RESEARCH COMMUNICATION

Receptor-mediated Mn^{2+} influx in rat hepatocytes: comparison of cells loaded with Fura-2 ester and cells microinjected with Fura-2 salt

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In single Fura-2 ester-loaded hepatocytes, stimulation by vasopressin, but not emptying of the agonist-sensitive Ca^{2+} store by 2,5-di-(t-butyl)hydroquinone, resulted in an increase in the rate of Fura-2 fluorescence-quenching by Mn^{2+}. Similarly, in cells microinjected with Fura-2 salt, vasopressin stimulated Mn^{2+} entry while 2,5-di-(t-butyl)hydroquinone or thapsigargin did not. The pattern of Fura-2 quenching by Mn^{2+} only correlated with the movement of Mn^{2+} across the plasma membrane.

INTRODUCTION

Stimulation of plasma membrane receptors linked to phospholipase C isoenzymes leads to a complex array of Ca^{2+} fluxes within the cell and across its plasma membrane [1]. The initial discharge of Ca^{2+} from internal stores into the cytosol, by the second-messenger Ins(1,4,5)P_3, occurs in parallel with the opening of Ca^{2+}-selective and non-specific cation channels in the plasma membrane. These allow Ca^{2+} to enter the cell, thereby replenishing internal stores, maintaining Ca^{2+} spikes and enabling cell secretion [1].

It is well-established that the depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool acts as a signal for Ca^{2+} entry, as originally proposed by Putney [2,3]. The strongest argument for this so-called 'capacitative Ca^{2+} entry model' stems from the findings that the depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} store by inhibitors of the endoplasmic reticulum Ca^{2+}-ATPase, i.e. in the absence of phospholipase C activation, is sufficient to activate the Ca^{2+} influx pathway [3]. The sensing mechanism linking the pool depletion to Ca^{2+} influx is still somewhat unclear, although the participation of a small GTP-binding protein [4,5] or a phosphorylated messenger [6,7] has been reported.

Several aspects of receptor-mediated Ca^{2+} entry suggest that the capacitative Ca^{2+} entry pathway may not be the only mechanism controlling Ca^{2+} entry during phospholipase C-linked receptor activation. For instance, in bovine adrenal glomerulosa cells [8], human fibroblasts [9], adrenal chromaffin cells [10], JURKAT T lymphocytes [11] and PC-12 cells [12], there is a component of receptor-mediated Ca^{2+} entry that is not mimicked by simply emptying the agonist-sensitive internal store(s). Similarly, it has been proposed that hepatocytes also possess, in addition to the capacitative pathway, a separate pathway for Ca^{2+} entry that is dependent on receptor activation [13]. This receptor-dependent pathway is characterized by its ability to pass Mn^{2+} in addition to Ca^{2+}, whereas pool depletion by 2,5-di-(t-butyl)hydroquinone (tBuHQ) and thapsigargin remains without effect on the entry of Mn^{2+} [14,15]. Mn^{2+} is a commonly used surrogate for Ca^{2+} in studies of receptor-mediated Ca^{2+} entry because (i) many channels have been shown to permeate Mn^{2+} [16,17], and (ii) its movement across the plasma membrane into the cytosol is readily detected as a quench of Fura-2 fluorescence [18].

The existence of two distinct pathways of receptor-mediated Ca^{2+} entry in hepatocytes has been challenged by a recent report [19] in which thapsigargin is shown to stimulate Mn^{2+} entry in hepatocytes loaded with Fura-2 salt by microinjection, rather than with the ester form. In that study the loading of hepatocytes with Fura-2 ester was reported to result in extensive compartmentalization of the indicator in subcellular organelles, including the Ins(1,4,5)P_3-sensitive Ca^{2+} store. It was therefore concluded that the apparent lack of stimulation of Mn^{2+} entry following internal pool depletion by tBuHQ or thapsigargin was only an artifact resulting from dye subcompartmentalization. This led those authors to question the existence of an additional receptor-dependent pathway of Ca^{2+} entry besides the pool-regulated, capacitative pathway [19].

Using a number of Ca^{2+}-channel antagonists, we have recently obtained further data incompatible with a model for receptor-mediated Ca^{2+} entry based solely on the capacitative pathway [20]. In view of this new evidence we decided to re-investigate the existence of multiple pathways of Ca^{2+} entry in single hepatocytes microinjected with Fura-2 salt.

MATERIALS AND METHODS

Chemicals

Fura-2-pentapotassium salt was purchased from Molecular Probes (Eugene, OR, U.S.A.). Thapsigargin and tBuHQ were from LC Services Corporation (Woburn, MA, U.S.A.) and Aldrich Chemie (Steinheim, Germany) respectively. Nonidet P-40 was obtained from Kebo AB (Stockholm, Sweden) and poly(L-lysine) (M_r 70000–150000) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were obtained from sources reported previously [13,14].

Microinjection of Fura-2 into hepatocytes

Hepatocytes freshly isolated from male Wistar rats were preincubated for 30–60 min in KH buffer (120 mM NaCl, 5.4 mM KCl, 4.2 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$,

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; tBuHQ, 2,5-di-(t-butyl)hydroquinone; DTPA, diethylenetriaminepenta-acetic acid; AM, acetoxymethyl ester.

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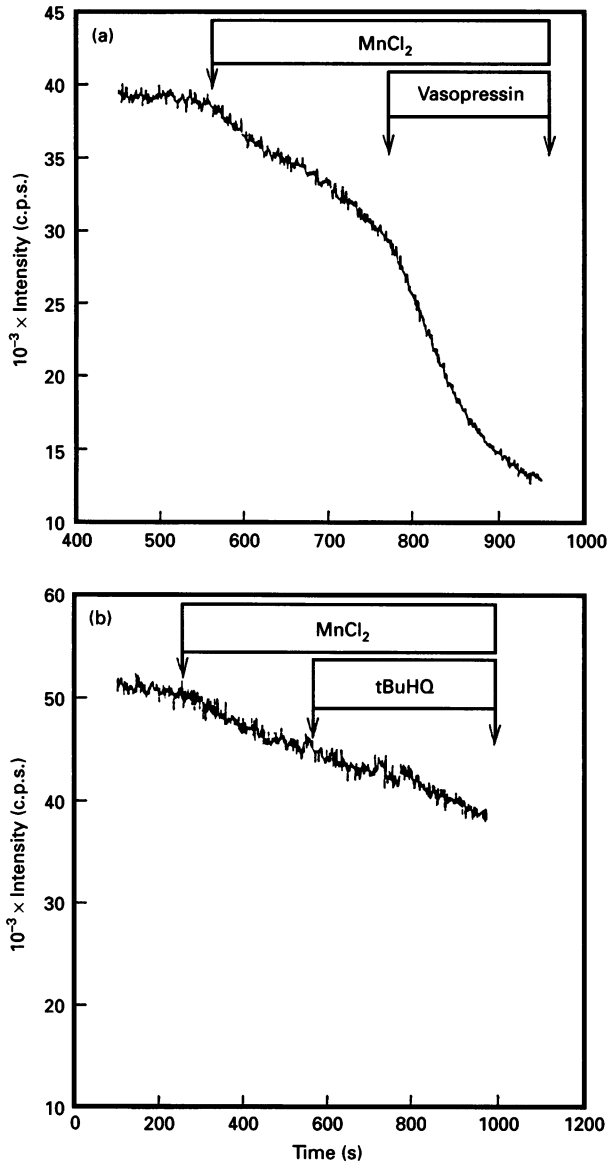

Figure 1 Mn^{2+} influx in single Fura-2 ester-loaded hepatocytes after stimulation with vasopressin or tBuHQ

Fura-2-loaded hepatocytes plated on coverslips were transferred to a perifusion chamber mounted on a Zeiss inverted microscope and perifused with KH medium maintained at 28 °C. After a stable baseline was reached at both 357 and 380 nm, the perifusion medium was switched to nominally Ca^{2+}-free KH medium containing 0.5 mM $MnCl_2$, as indicated by the first arrow. The traces are the average changes in Fura-2 emission (357 nm excitation wavelength) generated from four (**a**) or three (**b**) individual cells in each field. During the time indicated by the second and third arrows, the perifusion medium (containing $MnCl_2$) was supplemented with 10 nM vasopressin (**a**) or tBuHQ (20 μM) (**b**). The traces are representative of duplicate experiments performed on two separate batches of hepatocytes.

1.3 mM $CaCl_2$, 15 mM glucose and 20 mM Hepes, pH 7.4) supplemented with 2% (w/v) BSA before washing and resuspending the cells in KH buffer containing 0.2% BSA at a density of 3×10^5 cells/ml. The cells were then plated on to glass coverslips coated with poly(L-lysine) and were allowed to attach for 1 h at 37 °C. Microinjection was performed using an Eppendorf microinjector with an attached borosilicate glass micropipette. Microinjection pipettes were pulled with a Narashige micropipette puller (type PD/5) to tip diameters of 0.5 μm or less. The pipettes were filled with 30 mM Fura-2 salt dissolved in 150 mM KCl and 20 mM Hepes, pH 7.1. The injection time was less than 500 ms and the injected volume accounted for less than 1% of total cell volume. Under these conditions the concentration of microinjected Fura-2 was similar to that obtained by ester loading (Figures 1 and 2). The hepatocytes did not show any signs of damage (plasma membrane bleb formation and appearance of internal vacuoles) and were morphologically indistinguishable from uninjected cells. Although in all the experiments reported here each cell was only microinjected once, these cells withstood multiple consecutive injections without any apparent damage.

Loading of hepatocytes with Fura-2 acetoxymethyl ester (AM)

The conditions for loading the hepatocytes maintained in suspension with Fura-2 were as described in [20a]. Similar conditions were used when the loading procedure was performed on plated hepatocytes. Loading time was then increased to 20 min at 37 °C. The cells were subsequently washed with KH medium supplemented with 0.2% (w/v) BSA and incubated at room temperature for at least 10–20 min to enable complete de-esterification of the dye. The Fura-2-loaded cells were kept at room temperature and were used within 3 h of loading.

Measurement of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and Mn^{2+} influx in hepatocytes

Measurements of $[Ca^{2+}]_i$ and Mn^{2+} influx in cell populations were performed at 30 °C as previously reported [14,20] using a Sigma-ZWS II dual-wavelength spectrofluorimeter equipped with a xenon lamp. Single-cell measurements were performed at 28 °C using a Spex Fluorolog 2 (model CM1T11I) coupled to a Zeiss inverted microscope. Except for thapsigargin, which was directly added to the perifusion chamber, all additions were made by perifusing the cells with Ca^{2+}-free KH medium supplemented with the compounds of interest, as indicated in the Figures. The Fura-2 fluorescence signals from excitation at 380 and 357 nm were recorded separately to distinguish between alterations in $[Ca^{2+}]_i$- and Mn^{2+}-induced quenching of the dye. Emission was collected at 510 nm.

RESULTS AND DISCUSSION

In agreement with previous results obtained using cell populations [14,21], vasopressin stimulation produced a marked increase in the rate of Fura-2 fluorescence quenching by Mn^{2+} in single hepatocytes loaded with Fura-2 ester (Figure 1a). In contrast, the perifusion of the cells with tBuHQ did not enhance Fura-2 fluorescence quenching (Figure 1b); this shows that at the single-cell level also, emptying of the Ins(1,4,5)P_3-sensitive Ca^{2+} store is not sufficient to accelerate the rate of Mn^{2+} influx. One noticeable difference, however, was that in the single cells the initial rapid drop in fluorescence observed in populations of cells when Mn^{2+} is added to the cuvette [14,21] was absent; this suggests that the latter mainly consists of extracellular (leaked out) Fura-2.

In the next set of experiments Fura-2 ester-loaded cells were compared with cells that had been microinjected with Fura-2 salt. As shown in Figure 2, microinjection led to intracellular levels of the dye that were very similar to those registered in ester-loaded single cells. Most importantly, perifusing the microinjected hepatocytes with vasopressin markedly enhanced the rate of Fura-2 quenching by Mn^{2+} [Figure 2(a) and Table 1),

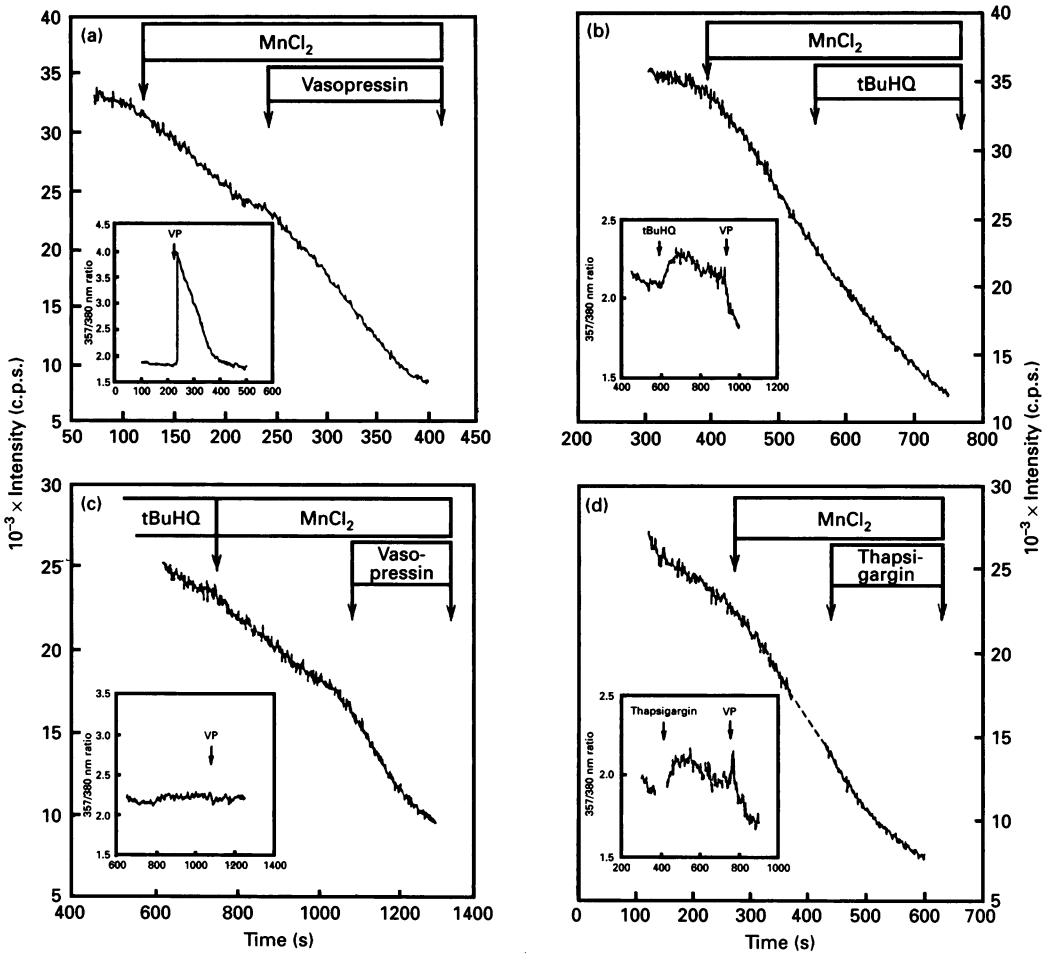

Figure 2 Mn^{2+} influx in single hepatocytes microinjected with Fura-2 salt

Hepatocytes were microinjected with Fura-2 pentapotassium salt as described in the Materials and methods section. The cells were allowed sufficient time for recovery, and the average Fura-2 fluorescence generated from four (**a** and **b**) or three (**c** and **d**) individual cells per field was recorded. The perifusion medium was then switched to nominally Ca^{2+}-free KH medium containing 0.5 mM $MnCl_2$, as indicated by the first arrow. The traces are the changes in Fura-2 emission using 357 nm as the excitation wavelength. During the time indicated by the second and third arrows, the perifusion medium was supplemented with 10 nM vasopressin (**a** and **c**), tBuHQ (20 μM) (**b**) or thapsigargin (0.75 μM) (**d**). The broken line in (**d**) represents the time during which the recording was interrupted to allow for the addition of thapsigargin to the perfusion chamber. In the experiment shown in (**c**), the hepatocytes were exposed to tBuHQ (20 μM) for 4.5 min in Ca^{2+}-free medium to deplete internal stores, before switching the medium to nominally Ca^{2+}-free KH medium containing 0.5 mM $MnCl_2$, as indicated by the first arrow and then vasopressin (10 nM)(second arrow). The computed 357/380 nm excitation ratios are shown in the inset of each panel. The traces are representative of three to seven experiments performed on five different batches of hepatocytes.

whereas emptying internal pools by infusing tBuHQ [Figure 2(b) and Table 1] or adding thapsigargin (Figure 2d) failed to stimulate Fura-2 quenching in the presence of Mn^{2+}. These observations fully support our previous findings and conclusions [13–15] that internal Ca^{2+}-pool emptying is not sufficient to activate the Mn^{2+} entry pathway but that the latter requires the stimulation of a phospholipase C-linked plasma membrane receptor.

As depicted in the insets of Figure 2, the treatments with tBuHQ and thapsigargin mobilized the Ins(1,4,5)P_3-sensitive Ca^{2+} store essentially to completion, as ascertained by the lack or minor Ca^{2+}-releasing effect obtained by a subsequent infusion of vasopressin. In the experiment shown in Figure 2(c), microinjected cells were perfused with tBuHQ for 4.5 min in Ca^{2+}-free KH medium. Perifusion medium was then switched to Ca^{2+}-free KH buffer supplemented with 0.5 mM Mn^{2+} to wash out tBuHQ from the chamber without allowing the cells to replenish their agonist-sensitive Ca^{2+} pool (Figure 2c, inset). When these hepatocytes were exposed to vasopressin, Mn^{2+} influx was stimulated (Figure 2c). That under these conditions, essentially complete emptying of internal pools by tBuHQ had been obtained is confirmed in the inset of Figure 2(c). Taken together, these results show that (i) the mode of introducing Fura-2 does not affect the results obtained and (ii) pool depletion is not a sufficient event to stimulate Mn^{2+} entry in hepatocytes.

Subcompartmentalization of ion indicators by cellular organelles during loading with their ester forms is a well-appreciated, yet often underestimated, phenomenon [22]. Organelles reported to sequester substantial amounts of indicator include the mitochondria [23], acidic organelles [24], the Ins(1,4,5)P_3-sensitive Ca^{2+}-storing organelle [19,25] and the Golgi apparatus [26]. Ca^{2+} indicators will also equilibrate with the nucleus through simple diffusion [26]. Glennon et al. [19] reported that in their laboratory the loading of hepatocytes with Fura-2 AM resulted in most of the dye ending up in various organelles, including the Ins(1,4,5)P_3-sensitive Ca^{2+} store. They also proposed that this compartmentalization was the reason for the failure of thapsigargin to stimulate an apparent Mn^{2+} influx in Fura-2 AM-loaded hepatocytes: Mn^{2+} entry was activated by thapsigargin

Table 1 Stimulation of Mn^{2+} influx in Fura-2 salt-microinjected hepatocytes requires receptor activation

Hepatocytes were microinjected with Fura 2 pentapotassium salt as described in the Materials and methods section. After allowing sufficient time for recovery, the average Fura-2 fluorescence generated by a field containing between two and eight cells was recorded and, after a stable baseline was reached at both 357 and 380 nm, the medium was switched to nominally Ca^{2+}-free KH medium containing 0.5 mM $MnCl_2$. The rates of Fura-2 fluorescence quenching (357 nm excitation) before and during stimulation by vasopressin (10 nM) or tBuHQ (20 μM) were compared. All data are expressed as fold stimulation of the rates of Fura-2 fluorescence quenching by vasopressin or tBuHQ over basal rate and are reported as mean ± S.E.M. of n experiments performed on five separate batches of hepatocytes.

Treatment	(n)	Mn^{2+} influx (fold stimulation)
Vasopressin (10 nM)	(5)	2.4 ± 0.2
tBuHQ (20 μM)	(7)	1.0 ± 0.1

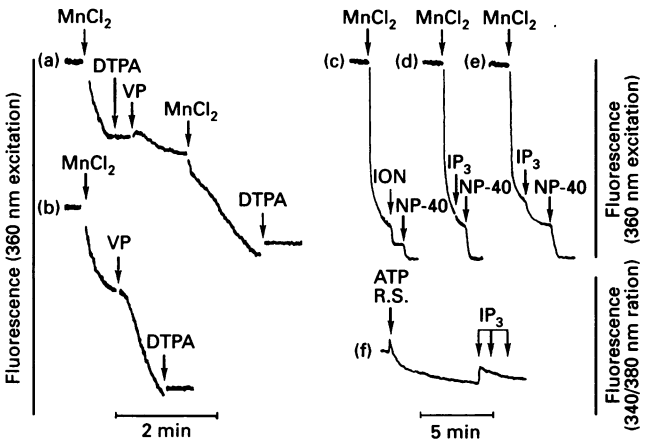

Figure 3 Role of intracellular Mn^{2+} and Fura-2 compartmentalization in the vasopressin-induced quench of Fura-2 in populations of isolated hepatocytes

Hepatocytes were loaded with Fura-2 AM as detailed in the Materials and methods section and incubated in 1.3 mM Ca^{2+}-containing KH medium. After washing by centrifugation, the cells were resuspended in nominally Ca^{2+}-free KH medium. (**a**) 1 min after resuspension, $MnCl_2$ (0.5 mM) was added as indicated by the first arrow. When the initial phase of Fura-2 quenching was complete, DTPA (0.625 mM) was added to remove all the extracellular Mn^{2+}, followed by vasopressin (VP, 10 nM). Quenching of Fura 2 fluorescence was reinitiated by a second addition of $MnCl_2$ (0.625 mM) followed again by DTPA (1 mM). (**b**) A typical control trace is shown where DTPA (0.625 mM) was added after vasopressin. Identical results were obtained when extracellular Mn^{2+} was removed by washing the cells. In (**c**), the Fura-2-loaded cells were washed by centrifugation and resuspended in an intracellular-like medium at 1×10^6 cells/ml before adding saponin (38 μg/ml) followed 1 min later by $MnCl_2$ (0.5 mM). After the rate of Fura-2 quenching began to slow down, ionomycin (10 μM) followed by Nonidet P-40 (NP-40, 0.1%) were added to the cuvette. In the experiments reported in (**d**)–(**f**), saponin (38 μg/ml) was given to the cells together with an ATP-regenerating system (ATP R.S.). When Ca^{2+} uptake by mitochondria and endoplasmic reticulum was complete, $MnCl_2$ (0.5 mM) or Ins(1,4,5)P_3 (IP_3; 5 μM) were added and [Ca^{2+}] (ratio 340/380 nm) and Fura-2 fluorescence quenching (360 nm excitation) were measured. In (**d**) and (**e**), Ins(1,4,5)P_3 (5 μM) and Nonidet P-40 (NP-40, 0.1%) were added after $MnCl_2$, as indicated by the arrows. The trace in (**e**) represents the largest stimulation of Fura-2 quenching by Ins(1,4,5)P_3 observed in five sets of experiments performed on separate batches of hepatocytes.

but Fura-2 only became accessible to intracellularly accumulated Mn^{2+} following the opening of the Ins(1,4,5)P_3-gated channels, i.e. through the stimulation of phospholipase C.

The following experiments were designed to address this particular issue. Hepatocytes loaded with Fura-2 ester were exposed to Mn^{2+} until the initial phase of quenching was complete (Figure 3a). Extracellular Mn^{2+} was then removed by chelation with diethylenetriaminepenta-acetic acid (DTPA), and cells then stimulated with vasopressin. This produced only a minor change in Fura-2 fluorescence, in contrast with the quenching produced on re-addition of extracellular Mn^{2+} (Figure 3a) or in the continuous presence of Mn^{2+} (Figure 3b). Furthermore, removal of extracellular Mn^{2+} by DTPA in vasopressin-stimulated hepatocytes immediately arrested the quenching of the fluorescence. Consequently, the decrease in Fura-2 fluorescence induced by phospholipase C-linked agonists reported here and in our previous work is caused by an increase in the permeability of the plasma membrane to bivalent cations, and is not due to a redistribution of intracellular Mn^{2+}.

Furthermore, as illustrated in Figures 3(c)–3(e), the addition of Mn^{2+} to a population of Fura-2-loaded hepatocytes (1×10^6 cells/ml) permeabilized with saponin (38 μg/ml) produced 85% quench of total Fura-2 fluorescence (360 nm excitation), as estimated from the quenching of the dye obtained by the addition of ionomycin. Some remaining Fura-2 appeared to be released by Nonidet P-40 (0.1%) (Figures 3c–3e), although most of the latter effect was due to a change in light scattering. When the hepatocytes were permeabilized in an intracellular-like medium [20] in the presence of an ATP-regenerating system, Ins(1,4,5)P_3 addition shortly after that of Mn^{2+} produced a small additional quench accounting for 5.1 + 1.8% (n = 5) of the total Fura-2 fluorescence [Figures 3(d) and 3(e)]. Ins(1,4,5)P_3 was able to release Ca^{2+} in the permeabilized cells, demonstrating that this concentration of saponin did not affect the ability of the cells to sequester Ca^{2+} into the Ins(1,4,5)P_3-sensitive pool (Figure 3f). Similar results were obtained when the hepatocytes were permeabilized with digitonin (20–30 μg/ml) instead of saponin (results not shown). The saponin concentration dependency for permeabilization of hepatocytes showed that the release of Fura-2 preceded the release of Acridine Orange and Rhodamine 123 in a similar manner, as reported in [27] (results not shown). Taken together, these results are in clear contrast with the findings reported by Glennon et al. [19] and show that with our Fura-2 AM-loading conditions, 85% of the Fura-2 is localized in the cytosol and approx. 5% in the Ins(1,4,5)P_3-sensitive compartment. Our findings are also in clear agreement with other reports, where the location of Fura-2 after loading of isolated rat hepatocytes with the ester form was investigated (see [27,28]). The rate of leakage of Fura-2 from the cytosol of hepatocytes is temperature-dependent, and we find it essential to keep the cells at room temperature after loading to avoid loss of the bulk of cytosolic Fura-2 to the medium.

While the difference in the degree of dye compartmentalization between this study and that by Glennon et al. [19] can easily be reconciled by differences in the loading and post-loading conditions, there is no direct explanation for the discrepancy in the effect of agonist-sensitive pool-emptying on Mn^{2+} entry in Fura-2-microinjected cells. However, one major experimental difference is that we used freshly isolated hepatocytes whereas Glennon et al. [19] stored their cells at 4 °C overnight. The latter condition is well known to induce a phenomenon often referred to as cold-shock. On re-warming, the cells undergo a number of degenerative processes leading to programmed cell death or apoptosis [29,30]. Programmed cell death is generally characterized by nuclear changes [31,32]; however, a number of ancillary signals such as alterations in plasma membrane composition and activation of certain cell-signalling pathways also participate in this mode of cell death [31–34]. It is possible that such changes could account for the observation that thapsigargin stimulates Mn^{2+} entry in cold-shocked hepatocytes.

In summary, whether hepatocytes are microinjected with Fura-2 salt or loaded with the ester form of this dye, emptying of the agonist-sensitive Ca^{2+} store with thapsigargin or tBuHQ does not completely mimic true receptor-mediated Ca^{2+} entry, as it is not sufficient to activate the Mn^{2+} influx pathway. It is concluded that the hepatocyte possesses multiple pathways that independently regulate Ca^{2+} entry during the stimulation of phospholipase C-linked receptors.

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