Phospholipase C inhibitor, U73122, releases intracellular Ca^{2+}, potentiates Ins(1,4,5)P_3-mediated Ca^{2+} release and directly activates ion channels in mouse pancreatic acinar cells

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It is recognized in many cellular systems that the receptor/G-protein activation of phospholipase C and Ins(1,4,5)P_3 production is the transduction pathway regulating the release of Ca^{2+} from internal stores. Ca^{2+} signals can now be monitored at the level of single cells but the biochemical detection of Ins(1,4,5)P_3 cannot match this resolution. It is often difficult or impossible to directly attribute responses evoked in single cells by putative phospholipase C-coupled agonists to changes in Ins(1,4,5)P_3 levels. U73122 is an aminosteroid that is reported to act as a specific inhibitor of phospholipase C and it has become an important tool in establishing the link between phospholipase C activation and cellular Ca^{2+} signalling. In the present study we use both patch-clamp electrophysiology and the imaging of fluorescent Ca^{2+} indicators to investigate the effect of U73122 in mouse pancreatic acinar cells. The study reveals that U73122 has effects other than the inhibition of phospholipase C. U73122 can directly activate ion channels. It can itself promote the release of Ca^{2+} from intracellular stores in permeabilized cells and in intact cells it triggers a release of Ca^{2+} that is initiated specifically at the secretory pole of these morphologically and functionally polarized cells. We also present evidence that U73122 can potentiate the response to Ins(1,4,5)P_3; this is seen both in permeabilized cells and in patch-clamp protocols in which cells are internally dialysed with submaximal concentrations of Ins(1,4,5)P_3. The effects of U73122 are therefore multiple and not specific for the inhibition of phospholipase C. Importantly, all the effects described influence Ca^{2+} signalling yet in many experimental protocols some of these effects can go unnoticed and might in error be attributed simply to the inhibition of Ins(1,4,5)P_3 production.

INTRODUCTION

The effects of a wide range of neurotransmitters and hormones are now known to be mediated by interaction with receptors that are coupled to heterotrimeric G-proteins, which in turn regulate activity in the enzyme phospholipase C. The activation of phospholipase C results in the hydrolysis of PtdIns(4,5)P_2 and the production of two intracellular messengers, Ins(1,4,5)P_3 and diacylglycerol. The role of Ins(1,4,5)P_3 in the regulation of intracellular Ca^{2+} levels is now well established and it is known to bind to a specific Ins(1,4,5)P_3 receptor resulting in the activation of an integral Ca^{2+} channel promoting the release of Ca^{2+} sequestered into internal stores [1]. It is now recognized that there is great diversity in the spatiotemporal pattern of Ins(1,4,5)P_3-mediated Ca^{2+} signalling in different tissues and much work is still being done to characterize the inositol polyphosphate-mediated Ca^{2+} signals in individual tissues and at different concentrations of agonists [2–4]. This work is often hindered by certain difficulties associated with the investigation of inositol polyphosphate-mediated Ca^{2+} signalling. The biochemical detection of Ins(1,4,5)P_3 requires the use of substantial amounts of tissue, yet Ca^{2+} signals can now be readily measured in single isolated cells. The measurement of Ins(1,4,5)P_3 production in populations of cells in many instances will not necessarily reflect the amplitude or dynamics of inositol polyphosphate production and metabolism in individual cells [3]. Another major problem is that to obtain measurable changes in inositol polyphosphates high concentrations of agonists have to be used. In fact, at the low concentration of agonists that can generate significant and reproducible Ca^{2+} signals in isolated cells (most probably the physiological concentrations) there is generally no measurable change in polyphosphate levels, leading some to question whether there is any significant change in Ins(1,4,5)P_3 concentration at these levels of stimulation [2,3,5,6]. It is therefore difficult, if not impossible, to correlate the Ca^{2+} signals in isolated cells directly with Ins(1,4,5)P_3 metabolism.

A potentially important breakthrough came when it was reported that the aminosteroid U73122 was a specific inhibitor of phospholipase C-mediated hydrolysis of PtdIns(4,5)P_2 [7,8]. In a study in rat pancreatic acinar cells the parallelism between the inhibition of Ins(1,4,5)P_3 production and the inhibition of Ca^{2+} signalling was very marked [9]. U73122 is now widely employed as a tool in the characterization of inositol polyphosphate-mediated Ca^{2+} signalling. More recently, however, a study on isolated rabbit pancreatic acinar cells reported that U73122 could itself act to release Ca^{2+} from intracellular stores and could in fact give rise to oscillations in intracellular Ca^{2+} levels [10]. It is not known whether the discrepancy between these two pancreatic acinar cells preparations can be explained as simply being due to species differences, though this is clearly an important point. The mouse pancreatic acinar cell preparation has been extensively employed in the investigation of the role of Ins(1,4,5)P_3 in Ca^{2+} signalling. In this tissue the combination of patch-clamp electrophysiological recording of Ca^{2+}-activated membrane currents and direct measurement of intracellular Ca^{2+} levels by means of fluorescent indicators have revealed important information about the role of Ins(1,4,5)P_3 in the generation of oscillating Ca^{2+} signals [11–13]. In particular the studies on mouse acinar cells have revealed the ability of Ins(1,4,5)P_3 at a fixed concentration to evoke discrete Ca^{2+} signals localized to one, the secretory pole, of these morphologically and functionally polarized cells [13]. The aim of the present study was to examine

Abbreviation used: ACh, acetylcholine.

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the effects of U73122 in the mouse tissue where inositol polyphosphate signalling had already been extensively investigated. The study reveals that in these cells U73122 [used at concentrations that are reported to block Ins(1,4,5)P_3 production] has a variety of effects other than the inhibition of phospholipase C: it directly activates ion channels, it promotes release of Ca^{2+} from internal stores and it potentiates Ins(1,4,5)P_3-mediated Ca^{2+} release. All of these effects interfere markedly with any role of U73122 as a phospholipase C inhibitor and make it extremely complex to interpret the results from the use of this agent.

MATERIALS AND METHODS

Cell preparation

Pancreata from male CD1 mice were injected and incubated with 200 units/ml collagenase (type CLSPA, Worthington Biochemical) as previously described to yield a mixture of single isolated acinar cells and small acinar cell clusters [11,13].

Patch-clamp electrophysiology

Current recordings were performed with an EPC-7 patch-clamp amplifier (List Medical Ltd., Darmstadt, Germany) in the whole-cell and cell-excised configuration of the patch-clamp technique [11–13].

The whole-cell current recordings were obtained by voltage steps from a holding potential of −48 mV to a potential of 0 mV. Each potential was held for 150 ms and the stepping frequency was 3 Hz. At the resolution shown in the figures the current traces at the two potentials appear continuous. The standard extracellular solution contained (mM): 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 1.0 $CaCl_2$, 10 glucose and 10 Hepes/NaOH at pH 7.2. The Ca^{2+}-free extracellular solution had no Ca^{2+} and 1 mM EGTA added. The standard intracellular pipette solution was (mM): 135 potassium glutamate, 20 NaCl, 1.13 $MgCl_2$, 0.1 EGTA, 2.0 ATP and 10 Hepes/KOH at pH 7.2. In one series of experiments (see figure legends) 10 mM EGTA was employed. The cells were continuously superfused with the extracellular solution at 20–22 °C throughout the whole-cell current recording. With the solutions employed the electrochemical equilibrium potential for Cl^- was at the holding potential of 48 mV and the equilibrium potential for currents in the non-selective cation channels was at the second, 0 mV, value. In this way the two current traces indicate predominantly Cl^- currents at 0 mV and cation currents at −48 mV.

Single-channel currents were recorded in excised inside-out patches of membrane. The pipette was filled with the standard extracellular solution and the solution bathing the cytosolic face of the membrane contained (mM): 145 KCl, 1.13 $MgCl_2$ and 10 glucose at pH 7.2. This solution had either no Ca^{2+} and 1 mM EGTA added (Ca^2-free) or the Ca^{2+} concentration was set at 5 μM by using a Ca^{2+}/EGTA mixture [14]. Single-channel currents were all filtered at 0.2 kHz low pass. With the solutions employed the equilibrium potential for currents in the non-selective cation channels was 0 mV.

Fluorescence measurements

Single acinar cells and small cell clusters were loaded with fura-red by incubation in 3–5 μM fura-red acetoxymethyl ester for 30 min. The fluorescence from the cells was recorded with a Noran Oddysy confocal microscope (Noran Instruments) with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Fura-red fluorescence was calibrated by using cells loaded with dye and exposed to either 10 mM EGTA or 10 mM Ca^{2+} in the presence of 1.5 μM ionomycin in a manner similar to that described by Grynkiewicz et al. [15].

Permeabilized cell preparation

The acinar cells and cell clusters were isolated enzymically as described above. After being washed, the cells were permeabilized by incubation at 25 °C for 7 min in a solution containing 140 mM KCl, 10 mM Hepes and 10 μM digitonin at pH 7.2. Studies with Trypan Blue demonstrated 100% permeabilization. Permeabilized cells were washed twice in the K^+/Hepes solution and transferred to K^+/Hepes supplemented with 10 mM phosphocreatine, 10 units/ml creatine phosphokinase, 10 mM glucose, 1 mM $MgCl_2$ and 10 μM oligomycin. ATP (2 mM) was then added to load the ATP-sensitive Ca^{2+} stores. After 15 min a 1.5 ml suspension of cells was transferred to a quartz cuvette. Fura 2 potassium salt (1 μM) was used as the fluorescent indicator of Ca^{2+} concentration. Fluorescence was monitored in a Perkin Elmer (LS5OB) spectrophotometer with excitation wavelengths of 340 and 380 nm (alternating every 40 ms) and an emission wavelength of 540 nm. The Ca^{2+} concentration was calculated as previously reported [15,16].

Materials

The reagents for the standard solutions were supplied by Sigma (Poole, Dorset, U.K.) unless otherwise stated. Fura-red and fura 2 were from Molecular Probes. U73122 and U73343 were supplied by Affiniti Research Product Ltd.

RESULTS

The initial approach employed was the recording of Ca^{2+}-activated currents in isolated mouse pancreatic acinar cells by means of whole-cell patch-clamp current recording. In the protocols employed, as previously reported, application of the phospholipase C-coupled agonists such as acetylcholine (ACh) or cholecystokinin is associated with the activation of an outward

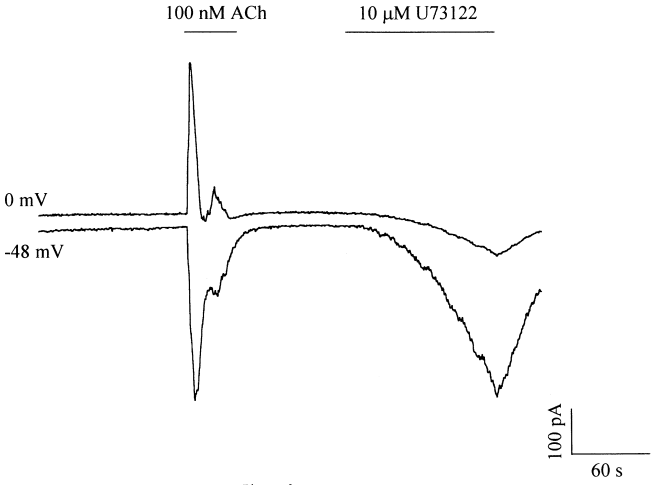

Figure 1 Whole-cell current recording from an enzymically isolated, single pancreatic acinar cell from mouse, comparing the effects of ACh and U73122

The trace shows currents recorded under voltage clamp at 0 and −48 mV. The application of the phospholipase C-coupled agonist ACh (100 nM) is associated with the concomitant activation of large outward (Cl^-) and inward (cation) currents. After the ACh was removed the putative phospholipase C inhibitor U73122 was applied, resulting in the activation of a large inward current in the absence of any outward current.

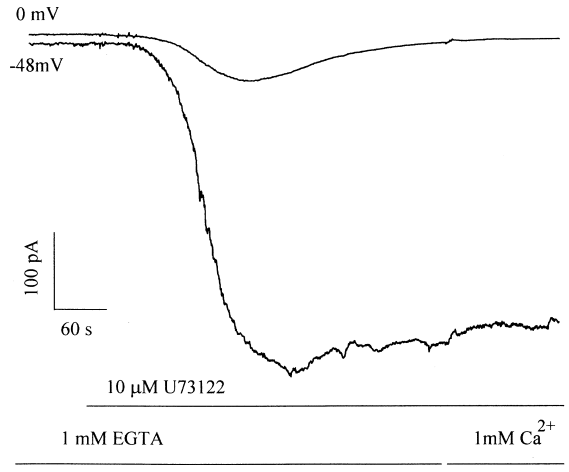

Figure 2 Whole-cell current recording from mouse pancreatic acinar cell showing the effect of U73122 in the absence of extracellular Ca^{2+}

Currents were recorded at 0 and −48 mV. The cell was initially superfused with a Ca^{2+}-free extracellular solution (no Ca^{2+} and 1 mM EGTA added). The application of 10 μM U73122 resulted in a large sustained inward current. When Ca^{2+} was restored to the extracellular solution (1 mM, no EGTA) there was no change in the amplitude of the inward current.

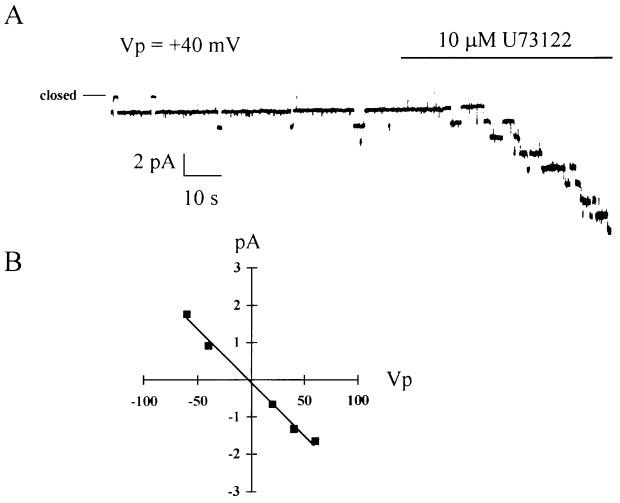

Figure 3 U73122 activates ion channels in excised membrane patches

(**A**) Single-channel current recording from an excised, inside-out patch of membrane from an enzymically isolated mouse pancreatic acinar cell. The recording pipette contained the standard extracellular 140 mM NaCl solution. The solution bathing the cytosolic face of the membrane patch was the standard 140 mM KCl solution with the free Ca^{2+} concentration buffered to 5 μM. The pipette was voltage clamped at +40 mV (the membrane potential inside with respect to outside was −40 mV); inward currents are seen owing to the opening of cation channels that do not discriminate between Na^+ and K^+ ions. The line at 'closed' indicates the zero current level, i.e. all channels closed. At the point indicated, 10 μM U73122 was included in the solution bathing the cytosolic face of the membrane; this is seen to evoke a marked increase in the open probability of the channels. (**B**) Plot of single-channel current amplitude as a function of the pipette potential. The conductance of these channels was approx. 25 pS.

current (seen at 0 mV owing to the opening of Cl^- channels) and an inward current (seen at −48 mV owing to the opening of a cation channel that does not discriminate between Na^+ and K^+) (see Figure 1). It has been established that these current activations directly correspond to, and are due to, the agonist-induced elevation in intracellular Ca^{2+} [13,17]. After a test application of ACh the intention was to apply U73122 and investigate for blockade of the ACh-induced response. U73122 did block responses to ACh (results not shown); however, as shown in Figure 1, the application of U73122 was itself associated with the activation of a large inward cation current ($n = 5$). The absence of any outward Cl^- current already suggested that the cation current activation was not mediated by an elevation in intracellular Ca^{2+}, which would have resulted in the concomitant activation of both outward and inward currents. The Ca^{2+} dependence of the U73122 was investigated by removal of Ca^{2+} from the extracellular medium. The sustained cation current evoked by U73122 did not require the presence of extracellular Ca^{2+}, again indicating that it was not a Ca^{2+}-dependent phenomenon. U73122 activated a sustained cation current in the absence of extracellular Ca^{2+}, and the restoration of Ca^{2+} to the external solution was without effect on the amplitude of the current response (Figure 2) ($n = 5$). In another series of experiments the Ca^{2+} chelator EGTA (10 mM) was included in the solution filling the patch pipette and thereby dialysing the cell interior. The presence of the Ca^{2+} chelator in the patch pipette resulted, as expected, in the blockade of the Ca^{2+}-dependent responses to the phospholipase C-coupled agonist cholecystokinin. It also blocked the U73122-evoked cation current activation (results not shown) ($n = 4$).

To test the effect of U73122 on ion channels directly it was applied to excised inside-out patches of membrane under single-channel current recording conditions. It has been reported that the Ca^{2+}-activated cation channels, on excision from the cells, develop a refractoriness to Ca^{2+}: with time their sensitivity to Ca^{2+} declines and the Ca^{2+} concentrations employed in excised patches to achieve channel activation do not reflect the sensitivity of the channels *in situ* [18]. Nevertheless it is possible to investigate the effects of agents on the kinetics of cation channel opening. In the absence of Ca^{2+} (no Ca^{2+} added and 1 mM EGTA present) in the solution bathing the intracellular, cytosolic face of the membrane, no single-channel currents were observed, as expected. This is because the ion channels require a background Ca^{2+} for activation. In this situation the application of U73122 did not initiate any single-channel currents. If background activity was restored to ion channels by bathing the intracellular face of the excised patches in 5 μM Ca^{2+}, then the application of U73122 is associated with a marked increase in the open probability of the channels (Figure 3A) ($n = 4$). There was no change in the amplitude of the single-channel currents. Figure 3(B) shows the current–voltage relationship for the single-channel currents, which reveals a single-channel conductance of 25 pS, the same as previously reported for the non-discriminating cation channels in this tissue [18].

It is known in these cells that internal dialysis of Ins(1,4,5)P_3, by inclusion in the patch-clamp pipette during whole-cell recording, can mimic agonist activation of membrane currents by releasing Ca^{2+} from intracellular stores and by promoting Ca^{2+} influx across the cell membrane [11,12]. When Ins(1,4,5)P_3 was internally perfused in this manner in the present study (Figure 4) it evoked the characteristic spikes in both inward and outward current that correspond to oscillations in intracellular Ca^{2+} levels [13,17]. When U73122 was applied during such responses to Ins(1,4,5)P_3 it was seen to give rise to a marked potentiation of both the inward, and now outward, currents, leading ultimately to sustained inward and outward current activations ($n = 5$).

We next investigated for any effect of U73122 in permeabilized mouse acinar cells. Figure 5 shows the effect of Ins(1,4,5)P_3 in releasing Ca^{2+} from internal stores in the mouse pancreatic acinar cells. Ca^{2+} release was maximal at 10 μM Ins(1,4,5)P_3. The

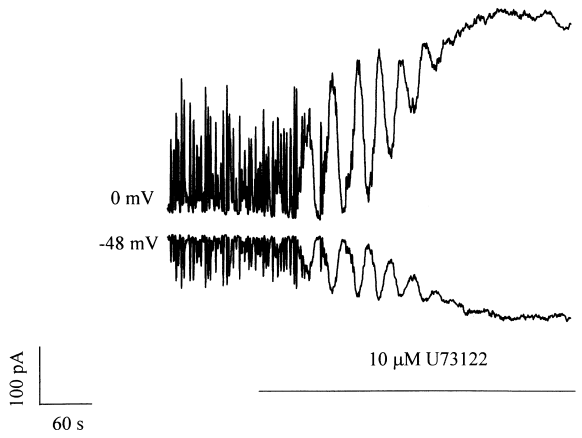

Figure 4 Whole-cell current recording from a mouse pancreatic acinar cell showing the potentiating effect of U73122 on Ins(1,4,5)P_3-induced oscillations

Currents were recorded at 0 and −48 mV. The recording pipette contained 10 μM Ins(1,4,5)P_3. As previously reported, Ins(1,4,5)P_3 internally perfused at this concentration resulted in the generation of repetitive spikes in both inward and outward current. At the point indicated, 10 μM U73122 was applied extracellularly, resulting in a marked potentiation of both inward and outward currents, leading ultimately to sustained activation of both current components.

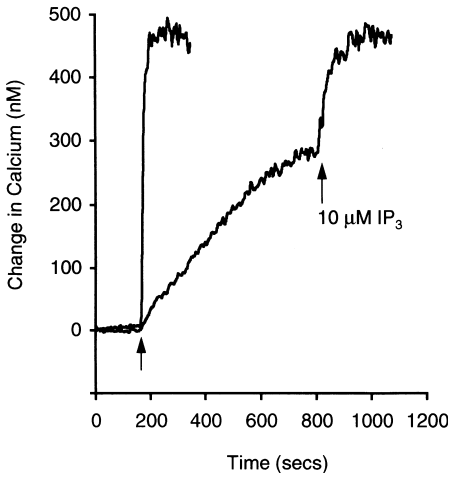

Figure 5 U73122 releases Ca^{2+} from Ins(1,4,5)P_3-sensitive stores in digitonin permeabilized mouse pancreatic acinar cells

Traces from two experiments are superimposed. The upper trace shows that when 10 μM Ins(1,4,5)P_3 (IP_3) was applied (first arrow) there was a rapid release of Ca^{2+} from intracellular stores. In the lower trace, 10 μM U73122 was applied at the first arrow; a slower release of Ca^{2+} resulted. This reached a plateau and at the second arrow 10 μM Ins(1,4,5)P_3 was added, releasing additional Ca^{2+}. The two traces were obtained from suspensions derived from the same cell preparation.

figure also shows the slower release of Ca^{2+} evoked by 10 μM U73122 from the same preparation of cells. U73122 is incapable of evoking release from cells in which the Ins(1,4,5)P_3-sensitive stores had been depleted by the earlier addition of a supramaximal concentration of Ins(1,4,5)P_3 ($n = 5$). U73122 is acting to release Ca^{2+} from the same intracellular stores as Ins(1,4,5)P_3. At 10 μM the Ca^{2+} release induced by U73122 reached a plateau and when directly compared with the effects of the maximal (10 μM) Ins(1,4,5)P_3, the aminosteroid released 49 ± 8 % (mean ± S.E.M.; $n = 8$) of the Ins(1,4,5)P_3-releasable stores. The effects of U73122,

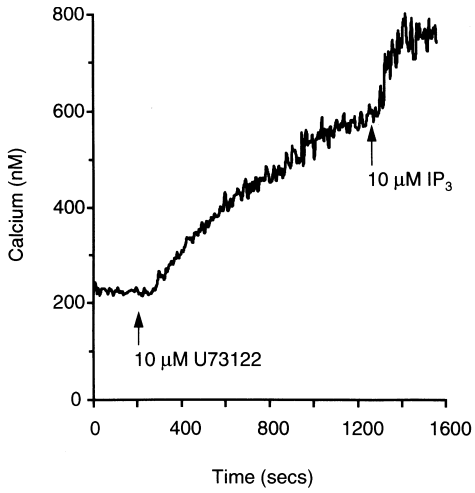

Figure 6 U73122 releases Ca^{2+} from digitonin-permeabilized rat pancreatic acinar cells

U73122 (10 μM) was applied at the point indicated by the arrow and resulted in Ca^{2+} release similar to that evoked in the mouse acinar preparation. The second arrow indicates when 10 μM Ins(1,4,5)P_3 (IP_3) was added.

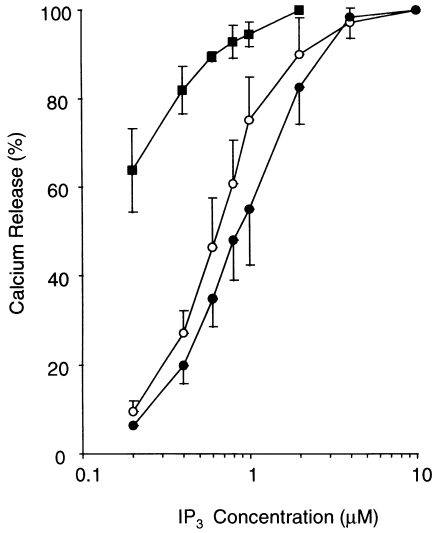

Figure 7 The effect of U73122 and thimerosal on the dose–response effect of Ins(1,4,5)P_3-induced Ca^{2+} mobilization in digitonin-permeabilized mouse pancreatic acinar cells

Ins(1,4,5)P_3 (IP_3)-mediated Ca^{2+} release in control cells (●) was compared with that evoked in cells pretreated for 2 min before experimentation with either 2.5 μM U73122 (○) or 10 μM thimerosal (■). Calcium release is expressed as a percentage of that mobilized by a supramaximal concentration of Ins(1,4,5)P_3 in the same cell suspensions. Each data point represents the mean ± S.E.M. for three to five experiments.

unlike those of Ins(1,4,5)P_3, were not antagonized by heparin ($n = 4$). In three experiments the effect of 10 μM U73122 was tested in rat pancreas where Ca^{2+}-measuring (fura 2) protocols had reported no effects of U73122 alone on resting Ca^{2+} levels [9]. We found that, as in the present study on mouse pancreatic acinar cells and as previously reported for rabbit pancreatic acinar cells [10], U73122 promoted Ca^{2+} release from permeabilized rat acinar cells (Figure 6).

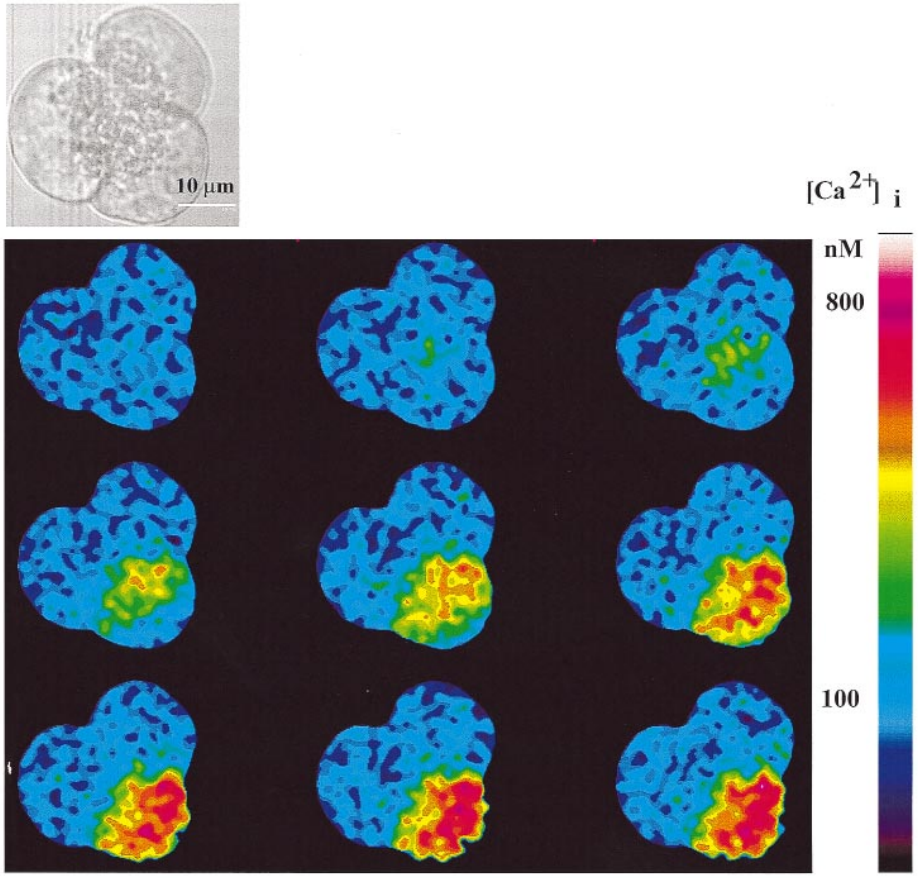

Figure 8 Spatiotemporal pattern of U73122-mediated Ca^{2+} release in intact mouse pancreatic acinar cells

The image at the top is a transmitted-light picture of three mouse pancreatic acinar cells. It shows that the granular poles of all three cells are oriented towards the centre of this cluster. These cells have been loaded with the Ca^{2+} indicator fura-red and the subsequent confocal images are of fura-red fluorescence psuedo-coloured to show the Ca^{2+} concentration within these cells before and during application of U73122. The first colour image (top left) is the control taken immediately before the application of U73122. The next image to the right shows the first detectable change in Ca^{2+}, 20 s after the application of the phospholipase C inhibitor. The subsequent images (left to right, top to bottom) were taken at 0.5 s intervals thereafter. The latency of the response to U73122 was variable and in the time course of this experiment only one cell was seen to respond. In this cell the elevation in Ca^{2+} concentration was first detected at the region corresponding to the granular or secretory pole before spreading to the rest of the cell. The pseudo-colour calibration scale is shown at the right.

A comparison of the dose–response curves for Ins(1,4,5)P_3-mediated Ca^{2+} release in the absence and the presence of U73122 (Figure 7) revealed that there was a potentiating effect of U73122 on Ins(1,4,5)P_3-mediated Ca^{2+} release, but this was modest in comparison with the effect of the known Ins(1,4,5)P_3 receptor-sensitizing agent thimerosal (10 μM) [19,20]. The control compound U73343 was without effect in releasing Ca^{2+} at 10 μM.

It has been reported that in rabbit pancreatic acinar cells U73122 can in its own right promote the release of intracellular Ca^{2+} [10]. We found no evidence of this in the whole-cell patch-clamp protocols, but to investigate directly for any changes in cytosolic Ca^{2+} level associated with the application of U73122 we used confocal microscopy to image the Ca^{2+} concentration in fura-red-loaded acinar cells. When U73122 was applied extracellularly to acinar cells there was a transient elevation of the intracellular Ca^{2+} level. This transient elevation was seen both in the presence ($n = 12$) and absence ($n = 3$) of external Ca^{2+} and hence reflects Ca^{2+} release from internal stores. Figure 8 shows an example of this elevation of the Ca^{2+} level. The confocal microscopy revealed that the elevation in Ca^{2+} originates specifically in the granular region of the cells, i.e. the secretory pole. In this respect the effect of U73122 is identical with that reported for responses evoked either by application of the phospholipase C-coupled agonist ACh [17] or for internally perfused Ins(1,4,5)P_3 [13], where responses are also initiated at the secretory pole of these cells.

DISCUSSION

U73122 has in many instances been reported to act as an inhibitor of phospholipase C and the production of Ins(1,4,5)P_3. In rat pancreatic acinar cells in particular, the parallelism between the inhibition of Ins(1,4,5)P_3 production and the blockade of agonist-induced Ca^{2+} signalling is particularly convincing [9]. Importantly, in that study, when Ca^{2+} was directly measured by using fura 2, U73122 had no effect on its own on resting Ca^{2+} levels. The work of the present study on mouse pancreatic acinar cells indicates that there are other effects of the aminosteroid that undoubtedly have effects on Ca^{2+} signalling but could pass unnoticed in many experimental protocols. Thus some effects described for U73122 on agonist-induced Ca^{2+} signalling could be attributable to factors other than the inhibition of Ins(1,4,5)P_3 production.

Patch-clamp experimentation reveals that U73122 directly activates the cation channels that give rise to inward current responses. This effect is not mediated by an elevation of Ca^{2+} levels as there is no concomitant activation of the Ca^{2+}-dependent Cl^- currents. Although Ca^{2+} is not the stimulus for current activation on application of U73122 it is required to provide a background activation of the ion channels for the potentiating effect of U73122 to be seen. Thus the total chelation of Ca^{2+} at the intracellular face of the membrane, either during whole-cell recording or in the excised patches, results in a blockade of the effect. The U73122 could be operating to increase the sensitivity of the channels to Ca^{2+} such that *in situ* their open probability is increased at resting cytosolic Ca^{2+} concentrations. The significance of this effect, which does not involve Ca^{2+} mobilization, might not at first seem obvious but in other experimental protocols, notably Ca^{2+} measurement by means of fluorescent indicators, this effect would not be at all apparent. However, the effect of cation channel opening and the consequent cell depolarization and change in intracellular electrolyte content cannot be excluded as having effects on Ca^{2+} homoeostasis and indeed Ca^{2+} signalling itself.

The other effects that we report in the mouse acinar cells relate directly to Ca^{2+} mobilization. In permeabilized mouse (and indeed rat) pancreatic acinar cells U73122 is shown to evoke a significant release of Ca^{2+} from intracellular stores. The evidence indicates that Ca^{2+} is being released from the same pool that is accessed by Ins(1,4,5)P_3. This effect of U73122 in releasing Ca^{2+} from intracellular stores has also been reported in permeabilized rabbit pancreatic acinar cells and in microsomal preparations from rat hepatocytes [9,21]. In the liver microsomes the effect of U73122 was demonstrated to be due to the inhibition of the Ca^{2+}-activated ATPase responsible for sequestering Ca^{2+}. The Ca^{2+} releasing properties reported in the present study are consistent with this finding. The question arises as to why we did not see such Ca^{2+}-mobilizing effects of U73122 in the patch-clamp protocols. The inward current activation seen was not due to an elevation in Ca^{2+} concentration. A similar situation was reported in the liver hepatocytes where, although U73122-mediated Ca^{2+} release was readily demonstrable in the microsomal preparation, in intact hepatocytes loaded with the Ca^{2+} indicator fura 2 no Ca^{2+} signals were detected in response to U73122 [21]. The most probable explanation is that in the intact cells, in contrast with permeabilized cells, the surface membrane Ca^{2+} pumps are functional and they are acting successfully to maintain a low intracellular Ca^{2+} concentration despite the slow release from intracellular stores induced by the aminosteroid. The release induced by U73122 could also be inhibited in these circumstances if this agent were acting simultaneously to lower the concentration of endogenous Ins(1,4,5)P_3 and the background Ca^{2+} 'leak' that it promotes. Another consideration must be applied to the patch-clamp protocols. Under conditions of whole-cell recording the interior of the cell is being dialysed by the solution filling the recording pipette. It is most likely that soluble cytosolic components are being diluted during whole-cell recording. The levels of endogenous Ins(1,4,5)P_3 are almost certainly being decreased and this could further contribute to a decreased rate of leakage from the stores in the presence of U73122.

Unlike the situation in the liver microsomes we found evidence that U73122 could potentiate the Ins(1,4,5)P_3-induced release of Ca^{2+}. The mechanism of action of U73122 involves the maleimide side chain of the molecule (in U73343 this moiety is substituted and the molecule is now ineffective in promoting Ca^{2+} release) and is most probably due its effect in alkylating thiol groups. In this respect it could be expected to be similar in action to another alkylating agent, thimerosal, which has been shown to sensitize for Ins(1,4,5)P_3-mediated Ca^{2+} release [19,20]. We directly compared the potentiating effects of thimerosal and U73122, both at concentrations that were threshold for Ca^{2+} release. The potentiation of Ins(1,4,5)P_3-mediated Ca^{2+} release by U73122 was apparent but modest in comparison with that of thimerosal. However, in the patch-clamp experiments a marked potentiation of Ins(1,4,5)P_3-mediated Ca^{2+} release was indicated by the marked increase in inward and outward currents on the application of U73122 to cells internally perfused with a submaximal concentration of Ins(1,4,5)P_3. The current transients initially became of longer duration and ultimately fused to give rise to sustained current responses. This cannot be explained as being due to the direct effect of U73122 in activating inward cation currents because, for the first time, it is seen that there is a concomitant activation of Ca^{2+}-dependent Cl^- currents. This also demonstrates that the U73122 is not blocking Cl^- current activation, which could have been suggested as a reason for the absence of any Cl^- currents in Figures 1 and 2.

In the intact rat hepatocytes [21] and in rat pancreatic acinar cells in fluorescent Ca^{2+}-measuring protocols [9], as in the patch-clamp protocols of the present study on mouse acinar cells, there was no evidence of a role for U73122 in Ca^{2+} mobilization. This might seem to be at odds with the findings in the permeabilized preparations of the same cells. That this could reflect an increased activity in the Ca^{2+} ATPase in plasma membrane has been discussed. In intact rabbit pancreatic acinar cells, however, U73122 has been shown to be associated with transient increases in Ca^{2+} and even on occasion with the generation of oscillations in intracellular Ca^{2+} concentration [10]. As discussed above, the whole-cell recording technique is associated with the internal dialysis of the cell interior and the possible dilution of cytosolic components such as endogenous Ins(1,4,5)P_3. When confocal microscopy was used to monitor intracellular Ca^{2+} in intact fura-red-loaded acinar cells, it was found that U73122 did indeed give rise to a Ca^{2+} signal. This Ca^{2+} signal was transient and was unaffected by the removal of extracellular Ca^{2+}. Importantly the Ca^{2+} signal induced by U73122 is initiated specifically at the secretory pole of the cells. In this respect U73122 is mimicking the spatial pattern induced by either the phospholipase C-coupled agonist ACh [22] or internally perfused Ins(1,4,5)P_3 [13]. Why should this be apparent in the Ca^{2+}-measuring protocols but not in the patch-clamp experiments? As discussed previously, we consider that patch-clamp, with its internal dialysis of the cell, probably results in some washing-out or dilution of cytosolic components such as endogenous Ins(1,4,5)P_3 itself. If the effect of U73122 in mobilizing intracellular Ca^{2+} were due to its sensitization of the Ins(1,4,5)P_3 receptor, then this effect might be seen only in the intact cells (the confocal experiments) where endogenous Ins(1,4,5)P_3 levels are unchanged. The initiation of the response at the secretory pole of the cells would be consistent with this observation as it is here that the stores that are most sensitive to Ins(1,4,5)P_3 are located [13]. The responses were always transient, in both the presence and the absence of extracellular Ca^{2+}, suggesting that all Ca^{2+} stores are not depleted and that capacitative Ca^{2+} entry has not been activated.

In summary we conclude that the effects of U73122 cannot be considered to be specific for the inhibition of phospholipase C. In protocols in intact cells such as during Ca^{2+} measurement with fluorescent indicators, U73122 is most probably having other effects that are not directly manifested but could cause significant perturbations in terms of Ca^{2+} homoeostasis and Ca^{2+} signalling. The direct activation of ion channels can both change the membrane potential of the cells and alter the intracellular electrolyte content. The U73122, by blocking the Ca^{2+} ATPase of

the intracellular stores, gradually depletes the stores. In intact cells if the activity of the surface membrane Ca^{2+} pumps increases to compensate for this, no Ca^{2+} signal attributable to U73122 might be detected. Although not detected, this effect in depleting intracellular stores undoubtedly affects Ca^{2+} signalling in a manner that could erroneously be attributed to inhibition of phospholipase C. The situation is made more complex by the possible sensitizing effect of U73122 at the Ins(1,4,5)P_3 receptor, as evidenced by the potentiation of the Ins(1,4,5)P_3-mediated Ca^{2+} release and Ca^{2+}-dependent current activation. In different tissues, and even in the same tissues under different protocols, the extent to which the various effects of U73122 manifest themselves depends on a balance of factors. U73122 is clearly not specific for the inhibition of phospholipase C and the production of Ins(1,4,5)P_3.

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