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The liver cell plasma membrane Ca^{2+} inflow systems exhibit a broad specificity for divalent metal ions

John N. CROFTS and Gregory J. BARRITT*
Department of Medical Biochemistry, Flinders University School of Medicine, Flinders Medical Centre, Bedford Park, SA 5042, Australia

1. The inflow of Mn^{2+} across the plasma membranes of isolated hepatocytes was monitored by measuring the quenching of the fluorescence of intracellular quin2, by atomic absorption spectroscopy and by the uptake of $^{54}Mn^{2+}$. The inflow of other divalent metal ions was measured using quin2. 2. Under ionic conditions which resembled those present in the cytoplasmic space, Mn^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Cd^{2+} each quenched the fluorescence of a solution of Ca^{2+}–quin2. 3. The addition of Mn^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} or Cd^{2+} to cells loaded with quin2 caused a time-dependent decrease in the fluorescence of intracellular quin2. Plots of the rate of decrease in fluorescence as a function of the concentration of Mn^{2+} reached a plateau at 100 μM-Mn^{2+}. 4. The rate of decrease in fluorescence induced by Mn^{2+} was stimulated by 20% in the presence of vasopressin. The effect of vasopressin was completely inhibited by 200 μM-verapamil. Adrenaline, angiotensin II and glucagon also stimulated the rate of decrease in the fluorescence of intracellular quin2 induced by Mn^{2+}. 5. The rate of decrease in fluorescence induced by Zn^{2+}, Co^{2+}, Ni^{2+} or Cd^{2+} was stimulated by between 20 and 190% in the presence of vasopressin or angiotensin II. 6. The rates of uptake of Mn^{2+} measured by atomic absorption spectroscopy or by using $^{54}Mn^{2+}$ were inhibited by about 20% by 1.3 mM-Ca^{2+}_o and stimulated by 30% by vasopressin. 7. Plots of Mn^{2+} uptake, measured by atomic absorption spectroscopy or with $^{54}Mn^{2+}$, as a function of the extracellular concentration of Mn^{2+} were biphasic over the range 0.05–1.0 mM added Mn^{2+} and did not reach a plateau at 1.0 mM-Mn^{2+}. 8. It is concluded that (i) hepatocytes possess both a basal and a receptor-activated divalent cation inflow system, each of which has a broad specificity for metal ions, and (ii) the receptor-activated divalent cation inflow system is the receptor-operated Ca^{2+} channel.

INTRODUCTION

The maintenance of an increased concentration of Ca^{2+} in the cytoplasmic space of the liver cell in the presence of agonists such as vasopressin, adrenaline and angiotensin II requires the stimulation by the agonist of Ca^{2+} inflow across the plasma membrane (Barritt *et al.*, 1981; Mauger *et al.*, 1984, 1985; Reinhart *et al.*, 1984). Studies conducted with organic Ca^{2+} antagonists (Hughes *et al.*, 1986*b*), divalent metal ion inhibitors (Hughes & Barritt, 1989*b*), other inhibitors (Altin & Bygrave, 1987) and the replacement of extracellular Na^+ by choline (Crofts & Barritt, 1989) have indicated that the liver cell plasma membrane possesses two types of Ca^{2+} inflow systems, a basal system and a receptor-operated Ca^{2+} channel. The results of several experimental approaches suggest that the stimulation of the receptor-operated Ca^{2+} channel involves the interaction of a GTP-binding regulatory protein with the Ca^{2+} channel (Hughes & Barritt, 1987, 1989*a*; Hughes *et al.*, 1987*b*).

Evidence has been obtained which indicates that Ca^{2+} inflow through the basal Ca^{2+} inflow system and the receptor-operated Ca^{2+} channel is not readily saturated by extracellular Ca^{2+} (Ca^{2+}_o) (Parker *et al.*, 1983; Hughes *et al.*, 1987*a*; Crofts & Barritt, 1989; but see Mauger *et al.*, 1984; Joseph *et al.*, 1985), does not require depolarization of the plasma membrane (Hughes *et al.*, 1986*b*; Sawanobori *et al.*, 1989; but see Taylor *et al.*, 1985; Savage *et al.*, 1989), does not involve Na^+–Ca^{2+} (Cittadini & van Rossum, 1978; Savage *et al.*, 1989; Crofts & Barritt, 1989) or H^+–Ca^{2+} exchange (Altin & Bygrave, 1987), and is not readily inhibited by low concentrations of organic inhibitors of voltage-operated Ca^{2+} channels or by organic inhibitors of other receptor-operated Ca^{2+} channels (Hughes *et al.*, 1986*b*; Hughes & Barritt, 1989*b*). Ca^{2+} inflow through the receptor-operated Ca^{2+} channel is inhibited by many of the metal ions which also inhibit voltage-operated Ca^{2+} channels (Hughes & Barritt, 1989*b*) and it has been suggested (Hughes & Barritt, 1989*b*) that there are some similarities between voltage-operated Ca^{2+} channels and the liver cell receptor-operated Ca^{2+} channel. Voltage-operated Ca^{2+} channels admit a variety of divalent metal ions as well as Ca^{2+} (reviewed by Hagiwara & Byerly, 1981*a*,*b*; Hurwitz, 1986). However, the specificity of the liver cell receptor-operated Ca^{2+} channel for the divalent metal ion admitted has not been reported.

The present experiments were designed to determine the ability of Mn^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Cd^{2+} to move inwards across the liver cell plasma membrane. Mn^{2+} inflow was measured by the ability of the cation to quench the fluorescence of intracellular quin2 (Hallam & Rink, 1985), by atomic absorption spectroscopy and by the use of $^{54}Mn^{2+}$. The inward movement of the other metal ions was measured by their ability to quench the fluorescence of intracellular quin2. Although there are some advantages in the use of fura-2 rather than quin2 in the measurement of the uptake of Mn^{2+} and other divalent cations by cells (Merritt & Hallam, 1988; Sage *et al.*, 1989; Merritt *et al.*, 1989; Alonso *et al.*, 1989), studies conducted in our laboratory have provided evidence which indicates that in isolated liver cells in suspension, fura-2 enters other intracellular compartments as well as the cytoplasmic space (J. N. Crofts & G. J. Barritt, unpublished work). Moreover, the relatively high concentration of the fluorescent metal ion chelator in cells loaded with quin2 in comparison with cells loaded with fura-2 provides a large number of high-affinity binding sites for the metal ion under study. This

Abbreviations used: DTPA, diethylenetriaminepenta-acetic acid; Ca^{2+}_o, extracellular Ca^{2+}; $[Mn^{2+}]_o$, concentration of extracellular Mn^{2+}; $[Mn^{2+}]_i$, concentration of intracellular free Mn^{2+}.

* To whom correspondence and reprint requests should be addressed.

should reduce the possibility of saturation of the fluorescent metal ion chelator and should also reduce the uptake of metal ion by intracellular organelles.

It has previously been shown that the fluorescence of the Ca–quin2 complex and of quin2 free acid is quenched by Mn^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} (Hesketh *et al.*, 1983; Arslan *et al.*, 1985; Cobbold & Rink, 1987). However, little is known of the effects of these divalent metal ions or of Cd^{2+} on the fluorescence of Ca–quin2 and quin2 free acid under the ionic conditions and at the concentration of free Ca^{2+} present in the cytoplasmic space. Therefore, the first part of this study was to define the time course and concentration-dependence for the decreases in fluorescence of a mixture of Ca^{2+} and quin2 under these conditions. The results of these and other experiments reported here indicate that the liver cell plasma membrane possesses a basal divalent cation inflow system and an agonist-stimulated divalent cation inflow system, both of which admit Mn^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+} and Cd^{2+}. The results also provide evidence which indicates that the receptor-activated divalent cation inflow system is the receptor-operated Ca^{2+} channel.

EXPERIMENTAL

Isolation and incubation of hepatocytes

Hepatocytes were isolated from fed male hooded Wistar rats by collagenase digestion using the method of Berry & Friend (1969) as described previously (Barritt *et al.*, 1981). The incubation medium contained 117 mM-NaCl, 4.7 mM-KCl, 1.2 mM-KH_2PO_4 1.2 mM-$MgSO_4$, 24 mM-$NaHCO_3$, 20 mM-Tes/KOH, pH 7.4, Ca^{2+} and other additions as indicated, and hepatocytes [30 mg wet weight (2×10^6 cells)/ml]. Hepatocytes were loaded with quin2 using the method of Tsien *et al.* (1982) as described previously (Hughes *et al.*, 1986*a*; Crofts & Barritt, 1989). $CaCl_2$ (0.5 mM) was present during the incubation with the acetoxymethyl ester of quin2 (100 μM final concentration) but was not present in media used for the wash and suspension of the loaded cells.

Measurement of fluorescence of quin2-loaded cells

The fluorescence of quin2-loaded cells or of solutions of quin2 free acid was measured at 37 °C using excitation and emission wavelengths of 340 nm and 490 nm respectively in an Aminco–Bowman spectrofluorimeter (SPF 74; American Instrument Co., Silver Spring, MD, U.S.A.) or an LS-50 spectrofluorimeter (Perkin–Elmer Ltd., Beaconsfield, Bucks., U.K.) equipped with a magnetic stirring device and a thermostatically controlled cell housing unit. Changes in the intracellular free Ca^{2+} concentration in cells loaded with quin2 were measured as described previously (Hughes *et al.*, 1986*a*; Crofts & Barritt, 1989).

For the measurement of the fluorescence of Ca–quin2 in solutions of quin2 free acid, quin2 free acid (50 μM) was dissolved in a medium which was a modification of that described by Burgess *et al.* (1983). This contained 20 mM-NaCl, 100 mM-KCl, 1 mM-$MgCl_2$, 25 mM-$NaHCO_3$, 20 mM-Tes/KOH and 1% (w/v) dimethyl sulphoxide, adjusted to pH 7.2. $CaCl_2$ (30 μM) was added to give a free Ca^{2+} concentration of approx. 200 nM. The concentration of free Ca^{2+} was estimated from the observed fluorescence intensity of the solution using the equation of Tsien *et al.* (1982), a value of 115 nM for the dissociation constant for the Ca–quin2 complex (Tsien *et al.*, 1982), the fluorescence intensity ($F_{max.}$) obtained in the presence of 0.5 mM-$CaCl_2$, and the fluorescence intensity ($F_{min.}$) obtained at pH 8.0 in the presence of 5 mM-EGTA.

Measurement of Mn^{2+} uptake using atomic absorption spectroscopy and $^{54}Mn^{2+}$

The total Mn^{2+} content of hepatocytes was measured by atomic absorption spectroscopy using a Perkin–Elmer model 5000 atomic absorption spectrometer. Samples were prepared using a modification of the method of Parker & Barritt (1981) for the measurement of cellular Ca^{2+}, except that 3 ml of cell suspension was added to 9 ml of washing buffer which consisted of 0.9% (w/v) NaCl, 10% (w/v) sucrose and 0.5 mM-EGTA, pH 7.4 (Blackmore & Exton, 1985).

The amount of $^{54}Mn^{2+}$ associated with the cells was determined by centrifuging a sample of the incubation mixture through a layer of silicone oil to separate the cells from the incubation medium as described previously for $^{45}Ca^{2+}$ (Barritt *et al.*, 1981). La^{3+} was omitted from the medium above the silicone oil. Amounts of $^{54}Mn^{2+}$ were determined using a Cobra Auto-Gama counter (Packard, Downers Grove, IL, U.S.A.).

Materials

$^{54}Mn^{2+}$ was purchased from Amersham (Australia), Surrey Hills, NSW, Australia; $MnCl_2$ from May and Baker, Dagenham, Essex, U.K.; $ZnSO_4$ from Baker and Adamson, New York, NY, U.S.A.; $NiCl_2$ from Merck, Darmstadt, Germany; $CoCl_2$ and $CdCl_2$ from Ajax Chemicals, Auburn, NSW, Australia, and ionomycin from Calbiochem Corporation, La Jolla, CA, U.S.A. All other reagents were obtained from the sources described previously (Crofts & Barritt, 1989).

RESULTS

Quenching by metal ions of the fluorescence of solutions of quin2 free acid

The ability of divalent metal ions to quench the fluorescence of quin2 free acid *in vitro* was investigated at 50 μM total quin2 free acid and 200 nM free Ca^{2+} under ionic conditions which resemble those present in the cytoplasmic space (Burgess *et al.*, 1983). The addition of Mn^{2+} caused a rapid decrease in fluorescence (Fig. 1*a*). Almost complete quenching of fluorescence was observed after six additions of 8.3 μM-Mn^{2+}. The metal ions Co^{2+}, Cd^{2+} and Zn^{2+} caused a rapid decrease in fluorescence similar to that induced by Mn^{2+} (results not shown).

Addition of Ni^{2+} also quenched the fluorescence of quin2 under ionic conditions which resembled those of the cytoplasmic space (Fig. 1*b*), but the effect of Ni^{2+} was considerably slower than that of Mn^{2+}. The effects of successive additions of Ni^{2+} (8.3 μM) to solutions which contained 200 nM free Ca^{2+} and 50 μM-quin2 free acid dissolved in water (the final pH was 4.0) or in 20 mM-Tes/KOH at pH 6.0, 7.2 or 8.0 were compared. In water at pH 4.0 the fluorescence intensity of Ca–quin2 was lower than that observed in Tes/KOH at the higher pH values tested and, in contrast with the results shown in Fig. 1(*b*), the decrease in fluorescence caused by each addition of Ni^{2+} was very rapid. In Tes/KOH at pH 6.0 the rate of decrease in fluorescence caused by the addition of Ni^{2+} was slower than that observed in water at pH 4.0. This effect was more pronounced for quin2 in Tes/KOH at pH 7.2 and 8.0 (results not shown).

Half-maximal quenching of fluorescence by Co^{2+} and Mn^{2+} was observed at about 25 μM metal ion (Fig. 2), whereas a concentration of about 30 μM-Ni^{2+}, -Zn^{2+} or -Cd^{2+} gave half-maximal quenching of fluorescence (Fig. 2). At a final concentration of 50 μM, Co^{2+} and Mn^{2+} were effective in completely quenching the fluorescence of 50 μM-quin2 free acid.

Divalent metal ion inflow to hepatocytes loaded with quin2

The addition of 100 μM-Mn^{2+} to hepatocytes loaded with quin2 and incubated in the absence of added Ca^{2+}_o caused a

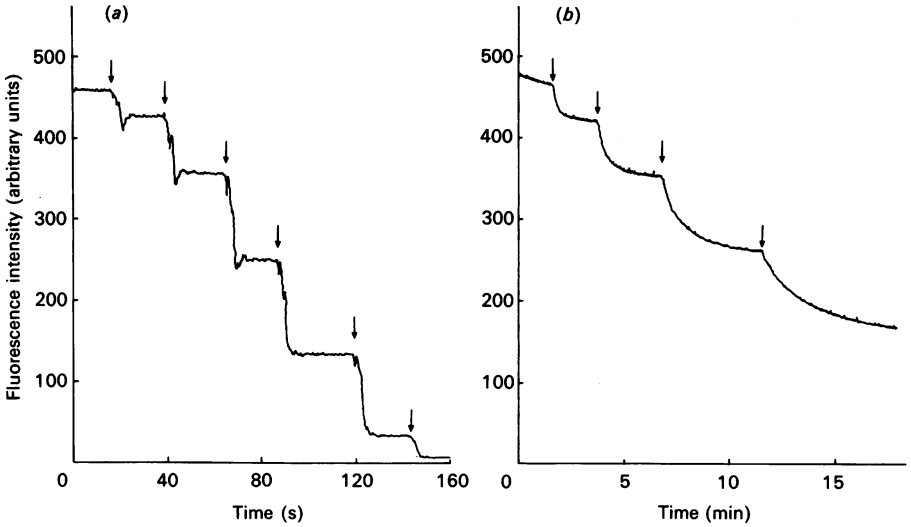

Fig. 1. Time courses for quenching of the fluorescence of a solution of Ca–quin2 by Mn^{2+} and Ni^{2+} under ionic conditions which mimic those of the cytoplasmic space

Successive additions (indicated by the arrows) of 8.3 μM (final concentration) $MnCl_2$ (*a*) or $NiCl_2$ (*b*) were made to a medium which contained 50 μM total quin free acid and 30 μM total Ca^{2+}. The concentration of free Ca^{2+} in the absence of Mn^{2+} or Ni^{2+} was 200 nM. The composition of the medium and the method for determination of the free Ca^{2+} concentration are described in the Experimental section. Each trace is representative of similar traces obtained in two separate experiments.

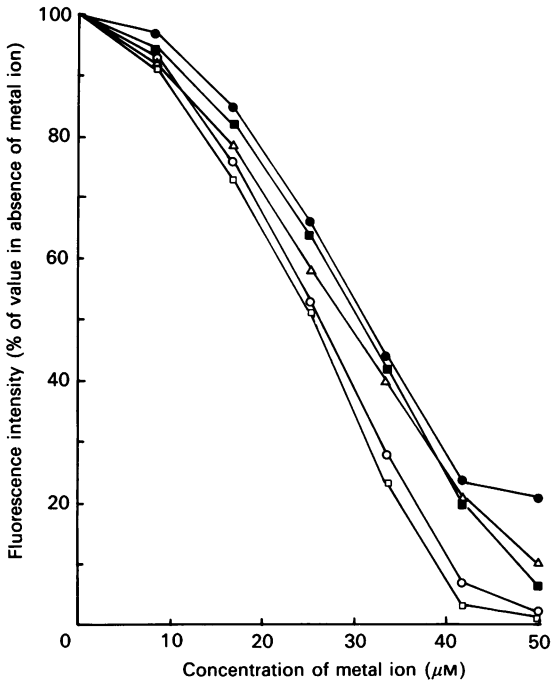

Fig. 2. Effect of divalent metal ion concentration on the fluorescence of a solution of Ca–quin2 measured under ionic conditions which mimic those of the cytoplasmic space

Successive additions of $CoCl_2$ (□), $MnCl_2$ (○), $NiCl_2$ (△), $CdCl_2$ (●) or $ZnCl_2$ (■) to a solution of 50 μM-quin2 free acid and 30 μM total Ca^{2+} (200 nM free Ca^{2+}) were made as described in the legend of Fig. 1. The intensity of the fluorescence of Ca–quin2 in the presence of a given metal ion is expressed as a percentage of the fluorescence of Ca–quin2 in the absence of any other metal ion. The data are the means obtained from two time-courses for each metal ion. The difference between the replicate time courses was less than 4%.

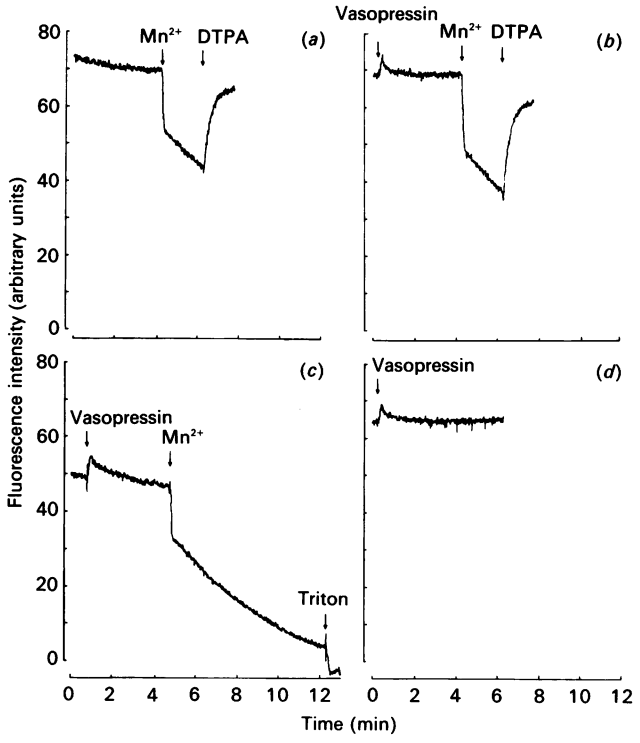

Fig. 3. Quenching by Mn^{2+} of the fluorescence of hepatocytes loaded with quin2

The loading of hepatocytes with quin2 and measurement of fluorescence intensity were performed as described in the Experimental section. The fluorescence intensities for unloaded cells incubated under the same conditions have been subtracted in order to generate the results shown. The additions were: (*a*) $MnCl_2$ (100 μM) and DTPA (200 μM); (*b*) vasopressin (13 nM), $MnCl_2$ (100 μM) and DTPA (200 μM); (*c*) vasopressin (13 nM), $MnCl_2$ (100 μM) and Triton X-100 (0.1%, w/v); and (*d*) vasopressin (13 nM); the traces shown are representative of nine experiments using three cell preparations (*a* and *b*) or four experiments using two cell preparations, each of which gave similar results.

Table 1. Stimulation by different agonists of the quenching by Mn^{2+} of the fluorescence of intracellular quin2 in quin2-loaded cells

The loading of hepatocytes with quin2 and measurement of rates of change in fluorescence in the presence of 100 μM-Mn^{2+} and in the presence or absence of agonist (added 3 min before Mn^{2+}) and presence or absence of 1.3 mM-Ca^{2+}_o were performed as described in the Experimental section and in the legend of Fig. 3. When glucagon and vasopressin were both present, glucagon was added 2 min before vasopressin. The values are the means ± S.E.M. of the number of determinations indicated in parentheses made using between three and nine separate cell preparations. The degree of significance for comparison of the rate of decrease in fluorescence observed in the presence of agonist with that observed in the absence of agonist, determined using Student's t test for unpaired samples, is $*P < 0.05$.

Agonist	Concentration of added Ca^{2+}_o (mM)	Rate of decrease in fluorescence (mV/min)
None	0	2.9±0.07 (18)
Vasopressin (10 nM)	0	3.5±0.08*(18)
Adrenaline (1 μM)	0	3.6±0.17* (6)
Angiotensin II (10 nM)	0	3.5±0.16* (6)
Glucagon (0.1 μM)	0	3.3±0.08*(12)
Glucagon + vasopressin	0	3.9±0.23* (8)
None	1.3	3.3±0.2 (6)
Vasopressin (10 nM)	1.3	4.4±0.1* (6)

very rapid decrease in fluorescence followed by a slower, steady decline (Fig. 3*a*). The addition of the metal chelator diethylenetriaminepenta-acetic acid (DTPA) 2 min after Mn^{2+} (cf. Merritt & Hallam, 1988) caused an increase in fluorescence (Fig. 3*a*). However, after 1.5 min the fluorescence was not restored to the value observed before the addition of Mn^{2+}. By contrast, the addition of 200 μM-DTPA to a solution of 30 μM-quin2 free acid, 1 mM-$CaCl_2$ and 100 μM-$MnCl_2$ in cell incubation medium completely restored the fluorescence quenched by the Mn^{2+} (results not shown). It is concluded from the results obtained in the presence of DTPA that the very rapid decrease in fluorescence induced by the addition of Mn^{2+} is due to the quenching by Mn^{2+} of the fluorescence of extracellular quin2, whereas the subsequent slower decrease in fluorescence is due to the quenching of the fluorescence of intracellular quin2.

In hepatocytes loaded with quin2 and treated with vasopressin in the absence of added Ca^{2+}_o, the rate of decrease in the fluorescence of intracellular quin2 induced by 100 μM-Mn^{2+} was greater than that observed in cells incubated in the absence of vasopressin (Fig. 3*b* compared with Fig. 3*a*, and Table 1). There was no change in the rapid decrease in fluorescence attributed to the quenching of extracellular quin2. A linear rate of decrease in fluorescence was maintained for a period of about 2 min. Over a period of 8 min, almost complete quenching of the fluorescence of quin2 was observed, as indicated by the small additional quenching caused by Triton X-100 (Fig. 3*c*). Little change in fluorescence was observed in the absence of Mn^{2+} (Fig. 3*d*).

A plot of the rate of quenching of intracellular quin2 as a function of the concentration of extracellular Mn^{2+} ($[Mn^{2+}]_o$) (measured in the presence of 1.3 mM-Ca^{2+}) reached a plateau at about 100 μM-Mn^{2+} (Fig. 4). The $[Mn^{2+}]_o$ which gave half-maximal stimulation of quenching of the fluorescence was 20 μM (Fig. 4). A plot with a shape similar to that shown in Fig. 4 was obtained for cells incubated (i) in the presence of 1.3 mM-Ca^{2+} and in the presence of 13 nM-vasopressin, (ii) in the absence of added Ca^{2+}_o and in the absence of vasopressin, and (iii) in the absence of added Ca^{2+}_o and in the presence of 13 nM-vasopressin (results not shown).

The possibility that the plateau observed in the plots of the rate of quenching of intracellular quin2 as a function of $[Mn^{2+}]_o$ is due to the saturation by Mn^{2+} of intracellular quin2 was tested using ionomycin. The addition of 10 μM-ionomycin after the addition of Mn^{2+} to cells loaded with quin2 markedly increased the rate of quenching of intracellular quin2 and allowed complete

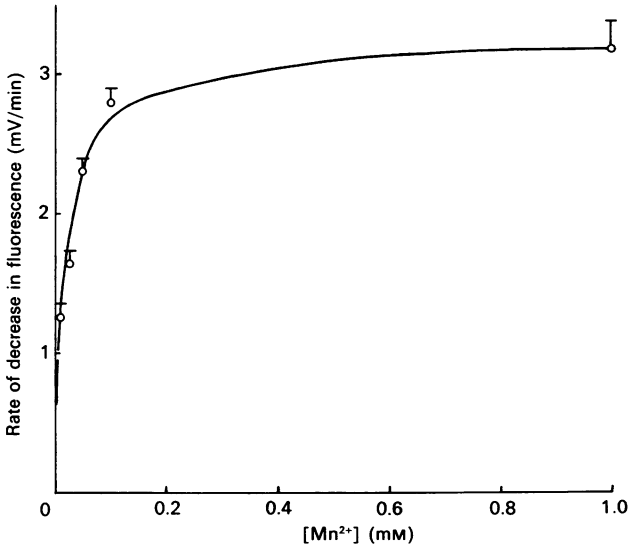

Fig. 4. Effect of increasing concentrations of Mn^{2+} on the quenching of the fluorescence of intracellular quin2

The rate of decrease in the fluorescence attributed to intracellular quin2 following the addition of a given concentration of $MnCl_2$ to hepatocytes loaded with quin2 was measured in the presence of 1.3 mM-Ca^{2+}_o as described in the legend of Fig. 3(*a*) and in the Experimental section. The results are the means ± S.E.M. of four or five determinations, each using a separate cell preparation.

quenching by Mn^{2+} of all intracellular quin2, as indicated by the failure of Triton X-100 to cause a further decrease in fluorescence (Fig. 5*a*). The addition of 200 μM-DTPA at 2 min after the addition of ionomycin in the presence of Mn^{2+} in order to chelate extracellular Mn^{2+} caused an increase in fluorescence similar in magnitude to the rapid initial decrease induced by addition of Mn^{2+} (results not shown). This result indicates that ionomycin induced little leakage of quin2 from the cells. The addition of 1% (v/v, final concentration) dimethyl sulphoxide in place of ionomycin did not alter the rate of decrease in fluorescence (results not shown). In the absence of Mn^{2+}, ionomycin caused a rapid increase in fluorescence which was followed by a small steady decrease (Fig. 5*b*).

In the absence of added Ca^{2+} and in the presence of 100 μM-

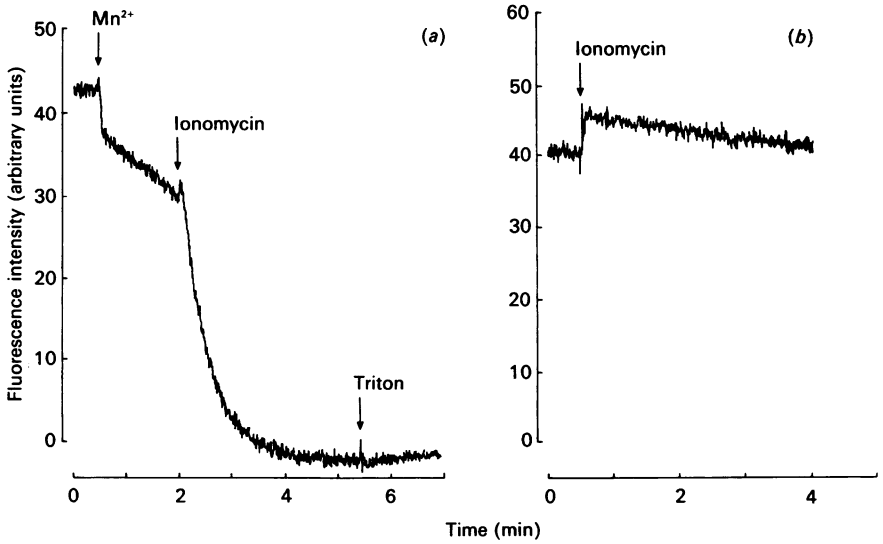

Fig. 5. Effect of ionomycin on the fluorescence of cells loaded with quin2 and incubated in the presence (*a*) and absence (*b*) of Mn^{2+}

The loading of hepatocytes with quin2 and measurement of fluorescence intensity were performed as described in the Experimental section. The fluorescence intensities for unloaded cells incubated under the same conditions have been subtracted in order to generate the results shown. The additions were: (*a*) $MnCl_2$ (100 μM), ionomycin (10 μM) and Triton X-100 (0.1%, w/v), and (*b*) ionomycin (10 μM). Dimethyl sulphoxide, the solvent for ionomycin, was present at a final concentration of 1% (v/v). The traces shown are representative of three experiments obtained using two cell preparations.

Table 2. Quenching by other metal ions of the fluorescence of intracellular quin2 in quin2-loaded cells in the presence and absence of vasopressin and angiotensin II

The loading of hepatocytes with quin2 and measurement of rates of change in fluorescence in the presence of the metal ion and in the presence or absence of vasopressin or angiotensin II and in the presence or absence of 1.3 mM-Ca^{2+}_o were performed as described in the Experimental section and in the legend of Fig. 3. The values are the means ± S.E.M. of the number of determinations indicated in parentheses made using two to four separate cell preparations. The degree of significance for comparison of the value obtained in the presence of hormone with that in the absence of hormone, determined using Student's *t* test for unpaired samples, is $*P < 0.05$.

		Rate of decrease in fluorescence (mV/min)			
Metal ion	Concentration of added Ca^{2+}_o (mM)	No hormone	Vasopressin (10 nM)	No hormone	Angiotensin II (10 nM)
Co^{2+} (1 mM)	0	2.3±0.1 (7)	2.8±0.2* (7)	2.5±0.1 (6)	3.0±0.2* (3)
	1.3	3.2±0.2 (6)	4.1±0.2* (6)		
Ni^{2+} (1 mM)	0	1.4±0.1 (4)	1.9±0.1* (4)	1.7±0.1 (3)	3.0±0.3* (3)
	1.3	2.3±0.1 (6)	3.3±0.1* (6)		
Cd^{2+} (100 μM)	0	2.2±0.2 (4)	3.3±0.1* (4)	2.0±0.1 (5)	2.7±0.1* (5)
	1.3	2.8±0.2 (6)	4.3±0.2* (6)		
Zn^{2+} (80 μM)	0	1.2±0.2 (4)	1.7±0.2* (4)	1.0±0.2 (3)	2.9±0.4* (3)
	1.3	1.3±0.2 (6)	2.0±0.1* (6)		

Mn^{2+}, the concentration of vasopressin which gave half-maximal stimulation of the Mn^{2+}-induced quenching of the fluorescence of intracellular quin2 was 0.5 nM (results not shown). Adrenaline, angiotensin II and glucagon, at saturating concentrations, each caused a stimulation of the quenching of the fluorescence of intracellular quin2 (Table 1). As previously shown by others (Berthon *et al.*, 1984; Charest *et al.*, 1985; Joseph *et al.*, 1985), these hormones increased the fluorescence of quin2-loaded cells incubated in the presence of 1.3 mM-Ca^{2+}_o and in the absence of Mn^{2+} both immediately after and at 2 min after addition of the agonist (results not shown). The effect of glucagon plus vasopressin on Mn^{2+}-induced quenching of fluorescence was equal to the sum of the effects of each agent alone (Table 1).

The stimulation by vasopressin of the quenching by Mn^{2+} of intracellular quin2 was completely inhibited by 200 μM-verapamil. In the absence of verapamil, the rates of decrease in fluorescence in the absence and presence of vasopressin were 2.48±0.11 and 3.26±0.04 mV/min respectively [mean ± S.E.M., $n = 4$ (two cell preparations)] ($P < 0.05$; Student's *t* test for unpaired samples), whereas in the presence of verapamil the rates of decrease in fluorescence in the absence or presence of vasopressin were 2.56±0.26 and 2.53±0.01 mV/min respectively [mean ± S.E.M., $n = 4$ (two cell preparations)]. The concentration of verapamil which gave half-maximal inhibition of vasopressin-stimulated quenching by Mn^{2+} of intracellular quin2 was 70 μM (results not shown).

The addition of Co^{2+}, Ni^{2+} or Zn^{2+} to hepatocytes loaded with quin2 and incubated in the absence of added Ca^{2+}_o also caused a biphasic decrease in fluorescence (results not shown) similar to that induced by Mn^{2+} (cf. Fig. 3*a*). For each metal ion, the decrease in fluorescence attributed to the quenching of the fluorescence of intracellular quin2 was stimulated by vasopressin

and angiotensin II (Table 2). Since the rate of quenching of quin2 by Ni^{2+} *in vitro* (Fig. 1*b*) is slow relative to that by the other metal ions, experiments were performed in order to test whether the interaction of Ni^{2+} with quin2 rather than the inflow of Ni^{2+} across the plasma membrane is the rate-limiting step in the quenching by Ni^{2+} of the fluorescence of intracellular quin2. When the concentration of added Ni^{2+} was reduced from 1 mM to 50 μM, vasopressin also caused an 80% increase in the rate of quenching by Ni^{2+} of the fluorescence of intracellular quin2. Moreover, in the absence of vasopressin the addition of 10 μM-ionomycin caused a substantial increase in the rate of quenching of fluorescence induced by 1 mM-Ni^{2+} (results not shown).

In the presence of 1.3 mM-Ca^{2+}_o, the rate of decrease in fluorescence attributed to the quenching of intracellular quin2 by a given metal ion in both the absence and presence of vasopressin was 8–75% greater than the value observed in the absence of added Ca^{2+}_o (Tables 1 and 2). Moreover, in the presence of 1.3 mM-Ca^{2+}_o the degree of stimulation by vasopressin of the divalent metal ion-induced decrease of fluorescence of intracellular quin2 was larger than that observed in the absence of added Ca^{2+}_o (Tables 1 and 2).

The lower rates of metal ion-induced quenching of quin2 fluorescence in cells incubated in the absence of added Ca^{2+}_o were associated with a lower basal value of $[Ca^{2+}]_i$ and a smaller increase in $[Ca^{2+}]_i$ induced by vasopressin compared with cells incubated in the presence of 1.3 mM added Ca^{2+}_o. For cells incubated in the absence of added $[Ca^{2+}]_o$, the basal value of $[Ca^{2+}]_i$ was 174 ± 13 nM ($n = 12$) and the maximum value of $[Ca^{2+}]_i$ induced by vasopressin was 365 ± 39 nM ($n = 12$), whereas in cells incubated in the presence of 1.3 mM-Ca^{2+}_o, the basal value of $[Ca^{2+}]_i$ was 240 ± 21 nM ($n = 9$) and the maximum value of $[Ca^{2+}]_i$ induced by vasopressin was 785 ± 78 nM ($n = 9$) (means $\pm$ S.E.M. for the number of experiments indicated). These results indicate that, in cells incubated in the absence of added Ca^{2+}_o, the Ins(1,4,5)P_3-releasable intracellular stores of Ca^{2+} (Joseph *et al.*, 1984; Burgess *et al.*, 1984) are partially depleted.

Mn^{2+} inflow measured by atomic absorption spectroscopy and $^{54}Mn^{2+}$ uptake

The time course for the increase in total $^{55}Mn^{2+}$ (measured by atomic absorption spectroscopy) in hepatocytes incubated in the absence of added Ca^{2+}_o and vasopressin and in the presence of 100 μM-$MnCl_2$ is shown in Fig. 6(*a*) (△). From these data the initial rate of Mn^{2+} uptake was calculated to be about 0.05 nmol/min per mg wet wt. of cells. The inclusion of 1.3 mM-Ca^{2+}_o in the incubation medium caused a small decrease in the rate of Mn^{2+} uptake whereas in either the absence or the presence of 1.3 mM Ca^{2+}_o, vasopressin caused a small stimulation of Mn^{2+} uptake. Results similar to those shown in Fig. 6(*a*) were obtained when Mn^{2+} inflow to hepatocytes was measured using $^{54}Mn^{2+}$ (Fig. 6*b*). Rates of Mn^{2+} inflow measured using this technique were faster than those observed using atomic absorption spectroscopy. In the absence of added Ca^{2+}_o and vasopressin the initial rate of uptake of $^{54}Mn^{2+}$ was about 0.10 nmol/min per mg wet wt. of cells.

Plots of the rates of uptake of Mn^{2+}, measured by atomic absorption spectroscopy or with $^{54}Mn^{2+}$, as a function of $[Mn^{2+}]_o$, were biphasic over the range 0.05–1.0 mM-Mn^{2+} (Fig. 7). No evidence for the saturation of Mn^{2+} uptake was observed over this concentration range. At 1 mM-Mn^{2+}_o the shape of the plots of the amount of $^{55}Mn^{2+}$ associated with the cells as a function of time was similar to the shape of the plots obtained at 0.1 mM-Mn^{2+} shown in Fig. 6(*a*) (results not shown). This indicates that the biphasic nature of the plot of the rate of Mn^{2+} uptake as a function of $[Mn^{2+}]_o$ is unlikely to be due to non-linearity of the time course for Mn^{2+} uptake at high $[Mn^{2+}]_o$.

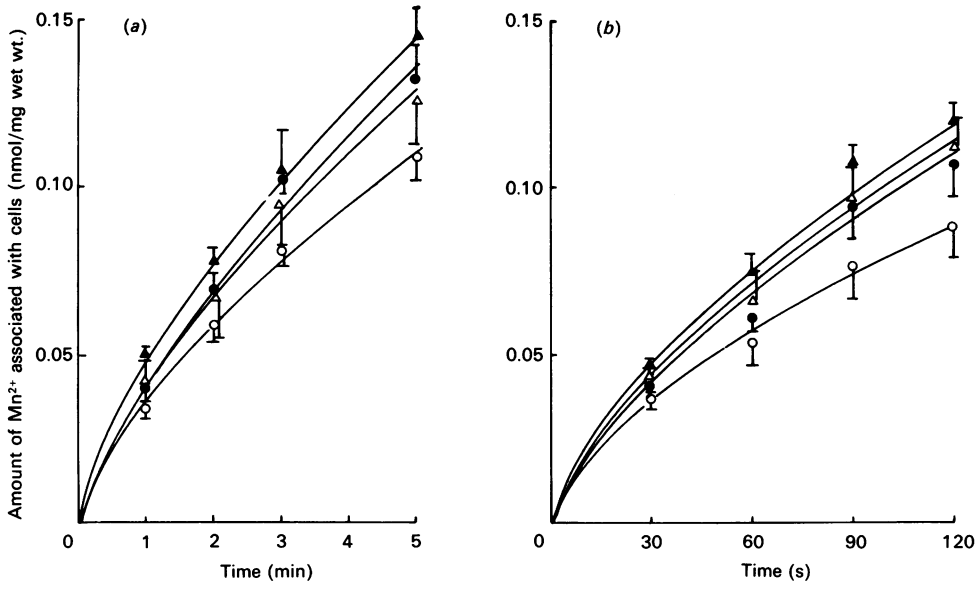

Fig. 6. Uptake of Mn^{2+} by hepatocytes measured by atomic absorption spectroscopy (*a*) or by $^{54}Mn^{2+}$ (*b*)

Isolated hepatocytes were incubated at 37 °C in the absence of vasopressin and added Ca^{2+} (△), in the presence of 13 nM-vasopressin and absence of added Ca^{2+} (▲), in the absence of vasopressin and presence of 1.3 mM-$CaCl_2$ (○), or in the presence of 13 nM-vasopressin and 1.3 mM-$CaCl_2$ (●). (*a*) Cells were incubated for 10 min before the addition of 100 μM-Mn^{2+} ($t = 0$ min on the graph). Where present, 13 nM-vasopressin was added 1 min before Mn^{2+}. For those cells incubated in the presence of Ca^{2+}, 1.3 mM-Ca^{2+} was added at the beginning of the incubation. The amounts of Mn^{2+} associated with the cells were measured by atomic absorption spectroscopy as described in the Experimental section. The value for endogenous Mn^{2+} has been subtracted. The results are the means $\pm$ S.E.M. of four determinations, each from a separate cell preparation. (*b*) Cells were incubated for 15 min before addition of 100 μM-$^{54}Mn^{2+}$ ($t = 0$ s on the graph). Where present, 13 nM-vasopressin was added 5 min before $^{54}Mn^{2+}$. For those cells incubated in the presence of Ca^{2+}, 1.3 mM-Ca^{2+} was added at the beginning of the incubation. The results are the means $\pm$ S.E.M. of four determinations obtained from two separate cell preparations.

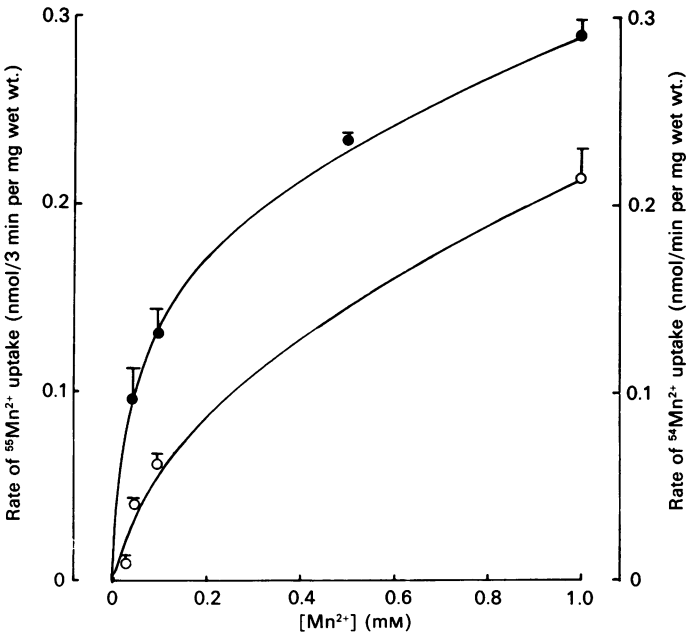

Fig. 7. Effect of increasing concentrations of Mn^{2+} on the rate of uptake of Mn^{2+} measured by atomic absorption spectroscopy or by $^{54}Mn^{2+}$

The amount of $^{55}Mn^{2+}$ taken up by the cells (●) 3 min after the addition of the indicated concentrations of $MnCl_2$ was measured by atomic absorption spectroscopy as described in the legend of Fig. 6 and in the Experimental section. The results are the means ± S.E.M. of four determinations obtained from two cell preparations. The amount of $^{54}Mn^{2+}$ taken up by the cells (○) 1 min after the addition of the indicated concentrations of $MnCl_2$ was measured as described in the legend of Fig. 6 and in the Experimental section. The results are the means ± S.E.M. of three determinations each from a separate cell preparation (0.01, 0.05 and 0.1 mM-Mn^{2+}) and ten determinations obtained from eight cell preparations (1 mM-Mn^{2+}).

In order to determine whether the presence of intracellular quin2 influences the shape of the plot of $^{54}Mn^{2+}$ uptake as a function of $[Mn^{2+}]_o$, rates of $^{54}Mn^{2+}$ uptake were measured in cells loaded with quin2, as described in the Experimental section, and incubated in the presence of $^{54}Mn^{2+}$, as described in the legend of Fig. 6. The plot of the rate of $^{54}Mn^{2+}$ uptake as a function of $[Mn^{2+}]_o$ obtained for cells loaded with quin2 was similar to the plot (Fig. 7) obtained for cells not loaded with quin2 (results not shown).

DISCUSSION

The results of studies of the interaction of divalent metal ions with Ca–quin2 *in vitro* indicate that, with the exception of Ni^{2+}, the quenching by divalent cations of Ca–quin2 fluorescence is rapid under conditions which resemble those present in the cytoplasmic space (cf. Arslan *et al.*, 1985). The relatively long time taken for Ni^{2+} to quench quin2 fluorescence at higher concentrations of Ni^{2+} and at neutral and alkaline pH may be due to the formation of nickel hydroxides and the slow dissociation of these complexes (Cotton & Wilkinson, 1988). The results also indicate that for each of Mn^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} and Cd^{2+} the concentration of metal ion that gives half-maximal quenching of fluorescence is 25–30 μM, a stochiometric concentration of the metal ion gives almost complete quenching, and within the range 10–40 μM added metal ion the decrease in fluorescence is a linear function of the concentration of added metal ion. These observations, together with the reported high affinities of quin2 for Mn^{2+} and Zn^{2+} (Hesketh *et al.*, 1983), provide evidence which indicates that quenching of the fluorescence of intracellular Ca–quin2 can be used as a measure of the concentration of metal ion in the cytoplasmic space, and hence can be used to estimate the rate of metal ion inflow in experiments conducted with intact cells.

The results obtained using three different experimental techniques for the measurement of Mn^{2+} uptake, i.e. quenching of the fluorescence of quin2, atomic absorption spectroscopy and $^{54}Mn^{2+}$ uptake, indicate that Mn^{2+} flows into hepatocytes in the absence of agonists. The observation that Co^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+} quenched the fluorescence of intracellular quin2 in hepatocytes incubated in the absence of agonist indicates that each of these cations also flows across the plasma membrane in the absence of an agonist.

The plot of the rate of Mn^{2+} uptake, measured using quin2, as a function of the extracellular Mn^{2+} concentration reached a plateau at 100 μM-Mn^{2+}, whereas plots obtained by measuring Mn^{2+} uptake by atomic absorption spectroscopy or using $^{54}Mn^{2+}$ showed no saturation at 1 mM extracellular Mn^{2+}. The possibility that the difference between these two sets of data is due to the saturation by Mn^{2+} of intracellular quin2 at high concentrations of extracellular Mn^{2+} is considered to be unlikely for the following reasons. (i) At each concentration of extracellular Mn^{2+} the quenching by Mn^{2+} of the fluorescence of intracellular quin2 continued for a number of minutes (cf. Fig. 3*c*). Moreover, the initial rate of fluorescence quenching was measured. (ii) From the results of the atomic absorption experiments (Fig. 6*a*), it can be calculated that the concentration of Mn^{2+} accumulated in the cell after 1 min of incubation of the cells with 1 mM extracellular Mn^{2+} is 200 μM. It has previously been shown that, under the conditions of quin2 loading employed in the present experiments, the concentration of intracellular quin2 is greater than 1 mM (Crofts & Barritt, 1989). Thus at 1 min after the addition of extracellular Mn^{2+} no more than 20% of the quin2 would be complexed to Mn^{2+}. (iii) Ionomycin, a divalent metal ion ionophore which facilitates the movement of Mn^{2+} as well as Ca^{2+} through membranes (Hesketh *et al.*, 1983; Hallam & Rink, 1985; Merritt & Hallam, 1988), caused a marked increase in the rate of Mn^{2+}-induced quenching of the fluorescence of quin2. This is likely to be due to an increase in the rate of entry of Mn^{2+} into the cytoplasmic space and a subsequent increase in the rate of formation of the Mn–quin2 complex in this space.

The plateau of the plot of the rate of Mn^{2+} inflow as a function of $[Mn^{2+}]_o$ in the quin2 quenching experiments may be due to the failure of intracellular quin2 to complex all Mn^{2+} which enters the cell at high, but not at low, $[Mn^{2+}]_o$. This may arise because the flux of Mn^{2+} from the cytoplasmic space to the endoplasmic reticulum and mitochondria becomes substantial as the $[Mn^{2+}]_i$ increases at high values of $[Mn^{2+}]_o$. This may mean that some Mn^{2+} flowing into the cell at high $[Mn^{2+}]_o$ is directed away from quin2 located in the cytoplasmic space. It has previously been shown that mitochondria can take up Mn^{2+} (Vinogradov & Scarpa, 1973). An alternative explanation for the plateau in the quin2 quenching experiments may be that, at high $Mn^{2+}{}_o$, some Mn^{2+} moves inwards across the plasma membrane without entering the cytoplasmic space to an intracellular compartment, such as the endoplasmic reticulum, which does not contain quin2 [cf. one of the mechanisms proposed to explain agonist-stimulated Ca^{2+} inflow to cells (Putney, 1986)]. It is considered unlikely that the presence of quin2 in the cytoplasmic space alters the process by which Mn^{2+} moves inward across the plasma membrane, because the shape of the concentration–response curve for Mn^{2+} inflow measured using $^{54}Mn^{2+}$ was not altered in cells loaded with quin2.

The results obtained using the fluorescence quenching, atomic absorption spectroscopy and $^{54}Mn^{2+}$ uptake techniques indicate

that vasopressin stimulates Mn^{2+} inflow. Although the degree of stimulation by vasopressin of Mn^{2+} inflow is small (about 30%), it is comparable with the degree of stimulation observed using other techniques for the measurement of Ca^{2+} inflow (Hughes *et al.*, 1986*a*; Crofts & Barritt, 1989). It is considered unlikely that, for the experiments conducted in the absence of added Ca^{2+}_o, the stimulation by vasopressin of Mn^{2+}-induced quenching of the fluorescence of intracellular quin2 is due to an increase in $[Ca^{2+}]_i$ induced by the hormone, since Mn^{2+} was added after the increase in $[Ca^{2+}]_i$ induced by vasopressin had returned to the basal value. Although it was possible to determine the vasopressin-stimulated component of Mn^{2+} inflow in the experiments conducted using quin2, it was not possible to accurately dissect out this parameter in the experiments conducted using atomic absorption spectroscopy or $^{54}Mn^{2+}$. This is chiefly due to the large inflow of Mn^{2+} which occurs in the absence of an agonist.

A somewhat surprising result is the observation that in the presence of 1.3 mM-Ca^{2+}_o the rate of quenching of intracellular quin2 by divalent metal ions is faster, and the degree of stimulation of quenching by agonist is greater, when compared with cells incubated in the absence of added Ca^{2+}_o. One explanation for these results may relate to the smaller amount of Ca^{2+} present in Ins(1,4,5)P_3-sensitive stores in cells incubated in the absence of added Ca^{2+}_o. It has recently been proposed that in certain types of cells the rate of Ca^{2+} inflow through receptor-operated Ca^{2+} channels is determined principally by the amount of Ca^{2+} present in Ins(1,4,5)P_3-sensitive stores and is inversely proportional to the amount of Ca^{2+} present in these stores (Hallam *et al.*, 1989; Putney *et al.*, 1989; Sage *et al.*, 1989; Takemura & Putney, 1989; Takemura *et al.*, 1989). If Mn^{2+} and Ca^{2+} both move through the same plasma membrane receptor-operated channel, this hypothesis would predict that the degree of stimulation of Mn^{2+} inflow by vasopressin in cells incubated in the absence of added Ca^{2+}_o would be less than that in cells incubated in the presence of 1.3 mM-Ca^{2+}_o. This prediction is consistent with the present results. However, it would also be predicted that, in the absence of vasopressin, Mn^{2+} inflow would be greater in cells incubated in the absence of Ca^{2+}_o than in cells incubated in the presence of 1.3 mM-Ca^{2+}_o [that is, inflow through the receptor-operated Ca^{2+} channel would be partially activated by depletion of Ins(1,4,5)P_3-sensitive Ca^{2+} stores]. Since this prediction is not consistent with the present results, it is considered unlikely that differences in the amount of Ca^{2+} present in the Ins(1,4,5)P_3-sensitive stores account for differences in the rate of quenching of intracellular quin2 in the presence and absence of added Ca^{2+}_o. An alternative explanation for the stimulation by Ca^{2+}_o of Mn^{2+}-induced quenching of the fluorescence of intracellular quin2 may involve the higher initial fluorescence of the quin2 caused by the presence of a higher $[Ca^{2+}]_i$ in cells incubated in the presence of Ca^{2+}_o.

The observation that vasopressin and angiotensin II stimulate the quenching of intracellular fluorescence induced by Co^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+} indicates that liver cells possess a receptor-activated divalent cation channel which admits these ions as well as Mn^{2+}. Two observations indicate that it is unlikely that the observed rate of quenching by Ni^{2+} of the fluorescence of intracellular quin2 reflects the rate of dissociation of nickel complexes or the interaction of Ni^{2+} with quin2 rather than the rate of movement of Ni^{2+} across the plasma membrane. Firstly, ionomycin, which should increase the rate of entry of Ni^{2+} to the cytoplasmic space (Merritt *et al.*, 1989), enhanced the rate of quenching by Ni^{2+} of intracellular quin2. Secondly, vasopressin stimulated the quenching by Ni^{2+} of intracellular quin2 at 50 μM as well as at 1.0 mM added Ni^{2+}. At the lower extracellular Ni^{2+} concentration employed, the amount of Ni^{2+} which enters the cytoplasmic space is likely to be in the range of the amount added in the first addition of Ni^{2+} to quin2 in the experiments conducted *in vitro* (Fig. 1*b*). At this low Ni^{2+} concentration, the decrease in fluorescence induced by Ni^{2+} was rapid.

The concentration of vasopressin which gave half-maximal stimulation of Mn^{2+} inflow (0.5 nM) is similar to that which has been reported to give half-maximal stimulation of changes in $[Ca^{2+}]_i$, Ca^{2+} fluxes and glycogen phosphorylase activity in hepatocytes (Rhodes *et al.*, 1983; Thomas *et al.*, 1984; Charest *et al.*, 1985; Hughes *et al.*, 1986*a*). This result, together with the observations that (i) the concentration of verapamil which gives half-maximal inhibition of vasopressin-stimulated Mn^{2+} inflow (70 μM) is comparable with that which gives half-maximal inhibition of Ca^{2+} inflow (Hughes *et al.*, 1986*b*), and (ii) that adrenaline, angiotensin II and glucagon, which stimulate Ca^{2+} inflow (Barritt *et al.*, 1981; Reinhart *et al.*, 1984; Mauger *et al.*, 1984, 1985), also stimulate Mn^{2+} inflow, indicate that the inflow of Mn^{2+} stimulated by vasopressin is through the receptor-operated Ca^{2+} channel. [Evidence has previously been provided which indicates that, under the conditions of the present experiments, verapamil does not displace vasopressin from its receptor (Hughes *et al.*, 1986*b*).]

Taken together, the results indicate that hepatocytes possess both a basal divalent cation inflow system and an agonist-activated divalent cation inflow system, each of which has a broad specificity for divalent cations. Previous studies have indicated that the basal system also admits Sr^{2+} (Hughes & Barritt, 1989*b*). The results obtained with Mn^{2+} indicate that the receptor-activated inflow system for divalent metal ions is the same as the receptor-operated Ca^{2+} channel through which agonist-stimulated Ca^{2+} inflow occurs. The relatively small inhibition by Ca^{2+} of Mn^{2+} inflow (measured by atomic absorption spectroscopy and $^{54}Mn^{2+}$) may be due to the ability of the channel to readily allow the simultaneous entry of both Ca^{2+} and Mn^{2+} [cf. the proposed simultaneous movement of Ca^{2+} and Ba^{2+} through the voltage-operated Ca^{2+} channel (Hess & Tsien, 1984; Friel & Tsien, 1989)].

It may be concluded that, with respect to the nature of the divalent metal ion admitted, the liver cell receptor-operated Ca^{2+} channel is similar to some voltage-operated Ca^{2+} channels which have been shown to admit Mn^{2+}, Zn^{2+} and Cd^{2+} as well as Ca^{2+}, Ba^{2+} and Sr^{2+} (Fukada & Kawa, 1977; Kawa, 1979; Hagiwara & Byerly, 1981*a*,*b*; Hinkle *et al.*, 1987), and to agonist-stimulated Ca^{2+} inflow channels in blood platelets (Hallam & Rink, 1985; Merritt & Hallam, 1988; Sage *et al.*, 1989; Alonso *et al.*, 1989), endothelial cells (Hallam *et al.*, 1988) and neutrophils (Merritt *et al.*, 1989) which have been shown to admit Mn^{2+}. However, the liver cell receptor-operated Ca^{2+} channel differs from the agonist-induced Ca^{2+} inflow system in the parotid cell, which does not appear to admit Mn^{2+} (Merritt & Hallam, 1988). Liver cells also differ from endothelial cells in that the latter do not appear to permit the inflow of Ni^{2+} across the plasma membrane (Hallam *et al.*, 1988).

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