA synthesis and augmented expression of genes critical to cellular proliferation ([442]. Although the report suggests a role for a P2X receptor, here we suggest a similar role for the $P2Y_2$ receptor in brain.

The following figures (Figure 5.1 and 5.2) summarize the responses induced by ischemia (OGD) and by UTP during or after ischemia in astrocytoma cells transfected with the $P2Y_2$ receptor.

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Figure 5.1 Proposed signal transduction pathway activated by the $P2Y_2$ receptor in 1321N1-h$P2Y_2$ receptor transfected cells: MAPK family and Akt/PKB -RGD, -proline rich dependence and possible EGFR transactivation.

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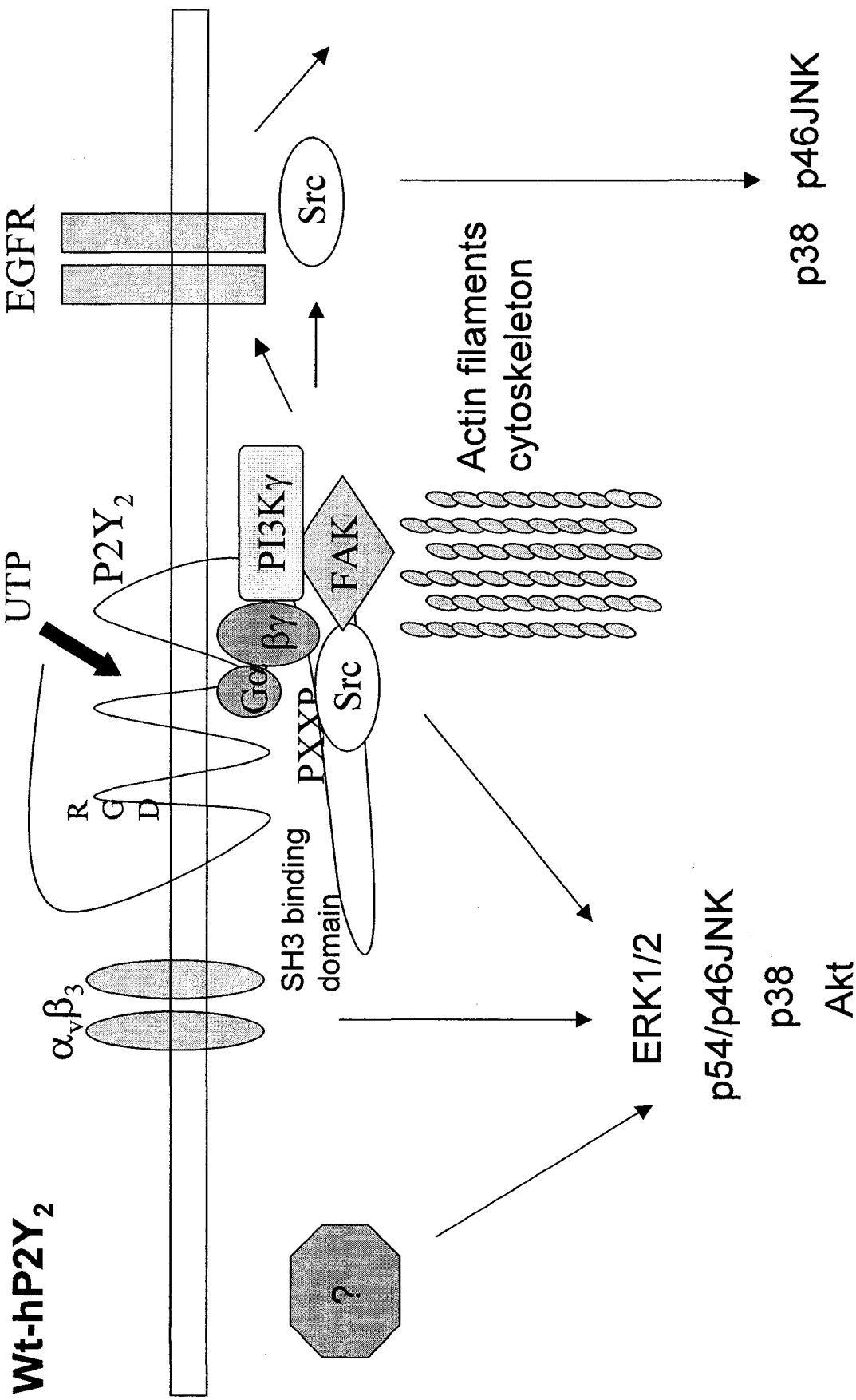

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Figure 5.2. Paracrine and autocrine signals during an OGD episode.

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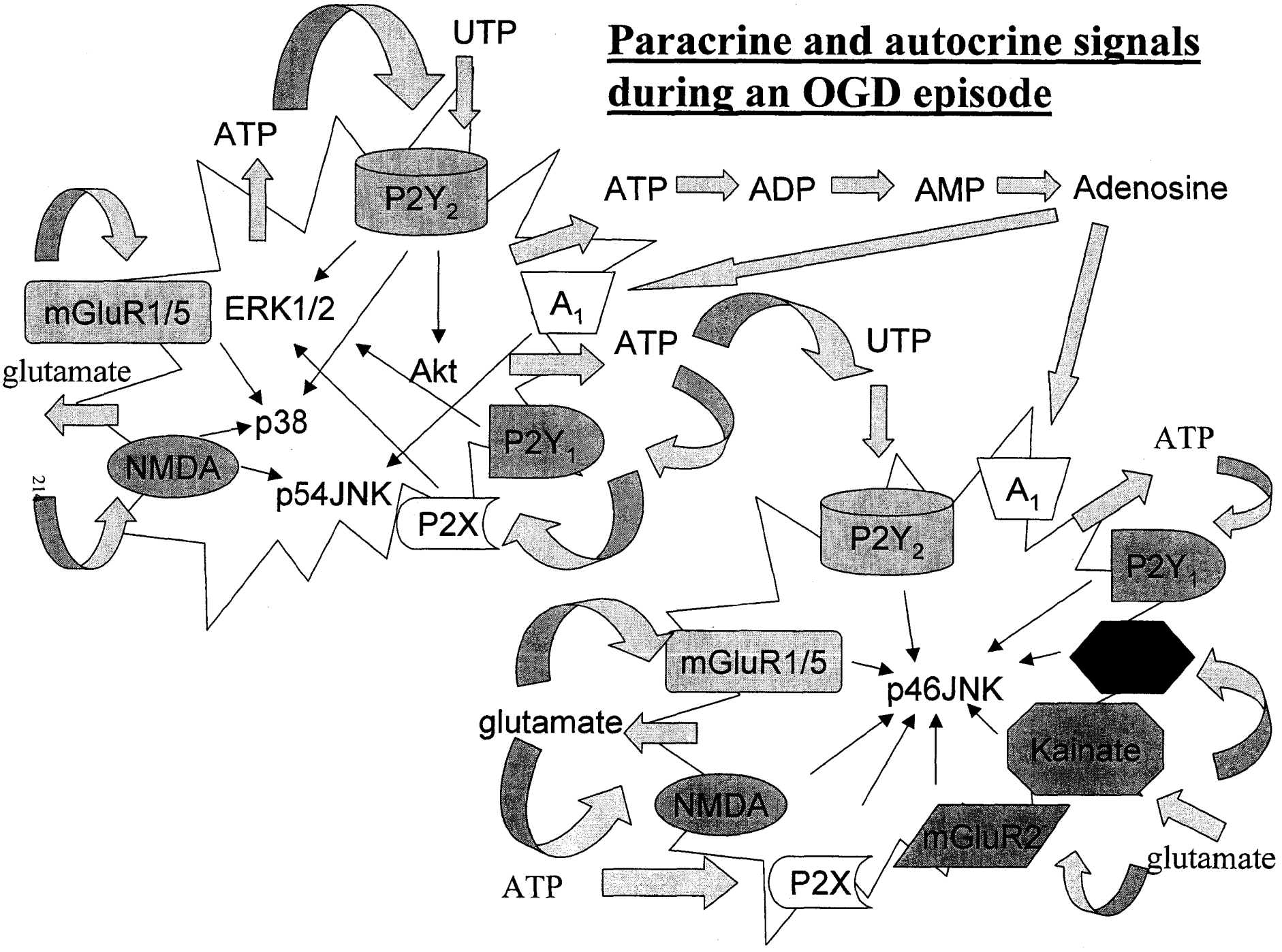

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