Effect of the aminosteroid, U73122, on Ca\(^{2+}\) uptake and release properties of rat liver microsomes

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The putative phospholipase C inhibitor, U73122, transiently increases the cytosolic free Ca\(^{2+}\) concentration in rabbit pancreatic acinar cells by stimulating the release of Ca\(^{2+}\) from intracellular stores [Willems, Van de Put, Engbersen, Bosch, Van Hoof & De Pont (1994) Pflügers Arch. 427, 233–243]. In order to elucidate the exact mechanism of action of U73122 we studied its effects on both Ca\(^{2+}\)-stimulated Mg\(^{2+}\)-dependent ATPase activity and Ca\(^{2+}\)-stimulated ATP-dependent Ca\(^{2+}\) uptake in rat liver microsomes. In addition, we studied its effects on Ca\(^{2+}\) release from steady-state loaded microsomes. The effects of U73122 were compared with those of thimerosal, described in the literature as inhibiting Ca\(^{2+}\)-ATPases and sensitizing inositol 1,4,5-trisphosphate-operated Ca\(^{2+}\) release channels, and thapsigargin, a specific inhibitor of sarcoplasmic and endoplasmic reticulum Ca\(^{2+}\)-ATPases. Both U73122 (IC\(_{50}\) = 9 \(\mu\)M) and thimerosal (IC\(_{50}\) = 11 \(\mu\)M) dose-dependently inhibited Ca\(^{2+}\)-stimulated Mg\(^{2+}\)-dependent ATPase activity, without significantly affecting Mg\(^{2+}\)-stimulated ATPase activity. Similarly, both U73122 (IC\(_{50}\) = 9 \(\mu\)M) and thimerosal (IC\(_{50}\) = 14 \(\mu\)M) dose-dependently inhibited ATP-dependent Ca\(^{2+}\) uptake. At concentrations beyond 20 \(\mu\)M, U73122 stimulated Ca\(^{2+}\) release from steady-state loaded microsomes at a rate considerably higher than obtained with a maximally inhibitory concentration of thapsigargin (1 \(\mu\)M). This observation, which was not reached with equally inhibitory concentrations of thimerosal, demonstrates that higher U73122 concentrations cause an additional increase of the passive Ca\(^{2+}\) leak. The data presented demonstrate that U73122 stimulates the release of actively stored Ca\(^{2+}\) primarily through inhibition of the internal Ca\(^{2+}\) pump.

Keywords: U73122; thimerosal; Ca\(^{2+}\)-ATPase; endoplasmic reticulum; hepatocytes.

A wide variety of external stimuli use receptors coupled to phospholipase C \(\gamma\) to pass information into the cell [1]. Activation of the enzyme occurs through the intermediation of a G\(_{\text{q}}\) protein [2] and results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate. Two second messengers are formed namely, inositol 1,4,5-trisphosphate \([\text{Ins}(1,4,5)P_3]\), releasing Ca\(^{2+}\) from intracellular stores [1], and 1,2-diacylglycerol, activating protein kinase C [3]. Monitoring of the free cytosolic Ca\(^{2+}\) concentration \([\text{Ca}^{2+}\]_c\) in individual cells has revealed that external stimuli, acting through this signal transduction pathway, can evoke complex patterns of intracellular Ca\(^{2+}\) signalling [4].

In order to explore the involvement of phospholipase C \(\gamma\) in the mechanism of action of Ca\(^{2+}\)-mobilizing stimuli, recent studies have used the aminosteroid U73122 [5–10]. U73122 was originally described as a potent inhibitor of platelet aggregation induced by a variety of agonists [5]. The inhibitory action of U73122 was paralleled by the inhibition of the agonist-stimulated increase in cellular Ins(1,4,5)P\(_3\) content and [Ca\(^{2+}\)\]_c. The finding that U73122 dose-dependently inhibited the GTP\(_{\gamma}\)-stimulated hydrolysis of phosphatidyl-inositol 4,5-bisphosphate by a soluble fraction from platelets led to the conclusion that the aminosteroid in question acts by a mechanism involving the inhibition of phospholipase C.

We have recently reported that U73122 transiently increased [Ca\(^{2+}\)\]_c, rather than inhibiting receptor-evoked Ca\(^{2+}\) mobilization in freshly isolated rabbit pancreatic acinar cells. Using permeabilized acinar cells it was demonstrated that U73122 released Ca\(^{2+}\) from internal stores by a mechanism possibly involving inhibition of the Ca\(^{2+}\)-ATPase [11]. The latter observation has urged us to investigate the mechanism of action of U73122 in more detail. To this end, we have used a well defined microsomal preparation obtained from rat liver [12]. The advantage of this preparation over the permeabilized cell system is the absence of any plasma membrane Ca\(^{2+}\)-ATPase activity [13]. The effects of U73122 were compared with those of thimerosal, described in the literature to inhibit Ca\(^{2+}\)-ATPases [14] and sensitize Ins(1,4,5)P\(_3\)-operated Ca\(^{2+}\) release channels [14, 15], and thapsigargin, a specific inhibitor of intracellular Ca\(^{2+}\)-ATPases [16]. The data presented demonstrate that U73122 releases Ca\(^{2+}\) from internal stores primarily through inhibition of the internal Ca\(^{2+}\) pump.

**MATERIALS AND METHODS**

Preparation of rat liver microsomes. A rat liver heavy microsomal fraction was prepared according to Reinhart and By-
grave [17] as previously described [13] with the exception that dihydrothreitol was omitted from the homogenization buffer. Isolated heavy microsomes were resuspended in a medium containing 250 mM sucrose, 10 mM KCl and 5 mM Hepes/KOH pH 7.0 to a final concentration of 9.0 mg protein \cdot ml \^{-1} (SD \pm 1.7, n = 11) and stored on ice until use.

ATPase assay. Rat liver microsomes (0.98 mg protein/\mu l, SD \pm 0.16, n = 6) were incubated in assay medium (final volume of 400 \mu l) containing (final concentrations) 150 mM KCl, 1 mM NaN\textsubscript{3}, 0.5 mM EGTA, 0.5 mM nitrilotriacetic acid, 0.5 mM Heeds, 20 mM Hepes/KOH pH 7.0 and the drug(s) in question, for 3 min at 37°C. The free Mg\textsuperscript{2+} and Ca\textsuperscript{2+} concentrations were adjusted to 1.5 mM and 1 \mu M, respectively, using a recent modification by Schoenmakers et al. [18] of the method originally described by Van Heeswijik et al. [19]. The total concentrations of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} were 4.72 mM and 0.35 mM, respectively. Ionomycin (1 \mu M) was included in the assay medium to prevent Ca\textsuperscript{2+} accumulation. The reaction was started by the addition of 3 mM Na\textsubscript{2}ATP. After 10 min, the reaction was stopped by adding 0.5 ml ice-cold 10\% (mass/vol.) trichloroacetic acid. The precipitated protein was pelleted by centrifugation at 5000 \times g for 5 min. The phosphate concentration in the supernatant was measured as described by Swarts et al. [20]. Ca\textsuperscript{2+}-stimulated Mg\textsuperscript{2+}-dependent ATPase activity, defined as the difference between the activities observed in the absence (total ATPase activity) and presence (Mg\textsuperscript{2+}-stimulated ATPase activity) of thapsigargin (1 \mu M), is expressed as rate of P\textsubscript{i} production/mass of protein.

Ca\textsuperscript{2+} uptake and release experiments. Rat liver microsomes (0.81 \mu g protein/\mu l, SD \pm 0.12, n = 6) were incubated in the above assay medium (final volume of 100 \mu l) lacking ionomycin but containing in addition 5 \mu C/ml "Ca\textsuperscript{2+}" and the drug(s) in question, for 3 min at 37°C. Ca\textsuperscript{2+} uptake was started by the addition of 3 mM Na\textsubscript{2}ATP. At the indicated time, the reaction was quenched in 1 ml ice-cold stop solution containing 150 mM KCl, 1 mM EGTA and 20 mM Hepes/KOH pH 7.0. The suspension was rapidly filtered through a nitrocellulose filter with a pore size of 0.45\,\mu m (Schleicher & Schüll). The filters were washed twice with 1 ml ice-cold stop solution, dissolved in scintillation fluid and the radioactivity measured. Total Ca\textsuperscript{2+} was calculated and expressed as molar amount Ca\textsuperscript{2+}/mass protein. Actively stored Ca\textsuperscript{2+} is defined as the difference in total Ca\textsuperscript{2+} retained on the filter after incubation in the absence and presence of thapsigargin (1 \mu M).

Preparation of rat hepatocytes. Hepatocytes were prepared from male Wistar Kyoto rats (180–220 g) according to the method of Berry and Friend [21]. Briefly, the portal vein of the liver of an anaesthetized rat was cannulated and perfused (40 ml/min) with 400 ml of a warmed (37°C) perfusion buffer containing 142 mM NaCl, 6.7 mM KCl, 20 mM glucose and 10 mM Hepes/KOH pH 7.4. Subsequently, the liver was perfused (40 ml/min) with 20 ml of a warmed (37°C) buffer containing 68.4 mM NaCl, 6.7 mM KCl, 4.76 mM CaCl\textsubscript{2}, 20 mM glucose, 0.5 mM EGTA, 5.8 mM glucose, an amino acid mixture according to Eagle, 1% (mass/vol.) BSA and 10 mM Hepes/KOH pH 7.6. Thereafter, another 80 ml of the latter buffer was recirculated for 10 min. Dispersed hepatocytes were harvested and resuspended (10' cells/ml) in a Hepes/Tris medium (pH 7.4) containing 133 mM NaCl, 4.2 mM KCl, 1 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2}, 5.8 mM glucose, an amino acid mixture according to Eagle, 1% (mass/vol.) BSA and 10 mM Hepes, adjusted to pH 7.4 with Tris. The intactness of the isolated cells was demonstrated by trypan blue exclusion. The cells were kept at room temperature until use.

Fluorescence measurement. Freshly isolated hepatocytes (10' cells/ml), resuspended in the above Hepes/Tris medium containing 1% (mass/vol.) BSA (loading medium), were incubated for 15 min at 37°C. After centrifugation at 40 g for 3 min, the cells were resuspended in loading medium containing 3 \mu M Fura-2/AM and 0.03\% (mass/vol.) pluronic (a nonionic surfactant) and incubated for another 30 min at 37°C. Excess Fura-2/AM was removed by washing the cells with the above Hepes/Tris medium containing 0.1\% (mass/vol.) BSA (incubation medium). Fura-2-loaded hepatocytes, resuspended in incubation medium (0.5 x 10' cells/ml), were transferred to a Shimadzu RF-5000 spectrofluorophotometer equipped with a magnetic stirrer and a thermostatted cuvette holder. Fluorescence was measured at 37°C. The fluorescence emission ratio at 490 nm was monitored after excitation at 340 nm and 380 nm. U73122 and U73343 were added from a dimethylsulfoxide stock.

Protein determination. The protein content was determined with a commercial Coomassie-blue kit (Bio-Rad) after treatment of the microsomes with 0.08\% Triton X-100. BSA (Bio-Rad) was used as a standard.

Analysis of the data. The IC\textsubscript{50} values were determined by fitting the concentration/response relationship to a logistic equation using the nonlinear regression computer program InPlot (GraphPAD Software for Science, San Diego CA).

Materials. Collagenase B was purchased from Boehringer (Mannheim), Fura-2/AM and pluronic were obtained from Molecular Probes Inc.; thapsigargin from LC Services Corporation (Woburn MA); Hepes from Research Organics Inc. (Cleveland OH); ionomycin from Calbiochem (La Jolla CA) and Triton X-100 from BDH Chemicals Ltd (Poole, UK). "Ca\textsuperscript{2+}" (740 kBq/\mu l) was obtained from New England Nuclear, and vasopressin, Na\textsubscript{2}ATP, thimerosal, Heedta and nitrilotriacetic acid from Sigma. Ammonium heptamolybdate, NaN\textsubscript{3}, dimethylsulfoxide and EGTA were purchased from Merck. U73122 and U73343 were generously supplied by Dr J. E. Bleasdale, The Upjohn Company (Kalamazoo MI). All other chemicals were of analytical grade.

RESULTS AND DISCUSSION

In order to investigate the mechanism by which the amino-steroid U73122 stimulates the release of Ca\textsuperscript{2+} from intracellular stores, we used rat liver microsomes. The advantage of this preparation over the permeabilized cell system used in our previous study is the absence of any thapsigargin-insensitive "Ca\textsuperscript{2+}-ATPase activity [13]. Rat liver microsomes rapidly accumulate Ca\textsuperscript{2+} through the action of a "Ca\textsuperscript{2+}-stimulated Mg\textsuperscript{2+}-dependent ATPase [12]. Maximal stimulation of this Ca\textsuperscript{2+}-translocating enzyme is achieved at an ambient free Ca\textsuperscript{2+} concentration of 1.0 \mu M [13]. The activity of the enzyme can be defined as the difference between ATP hydrolysis in the absence and presence of the sesquiterpene lactone, thapsigargin, which acts as a specific inhibitor of all members of the family of the sarcoplasmic and endoplasmic reticulum Ca\textsuperscript{2+} pumps [16].

\begin{figure}
\includegraphics[width=\textwidth]{figure1.png}
\caption{Fig. 1 shows that only 14\% (13.9 nmol P\textsubscript{i} \cdot mg protein \cdot min\textsuperscript{-1}, SEM \pm 1.3, n = 6) of the total P\textsubscript{i} production (100.9 nmol P\textsubscript{i} \cdot mg protein \cdot min\textsuperscript{-1}, SEM \pm 11.9, n = 6), measured at an ambient free Ca\textsuperscript{2+} concentration of 1.0 \mu M, is due to the activity of the "Ca\textsuperscript{2+}-stimulated ATPase. The residual enzyme activity (87.0 nmol P\textsubscript{i} \cdot mg protein \cdot min\textsuperscript{-1}, SEM \pm 10.9, n = 6) represents the Mg\textsuperscript{2+}-stimulated ATPase, which we have previously shown to be insensitive to thapsigargin [13]. Both U73122 (Fig. 1A) and the "Ca\textsuperscript{2+}-ATPase inhibitor, thimerosal [14] (Fig. 1B), dose-dependently inhibited the activity of the "Ca\textsuperscript{2+}-stimulated enzyme, without significantly affecting the Mg\textsuperscript{2+}-stimulated ATPase activity. In the presence of the inactive analog U73343 the total ATPase activity remained largely un-}
\end{figure}
U73122 and thimerosal can completely inhibit the activity of the
and thimerosal, respectively. These data demonstrate that both
analysis of the Ca2+-stimulated ATPase activities, measured in
(30 µM U73122 (Fig. 1 A) or 100 µM thimerosal (Fig. 1 B). Nonlinear regression
produced in triplicate. Total ATPase activity (100%) amounted to 100.9 nmol P2 • mg protein−1 • min−1 (SEM ± 1.9, n = 6).
changed (Fig. 1 A). Virtually complete inhibition of Ca2+-stimu-
U73122 activity was reached with either 30 µM U73122 (Fig. 1 A) or 100 µM thimerosal (Fig. 1 B). Nonlinear regression
analysis of the Ca2+-stimulated ATPase activities, measured in
the presence of different concentrations of either U73122 or thi-
merosal, revealed IC50 values of 9 µM and 11 µM for U73122 and thimerosal, respectively. These data demonstrate that both
U73122 and thimerosal can completely inhibit the activity of the
Ca2+-stimulated Mg2+-dependent ATPase in rat liver microsomes, without significantly affecting the activity of the Mg2+-
stimulated ATPase, which is relatively large (85% of total ATPase activity) in this preparation.

Rat liver microsomes rapidly accumulated Ca2+ in an en-
energy-dependent manner when incubated at an ambient free Ca2+
concentration of 1 µM (Fig. 2). Actively stored Ca2+, defined as the difference in Ca2+ retained on the filter after loading in the
absence (11.5 nmol Ca2+/mg protein, SEM ± 1.3, n = 6) and presence (1.2 nmol Ca2+/mg protein, SEM ± 0.1, n = 6) of 1 µM
thapsigargin, reached a steady-state of 10.3 nmol Ca2+/mg protein
(SEM ± 1.2, n = 6) at about 5 min and remained unchanged for the next 5 min. The vesicular nature of the energy-dependent
Ca2+ store was demonstrated by the action of the Ca2+ iono-
phore, ionomycin (1 µM), reducing the amount of Ca2+ retained on the filter to the same level as obtained in the absence of ATP. Furthermore, the intracellular origin of the Ca2+-accumulating vesicles was demonstrated by complete inhibition of active Ca2+
uptake by thapsigargin (1 µM). U73122 virtually completely in-
hibited ATP-dependent Ca2+ uptake when added at a concentra-
tion of 60 µM at which it completely inhibited the Ca2+-stimu-
lated Mg2+-dependent ATPase activity. At the same concentra-
tion, U73343 (60 µM) only slightly decreased the steady-state Ca2+
uptake level. The latter finding is in agreement with the observation that, in some instances, the inhibitory action of
U73122 was not completely abolished by substitution of the male-
ime group for the less electrophilic succinate group [5].

In order to study the dose-dependency for the inhibitory ef-
effect of U73122 and thimerosal on the initial rate of Ca2+ uptake, incubations were stopped 20 s following the addition of ATP. Rat liver microsomes actively accumulated Ca2+ at a rate of
9.2 nmol Ca2+ · mg protein−1 · min−1 (SEM ± 1.2, n = 4) when incubated at an ambient free Ca2+ concentration of 1.0 µM. U73122 dose-dependently reduced the amount of Ca2+ stored within 20 s following the initiation of Ca2+ uptake (Fig. 3 A). The minimal effective concentration of U73122 was 3 µM, whereas at a concentration of 30 µM the drug virtually com-

Fig. 1. Inhibitory effect of U73122 and thimerosal on the Ca2+-stimu-
lated Mg2+-dependent ATPase activity in rat liver microsomes. Rat
liver microsomes (0.98 µg protein/µl assay medium, SEM ± 0.07, n = 6) were incubated in the presence of 3 mM Na2ATP for 10 min at 37°C. The ambient free Mg2+ and Ca2+ concentrations were set at 1.5 mM and
1.0 µM, respectively. Ionomycin (1 µM) was routinely included in the
assay medium to prevent Ca2+ accumulation. Ca2+ -stimulated ATPase activity was reached with either 30 µM U73122
or 1 µM thimerosal (Fig. 1 B). Nonlinear regression

Fig. 2. Effect of U73122 on ATP-dependent Ca2+ uptake in rat liver
microsomes. Rat liver microsomes (0.81 µg protein/µl assay medium) were incubated in the absence and presence of 3 mM Na2ATP at 37°C. The ambient free Mg2+ and Ca2+ concentrations were set at 1.5 mM and
1.0 µM, respectively. The "Ca2+" concentration in the assay medium was
5 µM/lml. Following incubation in the absence or presence of the drug
in question for 5 min, active Ca2+ uptake was started by the addition of
ATP. The reactions were stopped at the indicated times. Total Ca2+ re-
tained on the filter was calculated and expressed in nmol · mg protein−1. In
each individual experiment, the control uptake value at 10 min following
addition of ATP was set at 100%, to which all other values were related.
Where indicated by error bars, the values presented are the mean ± SEM of three independent incubations. In the absence of any drug, steady-
state Ca2+ uptake (100%) amounted to 11.5 nmol Ca2+ · mg protein−1
(SEM ± 1.3, n = 6).
completely inhibited active Ca\(^{2+}\) uptake. The IC\(_{50}\) for U73122 was calculated to be 9 µM.

Similarly to U73122, thimerosal dose-dependently reduced the amount of actively stored Ca\(^{2+}\) during the first 20 s of the uptake process (Fig. 3B). The minimal effective concentration was 3 µM, whereas virtually complete inhibition was reached with 30 µM thimerosal. The IC\(_{50}\) for thimerosal was calculated to be 14 µM. The close correlation between the IC\(_{50}\) value for the inhibition of Ca\(^{2+}\)-stimulated ATPase activity and the IC\(_{50}\) value for the inhibition of ATP-dependent Ca\(^{2+}\) uptake demonstrates that both U73122 and thimerosal interfere with active Ca\(^{2+}\) uptake. The IC\(_{50}\) for thimerosal (100 µM) was set at 100%, to which all other values were related. Where indicated by error bars, the values presented are the mean ± SEM of three independent incubations.

Ca\(^{2+}\) uptake by inhibition of the Ca\(^{2+}\) pump. Inhibitory effects of thimerosal on the activity of the internal Ca\(^{2+}\)-ATPase have been reported in permeabilized A7r5 smooth muscle cells [15], skeletal muscle sarcoplasmic reticulum [14], rat cerebellar microsomes [14] and permeabilized HeLa cells [22]. Thimerosal inhibited the endoplasmic reticulum Ca\(^{2+}\) pump with an IC\(_{50}\) of 11 µM, which is in good agreement with the IC\(_{50}\) of 9 µM reported in the literature [15].

Addition of thapsigargin (1 µM) to liver microsomes, loaded with Ca\(^{2+}\) for 10 min to steady state, resulted in the gradual loss of actively stored Ca\(^{2+}\) (Fig. 4). Similarly, thimerosal caused the time-dependent loss of actively stored Ca\(^{2+}\) when added at a concentration of 100 µM at which it completely inhibited active Ca\(^{2+}\) uptake. The rate of Ca\(^{2+}\) loss induced by 100 µM thimerosal was equal to that observed with a maximally inhibitory concentration of thapsigargin (1 µM), indicating that thimerosal, at concentrations up to 100 µM, solely acts by inhibiting the microsomal Ca\(^{2+}\) pump. By contrast, thimerosal has been reported to increase the aspecific Ca\(^{2+}\) leak in permeabilized A7r5 smooth muscle cells when added at concentrations beyond 45 µM [15]. Thimerosal-stimulated Ca\(^{2+}\) release has also been reported in bovine adrenal cortex microsomes [23]. In the latter study, the Ca\(^{2+}\)-mobilizing effect of thimerosal was found to be insensitive to heparin, ruling out a possible participation of the Ins(1,4,5)P\(_3\)-operated Ca\(^{2+}\) release channel being sensitized by thimerosal to endogenous concentrations of Ins(1,4,5)P\(_3\) [14, 15]. U73122 (60 µM) stimulated the release of actively stored Ca\(^{2+}\) at a rate considerably higher than that obtained with either thapsigargin (1 µM) or thimerosal (100 µM) (Fig. 4). This observation clearly demonstrates that a higher concentration of U73122 does not act by the mere inhibition of the Ca\(^{2+}\) pump but additionally increases the passive Ca\(^{2+}\) leak.

The dose-dependency for the stimulatory effect of U73122 on the release of actively stored Ca\(^{2+}\) was measured 2 min after the addition of the drug in question to steady-state loaded microsomes (Table 1). The effect of U73122 was clearly dose-dependent and exceeded that of thapsigargin (1 µM) and thimerosal (100 µM) when added at concentrations beyond 20–30 µM. In
Table 1. Dose dependence for the stimulatory effect of U73122 on Ca\(^{2+}\) release from steady-state loaded rat liver microsomes. Rat liver microsomes were loaded with Ca\(^{2+}\) to steady state as described in the legend to Fig. 2. At 10 min following initiation of active Ca\(^{2+}\) uptake, microsomes were treated with the drug in question at the indicated concentrations and the reactions were stopped 2 min later. In each individual experiment, the control value obtained with dimethylsulfoxide, is set at 100%, to which all other values are related. The values presented are the mean ± SEM of three independent incubations.

<table>
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<tr>
<th>[Drug]</th>
<th>Residual Ca(^{2+}) content retained on the filter with thapsigargin</th>
<th>U73122</th>
<th>U73343</th>
<th>thimerosal</th>
<th>ionomycin</th>
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<td>6</td>
<td>−</td>
<td>97.2</td>
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<td>10</td>
<td>79.7 ± 1.9</td>
<td>81.7 ± 2.1</td>
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<td>20</td>
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<td>53.5 ± 0.2</td>
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<tr>
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<td>−</td>
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<td>74.5 ± 1.9</td>
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![Fig. 5](image-url)  
Fig. 5. Inhibitory effect of U73122 on the vasopressin-evoked increase in average free cytosolic Ca\(^{2+}\) concentration in a suspension of rat hepatocytes. Isolated rat hepatocytes, loaded with Fura-2, were stimulated with 0.1 \(\mu M\) vasopressin at the time indicated. Vasopressin was added in addition to either 10 \(\mu M\) U73343 (A) or 10 \(\mu M\) U73122 (B). The fluorescence emission ratio at 402 nm was monitored as a measure of [Ca\(^{2+}\)], after excitation at 340 nm and 380 nm.

In contrast, thimerosal, at concentrations equally effective in inhibiting active Ca\(^{2+}\) uptake, did not increase the aspecific Ca\(^{2+}\) leak. U73343 did not significantly affect the steady-state Ca\(^{2+}\) uptake level.

In order to investigate whether U73122 affects intracellular Ca\(^{2+}\) stores in the intact cell, its effect on [Ca\(^{2+}\)]\(_{i}\), was measured using Fura-2-loaded hepatocytes. Fig. 5B shows that addition of 10 \(\mu M\) U73122 has no effect on [Ca\(^{2+}\)]. In contrast, we previously demonstrated that U73122 does increase [Ca\(^{2+}\)], in rabbit pancreatic acinar cells [11]. Thus far, such a Ca\(^{2+}\)-mobilizing effect of U73122 has only been reported in GH3 pituitary cells of the rat [24]. Moreover, Fig. 5B shows that the Ca\(^{2+}\)-mobilizing effect of vasopressin was completely blocked by 10 \(\mu M\) U73122. This observation is in agreement with its putative action as a phospholipase C inhibitor. The inactive analog U73343 (10 \(\mu M\)) did not affect the vasopressin-evoked increase in [Ca\(^{2+}\)], (Fig. 5A). Inhibitory effects of U73122 on agonist-evoked Ca\(^{2+}\) mobilization have been reported in a variety of cell types including human platelets [5], human polymorphonuclear neutrophils [5, 6] and rat osteosarcoma cells [8].

The main conclusion of the present study is that the putative phospholipase C inhibitor, U73122, releases Ca\(^{2+}\) from intracellular stores primarily through inhibition of the intracellular Ca\(^{2+}\) pump. In addition, higher concentrations of the aminosteroid increase the aspecific Ca\(^{2+}\) leak of the internal stores by a hitherto unknown mechanism. At present, it is not clear for what reason U73122 in intact cells acts as a phospholipase C inhibitor in some cell types (hepatocytes), whereas it primarily releases Ca\(^{2+}\) from intracellular stores in other cell types (pancreatic acinar cells).

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