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Involvement of protein kinase C in the UTP-mediated potentiation of cyclic AMP accumulation in mouse J774 macrophages

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1 We have investigated the effects of nucleotide analogues on cyclic AMP formation in mouse J774 macrophages and the mechanisms involved.

2 UTP, in the concentration range 0.1–100 μM, induced concentration-dependent potentiation of prostaglandin E_1 (PGE_1)-induced cyclic AMP formation, but had no effect on basal cyclic AMP formation. UDP showed an equal potency, while 2-methylthio ATP, α,β-methylene ATP and β,γ-methylene ATP gave either a slight increase or had no effect at concentrations up to 100 μM. ATP, although 100 fold less effective than UTP, also caused cyclic AMP potentiation, but had no effect on agonist-stimulated or basal cyclic AMP levels.

3 The cyclic AMP potentiation effect of UTP correlated with increased $[Ca^{2+}]_i$ and inositol phosphate (IP) formation over the same concentration range.

4 Ionomycin, which evokes an increase in $[Ca^{2+}]_i$ without affecting IP formation, did not cause an increase in cyclic AMP content, indicating that UTP-induced cyclic AMP regulation is not due to activation of Ca^{2+}-sensitive adenylyl cyclase isoforms.

5 Although reduced, UTP potentiation was seen in cells incubated in a Ca^{2+}-free and/or BAPTA-containing medium. Under these conditions, the UTP-increased IP accumulation was similarly reduced.

6 Exposure of cells to phorbol 12-myristate 13-acetate (PMA) also increased PGE_1 stimulation of cyclic AMP levels, and the UTP-induced potentiation of cyclic AMP formation was inhibited by either staurosporine or Ro 31-8220. Pretreatment of cells with PMA for 4–24 h resulted in marked attenuation of UTP-stimulated cyclic AMP potentiation.

7 Pretreatment with pertussis toxin (24 h, 100 ng ml^{-1}) did not significantly affect UTP-induced cyclic AMP potentiation and IP formation, although it increased the cyclic AMP response to PGE_1.

8 Analysis of J774 cells by Western blotting with antibodies specific for different protein kinase C (PKC) isoforms shows the presence of the βI, βII, δ, ε, η, μ, λ and ζ isoforms. Moreover, UTP significantly increased the level of PKC βI, βII, δ, ε, μ, λ and ζ immunoreactivity in the membrane fraction and decreased the cytosolic reactivity of PKC βII, δ, ε and ζ.

9 Immunoblot studies also indicate the presence of type II adenylyl cyclase.

10 These results indicate that PKC is required for the potentiation of adenylyl cyclase activity by macrophage pyrimidinoceptors, which exhibit a higher specificity for UTP and UDP than for ATP.

Keywords: Uridine 5′-triphosphate (UTP); protein kinase C; adenylyl cyclase; J774 macrophages; pyrimidinoceptor

Introduction

Since 1993, at least eight G protein-coupled P2Y purinoceptor subtypes have been cloned and their signalling cascades have been recently reviewed (Barnard *et al*., 1994; Boarder *et al*., 1995; Harden *et al*., 1995; Fredholm *et al*., 1996, 1997). P2Y purinoceptors are widely-distributed phospholipase C (PLC)-activating receptors. The rank order of agonist potency for the $P2Y_1$ subtype is 2-methylthio ATP > adenosine-5′-*O*-(β-thiodiphosphate) (ADPβS) > adenosine 5′-triphosphate (ATP) >> uridine 5′-triphosphate (UTP), α,β-methylene ATP, β,γ-methylene ATP, while the $P2Y_2$ subtype (previously known as the P_{2U} subtype) is stimulated by ATP and UTP, but not by 2-methylthio ATP or α,β-methylene ATP. UTP may also act at certain receptors that are not activated by purine nucleotides, but are selective for uridine nucleotides (previously known as pyrimidinoceptors) (Seifert & Schultz, 1989). Recently, two pyrimidinoceptors have been cloned and sequenced and designated as the $P2Y_4$ and $P2Y_6$ subtypes (reviewed in Burnstock & King, 1996). Second-messenger signalling cascades known to be activated by the pyrimidinoceptors include the phosphoinositide (PI)-specific PLC, phospholipase A_2 (PLA_2) and/or intracellular Ca^{2+} increase, as seen in HL-60 cells (Seifert & Schultz, 1989), C_6-2B glioma (Lazarowski & Harden, 1994), NG 108-15 neuroblastoma (Lin, 1994) and RAW 264.7 macrophages (Lin & Lee, 1996).

As well as being involved in the activation of PLC and PLA_2 in many cells, P_2 purinoceptors have been linked to changes in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels in certain cell types. Direct activation or potentiation of adenylyl cyclase (AC) by ATP was demonstrated in microvascular endothelial cells from the adrenal medulla (Allsup & Boarder, 1990), Jurkat T cells (Tokumitsu *et al*., 1991), type II pneumocytes (Griese *et al*., 1991), aortic smooth muscle cells (Tada *et al*., 1992), C2C12 myotubes (Henning *et al*., 1993), NG 108-15 cells (Matsuoka *et al*., 1995) and MDCK epithelial cells (Post *et al*., 1996). However, the subtypes involved have not been well documented. In contrast, ATP-mediated inhibition of AC via unidentified P2Y purinoceptor(s) has also been demonstrated in hepatocytes (Okajima *et al*., 1987), renal LLC-PK_1 epithelial cells (Anderson *et al*., 1991), ventricular myocytes (Yamada *et al*., 1992), FRTL-5 thyroid cells (Sato *et al*., 1992) and C_6 glioma cells (Boyer *et al*., 1993; Lin & Chuang, 1994). Recently, Webb *et al*. (1996) have shown that a $P2Y_1$ receptor on rat brain microvascular endothelial cells appears to be coupled to AC inhibition.

PLC and AC are major transmembrane-signalling systems in most cells. An interaction between these signalling cascades

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could be of considerable importance in the modulation of cellular responsiveness to many receptor ligands. AC comprises a heterogeneous multigene family, the members of which are variously regulated by the α and $\beta\gamma$ subunits of G proteins, by Ca^{2+} and by protein kinase C (PKC) (Iyengar, 1993; Mons & Cooper, 1995; Taussig & Gilman, 1995; Sunahara *et al*., 1996). Because of the importance of cyclic AMP in regulating macrophage functions, such as cytokine release and apoptosis (Spriggs *et al*., 1991), and the potential interaction between the P_2 purinoceptor and the AC pathway, we explored the regulatory role of nucleotide analogues on cyclic AMP signal transduction and the convergence of the cyclic AMP and PLC pathways in macrophages. Pharmacological studies and AC isozyme identification were performed on the J774 mouse macrophage, a well-differentiated macrophage cell line that has been widely used as a model system for studying the regulation of macrophage function. We found UTP to be the most potent nucleotide in potentiation prostaglandin E_1 (PGE_1)-induced cyclic AMP production in these cells. Mechanisms relating PLC stimulation and PKC activation to modulation of cyclic AMP formation could explain this result.

Methods

Cell cultures

Mouse J774 macrophages, obtained from the American Type Culture Collection (Bethesda, MD), were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM)/10% foetal bovine serum/100 u ml^{-1} penicillin and 100 μg ml^{-1} streptomycin in a humidified atmosphere of 95% air/5% CO_2.

Measurement of cyclic AMP levels

Confluent cells on 35 mm culture plates were washed with physiological saline solution (PSS, composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 1.8, $MgCl_2$ 1.2, KH_2PO_4 1.2, glucose 11 and HEPES 20, pH 7.4) and incubated for 20 min at 37°C in the presence of 500 μM 3-isobutyl-methylxanthine (IBMX) and 500 μM Ro 20-1724. The reaction was started by addition of the test reagents and continued for 10 min at 37°C, unless otherwise indicated; it was then terminated by aspirating the reaction mixture and immediately adding 0.1 N HCl. The cells were scraped off and the suspension centrifuged. The supernatant was neutralized and cyclic AMP levels assayed with the [^{3}H]-cyclic AMP assay kit. Cell pellets were assayed for protein content by the Bradford method with bovine serum albumin as the standard and the cyclic AMP level expressed in pmol mg^{-1} protein.

Measurement of PI turnover

PI hydrolysis was measured by the accumulation of inositol phosphates (IP) in the presence of 10 mM LiCl, as described previously (Lin & Chuang, 1994). Confluent cells on 35 mm Petri dishes were labelled with [^{3}H]-*myo*-inositol (2.5 μCi/dish) in the growth medium for 24 h, washed with PSS containing 10 mM LiCl and incubated at 37°C for 20 min. After this preincubation, the indicated drugs were added and incubation continued for another 30 min. The reaction was terminated by aspiration of the reaction solution and addition of ice-cold methanol. The cells were scraped off and the [^{3}H]-inositol phosphate (IP) isolated with an AG-1X8 column (formate form, 100–200 mesh, Bio-Rad, Richmond, CA) and elution with 0.2 N ammonium formate/0.1 N formic acid.

Immunoblotting analysis

Cells were placed on ice, rinsed with PBS, resuspended in homogenization buffer (20 mM Tris-HCl, 0.5 mM EGTA, 2 mM EDTA, 2 mM DTT, 0.5 mM p-methylsulphonyl fluoride and 10 μg ml^{-1} leupeptin, pH 7.5) and sonicated. The lysate was separated into cytosolic and particular fractions by centrifugation at 40,000 *g* for 45 min. The protein level of cell homogenate was assayed by the Bradford method. Equal amounts of each protein fraction (80 μg) were separated by 9% polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate (SDS–PAGE), then electrotransferred on to nitrocellulose membranes (Amersham ECL grade). In some experiments, the homogenates of rat whole brain, human neuroblastoma U105 and human Jurkat cells were prepared, separated by gel electrophoresis and electrotransferred. The blots were then blocked with TBST buffer (100 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5) containing 2% nonfat dry milk powder for 12 h at 4°C, washed with TBST buffer, and incubated with PKC isoform- or ACII-specific primary antibody for 1.5 h. After further washing, the blots were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody for 1 h. After further washing, the blots were processed for visualization using the enhanced chemi- luminescence (ECL) system according to the manufacturer's recommendations (Amersham), then were exposed to Kodak XAR-5 film to obtain the fluorographic images.

Measurement of $[Ca^{2+}]_i$

Cells grown on glass slides were loaded with 3 μM fura-II/AM and pluronic F-127 (0.02% v/v) in DMEM at 37°C for 45 min. The fluorescence was monitored on a PTI M-series spectrofluorometer with dual excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescence at the two excitation wavelengths, by use of a K_d value of 224 nM for the fura-II/Ca^{2+} equilibrium, as described by Grynkiewicz *et al*. (1985).

Materials

DMEM, foetal bovine serum, penicillin and streptomycin were obtained from Gibco BRL (Grand Island, NY). [^{3}H]-*myo*-inositol (20 Ci $mmol^{-1}$) was purchased from New England Nuclear (Boston, MA). The [^{3}H]-cyclic AMP assay system was obtained from Amersham (Arlington Heights, IL). α,β-Methylene ATP, β,γ-methylene ATP, 2-methylthio ATP and 4-[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone (Ro 20-1724) were obtained from Research Biochemicals (Natick, MA) and {3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide methane sulfonate} (Ro 31-8220) from Calbiochem (La Jolla, CA). Mouse monoclonal antibodies specific for PKC α, γ, δ, ε, θ, λ, μ, ι and ζ were purchased from Transduction Laboratories (Lexington, KY), and rabbit polyclonal antibodies specific for PKC βI, βII and η, and ACII were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-coupled anti-mouse and anti-rabbit antibody and the ECL detection agent were purchased from Amersham International. All materials for SDS–PAGE were obtained from Bio-Rad Laboratories (Hercules, CA). Other chemicals were obtained from Sigma (St. Louis, MO).

Statistical analysis

Each experiment was performed in duplicate, and the data represent the mean ± s.e.mean of several independent experiments. Student's *t* test for paired observations or analysis of variance (ANOVA) corrected for multiple comparisons by Bonferoni method was used. Statistical significance was accepted when $P<0.05$. The *n* values represent the number of independent experiments. The error bar was omitted when it fell within the size of the symbol representing the mean value.

Results

Nucleotide analogue-induced IP accumulation and cyclic AMP potentiation

Of the nucleotide analogues tested, UTP and UDP were most effective in stimulating PI turnover. At concentrations higher than 0.3 μM, they were equipotent and increased IP accumulation in a concentration-dependent manner up to approximately 11 fold at 100 μM (the EC_{50} was 1.6 μM for UTP and 1.9 μM for UDP), while ATP only caused IP accumulation (417 ± 70% of control) at 300 μM (Figure 1a). 2-Methylthio ATP, α,β-methylene ATP, β,γ-methylene ATP, ADP, AMP and adenosine did not cause IP production at 100 μM. Benzoylbenzoic ATP, only caused a 95% increase in IP accumulation at 100 μM (data not shown).

In the presence of 500 μM isobutylmethylxanthine (IBMX) and 500 μM Ro 20-1724, PGE_1 (1 μM) increased cyclic AMP levels from the basal level of 27 ± 4 pmol mg^{-1} protein ($n=4$) to 1269 ± 100 pmol mg^{-1} protein ($n=4$) within 10 min. Although having no effects on basal cyclic AMP levels, UTP and UDP concentration-dependently potentiated the PGE_1-induced cyclic AMP response in the same concentration ranges used for IP stimulation (Figure 1b). At 100 μM, UTP and UDP potentiated the PGE_1 response 2.8 and 2.4 fold, respectively, while ATP was at least 100 fold less effective. 2-Methylthio ATP and ADP only caused about 30% potentiation at 100 μM, while α,β-methylene ATP and β,γ-methylene ATP had no potentiation effects on either basal or PGE_1-induced cyclic AMP accumulation at 100 μM (data not shown). Adenosine also failed to affect cyclic AMP levels in the absence of IBMX at 100 μM (data not shown). Because of the high potency and obvious potentiation by UTP of cyclic AMP accumulation, the underlying mechanism of the UTP-mediated event was investigated in the following experiments.

As shown in Figure 2a, PGE_1 alone caused concentration-dependent cyclic AMP accumulation; over the range of 10 nM – 1 μM PGE_1, this was enhanced by 100 μM UTP. Upon stimulation with 1 μM PGE_1 alone, the cyclic AMP level increased rapidly and peaked at 5 min, while in the presence of UTP (100 μM), the response was enhanced and peaked after 2.5 min (Figure 2b).

PKC activation involvement of AC potentiation

To understand the regulation of AC activity by PKC and the underlying mechanism of UTP-mediated potentiation, the effects of a PKC activator, phorbol 12-myristate 13-acetate (PMA), and two PKC inhibitors, Ro 31-8220 and staurosporine, were investigated. Pretreatment of cells with 1 μM Ro 31-8220 or staurosporine had no effect on the PGE_1 response, but inhibited the UTP-induced potentiation effect by 84 ± 13% ($n=4$) and 80 ± 8% ($n=3$), respectively (Figure 3a). Pretreatment of J774 cells with PMA for 20 min, while itself had no

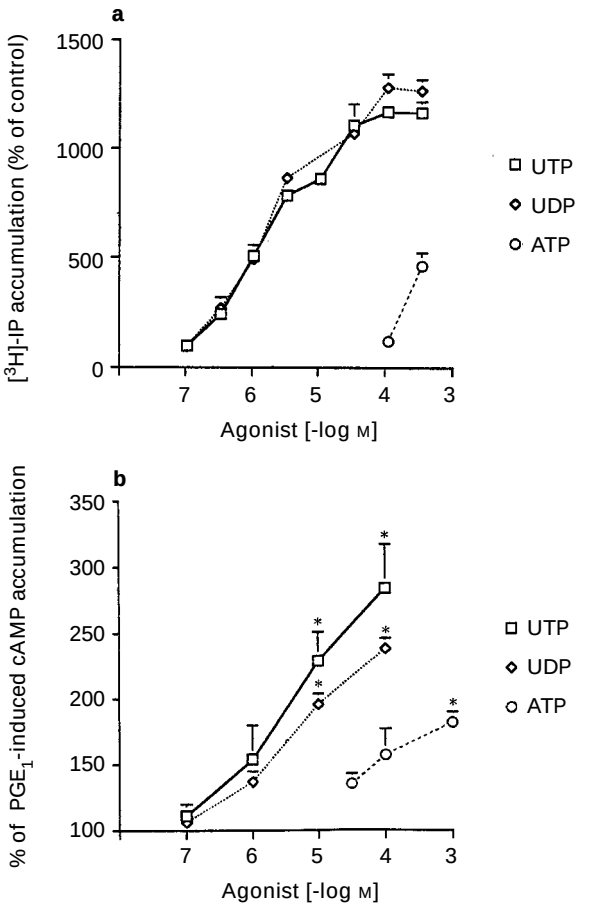

Figure 1 Effect of UTP, UDP and ATP on PI turnover and PGE_1-mediated cyclic AMP accumulation in J774 macrophages. (a) Cells were labelled with [^{3}H]-*myo*-inositol and IP accumulation after UTP, UDP or ATP treatment was measured as described in Methods. (b) In the presence of 500 μM IBMX and 500 μM Ro 20-1724, cells were stimulated with 1 μM PGE_1 and the indicated concentrations of UTP, UDP or ATP for 10 min. Cytosolic cyclic AMP levels were assayed as described in Methods. The data represent the mean of three independent experiments performed in duplicate; vertical lines show s.e.mean. *Significantly different ($P<0.05$) from controls without nucleotide treatment according to analysis of variance (ANOVA) followed by Bonferroni test.

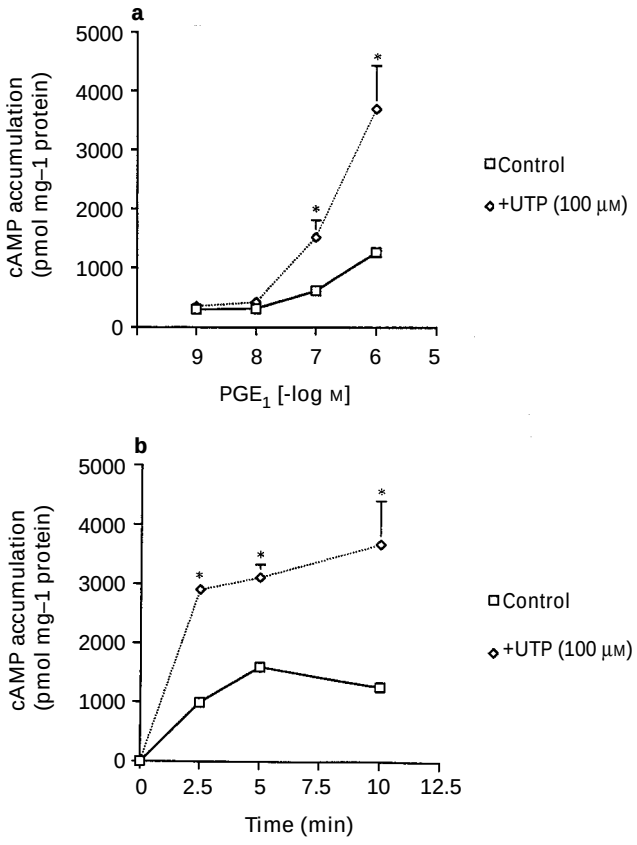

Figure 2 Concentration- and time-dependent effects of UTP-induced potentiation of cyclic AMP accumulation. (a) J774 cells were treated with the indicated concentrations of PGE_1 in the absence and presence of 100 μM UTP for 10 min and cyclic AMP accumulation measured. (b) PGE_1 (1 μM), without or with UTP (100 μM), was added to J774 cells for the indicated period, then cyclic AMP accumulation was determined. The data represent the mean of three independent experiments performed in duplicate; vertical lines show s.e.mean. *According to Student's t test, $P<0.05$ compared to the control response without UTP treatment.

significant effect on resting levels of cyclic AMP, had a biphasic effect on PGE_1-stimulated cyclic AMP accumulation (Figure 3b). At the lower concentrations of 1 and 10 nM, PMA caused approximately 30–40% attenuation of the PGE_1 response, which disappeared in the presence of 1 μM staurosporine (data not shown), while at 100 nM and 1 μM PMA, $42 \pm 7\%$ ($n=3$) and $145 \pm 20\%$ ($n=5$) increases were seen, respectively. The inactive phorbol ester, 4α-PMA did not elicit any potentiation effect at concentrations up to 1 μM (data not shown).

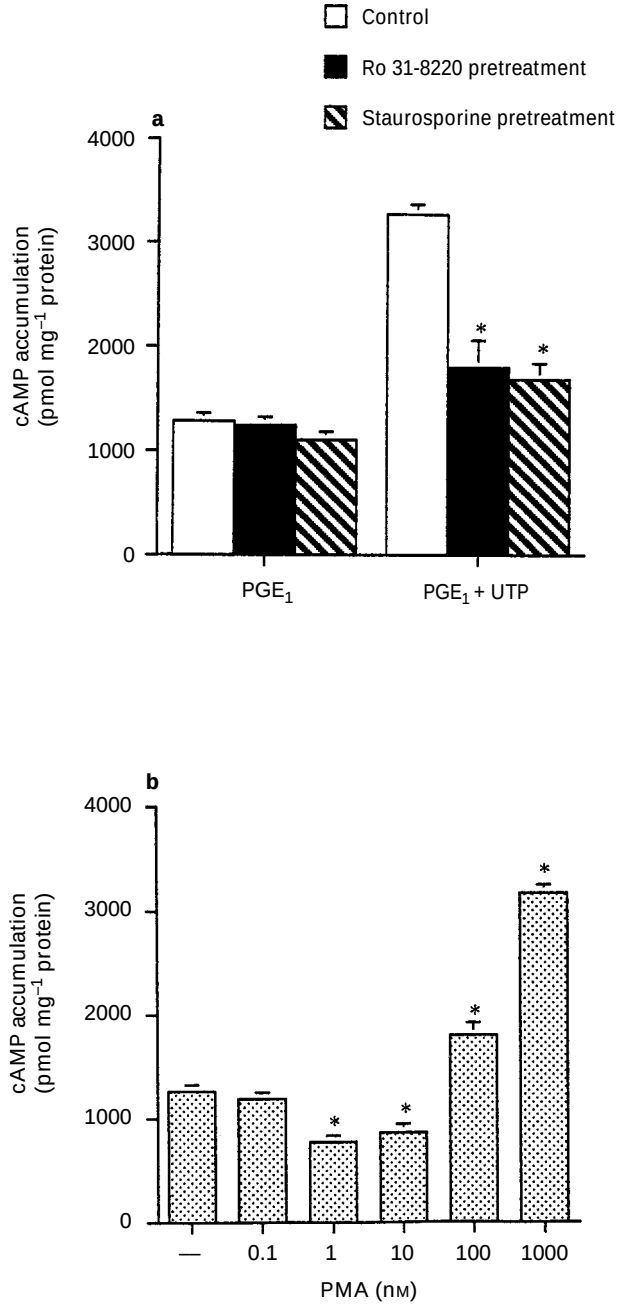

Figure 3 Effect of PKC inhibitors on UTP-potentiated and PGE_1-stimulated cyclic AMP accumulation. (a) Cells were pretreated with vehicle (DMSO), Ro 31-8220 (1 μM) or staurosporine (1 μM) for 20 min before stimulation with PGE_1 (1 μM), in the absence or presence of 100 μM UTP, for 10 min and the accumulation of cyclic AMP measured. (b) Different concentrations of PMA were added to cells for 20 min before stimulation with PGE_1 (1 μM) for 10 min. *Significantly different ($P<0.05$) from the response in the absence of the PKC inhibitor (a) or PMA (b) pretreatment. The data represent the mean $\pm$ s.e.mean of 3–4 independent experiments performed in duplicate.

To assess further the role of PKC in cyclic AMP potentiation, PKC down-regulation was induced by pretreatment with 1 μM PMA for various periods up to 24 h before UTP and PGE_1 treatment. As shown in Figure 4, in contrast to the potentiation caused by 20 min pretreatment (Figure 3b), longer exposure of cells to 1 μM PMA for 2 or 4 h had no significant effect on the PGE_1 response, while PMA incubation periods of 8 or 24 h resulted in a $26 \pm 11\%$ ($n=4$) or $31 \pm 10\%$ ($n=4$) decrease, respectively, in the response. Under these conditions, the longer the period of PMA pretreatment, the lower was the potentiation effect. Indeed, the UTP potentiation effect was virtually abolished after 4–24 h of PMA pretreatment.

Effects of Ca^{2+}, ionomycin and PTX on UTP-induced cyclic AMP potentiation

As shown in Figure 5a and b, at concentrations between 0.1 and 100 μM, UTP caused a concentration-dependent and rapid increase in $[Ca^{2+}]_i$ which was markedly reduced in Ca^{2+}-free PSS (no added $CaCl_2$ and addition of 1 mM EGTA). UDP showed a similar potency in increasing $[Ca^{2+}]_i$, while ATP induced significant $[Ca^{2+}]_i$ rise only at concentrations up to 100 μM (Figure 5b).

To assess the regulatory role of $[Ca^{2+}]_i$ on AC activity in J774 cells, the effects of Ca^{2+}-free PSS and BAPTA/AM were determined. Although the cyclic AMP response to PGE_1 was unaffected by the use of Ca^{2+}-free PSS or 30 μM BAPTA/AM, the potentiation effect of UTP was reduced by $51 \pm 9\%$ ($n=3$) and $69 \pm 3\%$ ($n=3$), respectively (Figure 6). The potentiation effect of PMA was not affected by removal of extracellular Ca^{2+}, and, under these conditions, the potentiation effects of UTP and PMA were non-additive. Ionomycin (1 μM) had no effect on either the PGE_1 response or UTP potentiation.

To determine whether the UTP potentiation effect was due to interference by the G_i-regulated inhibitory pathway with AC, cells were pretreated with pertussis toxin (PTX) (100 ng ml^{-1}, 24 h) to uncouple this pathway by ADP-ribosylation of the Giα protein. As shown in Figure 6, although PTX significantly increased the PGE_1 response by $59 \pm 10\%$ ($n=4$), the UTP potentiation of the effect was not affected.

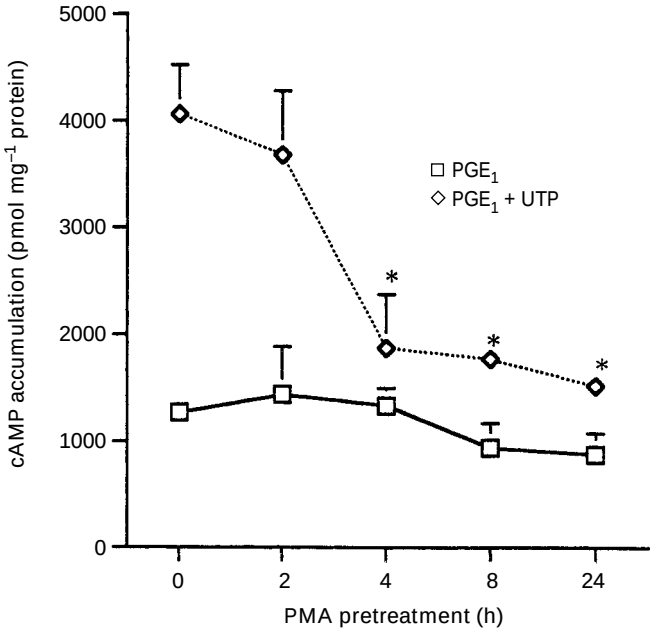

Figure 4 Effect of PMA pretreatment on PGE_1- and UTP-mediated potentiation of cyclic AMP accumulation. Cells were pretreated with 1 μM PMA for 2, 4, 8 or 24 h, then PGE_1 (1 μM), without or with UTP (100 μM), was added for 10 min. Data represent the mean of 3 independent experiments performed in duplicate; vertical lines show s.e.mean. *According to Bonferroni test, significantly different ($P<0.05$) from the control response without PMA pretreatment.

Effects of UTP-mediated IP formation on cyclic AMP potentiation

To understand the correlation between UTP-mediated PI turnover and AC potentiation, the effects of various pharmacological manipulations on UTP-induced IP accumulation were studied. Figure 7 shows that the IP accumulation induced by 100 μM UTP was inhibited 73$\pm$3% and 64$\pm$2% in Ca^{2+}-free PSS and 30 μM BAPTA/AM, respectively. In contrast, PTX, staurosporine and Ro 31-8220 had no effect.

Reactive blue, an antagonist more selective for $P2Y_1$ than $P2Y_2$ purinoceptors, inhibited both the IP accumulation and cyclic AMP potentiation caused by UTP in a concentration-dependent and parallel manner (Figure 8).

Presence of ACII in J774 cells

By use of ACII-specific antibody and Western blot analysis, ACII was detected in J774 cells and rat brain (Figure 9).

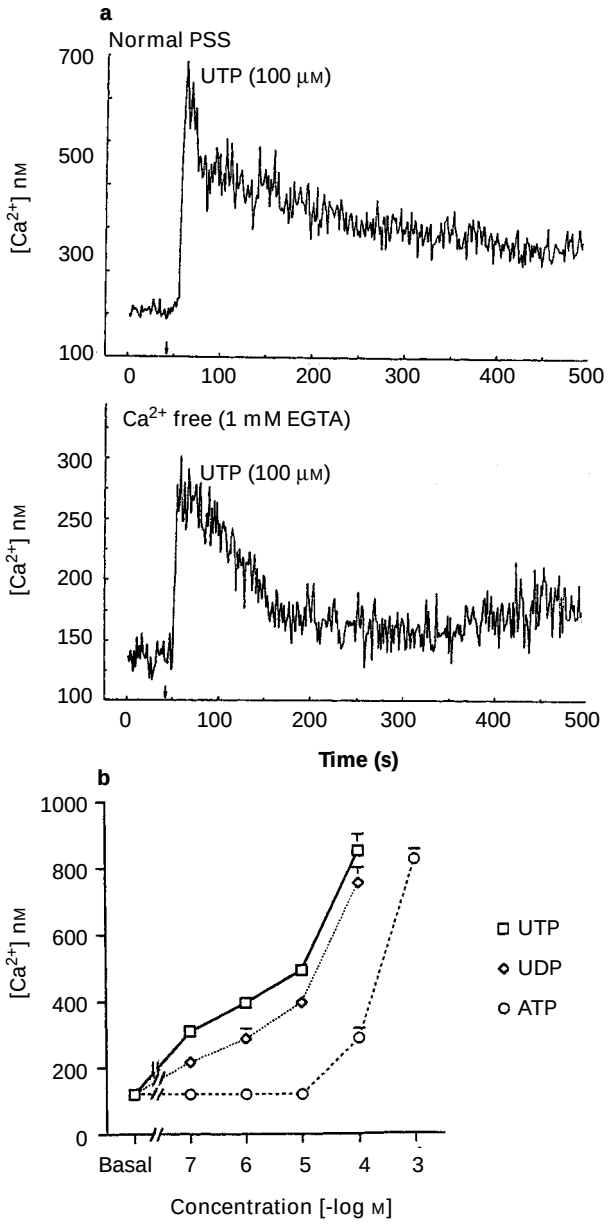

Figure 5 Effect of UTP, UDP and ATP on intracellular Ca^{2+} levels. (a) Typical $[Ca^{2+}]_i$ responses to UTP in normal and Ca^{2+}-free PSS. Note the different scale of the traces. (b) Dose-dependence of the $[Ca^{2+}]_i$ increase elicited by UTP, UDP and ATP. Data represent the mean of at least three experiments; vertical lines show s.e.mean.

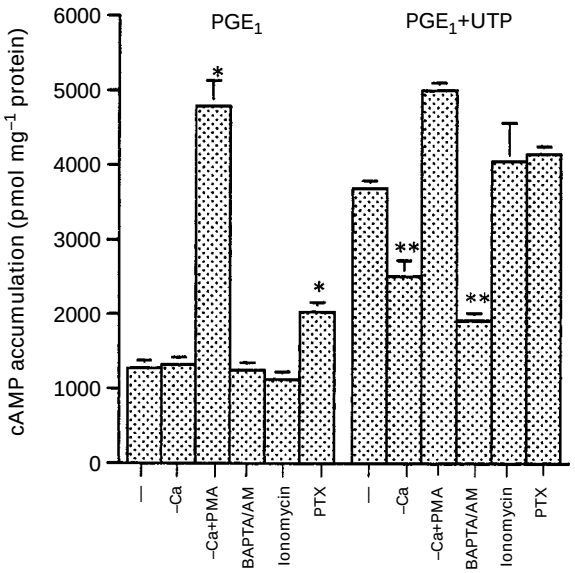

Figure 6 Effect of extracellular Ca^{2+}, BAPTA/AM, ionomycin or pertussis toxin (PTX) on UTP- and PMA-mediated potentiation of cyclic AMP accumulation. Cells were pretreated with Ca^{2+}-free PSS ($-$Ca), in the presence or absence of 1 μM PMA, 30 μM BAPTA/AM, or 100 ng ml^{-1} pertussis toxin for 20 min, 10 min or 24 h, respectively, before stimulation with PGE_1 (1 μM), in the absence or presence of 100 μM UTP, and/or 1 μM ionomycin for 10 min. The data represent the mean$\pm$s.e.mean of at least 3 independent experiments performed in duplicate. According to Student's t test, $^*P<0.05$ compared to the basal level in normal PSS without pretreatment, and $^{**}P<0.05$ compared to the potentiating effect of UTP in normal PSS.

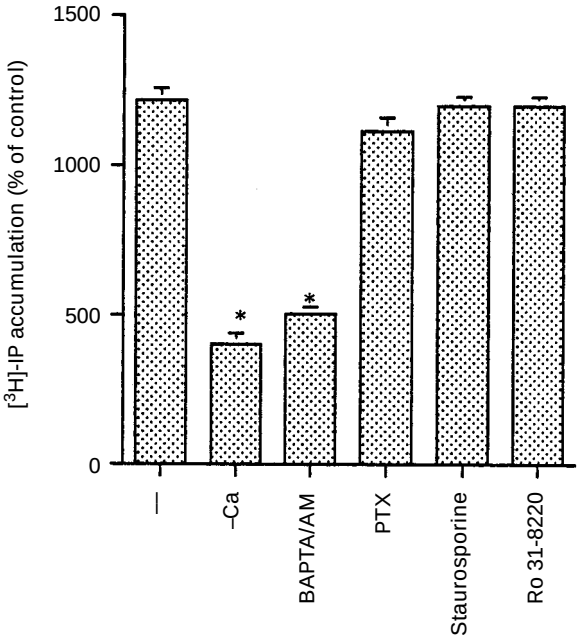

Figure 7 Effect of pharmacological manipulations on UTP-induced IP accumulation. [^{3}H]-*myo*-inositol-labelled cells were pretreated with Ca^{2+}-free PSS ($-$Ca), 30 μM BAPTA/AM, 1 μM staurosporine or 1 μM Ro 31-8220 for 20 min or with 100 ng ml^{-1} pertussis toxin (PTX) for 24 h before stimulation with 100 μM UTP for 30 min. [^{3}H]-IP accumulation was assessed as described in Methods. The data represent the mean$\pm$s.e.mean of 3 independent experiments performed in duplicate. *According to Student's t test, $P<0.05$ compared to the control UTP response without pretreatment.

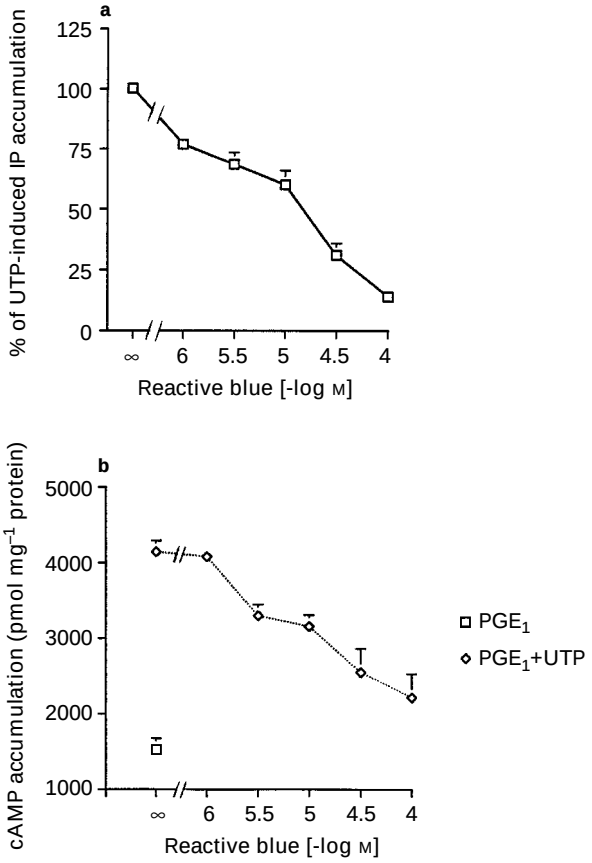

Figure 8 Effect of reactive blue on UTP-induced IP accumulation and potentiation of cyclic AMP accumulation. Cells were pretreated with reactive blue, at the indicated concentrations, for 20 min before (a) 100 μM UTP stimulation of IP accumulation or (b) PGE_1 stimulation (1 μM), with or without 100 μM UTP, for cyclic AMP determination. The data represent the mean of 3 independent experiments performed in duplicate; vertical lines show s.e.mean.

Effects of UTP on PKC isoform translocation

To determine which PKC isoforms were activated by UTP, we performed Western blotting with antibodies specific for twelve different PKC isoforms. Positive results were obtained with anti-βI, βII, δ, ε, η, μ, λ and ζ antibodies; results with antibodies directed against other PKC isoforms (α, γ, θ and ι) were either weak or undetectable. With respect to those not detected, we used human neuroblastoma U105 and Jurkat cells as positive controls to prove the effectiveness of α, γ, ι and θ antibodies. In membrane fractions, UTP (100 μM) increased immunoreactivity for βI, βII, δ, ε, μ and λ after either 3 or 10 min treatment and for ζ after 10 min, while their immunoreactivities in cytosolic fraction showed a transient decrease after 3 min (PKC, δ, ε), a delayed decrease after 10 min (PKC ζ), or a more sustained reduction over 10 min (PKC βII) (Figure 10). The cytosolic immunoreactivity for PKC βI, μ and λ was unaffected over 10 min. Both the membrane and cytosolic immunoreactivity for PKC η were not changed by UTP treatment (data not shown).

Discussion

In this study, we found that agonist-induced cyclic AMP production in mouse J774 macrophages is potentiated by UTP, which alone had no effect. All the studies on cyclic AMP accumulation described here were carried out in the presence of cyclic nucleotide phosphodiesterase blockers (IBMX and Ro 20-1724), and hence the observed effects

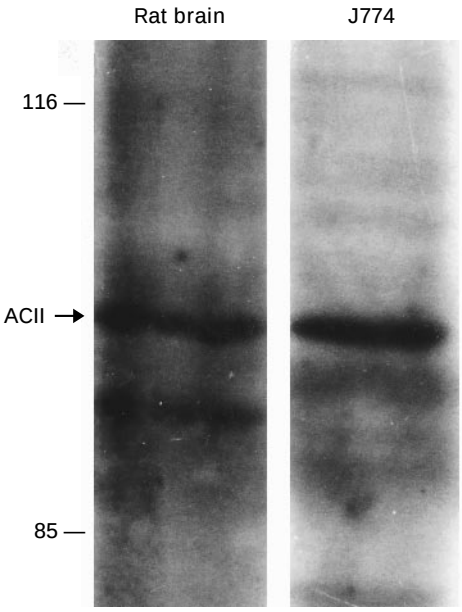

Figure 9 Immunoreactivity of adenyl cyclase (AC) II in J774 cells. A J774 cell homogenate (100 μg) and rat whole brain (80 μg) was immunoblotted with polyclonal antibody against ACII, as described in Methods.

relate to changes in the rate of synthesis of cyclic AMP, rather than to its degradation. Based on the ability of extracellular nucleotide analogues to potentiate cyclic AMP accumulation and stimulate PI-PLC, i.e. UTP and UDP are much more potent than ATP and other purine nucleotides, we concluded that pyrimidinoceptors are present and mediate the signalling crosstalk between the PI-PLC and AC cascades in these cells. Although ATP-induced cyclic AMP accumulation has been found in other types of cell, the effects of UTP on the AC signalling system have not been documented. Thus, this study is the first to show the potentiation effect of uridine nucleotides on cyclic AMP formation and suggests possible physiological roles for pyrimidinoceptors in macrophages and the immune system. The existence of pyrimidinoceptors in J774 cells agrees with our recent findings in mouse RAW 264.7 macrophages (Lin & Lee, 1996).

Recent molecular cloning studies have revealed that several different subtypes of AC exist in mammalian cells (Iyengar, 1993; Taussig & Gilman, 1995). Activation of PKC has been shown to either potentiate or inhibit agonist-stimulated AC activity in a variety of cell types (Bouvier, 1992). The target of PKC action varies with cell type and PKC appears to have multiple sites of action within the AC signalling system. PKC activation can directly phosphorylate and thus regulate the activity of certain AC isoenzymes, such as types II and VII (Yoshimasa *et al*, 1987; Yoshimura & Cooper, 1993; Jacobowitz & Iyengar, 1994; Kawabe *et al*., 1994), while types I and III AC show a modest stimulation of activation by PKC (Iyengar, 1993). PKC can also act on an activated component of the second messenger systems, such as the Gs or Giα-protein, and thus regulate its coupling to AC (Taussig *et al*., 1993; Morimoto & Koshland, 1994).

Results from the present study suggest that UTP-induced activation of PKC is probably the mechanism of sensitization. Three lines of evidence support PKC-mediation of the UTP effect. Firstly, pharmacological activation of PKC by PMA mimicked the potentiation effect of UTP (Figure 3b) and their responses were non-additive (Figure 6). Secondly, the potentiation effect of UTP was greatly reduced by the PKC inhibitors, staurosporine and Ro 31-8220 (Figure 3a). Thirdly, UTP was less effective in stimulating PGE_1 stimulation after down-regulation of PKC (Figure 4). Although activation of PKC has been shown, in many other systems, to influence cyclic AMP

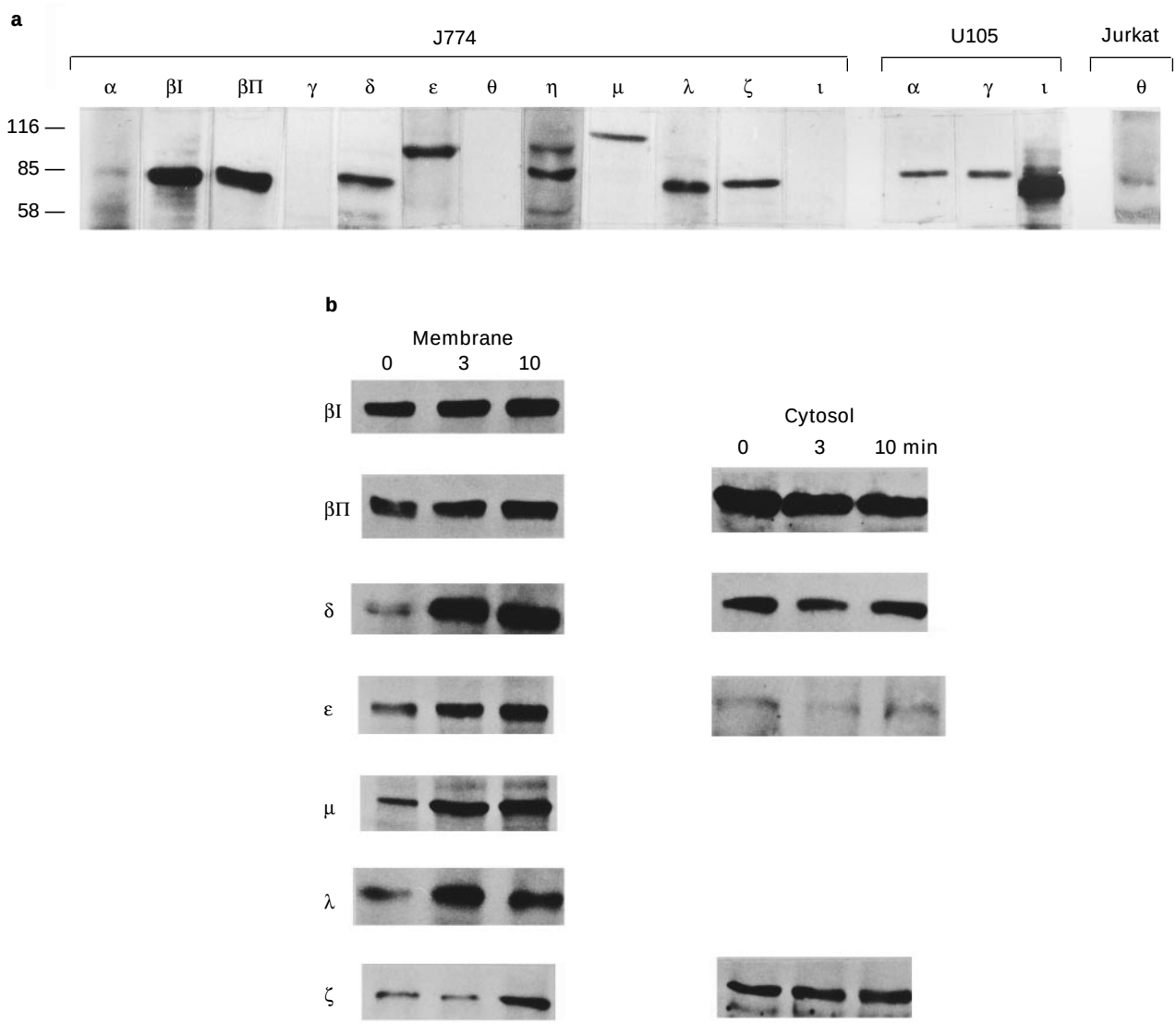

Figure 10 Effect of UTP treatment on the translocation of PKC isoforms in J774 cells. (a) The presence of PKC isoforms in J774 cells. The expression of PKC α, γ, ι in U105 neuroblastoma cells and PKC θ in Jurkat cells were shown as positive controls. (b) Homogenate from J774 cells, exposed to 100 μM UTP for 3 or 10 min, was fractionated into cytosolic and membrane fractions and immunoblotted with antibodies against each PKC isoform, as described in Methods. These results are representative of three experiments.

production, this is the first demonstration of such an effect produced by pyrimidinoceptor activation.

PKC is a calcium- and phospholipid-dependent serine/threonine protein kinase known to be involved in intracellular signalling pathways. PKC isoforms are classified into three groups, these being the Ca^{2+}-dependent conventional PKCsα, βI, βII, γ, the Ca^{2+}-independent novel PKCs δ, ε, η, μ and the atypical PKCs ζ, λ, ι, which are not activated by either Ca^{2+} or DAG (Hug & Sarre, 1993; Dekker & Parker, 1994; Nishizuka, 1995; Jaken, 1996). The complex and multiple sites of action of PKC, which vary with cell type, may be ascribed to both the PKC isoform present and the type of AC. In J774 macrophages, the βI, βII, δ, ε, η, μ, λ and ζ isoforms of PKC are expressed, thus total PKC activity will necessarily be a composite of the activation of several different isoforms with differing Ca^{2+} and diacylglycerol dependencies.

Because UTP-induced cyclic AMP potentiation and the increase in $[Ca^{2+}]_i$ showed very similar concentration-response curves in J774 cells, such as Ca^{2+}-sensitive AC might possibly contribute to UTP-induced cyclic AMP potentiation. However, we observed that the Ca^{2+} ionophore, ionomycin, had no effect either on the basal or PGE_1-stimulated cyclic AMP levels. Moreover, in the presence of ionomycin, the cyclic AMP potentiation effect was still seen (Figure 6). We therefore conclude that the effect of UTP on cyclic AMP levels cannot be directly ascribed to the increase in $[Ca^{2+}]_i$ and also rules out the action of types I and III AC. Our Western blot analysis showed AC II to be present in J774 cells, thus supporting the concept of PKC-dependent potentiation of the effects of UTP and also suggesting an important pathway for the integration of PKC activation and PGE_1-induced cyclic AMP signalling in J774 cells. The effects of the PKC activator, PMA, on cyclic AMP accumulation are also consistent with the presence of the type II isozyme of AC.

Preincubation with PTX caused increased stimulation by PGE_1, presumably due to the removal of a tonic level of inhibition mediated by G_i. In addition, the inability of PTX to reduce UTP- (Figure 6) and PMA- (data not shown) induced cyclic AMP potentiation indicates that this PKC-dependent mechanism is unrelated to G_i inactivation. Thus, the previous demonstration that PKC can potentiate AC catalytic activity by phosphorylation and inactivation of G_i, subsequently removing the inhibitory tonus of G_i on AC, is not the underlying mechanism for the effects of UTP and PMA in J774 cells (Katada *et al*, 1985; Chen & Iyengar, 1993; Taussig *et al*., 1993).

Apart from the PKC-dependent mechanism discussed above, other possible mechanisms involved in UTP- and ATP-induced AC potentiation require consideration. Firstly, the potentiation effect of ATP appears not to be due to the action of adenosine, because adenosine, at concentrations up to 100 μM, had no effect on the basal and PGE_1-stimulated cyclic AMP accumulation (data not shown). Secondly, in MDCK-D_1 epithelial cells, ATP and UTP have been shown to enhance cyclic AMP production via an autocrine/paracrine mechanism by formation of PGE_2 (Post *et al*., 1996). This mechanism seems not to be involved in J774 cells, as indomethacin, a cyclooxygenase inhibitor, has no effect on UTP-mediated cyclic AMP enhancement (data not shown).

In conclusion, extracellular UTP induces phosphoinositide turnover, Ca^{2+} mobilization and cyclic AMP potentiation in J774 macrophages by activating UTP/UDP-specific pyrimidinoceptors. Cyclic AMP potentiation is secondary to translocation of PKC, at least the βI, βII, δ, ε, μ, λ and ζ isoforms, thus leading to modulation of AC II activity. The pharmacological correlation between PI turnover and cyclic AMP potentiation suggests that diacylglycerol, the endogenous PKC activator, primarily comes from the PI-PLC signalling cascades. We believe that the UTP-elicited crosstalk between PI-PLC and AC pathways could be important in understanding the physiological role of UTP in macrophages.

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