Cytosolic calcium pre-elevation amplifies agonist-induced calcium release in human leukaemic HL-60 cells

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Histamine, ATP, and two microsomal Ca^{2+} -pump inhibitors, thapsigargin (TG) and cyclopiazonic acid (CPA), were able to release intracellular Ca^{2+} in human leukaemic HL-60 cells. The relationships between the agonist-, TG- and CPA-sensitive Ca^{2+} pools were investigated with optimal concentrations of these agents in Ca^{2+} -free medium. CPA failed to release Ca^{2+} after the Ca^{2+} stores of the cells had been discharged by TG, and vice versa, suggesting that the TG- and CPA-sensitive pools exactly overlap. Using this protocol, it was further demonstrated that (a) histamine and ATP utilized the same agonist-sensitive pool, and (b) the CPA- or TG-sensitive pool was much larger than, and encompassed, the agonist-sensitive pool. Although optimal (30 μM) CPA treatment for 5 min totally emptied the agonist-sensitive pool, a brief exposure (1.5 min) to a sub-optimal

concentration (3 μM) of CPA, which only slightly raised cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), substantially enhanced subsequent agonist-induced Ca^{2+} release. Brief pretreatments with sub-optimal concentrations of TG or ionomycin, which caused moderate $[\text{Ca}^{2+}]_i$ elevation, also caused such enhancement. However, sub-optimal CPA pretreatment had no prominent effect on Ca^{2+} release, which was InsP_3 -independent: it did not enhance TG-induced Ca^{2+} release, and only relatively weakly augmented ionomycin-induced Ca^{2+} release. Our results represent a novel observation showing that low concentrations of CPA, TG and ionomycin can potentiate subsequent agonist-induced Ca^{2+} release, and suggest that a 'priming' moderate $[\text{Ca}^{2+}]_i$ elevation can amplify subsequent InsP_3 -dependent Ca^{2+} release in HL-60 cells.

INTRODUCTION

Many agonists release Ca^{2+} from intracellular non-mitochondrial Ca^{2+} stores by generating inositol 1,4,5-trisphosphate (InsP_3) [1,2]. In many systems, InsP_3 or agonists do not release all the internal Ca^{2+} . For instance, in permeabilized Jurkat T cells, rat lacrimal acinar cells and DDT₁MF-2 smooth-muscle cells, addition of InsP_3 only partially releases internal Ca^{2+} [3–5]. Similarly, in intact Jurkat T cells, rat lacrimal acinar cells, NG108-15 neuroblastoma cells and bovine endothelial cells, agonists only mobilize a fraction of the intracellular Ca^{2+} pools [3,4,6,7]. In all these cases, the InsP_3 - (or agonist-) insensitive Ca^{2+} store, as well as the InsP_3 - (or agonist-) sensitive Ca^{2+} store, could be released by thapsigargin (TG), which has been shown to be a selective inhibitor of microsomal Ca^{2+} pumps [8,9]. Because of the spontaneous Ca^{2+} leak from internal stores and the prevention of Ca^{2+} re-uptake by TG, this agent can mobilize intracellular Ca^{2+} . Agonists can also raise cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) by inducing Ca^{2+} influx from extracellular space. Agonist-induced Ca^{2+} entry can be stimulated by the empty state of the Ca^{2+} store after InsP_3 -induced discharge [10], or via 'receptor-mediated Ca^{2+} entry', by which agonist–receptor interaction directly activates plasmalemmal Ca^{2+} channels [11].

In vivo, it is likely that Ca^{2+} signals are modulated by other hormones, endogenous substances, drugs or other factors such as membrane potential. There have indeed been a number of studies of amplification of Ca^{2+} -dependent physiological processes, Ca^{2+} signalling in general, Ca^{2+} entry and Ca^{2+} release. Angiotensin II amplifies noradrenaline-induced contraction in rabbit arterial smooth muscle [12]. Endothelin-1 or depolarization magnifies α -adrenergic vasoconstriction in dog mesenteric artery and vein [13]. It has been shown that 1,25-

dihydroxycholecalciferol enhances in GH_4C_1 rat pituitary cells the Ca^{2+} signal induced by thyrotropin-releasing hormone and depolarization [14]. Cyclic-AMP-elevating agonists or drugs amplify bradykinin-induced Ca^{2+} entry in pig aortic endothelial cells [15]. TG has been shown to potentiate caffeine-induced Ca^{2+} release in Jurkat T cells [3]. Bian et al. [5] showed that in permeabilized DDT₁MF-2 smooth-muscle cells InsP_3 -induced Ca^{2+} release (IICR) could be magnified upon GTP addition. Rooney et al. [16] showed that another microsomal- Ca^{2+} -pump inhibitor, t-butyl hydroperoxide, could amplify IICR in permeabilized rat hepatocytes. It has also been demonstrated that in a number of different cell types Ca^{2+} itself amplified IICR [17–21].

In the present study, using human leukaemic HL-60 promyelocytes, we describe a novel amplification phenomenon of agonist-induced Ca^{2+} release: (a) examination of the relation between the agonist-sensitive Ca^{2+} pool and the Ca^{2+} pool sensitive to TG or cyclopiazonic acid (CPA) (another microsomal- Ca^{2+} -pump inhibitor, chemically unrelated to TG [22,23]) revealed that the agonist-sensitive pool was encompassed by the much bigger TG/CPA-sensitive pool; (b) importantly and interestingly, a brief treatment with sub-optimal concentrations of TG or CPA, moderately raising $[\text{Ca}^{2+}]_i$, substantially amplified subsequent agonist-induced Ca^{2+} release. This amplification phenomenon is unique amongst previous observations on Ca^{2+} -signal amplification, because it demonstrates that two intracellular Ca^{2+} -pump inhibitors (and hence Ca^{2+} -store depletors), namely TG and CPA, paradoxically enhance agonist-induced Ca^{2+} release. We present evidence here that this amplification effect could possibly be due to a pre-elevation of $[\text{Ca}^{2+}]_i$. Also, the physiological significance of this amplification phenomenon will be discussed.

Abbreviations used: TG, thapsigargin; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; CPA, cyclopiazonic acid; HBSS, Hanks' buffered saline solution; IICR, InsP_3 -induced Ca^{2+} release.

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MATERIALS AND METHODS

Materials

ATP, histamine, TG, ionomycin, and fura 2-AM [acetoxymethyl ester) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. CPA was purchased from Calbiochem, Richmond, CA, U.S.A. Fetal-calf serum was purchased from Gibco BRL (Gaithersburg, MD, U.S.A.).

Cell culture

HL-60 cells, obtained from American Type Culture Collection, were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal-calf serum, 5 µg of gentamycin/ml and penicillin (100 units/ml)/streptomycin (100 µg/ml) in a humidified atmosphere with 5% CO₂ at 37 °C. The cells were split in a 1:5 ratio every 2 or 3 days.

[Ca²⁺]_i measurement

The method to measure [Ca²⁺]_i was modified from the protocol described by Lambert and Nahorski [24]. Hank's buffered saline solution (HBSS), composed of 138 mM NaCl, 5.3 mM KCl, 0.8 mM MgSO₄, 1.2 mM CaCl₂, 0.44 mM KH₂PO₄, 0.34 mM Na₂HPO₄, 5 mM glucose and 25 mM Hepes, buffered at pH 7.4, was used for cell washing. HL-60 cells grown to a density of around 1.5 × 10⁶/ml were harvested, washed once in Ca²⁺-containing HBSS and resuspended in serum-free RPMI 1640 medium at a cell density of about 2 × 10⁷/ml. Fura 2-AM was added to this cell suspension at a final concentration of 5 µM. The cell suspension was then incubated at 37 °C for 45 min. Thereafter, the fura 2-loaded cells were washed twice in Ca²⁺-containing HBSS and resuspended in the same buffer solution at a cell density of 2.8 × 10⁶/ml. Before experimentation on each sample, 1 ml of this cell suspension was centrifuged and the cell pellet was resuspended in 2 ml Ca²⁺-free HBSS. Ca²⁺-free HBSS was used in [Ca²⁺]_i measurement to detect Ca²⁺ release from intracellular stores. This 2 ml cell suspension was then transferred to a quartz cuvette which contained a mini stirrer, and warmed at 37 °C for about 3 min before addition of agonists or drugs. About 30 s before agonist or drug stimulation, EGTA (final concn. 100 µM) was added to the cell suspension to chelate possible contaminating Ca²⁺. Fura 2 fluorescence was detected with a Hitachi F-4000 fluorescence spectrophotometer. Excitation and emission wavelengths were set at 340 nm and 500 nm respectively. For calibration, $F_{\max}$ was obtained by adding 1.2 mM CaCl₂ to each sample and then lysing the cells with 0.1% Triton X-100, and $F_{\min}$ by chelating Ca²⁺ with 20 mM EGTA. [Ca²⁺]_i was calculated as described by Grynkiewicz et al. [25]. The basal [Ca²⁺]_i ranged from 40 to 80 nM.

Statistical analysis

Results are expressed as means ± S.E.M. Student's paired *t* test was used, and differences were considered significant when *P* < 0.05.

RESULTS

Relationships between the agonist-, TG-, CPA- and ionomycin-sensitive Ca²⁺ pools

In all the experiments in this work, Ca²⁺-free medium was used, since we aimed to test the intracellular Ca²⁺ mobilization of HL-60 cells in response to agonists and several pharmacological tools. Figures 1(a)–1(e) show the Ca²⁺ release produced by

optimal concentrations of histamine, ATP, CPA, TG and ionomycin that produced the maximal [Ca²⁺]_i elevation. ATP and histamine raise [Ca²⁺]_i, most probably by generating InsP₃ [26,27]. TG and CPA mobilize internal Ca²⁺ by inhibiting microsomal Ca²⁺ pumps and hence impairing the recycling of Ca²⁺ back to the internal pools [8,9,22,23]. Ionomycin, by acting as a Ca²⁺ ionophore [28], causes internal Ca²⁺ release. As shown in Figure 1(f), ATP was more efficacious than histamine in mobilizing Ca²⁺ (150 nM versus 60 nM). Histamine elicited a relatively small Ca²⁺ release in HL-60 cells, presumably due to its weak stimulation of InsP₃ generation [27]. Further, ATP was more potent than histamine (EC₅₀ approx. 1.5 µM and 10 µM respectively). Although both microsomal-Ca²⁺-pump inhibitors had similar efficacy, TG was much more potent than CPA (EC₅₀ approx. 0.8 nM and 5 µM respectively) (Figure 1g). The ryanodine

Figure 1 Ca²⁺ signals elicited by maximal concentrations of agonists and pharmacological agents

Fura-2-loaded cells in 0.1 mM EGTA-supplemented Ca²⁺-free HBSS were challenged with (a) 100 µM histamine (HA), (b) 100 µM ATP, (c) 30 µM CPA, (d) 30 nM TG or (e) 3 µM ionomycin (IONO). Similar traces were obtained in at least six other separate experiments. Concentration–response curves of histamine (○) and ATP (●) are shown in (f); those for TG (■), CPA (□) and ionomycin (Δ) are shown in (g). The increase in [Ca²⁺]_i is calculated as the difference between the [Ca²⁺]_i peak and the basal [Ca²⁺]_i. Each point is the mean ± S.E.M. of 3–4 separate experiments.

Figure 2 Relations of the CPA-, TG- and ionomycin-releasable pools

Fura-2-loaded cells in 0.1 mM EGTA-supplemented Ca^{2+} -free HBSS were challenged with 30 μM CPA, 30 nM TG and 3 μM ionomycin in different sequences. (a) After TG-induced Ca^{2+} release, CPA and ionomycin (IONO) were added sequentially; (b), as in (a), but the order of addition of TG and CPA was reversed; (c) after ionomycin treatment, addition of either TG or CPA caused minimal Ca^{2+} release. Similar traces were obtained in four other separate experiments.

receptor mediates voltage-sensitive Ca^{2+} release and Ca^{2+} -induced Ca^{2+} release in skeletal- and cardiac-muscle-cells, respectively [29]. Although ryanodine-sensitive pools have been observed in non-muscle cells [30,31], we failed to detect any Ca^{2+} release induced by ryanodine (0.1–10 μM) or the ryanodine receptor agonist caffeine (10 mM) in HL-60 cells (results not shown).

The relations between the agonist-, TG-, CPA- and ionomycin-sensitive Ca^{2+} pools were investigated by using optimal concentrations of these agents. CPA failed to release Ca^{2+} after the cells had been exposed to TG, and vice versa (Figures 2a and 2b). In these cases, after CPA or TG treatment, ionomycin could still mobilize a substantial amount of Ca^{2+} . However, if the cells were pretreated with ionomycin, CPA or TG could only release a minimal amount of Ca^{2+} (Figure 2c). These data suggest that (a) the TG- and CPA-sensitive pools exactly overlap, and possibly these two drugs recognize the same microsomal Ca^{2+} pump(s), and (b) the ionomycin-releasable pool is bigger than, and to a great extent encompasses, the TG- or CPA-sensitive pool.

After Ca^{2+} release induced by histamine, the Ca^{2+} response to ATP was slightly diminished (to $78.6 \pm 4.5\%$ of the control, i.e. cells not pretreated with histamine; $n = 5$; $P < 0.05$) (Figures 3a and 3b). After stimulation with ATP, the Ca^{2+} release induced by histamine was much decreased (to $32.5 \pm 12.9\%$ of the control, i.e. cells not pretreated with ATP; $n = 5$; $P < 0.05$) (Figures 3a and 3b). These observations indicate that histamine and ATP utilized the same agonist-sensitive pool, which was of very limited size. Histamine and ATP failed to release Ca^{2+} after CPA treatment (Figures 3c and 3d), but prior exposure to histamine and ATP seemed to elicit little effect on the CPA-sensitive pool size (Figures 3a and 3b). Therefore the CPA-sensitive pool is much larger than, and encompasses, the agonist-sensitive pool. Similar results (not shown) were obtained when TG was used instead of CPA.

Amplification of agonist-induced Ca^{2+} release by pretreatment with low concentrations of CPA, TG and ionomycin

Although optimal (30 μM) CPA treatment for 5 min totally emptied the agonist-sensitive pool (Figures 3c and 3d), a brief exposure (1.5 min) to a sub-optimal concentration (3 μM) of CPA surprisingly enhanced histamine-induced Ca^{2+} release, with an amplification effect especially prominent at the low agonist-

Figure 3 Relationships between the agonist- and CPA-sensitive pools

Fura-2-loaded cells in 0.1 mM EGTA-supplemented Ca^{2+} -free HBSS were challenged with 100 μM histamine (HA), 100 μM ATP and 30 μM CPA in different sequences. (a) and (b), addition of CPA was after depletion of the Ca^{2+} store by agonists; (c) and (d), after depletion by CPA, agonists were added. Similar traces were obtained in two other separate experiments.

concentration range (Figure 4). Thus pretreatment with 3 μM CPA shifted the histamine concentration-response curve to the left, lowering the EC_{50} from 10 to 4 μM and also increasing the maximal response about 1.5-fold. Figure 5 shows that pretreatment with 3 μM CPA also amplified ATP-induced Ca^{2+} release and shifted the ATP concentration-response curve to the left, lowering the EC_{50} from 1.5 to 0.5 μM . The amplification

Figure 4 Amplification of histamine-induced Ca^{2+} release by CPA pretreatment

Fura-2-loaded cells in 0.1 mM EGTA-supplemented Ca^{2+} -free HBSS were pretreated with either vehicle (dimethyl sulphoxide) or 3 μ M CPA for approx. 1.5 min, at which time the CPA-elevated $[Ca^{2+}]_i$ reached the peak, before stimulation with various concentrations of histamine. (a)–(c), typical traces of amplification patterns of 1 μ M, 10 μ M and 100 μ M histamine-induced Ca^{2+} release. (d), Histamine-induced $[Ca^{2+}]_i$ elevation in the absence (■) and presence (□) of CPA presented graphically; each point is the mean $\pm$ S.E.M. of 3–4 separate experiments. In the absence of CPA pretreatment, the increase in $[Ca^{2+}]_i$ is calculated as the difference between the histamine-induced $[Ca^{2+}]_i$ peak and the basal $[Ca^{2+}]_i$. In the case of CPA pretreatment, the increase in $[Ca^{2+}]_i$ is the difference between the histamine-induced $[Ca^{2+}]_i$ peak and the CPA-elevated $[Ca^{2+}]_i$ peak. *Significantly different ($P < 0.05$) from the Ca^{2+} release by the same histamine concentration in the absence of CPA pretreatment.

effect was prominent at the low agonist-concentration range, but the maximal response, which may represent an approximation of the agonist pool size, was not significantly enhanced, suggesting that there might not have been an augmentation of the agonist-sensitive pool size. That Ca^{2+} release induced by optimal concentration of histamine could be enhanced may be explained by the fact that histamine, even at optimal concentration, mobilized only a small fraction of the agonist-sensitive pool.

The concentration-dependent effect of CPA on amplification of agonist-induced Ca^{2+} release is shown in Figure 6, and the data summarized in Figure 7(a) show a bell-shaped relationship. The histamine-induced Ca^{2+} release was positively correlated with the $[Ca^{2+}]_i$ elevated by CPA at concentrations at and below 3 μ M. Higher concentrations of CPA did not produce any amplification effect, or became inhibitory. We also examined whether other $[Ca^{2+}]_i$ -elevating agents, such as TG and ionomycin, could amplify agonist-induced Ca^{2+} release. Figures 7(b) and 7(c) indicate that bell-shaped relationships were also obtained. Optimal concentrations of CPA, TG and ionomycin induced 2.9-, 3.8- and 2.4-fold increases, respectively, in agonist-induced Ca^{2+} release. Remarkably, on the left sides of the three bell-shaped curves, the histamine-induced Ca^{2+} release was positively correlated with the $[Ca^{2+}]_i$ elevated by all three pharmacological agents. More remarkably common for these

Figure 5 Amplification of ATP-induced Ca^{2+} release by CPA pretreatment

Fura-2-loaded cells in 0.1 mM EGTA-supplemented Ca^{2+} -free HBSS were pretreated with either vehicle (dimethyl sulphoxide) or 3 μ M CPA for approx. 1.5 min, at which time the CPA-elevated $[Ca^{2+}]_i$ reached the peak, before stimulation with various concentrations of ATP. (a)–(c), typical traces of amplification pattern of 0.3 μ M, 3 μ M and 100 μ M ATP-induced Ca^{2+} release. (d), ATP-induced $[Ca^{2+}]_i$ elevation in the absence (■) and presence (□) of CPA presented graphically; each point is the mean $\pm$ S.E.M. of 3 separate experiments. In the absence of CPA pretreatment, the increase in $[Ca^{2+}]_i$ is calculated as the difference between the ATP-induced $[Ca^{2+}]_i$ peak and the basal $[Ca^{2+}]_i$. In the case of CPA pretreatment, the increase in $[Ca^{2+}]_i$ is the difference between the ATP-induced $[Ca^{2+}]_i$ peak and the CPA-elevated $[Ca^{2+}]_i$ peak. *Significantly different ($P < 0.05$) from the Ca^{2+} release by the same ATP concentration in the absence of CPA pretreatment.

three drugs were that (a) significant and invariable amplification seemed to require a 'priming' $[Ca^{2+}]_i$ elevation greater than 10 nM, and (b) a priming $[Ca^{2+}]_i$ elevation of 12–40 nM produced the optimal amplification effect. These observations strongly suggest that the amplification effect was not a selective effect of CPA or microsomal- Ca^{2+} -pump inhibitors, but could possibly be due to a 'priming' moderate elevation of $[Ca^{2+}]_i$ (see the Discussion section).

Effect of the CPA pretreatment period on the degree of amplification

The amplification effect by CPA appears intriguing. In view of the depletion of intracellular stored Ca^{2+} by CPA and the fact that the agonist-releasable pool is within the CPA-sensitive pool, it is expected that even a brief exposure of cells to sub-maximal CPA concentrations might have drained some Ca^{2+} out of the agonist-sensitive pool. In fact, it was not the case, because Ca^{2+} release induced by maximal ATP, which is an estimate of the agonist-releasable pool size, was not diminished after a brief treatment with 3 μ M CPA (see Figure 5c). However, the treatment of cells with 3 μ M CPA, if prolonged (11–13 min), caused

Figure 6 Effects of different CPA concentrations on histamine-induced Ca^{2+} release

Fura-2-loaded cells in 0.1 mM EGTA-supplemented Ca^{2+} -free HBSS were pretreated with dimethyl sulphoxide or various concentrations of CPA for approx. 1.5 min before histamine (HA; 6 μM) stimulation. Traces shown are representative of results from three separate experiments, which are summarized graphically in Figure 7(a).

a decrease in agonist-releasable pool size (Figures 8f and 8g). Therefore, the enhancement effect on histamine-induced Ca^{2+} release diminished with the time of CPA treatment, and was abolished when the cells had been exposed to CPA for 11–13 min, as the agonist-sensitive pool became progressively exhausted (Figures 8a–8d). Furthermore, Figures 8(d) and 8(e) indicate that prolonged treatment with 3 μM CPA also decreased the size of the ionomycin-releasable pool, which represents the biggest Ca^{2+} pool that we have so far examined in HL-60 cells.

Can InsP_3 -independent Ca^{2+} release be amplified by CPA?

We have shown that low concentrations of CPA could amplify agonist-induced Ca^{2+} release, which was dependent on InsP_3 generation [26,27]. We then investigated whether CPA pretreatment could amplify Ca^{2+} release that was independent of InsP_3 . Ionomycin releases intracellular Ca^{2+} simply by an ionophoric mechanism [28]. It has been shown that TG and CPA release Ca^{2+} from internal stores without generating InsP_3 [8,32]. Results in Table 1 clearly show that CPA pretreatment did not potentiate subsequent TG-induced Ca^{2+} release, and vice versa. Also, in contrast with the substantial amplification of the agonist-induced Ca^{2+} release by CPA, the ionomycin-induced Ca^{2+} release was relatively weakly amplified.

Low concentrations of ATP and histamine exhibit synergism in releasing intracellular Ca^{2+}

Since moderate $[\text{Ca}^{2+}]_i$ elevation induced by CPA, TG or ionomycin magnifies agonist-induced Ca^{2+} release, as demon-

Figure 7 Effects of various concentrations of CPA, TG and ionomycin on histamine-induced Ca^{2+} release

Fura-2-loaded cells in 0.1 mM EGTA-supplemented Ca^{2+} -free HBSS were pretreated with dimethyl sulphoxide or various concentrations of CPA or TG for approx. 1.5 min, or ionomycin for 0.5 min (at which time the ionomycin-elevated $[\text{Ca}^{2+}]_i$ reached the peak), before histamine (6 μM) stimulation. $\circ$, $[\text{Ca}^{2+}]_i$ elevated by CPA, TG or ionomycin; $\bullet$, represents histamine-induced $[\text{Ca}^{2+}]_i$ elevation, which is the difference between the histamine-elevated $[\text{Ca}^{2+}]_i$ peak and the basal $[\text{Ca}^{2+}]_i$ or the $[\text{Ca}^{2+}]_i$ elevated by CPA, TG, or ionomycin. Each point is the mean $\pm$ S.E.M. of 3 separate experiments: *significantly different ($P < 0.05$) from the histamine-induced Ca^{2+} release without drug pretreatment.

strated by the results in Figure 7, it is of great interest to test whether moderate $[\text{Ca}^{2+}]_i$ elevation induced by low concentrations of ATP could enhance Ca^{2+} mobilization triggered by low concentrations of histamine, or vice versa. However, unlike the $[\text{Ca}^{2+}]_i$ elevation induced by 3 μM CPA, which remained relatively stable at the peak for some 10 s or so, and then declined slowly (see Figures 3–5 and 8; low concentrations of TG and ionomycin showed similar Ca^{2+} -transient profiles), that induced by low concentrations of histamine or ATP was very transient (Figures 4–6, 8 and 9). It is therefore impossible to stimulate the cells with the second agonist at a relatively stable level of $[\text{Ca}^{2+}]_i$ pre-elevated by the first agonist. To resolve this problem, we adopted an alternative to stimulate the cells with both agonists simultaneously to test whether they could synergistically mobilize Ca^{2+} . Figure 9 clearly shows that sub-optimal, but not optimal,

Figure 8 Time course of amplification of histamine-induced Ca^{2+} release

Fura-2-loaded cells in 0.1 mM EGTA-supplemented Ca^{2+} -free HBSS were pretreated with either dimethyl sulphoxide (DMSO) or 3 μM CPA for various times before histamine (HA; 6 μM) stimulation (a–c). In (d) and (e) 3 μM ionomycin (IONO) was added finally to test the size of the ionomycin-releasable pool after prolonged CPA treatment. In (f) and (g), 100 μM ATP was added after treatment for 11–13 min with DMSO or CPA to check the status of the agonist-sensitive pool. Similar traces were obtained in at least two other separate experiments.

Table 1 Effects of CPA or TG pretreatment on subsequent InsP_3 -dependent and -independent Ca^{2+} release

Fura-2-loaded cells in 0.1 mM EGTA-supplemented Ca^{2+} -free HBSS were pretreated with dimethyl sulphoxide, 3 μM CPA or 0.6 nM TG for approx. 1.5 min before addition of 6 μM histamine, 3 μM CPA, 0.6 nM TG or 1 nM ionomycin. $[\text{Ca}^{2+}]_i$ elevated is the difference between the histamine-, TG-, CPA- or ionomycin-elevated $[\text{Ca}^{2+}]_i$ peak and the basal $[\text{Ca}^{2+}]_i$ or the $[\text{Ca}^{2+}]_i$ pre-elevated by TG or CPA. Results are means $\pm$ S.E.M. of 3–4 separate experiments: * indicates significant difference ($P < 0.05$).

Treatments	Elevation of $[\text{Ca}^{2+}]_i$ (nM)
Histamine alone	10.3 ± 1.2
Histamine with CPA pretreatment	50.0 ± 9.7 *
TG alone	26.8 ± 4.5
TG with CPA pretreatment	25.8 ± 3.1
CPA alone	32.3 ± 1.0
CPA with TG pretreatment	39.8 ± 4.0
Ionomycin alone	11.0 ± 2.0
Ionomycin with CPA pretreatment	20.7 ± 2.4 *

concentrations of histamine and ATP exhibited synergism ($P < 0.05$) in releasing intracellular Ca^{2+} (1.9-fold of the sum of Ca^{2+} release triggered by each agonist alone).

DISCUSSION

Amplification of Ca^{2+} -dependent physiological processes or Ca^{2+} signalling has frequently been observed, and in many such reports the molecular mechanisms have begun to be elucidated. For example, in dog mesenteric artery and vein, sub-optimal

concentrations of KCl-, Bay K 8644- or endothelin-1-induced amplification of α -adrenergic vasoconstriction in Ca^{2+} -containing medium may possibly be due to enhanced Ca^{2+} influx [13]. Data from Henrion et al. [12] suggest a role of protein kinase C in angiotensin II-mediated enhancement of contraction induced by noradrenaline in rabbit facial artery. Adenosine enhances bradykinin-induced Ca^{2+} entry in pig endothelial cells, possibly by inducing cyclic AMP elevation and subsequent membrane hyperpolarization [15]. Bian et al. [5] observed that in saponin-permeabilized DDT₁MF-2 smooth-muscle cells GTP magnifies IICR, very possibly because this nucleotide mediates a transfer of Ca^{2+} from the InsP_3 -insensitive Ca^{2+} store to the InsP_3 -sensitive Ca^{2+} store, hence enlarging the latter's pool size. Enhanced Ca^{2+} influx of course cannot explain the amplification phenomenon that we observed, since our experiments were performed in the absence of extracellular Ca^{2+} . As explained above, there is no evidence of an enlargement of the InsP_3 -sensitive pool size after CPA treatment in our case. We have not studied the effects of protein kinase C and cyclic AMP on the amplification of agonist-induced Ca^{2+} release in our system, and therefore their roles in amplification cannot be excluded. But we consider the involvement of protein kinase C and cyclic AMP unlikely, since there is no compelling evidence to suggest that CPA, TG and ionomycin modulate the cytosolic levels of these intracellular messengers.

It is possible that the amplification of agonist-induced Ca^{2+} release was due to the partial inhibition of the microsomal Ca^{2+} pump, so that Ca^{2+} released by agonists was prevented from being re-sequestered into the internal stores. If this hypothesis is correct, it can be expected that CPA pretreatment will not only amplify Ca^{2+} release by agonists, but also that by other means, for example, that by ionomycin. Indeed, CPA pretreatment also potentiated ionomycin-induced Ca^{2+} release. However, this

Figure 9 Sub-optimal concentrations of histamine and ATP synergize in releasing intracellular Ca^{2+}

Fura-2-loaded cells in 0.1 mM EGTA-supplemented Ca^{2+} -free HBSS were stimulated with sub-optimal (a and b) or optimal (d and e) concentrations of histamine (HA) or ATP, or a combination of the two agonists (c and f). Traces shown are representative of results from three separate experiments.

mechanism can only partially explain the amplification of agonist-induced Ca^{2+} release, because (a) ionomycin, which does not inhibit microsomal Ca^{2+} pumps, also caused significant amplification of agonist-induced Ca^{2+} release, and (b) the CPA amplification of agonist-induced Ca^{2+} release is much more substantial than the CPA amplification of ionomycin-induced Ca^{2+} release (4.9-fold versus 1.9-fold). The latter phenomenon not only suggests that the prevention of Ca^{2+} re-uptake plays a minor role in amplification of agonist-induced Ca^{2+} release, but also hints that amplification of agonist-induced Ca^{2+} release is largely dependent on InsP_3 formation.

For all the three drugs (CPA, TG and ionomycin) that caused amplification, the relation between the drug-induced slight $[\text{Ca}^{2+}]_i$ elevation and the subsequent amplified agonist-induced Ca^{2+} release (left sides of bell-shaped curves) is, both qualitatively and quantitatively, very similar. This strongly suggests that it is Ca^{2+} , but not a particular drug, which causes the amplification effect. As explained above, only InsP_3 -dependent Ca^{2+} release can be prominently amplified. Therefore, one of the mechanisms of the amplification of agonist-induced Ca^{2+} release in the HL-60 cell system might be that a moderate $[\text{Ca}^{2+}]_i$ pre-elevation could substantially magnify IICR. Indeed, there has been growing evidence that Ca^{2+} concentration regulates IICR in a biphasic manner [17–21]. Pioneering studies in permeabilized guinea-pig

taenia caeci smooth-muscle cells, rat brain synaptosomal vesicles and dog cerebellum endoplasmic-reticulum vesicles reveal that Ca^{2+} at 300 nM or below enhances, whereas over 300 nM inhibits, IICR [17–19]. A recent study in rat hepatocytes reveals similar findings [20]. Nevertheless, these studies on the regulation of IICR by Ca^{2+} have to be treated with caution, because those workers used either permeabilized cells or microsomal vesicle preparations, which might not totally reflect the physiological environment of the cytosol in intact cells. Our observation in intact HL-60 cells, that a moderate elevation of $[\text{Ca}^{2+}]_i$ substantially amplifies subsequent agonist-induced Ca^{2+} release, provides evidence supporting the notion that a mild $[\text{Ca}^{2+}]_i$ elevation can exert a magnifying effect on IICR. In the aforementioned reports [17–20] showing that Ca^{2+} can exert a magnifying effect on IICR, it was demonstrated that Ca^{2+} at around 300 nM was the optimal concentration for IICR enhancement. However, our results show that the optimal condition for amplification was a $[\text{Ca}^{2+}]_i$ elevation of 12–40 nM above the basal value, which ranged from 40 to 80 nM. A larger elevation (120 nM above basal level) failed to produce amplification. Perhaps the failure was due to the depletion of the agonist-sensitive pool by high concentrations of CPA, TG or ionomycin; alternatively, the InsP_3 receptors of HL-60 cells might be optimally 'sensitized' by a 12–40 nM elevation of $[\text{Ca}^{2+}]_i$, so that a higher $[\text{Ca}^{2+}]_i$ rise becomes less effective or ineffective. Perhaps InsP_3 receptors in different cell types display different Ca^{2+} -sensitivity. It is noteworthy that, in 3T6 fibroblasts, Zhang et al. [21] reported a biphasic effect of Ca^{2+} on IICR, the optimal concentration of Ca^{2+} for IICR being 60 nM. In addition to the explanation just proposed, there is a possibility that the amplification of agonist-induced Ca^{2+} release was caused by an enhanced InsP_3 formation upon agonist stimulation. This possibility is currently under investigation.

Of particular relevance to our study, Blackmore [33] recently reported that in mature human sperm TG elevated $[\text{Ca}^{2+}]_i$ and potentiated the effect of progesterone to raise $[\text{Ca}^{2+}]_i$. However, his data reveal that progesterone, even at high concentration, could not raise $[\text{Ca}^{2+}]_i$ in Ca^{2+} -free medium (that is, it does not seem that progesterone can release intracellular Ca^{2+}), and the potentiation effect was only observed in Ca^{2+} -containing medium. Therefore, the potentiation effect observed by Blackmore [33] presumably has a mechanism different from that reported here, since we carried out our experiments in Ca^{2+} -free medium and showed that internal Ca^{2+} release could be amplified.

In vivo, it is likely that cells are exposed to sub-optimal concentrations of agonist, and very often cells are acted on by several agonists simultaneously. That a moderate $[\text{Ca}^{2+}]_i$ elevation can exert a substantial enhancing effect on subsequent Ca^{2+} release by sub-optimal concentrations of agonist, as demonstrated in our study, bears the physiological implication that agonists might act synergistically *in vivo* through amplification of Ca^{2+} signalling, and may partly account for the synergistic effect of low concentrations of ATP and histamine on Ca^{2+} mobilization.

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