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Biphasic and differential modulation of Ca^{2+} entry by ATP and UTP in promyelocytic leukaemia HL60 cells

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ATP and UTP cause mobilization of Ca^{2+} from the intracellular stores with similar potency in several cell types including both undifferentiated and differentiated HL60 cells. We show here that, in HL60 cells with Ca^{2+} stores that had been fully and irreversibly emptied using the endomembrane Ca^{2+}-ATPase inhibitor thapsigargin, both nucleotides produced a biphasic effect on Ca^{2+} entry, first rapid inhibition and then delayed (about 15 s) activation. ATP was more effective at producing the initial inhibition of Ca^{2+} entry, whereas UTP was more effective at activating the delayed Ca^{2+} entry. Previous incubation with UTP desensitized the Ca^{2+} mobilization and the delayed activation of Ca^{2+} entry induced by ATP but not the inhibition of Ca^{2+} entry. The ATP analogue 2-methylthioATP (2-MeSATP) barely mobilized stored Ca^{2+} but inhibited Ca^{2+} entry. These results could be explained by the presence of two receptors: (i) a P_{2u} receptor sensitive to ATP and UTP, responsible for activation of phospholipase C and Ca^{2+} mobilization, early inhibition of Ca^{2+} entry and delayed activation of Ca^{2+} entry and (ii) a P_{2y}-like receptor sensitive to ATP and 2-MeSATP which produces only inhibition of Ca^{2+} entry. The inhibition of Ca^{2+} entry by nucleotides increased greatly during differentiation. Given that Ca^{2+} mobilization by nucleotides is not modified by differentiation, this suggests that a component of the mechanism of inhibition of Ca^{2+} entry is gradually expressed during differentiation of HL60 cells.

INTRODUCTION

Extracellular ATP is known to activate the inositol–phospholipid signalling cascade in both undifferentiated (uHL60) and fully differentiated (dHL60) HL60 promyelocytic leukaemia cells [1,2]. This effect is mediated by plasma-membrane receptors for ATP showing a pattern of agonist selectivity clearly different from that of other subtypes of P_2 purinergic receptors [3–6]. The most characteristic pharmacological property of these ATP receptors is the fact that the pyrimidine nucleotide UTP shows similar potency to ATP [3–6]. Adenosine 5′-[γ-thio]triphosphate (ATP[S]) is also a potent agonist but 2-methylthioATP (2-MeSATP) (a P_{2y} agonist) and adenosine 5′-[α,β-methylene]-triphosphate (pp[CH_2]pA (a P_{2x} agonist) show little or no activity [3–7]. Nucleotide receptors showing activation with similar potency with ATP and UTP have been described in many other cell types [3–6]. This has led recently to their classification as a separate subtype of P_2 purinergic receptors named P_{2u} [3–6], or they are referred to simply as 'nucleotide' receptors [5,6] because they are not specific for purines. Alternatively, it has been proposed that UTP could bind to a separate pyrimidinoceptor [8,9]. In fact, recent ligand-binding studies in human neutrophils using [^{35}S]ATP[S] have shown that a good correlation exists between functional response and ligand displacement for ATP and ATP[S], but not for UTP, which has no affinity for the binding site [10,11]. However, in human neutrophils ATP[S] desensitizes the cells to further Ca^{2+} mobilization induced by both UTP and ATP, but not to the Ca^{2+} mobilization from the intracellular stores induced by other agonists such as chemotactic peptide [7]. More recently, moreover, a mouse neuroblastoma P_{2u} receptor has been cloned and expressed in *Xenopus* oocytes, demonstrating that a single gene product is able to respond to both ATP and UTP causing Ca^{2+} mobilization [12]. This receptor is probably very similar to the ATP- and UTP-sensitive one expressed after injection into *Xenopus* oocytes of fractionated mRNA from HL60 cells [13].

We have shown that both uHL60 and dHL60 cells possess a Ca^{2+}-entry pathway that is activated by emptying the intracellular Ca^{2+} stores [14], a mechanism first proposed by Putney [15,16] (capacitative Ca^{2+} entry). This Ca^{2+}-entry mechanism [referred to in this paper as store-operated Ca^{2+} pathway (SOCP)] is activated when the Ca^{2+} stores are emptied by either agonists or thapsigargin, an inhibitor of the endomembrane Ca^{2+}-ATPase [14,17]. We have also shown that both the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol dibutyrate (PDBu) transiently inhibit this Ca^{2+}-entry pathway via a mechanism mediated by phosphorylation, in both human neutrophils and dHL60 cells [17–19]. No inhibition by either fMLP or PDBu occurred in uHL60 cells but developed gradually during differentiation. The lack of effect of fMLP in uHL60 cells could be attributed to the lack of high-affinity fMLP receptors in these cells [20]. However, the lack of effect of PDBu suggests that either a necessary protein kinase C subtype or a component of the mechanism of inhibition was absent from uHL60 cells. We therefore decided to study the effect of nucleotides, the receptors of which are known to be already present in uHL60 cells [1,2], on the inhibition of SOCP during differentiation of these cells.

We show here that the transient $[Ca^{2+}]_i$ increase produced

Abbreviations used: uHL60 cells, undifferentiated HL60 cells; dHL60 cells, HL60 cells, differentiated for 7-days; $[Ca^{2+}]_i$, cytosolic free Ca^{2+}-concentration; ATP[S], adenosine 5′-[γ-thio]-triphosphate; 2-MeSATP, 2-methylthioadenosine 5′-triphosphate; pp[CH_2]pA, adenosine 5′-[α,β-methylene]triphosphate; fMLP *N*-formyl-methionyl-leucyl-phenylalanine; PDBu, phorbol 12,13-dibutyrate; SOCP, store-operated Ca^{2+} entry pathway; Me_2SO, dimethyl sulphoxide.

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by mobilization of stored Ca^{2+} on stimulation with ATP or UTP was almost indistinguishable in uHL60 and dHL60 cells. In contrast, the inhibition of Ca^{2+} entry induced by these nucleotides increased greatly during differentiation. In addition, a slowly developing activation of Ca^{2+} entry was observed once the inhibition was terminated. This Ca^{2+} entry was observed in cells with stores that had been previously emptied of Ca^{2+} by thapsigargin. This suggests that it may occur either via a pathway different from SOCP or by further activation of SOCP by a mechanism other than emptying the Ca^{2+} stores. Activation of Ca^{2+} entry was induced with much greater selectivity by UTP than by ATP. ATP, in contrast, showed greater selectivity for inhibition of SOCP.

MATERIALS AND METHODS

HL60 human leukaemia cells were obtained from Dr. Faustino Mollinedo, Centro de Investigaciones Biológicas del CSIC, Madrid, Spain. They were propagated and cultured as described elsewhere [21]. Differentiation to granulocytes was achieved by culture in medium containing 1.3% dimethyl sulphoxide (Me_2SO) [22] for 1–7 days.

All the $[Ca^{2+}]_i$ measurements were taken in suspensions of Fura-2-loaded cells. Cells were collected and washed twice with standard medium of the following composition (in mM): $CaCl_2$, 0.2; NaCl, 145; KCl, 5; $MgCl_2$, 1; glucose, 10; Hepes (sodium salt), 10, pH 7.4. They were then resuspended at 2% cytocrit in the same medium, and Fura-2 acetoxymethyl ester (Fura-2/AM; final concentration, 4 μM) was added from a 2 mM stock in Me_2SO. After 30–45 min at room temperature, the cells were washed twice with standard medium to remove extracellular Fura-2, resuspended at 1% cytocrit in the same medium, and stored at room temperature until used. Fluorescence was recorded in 0.5 ml portions of the cell suspension under magnetic stirring in a fluorescence spectrophotometer constructed by Cairn Research Ltd. (Newnham, Sittingbourne, Kent, U.K.), which allows quasisimultaneous (30–300 Hz) measurements of fluorescence excited at up to six different wavelengths. Fluorescence emitted was filtered using a high-pass 520 nm filter and the readings integrated at 1 s intervals. $[Ca^{2+}]_i$ was estimated from the ratio of fluorescence excited at 340 and 380 nm ($R_{340/380}$ [23]). Measurements were taken at 37 °C. Mn^{2+} uptake was followed by monitoring quenching of Fura-2 fluorescence excited at 360 nm, which is insensitive to variations in $[Ca^{2+}]_i$ [23].

Fura-2/AM was obtained from molecular Probes, Eugene, OR, U.S.A. fMLP, ATP, UTP, ATP[S], pp[CH_2]pA and staurosporin were from Sigma, Madrid, Spain. 2-MeSATP and pertussis toxin were obtained from Research Biochemicals International, Natick, MA, U.S.A. Thapsigargin and chelerythrine were purchased from Alomone Laboratories, Jerusalem, Israel. Ionomycin was from Calbiochem, La Jolla, CA, U.S.A. Other chemicals were obtained from either Sigma or Merck, Darmstadt, Germany.

Results are presented as means ± S.E.M. for the indicated number of experiments performed.

RESULTS

Figure 1(a) shows the effects of different concentrations of ATP and UTP on $[Ca^{2+}]_i$ in both uHL60 and dHL60 cells. No significant differences in the shape of the $[Ca^{2+}]_i$ peaks or the concentration-dependence for ATP and UTP were found. This is consistent with previously reported data showing similar Ca^{2+} mobilization by ATP in uHL60 and dHL60 cells [1,2] and equipotency of ATP and UTP in activating inositol trisphosphate production in uHL60 cells [24].

However, when the effects of ATP and UTP were tested in cells previously treated with thapsigargin (to empty the intracellular Ca^{2+} stores) a different picture emerged. Treatment with thapsigargin in Ca^{2+}-containing medium leads to an increase in $[Ca^{2+}]_i$ which reaches a steady state as a consequence of the balance between increased Ca^{2+} entry via SOCP and Ca^{2+} pumping by the plasma-membrane Ca^{2+}-ATPase [17–19]. Under these conditions, any inhibition or activation of Ca^{2+} entry will be seen as a decrease or increase respectively in $[Ca^{2+}]_i$. Figure 1(b) shows that nucleotides produced a double effect on $[Ca^{2+}]_i$ of thapsigargin-treated cells: a fast and transient decrease and a late and also transient increase which returns to near resting levels about 5 min after the addition of the agonist. The intensity of these two effects varied depending on cell differentiation, the nucleotide type and its concentration. In uHL60 cells, low ATP concentrations activated only the $[Ca^{2+}]_i$-increasing mechanism but concentrations above 5 μM developed a biphasic effect on $[Ca^{2+}]_i$. In contrast, UTP at any concentration selectively activated the $[Ca^{2+}]_i$-increasing mechanism. In dHL60 cells, the nucleotide-induced $[Ca^{2+}]_i$-decreasing mechanism was stronger. However, concentrations of ATP or UTP below 1 μM still activated only the $[Ca^{2+}]_i$ increase, which reached near-maximum activation at 1 μM. At higher concentrations the $[Ca^{2+}]_i$-decreasing mechanism was activated, maximum effects being obtained with concentrations around 20 μM of either nucleotide. At any concentration, ATP was more efficient than UTP at activating the initial $[Ca^{2+}]_i$ decrease, whereas UTP activated the late $[Ca^{2+}]_i$ increase more strongly than did ATP.

Figure 2 shows the development of both effects during differentiation. Development of the nucleotide-induced $[Ca^{2+}]_i$ decrease during differentiation is observed with both ATP and UTP, reaching maximum response after 4 days of differentiation. ATP produced a larger or longer $[Ca^{2+}]_i$ decrease than UTP at each stage of differentiation. Conversely, UTP produced a greater $[Ca^{2+}]_i$ increase than ATP at each stage of differentiation. On the other hand, the delayed $[Ca^{2+}]_i$ increase was already present in uHL60 cells and maximum response was seen after only 1 day of differentiation.

Cross-desensitization of a response to one agonist by previous addition of a different one is usually considered good proof that the two agonists act on the same receptor. Figure 3(a) shows that addition of UTP to uHL60 cells prevented any further Ca^{2+} mobilization induced by subsequent addition of ATP, which in fact produced instead a small $[Ca^{2+}]_i$ decrease. Vice versa, previous addition of ATP almost completely abolished Ca^{2+} mobilization induced by UTP. Similar results were obtained in dHL60 cells (results not shown). However, when the effects of the consecutive addition of the nucleotides were examined in thapsigargin-treated uHL60 cells (Figure 3b) or dHL60 cells (Figure 3c) the picture was different. UTP suppressed only the late $[Ca^{2+}]_i$ increase induced by the subsequent addition of ATP and not the ATP-induced $[Ca^{2+}]_i$ decrease. In fact, after UTP, ATP produced a permanent $[Ca^{2+}]_i$ decrease in dHL60 cells. On the other hand, the response to UTP almost completely disappeared when it was added after ATP. Therefore our results show that ATP and UTP desensitize each other selectively for the phenomena of Ca^{2+} mobilization and the delayed increase in $[Ca^{2+}]_i$ observed in thapsigargin-treated cells, but UTP is unable to desensitize the ATP-induced $[Ca^{2+}]_i$-decreasing mechanism.

Figure 4 shows on an expanded time scale the initial moments after the addition of either ATP or UTP. In dHL60 cells the activation by ATP of the $[Ca^{2+}]_i$-decreasing mechanism was fast. The delay between ATP addition and the first point with lower $[Ca^{2+}]_i$ was 1.2 ± 0.3 s ($n = 17$). For UTP, a longer delay of 3.6 ± 0.3 s ($n = 17$) was found. In uHL60 cells ATP produced

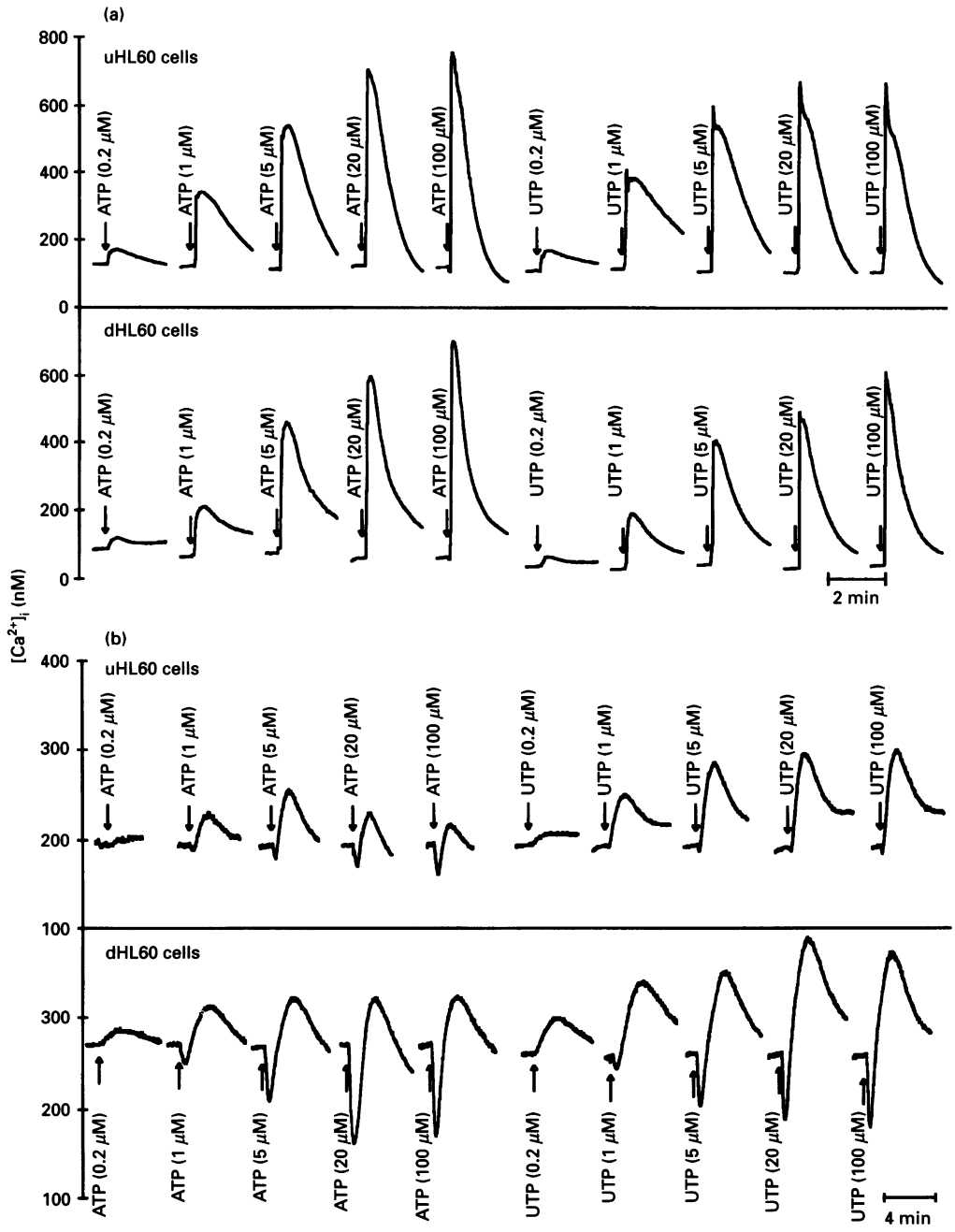

Figure 1 Effects of different concentrations of ATP and UTP on $[Ca^{2+}]_i$ of both uHL60 and dHL60 cells either untreated (a) or treated with thapsigargin (b)

In (**a**) Fura-2-loaded uHL60 and dHL60 cells were incubated in standard medium containing 1 mM Ca^{2+}. Then different concentrations of ATP and UTP were added, as indicated. In (**b**) Fura-2-loaded uHL60 and dHL60 cells suspended in standard medium containing 0.2 mM Ca^{2+} were treated with 0.5 μM thapsigargin for 10 min. This treatment leads to complete emptying of the intracellular Ca^{2+} stores and to an increase in $[Ca^{2+}]_i$ to about 200–300 nM as a result of stimulation of Ca^{2+} entry via SOCP. Then different concentrations of ATP and UTP were added, as indicated. The experiments shown are representative of three to six different ones of each type.

only a small biphasic effect, and UTP a delayed $[Ca^{2+}]_i$ increase which started 16 ± 1 s ($n = 13$) after UTP addition. Note that this increase in $[Ca^{2+}]_i$ is completely different from that occurring after Ca^{2+} mobilization from intracellular stores in cells not treated with thapsigargin. In that case, increase in $[Ca^{2+}]_i$ after the addition of the agonist shows no measurable delay and reaches maximum $[Ca^{2+}]_i$ in less than 5 s (result not shown).

The lack of an immediate $[Ca^{2+}]_i$ increase after UTP addition in Figure 4 (and see also Figures 1–3) indicates that the intracellular Ca^{2+} stores are completely empty in these thapsigargin-treated cells. In order to obtain further evidence on this critical point we have studied the effect of nucleotides in the presence of both thapsigargin and the Ca^{2+} ionophore ionomycin, to ensure complete emptying of the stores. Figure 5 shows that addition of 200 nM ionomycin to cells suspended in medium containing 0.2 mM Ca^{2+} produced an increase in $[Ca^{2+}]_i$ up to a steady-state level of about 400 nM. This increase in $[Ca^{2+}]_i$ is probably due to ionomycin-mediated Ca^{2+} entry, which shifts the

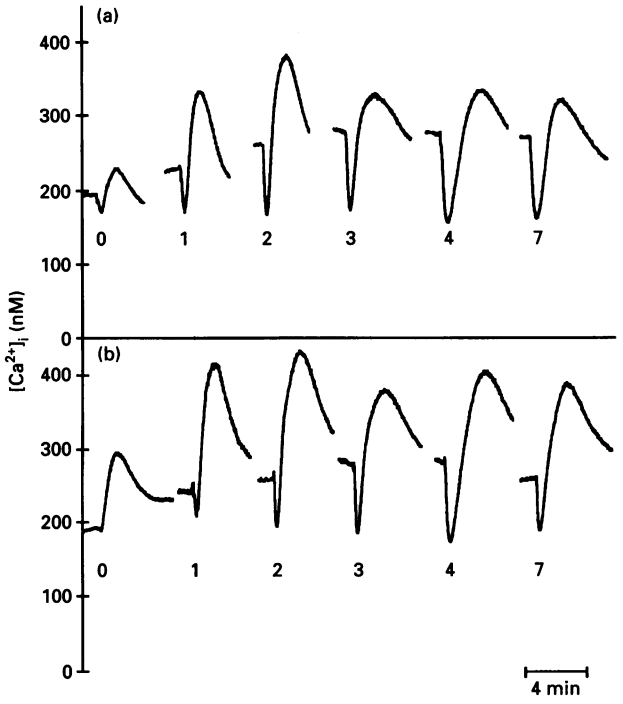

Figure 2 Development during differentiation of the effects of ATP and UTP in thapsigargin-treated HL60 cells

uHL60 cells were incubated with Me_2SO for 0, 1, 2, 3, 4 or 7 days, as indicated. They were then loaded with Fura-2 and treated with 0.5 μM thapsigargin for 10 min in standard medium containing 0.2 mM Ca^{2+} before the addition of either 20 μM ATP (**a**) or 20 μM UTP (**b**). The experiments shown are representative of six different ones of each type.

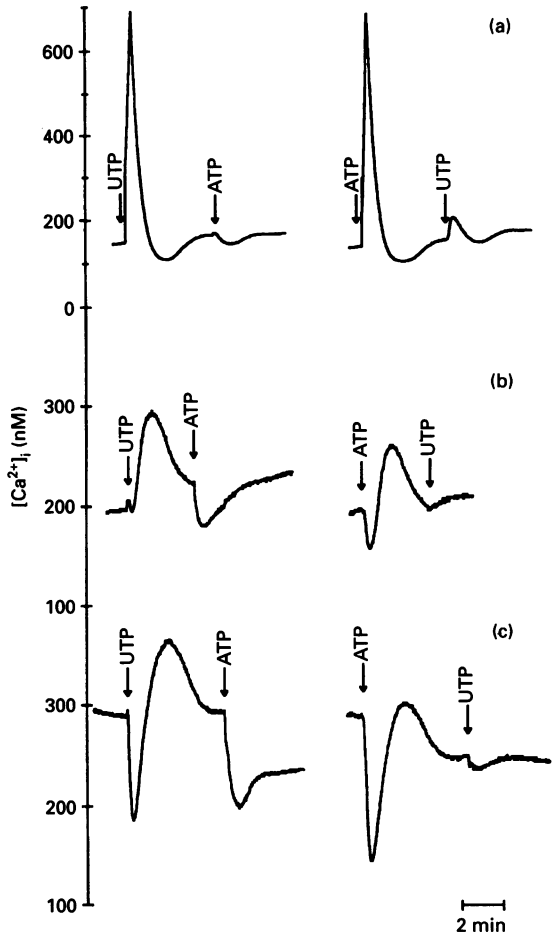

Figure 3 Desensitization of the response to UTP by ATP and vice versa

In (**a**), Fura-2-loaded uHL60 cells incubated in standard medium containing 1 mM Ca^{2+} were treated sequentially with 100 μM UTP and 100 μM ATP (left), or vice versa (right). In (**b**) and (**c**), either Fura-2-loaded uHL60 cells (**b**) or dHL60 cells (**c**) were treated with 0.5 μM thapsigargin for 10 min in standard medium containing 0.2 mM Ca^{2+}, followed by the sequential addition of 100 μM UTP and 100 μM ATP, or vice versa. The experiments shown are representative of four different ones of each type.

steady state between Ca^{2+} entry and Ca^{2+}-pumping to higher $[Ca^{2+}]_i$ levels. At the same time, ionomycin should also release any residual Ca^{2+} from the stores. In separate experiments, 200 nM ionomycin was shown to produce a maximum release of Ca^{2+} from the stores in cells suspended in Ca^{2+}-free medium, in such a way that 1 μM ionomycin added subsequently produced no further Ca^{2+} release (results not shown). Figure 5 shows that, under these conditions, both ATP and UTP were still able to produce a biphasic $[Ca^{2+}]_i$ response, indicating that the delayed stimulation of Ca^{2+} entry is not due to further emptying of the stores, which were completely empty already.

The effects of the nucleotides shown in Figures 1–5 could be due to modifications in either Ca^{2+} entry or Ca^{2+} pumping. We have therefore measured the effect of ATP on unidirectional Ca^{2+} and Mn^{2+} (used as a Ca^{2+} surrogate) fluxes. Figure 6(a) shows the effect of the previous addition of ATP on the $[Ca^{2+}]_i$ overshoot produced on addition of 1 mM Ca^{2+} to the extracellular medium of thapsigargin-treated cells incubated in low-Ca^{2+} (0.1 mM) medium. When ATP was added 5 s before Ca^{2+}, a strong inhibition of Ca^{2+} entry was observed [Ca^{2+} entry measured 15 s after the addition of Ca^{2+} was $46\pm4\%$ ($n = 9$) of that in control cells]. When ATP was added 30 s before Ca^{2+}, the initial rate of Ca^{2+} entry returned to near the values in the control [Ca^{2+} entry was $86\pm10\%$ ($n = 5$) of that in control cells]. Finally, when ATP was added 1 or 2 min before Ca^{2+}, the rate of Ca^{2+} entry was greater than in control cells [141 ± 14 ($n = 4$) or $149\pm11\%$ ($n = 9$) respectively]. Figure 6(b) shows the effect of the addition of UTP during the rising phase of $[Ca^{2+}]_i$ after extracellular Ca^{2+} addition. UTP was added 15 s after the addition of 1 mM Ca^{2+} to thapsigargin-treated cells, showing how the biphasic change in Ca^{2+} permeability that they induce modifies the rate of $[Ca^{2+}]_i$ increase. ATP had the same effects as UTP (results not shown).

Figure 7 shows the effect of nucleotides on Mn^{2+} entry during both the initial phase of inhibition and the late phase of activation. Figure 7(a) shows the effect of different concentrations of ATP on Mn^{2+} entry in thapsigargin-treated cells. ATP was added 5 s before Mn^{2+} in order to study the concentration-dependence of the inhibition of Mn^{2+} entry by ATP. Half-maximal effect was obtained with about 2 μM ATP, which produced $51\pm7\%$ ($n = 4$) of the inhibition produced by 300 μM ATP, whereas 1 and 20 μM ATP produced 24 ± 9 and $69\pm12\%$ ($n = 4$) respectively. The previously reported inhibition of Mn^{2+} entry by 1 μM fMLP [17] is also shown in Figure 7 for comparison. Figure 7(b) shows the effect of UTP on Mn^{2+} entry studied 1 or 2 min after the addition of the nucleotide, once the delayed activation had been developed. Mn^{2+} entry 1 or 2 min after the addition of UTP was faster than in the controls. Stimulation of Mn^{2+} entry by UTP measured 10 s after the addition of Mn^{2+} amounted to 90 ± 15 ($n = 4$) and $130\pm20\%$ ($n = 4$) 1 and 2 min after UTP addition respectively. The stimulation by UTP was maximal in cells differentiated for 2 or 3 days (as shown) and smaller in both

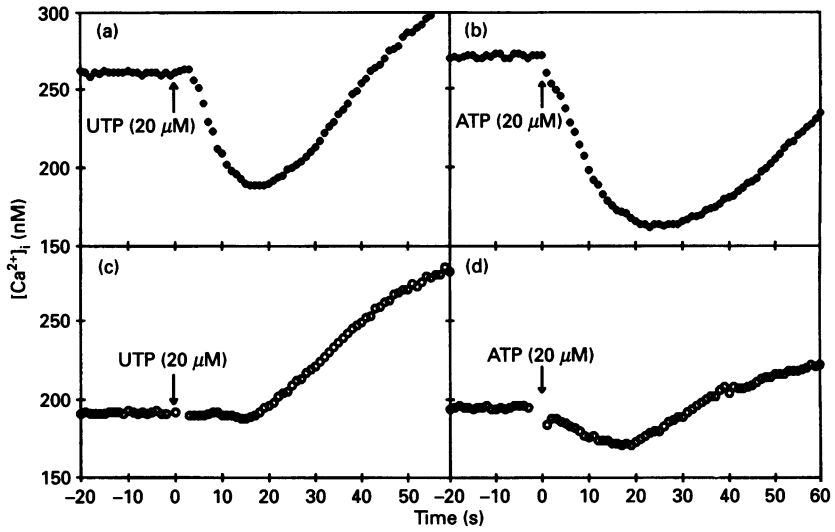

Figure 4 Early effects of ATP and UTP on $[Ca^{2+}]_i$ in thapsigargin-treated HL60 cells

Fura-2-loaded uHL60 (**c, d**) or dHL60 (**a, b**) cells were treated with 0.5 μM thapsigargin for 10 min in standard medium containing 0.2 mM Ca^{2+}, before the addition of either 20 μM UTP (**a, c**) or 20 μM ATP (**b, d**), as indicated. The experiments shown are representative of 13–17 different ones of each type.

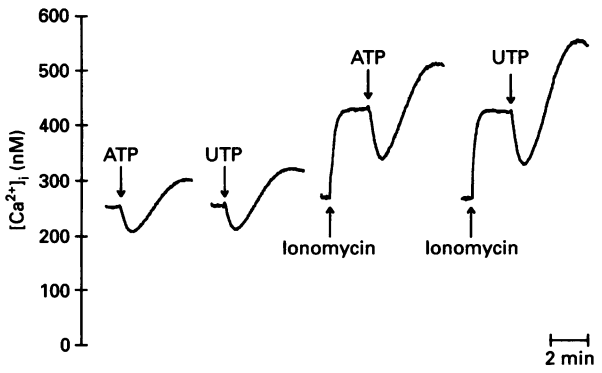

Figure 5 Effect of ionomycin on the biphasic $[Ca^{2+}]_i$ response of thapsigargin-treated cells to nucleotides

Fura-2-loaded dHL60 cells were treated with 0.5 μM thapsigargin for 10 min in standard medium containing 0.2 mM Ca^{2+} before the addition of 20 μM ATP, 20 μM UTP or 200 nM ionomycin, as indicated. The traces shown are averaged from four different experiments of each kind.

undifferentiated and fully differentiated cells (results not shown), in the last case probably because of the development of the inhibition.

We have also studied the effects of several nucleotide analogues on the $[Ca^{2+}]_i$ of thapsigargin-treated cells. Figure 8 shows that 100 μM ATP[S] produces an effect that is almost identical with that of ATP (compare with Figure 1b). Similarly, ATP[S] produced a $[Ca^{2+}]_i$ peak identical with that produced by 100 μM ATP in the absence of thapsigargin (results not shown). The P_{2y} agonist 2-MeSATP only produced marked inhibition of Ca^{2+} entry, but had, in contrast, little effect on Ca^{2+} mobilization. In the absence of thapsigargin, 100 μM 2-MeSATP produced a $[Ca^{2+}]_i$ increase of only 13 ± 1 % ($n = 3$) of that produced by ATP

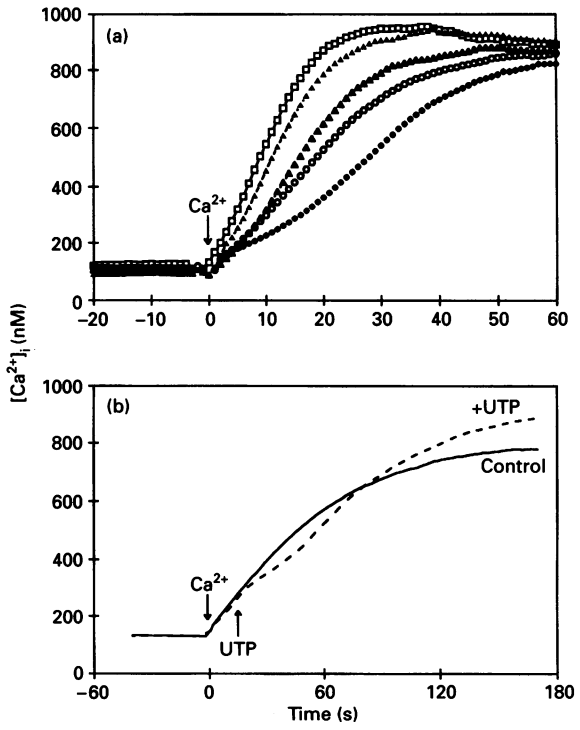

Figure 6 Time-course of the effects of ATP on the $[Ca^{2+}]_i$ overshoot induced by addition of Ca^{2+} to thapsigargin-treated HL60 cells

Fura-2-loaded dHL60 cells were treated with 0.5 μM thapsigargin for 10 min in standard medium containing 0.1 mM Ca^{2+}. Then, in (**a**), 1 mM Ca^{2+} was added at $t = 0$ to either control cells (○) or cells to which 100 μM ATP had been added at $t = -5$ s (●), $t = -30$ s (△), $t = -1$ min (▲) or $t = -2$ min (□). This experiment is representative of five to nine similar ones of each type. In (**b**) 1 mM Ca^{2+} was added at $t = 0$ to thapsigargin-treated cells. Then, 15 s later, 100 μM UTP was added (+UTP). The traces are averaged from three different experiments of each kind. The addition of 100 μM ATP produced similar effects to those of UTP (results not shown).

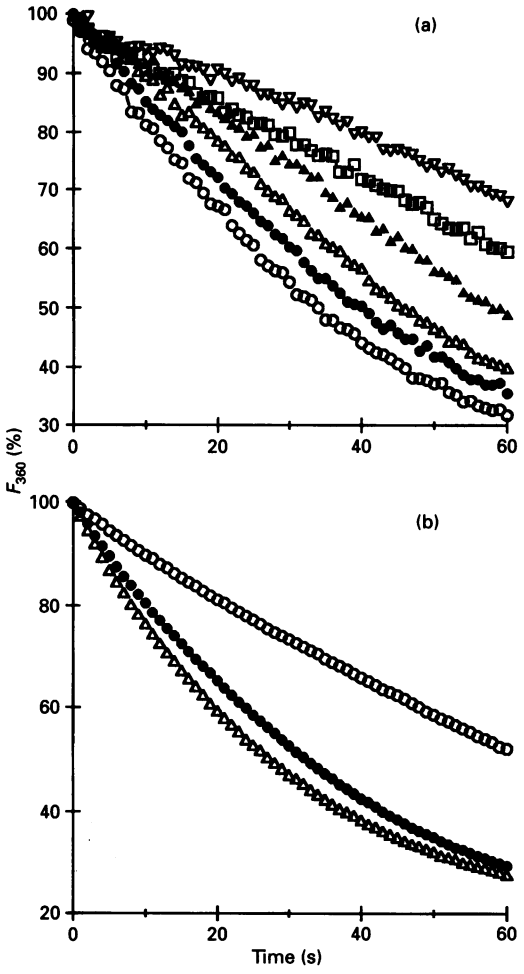

Figure 7 Effect of nucleotides on Mn^{2+} entry during both the rapid inhibitory phase (a) and the delayed activation phase (b) in thapsigargin-treated HL60 cells

In (**a**), Fura-2-loaded dHL60 cells were treated with 0.5 μM thapsigargin for 10 min in standard medium containing 1 mM Ca^{2+}. Then, at $t = 0$, 0.2 mM Mn^{2+} was added to either control cells (○) or cells that had been treated 5 s before (at $t = -5$ s) with 1 μM ATP (●), 5 μM ATP (△), 20 μM ATP (▲), or 300 μM ATP (□). ▽, Mn^{2+} entry in cells treated with 1 μM fMLP 10 s before the addition of Mn^{2+}. This experiment is representative of four similar ones of each type. In (**b**), HL60 cells differentiated for 2 days with Me_2SO were loaded with Fura-2 and treated with 0.5 μM thapsigargin for 10 min in standard medium containing 0.2 mM Ca^{2+}. Then, at $t = 0$, 0.2 mM Mn^{2+} was added to either control cells (○) or cells that had been treated with 20 μM UTP for either 1 (●) or 2 (△) min. The traces shown are averaged from four different experiments. In both (**a**) and (**b**) the traces were normalized to 100% F_{360} just after addition of Mn^{2+} at $t = 0$.

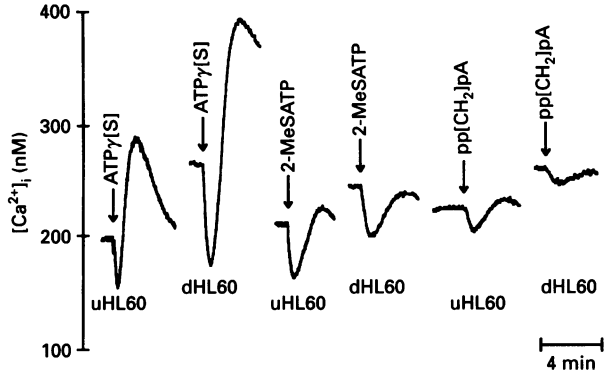

Figure 8 Effects of ATP[S], 2-MeSATP and pp[CH_2]pA on $[Ca^{2+}]_i$ in thapsigargin-treated HL60 cells

Fura-2-loaded uHL60 and dHL60 cells were treated with 0.5 μM thapsigargin for 10 min in standard medium containing 0.2 mM Ca^{2+}. Then 100 μM ATP[S], 100 μM 2-MeSATP or 100 μM pp[CH_2]pA was added as indicated. This experiment is representative of three to five similar ones of each type.

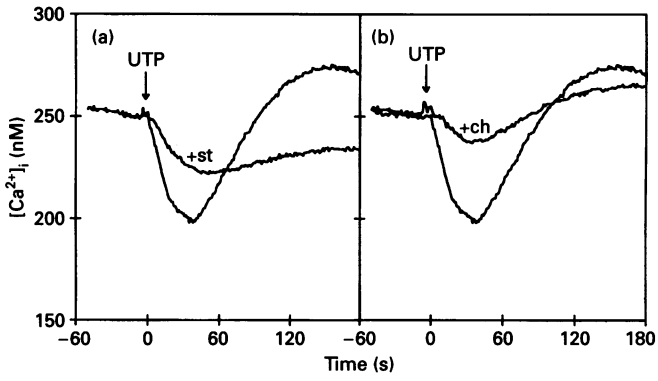

Figure 9 Effects of the protein kinase inhibitors, staurosporin and chelerythrine, on the actions of UTP on $[Ca^{2+}]_i$ in dHL60 cells

Fura-2-loaded dHL60 cells were treated with thapsigargin for 10 min in standard medium containing 0.2 mM Ca^{2+}. Then 100 μM UTP was added to either control cells or cells incubated for either 5 min with 100 nM staurosporin (**a**; +st) or 30 min with 5 μM chelerythrine (**b**; +ch). This experiment is representative of three similar ones of each type.

in dHL60 cells (results not shown). Similarly, the P_{2x} agonist pp[CH_2]pA produced only inhibition of Ca^{2+} entry, although less intense than that of 2-MeSATP (Figure 8), and was unable to mobilize Ca^{2+} from the stores. At a concentration of 100 μM it produced a $[Ca^{2+}]_i$ increase of only 4 ± 1% ($n = 3$) of that produced by ATP in dHL60 cells not treated with thapsigargin (results not shown).

As reported previously for the inhibition of Ca^{2+} entry induced by fMLP [18], inhibition by ATP is also dependent on $[Ca^{2+}]_i$, although the inhibition was not completely abolished at low $[Ca^{2+}]_i$. In dHL60 cells treated with 0.5 μM thapsigargin in medium containing 1 mM Ca^{2+} ($[Ca^{2+}]_i = 980 \pm 23$ nM; $n = 9$), 100 μM ATP produced 49 ± 3% ($n = 3$) inhibition of Mn^{2+} entry when measured 10 s after Mn^{2+} addition and 37 ± 1% when measured 20 s after Mn^{2+} addition. In contrast, in thapsigargin-treated dHL60 cells suspended in Ca^{2+}-free medium ($[Ca^{2+}]_i = 34 \pm 1$ nM; $n = 9$), 100 μM ATP produced only 19 ± 2% ($n = 3$) inhibition of Mn^{2+} entry when measured 10 s after Mn^{2+} addition and 4 ± 1% when measured 20 s after Mn^{2+} addition (results not shown). Therefore inhibition of Mn^{2+} entry by ATP was much greater and long-lasting in cells with high $[Ca^{2+}]_i$.

We have shown previously that the inhibition of SOCP and fMLP and PDBu is sensitive to protein kinase inhibitors [17]. Inhibition by fMLP was not very sensitive to staurosporin and completely prevented by chelerythrine, whereas inhibition by PDBu was sensitive to both inhibitors [17]. Figure 9 shows the effects of these protein kinase inhibitors on the biphasic effect of UTP in thapsigargin-treated cells. Staurosporin only partially prevented the UTP-induced inhibition of Ca^{2+} entry, but completely blocked the late stimulation of $[Ca^{2+}]_i$ increase. On the

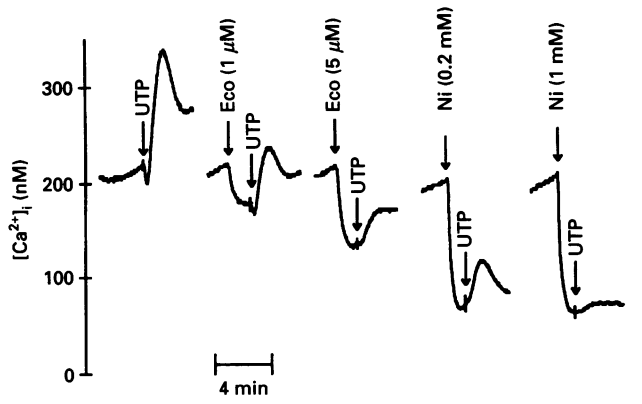

Figure 10 Effects of econazole and Ni on the activation of Ca^{2+} entry induced by UTP in thapsigargin-treated 1-day-differentiated HL60 cells

HL60 cells were incubated with Me_2SO for 1 day, loaded with Fura-2 and incubated with 0.5 μM thapsigargin for 10 min in standard medium containing 0.2 mM Ca^{2+}. Then either 20 μM UTP or different concentrations of econazole (Eco) or Ni were added as indicated. This experiment is a representative of five similar ones of each type.

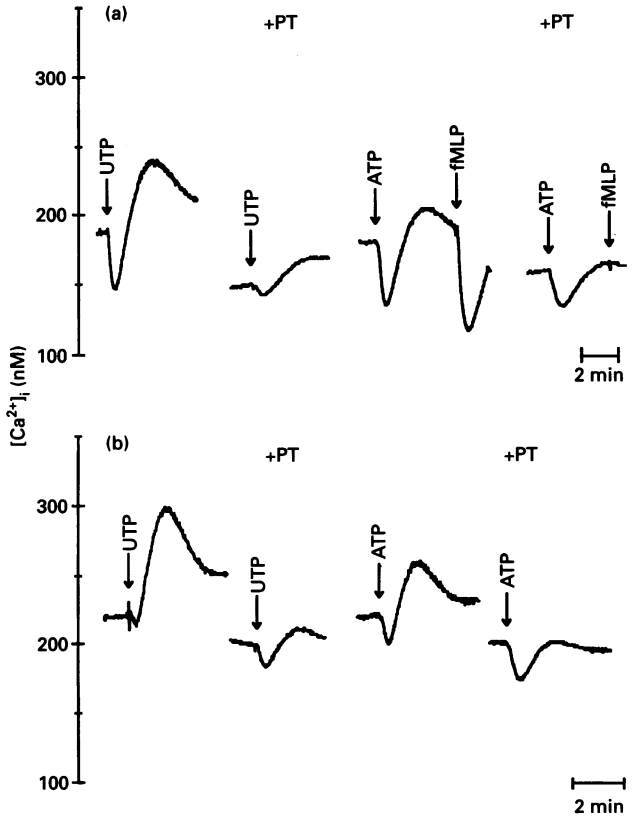

Figure 11 Inhibition by pertussis toxin of the effects of UTP and ATP in thapsigargin-treated HL60 cells

Either Fura-2-loaded uHL60 cells (**b**) or dHL60 cells (**a**) were treated with thapsigargin for 10 min in standard medium containing 0.2 mM Ca^{2+}. Then 100 μM UTP, 100 μM ATP or 1 μM fMLP was added as indicated. For traces labelled + PT, cells were previously incubated for 24 h with 0.5 μg/ml pertussis toxin. This experiment is a representative of three similar ones of each type.

other hand, chelerythrine almost completely prevented the UTP-induced inhibition of Ca^{2+} entry, but it was only partially effective against the late stimulation of Ca^{2+} entry. Similar results were obtained when ATP was used as the agonist (results not shown). These results suggest that both effects could be mediated by phosphorylation.

The nucleotide-induced delayed activation of Ca^{2+} entry could take place either via a new Ca^{2+} pathway or by further activation of SOCP by a mechanism other than emptying the Ca^{2+} stores. To differentiate between these two possibilities we have studied the effect of econazole and $NiCl_2$, two known inhibitors of SOCP [14,25], on the delayed $[Ca^{2+}]_i$ increase induced by UTP in 1-day-differentiated HL60 cells. Figure 10 shows that both econazole and Ni^{2+} produced a rapid decrease in $[Ca^{2+}]_i$ in thapsigargin-treated cells as a result of inhibition of SOCP. The subsequent addition of UTP showed that the stimulated $[Ca^{2+}]_i$ increase was also similarly sensitive to both inhibitors. These results demonstrate that the inhibitors used do not allow us to distinguish between the two mechanisms proposed above.

Finally, the effects of pertussis toxin on the nucleotide-induced $[Ca^{2+}]_i$ changes in thapsigargin-treated cells were studied. It has been shown that pertussis toxin only partially inhibits Ca^{2+} mobilization induced by ATP in these cells [2,26]. In our hands, the $[Ca^{2+}]_i$ peaks induced by 100 μM ATP and 100 μM UTP were reduced to 52 ± 6 ($n = 4$) and $49 \pm 2\%$ ($n = 3$) respectively by treatment with pertussis toxin. Figure 11 shows that pertussis toxin also partially inhibits $[Ca^{2+}]_i$ changes induced by ATP and UTP in thapsigargin-treated cells, although the toxin was apparently more effective against the late stimulation of Ca^{2+} entry than against the initial inhibition of Ca^{2+} entry. Partial prevention by pertussis toxin of the inhibition of Ca^{2+} entry by ATP contrasts with the full abolition of the inhibition produced by fMLP, tested in the same cells (Figure 11).

DISCUSSION

Nucleotides affect $[Ca^{2+}]_i$ of HL60 cells by four different mechanisms: (i) mobilization of stored Ca^{2+}; (ii) activation of Ca^{2+} entry via SOCP after emptying of the Ca^{2+} stores; (iii) early inhibition of Ca^{2+} entry via SOCP, probably mediated by phosphorylation; (iv) gradual stimulation of Ca^{2+} entry by a mechanism other than emptying of the stores. Using cells with intracellular Ca^{2+} stores that had been emptied by treatment with thapsigargin in order to avoid mechanism (i) and to fully and irreversibly activate mechanism (ii), mechanisms (iii) and (iv) are more conveniently demonstrated. The actual effects obtained depended on the nucleotide, its concentration and the stage of differentiation of HL60 cells. ATP was the most effective at inhibiting Ca^{2+} entry and this effect increased greatly with differentiation, the maximum being obtained after 4 days of incubation with Me_2SO. In contrast, UTP was more effective at producing the late activation of Ca^{2+} entry, which developed to a lesser extent but more quickly with differentiation: significant activation was already observed in uHL60 cells and maximum activation was seen after only 1 day of differentiation. In uHL60 cells, UTP selectively activated this late Ca^{2+}-entry mechanism without any preceding inhibition. Then inhibition of Ca^{2+} entry by UTP developed along with differentiation in such a way that almost complete inhibition was observed after 4 days of differentiation. For both nucleotides, full inhibition of Ca^{2+} entry required higher concentrations (20 μM) than full activation of Ca^{2+} entry (1–5 μM).

Inhibition of Ca^{2+} entry by nucleotides strongly resembles the transient inhibition produced by fMLP in both neutrophils and dHL60 cells, which appears to be mediated by phosphorylation

[17–19]. fMLP did not have any effect in uHL60 cells, probably because of the lack of high-affinity fMLP receptors which only develop during differentiation [20]. However, nucleotide receptors are present in both uHL60 and dHL60 cells and stimulation of these receptors produces Ca^{2+} mobilization that is identical in intensity and concentration-dependence in both sets of cells [1,2] (see also Figure 1a). Therefore development of inhibition by nucleotides during differentiation should not be attributed to expression of new receptors, but to expression of a component of the inhibition mechanism that is absent from or scarcely present in uHL60 cells. Our results resemble somewhat the previously described development during differentiation of ATP- and fMLP-induced phospholipase D activation and primary granule secretion [27]. In that case, development of the response to fMLP was attributed in part to expression of fMLP receptors during differentiation, but development of the response to ATP could only be attributed to induction of a component of the transduction cascade of the receptor-regulated signalling pathway [27].

The mechanism of inhibition of Ca^{2+} entry by nucleotides shows similarities to and differences from that produced by fMLP [17]. The similarities included the following: the inhibition is transient, greatly decreased in low-Ca^{2+} medium (although not abolished) and almost completely prevented by chelerythrine but only partially by staurosporin. One difference is that it is only partially sensitive to pertussis toxin, which completely abolishes the inhibition by fMLP. The above results were consistently obtained in 7-day-differentiated HL60 cells. In 2-day-differentiated HL60 cells, chelerythrine continued to be an efficient inhibitor, but staurosporin had either no effect or even potentiated the inhibition of SOCP by nucleotides (results not shown). We do not have an explanation for this finding.

Activation of Ca^{2+} entry by nucleotides develops only after a long delay of more than 15 s. This activation is not the consequence of further emptying of the Ca^{2+} stores, which are already completely empty after a 10 min incubation with thapsigargin [see Figure 5, and note also the lack of Ca^{2+} mobilization by the nucleotides in thapsigargin-treated cells (Figures 1–5)]. It should therefore be attributed to either the activation of a Ca^{2+}-entry pathway different from SOCP or an additional activation of Ca^{2+} entry via SOCP by a mechanism other than emptying the stores. Activation of Ca^{2+} entry was sensitive to Ni^{2+} and econazole, which have been shown to inhibit SOCP in neutrophils [25]. This is consistent with stimulated Ca^{2+} entry taking place via SOCP, but does not prove it, as Ni^{2+} is a non-specific Ca^{2+}-channel blocker and sensitivity to econazole has also been described for voltage-dependent Ca^{2+} channels [28] and for Ca^{2+}-dependent K^+ channels [29]. The delay in activation suggests that a second messenger may be necessary to activate Ca^{2+} entry. Activation of Ca^{2+} entry was consistently sensitive to pertussis toxin, suggesting that activation of a pertussis-toxin-sensitive G-protein is necessary to stimulate Ca^{2+} entry. A simple explanation, although speculation at present, of this stimulated Ca^{2+} entry could lie in the presence of a resting level of phosphorylation/inhibition of SOCP. In fact, inhibition of SOCP by the addition of protein phosphatase inhibitors [19] suggests that a phosphorylation/dephosphorylation cycle occurs under resting conditions. Agonists could then activate the first phosphorylation (producing inhibition) and then dephosphorylation via activation of the protein phosphatase involved, as described in other cases [30,31]. This process would probably lead to a transiently greater dephosphorylation (and activation of SOCP) than under resting conditions.

We also show here a clear difference between the effects of ATP and UTP on Ca^{2+} entry, which contrasts with their similar effects on Ca^{2+} mobilization. ATP activates more strongly than UTP the mechanism leading to inhibition of SOCP, whereas UTP is a stronger agonist for activation of the delayed Ca^{2+} entry. In fact, UTP does not produce inhibition of SOCP in uHL60 cells at any concentration. However, selectivity was not absolute; ATP always produced a certain degree of stimulation of Ca^{2+} entry and high concentrations of UTP inhibited Ca^{2+} entry in dHL60 cells. In addition, UTP desensitized the effect of a subsequent ATP addition on both Ca^{2+} mobilization and delayed activation of Ca^{2+} entry, but it was unable to prevent the ATP-induced inhibition of SOCP. In contrast, the previous addition of ATP prevented all the effects of a subsequent addition of UTP. Our results are therefore difficult to reconcile with both the presence of only one receptor in HL60 cells for both ATP and UTP and the presence of fully independent receptors for both agonists.

Given that there is strong evidence for the existence of a receptor able to recognize both ATP and UTP (see the Introduction), we believe that the best explanation for our results could be provided by a model with two receptors. The first one would be a P_{2u} receptor able to bind both ATP and UTP, leading to activation of phospholipase C and Ca^{2+} mobilization as well as inhibition of SOCP and activation of the delayed Ca^{2+} entry. The second receptor would recognize only ATP and its analogues, particularly 2-MeSATP, but not UTP. It would therefore be similar in the nucleotide potency order to a P_{2y} receptor. Activation of this receptor would only lead to inhibition of Ca^{2+} entry via SOCP. This P_{2y}-like receptor would mediate the effects of the ATP analogues, which produce inhibition of SOCP but little Ca^{2+} mobilization from the stores, and would be responsible for the much faster time course of inhibition of SOCP produced by ATP than that produced by TUP in dHL60 cells (Figure 4).

In summary, the results shown in the present paper indicate that Ca^{2+} homoeostasis after binding of a nucleotide to its receptor in the plasma membrane is achieved by several phenomena which develop consecutively and partially overlap. First, production of inositol trisphosphate leads to Ca^{2+} release from intracellular Ca^{2+} stores, leading to activation of Ca^{2+} entry via SOCP. A few seconds later, however, SOCP is transiently inhibited by a mechanism probably mediated by phosphorylation. The inhibition lasts for about 30 s and then Ca^{2+} entry is activated via both SOCP and a Ca^{2+} pathway independent of whether the Ca^{2+} stores are empty. This activation lasts for a few minutes and then Ca^{2+} permeability returns to the resting state.

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