001

A KEY ROLE FOR THE P2Y1 RECEPTOR IN A TISSUE FACTOR-INDUCED THROMBOEMBOLISM. STUDIES IN P2Y1-KNOCK-OUT MICE AND IN MICE TREATED WITH A P2Y1 ANTAGONIST

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The P2Y1 receptor plays an essential role in thrombotic states induced by intravenous infusion of collagen and adrenaline as shown in P2Y1 knock-out mice (Léon et al., J Clin Invest 1999). The aim of the present study was to assess the role of this receptor in a thrombin-dependent thromboembolism. Human thromboplastin was injected into P2Y1-deficient mice. The effects on platelet counts and mortality were determined and plasma thrombin-antithrombin III (TAT) complexes were quantified. P2Y1-deficient mice were found to be resistant to thromboembolism induced by injection of thromboplastin. Whereas platelet count dramatically decreased in wild-type mice, no significant drop in platelet number was observed in P2Y1-null mice. Platelet consumption in wild-type mice was most probably due to thrombin generation since this effect was abolished by prior sub-cutaneous injection of hirudin. Thromboplastin injection also led to a rise in TAT complexes in plasma, again reflecting thrombin generation. TAT complexes increased less strongly in P2Y1-knockout mice than in wild-type mice, indicating that less thrombin was generated. Similar results were obtained in wild-type mice after intravenous administration of N6-methyl 2'-deoxyadenosine-3'-5'-bisphosphate (MRS2179), a selective P2Y1 receptor antagonist. Injection of the antagonist 30 seconds prior thromboplastin prevented death of mice and decrease in platelet count, and TAT complexes were lower than in control mice injected with physiological saline. Overall, our results demonstrate a role of the P2Y1 receptor in thrombotic states involving thrombin generation and add evidence for the potential relevance of this receptor as a target for antithrombotic drugs.

002

CLONING AND FUNCTIONAL CHARACTERIZATION OF THE MURINE $P2Y_4$ AND $P2Y_6$ RECEPTORS: THEIR POSSIBLE ROLE IN GASTROINTESTINAL EPITHELIAL CELL ION TRANSPORT

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Extracellular nucleotides regulate epithelial cell calcium-dependent chloride secretion via multiple receptors. The $P2Y_2$ receptor is the predominant transducer of chloride transport responses to nucleotides in the airways, but the P2 receptors regulating chloride transport in gastrointestinal epithelia have not been identified. Recent studies indicated potent UDP- and UTP-promoted chloride secretory responses in mouse gall bladder and jejuna epithelia, respectively, which were unaffected by $P2Y_2$ receptor gene disruption (Cressman et al. J. Biol. Chem. 274, 26461-68, 1999). Degenerative primers corresponding to conserved regions among the (h)(r)(m)$P2Y_2$, (h)(r)$P2Y_4$, and (h)(r)$P2Y_6$ receptors were combined to PCR-amplify candidate uridine nucleotide-selective receptor sequences in both gall bladder epithelial cell transcripts and chromosomal DNA from $P2Y_2$(-/-) mice. Subsequent screening of a murine genomic library with a gall bladder-derived probe resulted in isolation of a full-length gene product 92% identical to the rat $P2Y_6$ receptor. A gene product 91% identical to the rat $P2Y_4$ receptor also was isolated with a chromosomal DNA-derived probe. The cloned murine $P2Y_6$ and $P2Y_4$receptors were stably expressed in 1321N1 human astrocytoma cells, and the nucleotide-promoted inositol phosphate and calcium responses characterized. The (m)$P2Y_6$ receptor is highly selective for uridine nucleotides (UDPUTPADP=GDPCDP), while the (m)$P2Y_4$ receptor is potently although not selectively activated by UTP (UTPATPITPGTPCTP). The nucleotide selectivities of the cloned (m)$P2Y_6$ and (m)$P2Y_4$ receptors resemble that for nucleotide-promoted chloride transport in gall bladder and jejuna epithelial cells, respectively. The $P2Y_4$ and the $P2Y_6$ receptors may be target candidates for correcting gastrointestinal epithelial ion transport deficiencies such as that of cystic fibrosis.

003

MOLECULAR CLONING AND CHARACTERIZATION OF THE MOUSE P2Y4 NUCLEOTIDE RECEPTOR

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In order to isolate the mouse P2Y4 (mP2Y4) receptor, a mouse genomic library was screened with a human P2Y4 (hP2Y4) probe. An open reading frame encoding a protein of 363 amino acids was isolated. This protein showed 82% and 95% sequence identity with the human and the rat P2Y4 receptors, respectively. Tissue distribution was determined by RT-PCR analysis on RNA extracted from 16 different mouse tissues. The P2Y4 messenger RNA was detected in mouse liver, intestine, stomach, bladder and lung. In 1321N1 transfected cells, the mP2Y4 receptor was equally activated by UTP and ATP, like the rat P2Y4 (rP2Y4) receptor. However, the murine receptor was antagonized by pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) and Reactive blue 2, but not by suramin. PPADS is an antagonist of the human receptor but had no effect on rP2Y4.Thus in terms of antagonist profile, the mP2Y4 receptor is closer to the human ortholog, while in terms of agonist stimulation, it is more similar to the rP2Y4 receptor.

004

THE ROLE OF P2X1 RECEPTORS IN THE BLADDER

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P2X1 receptors are present on many excitable cells, widely expressed in smooth muscle, and have been implicated in bladder function. In order to investigate the role of this receptor in the bladder, we have used P2X1 receptor deficient mice. In the bladder of wild type mice (+/+), immunoreactivity for P2X1 is restricted to the smooth muscle layer and is absent in P2X1 receptor deficient mice (-/-). In organ bath studies, ATP, lb,g-meATP and a,b-meATP (100 microM for each agonist) evoked contractions in (+/+) and (+/-) bladder, (a,b-meATP had an EC50 value of 6 microM). These agonists failed to evoke contractions of (-/-) bladder indicating that the P2X1 receptor is an essential component of the native P2X receptor in bladder. There was no difference in responses of (+/+), (+/-) and (-/-) to carbachol (EC50 = 5 microM). Nerve stimulation evoked contractions of the bladder, the P2X1 mediated component accounted for 70% and 52% of the responses to stimulation with 10 and 100 pulses at 10 Hz respectively. The residual component of contraction was abolished by atropine. These results show that the P2X1 receptor contributes substantially to the neurogenic contraction of the bladder and may play a significant role in the control of micturition.

005

$P2X_3$-DEFICIENT MICE DISPLAY URINARY BLADDER HYPOREFLEXIA AND REDUCED NOCIFENSIVE BEHAVIOR

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$P2X_3$ is one of several ligand-gated ion channel receptors for ATP. The particular localization of $P2X_3$ to C-fiber sensory neurons in ganglia such as the DRG suggests that $P2X_3$ mediates the afferent action of ATP in a variety of organ systems. ATP and $P2X_3$ have been widely implicated in nociceptive neurotransmission (1), and recent evidence suggests that ATP may act as a physiological regulator of sensory neurotransmission in visceral hollow organs such as bladder (2). To elucidate the potential roles of $P2X_3$ we have generated a $P2X_3$ knockout (-/-) mouse. $P2X_3^{-/-}$ mice lack $P2X_3$ immunoreactivity as well as $P2X_3$-mediated, ATP- and α, β-methylene ATP-evoked currents in dissociated DRG neurons. $P2X_3^{-/-}$ mice show significantly less pain-related behavior in response to exogenous ATP; nocifensive paw-lifting following intraplantar injection of ATP was decreased by 45% with 500 nmol ATP, and nearly abolished with 100nmol. Of particular interest, $P2X_3^{-/-}$ mice exhibit a marked urinary bladder hyporeflexia upon cystometric evaluation, characterized by decreased voiding frequency, increased voiding volume, but normal bladder pressure responses. Taken together, these data support a role for $P2X_3$ in mediating sensory neurotransmission, not only in the context of nociception, but especially in the regulation of afferent signaling pathways controlling reflex responses to volume changes in the urinary bladder. These data suggest the potential therapeutic utility of $P2X_3$ antagonists in correcting disorders associated with abnormal urinary storage and voiding.

1. Curr. Opin. Neurobiol. 1996, 6, 526-532; 2. BJU Int. 1999, 84, 854-860; 3. J. Anat. 1999, 194, 335-342.

006

ATTENUATION OF ENDOTHELIAL CELL INFLAMMATION FOLLOWING ADENOVIRUS-MEDIATED GENE TRANSFER OF THE HUMAN A_{2A} ADENOSINE RECEPTOR

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Excessive inflammation of the vascular endothelium is a key feature common to the pathology of cardiovascular diseases like septic shock and atherosclerosis. It has been shown previously that the A_{2A} adenosine receptor (A_{2A}AR) present in endothelial cells (ECs) plays an important anti-inflammatory role by A) reducing induction of the adhesion molecules E-selectin and vascular cell adhesion molecule-1 (VCAM-1), and B) inhibiting the release of interleukin (IL)-6 and IL-8 in response to pro-inflammatory mediators. To determine whether increasing A_{2A}AR expression could enhance these therapeutically important effects, recombinant adenoviruses have been generated that can direct efficient co-expression of a myc epitope-tagged human A_{2A}AR and green fluorescent protein (GFP). Infection of human umbilical vein ECs (HUVECs) with 100 plaque-forming units/cell of adenovirus results in recombinant protein expression in over 95% of virus-exposed cells, as determined by fluorescence microscopy to detect GFP expression. Parallel co-expression of the human A_{2A}AR was determined by immunoblotting and immunoprecipitation of epitope-tagged receptors from cell extracts. Importantly, under these conditions, increased A_{2A}AR expression blocks the induction of E-selectin normally observed in HUVECs in response to lipopolysaccharide, a potent pro-inflammatory molecule involved in the pathogenesis of septic shock. These results suggest that increasing the signalling capacity of the A_{2A}AR in ECs represents an effective therapeutic strategy for reducing vascular inflammation in disease states.

007

CHANGES IN INFLAMMATION CELLS IN BRONCHOALVEOLAR LAVAGE FLUID WITH AEROSOLIZED ADENOSINE IN A MOUSE MODEL OF ALLERGIC ASTHMA

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In this study, we investigated the effect of adenosine on dynamic changes in inflammatory cells in bronchoalveolar lavage fluid (BALF) using an *in vivo* mouse model for allergic asthma. Mice were sensitized with two i.p. injections of ragweed (200 mg) on days 1 and 6. Aerosolization was performed with 0.5% ragweed for 20 min in the morning and afternoon on days 11,12 and 13. Mice were divided into following groups: **Sensitized group**: mice were sensitized and challenged with ragweed as mentioned above, and aerosolized with 0.9% saline for 2 min after the last allergen challenge; **Adenosine group**: sensitized mice were aerosolized with adenosine (6 mg/ml, 2 min); **Theophylline group**: sensitized mice were nebulized with theophylline (12 mg/ml, 3 min), 15 min later followed by aerosolized adenosine; **Control group**: mice received vehicles for sensitization, challenge and aerosolization. At various time-points (1,3,6,24,48,72 hrs), BALF was collected after the treatments. Marked inflammatory cell recruitment was observed in sensitized group compared with control group. In adenosine group, the cell numbers in BALF increased significantly at every time-point. The differential cell count showed that eosinophils, which were virtually absent in control group, were recovered in BALF at every time-point. Adenosine enhanced eosinophil accumulation in BALF. Lymphocytes were observed into the airway at every time-point. Adenosine significantly increased lymphocytes infiltrating into the BALF at 1, 6, and 72 hrs. Neutrophils increased at 1, 24, 48, and 72 hr time-points from sensitized group, and adenosine also enhanced the recruitment of neutrophils infiltrating into BAL. Macrophages initially decreased and returned to normal level after 48 hrs in sensitized group. Adenosine stimulated monocytes recruitment in BALF. Theophylline attenuated the effects of adenosine on BALF cellularity and inhibited eosinophils infiltration at 72 hr, decreased the lymphocytes at 3, 6, 24, and 48 hrs, and decreased neutrophils at 24 and 48 hrs. The data indicated that adenosine aerosol promoted inflammatory cells infiltrating into BALF in ragweed sensitized mice. Theophylline inhibited inflammatory cells recruitment in BALF at different time-points. (Supported by HL 50049)

008

CYTOKINES INDUCE UPREGULATION OF VASCULAR P2Y2-RECEPTORS AND INCREASED MITOGENIC RESPONSES TO UTP AND ATP

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P2Y2-receptors which mediate contractile and mitogenic effects of extracellular nucleotides in vascular smooth muscle cells (VSMC) are upregulated in the synthetic phenotype of VSMC and in the neointima after balloon angioplasty suggesting a role in the development of atherosclerosis. Since released cytokines in atherosclerotic lesions mediate multiple effects on gene transcription in VSMC, we speculated that cytokines could be involved in the regulation of P2Y2 receptor expression. Using a competitive RT-PCR, we detected that IL-1 beta induced a time- and dose- dependent upregulation of P2Y2 receptor mRNA, which was dramatically enhanced when combined with IFN-gamma or TNF-alfa. Lipopolysaccharide (LPS) also significantly increased the expression of P2Y2 receptor mRNA. The upregulation of P2Y2 receptor mRNA was paralleled at the functional level since IL-1 beta significantly increased the UTP-stimulated DNA synthesis and the release of intracellular Ca2+. Actinomycin D completely blocked the upregulation of P2Y2 receptor mRNA expression by IL-1 beta indicating de novo mRNA synthesis. There was no cAMP accumulation in the cells stimulated with IL-1 beta. The cyclooxygenase inhibitor indomethacin and the PKC inhibitor RO-31-8220 inhibited IL-1 beta induced upregulation of P2Y2 receptor mRNA expression, whereas rapamycin and PD098059 had no effects. Furthermore, neither P38 MAPK inhibitor SB20358 alone nor its combination with PD098059 blocked the effect of IL-1 beta on the expression of P2Y2 receptor mRNA. Our results demonstrate that inflammatory mediators upregulate vascular P2Y2 receptor both at the transcriptional and the functional level through PKC and cyclooxygenase but not cAMP, ERK-1/2 or P38 dependent pathways. This may result in increased growth stimulatory or contractile effects of extracellular UTP and ATP, which may be of importance in the development of vascular disease.

009

ADENOSINE A2A RECEPTOR MEDIATES ETHANOL- AND METHOTREXATE-INDUCED HEPATIC COLLAGEN RELEASE

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We have previously shown that adenosine mediates many of the anti-inflammatory actions of methotrexate as well as its toxicities. Since adenosine increases fibrous matrix in wounds, we speculated that hepatic fibrosis, the most feared of methotrexate's adverse effects and more frequently encountered in patients consuming ethanol, may also be mediated by increased adenosine production in the liver. We studied adenosine release by HPLC following 4 hour incubations of HepG2 cells with methotrexate, ethanol or their combination in pharmacologically relevant doses. Both methotrexate (from $0.121 \pm 0.057 \mu M$ to $0.205 \pm 0.022 \mu M$, control vs. MTX, $n=6$, $p<0.026$) and ethanol (up to 0.309 ± 0.133 μM, $n=6$, $p<0.019$) promote adenosine release from HepG2 cells in a dose-dependent fashion and their combination appears to be additive in effect. We then studied collagen production by hepatic stellate cells following 4 hour incubations with adenosine A2A receptor agonist CGS-21680. CGS-21680 dramatically increased collagen production in a dose-dependent fashion up to $3223 \pm 917\%$ control ($n=3$, $p<0.019$). The A1 and A2B antagonists minimally reversed the effect of CGS-21680 on collagen production whereas the specific A2A antagonist, CSC completely reversed the effect of all concentrations of CGS-21680 tested ($p<0.032$). Adenosine stimulated collagen production via the cAMP-protein kinase A pathway since the PKA inhibitor KT-5720 abrogated the CGS-21680-mediated increase in collagen production (from $339 \pm 99\%$ of control to $181 \pm 69\%$ of control, $n=4$, $p<0.013$). We conclude that adenosine, acting through the A2A receptor, mediates methotrexate and ethanol-induced collagen production in the liver and is responsible for cirrhosis in vivo.

010

IS ADENOSINE INVOLVED IN THE ETIOPATHOGENESIS OF DUCHENNE-TYPE MUSCULAR DISTROPHIES? STUDIES ON MYOBLASTIC C2C12 CELLS

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Due to altered purine catabolism, patients with Duchenne Muscular Dystrophy (DMD) present increased blood and muscles levels of adenosine. Based on this, we have hypothesized that abnormal accumulation of adenosine in DMD may trigger apoptosis of muscle cells and thus contribute to muscle degeneration. To verify this hypothesis, we have taken advantage of C2C12 myoblastic cells, which can be differentiated in vitro into multinucleated cells (myotubes). Exposure of proliferating myoblasts to adenosine or 2-chloro-adenosine resulted in apoptotic cell death. Cytotoxicity by either nucleoside did not depend upon extracellular adenosine receptors but, at least in part, by entry into cells via membrane transporters. The adenosine kinase inhibitor 5-iodotubercidin prevented 2-chloro-adenosine- (but not adenosine-)-induced effects, suggesting that an intracellular phosphorylation reaction plays a key role in 2-chloro-adenosine-mediated cytotoxicity. Conversely, adenosine cytotoxicity was aggravated by the addition of homocysteine, suggesting that adenosine effects may be due to intracellular accumulation of S-adenosyl-homocysteine, which blocks methylation-dependent reactions. Both nucleosides markedly disrupted myotube structure via an effect on the actin cytoskeleton, although there were marked differences in the cytopathic mechanisms and in the morphological alterations induced by these two nucleosides. Also in myotubes, inhibition of adenosine-kinase prevented 2-chloro-adenosine- (but not adenosine) -induced alteration of myotube structure, whereas homocysteine aggravated the cytopathic effects of adenosine. These results show that adenosine and 2-chloro-adenosine induce apoptosis of myogenic cells via different metabolic pathways, and are consistent with the hypothesis that excessive adenosine accumulation in dystrophic muscle may represent a novel pathogenetic pathway in muscle diseases.

011

P2Y2 NUCLEOTIDE RECEPTOR SIGNALING IN U937 CELLS: ACTIVATION, DESENSITIZATION AND COUPLING TO MITOGEN-ACTIVATED PROTEIN KINASES

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P2Y2 receptor activation has been shown to induce phenotypic differentiation of human promonocytic U937 cells that is associated with the inflammatory response. We investigated pro-inflammatory signal transduction pathways coupled to P2Y2 receptor activation in U937 cells, namely the activation of the mitogen-activated protein kinase (MAPK) cascade, and the desensitization of the receptor by prolonged nucleotide treatments. Results indicated that P2Y2 receptors couple to MAP kinases via phosphatidylinositol 3-kinase (PI3K) and c-src, but not protein kinase C (PKC). ERK1/2 activation was independent of calcium mobilization. UTP-induced desensitization caused increases in the EC50 values for receptor activation while decreasing the maximal calcium mobilization by a supramaximal dose of ligand. The PKC inhibitor GF 109203X did not inhibit UTP-induced desensitization, while the protein phosphatase inhibitor okadaic acid blocked receptor resensitization. Calcium signaling desensitization was readily reversible, however, cultures pretreated for 1 hr or longer were unable to restore full signaling capacity even after 30 min. This sustained desensitized state correlated with a decrease in P2Y2 receptor mRNA levels as measured by RT-PCR. ERK1/2 activation was also desensitized by a 5 min pretreatment with UTP but was not restored after 30 min incubation without agonist. We speculate that the desensitization of the P2Y2 receptor may involve covalent modifications (phosphorylation) to functionally uncouple the receptor from calcium signaling, that transcriptional regulation may play a role in long-term desensitization, that ERK1/2 activation is independent of the calcium mobilization signaling, and that the desensitized receptor may initiate critical signaling towards monocytes activation.

012

MECHANISM OF ADENOSINE/HOMOCYSTEINE-INDUCED ENDOTHELIAL CELL APOPTOSIS

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Apoptosis is important in vascular injury and in regulation of angiogenesis. Extracellular ATP and adenosine cause apoptosis of cultured bovine pulmonary artery endothelial cells (BPAEC) through a mechanism requiring cellular uptake of adenosine (Am J Physiol. 273:L485-L494, 1997) and inhibition of S-adenosylhomocysteine (SAH) hydrolase (Am J Physiol. 275:L379-L388, 1998). The purpose of this study was to investigate this mechanism of BPAEC apoptosis. Using confocal immunofluorescence microscopy, we found that adenosine/homocysteine (100 microM) disrupts focal adhesion complexes after 4 hours, with loss of focal adhesion kinase (FAK), vinculin, and paxillin proteins from focal adhesion complexes and later caspase-induced degradation of focal adhesion complex proteins. Rho A GTPase modulates focal adhesion complex formation and terminates in a CAAX motif. Since CAAX proteins undergo methylation by prenylcysteine carboxymethyl transferase (PCMT), we determined whether PCMT inhibition might be playing a role in adenosine/homocysteine-induced endothelial cell apoptosis. Inhibitors of PCMT, N-acetyl-farnesyl-cysteine and N-acetyl-geranylgeranyl cysteine, potentiate adenosine/homocysteine-induced apoptosis. Transient over-expression of PCMT in BPAEC inhibits adenosine/homocysteine-induced apoptosis. We conclude that adenosine/homocysteine causes apoptosis of endothelial cells via a mechanism involving inhibition of SAH hydrolase and PCMT and characterized by disruption of focal adhesion complexes. Adenosine/homocysteine-induced endothelial cell apoptosis may be important in the pathogenesis of vascular injury observed in acute lung injury and in atherosclerosis.

013

ALTERATION IN EXTRACELLULAR ADENOSINE BUT NOT INTRACELLULAR ATP DURING A TEMPERATURE INCREASE IN HIPPOCAMPAL SLICES

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An increase in extracellular adenosine has been measured from hippocampal slices after an ischemia-like stimulus, field electrical stimulation, or application of excitatory amino acids (Pedata et al. 1991, 1993). The increase in extracellular adenosine is temporally correlated with a profound decrease in synaptic transmission (Latini et al, 1998). Recently we demonstrated that a modest, transient increase in the recording temperature of rat hippocampal slices (from 32.5-38.5ºC) also results in a profound decrease in excitatory transmission, an effect mediated by adenosine A1 receptors (Masino and Dunwiddie, 1999). In the present study we simultaneously recorded the field EPSP and measured adenosine efflux in the superfusate from adult hippocampal slices before, during, and after a temperature increase from 32.5 to 38.5ºC. We also quantified tissue ATP content under control conditions, during increased temperature, and upon exposure to hypoxia sufficient to inhibit the synaptic response by an amount comparable to that seen with the temperature increase. During the increased temperature, a significant increase in adenosine efflux was observed that was temporally correlated with the decrease in the amplitude of the field EPSP. In contrast, we did not observe any significant change in tissue ATP levels upon increasing the temperature for 15 minutes, whereas ATP was significantly decreased by hypoxia lasting 3-4 minutes. These experiments further characterize a modest temperature increase as a non-pathological manipulation that significantly increases extracellular adenosine and reduces synaptic transmission, yet does not measurably alter tissue ATP content. Supported by RO1 NS 29173 and the Veterans Administration Medical Research Service.

014

EXTRACELLULAR NUCLEOTIDE-INDUCED NEUTROPHIL STIMULATION IS MEDIATED THROUGH GENERATION OF LEUKOTRIENE B4 AS AN ESSENTIAL INTERMEDIATE

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Neutrophil stimulation and subsequent adh5esion to endothelium play a central role in inflammatory diseases. Extracellular nucleotides are released from activated platelets and damaged cells during vascular injury and stimulate peripheral blood leukocytes. ATP-induced stimulation of neutrophils results in chemotaxis, elastase release, and expression of adhesive proteins on the cell surface. The molecular mechanism of extracellular nucleotide-mediated leukocyte activation is not well understood. Here we demonstrate that extracellular nucleotides generate leukotriene B4 (LTB4) in human peripheral blood polymorphonuclear cells. ATP and UTP caused the human polymorphonuclear cells to release elastase in a concentration-dependent manner and was completely inhibited by phenidone, an inhibitor of both the lipoxygenase and cycloxygenase pathways, or MK-886, a selective inhibitor of the leukotriene biosynthetic pathway. Peripheral blood polymorphonuclear leukocytes from mice lacking 5-lipoxygenase, with impaired leukotriene biosynthesis, failed to release elastase in response to ATP and UTP. Furthermore, U-75302 a selective antagonist of the leukotriene B4 receptor, abolished nucleotide-induced elastase release from isolated human polymorphonuclear cells. These results demonstrate that the extracellular nucleotide-mediated elastase release from the human polymorphonuclear cells is mediated through the formation of leukotriene B4 as an essential intermediate. To date, an increase in intracellular calcium through receptor stimulation has been thought to be sufficient for leukocyte stimulation. Our study demonstrates that although intracellular calcium is increased by either nucleotides or leukotriene B4, additional intracellular signaling through the leukotriene B4 receptor is essential for neutrophil stimulation and granule release. These studies provide novel insights into the molecular mechanisms of inflammatory diseases.

015

P2X7 AND INTERLEUKIN 1 β IN HUMAN MACROPHAGES

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The effect of P2X7 stimulation on interleukin-1 β (IL-1 β) was investigated in human monocytes. Levels of IL-1 β in supernatants and cell lysates of adherent monocytes were determined by ELISA. The cells were primed with interferon γ for 16 hours followed by lipopolysaccharide for 2 hours. In these conditions, adherent monocytes produced IL-1 β that remained essentially cell-associated. Flow cytometric analysis revealed that 60 % of the adherent monocytes were positive for P2X7 and that they colocalized with IL-1 β containing cells and markers (CD14) specific to macrophages.

When stimulated with the P2X7 agonist benzoyl-ATP (BzATP), macrophages released the mature IL-1 β into the supernatant. This release was abolished by pretreatment with monoclonal antibodies against human P2X7 receptor or interleukin-1 β-converting enzyme (ICE) inhibitor. Preincubation with cycloheximide prevented the IL-1 β release induced by BzATP suggesting that one or more newly synthesized proteins are necessary for this release.

Activation of P2X7 receptor with BzATP stimulus induced an additional production of IL-1 β compared to unstimulated cells. A two-fold increase could be observed after 30 minutes of stimulation. The rise in IL-1 β content resulted from newly translated IL-1 β protein since it was inhibited by cycloheximide. This new synthesis was also abolished by monoclonal antibodies against human P2X7 receptor.

We conclude that in activated human macrophages, stimulation of P2X7 receptors induces the synthesis of IL-1 β and activates the expression of at least one other protein that is required for the release of mature IL-1 β.

016

INFLAMMATORY CYTOKINES REGULATE EXPRESSION OF A2A AND A2B ADENOSINE RECEPTORS, A POSSIBLE MECHANISM FOR REGISTANCE TO METHOTREXATE

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We and others have reported that the potent anti-inflammatory autocoid adenosine, acting at its receptors, mediates the effects of anti-inflammatory drugs such as methotrexate. Since the regulation of anti-inflammatory adenosine receptors during inflammation is not completely understood we studied the effects of inflammatory cytokines on expression of adenosine receptors A_{2A} and A_{2B} in a human monocytic cell line, THP-1 cells. Cultured THP-1 cells were exposed to IL-1 (10U/ml), TNF-α (100U/ml), or IFN-γ (100U/ml) for 3 hours or overnight at 37°C. Expression of adenosine A_{2A} and A_{2B} receptors was examined by semiquantitative RT-PCR, Western blot analysis, and stimulated intracellular cAMP concentrations (ELISA). IL-1 and TNF-α significantly upregulated expression of message for both A_{2A} and A_{2B} receptors by 144±12% and 167±16% of control (overnight) for A_{2A} ($n=9$, $P<0.01$), and 132±7% and 141±9% of control (overnight) for A_{2B} ($n=9$, $P<0.001$), respectively, whereas IFN-γ had opposing effects on expression of the two receptors: downregulation of A_{2A} (65±8% of control, $n=9$, $P<0.01$) and upregulation of A_{2B} (140±8% of control, $n=9$, $P<001$). Protein expression mirrored in RNA expression (data not shown). Moreover, consistent with cytokine effects on A_{2A} receptor expression, CGS-21680 stimulated an increased cAMP in a dose-dependent fashion in IL-1 and TNF-α-treated cells (EC_{50} approximately 30nM) but not in resting and IFN-γ-treated cells. Since adenosine mediates many of the anti-inflammatory effects of methotrexate these observations suggest that local changes in the cytokine milieu may regulate the therapeutic response to methotrexate by altering the balance of adenosine receptors on inflammatory cells.

017

THE EFFECTS OF ADENOSINE AND ITS ANTAGONISTS ON ELASTASE RELEASE FROM HUMAN NEUTROPHILS

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Neutrophil elastase, a powerful serine protease released from neutrophil primary granules upon stimulation, plays a central role in the pathogenesis of tissue injury during neutrophilic inflammation. To investigate the effect of adenosine on neutrophil elastase release, we examined the effect of adenosine and its antagonists on chemoattractant induced release of elastase activity from human neutrophils. Elastase activity was determined by fluorometrically using fluorescence dye conjugated elastin.

Adenosine and its analogues dose-dependently inhibited the release of elastase activity. Therapeutic concentrations of theophylline (10-100μM), a nonspecific antagonist of adenosine receptor, not only abolished this adenosine effect, but also enhanced elastase release from neutrophils without exogenous adenosine. This effect was reversed by pretreatment with adenosine deaminase (ADA), suggesting that endogenous adenosine is important to modulate elastase release. After removal of extracellular adenosine with ADA, theophylline inhibited elastase release at higher concentrations probably due to its phosphodiesterase inhibitory effect. Thus, theophylline has dual function on elastase release depending on the existence of extracellular adenosine. Our results suggest that adenosine is a physiological modulator of elastase release and theophylline may enhance neutrophil mediated inflammation by inhibiting the effect of adenosine.

018

ADENOSINE, ACTING VIA A_{2A} RECEPTORS, MEDIATES THE ANTIINFLAMMATORY EFFECTS OF METHOTREXATE IN A MODEL OF ACUTE INFLAMMATION

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We have previously demonstrated that adenosine, acting at A_2 receptors, mediates the antiinflammatory effects of metothrexate in the murine air pouch model of acute inflammation. To confirm the role of adenosine as an antiinflammatory agent we examined inflammation in mice with genetically disrupted adenosine A_{2A} receptors (KO) and wild type controls (WT). Peritoneal inflammation was induced by injection of 0.5ml of thioglycollate (10% w/v), four hours later exudates were harvested and leukocytes quantitated. Mice received intraperitoneal injections of either saline or methotrexate (MTX) (0.75mg/kg weekly for four weeks) or dexamethasone (1.5mg/kg on day of the experiment). Leukocyte accumulation in the peritoneum did not differ between KO and WT mice (3.52 ± 0.32 vs 2.95 ± 0.31 million cell/ml, KO vs WT, p=NS, n=21 and 18, respectively). MTX reduced leukocyte accumulation in WT mice (26.5 ± 5.4% inhibition, $p<0.01$, n=7) but not in KO mice (3.7 ± 9.6% inhibition, p=NS, n=7). In contrast, dexamethasone, a potent glucocorticoid (the antiinflammatory effect of which is due in part to inhibition of the translocation of the transcriptional regulator NFκB) inhibited leukocyte accumulation similarly in both WT and KO mice (38.11 ± 9.5% and 29.8 ± 14.1% inhibition, $p<0.01$, n=4 and p_{2A} receptors, mediates the effects of MTX in peritoneal inflammation in mice.

019

P2 RECEPTORS MEDIATE CHLORIDE SECRETORY REFLEXES IN GUINEA PIG DISTAL COLON

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Mechanical stimulation of colonic mucosa releases 5-hydroxytryptamine (5-HT) from enterochromaffin cells. 5-HT activates intrinsic afferent neurons in a reflex that triggers chloride secretion. While electrophysiological recordings support the presence of excitatory P2 receptors on submucosal neurons, it is unknown whether reflex-evoked chloride secretion is mediated by ATP. To examine this possibility, the mucosa/submucosa was brushed causing a tetrodotoxin-sensitive increase in short-circuit current (Isc) indicative of chloride secretion. The stimulus-evoked increase in Isc was reduced by maximal concentrations of P2 receptor antagonists, pyridoxalphosphate- 6-azophenyl-2',4'-disulphonic acid (PPADS) and reactive blue 2. ATP (500 nM) caused an immediate biphasic increase in Isc of 178±19 uA/cm2 and 48±5 uA/cm2. The initial response to ATP was reduced by 50-80% in the presence of tetrodotoxin or atropine. The tetrodotoxin-resistant component was due to direct effects on non-neural cells. The P2x1 antagonist, NF 279, and the agonist, β,γ-methylene-L-ATP, had little effect on mechanically-evoked 5-HT release. Western blotting with P2x1 receptor antiserum revealed a prominent 48 kDa band which disappeared with control peptide. Although immunoreactivity for P2x1 receptors was identified in colonic submucosa, the pharmacology does not support a role for P2x1 receptors in secretory reflexes. This is consistant with P2x1 receptor immunoreactivity being restricted to smooth muscle in ileal mucosa (PNAS 93:8063,1996). The results suggest that exogenous or endogenous ATP released by mucosal stroking stimulates chloride secretion by activating P2 receptors on cholinergic neurons or on their synaptic inputs. Supported by NIH DK37240 and DK44179.

020

PKC AND ERK DEPENDENT ACTIVATION OF IKK BY LPS IN MACROPHAGES: ENHANCEMENT BY PYRIMIDINOCEPTOR-MEDIATED ACTIVATION OF CALMODULIN-DEPENDENT PROTEIN KINASE

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Extracellular nucleotides released from cytolytic cells and accumulated in inflammatory loci might play an important role in inflammatory response. We have previously reported that UTP via acting on macrophage pyrimidinoceptors can transcriptionally potentiate the synthesis of inflammatory mediators, including NO, PGE2 and IL-6, in response to bacterial endotoxin lipopolysaccharide (LPS). We also have clarified the ability of UTP to enhance LPS-induced activation of NF-κB (an essential nuclear transcription factor required for the expression of iNOS, COX-2 and IL-6 genes), phosphorylation and degradation of IκBα. In this study, we further investigated the molecular mechanisms by which UTP and LPS activate IKK and induce NF-κB Rel A (p65) phosphorylation. Treatment of J774 macrophages with LPS elicited a time-dependent increase in IKKα/βactivity. Although UTP itself did not significantly induce IKKβactivation, it increased IKKα activity and potentiated the IKK response of LPS. Similarly, LPS and UTP caused time-dependent increase of p65 phosphorylation at Ser536. PDTC (NF-κB inhibitor), Ro 31-8220 (PKC inhibitor) and PD 098059 (MEK inhibitor) inhibited both the LPS and UTP-induced IKK activity. Moreover, BAPTA/AM, a calcium chelator, inhibited UTP-stimulated IKKα activity, but did not affect the LPS stimulating effect. Furthermore, the UTP-induced potentiation of LPS-induced IKKα/β activity was inhibited by KN-93 (a CaMK inhibitor), Ro 31-8220 and PD 098059. Taken together, these data suggest that UTP can potentiate the LPS-induced IKKα/β activity and contribute to IκB as well as p65 phosphorylation through CaMK-, PKC- and ERK-dependent pathways.

021

A2A-ADENOSINE RECEPTOR MEDIATED INHIBITION OF RENAL INJURY AND NEUTROPHIL ADHESION FOLLOWING ISCHEMIA REPERFUSION OF KIDNEYS

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We sought to determine the mechanisms responsible for dramatically reduced renal tissue injury by agonists of A2A-adenosine receptors (A2A-ARs) in models of ischemia/reperfusion (I/R) injury. DWH-146e, a selective A2A-AR agonist, was administered subcutaneously to Sprague-Dawley rats and C57BL/6 mice via osmotic minipumps and animals were subjected to I/R. I/R led to an increase in plasma creatinine and kidney neutrophil infiltration. Infusion of DWH-146e at 10 ng/kg/min produced a 70% reduction in plasma creatinine as well as a decrease in neutrophil density in outer medulla and cortex and myeloperoxidase activity in the reperfused kidney. Myeloperoxidase activity in kidney correlated with the degree of renal injury. P-selectin and ICAM-1 immunoreactivity was most prominent in endothelial cells of peritubular capillaries and interlobular arteries of cortex and outer and inner medulla of vehicle-treated mice whose kidneys were subjected to I/R. DWH-146e treatment led to a pronounced decrease in P-selectin and ICAM-1 like immunoreactivity. These data are consistent with our hypothesis that A2A-AR agonists limit I/R injury due to an inhibitory effect on neutrophil adhesion.

022

ADENOSINE-INDUCED CASPASE ACTIVITY IN MOUSE NEUROBLASTOMA CELLS

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Adenosine is an endogenous neuromodulator, which has also been implicated in induction of apoptosis in several cell types. In this study we have investigated the effect of extracellular adenosine on the activation of caspases. Caspases are activated when a cell receives an apoptotic signal. We found that adenosine did not induce caspase activity after 16 hours in N1E-115 cells at 30 μM but an 8-fold increase was observed at 100 μM. The effect of adenosine on caspase activity was independent of the activation of adenosine receptors, because incubation of cells with several selective and non-selective receptor agonists did not induce caspase activity. Interestingly, inhibition of the conversion of adenosine to AMP with the adenosine kinase inhibitor AMDA reduced the 8-fold increase to an only 2-fold increase in caspase activity at 100 μM of adenosine. Blocking the breakdown of adenosine to inosine with deoxycoformycin resulted in a 9-fold increase in caspase activity at 30 μM adenosine. This indicates that the presence of adenosine and its conversion to AMP is probably necessary for the activation of caspases in N1E-115 cells.

023

UTP-MEDIATED INTERCELLULAR CALCIUM WAVES IN AIRWAY EPITHELIA

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Mechanical stimulation of a single cell results in cell to cell propagation of cytosolic calcium transients in several cell types including airway epithelia. It has been proposed that mechanically induced Ca^{2+}-waves in airway cells spread from cell to cell by intercellular movement of inositol trisphosphate (IP_3) via gap junctions. We have previously reported that intercellular Ca^{2+}-waves in polarized airway epithelial cells are mediated by autocrine/paracrine activity of secreted 5'-nucleotides acting on P2 receptors. We have also shown that apical mechanical stimulation induces nucleotide release to both apical and basolateral epithelial compartments. Here we report an extension of this study investigating weather UTP also contributes to the mechanically induced Ca^{2+}-wave propagation. To study this issue we generated a unique airway epithelial model: a nasal epithelial cell line from $P2Y_2$-receptor deficient mice reconstituted with a P2Y-receptor specific for UTP (human $P2Y_4$). In these cells, UTP, but neither other triphosphate nucleotides nor UDP, stimulated substantial elevations in cytosolic Ca^{2+}-level.

Mechanical stimulation of a single cell did not induce Ca^{2+}-wave in the $P2Y_2$(-/-) cells in the presence of PAPS, a $P2Y_1$-receptor antagonist, whereas large Ca^{2+}-waves were observed in $P2Y_4$-R transduced cells. Desensitization of the $P2Y_4$-receptors with UTP pretreatment, or selective removal of UTP by UDP-glucose-pyrophosphorylase abolished the Ca^{2+}-wave propagation in the $P2Y_4$-R transduced cells.

These results strongly suggest that focal mechanical stimulation induced cellular UTP release, which contributed to the generation of Ca^{2+}-waves.

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024

MECHANICALLY INDUCED CALCIUM WAVES MEDIATING PURINERGIC COUPLING BETWEEN MYO- AND SECRETORY EPITHELIAL CELLS IN MAMMARY GLAND

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In mouse mammary epithelial cells in culture, mechanical stimulation induced intercellular calcium wave that was caused by the release of nucleotides (ATP, UTP, UDP) from the touched cell and activation of P2Y2 purinergic receptors on the surrounding cells. In mammary gland, myoepithelial cells envelop alveoli of secretory epithelial cells and contract in response to oxytocin to expel milk in alveoli to the duct. To investigate whether the contraction of myoepithelial cells functions as a mechanical stimulation to secretory epithelial cells and initiates calcium waves, we have developed a new culture method of mammary alveoli. The culture contained a lot of myoepithelial cells as well as secretory epithelial cells. In this co-culture, application of oxytocin induced calcium increase and contraction of myoepithelial cells at first, and then it caused calcium waves through the secretory epithelial cells. The calcium wave was suppressed by suramin, a blocker of P2 purinergic receptors. Furthermore, myoepithelial cells were found to possess P2Y1 purinergic receptors and respond to the calcium wave and then contract again. These findings indicate that myoepithelial and secretory epithelial cells interact mutually with mechanical and purinergic coupling and form a positive feedback system to expel milk.

025

MEDIATION OF ERYTHROCYTE ATP RELEASE BY EXPRESSION OF ABC PROTEINS: CFTR, DF508 CFTR AND MRP

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The DF508 mutation of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is found in the majority of Cystic Fibrosis (CF) diagnoses. The DF508 CFTR mutation results in the failure of the mutated protein to integrate into the apical plasma membrane of polarized epithelial cells. We have observed increased expression of DF508 CFTR in red blood cell (RBC) membranes prepared from blood samples of individuals homozygous for the DF508 CFTR.

Prior studies found no abnormality in chloride transport from CF erythrocytes. Elevation in the RBC ATP has been noted for homozygous CF subjects. We have analyzed the content and ATP release from DF508 CFTR RBCs and found that both parameters are elevated compared to RBCs with non-mutated CFTR.

Conclusions: the CFTR protein is expressed in the red blood cell membrane. The DF508 CFTR shows increased expression in the RBC membrane compared to non-mutated CFTR. A second ABC protein, MRP, is also up regulated in the RBC membranes from CF subjects. The presence of elevated amounts of DF508 CFTR and MRP are associated with elevated total blood ATP and elevated RBC ATP efflux rates. The elevated RBC ATP and elevated extracellular ATP have potentially deleterious sequelae including over stimulation of vascular P2 purinergic receptors and increased RBC rigidity. Pharmacologic correction of these RBC and blood alterations offers a new frontier in treatment of CF pathophysiology.

026

SITE DIRECTED MUTAGENESIS OF CONSERVED POSITIVE CHARGES IN THE EXTRACELLULAR LOOP OF THE HUMAN P2X1 RECEPTOR

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P2X receptors contain two transmembrane domains connected by a large extracellular loop which is thought to be involved in ligand binding. Many ATP binding proteins contain a conserved "Walker" motif which contains key lysine residues involved in ATP binding. No Walker motif is present in the P2X receptors, however, seven lysines within the extracellular loop are conserved throughout the family of P2X receptors. We therefore examined the importance of these lysines (K) in the human P2X1 receptor by mutating them to either arginine (R), thus maintaining the positive charge, or alanine (A), thus removing the positive charge. cRNA generated from the mutants was expressed in Xenopus oocytes and the sensitivity to ATP and suramin and the time course of response were determined by the two electrode voltage clamp technique.

The EC50 for ATP increased by 15 and 20 fold when lysines 70 and 309 are mutated to arginine (K70R and K309R) and by greater than 1000 fold when the same positions are mutated to alanine (K70A and K309A). The antagonistic action of suramin increased by approximately 6 fold for both K70R and K309R but not for K70A or K309A. Wild type currents decayed rapidly during continued agonist exposure. However, time from peak current to 50% decay was 7 and 10 fold longer in K309R and K309A respectively but not different in the position 70 mutants. These results implicate the involvement of lysines 70 and 309 in agonist binding and gating of the P2X1 receptor ion channel.

027

IDENTIFICATION OF POLYMORPHISMS WITHIN THE PROMOTER SEQUENCE OF THE P2X7 GENE IN CELLS ISOLATED FROM HUMAN DONORS WITH DIFFERENT ATP-RESPONSE PHENOTYPES

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We have previously identified wide heterogeneity in the macrophage response to ATP between individual donors within the general population(1). We report here that heterogeneity reflects the percentage of cells that express functional P2X7 receptors cells within an individuals macrophage population. Macrophage cultures isolated from high responders comprise of 80% P2X7 expressing cells whereas low responders comprise of <10%. This mosaic effect was attributed to possible genetic factors regulating expression of P2X7receptors within macrophage sub-populations as both high and low responder cultures contain cells that express functional receptors. Genetic regulation of ATP response phenotype was then proposed to reside in the P2X7 promoter region. A study was performed to search upstream of the published sequence of exon 1 of the P2X7 receptor gene for possible promoter sequences and polymorphisms within cells isolated from different ATP-responder phenotypes. A number of polymorphisms were thus identified in the putative promoter sequences of the P2X7 gene.

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028

DIFFERENTIAL EXPRESSION OF P2X RECEPTORS ASSEMBLING AS ATP-GATED ION CHANNELS IN THE COCHLEA

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Functional and molecular characterisation of ATP-gated ion channel expression in the rat and guinea-pig cochlea indicates a complexity of P2X receptor subunit assembly which confers differential ion channel phenotypes to cells involved in sound transduction and auditory neurotransmission. Regulation of P2X receptor subunit expression by cells in the cochlear partition results in variation in ATP-activated conductance properties, including differential sensitivity to 2MeSATP and a,ßMeATP, and allosteric modulation by divalent cations and protons. It is likely that purinergic signal transduction mediated via these receptors serves to decouple the active mechanical tuning mechanism in the cochlea at high sound levels by shunting the driving force for hair cell transduction. This process may contribute to noise-induced temporary shifts in hearing threshold. This purinergic regulation of the cochlear partition likely acts in concert with the dynamic dampening of hair cell sensitivity provided by cholinergic efferent innervation. Analysis of P2X receptor expression during cochlear ontogeny indicates that purinergic signalling involving several P2X receptor subunits is focused at the primary auditory neurones and associated innervation of the hair cells. These neurones have a pharmacology and desensitisation consistent with observed heterogeneous P2X subunit expression profiles.

Experiments were approved by the University of Auckland Animal Ethics Committee. Supported by the Health Research Council of New Zealand, Auckland Medical Research Foundation, Marsden Fund, and the New Zealand Lottery Grants Board.

029

MOLECULAR CLONING AND CHARACTERIZATION OF A NOVEL ATP P2X RECEPTOR SUBTYPE FROM EMBRYONIC CHICK SKELETAL MUSCLE

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We have cloned a new P2X ligand-gated ion channel receptor from embryonic chick skeletal muscle, which was tentatively named as chick $P2X_8$ ($cP2X_8$) receptor. The cloned cDNA encodes a protein with 402 amino acids. Electrophysiological study of the recombinant $cP2X_8$ receptor expressed in *Xenopus* oocytes showed that 10 μM ATP induced a fast inward current followed by rapid and long lasting desensitization in medium containing 1.8 mM Ca^{2+}. In medium with 0.3 mM Ca^{2+} ATP induced a biphasic response: a slower inward current succeeded the initial fast one. 2-Methylthio ATP, α,β-methylene ATP, and ATPγS were potent agonists, while ADP was a very weak agonist. ATP induced currents were blocked by 100 μM suramin and PPADS. Northern blot analysis and RT-PCR showed that $cP2X_8$ RNA transcripts were mainly expressed in skeletal muscle, brain, and heart of Day 10 chick embryos. A moderate level of expression was also detected in gizzard and retina. Whole mount in situ hybridization showed that $cP2X_8$ RNA transcripts were expressed mainly in neurotube, notochord, and stomach in Day 3 embryos. In Day 4 and Day 6 embryos, the $cP2X_8$ RNA transcripts were highly expressed in the myotome and premuscle mass. The physiological role of this receptor in the establishment of the skeletal muscle innervation will be studied.

030

THE EFFECT OF OMEGA-CONOTOXIN ON THE ATP-MEDIATED CURRENTS IN THE RAT DRG NEURONS

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ATP-evoked currents were investigated in acutely isolated DRG neurons of 8-10 day-old rats by means of voltage-clamp and concentration-clamp techniques. The response to ATP in all the cells tested could be subdivided into two groups: with fast and slow kinetics of desensitization. These currents are known to be mediated by P2X3 receptors and P2X2/X3 heteropolymers respectively (Ueno S. et.al., British J.Pharmacology, 1999, 126: 429-436). We have found that omega-conotoxin GVIA, traditionally accepted selective blocker of N-type calcium channels inhibits also ATP-activated currents. This action of omega-conotoxin is concentration-dependent and subtype-specific with IC50 values of 45 +/- 15 nM for P2X3 and 9 +/- 3 μM for P2X2/X3 receptors.

The action of another type of omega-conotoxin, MVIIC, was much lower: in the concentration of 10 μM the latter toxin inhibited rapidly desensitizing response to ATP by 65% and slowly desensitizing response by 18%.

The effects of both toxins were totally reversible.

031

UNUSUAL ALLOSTERIC MODULATION OF COCHLEAR P2X RECEPTORS

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P2X2 receptors expressed by sensory and supporting cells of the organ of Corti mediate suppression of cochlear sensitivity via humoral ATP actions (Housley et al., J. Neurosci. 19:8377-8388, 1999). Of the cloned P2X subunits, only P2X2 assembles into ATP-gated ion channels whose conductance is positively modulated by Zn2+, Cu2+ and H+ (Xiong et al., J. Neurophysiol. 81:2088-2094, 1999). In voltage-clamped Xenopus oocytes expressing the P2X2 subunit, 15μM ATP-evoked inward currents had rapid onset and slow desensitisation. These currents were increased by half with Cu2+ (40μM) and doubled by Zn2+ (40μM). Acid pH (6.5) quadrupled the ATP-activated current, whereas alkaline pH (9.0) eliminated it. In contrast, Cu2+ and acid pH potentiated voltage-clamped guinea-pig outer hair cells (OHC) by only approximately 15% and 50% respectively. Alkaline pH reduced the ATP-gated OHC current by half. Zn2+ was either ineffective (1-10μM) or inhibitory (40-100μM). Similar allosteric modulation of the P2X receptor conductance was observed in (supporting) Deiters' cells. Both recombinant P2X2 receptors and these native P2X receptors had reduced ATP-gated currents in the presence of extracellular Ca2+ (1.8mM), and gradual desensitisation with repetitive ATP applications. This desensitization was largely reduced by removal of extracellular Ca2+. Given P2X2 receptor expression by OHC and Deiters' cells, a greater sensitivity to pH and Cu2+ was expected, and the lack of potentiation by Zn2+ was surprising. These results suggest heteromeric subunit assembly contributes to the phenotypic characteristics of cochlear ATP-gated ion channels.
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032

AMINO ACID SUBSTITUTIONS WHICH ALTER THE CALCIUM PERMEABILITY OF P2X2 RECEPTORS

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The mechanism underlying Ca2+ permeation was investigated by measuring the reversal potential of current permeating through recombinant P2X2 receptor/channel and its mutants expressed in human embryonic kidney (HEK) 293 cells. Amino acid residues facing the channel pore in the M2 segment (Asn333, Thr336, Leu338, Ser340, Gly342 and Asp349) were substituted with Ala except for Asp349 which was replaced with Asn, Gln or Glu. With 2 mM external Ca2+ as the only external cation, the reversal potential of current permeating through the wild type channel was about -60 mV. Among the mutant channels, a negative shift in the reversal potential was found with the mutant where Asn333 was replaced with Ala, and a positive shift was found with T336A channel. Asn333 was further replaced with other amino acid residues to examine the relation between the reversal potential and the polarity at this position. A negative shift in the reversal potential was also found when Asn333 was replaced with Gly, another neutral amino acid residue, instead of Ala. In contrast, the replacement with a negatively charged residue (Asp or Glu) resulted in a positive shift of the reversal potential. The replacement of Asn333 with another negatively polarized residue (Gln or Ser) did not affect the reversal potential. Similar results were also obtained when Ba2+ was used as the only external cation. The results suggest that Asn333 interact directly with Ca2+, presumably though its negative polarity, to serve as a selective filter for cations.

033

P2X RECEPTOR GENE EXPRESSION IN RAT SYMPATHETIC NEURONES

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ATP is cotransmitter of sympathetic neurones but also induces noradrenaline release by acting on somatic P2X receptors. Using RT-PCR the present study investigated the expression pattern of P2X receptors in rat sympathetic paravertebral ganglia of newborn (P1) and 5 day-old (P5) Wistar rats, as well as in cultured neurones and fibroblasts derived from paravertebral ganglia. Para-vertebral tissue sections were used either for RNA extraction or enzymatic cell dissociation and gentle trituration. Neuronal cultures were grown in DMEM/5% FCS supple-mented with insulin and NGF. Fibroblasts were enriched by percoll centrifugation before seeding. mRNA encoding P2X1-7 receptors was detected in the tissue sections both at P1 and P5. Of the P2X2 receptor both a full-length and alternatively spliced form with a deletion of 207 bp was found. In neuronal-free subcultured fibroblasts mRNA encoding the P2X3, P2X4, P2X5 and P2X7 receptor was proven; no evidence was found for the expression of P2X1, P2X2 or P2X6 receptors. In single-cell RT-PCR studies on cultured neurones, a proportion of approx. 60% and 25% of tyrosine hydroxylase (TH)-positive cells expressed P2X5 and P2X2 receptors, respectively. The cells included in these studies had been electrophysiologically characterised by responses to acetylcholine and ATP before harvesting. The identity of the amplified P2X5 and P2X2 fragments with the known P2X5 and P2X2 gene was verified by sequence analysis. The present data demonstrate the expression of multiple P2X receptors both in sympathetic neurones and fibroblasts. The single-cell experiments suggest a differential P2X receptor expression of individual sympathetic neurones.

034

IMMUNOHISTOCHEMISTRY OF P2X RECEPTORS IN THE ADULT RAT TESTIS

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We investigated the expression of P2X receptors in testes of adult rats by immunohistochemistry and western blotting with antibodies against all seven P2X receptor subtypes. Immunoraective cells were identified and monitored throughout all 14 stages of the cycle of the seminiferous epithelium.

Results of the immunohistochemical and western blotting experiments showed expression of $P2X_1$, $P2X_2$, $P2X_3$, $P2X_5$ and $P2X_7$ receptors. $P2X_4$ and $P2X_6$ receptors were absent from the testis. Blood vessels diplayed immunostaining for $P2X_1$ and $P2X_2$ receptors, the $P2X_1$ receptors being seen exclusively in blood vessels. Immunostaining for $P2X_2$ and $P2X_3$ receptors in the seminiferous tubules was found to be highly coincident regarding the stained cell types and their staging during sperm maturation. Immunopositive $P2X_2$ and $P2X_3$ receptors in testicular germ cells first appeared in early steps of gamete development (steps 1 through 8). The expression of $P2X_5$ receptors was seen through the mid steps of spermatid differentiation (step 10 - 13) and in pachytene spermtocytes through stages 10 - 13. $P2X_2$ and $P2X_3$ receptors reappeared in later steps of spermatid differentiation (steps 16 - 19). Immunoraectivity for $P2X_7$ receptors was seen only in differentiated spermatids that were about to be released (step 19). Sertoli cells showed staining for $P2X_2$, $P2X_3$ and $P2X_7$ receptors through stages 1 to 8 of the cycle of the seminiferous epithelium; however, only $P2X_7$receptor expression was present in stages 9 - 14. No immunostaining was detected on Leydig cells.

035

INVESTIGATION OF THE INTERACTIONS BETWEEN ATP-GATED $P2X_3$ RECEPTORS AND CAPSAICIN-GATED VR1 RECEPTORS COEXPRESSED IN OOCYTES

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We have investigated the possibility that $P2X_3$ and VR1 receptors interact when heterologously coexpressed in *Xenopus* oocytes. Under voltage clamp conditions, ATP (0.1-100 μM) activated fast inward, rapidly-desensitising currents and capsaicin (0.1-30 μM) evoked slow inward, slowly-inactivated currents. Where ATP and capsaicin were co-applied (each at 1 μM), a rapidly-desensitising current ($P2X_3$-like) was followed by a slowly-inactivated current (VR1-like). The potency and efficacy of ATP and capsaicin were unaltered by the presence and action of the other. Also, neither agonist altered the potency and efficacy of the other when $P2X_3$ or VR1 receptors were expressed separately.

Lowered extracellular pH significantly enhanced the potency of capsaicin at VR1 receptors (EC_{50}: 0.04±0.01 μM at pH 5.5; 0.19±0.03 μM at pH 6.5; 0.86±0.07 μM at pH 7.5), whilst the potency of ATP at $P2X_3$ receptors was not markedly altered. Acidic bathing solution (pH 5.5) failed to activate inward currents at P2X3 receptors, but evoked rapidly-desensitising inward currents via VR1 receptors. With VR1 and $P2X_3$ receptors coexpressed, a lowered pH selectively enhanced the VR1-like response to capsaicin by 215±18% (pH 6.5) and 281±32% (pH 5.5) whilst the $P2X_3$-like response remained unchanged.

In conclusion, the activation of $P2X_3$ receptors neither enhances or diminishes the activation of VR1 receptors, and *vice versa*. The capsaicin response, but not the ATP response, was altered by lowered pH to the same degree when their receptors are expressed alone alltogether. At the level of macroscopic whole-cell currents, there is no evidence for crosstalk between heterologously expressed $P2X_3$ and VR1 receptors.

036

LOCALISATION OF THE P2X1 RECEPTOR SUBUNIT IN THE DEVELOPING RAT COCHLEA

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Riboprobe-based in situ hybridization and immunohistochemistry were used to study the distribution of the adenosine 5'-triphosphate (ATP)-gated ion channel P2X1 receptor subunit (P2X1R) mRNA and protein in the developing rat cochlea. Cochleae from embryonic (E14, E16 and E18), postnatal (P0, P2, P6 and P10) and adult rats were examined. P2X1R expression was evident from E16 until P6. P2X1R mRNA was associated with the otic capsule and adjacent mesenchyme (from E16 to P6), spiral limbus (from P0 to P6) and in the area around the point of the insertion of Reissner's membrane (from P2 to P6). Similar to mRNA localisation, P2X1R immunoreactivity was detected in the otic capsule, associated mesenchyme (from E16 to P6), spiral limbus (from P0 to adult), marginal cells adjacent to insertion of Reissner's membrane (from P0 to P6), and epithelial cells of Reissner's membrane (from P2 to P6). In addition, immunoreactivity was apparent in the spiral ganglion (from E16 to E18) and intraganglionic spiral bundle (from E18 to P6). Associated presumptive synaptic labelling occurred at the base of outer hair cells and inner hair cells (from P0 to P6, with the most intense staining at P2). These findings suggest that P2X1R plays a role in cochlear development , including differentiation of hair cell innervation. These data extend the functional significance of extracellular ATP signalling in the inner ear.

Approved by the University of Auckland Animal Ethics Committee.

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037

RECEPTOR ACTIVATION-DEPENDENT CHANGES IN AGONIST POTENCY FOR THE P2X7 RECEPTOR

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We have determined the effect of culture conditions, apyrase and prior receptor activation on agonist potency for recombinant P2X7 receptors. Receptor function was determined by measuring agonist-stimulated accumulation of ethidium into HEK293 cells expressing recombinant P2X7 receptors and grown in 96 well plates (Michel et al., 1999, Naunyn Schmeiderg's Arch,. Pharmacol., 359,102-109). The potency of 2'&3'-O-benzoylbenzoyl-ATP (BzATP) for the human P2X7 receptor varied 20 fold depending upon culture conditions and assay additions. Two extremes of potency existed. If cells were pretreated with apyrase, the BzATP EC50 BzATP was approximately 10μM. However, if cells were activated by either endogenous or exogenous agonist and washed, the subsequent BzATP EC50 increased to 0.5microM. BzATP potency for the P2X7 receptor only increased after activation of the cells with P2X7 receptor agonists but not with UTP, ADP or adenosine. The increase in BzATP potency was observed after short (5-60s) exposures to BzATP and was detectable within 5min of agonist exposure. The effect persisted for at least 1hr in assay buffer but could be reversed by incubating cells in apyrase and 10mM MgCl2. The activation-dependent increase in agonist potency was calcium-dependent. Similar changes in agonist potency were observed at rat and mouse P2X7 receptors. It is known that changes in ionic-selectivity of P2X7 receptors occur following receptor activation. This study demonstrates that agonist potency can also increase after prior receptor activation, a phenomenon which may be of importance in pathophysiological situations.

038

EXPRESSION AND FUNCTIONAL CHARACTERISATION OF THE RAT P2X7-GFP CHIMERIC PROTEIN.

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Receptor fusion with enhanced green fluorescent protein (eGFP) is a valuable tool to study receptor function in living cells. We have engineered a fusion protein between the P2X7 receptor and eGFP joined at the C-terminus, and used it to determine the subcellular distibution, density and regulation of this P2X channel. Plasmids containing either rP2X7-eGFP or rP2X7 were transiently transfected into HEK-293 cells using Lipolectamine. Approximately 25 % of the cells transfected with the rP2X7-eGFP construct showed green fluorescence, while the control wild type HEK-293, or cells transfected with the rP2X7 plasmid, showed no fluorescence. Cells expressing GFP fluorescence were also labelled with a monoclonal antibody directed against the hP2X7 receptor. Whole cell recordings were made from the transfected cells. BzATP evoked currents at the rP2X7 and rP2X7-eGFP channels with pEC50 values of 5.16±0.08 and 5.27±0.12, respectively. BzATP (5 min, 22oC), in sucrose buffer containing 0.5 mM CaCl2 and 0 mM MgCl2 also induced an ethidium influx through both channels, suggesting that the fusion between the eGFP and the rP2X7 proteins does not affect large pore formation. The pEC50 values for BzATP at the rP2X7 and the rP2X7-eGFP channels were 6.01±0.09 and 5.95±0.11, respectively. Confocal microscopy showed plasma membrane-bound as well as cytoplasmic distibution of the rP2X7-eGFP protein. Since the functional properties of P2X7 receptor are not affected by fusion with GFP, this construct should prove useful for study P2X7 function in living cells.

039

FUNCTIONAL CHARACTERISATION OF P2X7 CHIMERIC RECEPTORS

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Chimeric receptors were engineered to investigate the known differences in pharmacology between P2X7 receptor orthologues (Chessell et al., 1998, FEBS Lett., 439, 26-30). Mouse and human P2X7 receptor cDNAs were dissected at a unique Bgl II restriction site (position 255) within the extracellular domain. Corresponding N- and C-termini were ligated, directionally subcloned into pcDNA3.1, and stably transfected into HEK293 cells. Receptor function was determined using standard whole-cell patch clamp techniques. Concentration-effect curves to 2 and 3-O-(4-benzoylbenzoyl)-ATP (BzATP) were constructed on naïve cells by a 2 s application of increasing concentrations of agonist every 30 s. NaGlutamate was used to replace NaCl in the external solution, as Cl ions inhibit agonist responses at the P2X7 receptor, and only in this buffer could complete concentration-effect curves be constructed. EC50 values for BzATP were 7 and 3 μM for human and human-mouse receptors and 81 and 58 μM for mouse and mouse-human receptors, respectively. This study indicates that the agonist potency at the P2X7 receptor may be more dependent on the region preceding amino acid 255. The extracellular domains of the human and mouse P2X7 receptors are highly conserved within this region, therefore it may be possible to determine more closely those residues implicated in agonist activity. Antagonist potencies also vary dramatically between mouse and human P2X7 receptors (Chessell et al., 1998, Br.J.Pharmacol., 124, 1314-1320; Hibell et al., 1999, Br.J.Pharmacol., 126, 20P). The availability of chimeric receptors may enable the amino acids responsible for these species differences too to be identified.

040

THE RAT $P2X_7$ ISOFORM BEHAVES AS A TRIMER WHEN PURIFIED FROM XENOPUS OOCYTES

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We have previously demonstrated that blue native PAGE (Schägger et al., Anal. Biochem. 217, 220-230, 1994) has the capacity to correctly display the pentameric structure of the muscle-type nicotinic acetylcholine receptor (Nicke et al. EMBO J. 17, 3016-3028, 1998) and the ionotropic glycine receptor (Griffon et al. EMBO J. 18, 4711-4721, 1999). Based on the same method, we provided evidence that the $P2X_1$ and $P2X_3$ receptor isoforms exhibit a trimeric architecture distinct from the tetrameric and pentameric architectural pattern of glutamate and nicotinic acetylcholine receptors, respectively.

Here we studied the quaternary structure of $P2X_7$ by blue native PAGE. cDNAs encoding human and rat $P2X_7$ were isolated from rat brain and human B lymphocytes, respectively, and N-terminally tagged with a hexahistidyl sequence to allow for native purification by Ni^{2+} NTA chromatography. Rat and human His-$P2X_7$ carry 6 and 5 N-linked glycans, respectively, which remain in the core-glyosylated state *en route* to the plasma membrane. When resolved by blue native PAGE, rat His-$P2X_7$ migrated as a non-covalently linked homo-trimer, whereas no defined assembly state could be assigned to human His-$P2X_7$.

We conclude from these results that rat $P2X_7$ possesses a trimeric architecture like rat $P2X_1$ and $P2X_3$ analysed before. Further experiments are in progress to explain the unclear assembly behaviour of human $P2X_7$.

041

ELECTROPHYSIOLOGICAL CHARACTERIZATION OF RAT $P2X_1$ RECEPTOR ACTIVATION AND DESENSITIZATION AT SUB-MICROMOLAR ATP CONCENTRATIONS

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$P2X_1$ receptors exhibit rapid activation to micromolar concentrations of extracellular ATP followed by fast desensitization. In electrophysiological experiments on whole cells it is difficult to resolve kinetics of current activation at these ATP concentrations due to the restricted speed of solution exchange. This holds true especially for our two-electrode voltage-clamp experiments on *Xenopus* oocytes due to their large size (1.2 mm). Therefore, we analyzed the slow current activation and desensitization at ATP concentrations between 3 and 100 nM. At these concentrations receptor currents could still be resolved and analysis of onset and offset time course could be performed without influence of the solution exchange speed. At ATP concentrations of 3, 10, 30 and 100 nM inward currents amounted to 0.07±0.01, 0.28±0.05, 2.0±0.36 and 10.0±1.5% of the current response to 10 μM ATP (37.3±1.7 μA, N=7-11). Currents in this concentration range showed slow activation and desensitization with onset time constants from 2.5±0.17 to 0.55±0.07 s and desensitization time constants from 115±13 to 4.97±0.35 s for 10 to 100 nM ATP, respectively (N=14-17). We also analyzed the ability of low ATP concentrations to desensitize the $P2X_1$ receptor by pre-incubation of the oocytes in low ATP containing solutions and found that already 1 nM ATP leads to significant desensitization suggesting the existence of a high affinity desensitized state that is also known from other ligand-gated ion channels such as nACh or $5\text{-}HT_3$ receptors. Our results also correspond to radio-ligand binding experiments from others where ATP affinities close to 1 nM have been found.

042

EFFECT OF DIADENOSINE POLYPHOSPHATES ON HOMO- AND HETERO-OLIGOMERIC P2X RECEPTORS EXPRESSED IN C6BU-1 CELLS

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Dianedenosine polyphosphates (Ap_nA) exert their physiological effects activating nucleotide receptors (named P2X and P2Y) and as yet undefined specific receptor insensitive to ATP and its derivates, named receptor for dinucleotides or P4. So far, seven different P2X subunits have been cloned. Co-assembling of one (homo-oligomeric) or different subunits (hetero-oligomeric) form different functional ionotropic P2X receptors. In order to get a better knowledge on the pharmacology of Ap_nA, we are currently studying the effect of these compounds on P2X receptors. For this purpose, we expressed the different P2X subunits in C6BU-1 cells. A tumour cell line that has been proven to be insensible to ATP and Ap_nA. 48 h after transfection the cells were stimulated with different concentrations of ATP and/or Ap_nA and intracellular free Ca^{2+} levels were measured in a multiple excitation microfluorescence system using Fura-2 as a dye. C6BU-1 cells transiently transfected with the homo-oligomeric $P2X_5$ receptor responded to ATP, Ap_3A and Ap_5A. The order of magnitude was: $ATPAp_5AAp_3A$. $P2X_5$ receptor was insensitive to Ap_3A and Ap_6A. Cells transfected with the $P2X_6$ receptor gave inconsistent responses to ATP. All Ap_nA tested resulted in luck of response. We are currently studying the effect of Ap_nA on the $P2X_7$ receptor as well as different hetero-oligomeric P2X receptors transiently transfected in this cell line. These results will be compared with data obtained in native models to identify the receptors responsible for the physiological effects of Ap_nA.

043

THE N-GLYCAN AT N210 AFFECTS THE ATP SENSITIVITY OF $P2X_1$ RECEPTORS

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P2X isoforms share two membrane-spanning domains connected by a large ectodomain that carries several sites for N-glycosylation (N-X-S/T). We have previously shown that four of the five possible acceptor sites of rat $P2X_1$ are used in *Xenopus* oocytes.

To examine whether N-glycans contribute to assembly, surface appearance and ligand recognition, we eliminated individual N-glycosylation sites of $P2X_1$ by site-directed mutagenesis. The formation of the mutants was assessed by SDS-PAGE, blue native PAGE and two electrode voltage clamp measurements. Replacement of N153, N184, N210 or N300 by Q, one at a time, gave rise to $P2X_1$ subunits that lacked one N-glycan. In contrast, replacement of N284 by Q did not change the number of N-glycans, demonstrating that N284 of wild type $P2X_1$ is the site that is unused. From the four N-glycans of wild type $P2X_1$, solely that at N300 acquireed complex-type carbohydrates *en route* to the plasma membrane, whereas the other three N-glycans remained in the high-mannose form. $P2X_1$ mutants lacking one or two N-glycans were efficiently synthesised and migrated as trimers like wild type $P2X_1$ when analysed by blue native PAGE. Moreover, electrophysiological measurements confirmed that all the mutants were capable of forming functional cation channels. A more detailed analysis, however, revealed a significant decrease in apparent ATP affinity of the mutant lacking the N-glycan in position 210.

We conclude from these data that at least two N-glycans are dispensable for function and robust expression. The N-glycan in position 210, however, appears to modify the local structure of the $P2X_1$ receptor.

044

DO P2X RECEPTOR SPLICE VARIANTS PLAY A PHYSIOLOGICAL ROLE

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The diversity of P2X receptor subunits and the additional complexity imposed by the presence of alternatively spliced variants, together with the potential for heteropolymerization between different P2X subunits, are among the variables that act in concert to determine the phenotypic response of any given cell type activated by extracellular ATP. At least two isoforms of the mouse orthologue of the P2X4 receptor have been identified: mP2X4, and a splice variant of this receptor, mP2X4a, which lacks a 27 amino acid region in the extracellular domain corresponding to exon 6 of the known P2X receptor gene structures. PCR amplification of the region comprising the alternatively spliced sequence reveals expression of both P2X4 and P2X4a receptor in mouse brain. In situ hybridisation using isoform-selective oligonucleotide probes reveals a widespread distribution of both the P2X4 and P2X4a isoforms suggesting the possible formation of heteromeric P2X4/4a receptors in vivo. Bioinformatic analyses confirm the results of our in situ hybridisation studies. In a heterologous expression system devoid of endogenous P2 receptors, we observe a subtle interaction between the mP2X4 and mP2X4a subunits suggesting that mP2X4a, whilst forming a poorly functional homomeric receptor, is able to interact with a full-length mP2X4 to result in a functional heteromeric ion channel, although this is at the expense of the affinity of the heteromeric receptor for ATP. The eventual elucidation of the physiological role of the splice variants of P2X receptors will add a new and exciting element to the modulation of neurotransmission by purinoceptors.

045

HOW MANY P2 RECEPTORS TYPES CAN BE DETECTED IN THE GASTRIC MUCOSA

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Previous studies have indicated the presence of P2Y1-like receptors in the basolateral plasma membrane of the gastric parietal cells. These receptors are involved in the modulation of the gastric acid secretion. Preliminary investigations also suggest some modulation action of UTP on the gastric secretion but no information has been reported about P2X in the stomach.

In this study the first complete investigation of P2 receptors types on the gastric mucosa is presented. It has been carried out by RNA extraction and RT-PCR using specific primers to P2 receptors.

Total RNA was extracted from gastric mucosa of several species and some purified cell fractions using a genomic-free RNA extraction kit (Gstract RNA iso-lation kit II, Maxim Biotech, Inc.) based on the Chomczynski and Sacchi method. Samples of 1 µg of mRNA were reverse-transcribed using the cDNA cycle kit (Invitrogen) and subsequently, aliquots of the reverse transcriptase reaction product were amplified by PCR. For some P2 receptors the "in situ" RT-PCR technique has been applied because it allows the detection of low levels of gene expression.Sections (6 to 8 µm thick) were mounted onto poly-L-lysine-coated glass slides, deparaffinated, and processed for in situ PCR amplification following an adapted procedure. This was followed by PCR amplification using the specific primers and digoxigenin- dUTP as label. As a negative control, some sections were identically processed in parallel except for omission of the reverse transcription reaction. As positive control specific rat tissue for each type of P2 receptors has been used.

046

P2X7-INDUCED DEATH OF INTRACELLULAR MYCOBACTERIA WITHIN ATP-TREATED MACROPHAGES IS ASSOCIATED WITH THE FORMATION OF LARGE ACIDIFIED MULTIBACILLARY VACUOLES–AN ELECTRON MICROSCOPY (EM) STUDY

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We have previously reported that ATP stimulation of Mycobacterium-bovis BCG-infected macrophages results in mycobacterial killing via ligation of the P2x7 receptor. We have also previously demonstrated that neither reactive oxygen nor nitrogen radicals are required for this novel mycobactericidal activity. In this study we investigated the potential involvement of phagosome maturation and acidification in the ATP/P2x7-induced mycobacterial killing process.

Mouse macrophage (J774) cover slip cultures were infected with BCG and stimulated with ATP (3mM) for various times. Paired macrophage cultures were then either lysed to determine BCG viability (by CFU assessment) or treated with a weak base DAMP (Molecular Probes) for EM assessment of the acidity of the bacteria-containing phagosomes.

Reduced BCG viability was detected at 20 minutes, and was maximal at 60 minutes post ATP treatment of infected cells. Within non-ATP-treated cells, infected with viable BCG, the bacteria were observed by EM to occupy individual phagosomes that were not acidified. In contrast, in cells fed dead (heat killed) BCG, the bacteria were observed within multibacillary, acidified vacuoles. By 20 minutes post ATP stimulation, within cells infected with viable BCG, the bacteria still occupied individual phagosomes but these were now showing evidence of acidification. By 90 minutes post ATP stimulation the BCG were observed within multibacillary highly acidified vacuoles.

ATP/P2X7 stimulation of infected macrophages results in the early acidification of mycobacterial-containing phagosomes, and later formation of acidified multibacillary vacuoles. The kinetics of BCG viability following ATP-stimulation suggests that killing of the mycobacteria is associated with P2x7-induced phagosome acidification.

047

P2Z/P2X7 EXPRESSION AND ATP-INDUCED PERMEABILIZATION IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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P2Z/P2X7 receptors have been identified in peripheral blood mononuclear cells (PBMCs) by measuring membrane permeabilization, calcium signaling, mRNA expression, and staining by antibodies. Here, we used three-color flow cytometry analysis to study P2X7/P2Z expression, membrane permeabilization, and cell death induced by ATP in normal PBMCs. Membrane permeabilization was measured during a brief (10-15 min) exposure to ATP in the presence of a DNA-binding dye (Ethidium or Topro-3). Apoptosis was assessed by measuring DNA content of cells, 6 hrs after a 30 min exposure to ATP. Cells displayed variable degrees of dye uptake when exposed to 5-10 mM ATP. Monocytes displayed stronger staining while B, T, and NK cells displayed a more variable pattern. Permeabilization of both monocytes and lymphocytes was enhanced by a high-potassium low-sodium extracellular solution. No significant changes in permeabilization was observed in the presence of potassium channel blockers. Permeabilization was abolished by both high magnesium concentration and oxidized ATP, as expected for a P2Z-related phenomenon. Apoptosis was not detected in PBMCs 6 hrs after exposure to ATP, while 30-70% of murine macrophages displayed a P2Z-associated apototic DNA pattern when kept under the same conditions. P2X7 molecules were detected in monocytes, B, T, and NK cells. The precise correlation between the level of P2X7 expression and membrane permeabilization in each cell type is under investigation. We concluded that ATP-induced permeabilization and P2X7 expression are widespread phenomena in normal PBMCs but significant variability may exist among normal individuals.

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048

P2X7 RECEPTOR AND GIANT CELL FORMATION

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Cell fusion is a central phenomenon during the immune response that leads to formation of large elements called multinucleated giant cells (MGCs) of common occurrence at sites of granulomatous inflammmation. Multinucleation of monocyte-derived human macrophages in vitro was efficiently prevented by the P2X7 inhibitor oxidized ATP (oATP). However, as is the case with other purinergic antagonists, we could not esclude that other membrane molecules in addition to P2X7 also ligated and blocked by oATP. Here we have used a monoclonal antibody (MoAb) raised against the outer domain of P2X7. This MoAb completely blocked macrophage fusion, but very interestingly, did not prevent cell aggregation, suggesting that chemotaxis and surface molecules recognition were not affected. We have observed that fusion spontaneously occurs in in vitro cultures of mononuclear phagocytes clones that express high levels of the P2X7 receptor. Immunolocalization of P2X7 during MGC formation shown that in resting macrophages and MGC P2X7 is uniformly distributed on the plasma membrane, but during fusion it concentrate in discrete membrane cluster at the site of cell-to-cell interaction. In conclusion, our data support a role for P2X7 as a novel plasma membrane receptor involved in macrophage fusion and MGC formation.

049

THE P2X7 PURINERGIC RECEPTOR OF MICROGLIAL CELLS IS INVOLVED IN THE KILLING OF INTRACELLULAR BACILLUS CALMETTE GUERIN.

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P2X7 receptors is a newly identified plasma membrane receptor expressed by mouse and human mononuclear phagocytes. Stimulation with extracellular ATP is a potent cytotoxic stimulus for all cells expressing the P2X7 receptor. It has been recently observed that in case macrophages are infected by an intracellular parasite (e.g Bacillus Calmette Guerin (BCG), or Mycobacterium tubercolosis), stimulation with ATP causes killing of both the phagocytic cell and the intracellular parasite. Here, we study the intracellular mechanism(s) involved in the ATP-mediated killing of the intracellular pathogen. Under our conditions, ATP extracellular produced a decrease in bacilli viability that is accompained by cell death by apoptosis. BCG infection of microglial cells produced an increase on the expression of the P2X7 receptor and a ATP release.

The physiological role of the P2X7 receptor is currently unknown, however the observations reported above strongly suggest that this type of receptor plays an important role in elimination of phagocytes infected with intracellular pathogens.

050

HIGH GLUCOSE MODULATE P2X7 RECEPTOR-MEDIATED FUNCTION IN HUMAN PRIMARY FIBROBLASTS

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P2 receptors are plasma membrane molecules activated by ATP and involved in cellular functions such as vascular reactivity, apoptosis and cytokine secretion. We have previously shown that human skin fibroblasts of normal subjects express P2 receptors. It is increasingly appreciated that fibroblasts and their secretory products play a central role in the pathogenesis of atherosclerotic plaque and microvascular complications of diabetes. Aim of this study was to evaluate the effect of high glucose on fibroblast responses to ATP. In fibroblasts obtained from a skin biopsy from four healthy volunteers, identification of P2X7 was performed by RT-PCR and immunoblotting, measurement of plasma membrane potential, intracellular calcium concentration by fluorimetry, plasma membrane permeability increase by uptake of fluorescent markers. Chronic incubation in high glucose potentiated P2X7 receptor-dependent microvesiculation, chromatin clumping, and expansion of cytoplasmic vesicule network. High glucose also enhanced spontaneous apoptosis, IL-6 release under basal and stimulated conditions by ATP, sensitized fibroblasts to the depolarizing effect by BzATP and increased the [Ca2+]i in response to ATP. Immunofluorescence showed ring-like receptors aggregates at the periphery of the cell. These results show an intriguing relationship between hyperglycaemia and P2X7 receptor localization and function. Given that ATP is frequently released in the extracellular milieu, we hypotesize that ATP receptors might play a role in the pathogenesis of vascular complications of diabetes.

051

ENHANCED MACROPHAGE PERMEABILIZATION BY ATP UPON LEISHMANIA INFECTION

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Leishmania are intracellular protozoan parasites which only infects macrophages. The disease may range from localized skin lesions to the fatal visceral form. Normally, activation of the P2Z/P2X7 receptor in macrophages by appropriate ATP concentrations leads to membrane pores and increased permeability to molecules with up to 900D. Here, we proposed to investigate the sensitivity of macrophages to ATP-induced permeabilization upon infection with leishmania in vitro and in vivo. For in vitro experiments, peritoneal macrophages were infected with Leishmania amazonensis promastigotes for 48 h and then incubated for 10 minutes at 37° C with ATP /Lucifer yellow. The dye entrance was monitored by fluorometry of washed monolayers. In the presence of 500 - 4000 mM extracellular ATP, the infected cells were in average 30 % more fluorescent than the uninfected ones. For in vivo experiments, BALB/c mice were infected with L. donovani amastigotes for up to 35 days. Their spleen cells were isolated and incubated with several concentrations of ATP plus ethidium bromide (EB) for 10 min, and the permeability to EB was measured by flow citometry. We found that in the presence of 500 mM ATP the cells from infected animals were in average 26% more permeable to EB than cells from uninfected animals. Furthermore, the cells from infected animals showed enhanced sensitivity to ATP, responding to concentrations of ATP as low as 8 mM, whereas the cells from uninfected were unresponsive to 30 mM ATP, indicating that not only more cells were permeable, but also that the cells were expressing more receptors. These observations indicate that infection with leishmania parasites may induce an increased expression of P2Z receptors in macrophages, which may have implications in the adjuvancy of ATP for selective drug delivery into parasitized cells.

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P2Z/P2X7 DOWN-REGULATION:WOULD IT BE AN EVASION MECHANISM TRIGGERED BY TRYPANOSOMA CRUZI

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P2Z/P2X7 is usually associated with cellular death, but it seems to be involved in other cell functions, such as NO production, IL-1 beta release and death of intracellular parasites. Trypanosoma cruzi is an intracellular parasite and its infection induces elevated production of IFN-gamma in the acute phase. As long as this cytokine may up-regulate P2Z/P2X7 receptor in some cell types, we decided to investigate its modulation by using experimental T. cruzi infection as model.

After incubation with 5mM ATP during 10 min in the presence or absence of ethidium bromide, peritoneal cells from control and infected mice were analyzed by flow cytometry. The permeabilization pattern revealed a specific population in the infected mice, which displayed lower fluorescence intensity than the control, suggesting a down-regulation of the P2Z phenomenon. This result does not fit with the higher levels of IFN-gamma produced in the acute phase. Furthermore, this down-regulation can be observed in any time throughout the infection, even in the chronic phase. Phenotypic assays prior to ATP treatment show that the population modulated is mainly composed by macrophages. However, fluorescence microscopic analysis of macrophages infected in vitro showed no altered ATP-induced permeability, when compared to control. Altogether, our data suggest the existence of a factor secreted in the infection that might modulate the P2Z phenomenon. Therefore, if the P2Z/P2X7 receptor is somehow involved in microbicidal activity, our findings suggest an interesting mechanism used by T. cruzi to avoid the immune response. Functional assays to verify this hypothesis is currently under investigation.

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TRYPANOSOMA CRUZI-INDUCED THYMIC ATROPHY AND P2Z/P2X7 ACTIVATION

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P2Z/P2X7 receptor is able to induce cell death in some cell types. This receptor is expressed in many cells of the immune system and it may be of great importance in some pathophysiological situations, such as infection by microorganisms. In the acute phase of Trypanosoma cruzi infection, the thymus drastically atrophies, restoring its normal weight in the chronic phase. The decrease of cellularity is directly related to the loss of CD4/CD8 double positive (DP) thymocytes, but the molecular mechanism underlying this phenomenon remains unknown. Whether this atrophy has any consequence to the positive/negative selection and cardiac inflammation in chagasic myocarditis is currently unknown. Hence, we decided to investigate the possible involvement of P2Z/P2X7 receptor in thymocytes death in experimental T. cruzi infection.

We found a direct correlation between thymus atrophy and ATP-induced permeabilization in thymocytes. Furthermore, normal DP thymocytes slightly respond to ATP in short-term permeabilization assays while DP cells from infected mice become 4 folds more susceptible. We then decided to evaluate necrosis and apoptosis by incubating the cells in a 10-hour assay. ATP treatment induced neither necrosis nor apoptosis in normal DP thymocytes. In contrast, DP cells from infected mice displayed 8% apoptosis and 20% necrosis under the same conditions. Our findings indicate that the DP cells become susceptible to ATP during the atrophy phase of T. cruzi infection. Furthermore, our data suggest an important role of P2Z/P2X7 in the death of DP cells.

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EVIDENCE FOR THE INVOLVEMENT OF $P2X_3$-LIKE RECEPTORS IN THE MUSCARINIC CONTRACTION OF THE GUINEA-PIG ILEAL LONGITUDINAL SMOOTH MUSCLE

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One of the main current challenges in the P2 receptor field is to relate the cloned P2X and P2Y subtypes to the diverse responses mediated by native P2 receptors. It has been reported that purine nucleotides contract guinea-pig ileal longitudinal smooth muscle (GPI) by acting at two different P2 receptors. One appears to be a neuronal P2X-like receptor, the stimulation of which causes indirect (acetylcholine-mediated) contraction via postjunctional muscarinic M_3 receptors. The other is situated on the smooth muscle and has the characteristics of $P2Y_1$ receptors. The present study was addressed to elucidate the exact nature of the neuronal P2 receptor subtype in GPI. To this end, we examined the inhibitory potency of four key P2 receptor antagonists (pIC_{50} values are given for cloned $P2X_1$ and $P2X_3$ receptors): pyridoxalphosphate-6-azophenyl-2'-4'-disulfonate (PPADS; 6.0/5.9), the symmetrical 3'-urea of 8-(benzamido)naphthalene-1,3,5-trisulfonate (NF023; 6.6/4.5-5.1), suramin (6.0/4.9-5.5) and 8,8'-(carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino))bis(1,3,5-naphthalenetrisulfonate) (NF279; 7.7/5.8). Data are presented as pA_2 values derived from Schild analysis. Exogenous α,β-methylene ATP (αβmeATP; 0.1-300 μM) produced tetrodotoxin (1 μM)- and atropine (300 nM)-sensitive biphasic contractions consisting of a phasic, rapidly desensitizing response followed by a more sustained tonic response. PPADS (pA_2=6.2), NF279 (pA_2=6.0), suramin (pA_2=5.1) and NF023 (pA_2=4.6) concentration-dependently inhibited the αβmeATP-induced phasic responses, with potency decreasing in that order. These results suggest that the phasic, rapidly desensitizing contractions to αβmeATP in GPI are mediated by somatodendritic P2X receptors whose agonist and antagonist profile most favorably resembles the cloned homomeric $P2X_3$ subtype.

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HETERODIMERISATION OF SURAMIN AND PYRIDOXAL-PHOSPHATE PHARMACOPHORES AS A NOVEL APPROACH IN THE DESIGN OF SELECTIVE P2 RECEPTOR ANTAGONISTS

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The development of potent and selective antagonists is still an ambitious undertaking in P2 receptor pharmacology and medicinal chemistry. This report details the pharmacology of 6-[(4,6,8-trisulfo-1-naphthyl)iminocarbonyl-1,3-(4-methylphenylene)iminocarbonyl-1,3-phenylene-azo]-pyridoxal-5'-phosphate (SB9), a heterodimeric bivalent ligand consisting of pyridoxal-5'-phosphate and the suramin monomer. Inhibitory effects were studied on contractions of the rat vas deferens elicited by α,β-methyleneATP (αβmeATP; mediated by $P2X_1$-like receptors), contractions of the guina-pig ileal longitudinal smooth muscle elicited by adenosine 5'-O-(2-thiodiphosphate) (ADPβS; mediated by $P2Y_1$-like receptors), and the degradation of ATP by ecto-nucleotidases in folliculated Xenopus laevis oocytes. SB9 (0.1-1.0 μM) antagonized contractile responses produced by αβmeATP (0.1-300 μM) or ADPβS (0.1-300 μM) in a concentration-dependent manner. Schild analysis yielded linear regression lines of unit slope, indicating competitive antagonism. From the rightward shifts of the agonist concentration-response curves pA_2-values of 6.05±0.13 (vas deferens) and 6.98±0.07 (ileum) were derived. In both preparations, SB9 behaved as a slow onset, slow offset antagonist. Three oocytes degraded 30% of added ATP (100 μM) within 30 min. SB9 (300 μM) reduced this degradation by 60%. The results illustrate that SB9 is, in contrast to suramin and pyridoxal-5'-phosphate-6-azophenyl-2',4'-disulfonate (PPADS), a high affinity $P2Y_1$receptor antagonist with a remarkable selectivity for $P2Y_1$ vs. $P2X_1$receptors (about 10-fold) and ecto-nucleotidases (1000-fold). These properties make SB9 unique among the pyridoxal-5'-phosphate and suramin derivatives reported to date.

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STRUCTURE-ACTIVITY RELATIONSHIP OF A SERIES OF NOVEL, NON-NUCLEOSIDE ANALOGS AS ADENOSINE KINASE INHIBITORS

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Adenosine kinase (AK) is a key enzyme responsible for regulating intra- and extracellular levels of adenosine, an endogenous neuomodulator, and anti-inflammatory autocoid. In recent years, the development of AK inhibitors has emerged as a novel approach to exploiting the effects of endogenous adenosine for therapeutic benefit. Inhibition of AK augments the local levels and actions of extracellular endogenous adenosine at tissue sites which are undergoing accelerated adenosine release. AK inhibitors which have been reported to date are based on the purine nucleoside pharmacophore (for example, 5-iodotubercidin and 5'-deoy-5-iodotubercidin and their analogs). This report describes the identification and structure-activity relationships of s series of novel, potent and selective, non-nucleoside AK inhibitors. The 4-amino-7-aryl-substituted pteridine, (1) (AK IC50 = 440 nM) was discovered by high-throughput screening, and a number of analogs were prepared to improved its potency and selectivity. Initial work indicated that the 7-aryl ring was required for activity, and that changes in the substitutent on the ring modified activity. Substitution of the 6-position of the pteridine ring, as well as replacement of the nitrogen atom at the 5-position of (1) with a carbon, followed by substitution at the 5-position provided overa 100-fold improvement in activity. ABT-702 (4-amino-5(3-bromophenyl)-7-(6-morpholino-pyridin-3-yl)pyrido[2,3-d]pyrimidine) is one member of this series. ABT-702 is a potent (AK IC50 = 1 nM) and selective AK inhibitor with oral activity in animal models of pain and inflammation.

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UNDERSTANDING THE MECHANISM OF L-NUCLEOSIDES AS ANTIVIRAL AND ANTICANCER AGENTS: MOLECULAR MODELING APPROACH

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A series of L-nucleosides such as 3TC, FTC, L-FMAU, and L-OddC, etc. have been discovered as potent antiviral/anticancer agents and 3TC is being used as a combination chemotherapy along with AZT and a protease inhibitor, which is currently the primary therapy for HIV infection. Additionally, 3TC has recently been approved for the treatment of chronic hepatitis B virus (HBV) infection and FTC, L-FMAU, and L-OddC are currently undergoing various stages of clinical trials as potential anticancer and antiviral agents.

L-nucleosides have been known to be phosphorylated to the monophosphates by the cellular kinases, including thymidine kinase and deoxycytidine kinase. However, the mechanism of phosphorylation of these unnatural nucleosides at a molecular level are currently not well understood. In view of the fact that the 3D-structures of these enzymes are not yet known, it has been difficult to explain how the cellular enzyme may be able to phosphorylate the unnatural nucleosides without compromising the stereochemical requirements of the enzymes and/or the nucleosides.

In understanding the molecular level of these phosphorylations with respect to the antiviral/anticancer activity of L-nucleosides, we have performed the molecular modeling studies with reported X-ray structures of nucleosides in comparison to their D-counterparts. From these studies, we are able to partially explain how these L-nucleosides can be phosphorylated by the cellular enzymes. Furthermore, we are able to explain how the triphosphates of these L-nucleosides can interact with the HIV reverse transcriptase (supported by NIH grant AI 32351).

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MOLECULAR RECOGNITION OF MODIFIED ADENINE NUCLEOTIDES BY THE P2Y1-RECEPTOR. A SYNTHETIC, BIOCHEMICAL, COMPUTATIONAL AND NMR APPROACH.

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This paper describes the investigation regarding the origin of the remarkably high potencies of 2-thioether-adenine-nucleotides P2Y1-receptor (P2Y1-R) ligands over that of ATP. For this study, an integrated approach was employed combining the synthesis of new ATP analogues, their biochemical evaluation, and SAR analysis involving NMR experiments and theoretical calculations. The latter were performed to elucidate the conformation and the electronic nature of the investigated P2Y1-R ligands. ATP analogues synthesized included derivatives with electron donating groups at C2 or C8 positions. These compounds were tested for their potency to induce P2Y1 receptor-mediated activation of phospholipase C in turkey erythrocytes and Ca2+ response in rat astrocytes. 8-substituted ATP derivatives had little or no effect on phospholipase C or on calcium levels, whereas the corresponding 2-substituted ATP analogues potently increased the levels of inositol phosphates and [Ca2+]i. The molecular recognition of these derivatives by the P2Y1-R was analysed using ab-initio calculations. Parameters such as tautomerism, protonation energy, molecular electrostatic potential, and dipole moment, were examined on reduced models and correlated with the biochemical data for the parent compounds. The calculated electronic parameters cannot explain the biochemical results. Apparently, neither tautomerism nor electronic changes in the modified adenine determine binding specificity of adenine-nucleotides to P2Y1-R. It is suggested that the higher potency of the C2-substituted ATP derivatives, compared to ATP, may be due to interaction between the C2-side-chain heteroatom and the receptor and of the C2 alkyl side-chain with a hydrophobic pocket at the binding-site. In addition, NMR data indicate that the inactivity of the C8-substituted ATP analogues may be due to steric and conformational rather than electronic effects.

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NOVEL SHORT ACTING CORONARY VASODILATORS THAT ARE FUNCTIONALLY SELECTIVE FOR THE A2A RECEPTOR BASED ON 2-HETEROCYCLIC SUBSTITUTED ADENOSINE DERIVATIVES

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The design, synthesis, and pharmacological activity of three series of novel, functionally selective A2A agonists for coronary vasodilatation will be presented. Our strategy focused on pi system mimics for 2-acetylenic and 2-trans-olefinic adenosine derivatives [e. g. 2-(1-hexynyl) of HENECA and 2-(trans-hexenyl) of THENECA, respectively], because they are known to have high A2A affinity and binding selectivity. Three classes of 2-heterocyclic adenosine derivatives were prepared as pi system mimics: 2-(2-thienyl), 2-(N-1-pyrazolyl), and a 2-(4-pyrazolyl). A multistep process starting from 2-chloro or 2-iodo adenosine derivatives was utilized to prepare the A2A agonists. The highest affinity compound in vitro was CVT-3127 (2-[N-1-(4-carboethoxypyrazolyl)]adenosine, Ki = 194 ± 45 nM, pig striatum competitive binding assay [3H]- ZM 241385), as a 2-trans olefinic mimic. However, CVT-3127 demonstrated a long t0.5 for the reversal of the increases in coronary artery conductance in rat isolated hearts (t0.5 = 14.8 ± 3.6 min). In contrast, CVT-3033 and CVT-3146 were found to be short acting coronary vasodilators (t0.5 = 3.4 ± 0.5 and 5.2 ± 0.2 min, respectively) with good potency (EC50s 67.9 ± 16.7 nM and 6.4 ± 1.2 nM, respectively). These agents were shown to be functionally selective for the A2A response vs. the A1 AV nodal response.

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^{3}H MRE 3008F20: A NOVEL ANTAGONIST RADIOLIGAND FOR THE PHARMACOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF HUMAN A_3 ADENOSINE RECEPTORS.

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In the present study we characterized the human A_3 adenosine receptor on CHO cells by using a new potent A_3 adenosine receptor antagonist 5-N-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2-(2-furyl)-pyrazolo-[4,3-e]1,2,4-triazolo[1,5-c] pyrimidine [^{3}H]MRE 3008-F20. Saturation analysis reveals a single high affinity binding site, K_D=0.80 ± 0.06 nM, with a B_{max}= 300 ± 33 fmol/mg protein. MRE 3008-F20 displays high selectivity for human A_3 adenosine receptors when compared with its activity at human A_1 (K_i = 1294 ± 100 nM), A_{2A} (K_i = 165 ± 15 nM) and A_{2B} (K_i = 2471 ± 300 nM) adenosine receptors, respectively and binds to the rat A_3 receptors with a K_i 10 μM. The pharmacological profile of [^{3}H]-MRE 3008-F20 binding to hA_3 CHO cells was evaluated using typical adenosine receptor agonists and antagonists with a rank order of potency consistent with that typically found for interactions with the A_3 adenosine receptors. In the adenylyl cyclase assay the same compounds exhibited a rank order of potency similar to that observed in binding experiments. Thermodynamic data indicate that [^{3}H]-MRE 3008-F20 binding to hA_3 CHO is entropy and enthalpy-driven in agreement with the behaviour of adenosine antagonists to A_1 and A_{2A} receptors. These results show that [^{3}H]-MRE 3008-F20 is the first antagonist radioligand endowed with high affinity and selectivity for the human A_3 adenosine receptor and can be considered a useful tool to investigate the physiopathological role of the A_3 adenosine receptors.

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DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF 2-(4-SUBSTITUTED-N-PYRAZOLYL)-ADENOSINE DERIVATIVES AS NOVEL SHORT ACTING ADENOSINE A2A RECEPTOR AGONISTS

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The goal of this study was to design and synthesize a series of A2A adenosine receptor (A2A AdoR) agonists for coronary vasodilatation with high functional selectivity and a short duration of action. Our design was based on mimicking the known potent and selective A2A agonist, 2-(trans-hexenyl) of THENECA with a 2-(4-substituted-N-pyrazolyl)-adenosine derivative. Previously, Marumoto and coworkers demonstrated that 2-(3, 5-disubstituted-N-pyrazolyl)-adenosine derivatives were inactive at doses up to 100 micrograms as coronary vasodilators in an intracoronary open chest dog model (Chem. Pharm. Bull. 1975 Vol. 23, p. 759-774). This result suggests that such a substitution on adenosine abolish its affinity for the A2A AdoR. We attribute the lack of activity of the Marumoto compound to steric hindrance that limits the binding of this compound with the A2A AdoR. Inspection of the structure activity relationships of this class of A2A AdoR agonists suggests that an extended presentation of the 4-substituted pyrazole is preferred for binding. One member of this class of compounds, CVT-3146, was discovered to have good potency for coronary vasodilatation with an EC50 of 6.4 nM in rat isolated perfused hearts. Furthermore, CVT-3146 showed high functional selectivity for A2A AdoR mediated coronary vasodilatation vs A1 AdoR mediated prolongation of AV nodal conduction time. The compounds were prepared starting from 2-chloro adenosine in three linear steps. Further details on the design, synthesis, and structure activity relationships of this class of A2A AdoR agonists will be provided.

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DESIGN, SYNTHESIS AND PHARMACOLOGICAL EVALUATION OF 2(1-ALKYL-PYRAZOL-4-YL)ADENOSINE DERIVATIVES AS SHORT ACTING ADENOSINE A2A RECEPTOR AGONISTS

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The objective of this study was to design and synthesize a series of functionally selective and short acting A2A adenosine receptor (A2A AdoR) agonists for coronary vasodilatation. The design of one lead series, 2-(1-alkylpyrazol-4-yl)adenosine derivatives, was based on mimicking the 2-trans olefin group of THENECA [2-(trans-hexenyl)NECA] with respect to the pi system and presentation of the hydrophobic side chain to the A2A receptor. Molecular modeling illustrating how these novel agonists mimic THENECA will be shown (Tripos Force Field – Sybyl 6.6). These compounds were synthesized by coupling different 1-alkyl-4-iodopyrazoles with 2-stannyl-tri(2',3',5'-O-t-butyldimethylsilyl)-adenosine in the presence of palladium (0), followed by desilylation. Only modest micromolar affinity for the A2A receptor was obtained for this subclass with N-1 lipophilic side chains on the C-4-pyrazolyl group with more extended groups providing the best affinity (CVT-3033 Ki = 2895 and CVT-3032 Ki = 13651 nM, respectively – human A2A expressed in CHO cells). However, in spite of their modest affinity, both CVT-3033 and CVT-3032 have good potency and a short duration of effect for increasing coronary artery conductance in rat isolated hearts (EC50 = 67.9, t0.5 = 3.4 min and EC50 =66.5 nM , 4.1 min, respectively). Furthermore, high functional selectivity for A2A mediated coronary vasodilatation versus A1 adenosine receptor mediated prolongation of AV nodal conduction time was observed in this model. Detailed structure activity relationships (SAR) including exploration of more polar substituents capable of hydrogen bonding will be presented.

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DEFINING THE LIGAND BINDING DETERMINANTS OF P2Y RECEPTORS BY PROTEIN ENGINEERING

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Understanding how G protein coupled receptors recognise their ligands at the molecular level will aid the design of novel drugs and tools for studying receptor function.

It was proposed that it might be possible to define a ligand binding 'domain' of the nucleotide binding receptors by building a series of chimeras where multiple regions of these proteins were exchanged between related receptors. P2Y1 and P2Y2 were used as a model system. Their ligands (ADP and UTP respectively) are thought to bind to a site within the transmembrane region of the protein and this binding pocket is thought to comprise several non-contiguous regions of different helices.

Using comparative sequence analysis, regions of the proteins predicted to interact with nucleotide ligands were identified. Three dimensional models of receptors were used to aid the design of constructs in which the top portions of the transmembrane helices and the extracellular loops were exchanged between receptors. The ability of the resulting chimeras to be activated by nucleotides was tested in a yeast reporter gene assay and compared to the wild type receptors in this system. The results indicate that the ligand binding determinants are located in the upper regions of the helices and extracellular loops of these two receptors.

Through these experiments it has been demonstrated that G protein coupled receptors can be engineered within their helices to produce functional chimeras. Using this novel approach we have been able to define regions required for ligand induced activation for both P2Y1 and P2Y2.

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THE EFFECT OF NF023 AND P-5-P ON SYMPATHETIC PURINERGIC NEUROTRANSMISSION IN THE GUINEA-PIG ISOLATED VAS DEFERENS

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Despite much research into P2X receptor antagonists, the available compounds are not ideal, particularly for electrophysiological studies. For example, suramin has low potency, is very slow to equilibrate (over 30min) and is practically irreversible. PPADS is more potent and reversible, but we found that it not only blocked excitatory junction potentials (ejps), but also produced substantial depolarisation of smooth muscle cells in guinea-pig vas deferens. The aim of this study was to examine the effects of NF023 and P-5-P on membrane potential and ejp magnitude to determine if they more useful P2X receptor antagonists.

Intracellular microelectrodes were used to record the transmembrane potential and ejps produced by sympathetic nerve stimulation (1Hz) in smooth muscle cells of the guinea-pig isolated vas deferens.
NF023 produced a concentration-dependent inhibition of ejp magnitude (IC50=4.8uM), but had no effect on the resting membrane potential of the smooth muscle cells. Inhibition reached equilibrium within 10min and showed substantial reversal on washout of the drug.

P-5-P also depressed ejp magnitude in a concentration-dependent manner, but was less potent than NF023 (IC50=22uM). Again, inhibition reached equilibrium within 10min and showed substantial reversal on washout of the drug. However, at 100uM and above P-5-P significantly depolarised the smooth muscle cells. After reduction of ejps by NF023 or P-5-P (10uM), subsequent co-addition of ARL67156 (100uM) significantly increased their magnitude.

We conclude that for electrophysiological studies NF023 is preferable to other P2X receptor antagonists such as PPADS, suramin or P-5-P.

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COMPARISON OF THE PHARMACOLOGICAL PROPERTIES OF THE HUMAN AND RAT P2Y4 RECEPTORS

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At the human P2Y4 (hP2Y4) receptor, UTP is a potent, full agonist, but ATP has been reported to be a full agonist, a partial agonist or inactive. In contrast, UTP and ATP are full agonists at the rat P2Y4 (rP2Y4) receptor. Here the pharmacological selectivities of the hP2Y4 and rP2Y4 receptors stably expressed in 1321N1 human astrocytoma cells were determined by measuring increases in intracellular [Ca] under conditions that minimised metabolism, bioconversion, and endogenous nucleotide release.

In cells expressing the hP2Y4 receptor, UTP, GTP and ITP increased intracellular [Ca] with a rank order of potency of UTP(0.55) GTP(6.6) = ITP(7.4), (EC50,uM). ATP, CTP, XTP and AP4A (100uM), were inactive. In cells expressing the rP2Y4 receptor each nucleotide increased intracellular [Ca] with similar maximal effects and a rank order of potency of UTP(0.20) ATP(0.51) AP4A(1.2) = ITP(1.8) = GTP(2.3) CTP(7.2) XTP(22.9). As ATP was inactive at the hP2Y4 receptor, we assessed whether it displayed antagonist activity. When co-applied, ATP shifted the concentration-response curve to UTP rightwards in a concentration-dependent manner, with no change in the maximum. A Schild plot gave a pA2 value of 6.15 (KB=708nM) and slope ~1. CTP and AP4A (100uM) inhibited the response to UTP (300nM) by ~40% and ~50%, respectively, but XTP had no effect. Inhibition by ATP, CTP and AP4A reversed on washout.

Thus, ATP is a potent agonist at the rP2Y4 receptor, but is a competitive antagonist with moderate potency at the hP2Y4 receptor.

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IMPORTANCE OF URINARY BACTERIAL CONTAMINATION ON ADENOSINE STABILITY IN URINES OF HEALTHY VOLUNTEERS

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Introduction: The present study was undertaken in order to assess the effects of bacterial contamination on the stability of adenosine in urines of healthy volunteers.

Methods: Urinary adenosine concentrations were determined first in 24 h urines of healthy volunteers with and without stop solution and with and without antibiotic therapy (ofloxacine), second, in five samples of freshly voided urine after addition of increasing amounts of bacterially contaminated urine and third, in three samples both with and without bacteria and the adenosine deaminase inhibitor EHNA.

Results: Without stop solution or ofloxacine all native urines demonstrated a fall in urinary adenosine concentration over 24 hours. In urine specimen containing stop solution and/or ofloxacine no degradation of urinary adenosine occurred within 24 hours. In freshly voided urine adenosine concentrations showed an accelerated fall depending upon the extent of bacterial contamination. The adenosine deaminase inhibitor EHNA has no influence on the degradation of urinary adenosine.

Conclusions: Our experiments suggest that bacteria have the capacity to metabolize adenosine in urine. We propose that all future studies using adenosine concentration or adenosine excretion in urine for diagnostic purposes should take into account the variable instability of adenosine which is mainly dependent upon bacterial adenosine degradation.

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THE CHANGES OF ADENINNUCLEOTIDES CONTENTS IN BRAIN UNDER DIFFERENT EXPERIMENTAL STATES

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The goal of this investigation is to study the level of the content of ATP, ADP, AMP, c-AMP as well as the ratio ATP/ADP in the mitochondrial fraction of white rats' brain tissue in the condition of unilateral gangliosimpathectomy (GS, removal of the upper cervical sympathetic ganglia), reserpinisation and bandage of the right carotid artery.

The data obtained revealed significant decrease of ATP and increase of ADP content in white rats brain by unilateral GS in ectomised hemisphere which is accompanied by decrease of c-AMP content and ATP/ADP ratio on the background of decrease the ATP+ADP+AMP sum compared with nonectomised hemisphere. The reserpinisation, followed by exhaustion of catecholamines reserves and depression of adrenergic centres of brain, do not cause noticeable changes in studied parameters. In case of reserpinisation on the basis of gangliosympathectomy asymmetry is not observed which was revealed by gangliosympathectomy. By the bandage of the carotid artery the changes observed in the content of ATP and ATP/ADP ratio in the right hemisphere were less expressed. Those results confirm the important role of sympathic ganglion in the regulation of energogeneration systems and metabolism of macroergic nucleotides. The data represented correlated with the changes of mitochondrial phospholipid content, lipiddependent membranebound enzymes, particularly Na+,K+,Ca+,Mg+-ATP-ases activity, as well as by lactatdehydrogenase isoenzymes content and their activity. All these changes in total may lead to the formation of pronounced structural and functional abnormalities of membranes, particularly to the disorders in the activity of the membranebound, lipid-dependent enzymes.

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SYNTHESIS OF UTP DERIVATIVES AND ANALOGS WITH MODIFICATIONS IN THE SUGAR AND PHOSPHATE MOIETY AS P2Y RECEPTOR LIGANDS

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Three mammalian G protein-linked P2 receptors that are stimulated potently by uracil nucleotides have been cloned and characterized: P2Y2, P2Y4 and P2Y6[1,2]. Agonists and antagonists for these receptors are required, which exhibit:

-high affinity/potency at the receptors,
-receptor subtype selectivity, and
-stability towards hydrolysis by ectonucleotidases.

We had synthesized UTP derivatives modified in the pyrimidine moiety and found that large substituents in the 5-position of UTP were not tolerated by the P2Y2 receptors[3]. We have now synthesized a series of uridine nucleotide analogs with variations in the sugar and the triphosphate moiety. Uridine analogs bearing an acyclic (or aliphatic) spacer were prepared and phosphorylated using $POCl_3$ and tri-n-butylammonium diphosphate according to Ludwig's protocol[4]. In addition, nucleotide analogs bearing a phosphonic acid group and thus being more stable were prepared. A synthesis for compounds in which the triphosphate moiety was replaced by carboxylic acid groups (e.g. citric acid) was developed. The synthesized compounds were purified using anion exchange chromatography followed by preparative HPLC. The purity of the new compounds was assessed by capillary zone electrophoresis (CZE), and ^{31}P nmr spectroscopy.

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