Effects of vasopressin and La^{3+} on plasma-membrane Ca^{2+} inflow and Ca^{2+} disposition in isolated hepatocytes

Evidence that vasopressin inhibits Ca^{2+} disposition

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Vasopressin caused a 40% inhibition of ^{45}Ca uptake after the addition of 0.1 mM- $^{45}\text{Ca}^{2+}$ to Ca^{2+} -deprived hepatocytes. At 1.3 mM- $^{45}\text{Ca}^{2+}$, vasopressin and ionophore A23187 each caused a 10% inhibition of $^{45}\text{Ca}^{2+}$ uptake, whereas La^{3+} increased the rate of $^{45}\text{Ca}^{2+}$ uptake by Ca^{2+} -deprived cells. Under steady-state conditions at 1.3 mM extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$), vasopressin and La^{3+} each increased the rate of $^{45}\text{Ca}^{2+}$ exchange. The concentrations of vasopressin that gave half-maximal stimulation of $^{45}\text{Ca}^{2+}$ exchange and glycogen phosphorylase activity were similar. At 0.1 mM- Ca^{2+}_o , La^{3+} increased, but vasopressin did not alter, the rate of $^{45}\text{Ca}^{2+}$ exchange. The results of experiments performed with EGTA or A23187 or by subcellular fractionation indicate that the Ca^{2+} taken up by hepatocytes in the presence of La^{3+} is located within the cell. The addition of 1.3 mM- Ca^{2+}_o to Ca^{2+} -deprived cells caused increases of approx. 50% in the concentration of free Ca^{2+} in the cytoplasm ($[\text{Ca}^{2+}]_i$) and in glycogen phosphorylase activity. Much larger increases in these parameters were observed in the presence of vasopressin or ionophore A23187. In contrast with vasopressin, La^{3+} did not cause a detectable increase in glycogen phosphorylase activity or in $[\text{Ca}^{2+}]_i$. It is concluded that an increase in plasma membrane Ca^{2+} inflow does not by itself increase $[\text{Ca}^{2+}]_i$, and hence that the ability of vasopressin to maintain increased $[\text{Ca}^{2+}]_i$ over a period of time is dependent on inhibition of the intracellular removal of Ca^{2+} .

INTRODUCTION

An increase in the concentration of free Ca^{2+} in the cytoplasm ($[\text{Ca}^{2+}]_i$) is an early event in the processes by which vasopressin, α_1 -adrenergic agonists and angiotensin alter metabolism in the liver cell (Assimakopoulos-Jeannot *et al.*, 1977; Murphy *et al.*, 1980; Charest *et al.*, 1983; Berthon *et al.*, 1984). Two mechanisms responsible for the increase in $[\text{Ca}^{2+}]_i$ have been identified. These are the release of Ca^{2+} from the endoplasmic reticulum, catalysed by inositol triphosphate (Burgess *et al.*, 1984; Joseph *et al.*, 1984; Charest *et al.*, 1985), and activation of a receptor-activated plasma-membrane Ca^{2+} transporter (Barritt *et al.*, 1981; Parker *et al.*, 1983; Mauger *et al.*, 1984). Although it has been shown that enhanced plasma-membrane Ca^{2+} inflow is required for a maintained hormonal activation of glycogen phosphorylase *a* or glucose release (DeWitt & Putney, 1983; Reinhart *et al.*, 1984; Binet *et al.*, 1985; Charest *et al.*, 1985), the mechanisms involved, the fate of additional Ca^{2+} which enters the cell in response to hormone action, and whether this alone can increase $[\text{Ca}^{2+}]_i$, have not been clearly defined. These questions are particularly important in view of observations which indicate that hormone-induced changes in $[\text{Ca}^{2+}]_i$ in an individual hepatocyte are complex (Woods *et al.*, 1986). The aim of the present experiments was to investigate these questions using La^{3+} to simulate one component of vasopressin action, the increase in plasma-membrane Ca^{2+} inflow. It has

previously been shown that La^{3+} enhances Ca^{2+} inflow across the liver cell plasma membrane (Krell *et al.*, 1979; Parker & Barritt, 1981), but not Ca^{2+} outflow from intracellular organelles (Parker & Barritt, 1981). The results of the present study indicate that an increase in plasma-membrane Ca^{2+} inflow only leads to an increase in $[\text{Ca}^{2+}]_i$ if the processes that remove Ca^{2+} from the cytoplasm are concurrently inhibited.

EXPERIMENTAL

Isolation of hepatocytes and mitochondria, and measurement of $^{45}\text{Ca}^{2+}$ uptake and total cell Ca^{2+}

Methods and conditions for the isolation and incubation of hepatocytes were as described previously (Barritt *et al.*, 1981). The incubation medium contained, in a final volume of 6.0 ml, 117 mM-NaCl, 4.7 mM-KCl, 1.2 mM- KH_2PO_4 , 1.2 mM- MgSO_4 , 24 mM- NaHCO_3 and 20 mM-Tes/KOH (medium A), as well as CaCl_2 and other additions as indicated, and hepatocytes [30 mg wet wt. (2×10^6 cells)/ml]. The final pH was 7.4. After isolation the cells were maintained in this medium at 0 °C in the absence of added Ca^{2+} for 15 min before use.

Rates of $^{45}\text{Ca}^{2+}$ inflow were measured under two experimental conditions. The first used cells incubated under steady-state conditions. Hepatocytes were incubated at 37 °C in the presence of 1.3 mM- or 0.1 mM- Ca^{2+}_o for 15 min before addition of the agent under test.

Abbreviations used: Ca^{2+}_o , extracellular Ca^{2+} ; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; quin2, 2-([2-bis(carboxymethyl)amino-5-methylphenoxy]methyl)-6-methoxy-8-bis(carboxymethyl)-aminoquinoline.

Isotope exchange was initiated by the subsequent addition at the times indicated of tracer amounts (approx. 5 μCi) of $^{45}\text{CaCl}_2$. It has previously been shown by compartmental analysis that the initial rate of $^{45}\text{Ca}^{2+}$ uptake measured under these conditions is a reliable estimate of Ca^{2+} flux from the medium to the cell (Barritt *et al.*, 1981; Parker *et al.*, 1983). In the second protocol, the uptake of Ca^{2+} by Ca^{2+} -deprived cells was measured in hepatocytes incubated at 37 °C in the absence of added Ca^{2+} for 15 min before addition of the agent under test. Rates of Ca^{2+} uptake were estimated from measurement of the $^{45}\text{Ca}^{2+}$ associated with cells after addition of 1.3 mM- or 0.1 mM- $^{45}\text{Ca}^{2+}$ (5 μCi).

The amount of $^{45}\text{Ca}^{2+}$ associated with the cells was determined by centrifugation through a layer of silicone oil as described previously (Barritt *et al.*, 1981). The procedures for the determination of the amount of Ca^{2+} present in cells by atomic-absorption spectroscopy (Barritt *et al.*, 1981; Parker & Barritt, 1981) and for the isolation of 'heavy' mitochondria (Hughes & Barritt, 1984) were as described previously.

Integrity of hepatocytes in the presence of La^{3+}

It has previously been shown that incubation of hepatocytes in the presence of 100 μM - La^{3+} for periods of up to 60 min under conditions used in the experiments described in this paper causes no detectable damage to the cells, as assessed by light-microscopy, the proportion of cells that exclude Trypan Blue, the intracellular ATP concentrations and the quantity of lactate dehydrogenase released during the incubation (Parker & Barritt, 1981). Incubation of hepatocytes with 60 μM - La^{3+} for 15 min had no effect on the amount of [^{14}C]sucrose associated with the cell pellet after centrifugation through a layer of silicone oil. Examination by light-microscopy of cells suspended in incubation medium (medium A) containing 200 μM - La^{3+} revealed no clumps of cells or crystals of precipitate. The addition of La^{3+} to cell suspensions did not cause a detectable change in the pH of the suspension medium. From these results it is concluded that, under the experimental conditions used, La^{3+} does not cause hepatocytes to clump or form an observable precipitate with components of the incubation medium.

Fluorescence of cells loaded with quin2

Conditions for the loading of hepatocytes with the acetoxymethyl ester of quin2 and measurement of quin2 fluorescence were as described by Charest *et al.* (1983). Cells (approx. 50 mg wet wt.) were incubated at 37 °C with constant shaking (100 oscillations/min) in plastic vials (25 ml capacity) under an atmosphere of O_2/CO_2 (19:1) in 4.0 ml (final volume) of medium A containing 0.5 mM- CaCl_2 , 50 μM -EDTA and 15 mg of gelatin/ml, pH 7.4 (medium B).

After 15 min, the acetoxymethyl ester of quin2 (100 μM final concn., dissolved in 16 μl of dimethyl sulphoxide) or dimethyl sulphoxide alone was added, and the incubations were continued for a further 15 min. The cells were collected by centrifugation (50 *g* for 1 min) and resuspended in 4.8 ml of medium B, except that CaCl_2 was omitted and the concentration of gelatin was 5 mg/ml. The cells were then incubated at 37 °C for a further 15 min, collected by centrifugation and suspended at 22 °C in 4.8 ml of medium B which contained no added Ca^{2+} and gelatin at 5 mg/ml. Measurement of quin2 fluorescence was conducted between 5 and 30 min after

final suspension in medium B. The viability of the hepatocytes at the end of the loading process, assessed by Trypan Blue exclusion (Barritt *et al.*, 1981), was $86 \pm 3\%$ for unloaded cells and $85 \pm 2\%$ for cells loaded with quin2 (mean $\pm$ S.E.M., $n = 3$).

Fluorescence was measured at 37 °C in 1.5 ml samples of cell suspensions in an Aminco-Bowman spectrofluorimeter (SPF 74; American Instrument Co., Silver Spring, MD, U.S.A.), equipped with a constant-temperature accessory and a magnetic stirrer. The wavelengths for excitation and emission were 340 nm and 490 nm respectively, and the slit width was 0.5 mm. The output from the fluorimeter was channelled to a Brinkman 2543 potentiometric recorder (Brinkman Instruments, Westbury, NY, U.S.A.). For the calculation of values of $[\text{Ca}^{2+}]_i$ the fluorescence of unloaded cells was subtracted from the values obtained for cells loaded with quin2. Estimates of $[\text{Ca}^{2+}]_i$ were made as described by Tsien *et al.* (1982), by using 0.1% (w/v) Triton X-100 to lyse the cells and 1.3 mM- CaCl_2 and 44 mM-EGTA to estimate maximum and minimum fluorescence respectively, and a value of 115 nm for the dissociation constant for the combination of Ca^{2+} with quin2 (Tsien *et al.*, 1982). Correction for leakage of extracellular quin2 was made by using Mn^{2+} and calcium diethylenetriaminepentaacetate as described by Rink & Pozzan (1985). For measurement of the effects of La^{3+} on the fluorescence of quin2-loaded cells, gelatin was omitted from medium B.

Glycogen phosphorylase activity

Glycogen phosphorylase *a* activity was measured as described by Hutson *et al.* (1976). Samples of incubation medium (0.5 ml; 15 mg wet wt. of cells) were placed in plastic tubes which contained 1.3 ml of 550 mM-sucrose,

Fig. 1. Effect of vasopressin on $^{45}\text{Ca}^{2+}$ uptake by Ca^{2+} -deprived cells

The rate of $^{45}\text{Ca}^{2+}$ uptake by Ca^{2+} -deprived cells was measured in the absence (○) or presence (●) of 10 nM-vasopressin after the addition of 0.1 mM- $^{45}\text{Ca}^{2+}$ to Ca^{2+} -deprived cells as described in the Experimental section. Vasopressin (10 nM) was present for 5 min before the addition of $^{45}\text{Ca}^{2+}$ tracer. The values are means $\pm$ S.E.M. for two determinations made on each of three separate cell preparations. The differences between the mean values obtained in the presence and absence of vasopressin are significant (at the 5% level as assessed by a two-way analysis of variance, by using the F-ratio test).

Fig. 2. Effects of vasopressin (a) and ionophore A23187 (b) on the activity of glycogen phosphorylase *a* after the addition of Ca^{2+} to Ca^{2+} -deprived cells

(a) Hepatocytes were incubated in the absence of added Ca^{2+} for 25 min. Saline (○) or 10 nM-vasopressin (●, ■) was then added. After 5 min ($t = 5$ min), samples were removed for glycogen phosphorylase *a* measurements, and saline (●) or 1.3 mM- CaCl_2 (○) was added. The values are means of determinations made on each of two separate cell preparations. (b) Hepatocytes were incubated in the absence of added Ca^{2+} for 25 min. Dimethyl sulphoxide (1% v/v) (○) or 0.5 μM -A23187 (●, ■) was added. After 10 min ($t = 10$ min), samples were removed for measurement of glycogen phosphorylase *a* activity, and saline (●) or 1.3 mM- CaCl_2 (○, ■) was added. The values are means of determinations made on each of two separate cell preparations. Glycogen phosphorylase *a* activity was measured at the times shown as described in the Experimental section.

138 mM- NaF , 28 mM- Na_2EDTA and 138 mM-sodium β -glycerophosphate, pH 6.2. The tubes were immediately placed in liquid N_2 and stored frozen. For the assay, the samples were thawed and mixed, and then 50 μl was placed into glass tubes which contained 50 μl of a mixture of 1 mM-caffeine, 2% (w/v) glycogen, 100 mM-glucose 1-phosphate and 12.5 nCi of $[\text{U}-^{14}\text{C}]$ glucose 1-phosphate at pH 6.1. The tubes were incubated at 30 $^\circ\text{C}$. After 15 min, 50 μl of the reaction mixture was spotted on to squares (2.5 cm $\times$ 2.5 cm) of Whatman 3MM chromatography paper (0.38 mm thickness), and the $[\text{U}-^{14}\text{C}]$ glycogen was precipitated by dropping the squares into 60% (v/v) ethanol. The papers were washed with two changes of ethanol, rinsed with acetone and dried. The paper squares were placed in 15 ml of ACS II scintillation fluid (Amersham Australia Pty. Ltd., Sydney, N.S.W., Australia) and the amount of radioactivity was determined by liquid scintillation. A unit of glycogen phosphorylase *a* activity is expressed as 1 μmol of $[\text{U}-^{14}\text{C}]$ glucose 1-phosphate incorporated into glycogen/min per g wet wt. of cells.

Chemicals

The sources of most chemicals used have been described previously (Barritt *et al.*, 1981; Parker &

Barritt, 1981; Parker *et al.*, 1983; Hughes & Barritt, 1984). [Arginine]vasopressin, NaF , sodium β -glycerophosphate, caffeine and glycogen (type VII, from the mussel *Mytilus edulis*) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Glucose 1-phosphate was purchased from Boehringer Mannheim Australia Pty. Ltd., North Ryde, N.S.W., Australia. $[\text{U}-^{14}\text{C}]$ Glucose 1-phosphate and the acetoxymethyl ester of quin2 were purchased from Amersham Australia.

Treatment of data

Except where indicated otherwise, the results are expressed as means $\pm$ S.E.M. for the numbers of experiments indicated. Degrees of significance were determined by using Student's *t* test for unpaired (except where indicated otherwise) samples. Values of $P > 0.05$ were considered to be not significant.

RESULTS

Effects of vasopressin on $^{45}\text{Ca}^{2+}$ uptake and $[\text{Ca}^{2+}]_i$

Vasopressin inhibited the uptake of $^{45}\text{Ca}^{2+}$ after the addition of 0.1 mM- $^{45}\text{Ca}^{2+}$ to Ca^{2+} -deprived cells (Fig. 1). Most pronounced inhibition was observed between 45 and 120 s after addition of $^{45}\text{Ca}^{2+}$. The uptake of $^{45}\text{Ca}^{2+}$

Fig. 3. Effect of the addition of Ca^{2+} and vasopressin on the fluorescence of hepatocytes previously loaded with quin2 and incubated in the absence of added Ca^{2+}

Hepatocytes were loaded in the presence of 0.5 mM- CaCl_2 with the acetoxymethyl ester of quin2, washed and resuspended at 22 °C in Ca^{2+} -free medium, and incubated in the spectrofluorimeter cuvette at 37 °C in the absence of added Ca^{2+} ; fluorescence intensity was measured, and values of $[\text{Ca}^{2+}]_i$ were calculated as described in the Experimental section. Ca^{2+} (1.3 mM) and vasopressin (10 nM) were added at the times indicated. The numbers on the top of each trace are the values of $[\text{Ca}^{2+}]_i$ (nM; means $\pm$ S.E.M., $n = 3$) at that time point, calculated as described in the experimental section. The traces are from a representative experiment.

by Ca^{2+} -deprived cells exposed to 1.3 mM- Ca^{2+}_o was decreased by $8 \pm 3\%$ ($P < 0.01$, $n = 12$) in the presence of vasopressin (results not shown). Poggioli *et al.* (1985) have previously reported that vasopressin has no effect on $^{45}\text{Ca}^{2+}$ uptake measured under somewhat similar conditions. A similar degree of inhibition was observed when Ca^{2+} -deprived cells were incubated with 1.3 mM- Ca^{2+}_o for 15 s before addition of the $^{45}\text{Ca}^{2+}$ tracer (results not shown). When the time of incubation with 1.3 mM- Ca^{2+}_o was increased to 75 s, a 30% stimulation of $^{45}\text{Ca}^{2+}$ exchange was observed in the presence of vasopressin (results not shown).

Under steady-state conditions, vasopressin increased by 30% the rate of $^{45}\text{Ca}^{2+}$ exchange measured at 1.3 mM- Ca^{2+}_o at times of 0, 15, 30, 45 and 60 s after the addition of tracer ^{45}Ca (results not shown; cf. Keppens *et al.*, 1977). Maximal stimulation was observed at 2 min after addition of vasopressin, and the plateau was maintained for at least 15 min. The dose-response curve for this effect of vasopressin was similar to that for the stimulation of glycogen phosphorylase (results not shown). Similar results have been reported by Mauger *et al.* (1984, 1985), who used a filtration technique to separate cells from the incubation medium. No stimulation by vasopressin (added at 1, 5 or 15 min before $^{45}\text{Ca}^{2+}$) of the rate of $^{45}\text{Ca}^{2+}$ exchange was detected when the extracellular Ca^{2+} concentration was 0.1 mM (results not shown). For control cells, the rate of $^{45}\text{Ca}^{2+}$ uptake by Ca^{2+} -deprived cells was 2-fold greater than the rate of $^{45}\text{Ca}^{2+}$ exchange observed under steady-state conditions at 1.3 mM- Ca^{2+}_o (results not shown).

Adrenaline (100 nM) and glucagon (10 nM), added 5 min before trace amounts of $^{45}\text{Ca}^{2+}$, also produced increases of 40 and 20%, respectively, in the initial rate

Fig. 4. Effect of La^{3+} and ionophore A23187 on $^{45}\text{Ca}^{2+}$ uptake by Ca^{2+} -deprived cells

The rate of $^{45}\text{Ca}^{2+}$ uptake by Ca^{2+} -deprived cells was measured in the presence of LaCl_3 ($\square$), A23187 ($\bullet$), LaCl_3 plus A23187 ($\blacksquare$) or dimethyl sulphoxide [1% (v/v) final concn. ($\circ$, control)] as described in the Experimental section. The concentration of LaCl_3 was 60 μM and that of A23187 10 μM . Hepatocytes were incubated at 37 °C for 30 min before addition of 1.3 mM- $^{45}\text{Ca}^{2+}$. La^{3+} , and A23187 or dimethyl sulphoxide, were present for 15 and 5 min respectively before the addition of $^{45}\text{Ca}^{2+}$. The values are means $\pm$ S.E.M. for two determinations made on each of three separate cell preparations.

of $^{45}\text{Ca}^{2+}$ uptake measured at 1.3 mM- Ca^{2+}_o under steady-state conditions (results not shown). The combination of vasopressin and glucagon produced the same degree of stimulation as that caused by vasopressin alone (results not shown). The result contrasts with that for the effects of glucagon plus vasopressin reported by Mauger *et al.* (1985).

As shown by others (Binet *et al.*, 1985; Charest *et al.*, 1985; Joseph *et al.*, 1985), the addition of vasopressin to Ca^{2+} -deprived cells caused a transient increase in glycogen phosphorylase activity. The transient change, which reflects the release of Ca^{2+} from hormone-sensitive pools (Blackmore *et al.*, 1982; Binet *et al.*, 1985; Charest *et al.*, 1985; Joseph *et al.*, 1985), was complete 5 min after addition of the hormone (results not shown). The addition of 1.3 mM- Ca^{2+}_o to Ca^{2+} -deprived cells caused an increase of 75% in the activity of glycogen phosphorylase (Fig. 2a). When this experiment was repeated with cells incubated in the presence of vasopressin, added at zero time (5 min before addition of Ca^{2+} , and hence after vasopressin has released Ca^{2+} from the hormone-sensitive intracellular stores), a much larger

Fig. 5. Effect of La^{3+} and ionophore A23187 on the rates of $^{45}\text{Ca}^{2+}$ exchange measured under steady-state conditions at (a) 1.3 mM-Ca^{2+} and (b) 0.1 mM-Ca^{2+} .

(a) The amounts of $^{45}\text{Ca}^{2+}$ exchanged by hepatocytes incubated at 1.3 mM-Ca^{2+} under steady-state conditions in the presence of $60 \mu\text{M-LaCl}_3$ ($\square$), $10 \mu\text{M-A23187}$ ($\bullet$), LaCl_3 plus A23187 ($\blacksquare$) or dimethyl sulphoxide [1% (v/v) final concn. ($\circ$, control)] were measured as described in the Experimental section. Hepatocytes were incubated in the presence of 1.3 mM-Ca^{2+} for 30 min before addition of $^{45}\text{Ca}^{2+}$ tracer. La^{3+} and A23187 or dimethyl sulphoxide, were present for 15 and 5 min respectively, before the addition of $^{45}\text{Ca}^{2+}$ tracer. Other experiments (results not shown) have indicated that the concentration of dimethyl sulphoxide used has no effect on either the basal or the La^{3+} -stimulated rate of $^{45}\text{Ca}^{2+}$ exchange. The values are means $\pm$ S.E.M. for two determinations made on each of three separate cell preparations. The insert shows rates of $^{45}\text{Ca}^{2+}$ exchange (determined from plots of the amounts of $^{45}\text{Ca}^{2+}$ exchanged as a function of time) obtained in the presence of different concentrations of La^{3+} plotted as a function of the La^{3+} concentration. (b) The amount of $^{45}\text{Ca}^{2+}$ exchanged by hepatocytes incubated at 0.1 mM-Ca^{2+} under steady-state conditions in the absence ($\circ$) or presence ($\bullet$) of LaCl_3 was measured as described in the Experimental section. LaCl_3 ($60 \mu\text{M}$) was present for 15 min before the addition of $^{45}\text{Ca}^{2+}$ tracer. The values are means $\pm$ S.E.M. for two or three determinations made on each of four cell preparations.

increase in glycogen phosphorylase activity was observed after the addition of Ca^{2+} (Fig. 2a) (cf. Cooper *et al.*, 1985; Joseph *et al.*, 1985).

The preincubation of Ca^{2+} -deprived cells with ionophore A23187 also enhanced the increase in glycogen phosphorylase *a* activity observed after the addition of Ca^{2+} to Ca^{2+} -deprived cells (Fig. 2b). At this concentration, A23187 did not increase the uptake of $^{45}\text{Ca}^{2+}$ by Ca^{2+} -deprived cells (results not shown).

An increase of 50% in $[\text{Ca}^{2+}]_i$, monitored by the fluorescence of quin2, was observed after the addition of 1.3 mM-Ca^{2+} to Ca^{2+} -deprived cells (Fig. 3, upper trace). For cells previously treated with vasopressin, $[\text{Ca}^{2+}]_i$ increased by 130% after the addition of 1.3 mM-Ca^{2+} (Fig. 3, lower trace). The value of $[\text{Ca}^{2+}]_i$ in Ca^{2+} -deprived hepatocytes was lower than that in cells incubated at 1.3 mM-Ca^{2+} under steady-state conditions. The activities of glycogen phosphorylase *a* were 9.2 ± 0.6 ($n = 15$, $P < 0.0005$) and 13.1 ± 0.8 ($n = 19$) units respectively, and in cells loaded with quin2 the values of $[\text{Ca}^{2+}]_i$ were

190 ± 10 ($P < 0.05$, $n = 3$, paired *t* test) and $280 \pm 20 \text{ nM}$ respectively.

Effects of La^{3+} on $^{45}\text{Ca}^{2+}$ uptake and $[\text{Ca}^{2+}]_i$

In contrast with vasopressin, La^{3+} stimulated the uptake of Ca^{2+} by Ca^{2+} -deprived cells (Fig. 4). Under steady-state conditions La^{3+} increased the rate of $^{45}\text{Ca}^{2+}$ exchange at both 1.3 mM- (Fig. 5a) and 0.1 mM-Ca^{2+} (Fig. 5b). At 1.3 mM-Ca^{2+} , a concentration of $60 \mu\text{M-La}^{3+}$ gave a stimulation comparable with that observed with vasopressin. The effect of La^{3+} was rapid in onset and reached near-maximum values at 2 min (results not shown).

The addition of La^{3+} to hepatocytes incubated under steady-state conditions in the presence of 1.3 mM-Ca^{2+} did not increase the activity of glycogen phosphorylase (Fig. 6) or the value of $[\text{Ca}^{2+}]_i$ in quin2-loaded hepatocytes (results not shown). La^{3+} did not inhibit the ability of vasopressin to stimulate glycogen phosphorylase activity (Fig. 6). Pre-treatment of the cells with La^{3+} did

Fig. 6. Effect of La^{3+} on glycogen phosphorylase activity in hepatocytes incubated at 1.3 mM-Ca^{2+}_o under steady-state conditions

Hepatocytes were incubated in the presence of 1.3 mM-Ca^{2+}_o for 25 min. At this time ($t = 0 \text{ min}$) samples were removed for the measurement of glycogen phosphorylase *a* activity, and saline (○) or $120 \mu\text{M-LaCl}_3$ (●) was added. Vasopressin (10 nM) was added to both the control and LaCl_3 incubations at 15 min (indicated by the arrow). The values are means $\pm$ S.E.M. for two determinations made on each of three separate cell preparations. Glycogen phosphorylase *a* activity was measured at the times shown as described in the Experimental section.

not alter the magnitude of the activation of glycogen phosphorylase induced by vasopressin or the time-course of this process (Fig. 6; and results not shown). When Ca^{2+} was added to Ca^{2+} -deprived cells incubated in the presence of La^{3+} in experiments similar to that described in Fig. 2(a), no additional increase in glycogen phosphorylase activity was observed in the presence of La^{3+} compared with that in the absence of La^{3+} (results not shown).

Distribution of Ca^{2+} in hepatocytes treated with La^{3+}

Incubation of hepatocytes under steady-state conditions at 1.3 mM-Ca^{2+}_o in the presence of La^{3+} ($120 \mu\text{M}$) for 15 min increased the total amount of cellular Ca^{2+} (measured by atomic-absorption spectroscopy) from $0.86 \pm 0.04 \text{ nmol/mg wet wt.}$ for untreated cells to $1.11 \pm 0.09 \text{ nmol/mg wet wt.}$ for cells treated with La^{3+} ($P < 0.025$, $n = 8$). The stimulation by La^{3+} of $^{45}\text{Ca}^{2+}$ exchange under steady-state conditions at 1.3 mM-Ca^{2+}_o was not observed in the presence of A23187, although A23187 itself increased the initial rate of $^{45}\text{Ca}^{2+}$ exchange (Fig. 5a). The stimulation by La^{3+} of Ca^{2+} uptake by Ca^{2+} -deprived cells was not diminished by A23187, although the ionophore itself inhibited the uptake of $^{45}\text{Ca}^{2+}$ (Fig. 4).

Addition of EGTA to the incubation medium for cells incubated at 0.1 mM-Ca^{2+}_o under steady-state conditions

caused a loss of $^{45}\text{Ca}^{2+}$ from both untreated cells and cells treated with La^{3+} (Fig. 7a). Over a 5 min period, more $^{45}\text{Ca}^{2+}$ was removed from La^{3+} -treated than from untreated cells, but more than half of the additional Ca^{2+} associated with the cells in the presence of La^{3+} remained associated after treatment with EGTA (Fig. 7a). Addition of ionophore A23187 caused losses of 70% of the $^{45}\text{Ca}^{2+}$ associated with untreated cells and 20% of that associated with cells treated with $60 \mu\text{M-La}^{3+}$ (Fig. 7b).

Hepatocytes incubated at 0.1 mM-Ca^{2+}_o under steady-state conditions as described in the legend of Fig. 5(b) were homogenized and fractionated 1 min after the addition of $^{45}\text{Ca}^{2+}$. For untreated cells and cells treated with La^{3+} ($120 \mu\text{M}$), the amounts of $^{45}\text{Ca}^{2+}$ associated with the mitochondrial fraction were 0.80 ± 0.22 and $1.03 \pm 0.15 \text{ nmol/mg of protein}$ ($P < 0.05$, $n = 4$) respectively, and the amounts of $^{45}\text{Ca}^{2+}$ associated with the post-mitochondrial supernatant were 0.67 ± 0.15 and $1.16 \pm 0.27 \text{ nmol/mg of protein}$ ($P < 0.025$, $n = 4$) respectively.

DISCUSSION

The results indicate that La^{3+} increases the uptake of Ca^{2+} by hepatocytes under a variety of experimental conditions. Evidence that this represents an increase in net Ca^{2+} inflow across the plasma membrane, rather than an increase in the binding of Ca^{2+} to extracellular sites, is provided by the following observations. (i) Under steady-state conditions at 1.3 mM-Ca^{2+}_o , La^{3+} did not increase $^{45}\text{Ca}^{2+}$ exchange in the presence of A23187. (ii) Addition of EGTA does not rapidly remove additional $^{45}\text{Ca}^{2+}$ taken up in the presence of La^{3+} . (iii) The amount of $^{45}\text{Ca}^{2+}$ in the mitochondrial fraction of cells treated with La^{3+} is increased by 30% compared with untreated cells. (iv) Compartmental analysis of steady-state kinetic data conducted previously (Parker & Barritt, 1981) indicates that treatment with La^{3+} increases the amount of exchangeable Ca^{2+} in a compartment with the kinetic characteristics of intracellular organelles.

The results obtained at 1.3 mM-Ca^{2+}_o under steady-state conditions indicate that both vasopressin and La^{3+} increase plasma membrane Ca^{2+} inflow under these conditions. The onset of the effect of vasopressin is within the time-frame reported for the effects of the hormone on $[\text{Ca}^{2+}]_i$ and glycogen phosphorylase activity in isolated hepatocytes (Blackmore *et al.*, 1982; Charest *et al.*, 1983, 1985). The concentration of vasopressin which gives half-maximal stimulation of $^{45}\text{Ca}^{2+}$ exchange is similar to that which gives half-maximal increase in glycogen phosphorylase activity, but is an order of magnitude lower than the concentration which gives half-maximal stimulation of phosphatidylinositol bisphosphate hydrolysis (Creba *et al.*, 1983; Charest *et al.*, 1985) and the binding of vasopressin to plasma-membrane receptors (Cantau *et al.*, 1980). This indicates that the effect of vasopressin on plasma-membrane Ca^{2+} inflow is not a primary action of the hormone, but depends on other early events induced by the hormone. The hypothesis that the increased catalytic capacity of the Ca^{2+} -inflow transporter is caused directly by the hydrolysis of phosphoinositides and subsequent rearrangement of plasma-membrane phospholipids (Keryer & Rossignol, 1978; Michell, 1975) is not consistent with the observed dose-response curves.

Fig. 7. Effects of EGTA (a) and ionophore A23187 (b) on the amount of $^{45}\text{Ca}^{2+}$ associated with hepatocytes incubated in the presence or absence of La^{3+} .

Hepatocytes were incubated in the presence of $0.1 \text{ mM-}^{45}\text{Ca}^{2+}_o$ for 15 min. Saline (○) or LaCl_3 (●) was then added ($t = 0 \text{ min}$), and samples were removed at the indicated times after the addition of La^{3+} for measurement of the amount of $^{45}\text{Ca}^{2+}$ associated with the cells as described in the Experimental section. The concentrations of La^{3+} were $200 \mu\text{M}$ (a) and $60 \mu\text{M}$ (b). At the times indicated (arrow), 2.0 mM-EGTA (a) or $1 \mu\text{M-A23187}$ (b) was added both to untreated cells and to cells treated with La^{3+} . The values are means $\pm$ S.E.M. for two determinations made on each of two separate cell preparations (a) or means of values obtained from two cell preparations (b).

The results obtained at $0.1 \text{ mM-}^{45}\text{Ca}^{2+}_o$ under steady-state conditions and those with Ca^{2+} -deprived cells indicate that, in contrast with vasopressin, La^{3+} increases $^{45}\text{Ca}^{2+}$ uptake under these conditions as well as at $1.3 \text{ mM-}^{45}\text{Ca}^{2+}_o$. A concentration of La^{3+} , which at $1.3 \text{ mM-}^{45}\text{Ca}^{2+}_o$ under steady-state conditions gave the same degree of stimulation of $^{45}\text{Ca}^{2+}$ exchange as vasopressin, caused increases in $^{45}\text{Ca}^{2+}$ uptake of 150% in cells incubated at $0.1 \text{ mM-}^{45}\text{Ca}^{2+}_o$ under steady-state conditions, and of 70% after the addition of $1.3 \text{ mM-}^{45}\text{Ca}^{2+}$ to Ca^{2+} -deprived cells. These results indicate that the failure of vasopressin to stimulate $^{45}\text{Ca}^{2+}$ uptake at low $[\text{Ca}^{2+}]_o$ or in Ca^{2+} -deprived cells is not due to inability to detect a possible increase. The inhibition by vasopressin of $^{45}\text{Ca}^{2+}$ uptake by Ca^{2+} -deprived cells indicates that the hormone does not increase the inflow of Ca^{2+} across the plasma membrane under these conditions (cf. Poggioli *et al.*, 1985). However, other studies with verapamil indicate that under these conditions vasopressin does activate the putative receptor-activated plasma-membrane Ca^{2+} -inflow transporter (Hughes *et al.*, 1986). Therefore it is concluded that the inhibition by vasopressin of $^{45}\text{Ca}^{2+}$ uptake by Ca^{2+} -deprived cells is due to inhibition by the hormone of the uptake of $^{45}\text{Ca}^{2+}$ by organelles and intracellular proteins.

The stimulation by La^{3+} of plasma-membrane Ca^{2+} inflow was not associated with a detectable increase in $[\text{Ca}^{2+}]_i$. This indicates that the processes by which organelles and intracellular proteins accumulate Ca^{2+} are very sensitive to small increases in $[\text{Ca}^{2+}]_i$ and rapidly remove additional Ca^{2+} which enters the cytoplasm as a result of the increase in plasma-membrane Ca^{2+} inflow induced by La^{3+} . Although it is likely that the additional

Ca^{2+} that enters the cell in response to La^{3+} is associated with that part of the cytoplasm occupied by glycogen phosphorylase and quin2, the possibility that the Ca^{2+} is transferred directly from the extracellular medium to organelles, or is preferentially taken up by organelles near the plasma membrane (Taylor & Putney, 1985), cannot be completely excluded.

Ionophore A23187 did not inhibit the ability of La^{3+} to stimulate $^{45}\text{Ca}^{2+}$ uptake by Ca^{2+} -deprived cells, nor did this agent induce the release of all additional $^{45}\text{Ca}^{2+}$ accumulated in the presence of La^{3+} at $0.1 \text{ mM-}^{45}\text{Ca}^{2+}_o$; cf. effects of a combination of α_1 - and β -adrenergic agonists and elevated pH (Morgan *et al.*, 1983; Blackmore *et al.*, 1984). However, it has previously been shown that ionophore A23187 decreases the capacity of intracellular organelles to store Ca^{2+} (Blackmore *et al.*, 1978; Whiting & Barritt, 1982). These observations indicate that other mechanisms dispose of Ca^{2+} that enters the cytoplasm in response to La^{3+} . These may include the binding of Ca^{2+} to proteins and metabolites, the formation of calcium phosphate deposits in organelles (cf. Lehninger, 1970; Borle & Anderson, 1976) insensitive to A23187, and the outflow of Ca^{2+} across the plasma membrane (Morgan *et al.*, 1983).

The observations that vasopressin causes a substantial increase in $[\text{Ca}^{2+}]_i$ after the addition of Ca^{2+} to Ca^{2+} -deprived cells, but inhibits the flow of $^{45}\text{Ca}^{2+}$ to the cell from the extracellular medium, indicate that the increase in $[\text{Ca}^{2+}]_i$ is due to an inhibition by vasopressin of the inflow of Ca^{2+} to organelles, the outflow of Ca^{2+} across the plasma membrane and/or the binding of Ca^{2+} to sites (e.g. proteins and phospholipids) in the cytoplasm. The greater increase in $[\text{Ca}^{2+}]_i$ observed in the

presence of vasopressin cannot be due to the release of Ca^{2+} from hormone-sensitive intracellular stores, since this release occurred in the 5 min period before the addition of Ca^{2+} .

The results obtained with both vasopressin and La^{3+} indicate that (a) an increase in plasma-membrane Ca^{2+} inflow does not, by itself, increase $[\text{Ca}^{2+}]_i$ and (b) the ability of vasopressin to maintain an increase in $[\text{Ca}^{2+}]_i$ over a period of time is dependent on inhibition of the intracellular removal of cytoplasmic Ca^{2+} . This may be particularly important in the long-term actions of vasopressin, such as its effects on cell growth (Russell & Bucher, 1983). Inhibition of the removal of cytoplasmic Ca^{2+} may be one of the functions of inositol trisphosphate or other intracellular messengers generated by vasopressin. Inositol trisphosphate has been shown to inhibit Ca^{2+} uptake by permeabilized hepatocytes (A. M. Auld & G. J. Barritt, unpublished work). The intracellular mechanisms by which vasopressin inhibits Ca^{2+} uptake by mitochondria and other putative intracellular stores require investigation.

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