Calcium homeostasis in mouse fibroblast cells: affected by U-73122, a putative phospholipase C_β blocker, via multiple mechanisms

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1 The inhibitory effects of the putative phospholipase C_β inhibitor, U-73122, on ligand-induced and thapsigargin-induced [Ca²⁺]_i transients were investigated in mouse fibroblast cells (the L line).

2 Ca²⁺ release from intracellular stores was stimulated either by ATP (and also by UTP or ADP) working through the activation of a P_{2U} receptor, or by lysophosphatidic acid, which elicited a more pronounced response.

3 U-73122 inhibited the Ca²⁺ mobilization produced by all the agonists in a dose-dependent manner, consistent with a mode of action involving phospholipase C inhibition.

4 In addition, however, U-73122 slowed the kinetics of intracellular Ca²⁺ release induced by the Ca²⁺-ATPase inhibitor, thapsigargin, and reduced the influx of Ca²⁺ across the plasma membrane, following stimulation of store-dependent influx by the latter.

5 We conclude that U-73122 has multiple sites of action, all of which can lead to a change in Ca²⁺ homeostasis. Thus, particular caution is recommended when employing this agent and when interpreting the results obtained.

Keywords: U-73122; fibroblast; calcium homeostasis; phospholipase C; store-dependent Ca²⁺ influx; thapsigargin-induced Ca²⁺ release

Introduction

The release of intracellular Ca²⁺, induced by the activation of many surface receptors, is a multistep process that includes, in sequence: (1) ligand binding and the activation of the receptor and its interaction with a G protein, (2) the dissociation of the G protein and guanine nucleotide exchange, (3) the activation of phospholipase C_β (PLC) by the α subunit, with hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂), (4) the release into the cytosol of the second messenger, inositol 1,4,5-trisphosphate (IP₃) and its binding to the intracellular receptors located on the rapidly exchanging Ca²⁺ stores, and (5) the release of stored Ca²⁺ (Berridge, 1993). Experimental investigation of the role of the various molecular components in this process has been greatly assisted by the existence of specific blockers and activators, effective at different stages of the signal transduction pathway. For example, aluminium fluoride can be used to stimulate IP₃ generation via an activation of the G proteins and, if there is access to the cell cytosol, heparin can be applied to block the IP₃ receptor. However, we are still limited by our inability to prevent the generation of IP₃ in intact cells. It was therefore of interest when the aminosteroid, U-73122, was reported to be an inhibitor of the PLC enzyme (Smith *et al.*, 1990; Bleasdale *et al.*, 1990). This membrane permeable compound was shown to inhibit the production of radio-labelled inositol phosphates (lithium blocked metabolism) in SK-N-SH neuroblastoma cells, when stimulated with oxotremorine, as well as to prevent the induced transients of cytosolic free Ca²⁺ ([Ca²⁺]_i) (Thompson *et al.*, 1994). Furthermore, in rat pancreatic acinar cells, which exhibit cholecystokinin-evoked [Ca²⁺]_i oscillations, U-73122 was found to block the oscillatory activity (Yule & Williams, 1992). Thus, U-73122 was concluded to be a valuable tool with which to test the involvement of IP₃ generation in physiological processes.

In the course of our studies on Ca²⁺ homeostasis in mouse fibroblasts (L cells), we have found that U-73122 is indeed

very effective in inhibiting the agonist-induced [Ca²⁺]_i transients sustained by release of the cation from intracellular stores. However, we also found that the compound can affect Ca²⁺ homeostasis in ways which appear to be unrelated to an interaction with PLC. In particular, the drug slowed the kinetics of intracellular Ca²⁺ release induced by the endoplasmic-sarcoplasmic reticulum Ca²⁺ ATPase (SERCA) inhibitor, thapsigargin (TG), and reduced the influx of Ca²⁺ that follows the emptying of intracellular stores (Clementi *et al.*, 1992). These additional effects of U-73122 can clearly complicate the interpretation of experiments in which the compound is used.

Methods

Cell culture

Mouse L fibroblasts (ATCC CCL 1, clone 929, a kind gift from Dr A. Mantovani, Mario Negri Inst. of Pharmacology, Milan) were plated in 10 cm plastic dishes and maintained in Dulbecco's modified Eagle's medium, supplemented with 10% foetal bovine serum, 2 mM glutamine and antibiotics, and at 37°C in a humidified, 5% CO₂ atmosphere. After culturing for 5–7 days, the cells were removed by light trypsinization and a stream of Ca²⁺-free saline.

Determination of [Ca²⁺]_i

Detached cells were loaded with fura-2 AM (2 μM from Calbiochem, San Diego, CA, U.S.A.) for 45 min in a HEPES-buffered saline (KRH): (mmol⁻¹) NaCl 125, KCl 5, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 2, glucose 6, HEPES, 25 (pH 7.4), then centrifuged and resuspended in KRH containing 250 μM sulfinpyrazone. They were stored at room temperature, at a density of 2 × 10⁶ ml⁻¹ until use. For each experiment, the cells were diluted in 1:2 in KRH and transferred to a quartz cuvette of either 1.5 or 3 ml volume

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(volume of cell suspension 0.75 or 1.5 ml, respectively). Fluorescence measurements were made with both LS-5 and LS-50 fluorimeters (Perkin-Elmer) and with both single- and dual-wavelength modes, at 37°C. The cells were constantly stirred at a moderately slow rate. Each trace was calibrated at the end with EGTA and Ca^{2+} after Triton X-100 permeabilization. Data from the LS-50 was transferred to a spreadsheet programme for offline analysis and plotting. $[\text{Ca}^{2+}]_i$ was estimated by the equation:

$$[\text{Ca}^{2+}]_i = K_d \times (F - F_{\min}) / (F_{\max} - F)$$

Mn^{2+} quenching of fura-2 fluorescence

As described previously (Clementi *et al.*, 1992), cells loaded with fura-2 were resuspended in Ca^{2+} -free KRH with reduced phosphate (0.6 mM KH_2PO_4) and no sulphate ions. MnCl (50 μM) was added to the cells at the start of the experiment.

Materials

U-73122 and U-73343 (1-[6-[[17 β -3-methoxyestra-1-3-5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione and 1-[6-[[17 β -3-methoxyestra-1-3-5(10)-trien-17-yl] amino] hexyl]-2,5-pyrrolidine-dione, respectively) were purchased from Biomol Research Labs (Plymouth Meeting, PA, U.S.A.) and dissolved in dimethylsulphoxide (DMSO) at 2 mM. TG (Calbiochem) was 100 μM in DMSO. Unless stated otherwise, all additional chemicals were purchased from Sigma, St. Louis, MO, U.S.A.

Results

Suspensions of L fibroblast cells respond to ATP via a putative $\text{P}_{2\text{U}}$ (or P_{2Y_2}) type receptor (Cockcroft & Gomperts, 1979; Watson & Girdlestone, 1993; Abbracchio & Burnstock, 1994; Grierson & Meldolesi, 1995) with a release of stored intracellular Ca^{2+} to produce a rapid elevation of $[\text{Ca}^{2+}]_i$. At maximal ATP concentration, 0.1 mM, the peak amplitude was 320 ± 23 nM ($n = 12$; see Figure 1). Results similar to those obtained with ATP were observed when UTP was employed at the same concentrations whereas ADP induced only minor responses even when used at 0.5 mM concentration (not shown). There appeared to be no major contribution from external Ca^{2+} to the ATP-induced rise, since the response was apparently unchanged in Ca^{2+} -free (1 mM Ca^{2+} /2 mM EGTA) saline. Furthermore, prior depletion of the internal Ca^{2+} stores by the SERCA-type Ca^{2+} -ATPase inhibitor, TG, prevented the ATP-induced Ca^{2+} transient (not shown). The cells also responded, with a release of intracellular Ca^{2+} , to low concentrations of lysophosphatidic acid (LPA) (Jalink *et al.*, 1990; Durieux & Lynch, 1993). This phospholipid was effective over the range 0.01–10 μM producing, at maximal concentrations, a Ca^{2+} transient of greater amplitude and duration than that produced by ATP (peak amplitude 435 ± 33 nM, $n = 5$; Figure 1).

Inhibition of Ca^{2+} transients by U-73122

When the aminosteroid, U-73122, was added to the cells prior to stimulation, a dose-dependent inhibition of agonist-induced $[\text{Ca}^{2+}]_i$ transients was produced, irrespective of which agonist was used. Figure 2a illustrates the inhibition by U-73122 of the Ca^{2+}_i transients induced by 100 μM ATP, and similar families of curves were produced for other agonists, i.e. 100 μM UTP and 10 μM LPA. For LPA, an attenuation was observed over the range 1 to 8 μM , with an apparent EC_{50} around 3.5 μM (Figure 2b) and complete inhibition at the highest concentration. For ATP the inhibition curves were shifted further to the left and an apparent EC_{50} , for U-73122, of around 1.5 μM was obtained (Figure 2b). This difference could have been expected because of the stronger response induced by LPA with respect to ATP. In agreement

Figure 1 Concentration-dependence of the $[\text{Ca}^{2+}]_i$ responses by ATP and lysophosphatidic acid (LPA) in mouse L fibroblasts. Data shown are averages of results obtained in a total of 12 (ATP, ●) and 5 (LPA, ■) experiments. $[\text{Ca}^{2+}]_i$ of resting cells was 108 ± 25 nM. The concentration values in the abscissa scale run from 10^{-7} to 10^{-4} M for ATP and from 10^{-8} to 10^{-5} M for LPA.

Figure 2 Inhibition by U-73122 of ATP and lysophosphatidic acid (LPA)-induced Ca^{2+} mobilization. (a) Inhibition by various concentrations of U-73122 of ATP-induced Ca^{2+} mobilization. The series of $[\text{Ca}^{2+}]_i$ transients were stimulated by the maximal concentration (100 μM) of ATP added at the arrow in the presence of 0, 2, 4 and 6 μM U-73122. The traces shown are representative of the results obtained in at least three experiments. (b) Inhibition curves for U-73122 and U-73343 on Ca^{2+} mobilization by 100 μM ATP (● and ◆, respectively) and for U-73122 on 10 μM LPA (■). Data are expressed as a percentage of the control response (vehicle alone) amounting to 310 and 415 nM for ATP and LPA, respectively. Values shown in (b) are means $\pm$ s.e. of three separate experiments.

with earlier reports (Thompson *et al.*, 1994), we found that U-73122 was very sensitive to the protein concentration and, therefore, the EC_{50} increased with cell density (a five fold increase of density induced an approximate doubling of the apparent EC_{50} , not shown). It has been suggested that the closely related compound U-73343, which has a succinimide side chain instead of the maleimide chain of U-73122, may provide a control for U-73122 (Yule & Williams, 1992). We found that this compound still attenuated the ATP-induced peak $[\text{Ca}^{2+}]_i$, however, to a maximum of only about 18% (Figure 2b). With LPA the attenuation was similar (maximum 15% not shown), while DMSO, the vehicle for both these compounds, had no inhibitory effect up to a maximal concentration of 0.4% (not shown).

Effect of U-73122 on TG-induced Ca^{2+} release

The release of stored intracellular Ca^{2+} by ionomycin was unaffected by treatment with U-73122 (not shown). In contrast, close examination of the kinetics of Ca^{2+} release after 0.1 μM TG addition revealed that, when compared with controls, release of Ca^{2+} in the presence of U-73122 was slowed. In control experiments, in a Ca^{2+} -free medium, TG induced an increase in $[\text{Ca}^{2+}]_i$ which peaked after 16 ± 4 ($n = 5$) s and then began to decline slowly (Figure 3). In the presence of 5 μM U-73122 on the other hand, the time required to attain the peak response was retarded by 10–20 s (mean 30 ± 8 ; $n = 5$) and the overall shape of the transient was also more rounded, although there was no significant change in peak height (Figure 3). In some preparations, the recovery of Ca^{2+}_i to resting levels, after TG treatment, was retarded in the presence of U-73122. In contrast, U-73343 had no effect on the release kinetics of TG. Previous experiments have suggested that mouse L cells exhibit a constitutive activation of ATP P_{2U} receptors, from low levels of endogenous ATP released into the KRH, with a moderate stimulation of PIP_2 breakdown (Grierson & Meldolesi, 1995). We considered the possibility that the slowing effect of U-73122 on TG-induced release was caused by a decrease in the cytosolic concentration of IP_3 , via the known effect of U-73122 on PLC. Indeed, when the activation of ATP receptors was prevented by adding the ATPase, apyrase, to the KRH, it was found that the rate of Ca^{2+} release was similarly retarded when compared with untreated control (not shown).

Effect of U-73122 on Ca^{2+} influx

When the intracellular Ca^{2+} pools were depleted by TG in the presence of EGTA, the re-introduction of Ca^{2+} to the medium resulted in an overshoot in the $[\text{Ca}^{2+}]_i$ rise which then declined to an elevated plateau (Figure 4a). These events are known to be due to the activation of a Ca^{2+} influx that is carefully related to the depletion of the stores (Clementi *et al.*, 1992). It was found that if U-73122 was administered prior to Ca^{2+} re-introduction, the Ca^{2+} -overshoot above the step rise in fluorescence, due to extracellular fura-2, became hardly appreciable (Figure 4a). This inhibition by U-73122 of Ca^{2+} influx across the plasma membrane was further investigated by using the manganese-quench technique. In these experiments, increased cationic influx was indicated by an increase in the rate of fluorescence quenching at the Ca^{2+} -insensitive wavelength of fura-2, 365 nm. Figure 4b shows that under control conditions, the Mn^{2+} -induced reduction in cellular fluorescence was 50% over 3 min. This value was halved in the presence of 5 μM U-73122, thus confirming that cationic influx was inhibited.

Discussion and Conclusions

In this investigation, we have shown that the putative PLC inhibitor, the aminosteroid, U-73122, has multiple effects on Ca^{2+} homeostasis. It appears not only to inhibit the effects

that result from the generation of IP_3 , i.e. the release of Ca^{2+} from intracellular stores, but also to affect the release that follows SERCA blockade, which is widely believed to be IP_3 -independent and to involve a store-dependent Ca^{2+} influx across the plasma membrane.

Figure 3 The effect of U-73122 on the thapsigargin (TG)-induced $[\text{Ca}^{2+}]_i$ transients. U-73122 or vehicle was administered at the arrow and this was followed by 100 nM TG (arrowhead). With vehicle alone (upper trace) TG induced a fairly rapid release of intracellular Ca^{2+} whereas in the presence of 5 μM U-73122 (lower trace), release was markedly slower. In this example, the rates of $[\text{Ca}^{2+}]_i$ rise are 21.6 and 9.2 nM s^{-1} , respectively, with the peak delayed in the U-73122-treated cells by approximately 14 s. Traces shown are representative of 5 separate experiments.

Figure 4 The effect of U-73122 on the thapsigargin (TG)-induced Ca^{2+} influx. (a) Following the depletion of internal Ca^{2+} pools by TG (0.1 μM , arrows) in the presence of 1 mM EGTA, the addition of vehicle (V) and the restitution of $[\text{Ca}^{2+}]_o$ (2 mM) induces an overshoot in the $[\text{Ca}^{2+}]_i$ transient (upper trace). In the lower trace U-73122 (5 μM) was added to the cuvette at 'U'. The cellular response when $[\text{Ca}^{2+}]_o$ was re-added shows that the Ca^{2+} overshoot was largely blocked inasmuch as the visible step rise in fluorescence is primarily due to extracellular fura-2. (b) Shows the effect of U-73122 on Mn^{2+} quenching of 365 nm fluorescence. The upper trace shows the addition of 50 μM Mn^{2+} at the arrowheads, followed by 5 μM U-73122 (U). The lower trace shows the effect of vehicle alone (V). Traces shown are representative of 5 (a) and 3 (b) separate experiments.

U-73122 was found to produce inhibition of $[\text{Ca}^{2+}]_i$ transients induced by ATP and LPA, and the active range of concentrations was similar to that reported for SK-N-SH neuroblastoma cells and rat pancreatic acinar cells (Thompson *et al.*, 1994; Yule & Williams, 1992). These effects appear to be consistent with the proposed mechanism of action of the aminosteroid, i.e., the disruption of the G-protein/phospholipase C_β coupling and inhibition of IP_3 synthesis (Thompson *et al.*, 1994). An additional effect of U-73122, namely disruption of the processing of phosphoinositides upstream of PIP_2 hydrolysis, could decrease PIP_2 availability (Vickers, 1993) and thus also contribute to the effect.

An explanation for the U-73122-induced slowing of Ca^{2+} release observed after SERCA blockade by TG is via a reduction in the intracellular concentration of IP_3 . Indeed, our results with apyrase have revealed an at least partial disappearance of the aminosteroid effect when the autocrine stimulation by ATP was prevented. Moreover, other investigators (for example, Short *et al.*, 1993) have noted that the emptying of the Ca^{2+} store by TG is faster when combined with IP_3 . However, it remains possible that U-73122 has a direct effect on the SERCA of these cells. Preliminary results (J. Grierson, unpublished observations) have shown that U-73122 can cause the emptying of the rapidly exchanging Ca^{2+} stores in a PC12 cell subclone (Clementi *et al.*, 1993).

TG-induced depletion of the rapidly exchanging Ca^{2+} pool has widely been employed to evoke the inward Ca^{2+} current which is used to replenish the store, known as 'capacitative entry' or 'store-dependent calcium influx' (SDCI) (Clementi *et al.*, 1992; Meldolesi, 1993). Many investigators believe that this current is initiated by a retrograde signal released from the pool, but the identity of such a message remains elusive (Meldolesi, 1993). The store dependent calcium influx of L cells appears to be relatively weak, in that there is no prolonged plateau of elevated $[\text{Ca}^{2+}]_i$, only a transient response

when Ca^{2+} is re-introduced into the medium. This over-shoot was selectively eliminated by U-73122. An inhibition of IP_3 is not likely to be involved in this instance, since previous studies have shown that TG does not alter IP_3 synthesis or its metabolism (Thastrup *et al.*, 1990; Ely *et al.*, 1991). Possible sites of action could therefore be at the plasma membrane Ca^{2+} channel or at some stage of the retrograde signalling. It may be pertinent that recent evidence suggests that the retrograde messenger could be a small G protein (Fasolato *et al.*, 1993), i.e. a protein partially homologous to the α subunit of the trimeric G protein with which U-73122 is believed to react.

In conclusion, U-73122 seems not to act at just one site, as suggested previously, to provoke a change in Ca^{2+} homeostasis. Although the coupling of activated G protein to PLC_β appears to be the most important process disrupted by this compound, changes occur in Ca^{2+} flux across the membrane and probably also in the function of the SERCA Ca^{2+} -ATPase. These last two effects could contribute significantly to the overall effects of the aminosteroid. For example, $[\text{Ca}^{2+}]_i$ oscillations are known to involve the rhythmic uptake and release of Ca^{2+} from the IP_3 -sensitive stores and to be sustained by the continuous supply of Ca^{2+} via trans-plasma-membral influx (Harootunian, 1988; Berridge, 1993; Meldolesi, 1993). Thus, the blockade of Ca^{2+} oscillations by U-73122 could result not only from the disruption of IP_3 synthesis, but also from the two other coordinate effects revealed in the present study. Thus, the use of U-73122 as an experimental tool requires special caution and careful characterization of all of the effects of the drug in the cell type investigated.

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