Agonist-stimulated divalent cation entry was studied in fura-2-loaded hepatocytes. In the presence of extracellular Mn\(^{2+}\), the Ca\(^{2+}\)-mobilizing hormone vasopressin produced a severalfold stimulation of the basal rate of fura-2 fluorescence quenching as a result of Mn\(^{2+}\) influx; this effect was blocked by the presence of Ni\(^{2+}\) in the incubation medium. Half-maximum and maximum stimulation of Mn\(^{2+}\) influx was observed with 0.1 and 0.8 \(\mu\)M vasopressin, respectively. Agonist-stimulated Mn\(^{2+}\) influx was also seen with angiotensin II, ATP, phenylephrine, and the combination of AlCl\(_3\) and NaF. The stimulation of Mn\(^{2+}\) influx did not occur immediately after addition of Ca\(^{2+}\)-mobilizing agents, but was characterized by a latency period of 20–30 s. In contrast to vasopressin, glucagon did not stimulate Mn\(^{2+}\) influx into hepatocytes, but produced both a 3-fold enhancement of the rate of vasopressin-stimulated Mn\(^{2+}\) entry and the abolishment of the latency period. The effects of glucagon were mimicked by forskolin and dibutyryl cAMP. Pretreatment of hepatocytes with pertussis toxin or depolarization of the cells altered neither the basal rate of Mn\(^{2+}\) entry nor the ability of vasopressin to stimulate this rate. Emptying of the inositol 1,4,5-trisphosphate-sensitive Ca\(^{2+}\) store by treatment with 2,5-di-(tert-butyl)-1,4-benzoquinone (tBuBHQ) did not enhance Mn\(^{2+}\) entry into hepatocytes; however, exposure of the cells to tBuBHQ for 2 min markedly enhanced the ability of vasopressin, alone or in combination with glucagon, to increase the rate of Mn\(^{2+}\) influx. Furthermore, pretreatment with tBuBHQ for 2 min abolished the latency of vasopressin-stimulated Mn\(^{2+}\) influx. It is concluded that Ca\(^{2+}\)-mobilizing hormones stimulate Ca\(^{2+}\) influx in hepatocytes, possibly through receptor-operated Ca\(^{2+}\) channels. The stimulation of divalent cation entry is transduced by a G protein, and the rate of influx appears to be controlled both by the intracellular level of cAMP and the empty state of an intracellular Ca\(^{2+}\) pool that may be inositol 1,4,5-trisphosphate-insensitive.

**Changes in the cytosolic free calcium concentration**

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\(^{1}\) The abbreviations used are: [Ca\(^{2+}\)], cytosolic free Ca\(^{2+}\) concentration; ROCC, receptor-operated Ca\(^{2+}\) channel; tBuBHQ, 2,5-di-(tert-butyl)-1,4-benzoquinone; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTPA, diethylenetriaminepentaacetic acid; Gi, inhibitory regulatory GTP-binding protein of adenylate cyclase.
based on the ability of Mn\textsuperscript{2+} to bind to the fluorescent Ca\textsuperscript{2+} indicators quin-2 and fura-2 with a higher affinity than does Ca\textsuperscript{2+} and thereby to strongly quench their fluorescence. Hence, when Mn\textsuperscript{2+} is present in the extracellular medium, an agonist-stimulated decrease in fluorescence of intracellular fura-2 will reflect the stimulation of Mn\textsuperscript{2+} entry via Ca\textsuperscript{2+} channels. In this study, this method was adapted to the study and characterization of receptor-operated Ca\textsuperscript{2+} influx in hepatocytes. We report here that in hepatocytes, a G protein is involved in the stimulation of this influx pathway and that the cation flux is controlled both by the intracellular concentration of cAMP and the empty state of an intracellular Ca\textsuperscript{2+} pool that may be inositol 1,4,5-trisphosphate-insensitive.

**EXPERIMENTAL PROCEDURES**

**Materials—** Collagenase (grade II), ATP, nifedipine, and fura-2 pentapotassium salt were purchased from Boehringer Mannheim. Fura-2/acetoxymethyl ester, verapamil, [Arg\textsuperscript{8}]vasopressin, angiotensin II, L-phenylephrine, glucose, dibutylryl cAMP, Bt-cAMP, forskolin, and neomycin sulfate were obtained from Sigma. 2,5-Di-(tert-butyl)-1,4-benzohydroquinone was from EGA Chemie (Steinheim, Federal Republic of Germany), and the H\textsuperscript{+} radioassay kit for the measurement of cAMP was purchased from Amersham Int. All other chemicals were of the highest purity commercially available.

**Measurement of Mn\textsuperscript{2+} Influx in Hepatocytes—** Freshly isolated hepatocytes from male Wistar rats (200-250 g, fed ad libitum) were loaded with fura-2 as reported elsewhere (9) and postincubated at 10\textsuperscript{6} cells/ml in KH buffer (modified Krebs-Henseleit buffer. 120 mM NaCl, 8.4 mM KCl, 4.2 mM NaHCO\textsubscript{3}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 1.3 mM MgSO\textsubscript{4}, 1.3 mM CaCl\textsubscript{2} supplemented with 15 mM glucose, 0.2% (w/v) bovine serum albumin, and 20 mM Hepes, pH 7.4) for at least 20 min at room temperature. At the end of this period, the intracellular concentration of fura-2 typically was 80-100 \textmu M, as estimated from the amount of fura-2 fluorescence (excitation at 334 nm) liberated from hepatocytes after two cycles of freeze-thawing and compared with the fluorescence of unloaded freeze-thawed hepatocytes to which known amounts of fura-2 pentapotassium salt had been added. Cellular autofluorescence accounted for <15% of the total signal from fura-2-loaded cells. The procedure for the measurement of Mn\textsuperscript{2+} influx is as follows. Fura-2-loaded hepatocytes were transferred to a quartz cuvette maintained at 30 °C, and the fluorescence emission was recorded using a Sigma ZFP 22 dual-wavelength spectrofluorometer fitted with a 500-nm cutoff filter (9). The fluorescence signals obtained at the 334- and 366-nm excitation wavelengths were recorded separately to allow us to distinguish between alterations in [Ca\textsuperscript{2+}], (reflected by an increase in the fluorescence at 334 nm excitation and a small decrease at 366 nm excitation) and Mn\textsuperscript{2+}-induced quenching of fura-2 (as evidence by the simultaneous and parallel loss of the fluorescence signal at both excitation wavelengths) (31). To quantitatively compare the rates of Mn\textsuperscript{2+}-induced quenching of fluorescence between different hepatocytes preparations bearing different extents of fura-2 loading, the initial fluorescence of all hepatocytes samples was amplified by the fluorometer to a set initial fluorescence reading. This procedure allowed us to obtain similar basal rates of Mn\textsuperscript{2+} influx between different preparations of hepatocytes and different intracellular fura-2 content.

**Pertussis Toxin Treatment—** Hepatocytes (5 x 10\textsuperscript{6} cells/ml) were incubated in KH buffer containing 2% bovine serum albumin and 2 \textmu g of pertussis toxin/ml for 4-4.5 h before washing and loading the cells with fura-2 as described above. Control hepatocytes incubated under identical conditions for 4.5 h in the absence of the toxin had the same basal and hormone-stimulated rates of Mn\textsuperscript{2+} influx as freshly isolated cells. Inhibition of the G protein by pertussis toxin was assessed by measuring the ability of angiotensin II (100 nM) to decrease the stimulation of cAMP formation by glucagon (32). In control cells, glucagon (100 nM)-stimulated cAMP formation was lowered by 35% in the presence of angiotensin II, whereas in pertussis toxin-treated cells, angiotensin II lowered cAMP levels by only 11-20%, indicating that at least 90% of the toxin-sensitive G protein was ADP-ribosylated (32).

**RESULTS**

Ca\textsuperscript{2+}-mobilizing Hormones Stimulate Mn\textsuperscript{2+} Entry in Hepatocytes—The effects of Mn\textsuperscript{2+} and Ni\textsuperscript{2+} on the fluorescence of fura-2-loaded hepatocytes (500 nm emission) at the two excitation wavelengths (334 and 366 nm) are shown in Fig. 1. Upon addition of MnCl\textsubscript{2} (500 \textmu M) to a suspension of fura-2-loaded hepatocytes, there was an immediate drop in the fluorescence intensity. This decrease was observed simultaneously and in parallel at both excitation wavelengths and was reversed by the cell-impermeant heavy metal chelator DTPA (1 mM), indicating that Mn\textsuperscript{2+} was binding to trace amounts of extracellular fura-2. Following this initial Mn\textsuperscript{2+}-dependent loss of fluorescence, which accounted for <10% of the total fluorescence decrease, there was a slow and steady decrease in fluorescence at both excitation wavelengths, presumably due to a slow basal leak of Mn\textsuperscript{2+} into the hepatocytes. This basal decrease was linear for at least 15 min at both wavelengths. The addition of 10 nM vasopressin markedly enhanced the rate of fura-2 fluorescence quenching (Fig. 1), which is consistent with a stimulated influx of Mn\textsuperscript{2+} into the hepatocytes. Subsequent addition of 4 mM NiCl\textsubscript{2} blocked the effect of the hormone, and the rate of fluorescence decrease immediately returned to basal levels. The addition of NiCl\textsubscript{2} to unstimulated hepatocytes (in the presence of Mn\textsuperscript{2+}) did not alter the basal rate of fura-2 fluorescence quenching at either excitation wavelength. Following complete quenching of fura-2 fluorescence by Mn\textsuperscript{2+} after stimulation by vasopressin, the addition of DTPA increased the fluorescence signal to approximately the same extent as observed when DTPA was added immediately after Mn\textsuperscript{2+} in the absence of hormone (see above). This demonstrates that most of the quenched dye was localized intracellularly and that hormone-stimulated loss of fura-2 fluorescence was not caused by an enhanced leakage of fura-2. The concentration-response curve illustrated in Fig. 2 shows that half-maximum and maximum stimulation of Mn\textsuperscript{2+} influx occurred with 0.1 and 0.8 nM vasopressin, respectively.

Agonist-stimulated influx of Mn\textsuperscript{2+} was not limited to vasopressin alone, but was also observed with other Ca\textsuperscript{2+}-mobilizing hormones, such as angiotensin II, ATP, and the \alpha\textsubscript{1}-adrenergic agonist phenylephrine (Fig. 3). After addition of angiotensin II (10 nM) or ATP (10 \mu M), the rates of Mn\textsuperscript{2+} influx, as calculated from the velocity of fura-2 fluorescence quenching after the initial lag period (see below), were essen-
Receptor-operated Ca\textsuperscript{2+} Influx in Hepatocytes

With all Ca\textsuperscript{2+}-mobilizing hormones tested, the stimulation of Mn\textsuperscript{2+} influx occurred after a latency period of 20-30 s (24.6 ± 6.9 s for vasopressin (10 nM); mean ± S.D., n = 11) (Figs. 1 and 3). Decreasing the concentration of vasopressin had a dual effect on Mn\textsuperscript{2+} influx; as the rate of hormone-stimulated Mn\textsuperscript{2+} entry decreased (Fig. 2), the delay before stimulation of Mn\textsuperscript{2+} influx increased (Figs. 4 and 5). As seen in Fig. 4, the stimulation of Mn\textsuperscript{2+} influx was never gradual, but always occurred as a threshold-like event. The possibility that his lag was caused by hormone-induced changes in [Ca\textsuperscript{2+}], that could have masked the Mn\textsuperscript{2+}-induced quenching of fura-2 can be excluded for the following reasons: (i) The lag occurred simultaneously and in parallel at both excitation wavelengths (cf. Fig. 1). (ii) The lag also occurred in hepatocytes to which vasopressin was added 10-15 s after tBuBHQ (20 μM), a potent and specific mobilizer of the inositol 1,4,5-trisphosphate-sensitive Ca\textsuperscript{2+} pool (9) (data not shown). Under these conditions, tBuBHQ elevates [Ca\textsuperscript{2+}], within 8 s to the same maximum level as obtained with vasopressin; and subsequent addition of vasopressin does not further increase [Ca\textsuperscript{2+}], but rather produces a rapid decrease in [Ca\textsuperscript{2+}], (9, 10). Hence, despite these major differences in the direction of the [Ca\textsuperscript{2+}], changes, both experimental conditions produced an identical pattern of agonist-induced fluorescence quenching.

Enhancement of Vasopressin-stimulated Mn\textsuperscript{2+} Influx by cAMP—In contrast with Ca\textsuperscript{2+}-mobilizing hormones, glucagon did not stimulate Mn\textsuperscript{2+} entry into hepatocytes (Fig. 3). However, when the addition of glucagon (10 nM) was followed by vasopressin (10 nM), Mn\textsuperscript{2+} influx was initiated without a detectable lag (Fig. 6). Furthermore, the combination of glucagon and vasopressin stimulated Mn\textsuperscript{2+} entry at a rate that was >3-fold greater than the rate measured with vasopressin alone (Table II). This effect of glucagon could be mimicked by raising the intracellular levels of cAMP with the adenylate cyclase activator forskolin or by the addition of the

![Fig. 2. Concentration response for vasopressin-stimulated Mn\textsuperscript{2+} influx in hepatocytes. Cells were incubated in KH buffer containing 500 μM MnCl\textsubscript{2}. After a stable signal for basal Mn\textsuperscript{2+} influx was obtained, vasopressin was added to the cell suspension. The rates of Mn\textsuperscript{2+} influx are calculated from the linear portion of the rate curve recorded after the initial delay. Each point represents the mean of two to three separate experiments.](http://www.jbc.org/)

![Fig. 3. Stimulation of Mn\textsuperscript{2+} influx in hepatocytes by Ca\textsuperscript{2+}-mobilizing hormones. Mn\textsuperscript{2+} influx was measured as described under "Experimental Procedures." At the indicated time points (arrows), angiotensin II (AII; 10 nM), ATP (10 μM), phenylephrine (PE; 2 μM), and glucagon (GLN; 10 nM) were added. For clarity, only the traces obtained at the 334-nm excitation wavelength are presented. Very similar traces were obtained with excitation at 366 nm.](http://www.jbc.org/)

![Fig. 4. Effect of different concentrations of vasopressin on delay in Mn\textsuperscript{2+} influx in hepatocytes. Vasopressin (VP) was added (arrow) at the concentrations (nanomolar) indicated at the end of each trace. For details, see the legend to Fig. 3.](http://www.jbc.org/)

![Fig. 5. Concentration dependence of delay in vasopressin-stimulated Mn\textsuperscript{2+} influx. For details, see the legend to Fig. 4. Each point is the mean of two to three separate experiments.](http://www.jbc.org/)
Fig. 6. Effects of glucagon and cyclic nucleotides on vasopressin-induced Mn\textsuperscript{2+} influx in hepatocytes. Mn\textsuperscript{2+} influx was measured as described under “Experimental Procedures.” For details, see the legend to Fig. 3. VP, vasopressin (10 nM); GLN, glucagon (10 nM); FORSK, forskolin (10 \mu M); Bt,cAMP, dibutyryl cAMP (25 \mu M).

<table>
<thead>
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<th>Addition</th>
<th>Mn\textsuperscript{2+} influx (arbitrary units/min)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>0.59 ± 0.06</td>
</tr>
<tr>
<td>Vasopressin (10 nM)</td>
<td>2.78 ± 0.58*</td>
</tr>
<tr>
<td>Glucagon (10 nM) + vasopressin (10 nM)</td>
<td>7.78 ± 2.14*</td>
</tr>
<tr>
<td>Dibutyryl cAMP (25 \mu M) + vasopressin (10 nM)</td>
<td>8.64 ± 1.61*</td>
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</table>

TABLE II
Enhancement of vasopressin-stimulated Mn\textsuperscript{2+} influx by glucagon

Fura-2-loaded hepatocytes were resuspended in KH buffer containing 500 \mu M MnCl\textsubscript{2}, and the emission signals from the 334- and 366-nm excitation wavelengths were recorded simultaneously, as described under “Experimental Procedures.” The data were calculated from the changes in fura-2 fluorescence obtained with the 334-nm excitation wavelength and are given as the means ± S.D. of three to five separate experiments. Asterisks indicate values significantly different from control as analyzed by Student’s unpaired t test (p < 0.001).

Addition Mn\textsuperscript{2+} influx
<table>
<thead>
<tr>
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<th>arbitrary units/min</th>
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<tbody>
<tr>
<td>None</td>
<td>0.59 ± 0.06</td>
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<td>Vasopressin (10 nM)</td>
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<td>Dibutyryl cAMP (25 \mu M) + vasopressin (10 nM)</td>
<td>8.64 ± 1.61*</td>
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</table>

permeable cAMP analogue dibutyryl cAMP (25 \mu M) to the incubation medium (Fig. 5 and Table II). Enhancement of Mn\textsuperscript{2+} entry and abolishment of the latency period were also observed when glucagon or dibutyryl cAMP was administered prior to other Ca\textsuperscript{2+}-mobilizing hormones, such as angiotensin II, ATP, and phenylephrine (data not shown). Similar effects were obtained with 8-Br cAMP (25 \mu M), whereas dibutyryl cGMP (25 \mu M) was without effect (data not shown). None of these cyclic nucleotide analogues stimulated Mn\textsuperscript{2+} entry by themselves.

Effect of Emptying Inositol 1,4,5-Trisphosphate-sensitive Ca\textsuperscript{2+} Pool by tBuBHQ on Mn\textsuperscript{2+} influx—Release of the inositol 1,4,5-trisphosphate-sensitive Ca\textsuperscript{2+} pool by tBuBHQ did not increase Mn\textsuperscript{2+} influx in the absence of hormonal stimulation (Fig. 7A). However, under these conditions, [Ca\textsuperscript{2+}], is elevated (9), and Ca\textsuperscript{2+} could have acted as a negative effector on Mn\textsuperscript{2+} inflow (25). To investigate this possibility, we incubated hepatocytes with tBuBHQ (25 \mu M) for 1.5 h, by which time [Ca\textsuperscript{2+}], had returned to basal level, and the inositol 1,4,5-trisphosphate-sensitive Ca\textsuperscript{2+} pool was empty (as evidenced by the lack of effect of vasopressin on [Ca\textsuperscript{2+}], (9)). Again, the rates of Mn\textsuperscript{2+} uptake after exposure of the cells to tBuBHQ were not significantly different from control (0.64 ± 0.02 versus 0.65 ± 0.15 arbitrary fluorescence units/min; mean ± S.D., n = 3). However, when vasopressin was added 2 min after tBuBHQ, the hormone stimulated an immediate influx of Mn\textsuperscript{2+} at a rate that was 2–3-fold higher than the rate observed with vasopressin alone (Fig. 7A). This effect of tBuBHQ on vasopressin-induced Mn\textsuperscript{2+} influx became detectable only after 15 s and was maximal after 1 min of tBuBHQ pretreatment. tBuBHQ did not stimulate cAMP formation in hepatocytes (data not shown). Pretreatment with tBuBHQ for 2 min also further enhanced the potentiation of vasopressin-induced Mn\textsuperscript{2+} entry by glucagon (Fig. 7B) or dibutyryl cAMP (data not shown).

Effects of Pertussis Toxin Pretreatment and Depolarization of Plasma Membrane on Vasopressin-stimulated Mn\textsuperscript{2+} Influx—Barratt and co-workers (28) have recently reported that vasopressin-stimulated Ca\textsuperscript{2+} entry involves activation of a pertussis toxin-sensitive GTP-binding regulatory protein. As shown in Table I, activation of G protein by the combination of NaF and AICl\textsubscript{3} significantly stimulated Mn\textsuperscript{2+} entry. Fluoride ions only slightly stimulated Mn\textsuperscript{2+} influx by themselves, and the presence of A13' was required for maximum effect. AICl\textsubscript{3} had no effect on Mn\textsuperscript{2+} influx in the absence of F\textsuperscript{−} (data not shown). Incubation of hepatocytes for 4–4.5 h with pertussis toxin (2 \mu g/ml) had no effect on either the basal or vasopressin-stimulated rate of Mn\textsuperscript{2+} influx (Table III), despite the fact that at least 90% of the pertussis toxin-sensitive G protein was ADP-ribosylated (see “Experimental Procedures”). Hence, our data suggest that the activation of receptor-operated Ca\textsuperscript{2+} influx does not involve a pertussis toxin-sensitive G protein.

Several studies (33, 34) have shown that vasopressin-induced Ca\textsuperscript{2+} influx is inhibited by high extracellular K\textsuperscript{+} concentrations or depolarization of the plasma membrane. The data presented in Table III, however, show that depolarization of the plasma membrane by incubating hepatocytes in modified KH buffer containing 120 mM KCl and 5.4 mM NaCl altered neither the basal rate of Mn\textsuperscript{2+} influx nor the ability of vasopressin to stimulate Mn\textsuperscript{2+} entry. Furthermore, none of the above treatments modified the ability of glucagon to...
Receptor-operated Ca\(^{2+}\) Influx in Hepatocytes

**TABLE III**

Lack of effects of depolarization of the hepatocytes and pretreatment with pertussis toxin on Mn\(^{2+}\) influx

Fura-2 loaded hepatocytes were resuspended in KH buffer or depolarization buffer, all containing 500 µM MnCl\(_2\), as indicated below; and the emission signals of the 334- and 366-nm excitation wavelengths were recorded simultaneously, as described under "Experimental Procedures." The data were calculated from the changes in fura-2 fluorescence obtained with the 334-nm excitation wavelength and are given as the means ± S.D. of three to six separate experiments.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Basal</th>
<th>After vasopressin (10 nM)</th>
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<tbody>
<tr>
<td>None</td>
<td>0.61 ± 0.18</td>
<td>3.11 ± 0.68</td>
</tr>
<tr>
<td>Pertussin toxin(^a)</td>
<td>0.68 ± 0.16</td>
<td>2.96 ± 0.41</td>
</tr>
<tr>
<td>Depolarization</td>
<td>0.65 ± 0.25</td>
<td>3.66 ± 0.70</td>
</tr>
</tbody>
</table>

* Hepatocytes were incubated with pertussis toxin (2 µg/ml) for 4.5 h before loading with fura-2, as described under "Experimental Procedures."

**DISCUSSION**

Although their existence had been suspected for many years, ROCCs have only recently found direct experimental support in a number of cell types (19). The identification of ROCCs and the study of their regulation have become possible by which Ca\(^{2+}\) uptake occurs. Consequently, this report demonstrates that in hepatocytes, at least part of the hormonestimulated Ca\(^{2+}\) uptake occurs through a specific influx pathway that shares several similarities with ROCCs.

Glucagon or agents that mimicked or increased cAMP production did not stimulate Mn\(^{2+}\) influx. Several previous reports (11, 12, 39, 40) have, however, demonstrated a stimulation of \(^{45}\)Ca\(^{2+}\) uptake following the addition of glucagon to isolated hepatocytes. Hence, our findings would support the existence of at least two separate routes of Ca\(^{2+}\) influx; one which is similar to a receptor-operated type of Ca\(^{2+}\) channel that is activated by Ca\(^{2+}\)-mobilizing hormones, but not by glucagon or cAMP, and a second pathway which is opened by the action of glucagon and cAMP but that does not admit Mn\(^{2+}\).

Glucagon abolished the delay in onset and stimulated the rate of vasopressin-induced Mn\(^{2+}\) influx. This effect was mimicked by the addition of cell-permeant analogues of cAMP and by raising the intracellular concentration of cAMP with forskolin. Thus, cAMP appears to act as a positive effector on the receptor-operated Ca\(^{2+}\) influx pathway, altering its ability to become activated by Ca\(^{2+}\)-mobilizing hormones. A similar effect has previously been reported in which glucagon and cAMP were found to markedly increase the uptake of both \(^{45}\)Ca\(^{2+}\) and \(^{40}\)Ca\(^{2+}\) elicited by vasopressin in isolated hepatocytes (39–42) and that of \(^{40}\)Ca\(^{2+}\) in the isolated perfused liver (36, 43). It has been known for some time that cAMP can increase the probability of opening of voltage-operated, L-type Ca\(^{2+}\) channels in cardiac cells (44). There is considerable evidence that the mechanism underlying this phenomenon involves phosphorylation of the L channel by a cAMP-dependent protein kinase (44, 45). Preliminary evidence would, however, suggest that cAMP does not act by stimulating phosphorylation of the proposed hepatic ROCCs since preincubation of hepatocytes with the protein kinase inhibitors staurosporine (100 nM) or H7 (150 µM) did not prevent the potentiation of the vasopressin-stimulated Mn\(^{2+}\) influx by glucagon. Thus, the interaction of cAMP with the hepatic receptor-operated Ca\(^{2+}\) influx pathway may be analogous in some aspects to the modulation of the ion channels of the rod outer segment membrane, which are regulated through the direct and cooperative binding of cGMP to the channel itself (46, 47).

The mechanism by which agonist-receptor interaction is coupled to the stimulation of Ca\(^{2+}\) influx is still unclear. Our finding that the hepatocyte receptor-operated Ca\(^{2+}\) influx pathway can be opened by the combination of Al\(^{3+}\) plus F– (which can activate GTP-binding proteins by forming AlF\(_3\)) (48, 49) suggests that hormone receptors (i) do not act as Ca\(^{2+}\) channels themselves and (ii) do not interact through direct coupling with the Ca\(^{2+}\) influx route; rather, a G protein is involved in the transduction of the signal for the stimulation of the Ca\(^{2+}\) influx process. Hughes and Barritt (27) reached the same conclusion when they found that the combination of Al\(^{3+}\) plus F– could stimulate \(^{40}\)Ca\(^{2+}\) uptake by hepatocytes. However, in contrast with the \(^{40}\)Ca\(^{2+}\) influx data of Barritt and co-workers (28), the G protein that mediates activation of receptor-operated Ca\(^{2+}\) influx in hepatocytes was not inhibited by pertussis toxin, and therefore, it is apparently not a member of the G protein family. Although the hepatic receptor-operated Ca\(^{2+}\) influx pathway could conceivably be activated directly via functional coupling with a G protein (a mechanism that has been postulated to operate for certain voltage-operated Ca\(^{2+}\) channels (40, 50)), it is possible that an agonist-generated second messenger, such as an inositol polyphosphate, diacylglycerol, or Ca\(^{2+}\) itself, is the mediator for the activation of hepatic receptor-operated Ca\(^{2+}\) influx. In this study, stimulation of hepatocytes with Ca\(^{2+}\)-mobilizing hormones was always accompanied by a delay before Mn\(^{2+}\) influx was stimulated. One possible explanation for this latency is that a threshold concentration of second messenger

\(^{2}\) G. E. N. Kass and S. Orrenius, unpublished observations.
has to accumulate before the influx pathway is activated. A similar phenomenon has been reported for the vasopressin-induced [Ca\(^{2+}\)] increase: at submaximally effective concentrations, the initiation of the [Ca\(^{2+}\)] transient is delayed by a lag that is inversely proportional to the concentration of vasopressin (51, 52). Activation of receptor-operated Ca\(^{2+}\) influx by Ca\(^{2+}\) itself can be excluded on the grounds of our finding that an increase in [Ca\(^{2+}\)] to levels similar to those obtained following exposure of hepatocytes to Ca\(^{2+}\)-mobilizing hormones is not sufficient to stimulate Mn\(^{2+}\) influx (Ref. 9 and this study). Protein kinase C does also not appear to mediate the activation of the hepatic receptor-operated Ca\(^{2+}\) influx pathway for the following reasons. (i) The effects of Ca\(^{2+}\)-mobilizing hormones were not mimicked by administration of the protein kinase C agonist 12-O-tetradecanoylphorbol-13-acetate (50 nm) and (ii) pretreatment of the cells with staurosporine (100 nm) and H7 (150 \(\mu\)M) did not prevent vasopressin-stimulated Mn\(^{2+}\) influx.\(^{2}\)

An alternative model for the control of Ca\(^{2+}\) influx is the capacitative Ca\(^{2+}\) entry hypothesis, which has been proposed by Putney and co-workers to account for the observations that (i) during Ca\(^{2+}\) influx, the cytosol is apparently by-passed in favor of direct movement of Ca\(^{2+}\) into an endoplasmic reticular pool (Ref. 53; but see recent modifications of this model in Refs. 54 and 55); and (ii) Ca\(^{2+}\) influx can be stimulated independently of hormone action or inositol phosphate turnover, but as a consequence of the emptying of the hormone-sensitive intracellular Ca\(^{2+}\) pool (29). The capacitative model for Ca\(^{2+}\) entry has recently found experimental support in human endothelial cells (56), platelets (57), and a human T cell line (Jurkat).\(^{5}\) The possibility that hepatocyte receptor-operated Ca\(^{2+}\) influx may also be regulated by the emptying of the endoplasmic reticular Ca\(^{2+}\) pool(s) was therefore investigated as part of this study. We had previously reported (9) that emptying the inositol 1,4,5-trisphosphate-sensitive Ca\(^{2+}\) pool in the absence of inositol phosphate turnover using tBuBHQ did not stimulate \(^{45}\)Ca\(^{2+}\) or Mn\(^{2+}\) influx. Furthermore, prolonged incubation with tBuBHQ to empty the inositol 1,4,5-trisphosphate-sensitive Ca\(^{2+}\) pool and to allow the return of [Ca\(^{2+}\)] to basal levels also did not enhance the rate of Mn\(^{2+}\) influx. From these findings, it can be concluded that the emptying of the hormone-sensitive Ca\(^{2+}\) pool is not sufficient to activate the receptor operated Ca\(^{2+}\) influx pathway in hepatocytes. The rate of Mn\(^{2+}\) influx in hepatocytes that were stimulated with vasopressin after exposure to tBuBHQ for 10–15 s was similar to that observed in control cells (9). A surprising finding was that when hepatocytes had been exposed to tBuDIIQ for longer than 30 s prior to the addition of vasopressin, the rate of Mn\(^{2+}\) uptake was markedly stimulated, and the lag period before influx occurred was essentially abolished. The reason for this effect is still unclear. One possible explanation would be that, as postulated by a number of investigators (e.g. Refs. 58–60), a second, inositol 1,4,5-trisphosphate-insensitive pool of Ca\(^{2+}\) exists in the cell and that after the emptying of the inositol 1,4,5-trisphosphate-sensitive Ca\(^{2+}\) pool by tBuBHQ, the second pool would empty itself by re-equilibrating with the inositol phosphate-sensitive pool. Stimulation of Mn\(^{2+}\) influx by Ca\(^{2+}\)-mobilizing hormones may therefore require two components; a hormone-generated agonist as well as the more delayed emptying of an intracellular Ca\(^{2+}\) pool that is inositol 1,4,5-trisphosphate-insensitive.

In conclusion, this study supports the existence of a receptor-operated Ca\(^{2+}\) influx pathway in hepatocytes that is similar to ROCCs found in other nonexcitable cells. A number of the properties of the hepatic receptor-operated Ca\(^{2+}\) influx

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\(^{2}\) S. C. Chow and M. Jondal, submitted for publication.

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**REFERENCES**


**Receptor-operated Ca\(^{2+}\) Influx in Hepatocytes**

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Receptor-operated Ca\textsuperscript{2+} Influx in Hepatocytes

Receptor-operated calcium influx in rat hepatocytes. Identification and characterization using manganese.

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