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Differential regulation of $P2Y_{11}$ receptor-mediated signalling to phospholipase C and adenylyl cyclase by protein kinase C in HL-60 promyelocytes

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1 The regulatory mode of the $P2Y_{11}$ purinoceptor-mediated signalling cascades towards phospholipase C and adenylyl cyclase was studied in HL-60 promyelocytes.

2 Treatment with the potent $P2Y_{11}$ receptor activator dATP evoked an elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and inositol 1,4,5-trisphosphate (IP_3) production that was sustained for longer than 30 min. However, the dATP-induced responses were significantly inhibited by the activation of protein kinase C after a short exposure to phorbol 12-myristate 13-acetate (PMA).

3 dATP also potently stimulated cyclic AMP production with half maximum effect seen at 23 ± 7 μM dATP. In addition, a 5-min pretreatment with PMA enhanced the dATP-stimulated cyclic AMP accumulation.

4 PMA potentiated the cyclic AMP production when adenylyl cyclase was activated directly by forskolin or indirectly by G protein activation after cholera toxin treatment. dATP also enhanced the forskolin-mediated cyclic AMP generation.

5 Treatment of the cells with 10 μM U-73122, which almost completely blocked the dATP-stimulated IP_3 production and $[Ca^{2+}]_i$ rise, had no effect on cyclic AMP accumulation, while 10 μM 9-(tetrahydro-2-furyl)adenine (SQ 22536), which inhibited the adenylyl cyclase activation, did not effect the dATP-stimulated phosphoinositide turnover.

6 Taken together, the results indicate that $P2Y_{11}$ receptor-mediated activation of phospholipase C and adenylyl cyclase occurs through independent pathways and is differentially regulated by protein kinase C in HL-60 cells.

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Abbreviations: BzATP, 3′-O-(4-benzoyl)benzoyl ATP; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; fura-2/AM, Fura-2 pentaacetoxymethylester; IP_3, inositol 1,4,5-trisphosphate; PMA, phorbol 12-myristate 13-acetate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; SQ 22536, 9-(tetrahydro-2-furyl)adenine

Introduction

Extracellular purines and pyrimidines are important signalling molecules that mediate diverse biological effects *via* cell-surface receptors termed P2 receptors (Ralevic & Burnstock, 1998). Based on differences in molecular structure, affinity of selective drugs, and signal transduction mechanisms, the P2 receptors fall naturally into the two families of ligand gated ion channels and G-protein-coupled receptors termed P2X and P2Y receptors, respectively. However, as new pharmacological tools and new molecular techniques have become available, each P2 receptor type has been subdivided into an increasing number of distinct subtypes (Abbrachio & Burnstock, 1994; Fredholm *et al*., 1994). To date seven mammalian P2X receptors ($P2X_{1-7}$) and five mammalian P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$) have been cloned and characterized as members of the P2 receptor superfamily. Pharmacological data have revealed that the $P2Y_1$ and $P2Y_{11}$ receptors are specifically activated by adenine nucleotides (Janssens *et al*., 1996; Communi *et al*., 1997), whereas the $P2Y_4$ and $P2Y_6$ receptors become highly activated by uridine nucleotides such as UDP and UTP (Communi *et al*., 1996; Bogdanov *et al*., 1998). In contrast, the $P2Y_2$ receptors are equally sensitive to ATP and UTP (Parr *et al*., 1995).

Many recent studies have addressed the issue that the $P2Y_{11}$ receptors are coupled to both the phospholipase C and adenylyl cyclase through distinct signalling cascades in cells stably expressing the receptors (Communi *et al*., 1997; 1999). Several lines of evidence indicate that the receptors are coupled to phospholipase C through pertussis toxin insensitive G proteins, while they seem to be direct coupled to adenylyl cyclase through G_s proteins (Communi *et al*., 1999). The pharmacological characterization has shown that the receptors are almost equally activated by ATPγS, BzATP, and dATP, and more potently so than by ATP, whereas UTP and UDP are ineffective; thus, this receptor behaves as a selective purinoceptor. In addition, it has been observed that the dual responsiveness of $P2Y_{11}$ was maintained in 1321N1 cells transfected with the gene, just as was the case with the stably expressed $P2Y_6$ receptor (Robaye *et al*., 1997; Communi *et al*., 1999). The $P2Y_{11}$ receptor is also expressed by HL-60 promyelocytes, and the comparable pharmacological features of the $P2Y_{11}$ receptor were detected there as well (Choi & Kim, 1997; Conigrave *et al*., 1998). However, little is known about the $P2Y_{11}$ receptor-mediated Ca^{2+} mobilization and the mechanism by which the receptor signallings are regulated.

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Therefore, the aim of this study was to investigate the mode of $P2Y_{11}$ receptor-mediated Ca^{2+} mobilization and the functional modulation of the receptor-mediated signalling cascades in HL-60 cells. Since HL-60 cells also express an endogenous $P2Y_2$ receptor coupled to phospholipase C (Klinker *et al*., 1996), the experiments were performed using selective agonists for the receptors. Here, we demonstrate that the $P2Y_{11}$ receptor-mediated dual responses occur through independent pathways and that they are differentially regulated by protein kinase C. The results also show evidence that the slow desensitization of the $P2Y_{11}$ receptor-mediated signalling results in a sustained elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) under continuous presence of extracellular nucleotides.

Methods

HL-60 cell culture

Human promyelocytic leukaemia HL-60 cells were maintained in RPMI 1640 medium (GIBCO, Gaithersburg, MD, U.S.A.) supplemented with 20% (v v^{-1}) heat-inactivated bovine calf serum (Hyclone, Logan, UT, U.S.A.) plus 1% (v v^{-1}) penicillin-streptomycin (GIBCO) under a humidified atmosphere of 5% CO_2 at 37°C. Fresh medium was added to culture flasks every 2 days, and cells were subcultured about once a week.

Measurement of intracellular Ca^{2+} level

The level of intracellular Ca^{2+} was measured using fura-2/AM as previously described (Suh *et al*., 1996). Briefly, cell suspensions were incubated in fresh serum-free RPMI 1640 medium was 3 μM fura-2/AM at 37°C for 40 min under continuous stirring. After this the cells were resuspended in Locke's solution of the following composition (mM): NaCl 154, KCl 5.6, $CaCl_2$ 2.2, $MgCl_2$ 1.2, glucose 10 and HEPES buffer 5 (adjusted to pH 7.4). In the Ca^{2+}-free Locke's solution, $CaCl_2$ was omitted and 100 μM EGTA included. Sulfinpyrazone (250 μM) was added to all solutions to prevent dye leakage (Di Virgilio *et al*., 1988). Changes in fluorescence ratio were measured at the dual excitation wavelengths of 340 and 380 nm and the emission wavelength of 500 nm. $[Ca^{2+}]_i$ was calculated using the equation:

$$[Ca^{2+}]_i = K_D[(R - R_{min})(R_{max} - R)^{-1}](S_{f2}\ S_{b2}^{-1})$$

where R_{max} and R_{min} are the ratios obtained when fura-2 is saturated with Ca^{2+} and when EGTA is used to remove Ca^{2+}, respectively. To obtain R_{min} and R_{max}, the fluorescence ratios of the cell suspension were measured successively at final concentrations of 4 mM EGTA, 30 mM Trizma base, and 0.1% Triton X-100, and then at a final concentration of 4 mM $CaCl_2$. S_{f2} and S_{b2} are the proportionality coefficients of Ca^{2+}-free fura-2 and saturated fura-2, respectively. Calibration of the fluorescence signal in terms of $[Ca^{2+}]_i$ was performed according to Grynkiewicz *et al*. (1985).

Measurement of inositol 1,4,5-trisphosphate

IP_3 concentration in the cells was determined by [^{3}H]-IP_3 competition assay in binding to IP_3 binding protein (Suh *et al*., 1999). To determine the IP_3 production, the HL-60 cells were stimulated with agonists for specific periods of time and the reaction was terminated by aspirating the medium off the cells followed by addition of 0.3 ml ice-cold 15% (w v^{-1}) trichloroacetic acid containing 10 mM EGTA. The samples were left on ice for 30 min to extract the water-soluble inositol phosphates and then centrifuged at $5000 \times g$ for 10 min at 4°C. The extract was then transferred to an Eppendorf tube and the trichloroacetic acid removed by four extractions with dimethyl ether. Finally the extract was neutralized with 200 mM Trizma base and its pH adjusted to about 7.4. Twenty μl of the cell extract was added to 20 μl of the assay buffer [0.1 M tris(hydroxymethyl)aminomethane buffer containing 4 mM EDTA and 4 mg ml^{-1} bovine serum albumin] and 20 μl of [^{3}H]-IP_3 (0.1 μCi ml^{-1}). Then 20 μl of solution containing the binding protein was added and the mixture incubated for 15 min on ice and centrifuged at $2000 \times g$ for 5 min. The pellet was resuspended in 100 μl of water, and 1 ml of scintillation cocktail was added to measure the radioactivity. IP_3 concentration in the sample was determined based on a standard curve and expressed as pmol mg $protein^{-1}$. The IP_3 binding protein was prepared from bovine adrenal cortex according to the method of Challiss *et al*. (1990).

Measurement of [^{3}H]-cyclic AMP

Intracellular cyclic AMP generation was determined by [^{3}H]-cyclic AMP competition assay in binding to cyclic AMP binding protein as described previously by Park *et al*. (1997) with some modification. To determine the cyclic AMP production induced by histamine or ATP analogues, the HL-60 cells were stimulated with agonists for 20 min in the presence of the phosphodiesterase inhibitor Ro 20-1724 (5 μM) and the reaction was quickly terminated by three repeated cycles of freezing and thawing. The samples were then centrifuged at $2500 \times g$ for 5 min at 4°C. The cyclic AMP assay is based on the competition between [^{3}H]-labelled cyclic AMP and unlabelled cyclic AMP present in the sample for binding to a crude cyclic AMP-binding protein prepared from bovine adrenal cortex according to the method of Brown *et al*. (1971). Each sample was incubated with 50 μl of [^{3}H]-labelled cyclic AMP (5 mCi) and 100 μl of binding protein for 2 h at 4°C. Separation of the protein-bound cyclic AMP from the unbound cyclic AMP was achieved by adsorption of the free cyclic AMP onto charcoal (100 μl) followed by centrifugation at $12{,}000 \times g$ at 4°C. The 200 μl of supernatant were then placed into an Eppendorf tube containing 1.2 ml of scintillation cocktail to measure the radioactivity. The cyclic AMP concentration in the sample was determined based on a standard curve and expressed as pmol per number of cells.

Analysis of data

All quantitative data are expressed as mean $\pm$ s.e.mean. Comparison between two groups was analysed using Student's unpaired *t*-test, and comparison among more than two groups was carried out using one-way analysis of variance (ANOVA). Differences were considered to be significant when the degree of confidence in the significance was 95% or better ($P<0.05$). Concentration-response curves for agonist were made in the presence of 3–4 different concentrations of antagonists. Allfit program was used to obtain the EC_{50} from the individual experiments (De Lean *et al*., 1978).

Materials

ATP, dATP, UTP, 3′-O-(4-benzoyl)benzoyl ATP (BzATP), thapsigargin, sulfinpyrazone, EGTA, EDTA, Trizma base, trichloroacetic acid, PMA, 4-α-PMA, cyclic AMP, and IP_3

were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{3}H]-IP_3 and [^{3}H]-cyclic AMP were obtained from NEN Life Science Products (Boston, MA, U.S.A.). GF 109203X, U-73122, and Ro 20-1724 were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) was from Tocris Cookson Ltd. (Avonmouth, Bristol, U.K.). Fura-2 pentaacetoxymethylester (fura-2/AM) was purchased from Molecular Probes (Eugene, OR, U.S.A.). SQ 22536 was from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA, U.S.A.).

Results

$P2Y_{11}$ receptor-mediated intracellular Ca^{2+} mobilization

Since we had previously shown, using HL-60 promyelocytes, that BzATP activated nonselective cation channels (Song *et al*., 1997) and that ATP and UTP activated the $P2Y_2$ receptors (Lee *et al*., 1997), we used dATP as the selective agonist of $P2Y_{11}$ receptors in the present study. dATP had no effect on the $[Ca^{2+}]_i$ up to a 100 μM concentration in endogenously $P2Y_2$ receptor-expressing cell lines including PC12 (Suh *et al*., 1995) and mouse pineal tumour PGT-β (Suh *et al*., 1997) cells, while UTP increased the $[Ca^{2+}]_i$ in the above cell lines with an EC_{50} of approximately 25 and 13 μM, respectively (data not shown). Thus, dATP seems not to activate the $P2Y_2$ receptor at concentrations of up to at least 100 μM. Using lower concentrations of dATP, we investigated the coupling between $P2Y_{11}$ receptors and phospholipase C or adenylyl cyclase in HL-60 promyelocytes. Figure 1A shows that stimulation of the cells with 30 μM dATP in the presence of 2.2 mM extracellular $CaCl_2$ caused an elevation in the $[Ca^{2+}]_i$ which was followed by a sustained phase lasting up to more than 30 min. In contrast, although the activation of $P2Y_2$ receptors by treatment with 30 μM UTP resulted in a comparable elevation in $[Ca^{2+}]_i$, this peak rapidly declined to the basal levels within 5 min. Pretreatment of the cells with PMA (30 nM) inhibited the dATP-stimulated Ca^{2+} response, while it had little effect on the UTP-stimulated response (Figure 1A, dotted traces). In the absence of extracellular Ca^{2+}, the dATP-induced Ca^{2+} mobilization returned also more slowly to the basal level than the UTP-induced one (Figure 1B, solid traces). Also, PMA (30 nM) significantly inhibited the dATP-induced Ca^{2+} mobilization, but only slightly decreased the UTP response (Figure 1B, dotted traces). Figure 1C shows that dATP elicited the rise in $[Ca^{2+}]_i$ in a concentration-dependent manner with an EC_{50} of 4.2 ± 0.5 μM. However, preincubation with 30 nM PMA significantly decreased the dATP-stimulated $[Ca^{2+}]_i$ rise. The concentration-dependence curve for PMA shows that the 30 μM dATP-induced response was inhibited by PMA treatment by maximally $\sim$90% at 1 μM PMA, while the UTP-induced response was barely inhibited.

To investigate whether P2X receptors were involved in the dATP- and UTP-stimulated $[Ca^{2+}]_i$ rise, the antagonism by the general P2X receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) on the nucleotide's responses were examined. When we pretreated the cells with 10 μM of PPADS for 5 min, both the dATP- and UTP-stimulated $[Ca^{2+}]_i$ rises were not effected (Figure 2A). Concentration-dependence also showed that up to 30 μM PPADS had little effect on the nucleotide-mediated Ca^{2+} mobilization. Thus the results indicate that the dATP- and UTP-induced $[Ca^{2+}]_i$ rise is not mediated through any of P2X subtypes.

Effect of PMA on $P2Y_{11}$ receptor-mediated IP_3 generation

We performed a similar analysis with regard to the effect of the nucleotide agonists on IP_3 generation. Stimulation of the cells with dATP resulted in IP_3 generation with the peak obtained within 15 s after stimulation, and the effect was maintained for more than 30 min. On the other side, the UTP-induced IP_3 generation was rapidly desensitized within 5 min (Figure 3A). Figure 3B shows that dATP caused IP_3 production in a concentration-dependent manner, the half maximal response being obtained at 18 ± 3 μM. However, PMA treatment (30 nM) significantly inhibited the dATP-stimulated IP_3 generation. Pretreatment of the cells with PMA inhibited dATP (30 μM)-induced IP_3 production in a concentration-dependent manner, whereas PMA had little effect on the UTP-induced response (Figure 3C). The results thus suggest that the signal transduction pathway from the $P2Y_{11}$ receptors to phospholipase C is specifically inhibited by activation of protein kinase C.

Effect of PMA on $P2Y_{11}$ receptor-mediated cyclic AMP generation

We also examined the PMA effect on $P2Y_{11}$ receptor-mediated adenylyl cyclase activation. The cyclic AMP accumulation induced by dATP occurred as a slow and sustained increase in intracellular cyclic AMP level as shown in the time-course data obtained with 100 μM dATP treatment. A significant increase in cyclic AMP was detectable 5 min after stimulation and the saturation of cyclic AMP production was reached about 20 min after stimulation (Figure 4A). UTP had little effect on the cyclic AMP generation. The dATP-stimulated increase in the cyclic AMP level occurred in a concentration-dependent manner with an EC_{50} seen at 23 ± 7 μM dATP (Figure 4B). Interestingly, co-stimulation of the cells with PMA (30 nM) and dATP (100 μM) synergistically enhanced the dATP-induced cyclic AMP generation $\sim 2-3$ fold at various dATP concentrations. Treatment with PMA 5 min before dATP stimulation also showed comparable cyclic AMP generation to that of simultaneous stimulation with PMA plus dATP (Figure 4B, inset), which might be due to the PMA's sustained stimulatory effect on the activation of adenylyl cyclase. The stimulatory effect of PMA was concentration-dependent, and maximum enhancement was obtained at a concentration of over 100 nM PMA (Figure 4C). However, inactive phorbol ester 4-α-PMA did not effect cyclic AMP accumulation. BzATP, ATP, ATPγS, and 2-MeSATP all elevated cyclic AMP production, and co-treatment with PMA increased the effect about 2–3 fold (Figure 4D). The results thus indicated that the $P2Y_{11}$ receptor-mediated adenylyl cyclase activation was synergistically potentiated by protein kinase C activation.

Synergistic activation of adenylyl cyclase

In order to further analyse the stimulatory effect of protein kinase C on adenylyl cyclase activity, we treated the cells with forskolin, which directly activates adenylyl cyclase, and then assessed the cyclic AMP production in the presence or absence of PMA. Higher effective concentration (100 nM) of PMA itself slightly elevated the cyclic AMP production (Table 1). In addition, PMA treatment enhanced cyclic AMP levels when adenylyl cyclase was activated directly by forskolin. This result suggests that protein kinase C could activate the adenylyl cyclase and potentiate the activity of adenylyl cyclase, even when it was already stimulated. This phenomenon was also seen in the cells in which G_s protein had been activated by

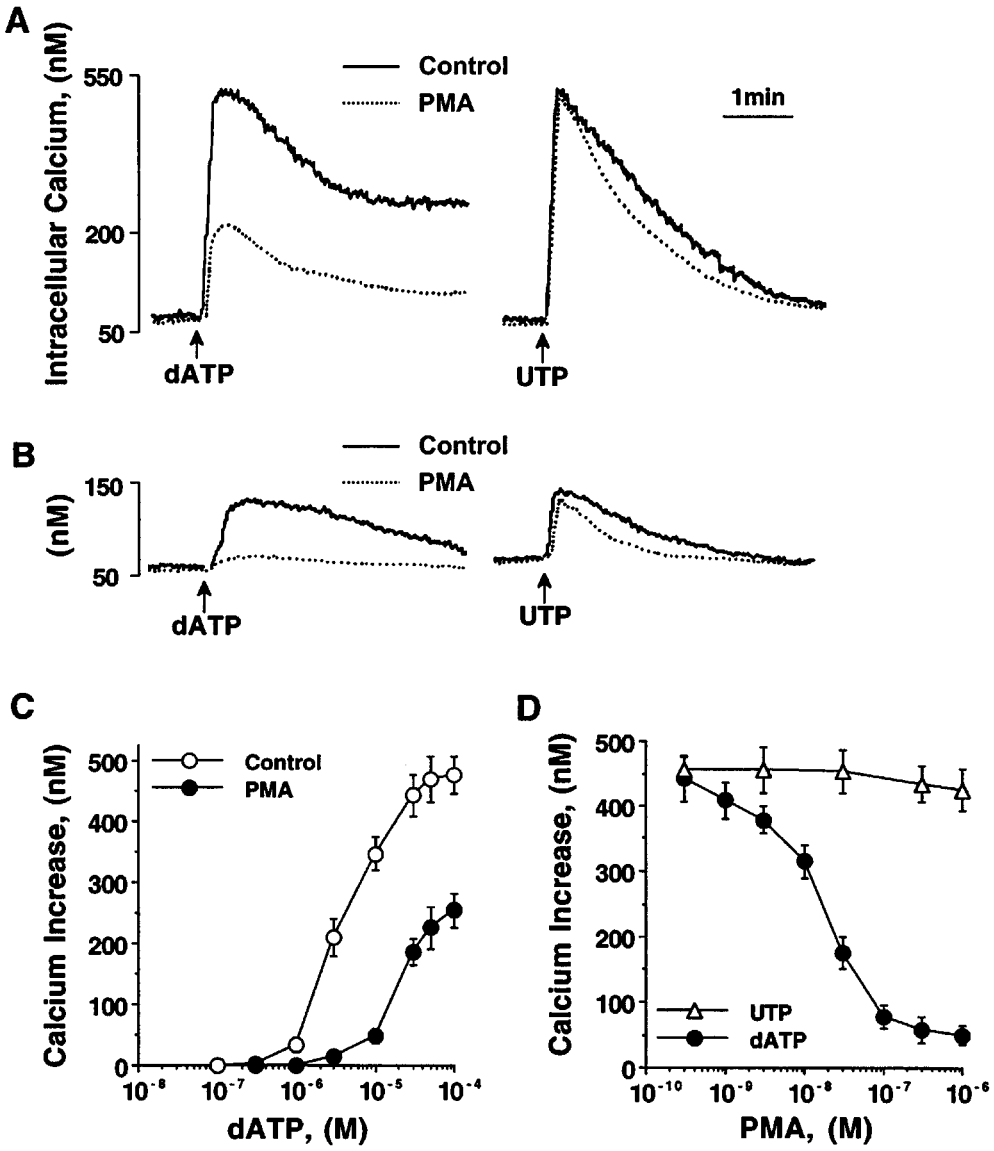

Figure 1 Effects of dATP and UTP on the $[Ca^{2+}]_i$ rise in HL-60 cells. Typical patterns of $[Ca^{2+}]_i$ rise in cells pretreated with vehicle (control) or 30 nM PMA for 5 min and obtained upon treatment with dATP (30 μM) and UTP (30 μM) in the presence (A) or absence (B) of 2.2 mM extracellular Ca^{2+}. (C) Concentration-dependent effect of dATP on $[Ca^{2+}]_i$ rise. Fura-2/AM-loaded cells were treated with vehicle (control) or 30 nM PMA for 5 min and then stimulated with various concentrations of dATP. Net increases in $[Ca^{2+}]_i$ were measured as described under Methods. (D) Concentration-dependent effect of PMA on dATP- and UTP-induced $[Ca^{2+}]_i$ rise. The cells, pretreated with various concentrations of PMA for 5 min, were stimulated with 30 μM dATP or UTP, and net increases in $[Ca^{2+}]_i$ were measured. The experiments were done three to five times and each point is the mean $\pm$ s.e.mean.

cholera toxin. Incubation of the cells with 2 μg ml^{-1} cholera toxin for 30 min increased cyclic AMP levels $\sim$3 fold. Co-treatment of the cells with PMA and cholera toxin enhanced this response. Furthermore, simultaneous treatment of the cells with 100 μM dATP and 10 μM forskolin resulted in an enhancement of the cyclic AMP accumulation. Taken together, these results demonstrate that the enhancement of the dATP-activated cyclic AMP accumulation by protein kinase C results from the potentiation of adenylyl cyclase activity, not the receptor activity.

Independent signalling pathways to phospholipase C and adenylyl cyclase from the P2Y$_{11}$ receptor

To investigate the interaction between the two dATP-mediated signalling pathways, we examined the effects of selective inhibitors of phospholipase C or adenylyl cyclase on each of the two pathways in HL-60 cells. Pretreatment of the cells with the phospholipase C inhibitor U-73122 inhibited the dATP-induced $[Ca^{2+}]_i$ rise (Figure 5A, inset). This occurred in a concentration-dependent manner with the maximum effect and half maximal concentration (IC_{50}) seen at approximately 15 and 2.6 μM U-73122, respectively (Figure 5A). As shown in Figure 5B, 10 μM U-73122 caused $\sim$95% decrease in the dATP-induced production of IP_3. However, the U-73122 treatment did not inhibit the dATP-induced cyclic AMP production (Figure 5C), indicating that blockage of the P2Y$_{11}$-mediated phospholipase C activation did not affect adenylyl cyclase activation. On the other hand, treatment of the cells with the adenylyl cyclase inhibitor SQ 22536 revealed selective inhibition of cyclic AMP generation (Figure 6A). Pretreatment of the cells with SQ 22536 inhibited the P2Y$_{11}$-stimulated adenylyl cyclase activation in a concentration-dependent manner with an IC_{50} at approximately 10 μM. However, the treatment did not affect the receptor-mediated IP_3

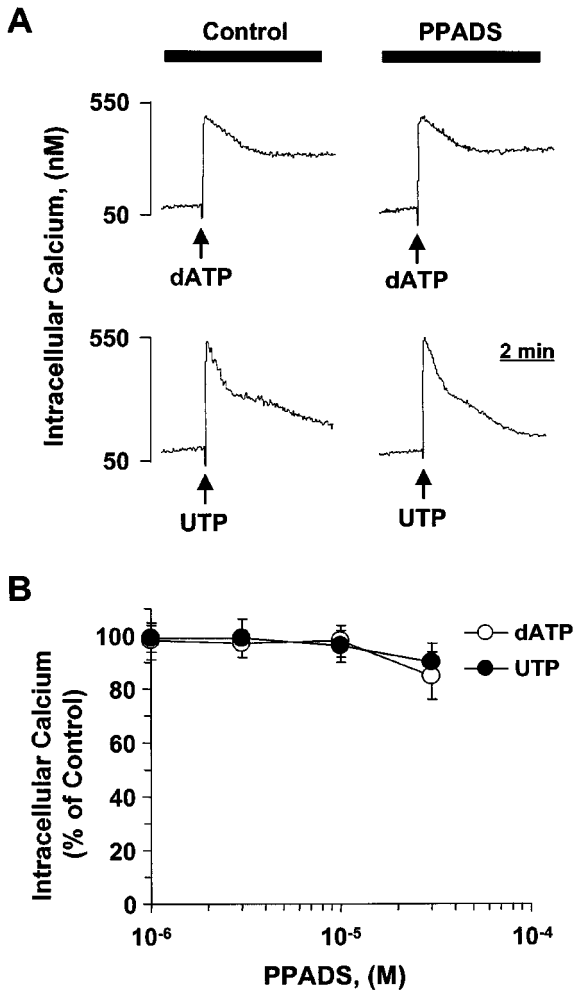

Figure 2 Effect of PPADS on dATP- and UTP-stimulated intracellular $[Ca^{2+}]_i$ rise. (A) Fura-2-loaded cells were incubated with vehicle (control) or 10 μM PPADS for 5 min and then treated with 100 μM dATP or UTP. Typical patterns of $[Ca^{2+}]_i$ rise were presented. (B) Concentration-dependent effect of PPADS on dATP- and UTP-stimulated $[Ca^{2+}]_i$ rise. Fura-2/AM-loaded cells were treated with various concentrations of PPADS, and then stimulated with 100 μM dATP or UTP. Net increases in $[Ca^{2+}]_i$ were presented as percentage of control (dATP or UTP alone). The experiments were done three times and each point is the mean$\pm$s.e.mean.

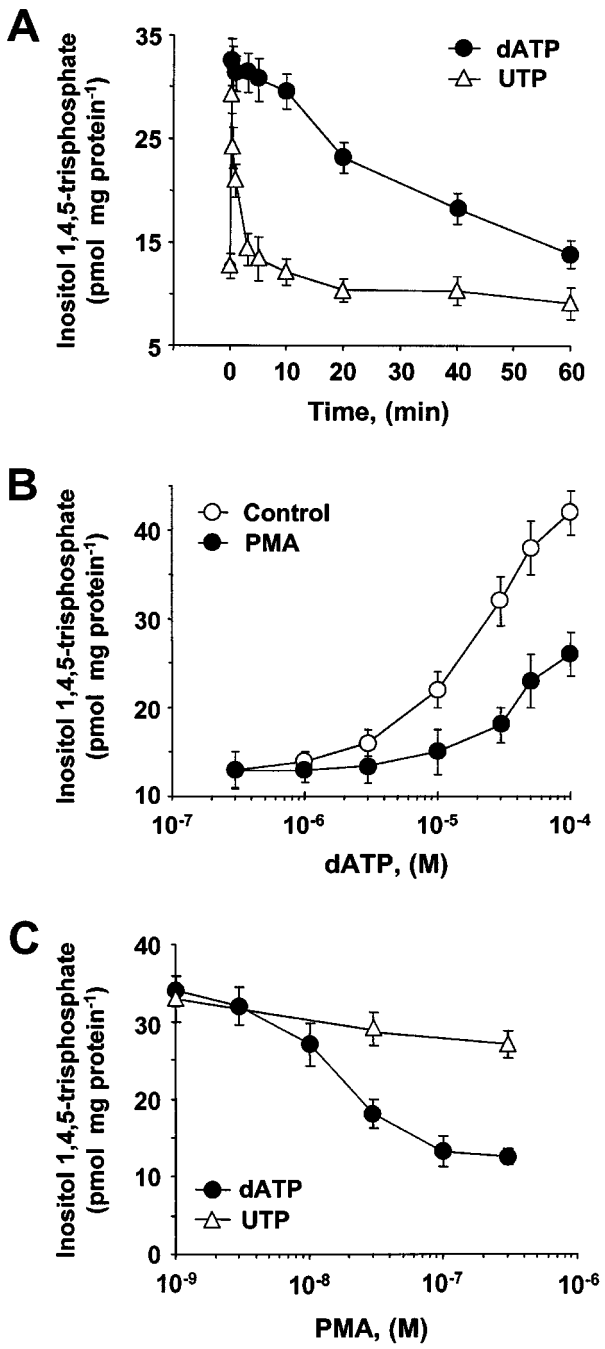

Figure 3 Effect of dATP and UTP on IP_3 production in HL-60 cells. (A) Time course of IP_3 generation evoked by dATP or UTP. The cells were stimulated with 100 μM dATP or UTP for the designated times (0, 0.25, 0.5, 1, 3, 5, 10, 20, 40, 60 min), and the reactions were stopped by addition of 15% (w v^{-1}) TCA containing 10 mM EGTA. (B) Concentration-dependent stimulation of IP_3 formation by dATP. The cells, pretreated with vehicle (control) or 30 nM PMA for 5 min, were treated with various concentrations of dATP for 15 s, and the IP_3 production was measured by competition assay as described under Methods. (C) Concentration-dependent effect of PMA on dATP- and UTP-stimulated IP_3 generation. The cells were pretreated with various concentrations of PMA for 5 min and then stimulated with 30 μM dATP or UTP for 15 s. IP_3 generation was measured as described under Methods. The experiments were done three times and each point is the mean$\pm$s.e.mean.

generation (Figure 6B). Therefore, the results indicate that P2Y$_{11}$ receptor-mediated activation of each enzyme occurs through independent signal transduction pathways.

Discussion

The P2Y$_{11}$ receptor is a P2 receptor which displays high selectivity for purine nucleotides (Communi *et al*., 1997) and which is endogenously expressed by HL-60 promyelocytes (Choi & Kim 1997; Jiang *et al*., 1997). We characterized the P2Y$_{11}$ receptor-mediated signals, the regulatory effect of protein kinase C on the receptor-induced signal transduction, and possible interactions between the receptor-mediated signalling pathways to phospholipase C and adenylyl cyclase in these cells. First, the intracellular Ca^{2+} mobilization induced by dATP stimulation of the P2Y$_{11}$ receptor was characterized by a relatively slower activity in reaching the peak and a longer duration of maintaining the elevated level in comparison to the P2Y$_2$ receptor. The sustained elevated $[Ca^{2+}]_i$ above basal level was maintained for more than 30 min in the presence of extracellular Ca^{2+}. This required the continuous presence of dATP in the stimulating solution, since the $[Ca^{2+}]_i$ rapidly dropped to the basal level following wash-out (data not shown). The slow decrease in the $[Ca^{2+}]_i$ observed after the

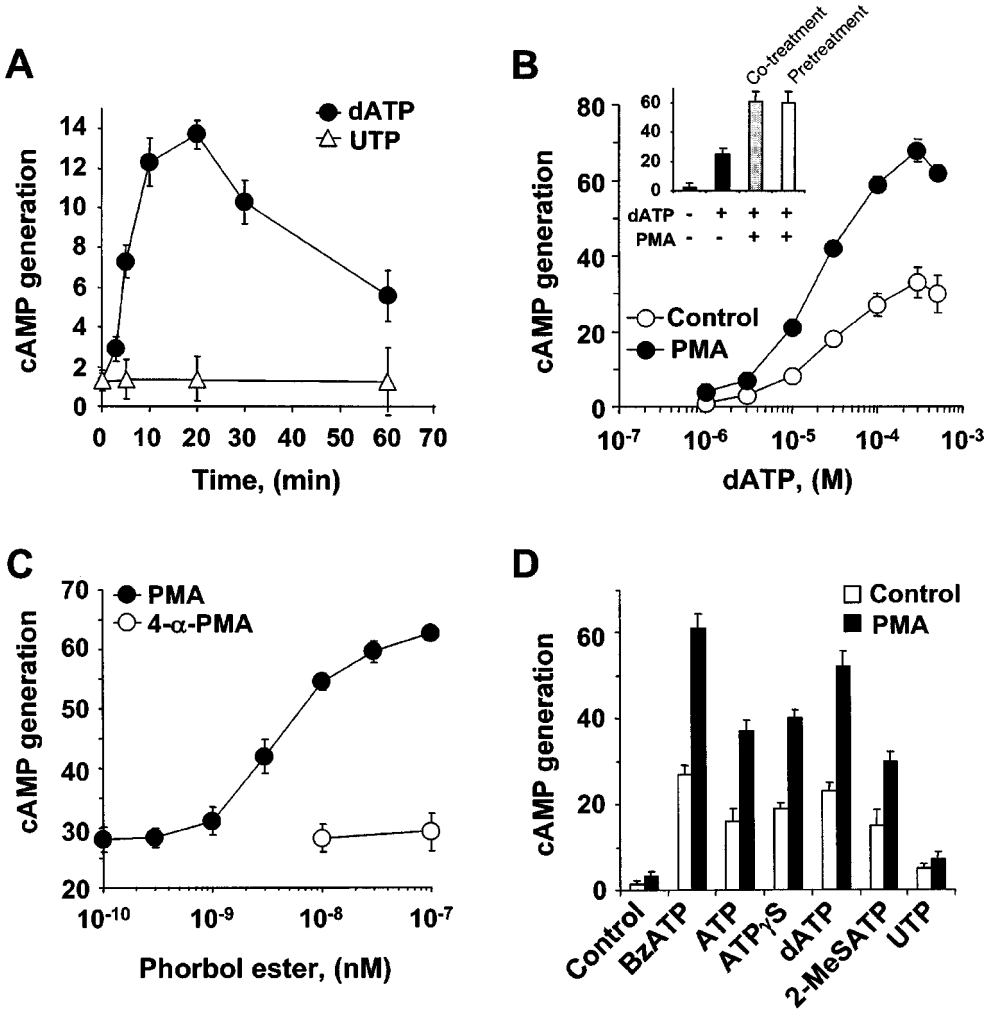

Figure 4 Effects of dATP and UTP on cyclic AMP generation in HL-60 cells. (A) Time-dependent changes in intracellular cyclic AMP levels stimulated by 100 μM dATP or UTP. The cells were incubated in 50 μM Ro 20-1724 for 20 min and then stimulated with dATP or UTP for the indicated times. The cyclic AMP accumulation was measured as described under Methods. (B) Concentration-dependence of dATP-stimulated cyclic AMP generation. The cells were treated with vehicle (control) or 30 nM PMA for 5 min and then stimulated with various concentrations of dATP for 20 min. (Inset) Cells were simultaneously stimulated with dATP (100 μM) and PMA (30 nM) or pretreated with PMA for 5 min and then stimulated with dATP, and then cyclic AMP production was measured. (C) Concentration-dependent effect of PMA on dATP-stimulated cyclic AMP generation. The cells were treated with 100 μM dATP and various concentrations of PMA or 4-α-PMA for 20 min. (D) PMA effect on cyclic AMP generation in HL-60 cells stimulated by various nucleotide analogues. The cells were treated with the analogues for 20 min in the presence or absence of 30 nM PMA, and the cyclic AMP levels were measured as described under Methods. Each point is the mean$\pm$s.e.mean of 3–5 experiments.

Table 1 The effect of PMA on cylic AMP generation

	Cylic AMP accumulation (pmol 10^6 cells^{-1})		
Treatment	*Control*	*Forskolin*	*Cholera toxin*
Basal	1.3$\pm$0.2	3.1$\pm$1.7	3.7$\pm$1.6
PMA	2.8$\pm$1.5	15.8$\pm$2.4	18.9$\pm$2.6
dATP	26.7$\pm$2.1	52.3$\pm$3.2	38.5$\pm$4.2

HL-60 cells preincubated with 50 μM Ro 20-1724 for 15 min were further treated with 10 μM forskolin or 2 μg ml^{-1} cholera toxin for 30 min in the presence or absence of 100 nM PMA and 100 μM dATP. The cylic AMP generation was measured as described in Methods and the data are expressed as mean$\pm$s.e.mean.

maximum rise seems to be due to the sustained elevation of IP_3 production in the presence of dATP; it was maintained above basal level for at least 1 h after stimulation in the absence of lithium (Figure 3A). This time-course of $[Ca^{2+}]_i$ rise and IP_3 generation is very different from the biphasic stimulation of the $P2Y_2$ receptor with UTP where the maximal effect was obtained within 15 s and was followed by a rapid decrease to basal level within 5 min. These results led us to conclude that the $P2Y_2$ receptor is being rapidly desensitized, whereas the $P2Y_{11}$ receptor is not. Although slow desensitization of P2 receptors has been observed for $P2Y_6$ and $P2Y_{11}$ receptors transiently expressed in 1321N1 cells (Robaye *et al.*, 1997; Communi *et al.*, 1999), it had not been documented that the continuous presence of the agonist was needed to maintain the sustained $[Ca^{2+}]_i$ elevation. Our present study is the first to demonstrate the effect of $P2Y_{11}$ receptor activation on $[Ca^{2+}]_i$ rise and to suggest distinct and characteristic time-courses for $P2Y_2$ and $P2Y_{11}$ receptor activation in HL-60 cells endogenously expressing these receptors.

The regulatory enzyme protein kinase C has attracted considerable interest due to its implication in a wide variety of cellular processes including extracellular signal transduction and tumour promotion. In our study, we found that protein kinase C activation upon PMA treatment had differential effects on the $P2Y_{11}$ receptor-mediated signalling pathways, namely potentiation of $P2Y_{11}$ receptor-induced cyclic AMP

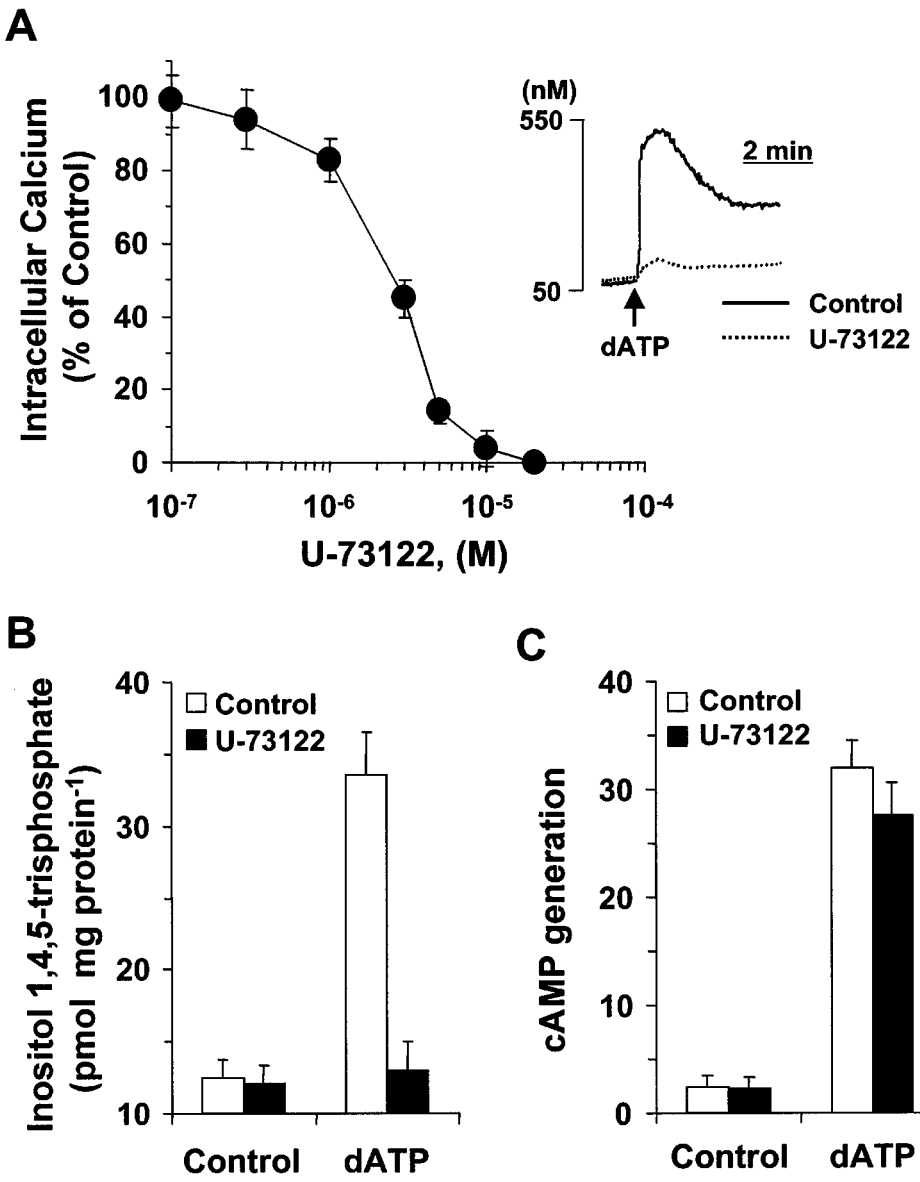

Figure 5 Effect of U-73122 on P2Y$_{11}$ receptor-mediated signalling in HL-60 cells. (A) Concentration-dependent inhibition of dATP-induced $[Ca^{2+}]_i$ rise by U-73122. Fura-2/AM-loaded cells were treated with various concentrations of U-73122 for 10 min and then stimulated with 100 μM dATP, and the peak $[Ca^{2+}]_i$ level was measured. (Inset) Typical pattern of $[Ca^{2+}]_i$ rise after treatment with dATP in the absence or presence of 10 μM U-73122. (B) Effect of U-73122 on dATP-stimulated IP_3 generation. The cells, pretreated with vehicle (control) or 10 μM U-73122 for 10 min, were stimulated with 100 μM dATP for 15 s, and the IP_3 generation was measured as described under Methods. (C) Effect of U-73122 on dATP-induced cyclic AMP generation. The cells, pretreated with vehicle (control) or 10 μM U-73122 for 10 min, were stimulated with 100 μM dATP for 20 min, and the cyclic AMP accumulation was measured as described under Methods. The experiments were done three times and each point is the mean $\pm$ s.e.mean.

production and inhibition of the receptor-mediated phosphoinositide turnover. Based on the ability of PMA to potentiate the forskolin- or cholera toxin-stimulated cyclic AMP accumulation and the finding that dATP also potentiated the forskolin-stimulated cyclic AMP production, we concluded that the site of action of protein kinase C in enhancing cyclic AMP generation is not the P2Y$_{11}$ receptor but rather the adenylyl cyclase directly. Many previous studies have shown that the activity of adenylyl cyclase is synergistically potentiated by protein kinase C activation when the adenylyl cyclase has already been activated by various stimuli (Jacobowitz & Iyengar, 1994; Yoshimura & Cooper, 1993). It has been shown that protein kinase C phosphorylation of adenylyl cyclase occurred in an isoenzyme-specific manner (Kawabe *et al*., 1994) and that this phosphorylation by protein kinase C was accompanied by a potentiation of adenylyl cyclase activity (Yoshimasa *et al*., 1987). Although the expression pattern of adenylyl cyclase isoenzymes in HL-60 cells is still unknown, the cyclic AMP elevating effect of PMA is consistent with that observed in embryonic kidney 293 cells transiently transfected with type II adenylyl cyclase (Yoshimura & Cooper, 1993) or in S49 mouse lymphoma cells with type VII adenylyl cyclase (Watson *et al*., 1994). Phorbol esters increased forskolin-stimulated adenylyl cyclase activity in these cells as well.

The inhibition of receptor-stimulated phospholipase C activation by PMA appears to be a general property of G protein-coupled phospholipase C signal transduction systems. Extracellular nucleotide-induced Ca^{2+} mobilization has been shown previously to be inhibited by phorbol esters in hepatocytes (Charest *et al*., 1985), fibroblasts (Fine *et al*.,

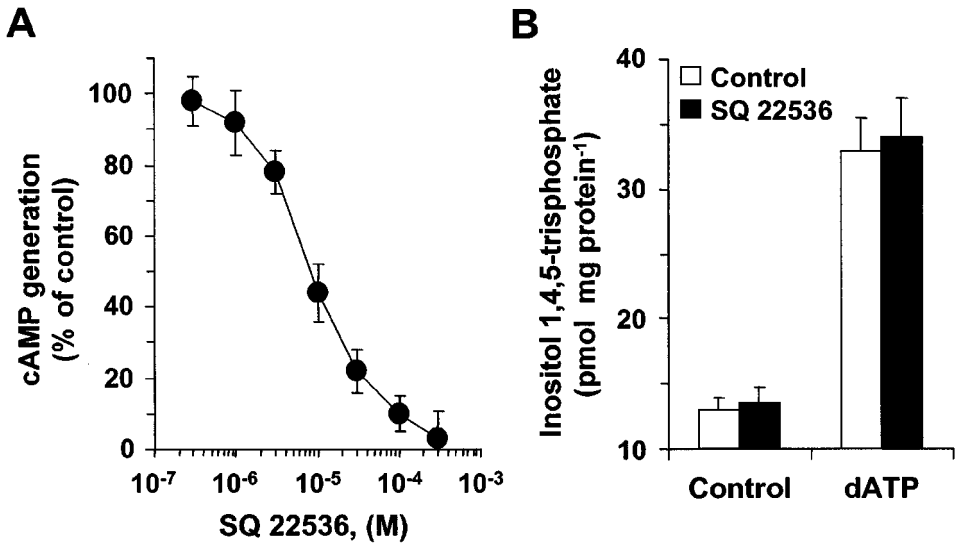

Figure 6 Effect of SQ 22536 on dATP-stimulated signalling pathways in HL-60 cells. (A) Concentration-dependent inhibition of the dATP-induced cyclic AMP production by SQ 22536. Fura-2/AM-loaded cells were treated with various concentrations of SQ 22536 for 10 min and then stimulated with 100 μM dATP, and cyclic AMP accumulation was measured. (B) Effect of SQ 22536 on dATP-stimulated IP_3 generation. The cells, pretreated with vehicle (control) or 100 μM SQ 22536 for 10 min, were stimulated with 100 μM dATP for 15 s, and the IP_3 generation was measured as described under Methods. The experiments were done three times and each point is the mean $\pm$ s.e.mean.

1989), and endothelial cells (Communi *et al*., 1995). In HL-60 cells, the activation of P2 purinergic receptors is also significantly attenuated upon protein kinase C activation. Previous studies have suggested that protein kinase C may act on the coupling between receptor and G-protein or the coupling between G-protein and phospholipase C or the phospholipase C isoenzyme (Orellana *et al*., 1987; Ali *et al*., 1997). However, the differences in the PMA effect between dATP- and UTP-stimulated responses suggest that the action site of protein kinase C is not on the phospholipase C. At present, the mechanism of inhibition is not well understood, but as with many G protein-coupled seven transmembrane receptors, it may involve phosphorylation of the intracellular regions of the receptor which thus could uncouple the receptor from the $G_{q/11}$ proteins.

The results of the present study also show that P2Y$_{11}$ and P2Y$_2$ purinoceptors display differential sensitivity to protein kinase C activation. The effect of dATP on the $[Ca^{2+}]_i$ rise was more strongly and more rapidly inhibited by PMA than was the UTP effect, when the extent of inhibition was measured at agonist concentrations that produced a comparable increase in $[Ca^{2+}]_i$. Previously, our studies with mouse pineal gland cells had also revealed that the UTP response through P2Y$_2$ was only slightly affected by PMA treatment, while the ADPβS elicited response to P2Y$_1$ receptor activation was dramatically inhibited (Suh *et al*., 1997). In addition, in bovine aortic endothelial cells, activation of protein kinase C profoundly inhibited IP_3 production upon P2Y receptor activation with 2-MeSATP and ADP, whereas it had no effect on the response to P2Y$_2$ receptor activation with UTP (Purkiss *et al*., 1994; Wilkinson *et al*., 1994). However, in bovine pulmonary artery endothelium, protein kinase C activation by PMA resulted in a negative feedback regulation of both the P2Y$_1$ and P2Y$_2$ receptor-mediated phosphoinositide turnover (Chen & Lin, 1999). This indicates that purine- and pyrimidine-selective purinoceptors might be differentially modulated by protein kinase C in different cell systems.

The interaction of the signal flows from the P2Y$_{11}$ receptor to phospholipase C and adenylyl cyclase was studied using selective inhibitors for each effector enzyme. Our results show no evidence of interactions between P2Y$_{11}$ receptor signalling pathways, since blockage of phospholipase C activation by U-73122 did not affect the receptor-mediated adenylyl cyclase activation, and the blockade of adenylyl cyclase by SQ 22536 did not affect the receptor-mediated phospholipase C activation. This may be the result of the different G-proteins coupled to the receptors. Previous studies reported that P2Y$_{11}$ receptors were coupled to phospholipase C through pertussis toxin-insensitive G proteins, while the receptor seemed to be coupled to adenylyl cyclase through G_s proteins (Choi & Kim, 1997; Communi *et al*., 1999). dATP-stimulated IP_3 generation and cyclic AMP production had similar concentration-response curves in HL-60 cells, suggesting that the two responses were contributed to by a single type of receptor. However, the $[Ca^{2+}]_i$ rise induced by dATP occurred at lower concentrations of dATP than the rise in IP_3 generation. But this could be the result of rapid and efficient Ca^{2+} mobilization even at lower concentrations of IP_3.

In conclusion, dATP induces phosphoinositide turnover, Ca^{2+} mobilization, and cyclic AMP production in HL-60 cells by activating purine selective P2Y$_{11}$ receptors. The present results are particularly significant insofar as they show that the regulatory mode of P2Y$_{11}$ receptors can differ and the P2Y$_{11}$ receptor-mediated activation of the phospholipase C and the adenylyl cyclase system can be independently evoked. Our results also support the conclusion that the P2Y$_{11}$ receptor-mediated Ca^{2+} mobilization is slowly desensitized as is the IP_3 generation. The pharmacological data also clearly show that the P2Y$_{11}$ and the P2Y$_2$ receptors are co-expressed but differentially regulated by protein kinase C in HL-60 cells.

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