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EVIDENCE OBTAINED USING SINGLE HEPATOCYTES FOR INHIBITION BY THE PHOSPHOLIPASE C INHIBITOR U73122 OF STORE-OPERATED Ca^{2+} INFLOW

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Abstract—The ability of 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122), an inhibitor of phospholipase C (Smith *et al.*, *J Pharmacol Exp Ther* **253**: 688–697, 1992), to inhibit agonist-stimulated and store-operated Ca^{2+} inflow in single hepatocytes was investigated with the aim of testing whether the activation of phospholipase C is a necessary step in the process of agonist-stimulated Ca^{2+} inflow in this cell type. U73122 inhibited the release of Ca^{2+} from intracellular stores and plasma membrane Ca^{2+} inflow induced by vasopressin. An inactive analogue of U73122, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidone-dione (U73433), did not inhibit vasopressin-induced release of Ca^{2+} from intracellular stores, but did partially inhibit Ca^{2+} inflow. Neither U73122 nor 'inactive' analogue U73433 inhibited the release of Ca^{2+} from intracellular stores when this was initiated by the photolysis of 'caged' guanosine (5′-[γ-thio]triphosphate (GTPγS) introduced to the cytoplasmic space by microinjection. However, both compounds inhibited GTPγS-stimulated Ca^{2+} inflow. U73122 also inhibited the actions of glycerophosphoryl-*myo*-inositol-4,5-diphosphate ($GPIP_2$), a slowly-hydrolysed analogue of inositol 1,4,5-triphosphate ($InsP_3$) which is released by photolysis of 'caged' 1-(α-glycerophosphoryl)-*myo*-inositol-4,5-diphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester, and thapsigargin in stimulating Ca^{2+} inflow. U73122 did not inhibit $GPIP_2$-stimulated release of Ca^{2+} from intracellular stores, but did partially inhibit the ability of thapsigargin to induce Ca^{2+} release. It is concluded that, while U73122 does inhibit phospholipase C_β in hepatocytes, complete inhibition of this enzyme *in situ* requires an intracellular concentration of U73122 higher than that achieved in the present experiments. Moreover, both U73122 and 'inactive' analogue U73433 have one or possibly two additional sites of action. These are likely to be the hepatocyte plasma membrane Ca^{2+} inflow channel protein (or a protein involved in the activation of this channel by the $InsP_3$-sensitive intracellular Ca^{2+} store), and a protein involved in thapsigargin action.

Key words: Ca^{2+} inflow; plasma membrane; hepatocytes; phospholipase C; U73122; U73433; inositol 1,4,5-trisphosphate

In non-excitable and in some excitable cells, agonists which bind to seven transmembrane-spanning receptors [1] and use Ca^{2+} as an intracellular messenger activate phospholipase C_β which hydrolyses phosphatidylinositol-4,5-bisphosphate to yield diacylglycerol and $InsP_3$† [2]. The receptors are coupled to phospholipase C_β through a trimeric GTP-binding protein (G protein) which is either $G_{q/11}$ or the pertussis toxin-sensitive proteins of the G_i family [3–5]. The $InsP_3$ subsequently releases Ca^{2+} from an intracellular store, most likely the endoplasmic reticulum (reviewed in Ref. 2). This, in turn, is thought to induce the inflow of Ca^{2+} across the plasma membrane through a process called 'capacitative' or 'store-operated' Ca^{2+} inflow (reviewed in Refs 6, 7). Both the $InsP_3$-induced release of Ca^{2+} from intracellular stores and increased inflow of Ca^{2+} across the plasma membrane are necessary steps in the process by which agonists increase the cytoplasmic free Ca^{2+} concentration [2].

The ability of agonists and $InsP_3$ to stimulate Ca^{2+} inflow can be mimicked by compounds such as thapsigargin, which inhibit the $(Ca^{2+} + Mg^{2+})$ATP-ase located in the endoplasmic reticulum, and release Ca^{2+} from this intracellular store [6, 8–11]. Moreover, in hepatocytes, agonist-stimulated Ca^{2+} inflow can also be mimicked by the microinjection of GTPγS, a slowly-hydrolysable analogue of GTP which activates trimeric and some monomeric G proteins [12, 13]. It has been proposed that two trimeric G proteins ($G_{q/11}$ and $G_{i2/3}$) are involved in the mechanism of activation of the hepatocyte receptor-activated Ca^{2+} channel [12–14]. Evidence for the requirement for a G protein in the activation of plasma membrane Ca^{2+} channels in mouse lacrimal acinar cells and in a mast cell line has also been reported [15, 16] although in these cell types GTPγS inhibits agonist-stimulated Ca^{2+} inflow [15, 16].

Bleasdale and his colleagues [17, 18] have developed an inhibitor of phospholipase C, U73122,

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† Abbreviations: U73122, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione; U73433, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino]hexyl]-2,5-pyrrolidine-dione; $InsP_3$, inositol 1,4,5-trisphosphate; 'caged' $GPIP_2$, 1-(α-glycerophosphoryl)-*myo*-inositol 4,5-diphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester; GTPγS, guanosine 5′-[γ-thio]triphosphate; G-protein, GTP-binding regulatory protein; $Ca^{2+}{}_o$, extracellular Ca^{2+}; UV, ultraviolet.

and a closely-related compound, U73433, which does not inhibit this enzyme. These agents have proven useful in elucidation of the role of phospholipase C in intracellular signalling in many cell types [17–30].

In our laboratory we have been interested in the roles of phospholipase C_β and $InsP_3$ in the mechanisms by which vasopressin and other agonists which bind to seven transmembrane-spanning receptors stimulate Ca^{2+} inflow across the plasma membrane in hepatocytes, and the mechanism of 'store-operated' Ca^{2+} inflow in this cell type [12–14]. The aim of the present studies was to determine whether U73122 inhibits the agonist-stimulated release of Ca^{2+} from intracellular stores (an indirect measure of the activity of phospholipase C_β) in hepatocytes and whether the inhibition of phospholipase C_β, and hence inhibition of $InsP_3$ production, also blocks agonist-stimulated Ca^{2+} inflow. The experiments have been performed using single hepatocytes, microinjection to introduce 'caged' GTPγS and 'caged' $GPIP_2$ to the cytoplasmic space of the cell, and photolysis to release GTPγS and $InsP_3$ from the 'caged' compounds. While the results show that U73122 inhibits phospholipase C_β in hepatocytes they also point to at least one other site of action of this compound. This is likely to be either the channel protein responsible for Ca^{2+} inflow across the plasma membrane, or a protein involved in coupling the release of Ca^{2+} from the endoplasmic reticulum to this channel protein.

MATERIALS AND METHODS

Materials. U73122 and U73433 were obtained from Sapphire Bioscience (Alexandria, NSW, Australia); fluo-3, fura-2 and 'caged' GTPγS from Molecular Probes (Eugene, OR, U.S.A.); 'caged' $GPIP_2$ from Calbiochem-Novabiochem (Alexandria, NSW, Australia); and thapsigargin from Sigma-Aldrich (Castle Hill, NSW, Australia). All other chemicals were obtained from the sources described previously [12].

Methods. Hepatocytes were isolated, attached to coverslips coated with collagen, and fluo-3 (10 mM in the pipette tip), fura-2 (10 mM in the pipette tip), 'caged' $GPIP_2$ (24 mM in the pipette tip), and 'caged' GTPγS (30 mM in the pipette tip) were introduced to the cytoplasmic space by microinjection, as described previously [12]. When present, U73122 or U73433 (dissolved in DMSO) was added to the extracellular medium at the time of injection of the cells with fura-2 or fluo-3 (20 min before beginning the measurement of fluorescence). The final concentration of DMSO was 0.25% (v/v). At this concentration, DMSO had no effect on vasopressin-stimulated Ca^{2+} inflow when added alone (results not shown).

Ca^{2+} inflow to single hepatocytes was monitored by measuring the increase in fluorescence of intracellular fluo-3 or fura-2 following the addition of extracellular Ca^{2+} ($Ca^{2+}{}_0$) to hepatocytes incubated in the absence of added $Ca^{2+}{}_0$, as described previously [12]. Fluorescence was measured using a TMD-EF inverted fluorescence microscope, a P1 photometer (Nikon Corporation, Tokyo, Japan) and the UMANS filter changing and data recording system, as described previously [12]. The excitation light source was a mercury lamp. Conditions for the excitation of fura-2 and the measurement of fura-2 fluorescence were as described previously [12]. For experiments involving 'caged' compounds, fluo-3 was employed for the measurement of intracellular free Ca^{2+} concentrations [31]. Excitation of fluo-3 was at 490 nm and a Nikon barrier filter B (wavelength 520 nm) was employed to isolate emitted light. Photolysis of 'caged' compounds was achieved by exposing the cell to the full spectrum of light from the mercury lamp for 3–5 sec while the shutter to the photometer was closed. The exposure of hepatocytes loaded with fluo-3 to UV light (in the absence of 'caged' GTPγS or 'caged' $GPIP_2$) caused a small increase in fluorescence immediately following the exposure to UV light. However, the observed rates of vasopressin- or thapsigargin-stimulated Ca^{2+} inflow in cells treated in this way were the same as those in cells treated in a similar manner but not exposed to ultraviolet light, indicating that exposure of the cells to ultraviolet light *per se* does not affect measurement of the rate of Ca^{2+} inflow [13].

RESULTS

In order to verify that U73122 inhibits phospholipase C_β in hepatocytes, experiments were initially conducted using concentrations of U73122 in the range of 0.1–10 μM. These concentrations have been reported to inhibit agonist-stimulated phospholipase C activity in other cell types [17–20]. However, when the inhibitor was present in the extracellular medium at 10 μM, only a partial inhibition of vasopressin-induced release of Ca^{2+} from intracellular stores was observed (results not shown), suggesting little inhibition of phospholipase C under these conditions. Thus, higher concentrations of U73122 were tested. At a concentration of 25 μM, U73122 completely inhibited the ability of vasopressin to induce the release of Ca^{2+} from intracellular stores, as monitored by the vasopressin-induced increase in the fluorescence of single hepatocytes loaded with fluo-3 (Fig. 1a). A similar dose-dependence for U73122 was observed with a different, freshly-shipped batch of inhibitor (results not shown).

U73122 also completely inhibited vasopressin-stimulated Ca^{2+} inflow, assessed by the increase in fluorescence following the addition of $Ca^{2+}{}_0$ to cells previously incubated in the absence of added $Ca^{2+}{}_0$ (Fig. 1a). The addition of $Ca^{2+}{}_0$ to cells injected with fluo-3 and not exposed to vasopressin caused no detectable increase in $[Ca^{2+}]_i$ (results not shown). U73433, which does not inhibit phospholipase C [18], did not inhibit vasopressin-induced release of Ca^{2+} from intracellular stores but did inhibit vasopressin-stimulated Ca^{2+} inflow by approximately 50% (Fig. 1b). When the experiments were conducted using fura-2 in place of fluo-3 as the fluorescent Ca^{2+} indicator, the effects of U73122 and U73433 on vasopressin-induced Ca^{2+} release from intracellular stores and on vasopressin-stimulated Ca^{2+} inflow were similar to those observed

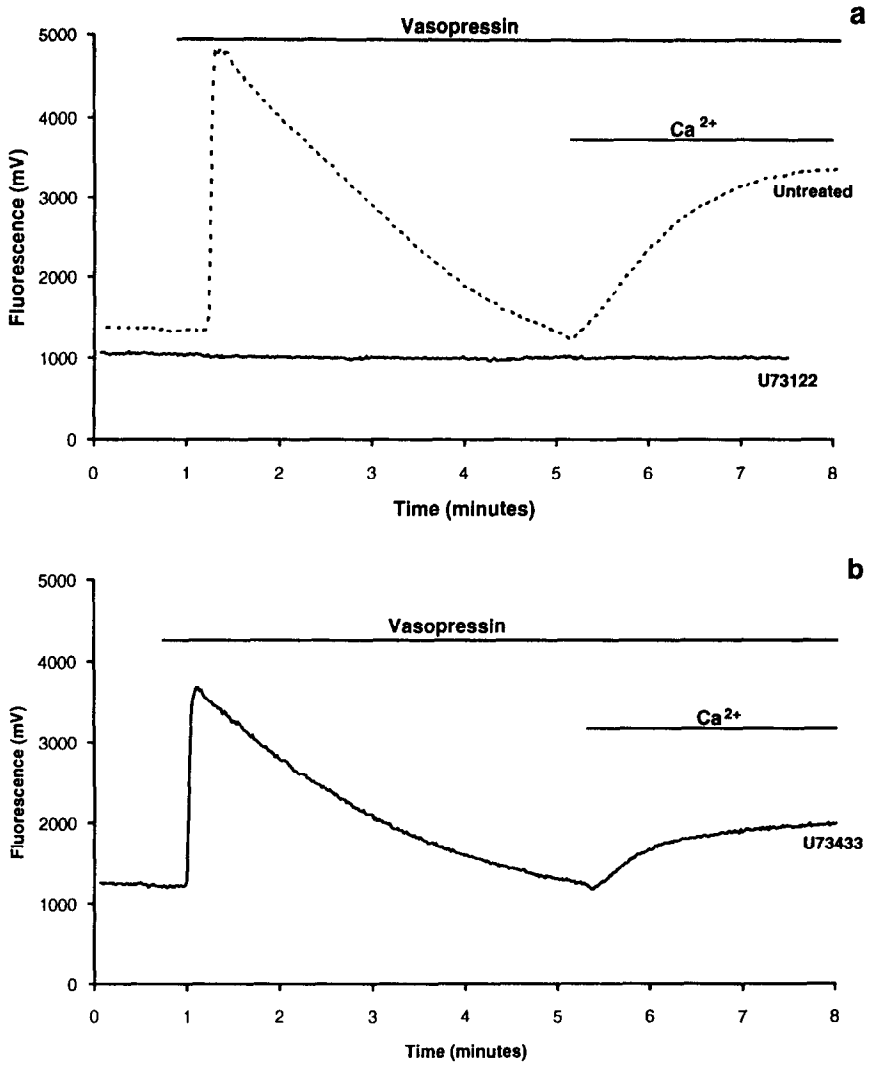

Fig. 1. Inhibition of vasopressin-stimulated plasma membrane Ca^{2+} inflow in single hepatocytes by (a) U73122, and (b) U73433. The solid line is a representative trace obtained from a single hepatocyte loaded with fluo-3, treated with (a) 25 μM U73122, or (b) 25 μM U73433, incubated initially in the absence of Ca^{2+}_0 then exposed to 40 nM vasopressin and 1.3 mM Ca^{2+} (added at the beginning of the periods indicated by the horizontal bars). The solid line shown in (a) is representative of the traces obtained for 13 of 18 cells tested. For the other 5 cells, the rate and amount of Ca^{2+} inflow, and the amount of Ca^{2+} released from the intracellular stores were similar to the values obtained for control (untreated) cells. The solid line in (b) is representative of the traces obtained for 5 of 7 cells tested. For the other 2 cells the rates and amounts of Ca^{2+} inflow, and amount of Ca^{2+} released from the intracellular stores were similar to the values obtained for control (untreated) cells. The broken line in (a) ('untreated') is a representative trace (for 9 of 11 cells tested) obtained from a single hepatocyte loaded with fluo-3 and treated with vasopressin and Ca^{2+}_0 in the absence of U73122 or U73433.

when fluo-3 was used as the Ca^{2+} indicator (results not shown).

The degree of inhibition by U73122 of vasopressin-stimulated release of Ca^{2+} from intracellular stores and Ca^{2+} inflow was not affected by varying the time the cells were exposed to U73122 from 2 to 20 min. While the results shown in Fig. 1 were obtained with cells incubated with U73122 for 20 min (as described in the Materials and Methods section), experiments in which cells were incubated with U73122 for shorter time periods (2 min) gave similar results (not shown).

When intracellular 'caged' GTPγS was used in place of vasopressin, it was found that U73122 did not inhibit the release of Ca^{2+} from intracellular stores initiated by exposure of the cells to ultraviolet light, which photolyses 'caged' GTPγS to yield GTPγS [31] (Fig. 2a). An increase in the time of exposure of the cells to U73122 from 20 to 60 min did not result in an inhibition of GTPγS-induced release of Ca^{2+} from intracellular stores (results not shown). It was not possible to test higher concentrations of the inhibitor since the compound

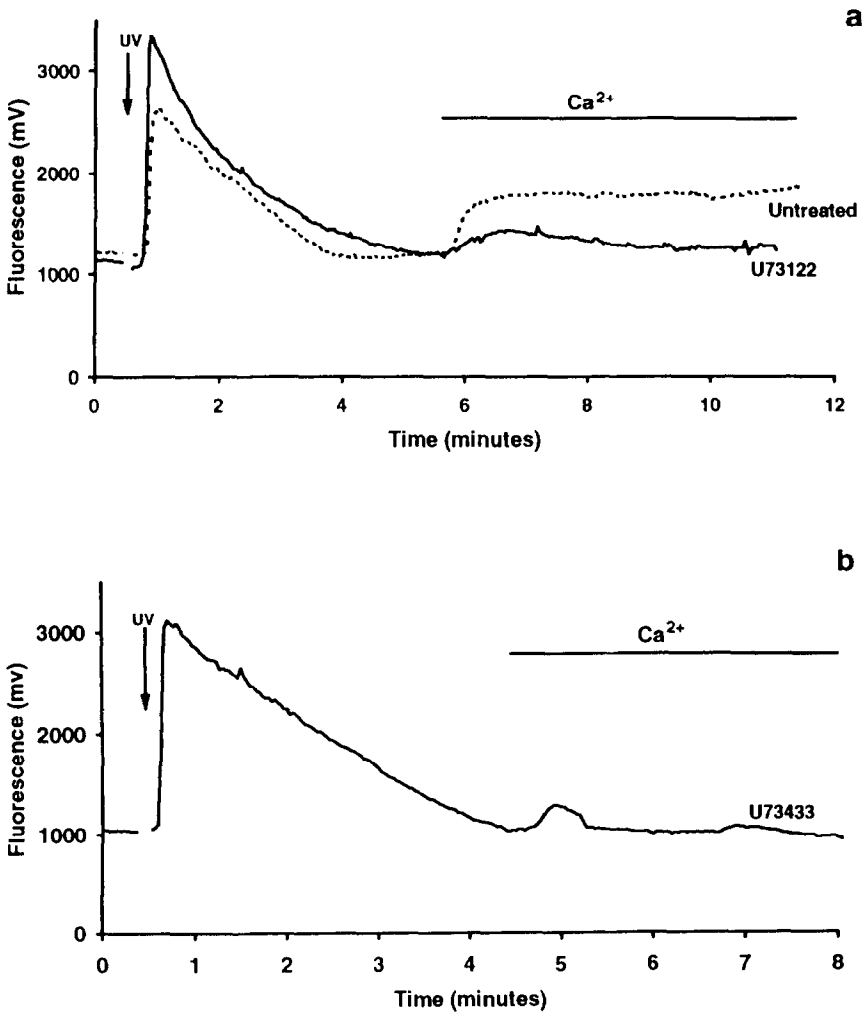

Fig. 2. Inhibition of plasma membrane Ca^{2+} inflow induced by GTPγS (released by the photolysis of 'caged' GTPγS) by (a) U73122, and (b) U73433. The solid line is a representative trace obtained from a single hepatocyte loaded with fluo-3 and 'caged' GTPγS, treated with (a) 25 μM U73122, or (b) 25 μM U73433, incubated initially in the absence of Ca^{2+}_0, then exposed to UV light (arrow) and 1.3 mM Ca^{2+}_0 (added at the beginning of the period indicated by the horizontal bar). The results shown are one representative trace of those obtained for (a) 6 of 9 cells tested (3 of the 9 cells showed no inhibition), and (b) 3 of 5 cells tested (2 of the 5 cells showed no inhibition). The broken line in (a) ('untreated') is a representative trace (for 7 of 12 cells tested) obtained from a single hepatocyte loaded with fluo-3 and then exposed to UV light and Ca^{2+}_0 in the absence of U73122 or U73433 (5 of the 12 untreated cells showed no Ca^{2+} inflow).

exhibited limited solubility in the extracellular medium. However, U73122 did substantially inhibit Ca^{2+} inflow induced by GTPγS (Fig. 2a). The 'inactive' analogue U73433 was also found to inhibit GTPγS-induced Ca^{2+} inflow, but had no substantial effect on the release of Ca^{2+} from intracellular stores induced by the photolysis of 'caged' GTPγS (Fig. 2b).

Further experiments were conducted using $GPIP_2$ (generated through the photolysis of intracellular 'caged' $GPIP_2$) to stimulate the release of Ca^{2+} from intracellular stores and to stimulate Ca^{2+} inflow [13]. U73122 had no effect on the $GPIP_2$-induced release of Ca^{2+} from intracellular stores (Fig. 3a). However, U73122 completely inhibited $GPIP_2$-stimulated Ca^{2+} inflow (Fig. 3a).

The results presented in Figs 1, 2 and 3(a) suggested that, in addition to phospholipase C_β, U73122 inhibits the hepatocyte plasma membrane receptor-activated Ca^{2+} inflow channel or a step required for the activation of this channel. This possibility was investigated further by testing the ability of U73122 to inhibit thapsigargin-induced Ca^{2+} inflow. U73122 completely inhibited thapsigargin-stimulated Ca^{2+} inflow (Fig. 3b) and also caused a substantial inhibition of the ability of thapsigargin to induce the release of Ca^{2+} from intracellular stores (Fig. 3b).

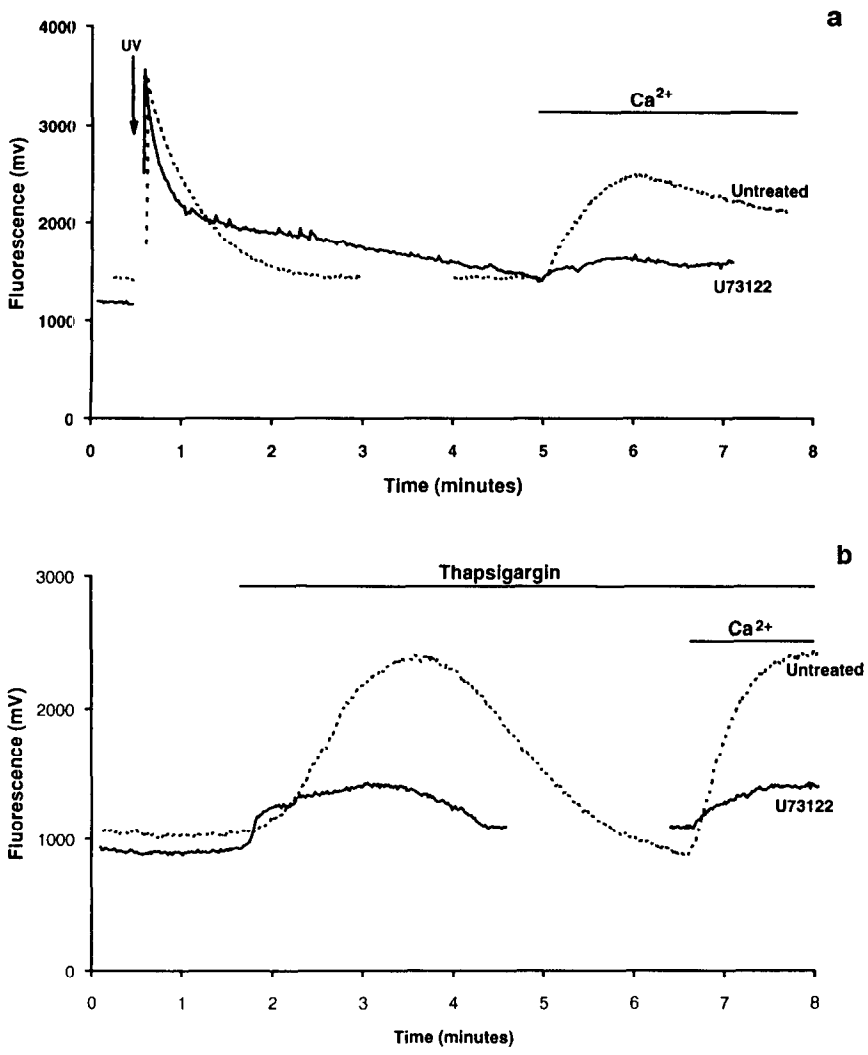

Fig. 3. Inhibition by U73122 of plasma membrane Ca^{2+} inflow stimulated by (a) $GPIP_2$ (released by the photolysis of 'caged' $GPIP_2$), and (b) thapsigargin. The solid line in (a) is a representative trace obtained from a single hepatocyte loaded with fluo-3 and 'caged' $GPIP_2$, treated with 25 μM U73122, incubated initially in the absence of $Ca^{2+}{}_0$, then exposed to UV light (arrow) and 1.3 mM $Ca^{2+}{}_0$ (added at the beginning of the period indicated by the horizontal bar). The result shown is a representative trace of those obtained for 3 or 4 cells tested (1 of the 4 cells showed no inhibition of Ca^{2+} inflow). The broken line ('untreated') is a representative trace (for 4 of 6 cells tested) obtained from a single hepatocyte loaded with fluo-3 and 'caged' $GPIP_2$ and exposed to UV light and $Ca^{2+}{}_0$ in the absence of U73122 (2 of the 4 untreated cells showed no Ca^{2+} inflow). Inspection of the results obtained from all cells tested in the presence of 'caged' $GPIP_2$ and in the absence or presence of U73122 indicated that U73122 had no effect on the increase in fluorescence observed following exposure to UV light. The solid line in (b) is a representative trace obtained from a single hepatocyte loaded with fura-2, treated with 25 μM U73122, incubated initially in the absence of $Ca^{2+}{}_0$, then exposed to 10 μM thapsigargin and 1.3 mM $Ca^{2+}{}_0$ (added at the beginning of the periods indicated by the horizontal bars). The trace shown is representative of one of 5 obtained for 7 cells tested. Two of the 7 cells showed normal Ca^{2+} release, one of these 2 cells also showed normal Ca^{2+} inflow. The broken line ('untreated') is a representative trace (for 6 of 6 cells tested) obtained from a single hepatocyte loaded with fura-2 and incubated in the presence of thapsigargin and Ca^{2+} but in the absence of U73122.

DISCUSSION

The conclusion that U73122 inhibits phospholipase C_β in hepatocytes is consistent with the observation that U73122, but not 'inactive' analogue U73433, inhibits the vasopressin-stimulated release of Ca^{2+} from intracellular stores. The requirement in hepatocytes for a relatively high concentration of U73122 to achieve this inhibition may reflect a low rate of diffusion of U73122 across the plasma membrane and/or rapid metabolism of the inhibitor in hepatocytes.

It was expected that U73122 would also inhibit the release of Ca^{2+} from intracellular stores induced by GTPγS since this agent should induce a constitutive stimulation of all trimeric G proteins [32], including $G_{q/11}$ which couples the vasopressin receptor to phospholipase C_β. The failure to observe complete inhibition of the GTPγS-stimulated release of Ca^{2+} from intracellular stores by U73122 may be due to the combination of two possible events. These are firstly that the inhibition of phospholipase C_β by U73122 at the concentration employed is incomplete, and secondly that there may be differences in the kinetics or mechanism of activation of phospholipase C_β by GTPγS (released by the photolysis of 'caged' GTPγS) on the one hand, and by the binding of vasopressin to its receptor on the other. The results obtained with GTPγS and U73122 are consistent with those reported for *Xenopus laevis* oocytes where it was also found that U73122 does not inhibit the action of phospholipase C which is required for GTPγS-induced ion channel currents [33].

The observations that (i) U73122 inhibits $GPIP_2$- and thapsigargin-stimulated Ca^{2+} inflow, processes which do not require phospholipase C action, and (ii) U73433, which is not an inhibitor of phospholipase C [18], inhibits both vasopressin- and GTPγS-stimulated Ca^{2+} inflow suggest that in hepatocytes there are one or more sites of action for U73122 in addition to its interaction with phospholipase C. Since U73122 completely inhibited vasopressin-, GTPγS-, $GPIP_2$- and thapsigargin-stimulated Ca^{2+} inflow and partially inhibited thapsigargin-induced release of Ca^{2+} from intracellular stores, it is concluded that U73122 inhibits one of the steps in the process of store-operated Ca^{2+} inflow. A likely candidate is the receptor-activated plasma membrane Ca^{2+} inflow channel itself. The results obtained with U73433 indicate that the process of store-operated Ca^{2+} inflow is inhibited by the 'inactive' analogue U73433 as well as by U73122. This suggests that the mechanism of inhibition of store-operated Ca^{2+} inflow by U73122 differs from that for the inhibition of phospholipase C action.

Two observations suggest that the process of store-operated Ca^{2+} inflow is more sensitive to inhibition by U73122 than is phospholipase C. Firstly, U73122 inhibited GTPγS-stimulated Ca^{2+} inflow but not GTPγS-stimulated release of Ca^{2+} from intracellular stores. Secondly, 'inactive' analogue U73433 partially inhibited vasopressin-stimulated Ca^{2+} inflow, but did not inhibit the vasopressin-stimulated release of Ca^{2+} from intracellular stores.

The observation that U73122 partially inhibits the ability of thapsigargin to release Ca^{2+} from intracellular stores suggests that there is a third site of action of this compound in hepatocytes (in addition to inhibition of phospholipase C_β and the process of store-operated Ca^{2+} inflow). Since U73122 inhibits thapsigargin-stimulated, but not vasopressin-stimulated, Ca^{2+} release from stores, it is concluded that the effect of the inhibitor on thapsigargin-stimulated Ca^{2+} release involves an inhibition of the interaction of thapsigargin with the $(Ca^{2+} + Mg^{2+})$ATP-ase on the endoplasmic reticulum (a thapsigargin-specific event). Alternatively, Irvine [34] has proposed that the formation of a multi-protein complex (involving endoplasmic reticulum proteins, the $InsP_3$ receptor protein and the plasma membrane receptor-activated Ca^{2+} channel) is required in the mechanism of stimulation of Ca^{2+} inflow. If this is indeed the case, inhibition by U73122 of one component of this complex (e.g. the plasma membrane Ca^{2+} channel) may affect the ability of thapsigargin to induce Ca^{2+} release from the endoplasmic reticulum and hence lead to an inhibition of this action of thapsigargin.

The present results provide further evidence which indicates that, while U73122 is a useful and reasonably specific inhibitor of phospholipase C in a number of types of cells [17–30], it does have other sites of action. In addition to inhibition of the action of one or more proteins involved in the process of store-operated Ca^{2+} inflow reported here, these other sites include phosphatidylinositol kinase and phosphatidylinositol 4-phosphate kinase [35], a step in the induction of ion currents by serum in *Xenopus* oocytes [33], a step in glucose-induced insulin secretion [36] and phospholipase D [37]. Notwithstanding these other sites of action, it seems likely that further investigation of the observation that U73122 inhibits store-operated Ca^{2+} inflow in hepatocytes may assist in elucidation of the mechanism of this process.

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