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Altered subcellular signaling in murine peritoneal macrophages upon chronic morphine exposure

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Abstract

Alterations in opioid signaling that take place in murine peritoneal macrophages in vitro are variably dependent on opiate exposure conditions. Acute exposure to morphine inhibits Fc-mediated phagocytosis by a pertussis toxin (PT)-sensitive mechanism, but has no effect on cAMP levels. In contrast, chronic exposure to morphine results in a "tolerant" state, wherein test and control values for both phagocytosis and cAMP are equivalent. However, drug withdrawal after chronic exposure to morphine results in inhibition of phagocytosis and a concomitant 4-fold increase in cAMP by a PT-insensitive mechanism. This increase is causally related to inhibition of phagocytosis since an artificial increase in cAMP inhibits phagocytosis in non-withdrawn cells exposed chronically to morphine. We suggest that macrophage opioid receptors signaling switches from a $G_{i/o}$-mediated mechanism that does not involve adenylate cyclase in acute exposure to a non-$G_{i/o}$-mediated adenylate cyclase superactivation during chronic exposure.

Keywords: Tolerance; Dependence; Immunosuppression; Cyclic AMP; Phagocytosis

1. Introduction

Studies on opioid effects on neural cells have revealed that the mechanisms responsible for transducing the opioid signal are strongly dependent on exposure conditions (Gintzler and Chakrabarti, 2000). In general, acute exposure to opioids inhibits neurotransmission, although stimulatory effects on release of transmitters such as acetylcholine (Neal et al., 1994) and met-enkephalin (Xu and Gintzler, 1992) have also been seen. This variability has been postulated to occur via differential coupling of opioid receptors to G_s- and G_i-like G proteins (Gintzler and Xu, 1991; Chakrabarti et al., 1995). Opioid activated G proteins then modulate the activity of ion channels or the levels intracellular messengers such as cAMP and IP_3 (Childers, 1991). In contrast, chronic opioid receptor signaling results in states of tolerance and dependence. The subcellular basis of this phenomenon seems to lie in a complex series of adaptations affecting different elements of the opioid signal transduction pathway, such as receptors, G proteins and adenylyl cyclase (Harrison et al., 1998).

Although acute exposure to morphine inhibits adenylyl cyclase in various cell lines, chronic exposure to the opiate results in control levels of the cyclic nucleotide that can be elevated 2- to 5-fold by a naloxone challenge (Sharma et al., 1975, 1977). Opiate withdrawal of chronically opiate-exposed CHO cells transfected with rat μ-opioid receptor cDNA (Avidor-Reiss et al., 1997) leads to immediate increases in cyclase activity (AC supersensitivity or cAMP

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overshoot), as occurs also in NG108-15 cells, where withdrawal-induced superactivation seems to require Gs, though not the Gsα subunit (Ammer and Schulz, 1998). In addition, in the myenteric plexus from sufentanyl-treated guinea pigs, electrical stimulation of cAMP formation is significantly larger in the presence of sufentanyl than in its absence, suggesting that in tolerance/dependence, in addition to loss of inhibitory potency of the opiate on cAMP formation, there is a reversal to enhancement (Wang and Gintzler, 1995). All these data point to an important role for alterations in the cAMP signaling in the variable development of opioid tolerance.

Significant evidence has accumulated in the last decade that morphine and other opioids have multiple effects on the immune system, including inhibition of processes such as nitric oxide synthesis (Iuvone et al., 1995), cytokine induction (Roy et al., 1999), antigen response by splenic cells (Rahim et al., 2002, 2003) and macrophage phagocytosis (Casellas et al., 1991; Rojavin et al., 1993; Szabo et al., 1993; Tomassini et al., 2003). These effects could be either direct, via opioid receptors on immune cells, as in the case of in vitro exposure using macrophage cultures or cell lines (Iuvone et al., 1995; Casellas et al., 1991; Tomassini et al., 2003), or indirect, when drug exposure takes place in vivo, and could be mediated by "systemic" opioid receptors in non-immune cells, as in the case of immune cells obtained from morphine pelleted mice (Rojavin et al., 1993; Rahim et al., 2002). Therefore, the in vivo situation has a complexity that is absent in in vitro studies. However, in the case of phagocytosis by murine peritoneal macrophages, the effect of morphine appears to be remarkably similar when comparing cells exposed to the opiate in vivo and in vitro (Rojavin et al., 1993).

Morphine effects on phagocytosis by murine macrophages are particularly interesting, since the effects observed are both time and concentration dependent. Acute morphine inhibits phagocytosis in murine peritoneal macrophages by a biphasic, dose-dependent mechanism, with inhibition observed with nanomolar concentrations of the drug, but with no inhibition of phagocytosis with micromolar concentrations (Tomei and Renaud, 1997). However, chronic exposure to morphine (6 h or greater) results in the development of a putative tolerant state, where cells phagocytize at control levels in the presence of the drug (Tomei and Renaud, 1997; Lázaro et al., 2000). Furthermore, drug withdrawal after chronic exposure results in an inhibition of phagocytosis comparable to that observed upon acute exposure, suggesting dependence (Lázaro et al., 2000). This withdrawal-induced inhibition of phagocytosis could be important in modulating immune defenses in opiate addicts, due to the key role of macrophages in the immune system. However, the mechanism underlying the effects on phagocytosis of morphine and opiate withdrawal is unknown. It is our aim to study some aspects of this mechanism, with emphasis on alterations in the cAMP pathway. Our results show remarkable parallelisms and interesting differences between effects of morphine withdrawal on cAMP levels in murine macrophages in comparison with known effects on neural cells.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich.

2.2. Animals

Four- to eight-week-old female C3HeB/FeJ mice (Jackson Labs) were utilized. They were kept in the Animal House facilities of the University of Puerto Rico, Río Piedras Campus, according to the rules and regulations for animal care of the National Institutes of Health.

2.3. Macrophage cultures

Macrophages were isolated from mice that had received an intraperitoneal injection of thioglycollate 5 days prior to sacrificing, as described previously (Lázaro et al., 2000). Cells were recovered by peritoneal lavage using sterile PBS and were cultured with complete RPMI medium: RPMI 1640 supplemented with 10% inactivated fetal bovine serum (Sigma, MO) and 1% antibiotic/antimycotic solution (Hyclone, UT). Macrophages were counted with a hemocytometer, and cell viability was determined by trypan blue exclusion (0.4% trypan blue). Only cell suspensions with a viability greater than 90% were used in these studies. For phagocytosis assays, 400 μL at a cell concentration of 3.7×10^4 cells/mL was added per well using 8-well Lab-Tek culture plates (Miles, IL). For the cAMP radioimmunoassays, 500 μL at a cell concentration of 2.0×10^5/mL was added per well using 24-well culture plates (Corning, NY). Cultures were incubated overnight at 37 °C under 5% CO_2, and washed twice the following day with complete RPMI to remove any non-adherent cells.

2.4. Phagocytosis assay

This was performed as previously described (Casellas et al., 1991; Tomei and Renaud, 1997). In brief, opsonized sheep red blood cells (SRBC) were prepared by washing SRBC from sheep blood (Colorado Serum) with PBS, and incubating with rabbit IgG anti-SRBC (ICN) in a 1:1000 dilution for 20 min at 37 °C in a water bath, followed by a PBS wash. Macrophage cultures were incubated with opsonized SRBC at a ratio of 100 SRBC per macrophage. Phagocytosis started upon transfer of cultures to a 37 °C incubator, and the process stopped after 20 min by hypotonic lysis of non-ingested erythrocytes, followed by methanol fixation and staining of the macrophages. Phago-

cytosis was measured by observing at least 300 cells per experiment by microscopy for the presence of ingested SRBC. To achieve maximal objectivity, slides were coded and observers were not aware of the meaning of the codes. Phagocytosis was expressed as % phagocytosis (i.e. % of cells that ingested at least one SRBC), and results from different experiments were standardized by setting the % phagocytosis of control cells as equal to 100%. Alternatively, results were also expressed in some experiments in terms of the phagocytic index (PI); namely, the mean number of erythrocytes ingested per macrophage. To do this, we counted the total number of SRBC ingested by 100 cells per variable. At least three independent replicates were performed of all experiments.

2.5. Effect of acute and chronic morphine and withdrawal on phagocytosis by murine peritoneal macrophages

Effect of acute exposure to morphine was tested by incubating cells with complete RPMI supplemented with 100 nM morphine 30 min prior to the addition of SRBC. Naloxone-reversibility of acute effects was tested by addition of 1 μM naloxone 30 min prior to addition of the opiate. Chronic exposure was performed by adding 100 nM morphine for 8 h prior to the addition of SRBC, and opiate withdrawal (WD) from chronically treated cells was effected by either washing three times with RPMI medium, or by displacing the drug by adding the antagonist naloxone at a final concentration of 1 μM. Percent phagocytosis was then scored at different times after withdrawal as above. We have demonstrated previously that essentially similar results are obtained when drug withdrawal is effected by either procedure (Lázaro et al., 2000).

2.6. Effect acute and chronic morphine and withdrawal on cAMP levels

Levels of cAMP were determined in opiate naïve cells, in cells exposed to acute morphine (100 nM, 0–30 min), and in cells exposed to chronic morphine (100 nM, 8 h), the latter with and without opiate withdrawal. Withdrawal was effected either by washing three times with opiate-free culture medium, or in some experiments by addition of 1 μM naloxone, and cAMP levels were determined at different times post-withdrawal. In some experiments, the effect of acute morphine on cAMP levels after PGE stimulation was also tested. Cell extracts were prepared by washing control and experimental samples twice with PBS, followed by addition of extraction solution (95% ethanol/20 mM HCl, 1 mL/well) at −20 °C for 16–24 h. The extraction solution is removed, and a second extraction is performed with fresh extraction solution at 5 °C for 1 h. The combined extracts are dried by speed-vacuum centrifugation (Savant) and stored at −20 ° until used in the RIA. At that time samples are reconstituted in 0.05 acetate buffer, pH 6.2. All experimental samples and standards were acetylated and the cAMP levels determined using a cAMP radioimmunoassay (RIA) kit according to the instructions of the manufacturer (DiaSorin, MN).

2.7. Effect of compounds that affect the cAMP pathway on phagocytosis by sub-chronically exposed macrophages

The intracellular level of cAMP was increased artificially in chronically exposed cells by addition of different concentrations (5 nM to 2 mM) of dibutyryl-cAMP, a membrane permeant cAMP analog, during the last 30 min of the opiate exposure period, followed by a phagocytosis assay. Controls included tolerant cells with no addition of dibutyryl-cAMP, and tolerant cells after opiate withdrawal by washing three times with RPMI medium. Since the enzyme protein kinase A (PKA) should be the immediate target of any morphine withdrawal-induced increase in cAMP, we tested the effect of the specific PKA inhibitor Rp-adenosine-3′,5′-cyclicmonophosphorothioate (Rp-cAMPT) on phagocytosis by tolerant cells after withdrawal. This inhibitor is a membrane-permeant molecule with a high specificity for PKA and resistance to phosphodiesterase degradation (Wang et al., 1991). A phagocytosis assay was done 1 h after withdrawal on cells exposed to 100 nM morphine for 24 h, followed by WD; and on cells to which 100 nM Rp-cAMPT was added 2 h prior to WD. Controls included phagocytosis assays on opiate naïve cells exposed acutely to morphine, and opiate-naïve cells to which inhibitor was added for 2 h.

2.8. Pertussis toxin (PT) sensitivity of morphine effects on macrophage phagocytosis

We wished to determine if the effect of morphine on macrophage phagocytosis was mediated by a $G_{i/o}$ protein, a known substrate of PT. Sensitivity to this toxin of the inhibition by acute morphine of phagocytosis was tested by incubation cells with 100 ng/mL PT for 24 h, followed by washing three times with toxin-free medium and incubation in the presence or absence of 100 nM morphine for 30 min; controls included a phagocytosis assay on acutely exposed cells not treated with the toxin. Similarly, PT-sensitivity of drug withdrawal-induced effects on phagocytosis was tested by treating cells with 100 nM PT for 24 h, with and without addition of 100 nM morphine for 8 h prior to finishing the 24-h PT exposure period. This was followed by drug displacement by addition of 10 μM naloxone, and a phagocytosis assay 30 min post-drug displacement. Controls included phagocytosis assays on opiate naïve cells exposed acutely to 100 nM morphine. The effect of pertussis toxin pre-treatment on cAMP levels after drug withdrawal from chronically exposed cells was also tested: cAMP levels were measured at different times (0–30 min) after drug withdrawal in cells that were pre-treated with PT, as above, and in chronically exposed cells not treated with toxin.

2.9. Statistics

Results were analyzed using ANOVA analysis or the Student's *t*-test, and differences were considered when $p < 0.05$. In all experiments, $n = 3$ or greater.

3. Results

3.1. Effect of acute and chronic exposure to morphine on phagocytosis by murine peritoneal macrophages

The results in Fig. 1A are consonant with our previous findings (Lázaro et al., 2000) that morphine effects on macrophage phagocytosis are exposure condition-dependent: when compared to control cells (C), acute (M30 min) exposure to 100 nM morphine inhibits phagocytosis by around 49% and this effect was totally reversed by the addition of naloxone (Nx+M). On the other hand, a chronic (M8 h) exposure to 100 nM morphine results in a % phagocytosis comparable to that of control cells, suggesting that these cells are in a putatively tolerant state; therefore, chronically exposed cells shall be referred to hereon as tolerant cells. However, when opiate is withdrawn from tolerant cells by addition of naloxone (WD), a 28% inhibition of phagocytosis is observed one h after WD (Fig. 1A) that is comparable, although somewhat lower, to that caused by acute exposure, suggesting dependence. This is in contrast to the results obtained when cells were exposed to 100 nM morphine for 30 min, washed to effect WD, and phagocytosis assayed 8 h later. Under these conditions, there was no significant difference between opiate treated and naïve controls (data not shown). The data obtained by means of % phagocytosis is strongly backed by the results obtained using the phagocytic index (PI, Fig. 1B). Acute morphine (M30) decreased the PI by approximately 63%, but this decrease was abolished by naloxone (Nx+M). In terms of chronic exposure, the PI of tolerant cells (M8 h) did not differ from that of control cells, but when opiate was withdrawn by naloxone addition (WD), the PI was decreased by approximately 53%. Therefore, two opiate-related inhibitory events are observed, but due to diametrically opposed circumstances: inhibition of phagocytosis by opiate naïve cells due to the presence of the drug, and inhibition of phagocytosis by tolerant cells due the absence of the drug.

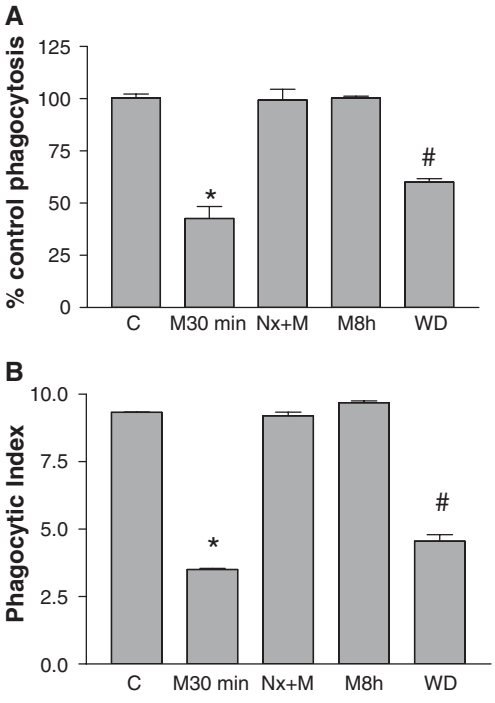

Fig. 1. Effect of acute and chronic morphine on phagocytosis by murine peritoneal macrophages. Prior to the phagocytosis assay, cultures of murine peritoneal macrophages were incubated in: RPMI medium without morphine (C = control cells), 100 nM morphine for 30 min (acute exposure, M30 min), 1 μM naloxone for 30 min followed by 100 nM morphine for 30 min (Nx+M), and 100 nM morphine during 8 h (chronic exposure, M8 h). Opiate withdrawal from chronically exposed cells (WD) was performed by addition of 1 μM naloxone followed by a phagocytosis assay after 1 h. Results are illustrated in terms of % phagocytosis (panel A) and of phagocytic index (panel B), $n = 4$. Panel A: $^{*}p < 0.001$ vs. C and vs. Nx+M; $^{\#}p < 0.001$ vs. M8 h, and < 0.01 vs. M30 min. Panel B: $^{*}p < 0.001$ vs. C and vs. Nx+M; $^{\#}p < 0.001$ vs. M30 and vs. M8 h.

3.2. Modulation of cAMP levels in macrophages by different conditions of morphine exposure and withdrawal

We wished to determine if modulation of cAMP levels was part of the mechanism responsible for inhibition of phagocytosis by acute morphine or by drug withdrawal. Measurements by RIA showed that the basal levels of cAMP in opiate naïve cells were 0.1–0.4 pM/10^5 macrophages, and were not affected by 30 min (acute) exposure to morphine concentrations ranging from 1 nM to 1 μM. To test the possibility that adenylyl cyclase (AC) needs to be ligand-activated to be inhibited by morphine, cAMP measurements were performed after stimulation by 10 μM PGE. In the presence of this AC activator, the level of cAMP increased 50–100 fold (5–40 pM/5×10^5 macrophages) when compared to control cells (0.1–0.4 pM/10^5 cells). However, PGE-stimulated activity was not affected by morphine concentrations ranging from 50 nM to 1 μM (data not shown). Therefore AC activity, both basal and ligand-stimulated, is insensitive to acute opiates in opiate-naïve macrophages.

The basal level of cAMP of tolerant cells does not differ from that in opiate-naïve cells; namely 0.1–0.45 pM/5×10^5 macrophages. However, in contrast to the observations made on cAMP levels after acute and chronic exposure, when opiate is withdrawn from chronically exposed cells by washing the cells three times with opiate-free medium, cAMP levels increase 4-fold and remain above tolerant, non-withdrawn levels for at least 30 min (Fig. 2A). Similar results were found when drug was displaced by addition of 1 μM naloxone at the end of the 8-h exposure period, and

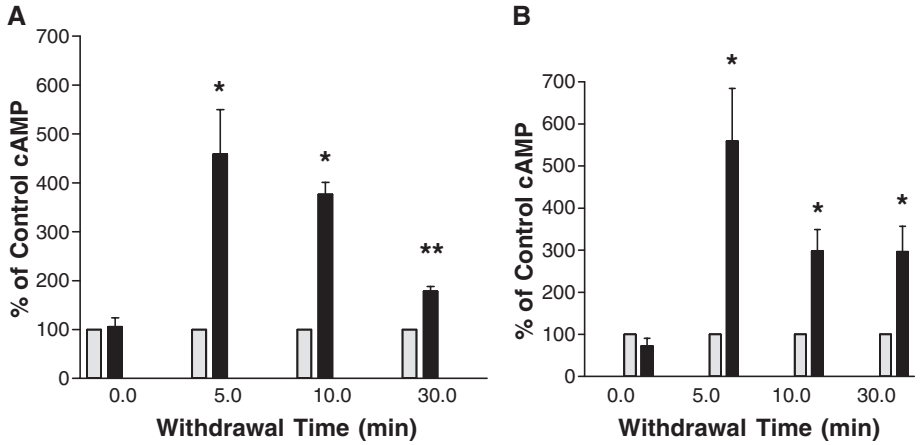

Fig. 2. Effect of drug withdrawal after chronic morphine exposure on cAMP levels in murine macrophages. (A) Cells were exposed for 8 h to 100 nM morphine, followed by washing three times with RPMI with 100 nM morphine (mock withdrawal, gray bars) and without opiate (drug withdrawal, dark bars). Cyclic AMP levels were then measured at the times indicated in the figure ($n=6$, *$p<0.01$, **$p<0.05$). (B) Same as (A), but cells were exposed to 100 ng/mL of pertussis toxin for 24 h, and 100 nM morphine was added during the last 8 h of incubation with the toxin ($n=3$, *$p<0.05$).

cAMP levels were measured at different times post drug-displacement. For example, the level of cAMP peaked 5 min after the addition of naloxone the level of cAMP, and this peak represented a 225% increase when compared to control cells ($p<0.01$, $n=3$).

3.3. Effect of compounds that affect the cAMP pathway on phagocytosis by chronically exposed cells

The above results suggest that an increase in cAMP levels may be part of the mechanism by which drug withdrawal from chronically exposed cells inhibits phagocytosis. In order to test this hypothesis, we determined if an artificial increase in the level of cAMP could mimic the effect of opiate withdrawal in chronically exposed cells that are maintained in the constant presence of morphine. Fig. 3A shows that addition of 10 nM dibutyryl-cAMP to cells exposed chronically to morphine causes an inhibition of phagocytosis comparable to that caused by WD; identical results were found with higher dibutyryl-cAMP concentrations (data not shown); therefore, an artificial increase in the level of cAMP imitates the effect of WD. Since the immediate target of cAMP is the enzyme protein kinase A (PKA); then, according to our hypothesis, inhibiting this enzyme should also block morphine WD-induced inhibition of phagocytosis. In Fig. 3B, it is shown that indeed

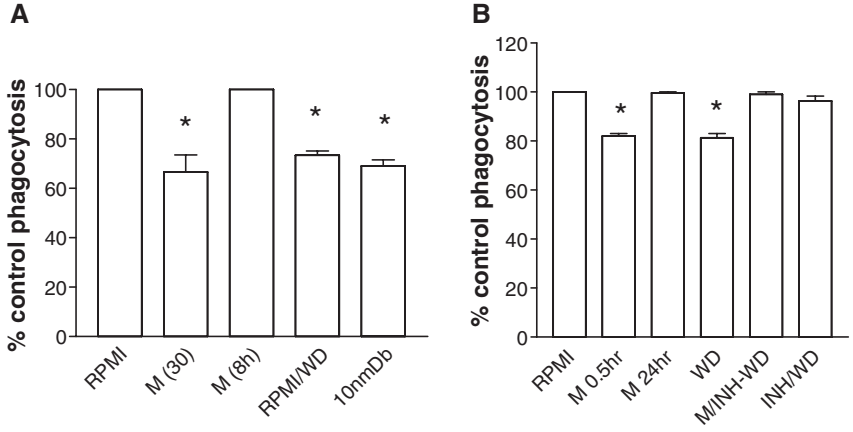

Fig. 3. Effect of compounds that modulate the cAMP pathway on phagocytosis by cells chronically exposed to morphine. (A) Effect of dibutyryl cAMP on phagocytosis by macrophages chronically exposed to 100 nM morphine. Phagocytosis assays were performed on: RPMI=cells exposed 30 min to culture medium; M(30)=cells exposed 30 min to 100 nM morphine; M(8 h)=cells exposed 8 h to 100 nM morphine; M/WD=same as M8 h, but assaying for phagocytosis 30 min after drug withdrawal by washing three times with RPMI; 10 nM Db=same as M(8 h), but with the addition of 10 nM dibutyryl cAMP 30 min before the end of the 8-h incubation period, with no WD ($n=3$, *$p<0.004$). (B) Effect of the PKA inhibitor Rp-adenosine-3′,5′-cyclicmonophosphorothioate on WD-induced inhibition of phagocytosis. Phagocytosis assays were performed on the following groups of cells: RPMI=opiate naïve cells; M0.5 h=cells exposed to 100 nM morphine for 0.5 h; M24 h=cells exposed to 100 nM morphine for 24 h; WD=same as M24 h, but drug withdrawn by washing three times with opiate-free medium, and phagocytosis assayed 1 h after WD; M/INH-WD=same as WD, but a 100-nM concentration of inhibitor was added 2 h prior to WD; INH/WD=opiate naïve cells in the presence of 100 nM inhibitor for 2 h prior to washing three times with fresh medium and assaying for phagocytosis 1 h after washing ($n=3$, *$p<0.05$).

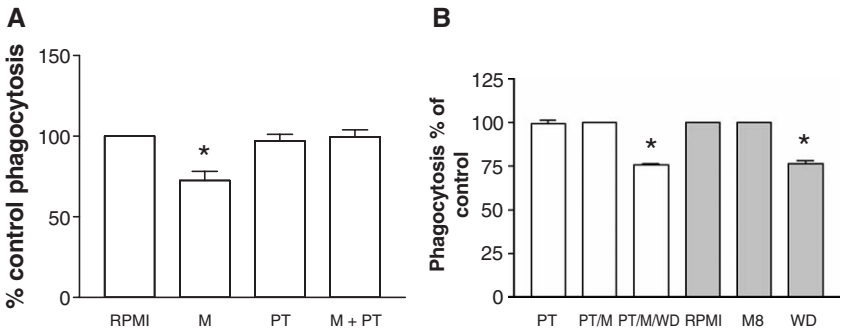

Fig. 4. Effect of pertussis toxin pre-treatment on phagocytosis by murine macrophages exposed to acute and chronic morphine. (A) Pertussis toxin (PT) pre-treatment blocks inhibition of phagocytosis by acute morphine. Macrophages were exposed 24 h to 100 ng/mL of PT, and then washed three times with toxin-free medium and incubated with 100 nM morphine (M+PT) or opiate-free RPMI (PT) for 30 min, followed by addition of SRBC and a phagocytosis assay. Controls included opiate naïve cells not treated with toxin (RPMI), and M=same as RPMI, but incubated with 100 nM morphine for 30 min ($n=3$, $^*p<0.01$). (B) Pertussis toxin pre-treatment does not block WD-induced inhibition of phagocytosis after chronic exposure. Cells were given the following treatments prior to a phagocytosis assay (open bars): PT=100 ng/mL of PT for 24 h; PT/M8=same as PT, but 100 nM morphine added 8 h prior to finishing the 24-h PT exposure period; PT/M/WD=same as PT/M8, but followed by DW for 30 min by addition of 10 μM naloxone. Controls (filled bars): RPMI=phagocytosis by opiate naïve cells, M8=cells exposed to morphine for 8 h, no PT treatment; WD=cells treated with morphine for 8 h followed by WD with naloxone, no PT treatment ($n=3$, $^*p<0.05$).

this is so: in this experiment treating tolerant (24-h exposure) macrophages with the PKA inhibitor Rp-cAMPT 2 h prior to drug withdrawal results in an abrogation of the inhibitory effect, again strengthening our hypothesis that the increase in cAMP is related to the observed inhibition of phagocytosis.

3.4. Pertussis toxin sensitivity of effects of acute and chronic opiates on AC activity and macrophage phagocytosis

Inhibitory effects by opiates are usually mediated by pertussis toxin (PT)-sensitive $G_{i/o}$ proteins. Therefore, we wished to test the PT sensitivity of the effect of acute and chronic opiates on AC activity and macrophage phagocytosis. PT pre-treatment clearly blocks inhibition of phagocytosis by acute morphine, which suggests that this inhibition is mediated by a $G_{i/o}$ protein (Fig. 4A). However, PT does not seem to affect the basal levels of cAMP, which did not differ significantly from those in control, non-PT treated cells (0.35 pM/5×10^5 cells); therefore, basal AC activity is insensitive to PT in opiate-naïve cells. In addition, PT-pretreatment did not block the development of putative tolerance, or of opiate withdrawal-induced inhibition of phagocytosis, which suggests that development of tolerance is $G_{i/o}$-independent (Fig. 4B). Furthermore, PT pre-treatment did not block the increase in cAMP level observed upon drug withdrawal in tolerant macrophages (Fig. 2B).

4. Discussion

When comparing our data with that reported for cell lines and neuromuscular preparations, the physiological consequences of withdrawal are diametrically opposed: in neural cells, WD from “addicted” tissues is stimulatory, resulting in increased firing activity (Sharma et al., 1975, 1977), whereas in tolerant murine macrophages drug withdrawal is inhibitory, at least in terms of phagocytosis. Similar inhibitory effects were found upon opiate withdrawal from tolerant/addicted enteric ganglia (Gintzler et al., 1987), which resulted in decreased met-enkephalin secretion. There are other examples of withdrawal-induced alterations in immune cells; for example, in a study of the development of SIV infection, it was found not to differ between addicted and control monkeys, but was precipitated upon opiate withdrawal, which suggests that withdrawal caused a depression in immune responses that resulted in a rise in SIV (Donahoe and Vlahov, 1998). In addition, splenocytes from opiate-addicted mice suffered a decreased in their capability to respond to antigens when the drug was withdrawn (Rahim et al., 2002). Lastly, in a study performed with human addicts, abnormalities in some immune parameters were apparent years after breaking the habit (Govitrapong et al., 1998). Therefore, although drug withdrawal has been relatively understudied in immune cells, it may be very important to understand how opiates affect immune defenses in opiate addicts.

Data presented here are consonant with previously published work showing that morphine effects on phagocytosis are dependent on exposure conditions, with acute exposures inhibiting phagocytosis, and chronic exposures resulting in development of a tolerant/dependent-like state (Tomei and Renaud, 1997; Lázaro et al., 2000). We are aware of the fact that in vivo tolerance and dependence are very complex phenomena, involving the nervous, endocrine and immune systems (Williams et al., 2001; Harrison et al., 1998), and that these complex interactions are absent in vitro at the cell culture level. Therefore, in this work we use the terms tolerance and dependence as operational ones, since our findings are not necessarily valid for the in vivo situation.

We have observed that the magnitude of the inhibitory effect of acute morphine and of opiate WD after chronic exposure ranges from 20% to 50% in different sets of experiments, both in previous work (Tomei and Renaud, 1997; Lázaro et al., 2000) and in the present paper. At the moment, we do not have an explanation for this variability. Nevertheless, our data shows that the pattern obtained is remarkably consistent when comparing different sets of experiments, regardless of the % inhibition observed. Still, it could be argued that an approximately 20–30% inhibition of phagocytosis may not be biologically relevant. However, we now demonstrate that when phagocytosis is scored in terms of the PI we detect a greater than 50–60% difference between control and experimental cells (Fig. 1B), and this difference is definitely biologically relevant. Therefore, the PI seems to be a better index of the true impact of the morphine treatment on phagocytosis.

Another point that needs to be addressed is the relevancy of the study using Fc-mediated phagocytosis by thioglycollate-elicited macrophages. Since these cells are partially activated, they may behave differently from resident cells, and this certainly should be taken into account when extrapolating our data to the in vivo situation. However, thioglycollate-elicited cells have been an accepted model for many years as a to study various macrophage functions, and continue to be used in studies ranging from phagocytosis (Ichinose et al., 1995, Li et al., 2000) to functional kinetics following inflammation (Wu et al., 2004), and adenosine modulation of TNF-α secretion (Kreckler et al., 2006).

The 3-fold increase in cAMP level observed in our work upon WD is comparable to that observed in a human promonocytic cell line (U937) cells upon HIV infection, and which has been ascribed to an HIV *env* protein-mediated increase in the intracellular level of cAMP (Thomas et al., 1997). This increase in U937 cells was shown to be responsible for inhibition of phagocytosis based in part on two observations: (1) a similar inhibitory effect was observed when control cells were treated with an analog of cAMP, 8-bromo-cAMP and (2) a PKA inhibitor restored phagocytosis in env protein transfected cells. Similar observations were made in our work that make us believe that the cAMP increase observed in our cells is causally related to the observed inhibition of phagocytosis upon drug withdrawal since: (1) an artificial cAMP increase using a very low concentration of dibutyryl cAMP (10 nM) has an identical effect on phagocytosis by tolerant cells that remain in morphine and (2) blocking PKA with a PKA-specific inhibitor (Rp-adenosine cyclic 3′,5′-phosphorothioate) also blocks inhibition of phagocytosis by tolerant cells in spite of WD. On the basis of our data and that of Thomas et al., 1997, it is tempting to speculate that in HIV-infected macrophages from opiate addicts phagocytosis could be greatly affected by both an env protein-mediated increase in cAMP and by a withdrawal-induced increase in this cyclic nucleotide. This combination of factors could result in an inhibition of phagocytosis greater than that caused by each factor by itself. Were it to be so, drug withdrawal could be an important co-factor in the modulation of immune defenses of HIV^+-opiate addicts.

In this work, we have made several intriguing observations that need to be contrasted with other results in the scientific literature. First of all, the fact that we did not detect any effect of acute morphine on basal or ligand-stimulated cAMP levels in opiate naïve cells is contrary to the situation in neural cell lines or CHO cells (Sharma et al., 1975; Avidor-Reiss et al., 1997), or in neuromuscular preparations (Wang and Gintzler, 1995), where acute morphine inhibits the activity of basal or stimulated AC, thus resulting in a decrease in the level of cAMP. In our system, we have demonstrated that the inhibitory effect of acute morphine on phagocytosis is mediated by a pertussis toxin-sensitive mechanism; therefore we postulate that the opioid receptor-mediated mechanism responsible for inhibition of phagocytosis upon acute exposure does not involve AC, but a G_i-mediated modulation of other effectors such as ion channels. Such a situation has been reported in rat peritoneal macrophages where 10 nM met-enkephalin inhibits phagocytosis by an increased calcium efflux, but with only a slight effect on the level of cAMP (Foris et al., 1986). In contrast, we found that expression of AC supersensitivity during chronic exposure is pertussis toxin-insensitive, since the cAMP "overshoot" took place upon drug withdrawal after chronic exposure even after pertussis toxin pre-treatment. This is consonant with the fact that G_s, which is pertussis toxin-insensitive, appears to play an important role in the induction of AC supersensitivity, as evidenced by the fact that in NG108-15 cells chemical inactivation of $G_{s\alpha}$ abolished the induction of AC supersensitivity (Ammer and Schulz, 1998). The fact that cAMP "overshoots" after drug withdrawal suggests that AC is inhibited by morphine during chronic exposure. This suggests a switch in AC coupling, from a morphine-insensitive situation in acute exposure, to a morphine-sensitive situation in chronic exposure. The inhibitory mechanism in the latter case is unknown, since the fact that it is not pertussis toxin sensitive suggests that it is not Gi/o-mediated. However, Gβγ subunits could be part of this regulatory mechanism, since different AC isotypes can either be inhibited or stimulated by Gβγ subunits (Bayewitch et al., 1998; Chakrabarti et al., 1998a). However, there is no information about which AC isotypes are present in murine macrophages.

Although the sub-cellular basis for the increase in cAMP levels (AC supersensitivity) manifested upon DW from tolerant cells is unknown at the moment, recent evidence suggests that opioid tolerance and dependence are not only the exclusive result of quantitative changes in opioid signaling, but also of altered consequences of coupling (Gintzler and Chakrabarti, 2000), and that chronic morphine can influence the activity and level of AC in an isoform-specific fashion. As an example, chronic systemic morphine increases the transcription of genes for AC IV and AC VII (Rivera and Gintzler, 1998). In addition, it has been found

that chronic opiate causes an increase in the phosphorylation state of AC, which results in increased sensitivity to $G_{s\alpha}$ in some isotypes (Chakrabarti et al., 1998b). This increased sensitivity, were it to take place in macrophages, would counterbalance any G_i-mediated inhibition of AC, thus resulting in "normal" levels of AC activity. Under these conditions withdrawal of morphine would result in increased AC activity, which would be manifested in an increase in cAMP levels.

The mechanism by which an increase in cAMP results in inhibition of phagocytosis is not known. We may speculate that increased production of cAMP results in an increase in a PKA-mediated event that inhibits particle ingestion by these cells. One possible target for PKA modulation is the assembly of the cytoskeleton. Actin filaments are known to play an important role in the formation of the phagocytic cup, and we surmise that their assembly could be affected by morphine withdrawal. This speculation is backed by the fact that opiate withdrawal from neurons obtained from "addicted tissues" results in a significant change of shape that obviously has to involve the re-distribution of cytoskeletal proteins (Spiga et al., 2003). Opioids have also been shown to affect the morphology of invertebrate and human immunocytes (Stefano et al., 1989) and the distribution of actin microfilaments in opossum kidney cells (Papakonstanti et al., 1998). Furthermore, there is a growing literature that supports a central role for PKA in cytoskeletal regulation (Howe, 2004). Therefore, we speculate that the subcellular basis for morphine effects on macrophage phagocytosis may lie at least in part in a cAMP-mediated modulation of the actin cytoskeleton.

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