Biochem. J. (1992) 284, 243–247 (Printed in Great Britain)

Evidence for two pathways of receptor-mediated Ca^{2+} entry in hepatocytes

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Receptor-mediated Ca^{2+} entry was studied in fura-2-loaded isolated hepatocytes. Emptying of internal Ca^{2+} stores by treatment with either the Ca^{2+}-mobilizing hormone vasopressin or the inhibitors of the microsomal Ca^{2+} pump, 2,5-di-(t-butyl)-1,4-benzohydroquinone (tBuBHQ) or thapsigargin, stimulated Ca^{2+} entry, as indicated by a rise in the cytosolic free Ca^{2+} concentration after Ca^{2+} was added to cells suspended in nominally Ca^{2+}-free medium. The enhancement of Ca^{2+} entry was proportional to the degree of depletion of the intracellular Ca^{2+} pool and occurred also after removal of vasopressin from its receptor. In contrast, the stimulation of Mn^{2+} entry by vasopressin required the continuous presence of the agonist, since it was prevented by the addition of vasopressin receptor antagonist. This effect was observed under conditions where refilling of the agonist-sensitive pool was prevented by using nominally Ca^{2+}-free medium. Unlike vasopressin, tBuBHQ or thapsigargin did not stimulate Mn^{2+} entry. These results suggest the existence of two pathways for receptor-mediated Ca^{2+} entry in hepatocytes, a 'capacitative' pathway that is sensitive to the Ca^{2+} content in the Ins(1,4,5)P_3-sensitive Ca^{2+} pool and does not allow Mn^{2+} entry, and a second pathway that depends on receptor occupation, seems to require a second messenger for activation, and permits influx of Mn^{2+}.

INTRODUCTION

The binding of many hormones and growth factors to their plasma membrane receptors is coupled through phospholipase C activation to the formation of two second messengers, diacylglycerol and the water-soluble Ins(1,4,5)P_3, which releases Ca^{2+} from intracellular stores, thereby transiently raising the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) [1]. In hepatocytes, as in other cell types, such Ca^{2+}-mobilizing hormones also promote Ca^{2+} fluxes across the plasma membrane, by stimulating both Ca^{2+} efflux [2–5] and Ca^{2+} entry [6–9]. This receptor-mediated Ca^{2+} entry, which is not dependent on depolarization, fulfills several functions in the cells, including refilling of internal Ca^{2+} pools mobilized by agonists, prolongation of the Ca^{2+} signal, localized signalling and maintenance of Ca^{2+} oscillations.

Various mechanisms of agonist-stimulated Ca^{2+} entry have been described. In several cell types receptor activation results in either a direct, or G protein-mediated, opening of Ca^{2+} channels; these have been termed receptor-operated Ca^{2+} channels [10,11]. In other cases there is strong evidence that hormone-generated inositol polyphosphates are involved (second-messenger-operated Ca^{2+} channels) [11–13]. Finally, a role for the depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} store in activating Ca^{2+} entry has been proposed (the capacitative Ca^{2+}-entry model) [14,15].

Although under physiological conditions the discharge of the internal Ca^{2+} pool only occurs after agonist stimulation, it is possible to deplete this store independently of receptor occupation, by using inhibitors of the microsomal Ca^{2+} translocase, such as 2,5-di-(t-butyl)-1,4-benzohydroquinone (tBuBHQ) [16] or thapsigargin [17]. Both inhibitors have been shown to mobilize the Ins(1,4,5)P_3-sensitive Ca^{2+} store in several cell types, without inducing production of inositol polyphosphates [18–21]. Thapsigargin has been reported to stimulate Ca^{2+} and Mn^{2+} entry from the extracellular medium [20,22–24], thereby supporting the capacitative hypothesis of Ca^{2+} entry. However, we have recently reported that, in contrast with Ca^{2+}-mobilizing hormones, tBuBHQ or thapsigargin did not produce Ca^{2+} influx across the plasma membrane in isolated hepatocytes as monitored by the Mn^{2+}-entry technique [24,25]. Consequently, the present study was carried out to clarify the role of the state of the hormone-responsive intracellular Ca^{2+} store in controlling Ca^{2+} entry, and we present evidence suggesting the existence of two distinct pathways of agonist-stimulated Ca^{2+} entry in hepatocytes.

MATERIALS AND METHODS

Materials

Fura-2 acetoxymethylester, verapamil, [Arg^8]vasopressin, [β-mercapto-$\beta\beta$-cyclopentamethylenepropionyl^1,OMe-Tyr^2,Arg^8]-vasopressin (vasopressin antagonist) and angiotensin II were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Collagenase (grade II) and nifedipine were purchased from Boehringer, Mannheim, Germany. tBuBHQ and thapsigargin were from EGA Chemie (Steinheim, Germany) and GIBCO BRL (Gaithersburg, MD, U.S.A.) respectively.

Measurement of $[Ca^{2+}]_i$ and Mn^{2+} influx in hepatocytes

Hepatocytes were isolated from male Wistar rats (180–230 g, fed *ad libitum*) as previously described [26]. Loading with fura-2 and measurement of $[Ca^{2+}]_i$ were performed as previously reported [21]. Under these conditions, greater than 80% of the intracellular fura-2 was located in the cytosolic compartment, as assessed by the quench of fura-2 fluorescence on addition of 75 μg of saponin/10^6 cells to hepatocytes incubated in the presence of 0.5 mM-$MnCl_2$. The procedure for monitoring Mn^{2+} influx was as described in [25], except that fura-2 fluorescence emission was recorded with a Sigma-ZWS II dual-wavelength spectrofluorimeter equipped with a xenon lamp. The fura-2 fluorescence signals from excitation at 340 and 360 nm were recorded separately to distinguish between alterations in $[Ca^{2+}]_i$ and Mn^{2+}-induced quenching of the dye. tBuBHQ and thap-

Abbreviations used: tBuBHQ, 2,5-di-(t-butyl)-1,4-benzohydroquinone; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; vasopressin antagonist, [β-mercapto-$\beta\beta$-cyclopentamethylenepropionyl^1,OMe-Tyr^2,Arg^8]vasopressin.

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sigargin were dissolved in dimethyl sulphoxide; the final concentration of solvent in the cell suspension never exceeded 0.5 % (v/v). Vasopressin and vasopressin antagonist were dissolved in water.

RESULTS

Effects of vasopressin, tBuBHQ and thapsigargin on Ca^{2+} entry across the plasma membrane in fura-2-loaded hepatocytes

Fig. 1*a* illustrates a typical transient Ca^{2+} response to vasopressin (1 nM) in fura-2-loaded hepatocytes suspended in nominally Ca^{2+}-free medium (without addition of EGTA). Restoration of extracellular Ca^{2+} 4 min after hormone addition produced a rapid elevation of $[Ca^{2+}]_i$, a 'Ca^{2+} overshoot' phenomenon, suggesting that the hormone activated a Ca^{2+}-influx pathway. This effect was effectively prevented by addition of Ni^{2+} (4 mM), a blocker of receptor-mediated Ca^{2+} entry [27,28] before Ca^{2+} (results not shown).

In order to determine whether the discharge of internal Ca^{2+} stores by vasopressin contributed to the stimulation of Ca^{2+} entry, excess vasopressin receptor antagonist (20 nM) was added to displace the hormone from its receptor. These experiments were performed in nominally Ca^{2+}-free medium to prevent refilling of the Ins(1,4,5)P_3-sensitive Ca^{2+} store. Addition of external Ca^{2+} produced an increase in $[Ca^{2+}]_i$ (Fig. 1*b*), similar to that observed with vasopressin alone. Moreover, Ca^{2+} entry refilled the Ins(1,4,5)P_3-sensitive Ca^{2+} store, as assessed by addition of another Ca^{2+}-mobilizing hormone, angiotensin II (10 nM) after removal of extracellular Ca^{2+} with EGTA (1.5 mM) (cf. Fig. 1*a* and 1*b*). Refilling of this pool was time-dependent, i.e. the magnitude of the angiotensin II-stimulated $[Ca^{2+}]_i$ rise increased with time after restoration of extracellular Ca^{2+} (results not shown). Prior treatment with vasopressin receptor antagonist

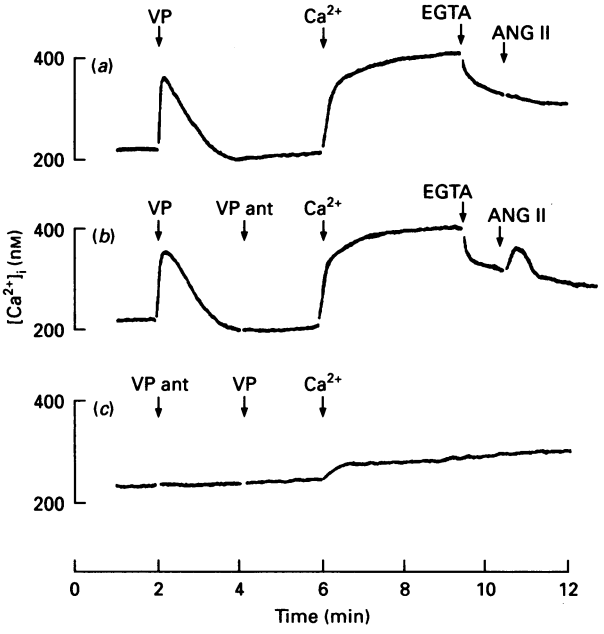

Fig. 1. Vasopressin-induced Ca^{2+} entry in isolated hepatocytes

Fura-2-loaded hepatocytes (10^6 cells/ml) maintained in Ca^{2+}-containing (1.3 mM) medium were washed by centrifugation and resuspended in nominally Ca^{2+}-free medium (no $CaCl_2$ or EGTA added) at 0 min. The following additions were made as indicated by arrows: vasopressin (VP, 1 nM), $CaCl_2$ (Ca^{2+}, 1.3 mM), EGTA (1.5 mM), angiotensin II (ANG II, 10 nM) and vasopressin antagonist (VP ant, 20 nM).

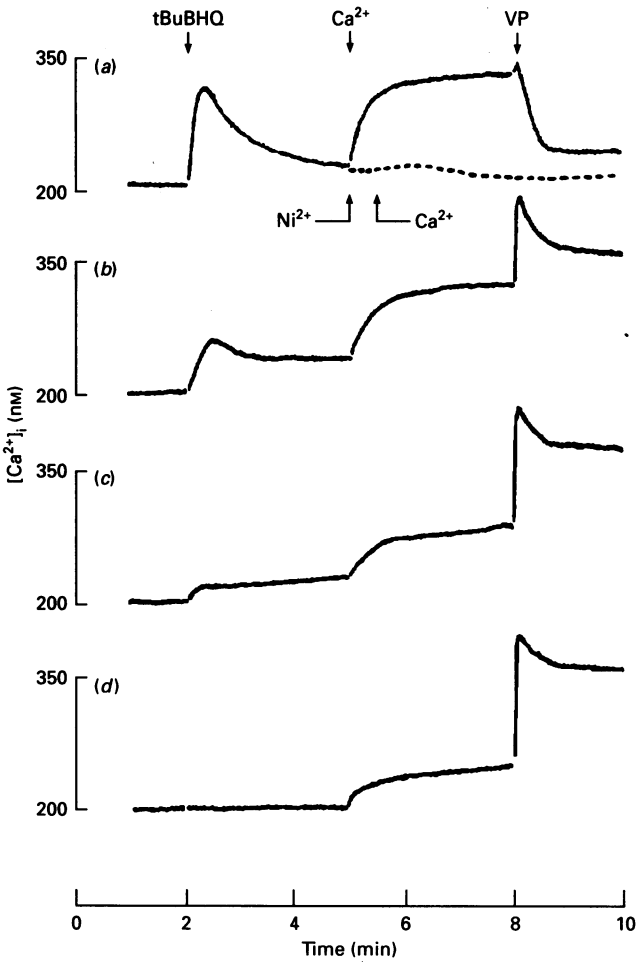

Fig. 2. Stimulation of Ca^{2+} entry by tBuBHQ

Fura-2-loaded hepatocytes (10^6 cells/ml) were resuspended in nominally Ca^{2+}-free medium at 0 min. The following additions were made as indicated by arrows: tBuBHQ (20, 2, 0.4 and 0 μM, for (*a*), (*b*), (*c*) and (*d*) respectively), $CaCl_2$ (Ca^{2+}, 1.3 mM) and vasopressin (VP, 10 nM). In (*a*) (broken line) $NiCl_2$ (Ni^{2+}, 4 mM) was added 15 s before Ca^{2+}.

effectively prevented the vasopressin-induced Ca^{2+} signal, as illustrated in Fig. 1(*c*), and subsequent Ca^{2+} addition induced only a small increase in $[Ca^{2+}]_i$. A similar $[Ca^{2+}]_i$ response to that shown in Fig. 1(*c*) was obtained when extracellular Ca^{2+} was restored to untreated cells (results not shown). These results show that: (i) emptying of the vasopressin-sensitive Ca^{2+} pool in isolated hepatocytes leads to the stimulation of Ca^{2+} entry across the plasma membrane, and (ii) refilling of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool occurs on hormone removal.

In an alternative approach to confirm the above findings, we used the inhibitors of the microsomal Ca^{2+}-ATPase, tBuBHQ and thapsigargin, to deplete the Ins(1,4,5)P_3-sensitive Ca^{2+} store independently of receptor occupancy. tBuBHQ caused a concentration-dependent transient increase in $[Ca^{2+}]_i$ in hepatocytes suspended in nominally Ca^{2+}-free medium (Fig. 2), as previously reported [21]. Addition of external Ca^{2+} after the tBuBHQ-induced $[Ca^{2+}]_i$ transient resulted in a rise in $[Ca^{2+}]_i$ (Fig. 2), which involved stimulation of Ca^{2+} entry, since it was effectively prevented by Ni^{2+} (Fig. 2*a*, lower trace). The rate and magnitude of Ca^{2+} entry was found to be proportional to the concentration of tBuBHQ and to the extent of Ca^{2+} mobilized by this compound (Fig. 2*a–d*). The amount of Ca^{2+} released by a subsequent addition of vasopressin (2 min after restoration of external Ca^{2+})

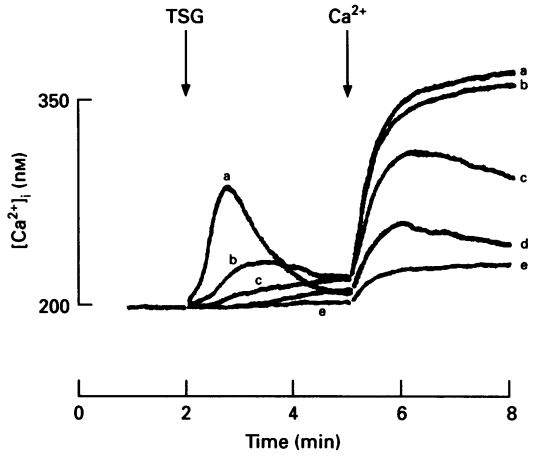

Fig. 3. Stimulation of Ca^{2+} entry by thapsigargin

Fura-2-loaded hepatocytes (10^6 cells/ml) were resuspended in nominally Ca^{2+}-free medium at 0 min. Thapsigargin was added at 2 min (TSG; 100, 10, 3, 1 and 0 nM, for traces a, b, c, d and e respectively); $CaCl_2$ (Ca^{2+}, 1.3 mM) was added at 5 min.

was smaller with increasing concentrations of tBuBHQ (Fig. 2), as expected from the continuous presence of the inhibitor, which prevented refilling of the hormone-sensitive store. With maximally effective concentrations of tBuBHQ (Fig. 2*a*), the addition of vasopressin after Ca^{2+} produced a rapid decrease in $[Ca^{2+}]_i$. This phenomenon has previously been observed, and was found to be due to the stimulation of Ca^{2+} extrusion across the plasma membrane by the hormone [5,21].

Thapsigargin elicited similar effects with regard to internal Ca^{2+} mobilization and Ca^{2+} entry (Fig. 3). The voltage-dependent Ca^{2+} channel antagonists verapamil and nifedipine (up to 50 μM) did not block the Ca^{2+}-entry phase elicited by vasopressin, tBuBHQ or thapsigargin (results not shown), supporting the contention that the Ca^{2+}-entry pathway involved is different from L-type voltage-dependent Ca^{2+} channels. It is worth noting that maximal Ca^{2+} entry was obtained with concentrations of the inhibitors that produced only partial discharge of the Ins(1,4,5)P_3-sensitive Ca^{2+} store (Fig. 3).

In order to obtain a quantitative estimate of Ca^{2+} entry by the different treatments described above, the rate of increase of $[Ca^{2+}]_i$ was measured during the first 10 s after restoration of extracellular Ca^{2+}. Table 1 shows that vasopressin alone or followed by receptor antagonist, and the inhibitors of the microsomal Ca^{2+} translocase all stimulated the initial rate of Ca^{2+} entry about 5-fold over the control, and no significant difference was observed between these treatments.

Effects of vasopressin, thapsigargin and tBuBHQ on Mn^{2+} entry in fura-2-loaded hepatocytes

Mn^{2+} not only permeates through many types of Ca^{2+} channels, but also binds strongly to fura-2 and, in contrast with Ca^{2+}, quenches the fluorescence emission of the dye at all excitation wavelengths. On the basis of these properties, Hallam & Rink developed a method to follow Ca^{2+} channel activity in intact cells loaded with fura-2 [27]. We have previously reported that, in the presence of 1.3 mM-Ca^{2+}, Ca^{2+}-mobilizing agonists such as vasopressin, angiotensin II, ATP and phenylephrine stimulate Mn^{2+} entry in hepatocytes [25]. In contrast, tBuBHQ alone did not stimulate this entry route, although pretreatment of hepatocytes with tBuBHQ markedly enhanced the onset and the rate of vasopressin-induced Mn^{2+} influx [25]. More recently, we have reported a similar effect for thapsigargin [24].

Table 1. Effect of vasopressin, tBuBHQ and thapsigargin on Ca^{2+} and Mn^{2+} entry in fura-2-loaded hepatocytes

Cells maintained in 1.3 mM-Ca^{2+}-containing medium were washed by centrifugation and resuspended in nominally Ca^{2+}-free medium. The following additions were performed (abbreviations and time after resuspension in nominally Ca^{2+}-free medium indicated in parentheses): vasopressin (VP; 2 min), vasopressin antagonist (VP ant; 3.5 min), tBuBHQ (2 min), thapsigargin (2 min) and 1.3 mM-$CaCl_2$ (5 min) or 0.5 mM-$MnCl_2$ (5 min). The rates of increase in $[Ca^{2+}]_i$ during the first 10 s after Ca^{2+} addition, or quench of intracellular fura-2 fluorescence (360 nm excitation) during the first 30 s after Mn^{2+} addition, expressed as arbitrary fluorescence units (AU)/min, are shown (mean ± S.D. of four to eleven experiments for each treatment).

	Ca^{2+} entry		Mn^{2+} entry	
Addition	(nmol·min^{-1}·litre^{-1})	Fold change	(AU/min)	Fold change
Control	120±57	1.0	6.1±1.8	1.0
1 nM-VP	590±96	4.9	23.8±3.3	3.9
1 nM-VP +20 nM-VP ant	572±73	4.8	7.8±0.9	1.3
10 μM-tBuBHQ	602±61	5.0	5.3±0.5	0.9
100 nM-thapsigargin	542±125	4.5	5.8±1.9	1.0

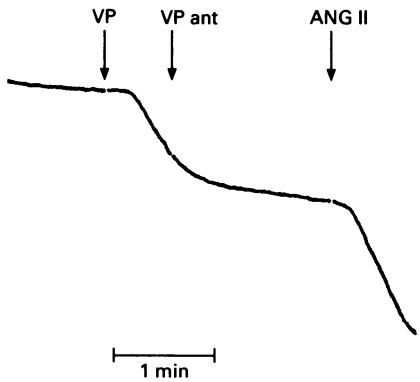

Fig. 4. Effect of vasopressin, vasopressin receptor antagonist and angiotensin II on Mn^{2+} entry

Fura-2-loaded hepatocytes (10^6 cells/ml) were resuspended in nominally Ca^{2+}-free medium in the presence of $MnCl_2$ (50 μM). Fluorescence (> 500 nm emission) was measured with excitation at 360 nm (at this wavelength changes in $[Ca^{2+}]_i$ do not alter fura-2 fluorescence). The following additions were made as indicated by arrows: vasopressin (VP, 1 nM), vasopressin antagonist (VP ant, 20 nM), and angiotensin II (ANG II, 10 nM).

To investigate the apparent requirement for hormone–receptor interaction for stimulating Mn^{2+} entry, vasopressin receptor antagonist was added to hepatocytes after emptying of the Ins(1,4,5)P_3-sensitive pool by the agonist; the cells were suspended in Ca^{2+}-free medium to prevent refilling of this pool. When Mn^{2+} was added 1.5 min after vasopressin antagonist, only a slight stimulation of the rate of Mn^{2+} entry occurred, whereas vasopressin alone (1 nM) increased by 4-fold the rate of Mn^{2+} influx (Table 1). The initial rate of Mn^{2+} entry after treatment with tBuBHQ or thapsigargin was similar to the control (Table 1).

Vasopressin receptor antagonist blocked ongoing agonist-induced Mn^{2+} entry, as illustrated in Fig. 4. Moreover, addition

of a second Ca^{2+}-mobilizing hormone (angiotensin II, 10 nM) restored the stimulation of bivalent cation influx (Fig. 4).

DISCUSSION

The stimulation of hepatocytes with Ca^{2+}-mobilizing hormones such as vasopressin, angiotensin II and phenylephrine results in the release of an intracellular non-mitochondrial Ca^{2+} store through the action of Ins(1,4,5)P_3. In addition, this group of hormones has been shown to enhance Ca^{2+} cycling across the plasma membrane and, in particular, to stimulate Ca^{2+} uptake by the cell [6–9]. In this report we have investigated the mechanism by which Ca^{2+}-mobilizing hormones regulate Ca^{2+} entry in hepatocytes.

In a first experimental approach Ca^{2+} entry was studied by measuring the $[Ca^{2+}]_i$ response on restoration of extracellular Ca^{2+} to cells depleted of the agonist-sensitive Ca^{2+} store with vasopressin or the microsomal Ca^{2+}-ATPase inhibitors. Under these conditions, vasopressin stimulated Ca^{2+} entry in isolated hepatocytes. Furthermore, Ca^{2+} entry was stimulated even after removal of vasopressin from its receptor, as well as by depletion of the agonist-sensitive Ca^{2+} store with tBuBHQ or thapsigargin. It can thus be concluded that the phenomenon of capacitative Ca^{2+} entry, i.e. the stimulation of Ca^{2+} entry as a consequence of the discharge of the internal Ca^{2+} store [15], also occurs in rat hepatocytes.

An additional approach to study the hormonal regulation of Ca^{2+} entry in hepatocytes was to use Mn^{2+} as a surrogate for Ca^{2+}. We have previously shown that Ca^{2+}-mobilizing hormones activate a Ni^{2+}-inhibitable Ca^{2+}-entry pathway that is permeable to Mn^{2+} [25]. In the present work we show that the activation of Mn^{2+} entry by Ca^{2+}-mobilizing hormones required the continuous presence of the agonist (Table 1 and Fig. 4), and was not stimulated after depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool by tBuBHQ or thapsigargin (Table 1; see also [24] and [25]).

Taken together, the observation of two influx responses activated by agonists, with different cation selectivity and subject to different regulation, supports the existence of two separate pathways for receptor-mediated Ca^{2+} entry in hepatocytes: a capacitative type of pathway that does not allow Mn^{2+} entry, and a second pathway that is permeable to Mn^{2+} and requires hormone–receptor interaction for activation. In agreement with this conclusion, recent work in thrombin-stimulated platelets showed temporal differences between $[Ca^{2+}]_i$ rise and Mn^{2+} entry that were also consistent with the existence of two Ca^{2+}-entry pathways, only one being permeable to Mn^{2+} [29].

The relative contribution of each of these pathways to hormone-stimulated Ca^{2+} entry in hepatocytes is at present not known. Although a direct comparison of the rates of Ca^{2+} entry shown in Table 1 is not possible owing to the different methodologies used, it is likely that both pathways are quantitatively important. In the Ca^{2+} overshoot experiments, the overall rate of Ca^{2+} entry stimulated by vasopressin is similar to the rate obtained with tBuBHQ, despite the fact that vasopressin stimulates a concomitant Ca^{2+} efflux response in addition to influx, whereas Ca^{2+} efflux is quantitatively not important with tBuBHQ [5]. Thus, the Ca^{2+} overshoot caused by vasopressin reflects the net balance between Ca^{2+} entry and Ca^{2+} extrusion across the plasma membrane, and therefore represents an underestimation of the true rate of Ca^{2+} entry. This explains why stimulation of cells with vasopressin, which activates both pathways, did not result in an increased rate of Ca^{2+} entry as compared with tBuBHQ, thapsigargin, or vasopressin followed by its receptor antagonist (Table 1, left column). Furthermore, activation of both pathways by vasopressin may explain why this hormone produced a small but significant stimulation of $^{45}Ca^{2+}$ uptake by isolated hepatocytes, whereas the effect of tBuBHQ was not detectable [21].

Although in hepatocytes Mn^{2+} entry was not stimulated by depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool, this is not a general characteristic of capacitative Ca^{2+}-entry pathways, since in several other cell types Mn^{2+} entry as a consequence of the discharge of the internal Ca^{2+} store has been observed [24,30,31]. Hence, it appears that the cation selectivity of receptor-mediated Ca^{2+} entry varies between different cell types.

An important feature of capacitative Ca^{2+} entry is the correlation between the depletion of internal Ca^{2+} pools and the rate of Ca^{2+} entry [22]. In human umbilical-vein endothelial cells a direct relationship between the rate of Mn^{2+} entry and depletion of the internal Ca^{2+} store was observed [31]. In agreement with the above reports, we found that the magnitude of Ca^{2+} entry was dependent on the extent of mobilization of the agonist-sensitive store (Fig. 2 and Fig. 3). However, our results also show that maximal Ca^{2+}-entry response occurs at concentrations of tBuBHQ and thapsigargin that do not completely deplete the internal Ca^{2+} pool, as reported by others [22]. How the information on the content of the Ca^{2+} stores is transduced to regulate plasma membrane Ca^{2+} permeabilitity in hepatocytes is still unclear, although some tentative models have been proposed [32].

With regard to the regulation of the pathway that requires continuous hormone–receptor binding for activation, either a receptor–G protein–Ca^{2+} channel ternary complex or a hormone-generated second messenger (inositol polyphosphate?) may be involved. The latter possibility appears more likely, since a direct interaction of a G protein with the entry mechanism would be difficult to reconcile with the latency observed for hormone-stimulated Mn^{2+} influx (cf. Fig. 4; see also [25]). In agreement with this possibility, it has been reported that, similarly to the treatment with phenylephrine, the microinjection of Ins(1,4,5)P_3 or its non-metabolizable analogue Ins(1,4,5)trisphosphorothioate stimulated Ca^{2+} entry in single hepatocytes [33]. It was concluded that Ins(1,4,5)P_3 itself was the signal for activation of extracellular Ca^{2+} entry, and that an Ins(1,4,5)P_3-operated Ca^{2+} channel in the plasma membrane was likely to be involved. In contrast with the present results, emptying of the Ins(1,4,5)P_3-sensitive Ca^{2+} store in that study did not stimulate Ca^{2+} entry. The reason for this apparent discrepancy is not known, but may involve differences in the control of Ca^{2+} entry between phenylephrine and vasopressin [34,35].

In conclusion, evidence is presented for the existence of two separate pathways for receptor-mediated Ca^{2+} entry in hepatocytes. One pathway is activated by the release of the Ins(1,4,5)P_3-mobilizable internal Ca^{2+} pool, resulting in capacitative Ca^{2+} entry. In contrast, the other pathway, which is permeable to Mn^{2+} in addition to Ca^{2+}, requires hormone–receptor interaction and is most likely activated by a second messenger. The molecular nature as well as the regulatory mechanisms of these two pathways of agonist-stimulated Ca^{2+} entry in hepatocytes require further investigation.

This work was supported by the Swedish Medical Research Council (Project no. 03X-2471) the Swedish Society of Medicine and a Fellowship from the Ministerio de Educación y Ciencia, Spain (to J. L.).

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Received 15 August 1991/28 October 1991; accepted 1 November 1991