Biochem. J. (1988) 256, 117–124 (Printed in Great Britain)

Characterization of Ca^{2+} fluxes in rat liver plasma-membrane vesicles

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Inside-out plasma-membrane vesicles isolated from rat liver [Prpic, Green, Blackmore & Exton (1984) J. Biol. Chem. **259**, 1382–1385] accumulated a substantial amount of $^{45}Ca^{2+}$ when they were incubated in a medium whose ionic composition and pH mimicked those of cytosol and which contained MgATP. The $V_{max.}$ of the initial $^{45}Ca^{2+}$ uptake rate was 2.9 ± 0.6 nmol/min per mg and the K_m for Ca^{2+} was 0.50 ± 0.08 μM. The ATP-dependent $^{45}Ca^{2+}$ uptake by inside-out plasma-membrane vesicles was about 20 times more sensitive to saponin than was the ATP-dependent uptake by a microsomal preparation. The $^{45}Ca^{2+}$ efflux from the inside-out vesicles, which is equivalent to the Ca^{2+} influx in intact cells, was increased when the free Ca^{2+} concentration in the medium was decreased. The Ca^{2+} antagonists La^{3+} and Co^{2+} inhibited the $^{45}Ca^{2+}$ efflux from the vesicles. Neomycin stimulated the Ca^{2+} efflux in the presence of either a high or a low free Ca^{2+} concentration. These results confirm that polyvalent cations regulate Ca^{2+} fluxes through the plasma membrane.

INTRODUCTION

An increase in the cytosolic Ca^{2+} concentration is involved in the signal-transduction mechanism in many cell types. Both Ca^{2+} release from an intracellular store and stimulation of Ca^{2+} influx through the plasma membrane contribute to the increase in the cytosolic Ca^{2+} concentration (see Berridge & Irvine, 1984; Putney, 1987; Mauger & Claret, 1988). In non-excitable cells, the mechanism involved in the hormone-stimulated Ca^{2+} influx remains unknown. Recent reports have suggested that receptor-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate may be involved in the regulation of the Ca^{2+} entry through the plasma membrane. Inositol 1,4,5-trisphosphate ($InsP_3$) opens Ca^{2+} channels in T lymphocytes (Kuno & Gardner, 1987), and its phosphorylated derivative inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) activates processes dependent on the presence of extracellular Ca^{2+} in sea-urchin eggs (Irvine & Moor, 1986). Also, the Ca^{2+} released from the intracellular compartment by $InsP_3$ may open non-specific channels permeable to Ca^{2+} in neutrophils (von Tscharner *et al.*, 1986).

In rat hepatocytes, the hormones which increase intracellular IP_3 also stimulate the $^{45}Ca^{2+}$ influx from the extracellular medium (Mauger *et al.*, 1984). Cyclic AMP is involved in this phenomenon, as it potentiates the response induced by the Ca^{2+}-mobilizing hormones (Mauger *et al.*, 1985; Poggioli *et al.*, 1986). We also observed that cytosolic Ca^{2+} inhibits the $^{45}Ca^{2+}$ influx in non-stimulated cells (Poggioli *et al.*, 1985). A better understanding of the mechanism(s) involved in resting and hormone-stimulated Ca^{2+} influx in rat hepatocytes requires study of the effect of intracellular messengers on the cytosolic face of the plasma membrane. Prpic *et al.* (1984) proposed a method to prepare plasma-membrane vesicles from rat liver. These vesicles appear to be essentially in the inside-out configuration and accumulate $^{45}Ca^{2+}$ in the presence of ATP. In addition, they display a hormone-stimulated polyphosphoinositide breakdown (Uhing *et al.*, 1986) and a Na^+/Ca^{2+} exchange (Schanne & Moore, 1986). This vesicle preparation is a good experimental model with which to study the influence of intracellular ions and messengers on the Ca^{2+}-permeability of the plasma membrane. The results of the present paper indicate that inside-out rat liver plasma-membrane vesicles accumulate substantial quantities of $^{45}Ca^{2+}$ in the presence of ATP, when incubated in a medium whose ionic composition mimics cytosol. The unidirectional $^{45}Ca^{2+}$ efflux from preloaded vesicles, equivalent to the Ca^{2+} influx in intact cells, is regulated by different polyvalent cations.

MATERIALS AND METHODS

Membrane isolation

Liver membranes were prepared from female Wistar rats weighing 200–250 g. Plasma membranes were isolated by Percoll-density-gradient centrifugation from a 1500 *g* sedimentation fraction of the homogenate essentially as described by Prpic *et al.* (1984). The resulting fraction was resuspended in a medium containing 250 mM-sucrose, 25 mM-Hepes/KOH, pH 7.4, 1 mM-dithiothreitol and 0.2 mM-phenylmethanesulphonyl fluoride, at about 1–2 mg of protein/ml. The microsomal fraction sedimenting at 35000 *g* was prepared as described by Dawson & Irvine (1984). The resulting fraction was resuspended in the same medium as the plasma-membrane vesicles, at 1–2 mg of protein/ml.

Measurement of $^{45}Ca^{2+}$ transport

$^{45}Ca^{2+}$ uptake was measured by adding 25 μl of the membrane fraction to 225 μl of prewarmed medium

Abbreviations used: $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_4$, inositol 1,3,4,5-tetrakisphosphate.

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containing, unless otherwise indicated: 100 mM-KCl, 20 mM-NaCl, 5 mM-$MgCl_2$, 0.96 mM-NaH_2PO_4, 25 mM-Hepes/KOH, pH 7.1 at 37 °C, 5 mM-NaN_3, 5 mM-phosphocreatine, 5 units of creatine kinase/ml, 0.5–1 μCi of $^{45}Ca^{2+}$/ml, 20 μM- or 100 μM-$CaCl_2$ with 200 μM-EGTA, 1.5 mM-Na_2ATP. In a few experiments, free Ca^{2+} concentrations were determined by the use of quin2, which was added at a final concentration of 1 μM. The Ca^{2+} concentration was calculated by using the dissociation constants of 60 nM for the quin2–Ca^{2+} complex and 1 mM for the quin2–Mg^{2+}complex (Tsien *et al.*, 1982). The reaction was stopped at appropriate times by diluting the mixture with 4 ml of 250 mM-sucrose/40 mM-NaCl, followed by filtration through a Whatman GF/C glass-fibre filter. The filter was washed three times with the washing medium, and counted for radioactivity in a scintillation spectrometer.

$^{45}Ca^{2+}$ efflux was measured from vesicles which had been loaded for 20 min in the presence of ATP as described above. A mixture of hexokinase and glucose was added to give final concentrations of 50 units/ml and 10 mM respectively. This resulted in a rapid degradation of ATP and stopping of $^{45}Ca^{2+}$ uptake. Samples were washed as described above for $^{45}Ca^{2+}$ uptake.

Measurement of $^{22}Na^+$ uptake

$^{22}Na^+$ uptake was measured under the same conditions as for $^{45}Ca^{2+}$, except that the incubation medium contained 60 mM-KCl, 60 mM-NaCl, 1–2 μCi of $^{22}Na^+$/ml, 0.2 mM-EGTA and no added $CaCl_2$.

Materials

$^{22}Na^+$ was from C.E.A. (Saclay, France); $^{45}Ca^{2+}$ and InsP_3 were from Amersham; phosphocreatine, creatine kinase, ATP (disodium salt), hexokinase (type V), saponin and neomycin were from Sigma; InsP_4 was from Calbiochem; GTP and guanosine 5′-[γ-thio]triphosphate were from Boehringer Mannheim.

RESULTS

Influence of the incubation-medium composition on the $^{45}Ca^{2+}$ accumulation into plasma-membrane vesicles

Previously published results demonstrating a Ca^{2+} accumulation into liver plasma-membrane vesicles had been performed in different incubation media. Chan & Junger (1983) or Prpic *et al.* (1984) used a sucrose medium buffered at pH 8, others used saline media essentially constituted of KCl and buffered from pH 6.8 to 8 (Kraus-Friedmann *et al.*, 1982; Epping & Bygrave, 1984; Schanne & Moore, 1986). We first studied the influence of the incubation medium on the ATP-dependent Ca^{2+} accumulation into rat liver plasma-membrane vesicles. Three different media were tested: two had an ionic composition resembling cytosol (see Burgess *et al.*, 1983) as described in the Materials and methods section, and were buffered at pH 7.1 or 8.0; the third iso-osmotic medium contained essentially 180 mM-sucrose and 50 mM-Tris/HCl, pH 8.0, as described by Prpic *et al.* (1984). Each medium also contained 20 μM-$^{45}Ca^{2+}$, 5 mM-$MgCl_2$ and 1.5 mM-ATP, which are necessary to obtain maximal $^{45}Ca^{2+}$ uptake (see below). Fig. 1 shows the kinetics of the ATP-dependent $^{45}Ca^{2+}$ accumulation into the vesicles incubated in the three different media. The ATP-independent $^{45}Ca^{2+}$ uptakes were maximal at 30 s, then remained constant for the next

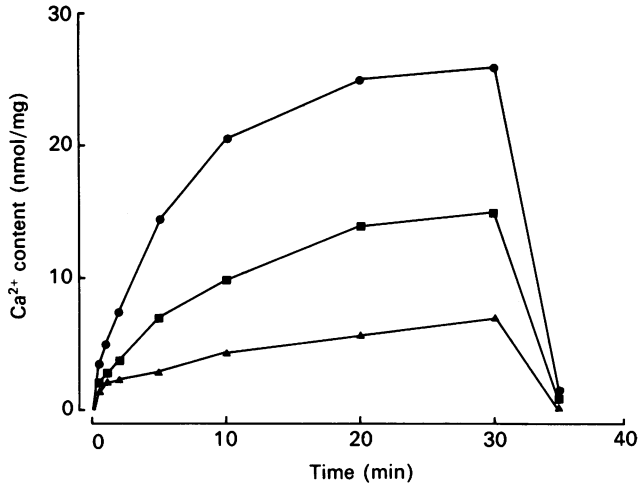

Fig. 1. Kinetics of $^{45}Ca^{2+}$ accumulation into plasma-membrane vesicles incubated in different media

The plasma-membrane vesicles were prepared as described in the Materials and methods section. They were incubated for the periods indicated at 37 °C in the sucrose medium buffered at pH 8.0 (▲) and in KCl media buffered at pH 8.0 (■) or at pH 7.1 (●) as described in the Materials and methods section. Each medium also contained 20 μM-$^{45}Ca^{2+}$ and 1.5 mM-ATP. Time 0 refers to the addition of the membrane vesicles to the prewarmed incubation media. Ca^{2+} ionophore A23187 was added to 10 μM after 30 min incubation. The values of $^{45}Ca^{2+}$ uptake and binding measured in the absence of ATP were subtracted from values measured in its presence. Each point is the mean of triplicate determinations in one typical experiment. Mean values for initial $^{45}Ca^{2+}$-uptake rate and $^{45}Ca^{2+}$ accumulation are given in the text.

30 min. They amounted to 1.2, 1.9 and 1.1 nmol/mg of protein in the sucrose medium, the KCl medium buffered at pH 8 and the KCl medium buffered at pH 7.1 respectively. The ATP-dependent $^{45}Ca^{2+}$ accumulations after 30 min incubation in the sucrose medium and in the KCl medium buffered at pH 8.0 and pH 7.1 were 7.7 ± 0.4, 13.4 ± 1.0 and 22.4 ± 2.2 nmol/mg of protein ($n = 5$) respectively. The initial $^{45}Ca^{2+}$-uptake rates measured during the first 2 min were 0.5 ± 0.1, 1.0 ± 0.1 and 2.4 ± 0.2 nmol/min per mg of protein ($n = 5$) for the sucrose medium and the KCl media buffered at pH 8.0 and pH 7.1 respectively. Addition of 4 mM-oxalate in the KCl medium buffered at pH 7.1 increased by 25% the $^{45}Ca^{2+}$ accumulation after 30 min incubation. Finally, Fig. 1 shows that the addition of 10 μM of the Ca^{2+} ionophore A23187 in the different incubation media completely discharged the accumulated $^{45}Ca^{2+}$. This indicates that ATP induced the accumulation of $^{45}Ca^{2+}$ into a closed compartment. The next experiments were performed in the internal KCl medium (pH 7.1). This medium allowed the Ca^{2+}–Mg^{2+}-ATPase to pump larger amounts of Ca^{2+} under more physiological conditions.

Identification of the membrane involved in Ca^{2+} accumulation

As the plasma-membrane fraction prepared as described by Prpic *et al.* (1984) contains some activity of the endoplasmic-reticulum-associated enzyme glucose-6-phosphatase, it was possible that a non-negligible part of

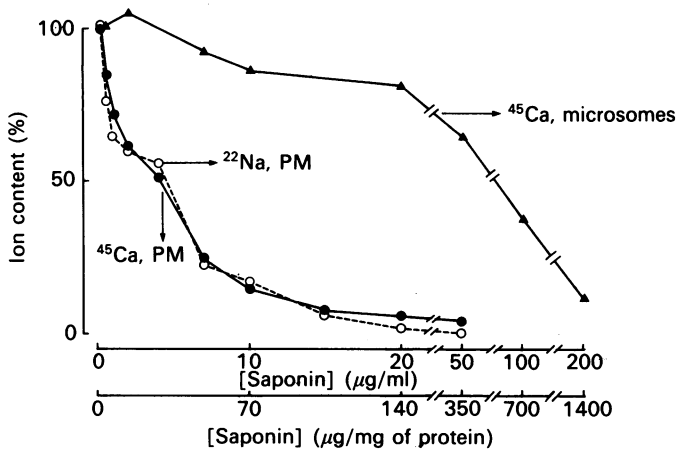

Fig. 2. Effect of increasing concentrations of saponin on $^{45}Ca^{2+}$ and $^{22}Na^{+}$ uptake by plasma-membrane vesicles or microsomes

The plasma-membrane (PM) vesicles and the microsomes were prepared as described in the Materials and methods section. They were preincubated for 5 min in the incubation media containing 20 μM-$^{45}Ca^{2+}$ or 60 mM-$^{22}Na^{+}$ and increasing concentrations of saponin. $^{45}Ca^{2+}$ or $^{22}Na^{+}$ uptake was initiated by the addition of 1.5 mM-ATP and stopped 10 min later. The protein concentration was adjusted in each case to 0.14 mg/ml. Data are the means of triplicate determinations in three different experiments. ATP-dependent $^{45}Ca^{2+}$ uptake in the absence of saponin was 19 ± 2 nmol/mg in the plasma-membrane vesicles (●) and 8.2 ± 0.5 nmol/mg in the microsomes (▲). ATP-dependent $^{22}Na^{+}$ uptake by the plasma-membrane vesicles was 48 ± 1 nmol/mg (○).

the $^{45}Ca^{2+}$ sequestered resulted from Ca^{2+} uptake by contaminating endoplasmic-reticulum vesicles (Moore *et al.*, 1975). This possibility was examined by testing the sensitivity of $^{45}Ca^{2+}$ uptake into the plasma-membrane vesicles to increasing concentrations of saponin. This steroid glycoside is known to permeabilize plasma membrane specifically by forming insoluble complexes with cholesterol. When it is used at low concentration, saponin does not affect the intracellular organelles (see Fiskum, 1985). Preincubation of membrane vesicles in the presence of saponin inhibited the $^{45}Ca^{2+}$ uptake induced by the addition of ATP. Fig. 2 shows that the ATP-dependent $^{45}Ca^{2+}$ uptake was inhibited by 50% when the vesicles were preincubated in the presence of 4 μg of saponin/ml and by 95% in the presence of 20 μg of saponin/ml. In comparison, microsomes (microsomal fractions) enriched with endoplasmic reticulum accumulated 8 nmol of $^{45}Ca^{2+}$/mg of protein when incubated in the presence of ATP in the same medium and at the same protein concentration as plasma-membrane vesicles. The ATP-dependent $^{45}Ca^{2+}$ uptake by microsomes was much less sensitive to saponin than was that by plasma-membrane vesicles. Preincubating microsomes with 20 μg of saponin/ml inhibited $^{45}Ca^{2+}$ uptake by only 20%, and 200 μg of saponin/ml was required to inhibit $^{45}Ca^{2+}$ uptake by 95% (see Fig. 2). This confirms that the intracellular membranes are less sensitive to saponin than is the plasma membrane. In order to characterize the saponin-sensitive fraction further, we measured the ATP-dependent $^{22}Na^{+}$ uptake into the plasma-membrane vesicles. It is well known that the Na^{+}/K^{+}-ATPase which actively transports Na^{+} and K^{+} is an enzyme present only in the cell plasma membrane (Evans, 1980). Plasma-membrane vesicles were incubated in the same medium as that used for the measurement of $^{45}Ca^{2+}$ uptake, except that it contained 60 mM-$^{22}Na^{+}$, 0.2 mM-EGTA and no added Ca^{2+}. Under these conditions, the addition of ATP induced the accumulation of 48 nmol of $^{22}Na^{+}$/mg of protein. Preincubation of the vesicles in the presence of saponin inhibited the $^{22}Na^{+}$ uptake, and Fig. 2 indicates that this occurred in the same range of concentrations as those inhibiting $^{45}Ca^{2+}$ uptake. Since the ATP-dependent $^{22}Na^{+}$ uptake was measured in the presence of EGTA, it was not secondary to a Ca^{2+} uptake by means of a Na^{+}/Ca^{2+} exchange. The most likely hypothesis is that the $^{22}Na^{+}$ uptake occurred through the Na^{+}/K^{+}-ATPase activity. The observation that $^{45}Ca^{2+}$ and $^{22}Na^{+}$ uptakes displayed identical sensitivities to saponin strongly suggests that the Ca^{2+} pump which allows the $^{45}Ca^{2+}$ uptake is present in the same membrane as the Na^{+}/K^{+}-ATPase, that is the plasma membrane. These results indicate that the contaminant endoplasmic reticulum present in the preparation accumulated negligible quantities of $^{45}Ca^{2+}$ when incubated in the internal medium.

Properties of the plasma-membrane ATP-dependent Ca^{2+} pump

The initial $^{45}Ca^{2+}$ uptake rate by inside-out plasma-membrane vesicles was measured after 2 min incubation in the internal medium buffered at pH 7.1. The effects of increasing concentrations of ATP, Mg^{2+} or Ca^{2+} were studied. The dose–response curve for ATP was obtained in a medium containing 5 mM-$MgCl_2$ and 20 μM-$^{45}Ca^{2+}$. Half-maximal and maximal responses occurred in the presence of 150 μM- and 1.5 mM-ATP respectively. The initial $^{45}Ca^{2+}$ uptake was measured in a medium containing 20 μM-$^{45}Ca^{2+}$ and 0.5 mM-, 1.0 mM-, 1.5 mM- or 2.0 mM-ATP in the presence of increasing concentration of $MgCl_2$. Free Mg^{2+} concentrations were calculated by using an apparent K_D for the ATPMg complex of 61 μM (Burgess *et al.*, 1983). In each condition tested, the $^{45}Ca^{2+}$ uptake increased with increasing concentration of Mg^{2+}. Half-maximal effect occurred in the presence of 3.4 ± 0.4 mM ($n = 4$) free Mg^{2+}. The initial $^{45}Ca^{2+}$-uptake rate was also measured as a function of the free Ca^{2+} concentration (Fig. 3). The incubation medium contained 1.5 mM-ATP, 5 mM-$MgCl_2$ and 0.2 mM-EGTA. $CaCl_2$ was added in order to obtain the indicated free Ca^{2+} concentrations, which were measured in parallel experiments by means of quin2. The data shown in Fig. 3 are consistent with Michaelis–Menten-type kinetics, with K_m value of 0.50 ± 0.08 μM ($n = 3$) and a $V_{max.}$ value of 2.9 ± 0.6 nmol/min per mg of protein ($n = 3$).

The $^{45}Ca^{2+}$ uptake determined in the presence of 1.5 mM-ATP, 5 mM-$MgCl_2$ and 20 μM-$^{45}Ca^{2+}$ was inhibited by the addition of increasing concentrations of vanadate. Half-maximal inhibition occurred in the presence of 0.6 μM-vanadate, and 0.1 mM inhibited the uptake by more than 95%.

Measurement of the $^{45}Ca^{2+}$ efflux from inside-out plasma-membrane vesicles

The $^{45}Ca^{2+}$ efflux was measured by adding a mixture of 50 units of hexokinase/ml and 10 mM-glucose to vesicles preincubated for 20 min in the KCl medium containing

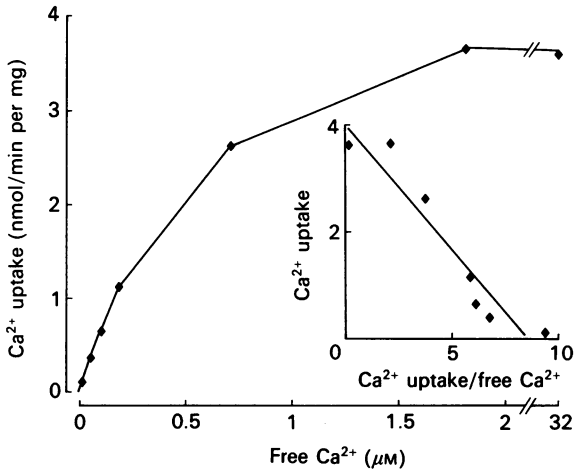

Fig. 3. Dose–response curve for the initial $^{45}Ca^{2+}$-uptake rate by plasma-membrane vesicles as a function of Ca^{2+} concentration

Plasma-membrane vesicles were preincubated for 5 min in the incubation media described in the Materials and methods section and containing 0.2 mM-EGTA and increasing concentrations of $CaCl_2$. The indicated free Ca^{2+} concentrations were determined in parallel experiments in the presence of 1 μM-quin2. $^{45}Ca^{2+}$ uptake was initiated by addition of 1.5 mM-ATP and was stopped 2 min later. Each point is the mean of triplicate determinations of the ATP-dependent uptake in one typical experiment. The inset shows the Hofstee plot of the data. Mean values for K_m and $V_{max.}$ are given in the text.

ATP and 20 μM-$^{45}Ca^{2+}$. This treatment inhibits the Ca^{2+} pumping into the vesicles by more than 98% within the first 30 s, as indicated by the inhibition of the initial $^{45}Ca^{2+}$-uptake rate when ATP was added in a medium already containing hexokinase and glucose (results not shown). Fig. 4(*a*) shows that the control efflux of $^{45}Ca^{2+}$ after addition of hexokinase and glucose had a $t_{\frac{1}{2}}$ of 8.6±0.6 min ($n = 4$). Fig. 4(*b*) shows that the $^{45}Ca^{2+}$-efflux rate increased linearly with the $^{45}Ca^{2+}$ content of the vesicles, which is consistent with a monoexponential loss of $^{45}Ca^{2+}$ from the vesicle.

As we have demonstrated that the cytosolic Ca^{2+} concentration regulates the Ca^{2+} influx through the plasma membrane in intact hepatocytes (Poggioli *et al.*, 1985), we investigated the influence of [Ca^{2+}] in the medium on the efflux from inside-out plasma-membrane vesicles. Fig. 4(*a*) indicated that the simultaneous addition of the mixture of hexokinase and glucose with 0.2 mM-EGTA, to decrease the free Ca^{2+} concentration to about 20 nM, enhanced the efflux rate of $^{45}Ca^{2+}$ from the vesicles. Decrease Ca^{2+} decreasing the $t_{\frac{1}{2}}$ to 5.5±0.5 min ($n = 4$). Fig. 4(*b*) shows that in the low-Ca^{2+} medium the $^{45}Ca^{2+}$-efflux rate was increased by 43%. The addition of 0.5 mM-EGTA, which decreased the Ca^{2+} concentration to about 10 nM, did not further increase the $^{45}Ca^{2+}$ efflux. Preincubating vesicles in the presence of ATP and 100 μM-$^{45}Ca^{2+}$ did not induce a larger accumulation of $^{45}Ca^{2+}$, and the efflux rate measured after the addition of hexokinase and glucose was similar to that measured in a medium containing 20 μM-Ca^{2+}.

In order to characterize a Ca^{2+}-binding site involved in

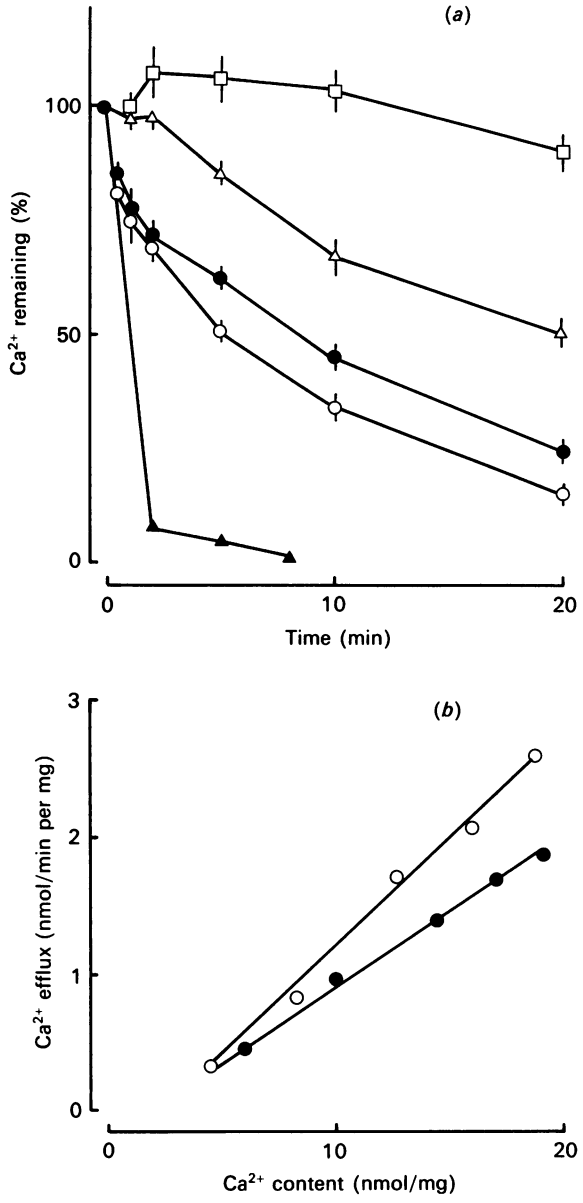

Fig. 4. Measurement of the $^{45}Ca^{2+}$ efflux from plasma-membrane vesicles

$^{45}Ca^{2+}$ effluxes were started by addition of hexokinase + glucose to vesicles preloaded for 20 min in the presence of ATP and 20 μM-$^{45}Ca^{2+}$ as described in the Materials and methods section. (*a*) Hexokinase and glucose were added at zero time, either alone (●) or with 30 μM-La^{3+} (△) or 1 mM-Co^{2+} (□) or 0.2 mM-EGTA (○) or 10 μM-A23187 (▲). The $^{45}Ca^{2+}$ contents of the vesicles were measured at the indicated times. Each point is the mean±S.E.M. for triplicate determinations in two to four different experiments and is expressed as a percentage of the ATP-dependent $^{45}Ca^{2+}$ accumulation at zero time, which refers to the value measured just before the addition of hexokinase and glucose, and was equal to 18±2 nmol/mg of protein. (*b*) $^{45}Ca^{2+}$-efflux rates were expressed as a function of the mean $^{45}Ca^{2+}$ content of the vesicles. Values were calculated from efflux experiments similar to those described in (*a*) after addition of hexokinase and glucose either alone (●) or with 0.2 mM-EGTA (○). Each point is the mean of triplicate determinations in one typical experiment.

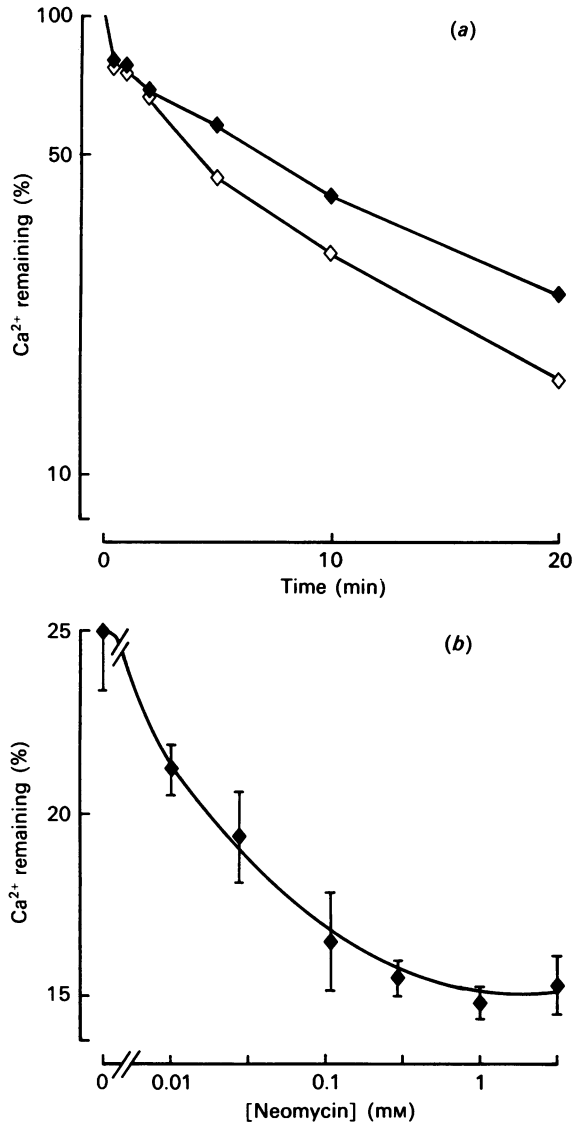

Fig. 5. Effect of neomycin on the $^{45}Ca^{2+}$ efflux from plasma-membrane vesicles

$^{45}Ca^{2+}$ effluxes were measured as described in the Materials and methods section from vesicles which had been loaded for 20 min in the presence of 20 μM-$^{45}Ca^{2+}$. (*a*) Hexokinase and glucose were added at zero time with (◇) or without (◆) 1 mM-neomycin. The $^{45}Ca^{2+}$ contents of the vesicles were measured at the indicated times. Each point is the mean of triplicate determinations in two different experiments; $^{45}Ca^{2+}$ remaining is expressed as percentage of the ATP-dependent $^{45}Ca^{2+}$ accumulation at zero time, which was 21 ± 3 nmol/mg. (*b*) Hexokinase and glucose were added at zero time with the indicated concentrations of neomycin. $^{45}Ca^{2+}$ remaining after 20 min is expressed as percentage of ATP-dependent $^{45}Ca^{2+}$ accumulation at zero time. Each point is the mean $\pm$ S.E.M. for triplicate determinations in three different experiments.

the regulation of Ca^{2+} fluxes, we investigated the effect of other polyvalent cations added simultaneously with hexokinase and glucose. Fig. 4(*a*) shows that 1 mM-Co^{2+} almost totally inhibited the $^{45}Ca^{2+}$ efflux. This effect was obvious as soon as 1 min after the addition of the cation, and could be detected in the presence of 0.1 mM-Co^{2+} (results not shown). The addition of 30 μM-La^{3+} partly inhibited the efflux (Fig. 4*a*). Addition of higher concentrations of La^{3+} increased the $^{45}Ca^{2+}$ binding to vesicles in the absence of ATP. This prevents the accurate estimation of the Ca^{2+} efflux. These effects of Co^{2+} and La^{3+} could not be due to ATP removal, and consequently to incomplete inhibition of the Ca^{2+} pumping, since (1) 1 mM-Co^{2+} totally inhibited the ATP-dependent $^{45}Ca^{2+}$ uptake, and (2) 30 μM-La^{3+} partly inhibited the initial $^{45}Ca^{2+}$ uptake, but led to a larger $^{45}Ca^{2+}$ accumulation at the plateau, suggesting an inhibition of the Ca^{2+} efflux. Finally, the addition of 1 mM-Mn^{2+} or Ca^{2+} at concentrations higher than 20 μM did not affect the $^{45}Ca^{2+}$ efflux (results not shown).

The effects of neomycin on the $^{45}Ca^{2+}$ efflux from plasma-membrane vesicles were also investigated, as it has been previously shown that this polycationic antibiotic affects Ca^{2+} fluxes in intact cells (Goodman *et al.*, 1974). Fig. 5(*a*) shows that addition of 1 mM-neomycin stimulated $^{45}Ca^{2+}$ efflux from vesicles which had been preincubated with ATP in the presence of 20 μM-$^{45}Ca^{2+}$. This effect appeared between 2 and 5 min after the addition of the antibiotic. The addition of EGTA at the same time as neomycin did not impair the action of the antibiotic, suggesting that it does not act simply by displacing Ca^{2+} from its binding site. The dose–response curve for the stimulatory effect of neomycin on the $^{45}Ca^{2+}$ efflux is shown in Fig. 5(*b*). The neomycin concentration which gave half-maximal effect, determined from three separate experiments, was 30 ± 10 μM.

Effects of intracellular messengers on $^{45}Ca^{2+}$ release from the vesicles

$InsP_3$ and its phosphorylated derivative $InsP_4$ are potential candidates for coupling activated membrane receptors to stimulated Ca^{2+} influx (Irvine & Moor, 1986; Kuno & Gardner, 1987). The effects of these inositol phosphates were investigated in plasma-membrane vesicles and microsomes. In the experiment shown in Fig. 6, microsomes or plasma-membrane vesicles were preincubated for 20 min in an incubation medium containing 0.1 mM-$^{45}Ca^{2+}$ and 0.2 mM-EGTA, which fixed the free Ca^{2+} to 0.2 μM, and supplemented with 5% poly(ethylene glycol) and 1.5 mM-ATP. Addition of 10 μM-GTP induced the release of 34% of the $^{45}Ca^{2+}$ accumulated into the microsomes within 5 min, and further addition of 3 μM-$InsP_3$ induced an additional release of 22% of the $^{45}Ca^{2+}$within 30 s. Fig. 6 also shows that the addition of GTP and $InsP_3$ did not affect the $^{45}Ca^{2+}$ content of the plasma-membrane vesicles. Addition of 1 μM-$InsP_4$ 5 min after the addition of GTP either alone or simultaneously with $InsP_3$ did not induce $^{45}Ca^{2+}$ release from plasma-membrane vesicles (results not shown). In isolated rat liver plasma membranes, GTP or its non-hydrolysable γ-thio and $\beta\gamma$-imido analogues stimulate hydrolysis of phosphatidylinositol 4,5-bisphosphate (Uhing *et al.*, 1986; Cockcroft & Taylor, 1987). Addition of 10 μM-guanosine 5′-[γ-thio]triphosphate to vesicles loaded in the presence of either 0.2 μM or 20 μM free $^{45}Ca^{2+}$ did not affect either the $^{45}Ca^{2+}$ accumulation or the $^{45}Ca^{2+}$ efflux.

DISCUSSION

The Ca^{2+}–Mg^{2+}-ATPase present in the plasma membrane of hepatocytes, as well as in other cells, plays a key

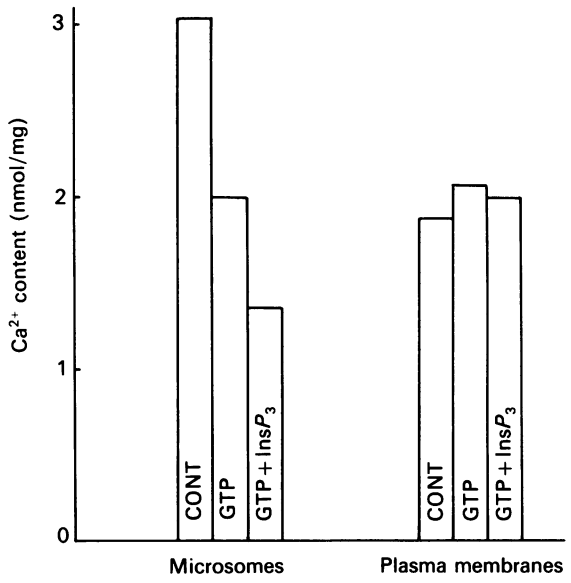

Fig. 6. Effects of GTP and InsP_3 on the $^{45}Ca^{2+}$ content of microsomes and plasma-membrane vesicles

Microsomes and plasma-membrane vesicles were prepared as described in the Materials and methods section. The fractions were preincubated for 20 min in the KCl medium containing 0.1 mM-$^{45}Ca^{2+}$ and 0.2 mM-EGTA and supplemented with 5% poly(ethylene glycol). GTP (10 μM) and InsP_3 (3 μM) were added 5 min and 30 s respectively before the measurement of the $^{45}Ca^{2+}$ content of the microsomes and the plasma-membrane vesicles (protein concn. 0.25 mg/ml). Data are duplicate determinations in one typical experiment representative of three; CONT, control.

role in the active extrusion of Ca^{2+} from the cytosol, thus allowing the maintenance of a large gradient between the two faces of the plasma membrane (Lotersztajn *et al.*, 1981; Penniston, 1983). This Ca^{2+} pump accounts for the accumulation of $^{45}Ca^{2+}$ into inside-out rat liver plasma-membrane vesicles prepared by the method described by Prpic *et al.* (1984). When incubated in a sucrose medium buffered at pH 8, the vesicles behaved essentially as described by Prpic *et al.* (1984), in that they accumulated similar amounts of $^{45}Ca^{2+}$ and that $^{45}Ca^{2+}$ uptake was dependent on ATP and free Mg^{2+}. In the present work, we have shown that incubating the vesicles in a medium resembling cytosol did not impair, but rather improved, the ATP-dependent $^{45}Ca^{2+}$ accumulation. The Ca^{2+} ionophone A23187 released the $^{45}Ca^{2+}$ accumulated in the presence of ATP, but does not affect the $^{45}Ca^{2+}$ uptake independent of ATP. This indicates that only the vesicles having an inside-out orientation accumulated $^{45}Ca^{2+}$. The form in which Ca^{2+} may be stored inside the vesicles, and particularly the ratio of bound to free Ca^{2+}, could not be deduced from the experiments described in the present work. However, the very fast $^{45}Ca^{2+}$ release initiated by the Ca^{2+} ionophore suggests that putatively bound Ca^{2+} rapidly dissociates from its binding sites.

We have shown that the $^{45}Ca^{2+}$ uptake displayed the same sensitivity to saponin as the ATP-dependent $^{22}Na^+$ uptake which occurred through the plasma-membrane marker Na^+/K^+-ATPase. The concentration of saponin necessary to inhibit most of the $^{45}Ca^{2+}$ accumulation into the vesicles was 50–100 μg/mg of protein (Fig. 2). This is in the same range as that necessary to permeabilize hepatocytes, namely 20 μg of saponin/mg of total cell protein, i.e. about 40 μg of saponin/mg of membrane protein (M. Hilly, M. Claret & J.-P. Mauger, unpublished work). Taken together, these observations strongly support the view that, under the present experimental conditions, most of the $^{45}Ca^{2+}$ was accumulated into plasma-membrane vesicles. This was confirmed by the lower sensitivity of $^{45}Ca^{2+}$ uptake to saponin found in the fraction enriched in endoplasmic-reticulum membranes (see Fig. 2).

Measurement of the initial $^{45}Ca^{2+}$-uptake rate mediated by the Ca^{2+} pump as a function of the free Ca^{2+} concentration indicated that the process follows simple Michaelis–Menten type kinetics: the apparent K_m is 0.5 μM. The free Ca^{2+} concentrations were measured by use of quin2, which is also used to determine the cytosolic free Ca^{2+} concentration in intact cells. This allows a direct comparison between Ca^{2+}-sensitivity of the Ca^{2+} pump and the cytosolic Ca^{2+} concentration. In intact cells, the resting Ca^{2+} concentration is about 0.1–0.2 μM (Charest *et al.*, 1983; Berthon *et al.*, 1984); this means that the pump works at about 20–30% of its maximal capacity. It can be activated when cytosolic Ca^{2+} concentration increases up to 5 μM, e.g. during stimulation by a Ca^{2+}-mobilizing hormone. This in turn stimulates the Ca^{2+} efflux from the hepatocytes (Combettes *et al.*, 1986).

The Ca^{2+} pump which extrudes Ca^{2+} from intact cells allows the accumulation of $^{45}Ca^{2+}$ into inside-out plasma-membrane vesicles. Consequently, the Ca^{2+} efflux from these vesicles is equivalent to the Ca^{2+} influx in intact cells, and the influence of intracellular components on the Ca^{2+} flux through the plasma membrane may be analysed. The $^{45}Ca^{2+}$ efflux was measured after addition of a mixture of hexokinase and glucose, which rapidly hydrolyses ATP and stops $^{45}Ca^{2+}$ pumping within 30 s. The observation that the ionophore A23187 considerably accelerated the $^{45}Ca^{2+}$ efflux (Fig. 4*a*) indicates that the diffusion of $^{45}Ca^{2+}$ through the membrane is the limiting step of the efflux process. Therefore the efflux rate of $^{45}Ca^{2+}$ is dependent on the properties of the plasma membrane, and not on the form (bound or free) of intravesicular Ca^{2+}. The other factor that would affect the $^{45}Ca^{2+}$ efflux from the vesicles is the concentration of free Ca^{2+} inside the vesicles. We have previously demonstrated that the Ca^{2+} influx into the hepatocytes is a saturable process when measured as a function of the extracellular Ca^{2+} concentration (Mauger *et al.*, 1984). However, comparison of the Ca^{2+} influx into the cells with the Ca^{2+} efflux from the vesicles is difficult, because we do not know the free Ca^{2+} concentration inside the vesicles. The observation that the $^{45}Ca^{2+}$ efflux is linearly related to the $^{45}Ca^{2+}$ content of the vesicles (Fig. 4*b*) suggests that the free Ca^{2+} concentration inside the vesicles remains low as compared with the K_m for extracellular Ca^{2+} when the influx is measured in the cells. This K_m is in the range of 0.1–0.4 mM in non-stimulated cells (Mauger *et al.*, 1984, 1985).

The $^{45}Ca^{2+}$ efflux from the vesicles is affected by different types of polycations. Ca^{2+} in the range 20 nM–20 μM partly inhibits the efflux (Fig. 4). We previously observed that, in intact cells, the cytosolic Ca^{2+} can regulate the Ca^{2+} influx (Poggioli *et al.*, 1985). The decrease in cytosolic Ca^{2+} from the resting value of about 0.2 μM to 50 nM stimulates the $^{45}Ca^{2+}$ influx. This effect

was reversed within 3 min when the cytosolic Ca^{2+} concentration was restored to 0.2–0.3 μM. These observations indicate that the plasma-membrane vesicles have retained their sensitivity to the intracellular Ca^{2+} during the isolation procedure.

La^{3+} and Co^{2+} inhibit $^{45}Ca^{2+}$ efflux from the vesicles. This effect is effective within the first 1 min and occurs in a medium containing 20 μM-Ca^{2+}. As concentrations of Ca^{2+} higher than 20 μM do not affect the $^{45}Ca^{2+}$ efflux, this suggests that La^{3+} and Co^{2+} inhibit the efflux in a different way from Ca^{2+}. In this context, it is noteworthy that the inhibitory effects of La^{3+} and Co^{2+} were also observed with vesicles incubated in the absence of Na^+ (results not shown). This excludes the involvement of a Na^+/Ca^{2+} exchange previously described in rat liver plasma-membrane vesicles and carrying part of the Ca^{2+} efflux from the vesicles (Schanne & Moore, 1986).

The aminoglycoside antibiotic neomycin stimulated Ca^{2+} efflux from inside-out vesicles. It is noteworthy that neomycin (Altin & Bygrave, 1987) and La^{3+} (Parker & Barritt, 1981) have opposite effects when they are applied to the extracellular or cytosolic face of the plasma membrane. However, the concentrations of neomycin which inhibited the Ca^{2+} influx in the liver and of La^{3+} which activated the influx in hepatocytes are higher than the concentrations used in our present work. Furthermore, as it is unlikely that neomycin and La^{3+} enter hepatocytes, it is difficult to compare the effects of these cations when they were added outside the cells or near the cytoplasmic face of the plasma membrane. The site of action of neomycin cannot be established from the present results. The observation that neomycin stimulated $^{45}Ca^{2+}$ efflux even in the presence of EGTA indicates that it does not act as a simple Ca^{2+} antagonist. The concentration of neomycin giving half-maximal stimulating effect on the Ca^{2+} efflux was 30 μM. This is in the same range as for the inhibitory effects of neomycin on phosphoinositide degradation (Downes & Michell, 1981) or synthesis (Marche *et al.*, 1983), or on the phospholipase C activity (Lipsky & Lietman, 1982) and the [^{3}H]gentamycin binding in renal membranes (Sastrasinh *et al.*, 1982). Whipps *et al.* (1987) have reported that, in isolated rat liver plasma membranes, Ca^{2+} and neomycin have an opposite action on the turnover of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate. An attractive hypothesis, which requires further investigation, would be that Ca^{2+} and neomycin affect the Ca^{2+} efflux from the plasma-membrane vesicles by interacting with the membrane polyphosphoinositides.

It has been suggested that InsP_3 and its phosphorylated derivative InsP_4 might be the intracellular messengers for the stimulation of Ca^{2+} influx (Kuno & Gardner, 1987; Irvine & Moor, 1986). The addition of InsP_3 and/or InsP_4 under conditions which have been shown to maximize the InsP_3-induced Ca^{2+} release from liver microsomes (Dawson *et al.*, 1986) does not affect Ca^{2+} fluxes through plasma-membrane vesicles (see Fig. 6). A paper by Guillemette *et al.* (1988) indicates that InsP_3 and GTP released Ca^{2+} from a fraction enriched with plasma membrane. However, these authors suggest that InsP_3 acts on a storage site that is co-purified with the plasma membrane. Our present results indicate that (1) the plasma-membrane vesicles preparation does not contain InsP_3-sensitive vesicles which could have accumulated $^{45}Ca^{2+}$ in the presence of ATP and (2) the plasma membrane is insensitive to the direct actions of InsP_3 and/or InsP_4. However, we cannot exclude that inositol phosphates stimulate the Ca^{2+} influx in hepatocytes by using some intermediates which would have been lost during the isolation of the membranes.

We thank J. Tansini for her technical assistance. This work was supported by grants MRT no. 85 T 0861 and FRM.

REFERENCES

Altin, J. G. & Bygrave, F. L. (1987) Biochem. J. **242**, 43–50
Berridge, M. J. & Irvine, R. F. (1984) Nature (London) **312**, 315–321
Berthon, B., Binet, A., Mauger, J.-P. & Claret, M. (1984) FEBS Lett. **167**, 19–24
Burgess, G. M., McKinney, J. S., Fabiato, A., Leslie, B. A. & Putney, J. W. (1983) J. Biol. Chem. **258**, 15336–15345
Chan, K. M. & Junger, K. D. (1983) J. Biol. Chem. **258**, 4404–4410
Charest, R., Blackmore, P. F., Berthon, B. & Exton, J. (1983) J. Biol. Chem. **258**, 8769–8773
Cockcroft, S. & Taylor, J. A. (1987) Biochem. J. **241**, 409–414
Combettes, L., Berthon, B., Binet, A. & Claret, M. (1986) Biochem. J. **237**, 675–683
Dawson, A. P. & Irvine, R. F. (1984) Biochem. Biophys. Res. Commun. **120**, 858–864
Dawson, A. P., Comerford, J. G. & Fulton, D. V. (1986) Biochem. J. **234**, 311–315
Downes, C. P. & Michell, R. H. (1981) Biochem. J. **198**, 133–140
Epping, R. J. & Bygrave, F. L. (1984) Biochem. J. **223**, 733–745
Evans, W. H. (1980) Biochim. Biophys. Acta **604**, 27–64
Fiskum, G. (1985) Cell Calcium **6**, 25–37
Goodman, F. R., Weiss, G. B. & Adams, H. R. (1974) J. Pharmacol. Exp. Ther. **188**, 472–480
Guillemette, G., Balla, T., Baukal, A. J. & Catt, K. J. (1988) J. Biol. Chem. **263**, 4541–4548
Irvine, R. F. & Moor, R. M. (1986) Biochem. J. **240**, 917–920
Kraus-Friedmann, N., Biber, J., Murer, H. & Carafoli, E. (1982) Eur. J. Biochem. **129**, 7–12
Kuno, M. & Gardner, P. (1987) Nature (London) **326**, 301–304
Lipsky, J. J. & Lietman, P. S. (1982) J. Pharmacol. Exp. Ther. **220**, 287–292
Lotersztajn, S., Hanoune, J. & Pecker, F. (1981) J. Biol. Chem. **256**, 11209–11215
Marche, P., Koutouzov, S. & Girard, A. (1983) J. Pharmacol. Exp. Ther. **227**, 415–420
Mauger, J.-P. & Claret, M. (1988) J. Hepatol., in the press
Mauger, J.-P., Poggioli, J., Guesdon, F. & Claret, M. (1984) Biochem. J. **221**, 121–127
Mauger, J.-P., Poggioli, J. & Claret, M. (1985) J. Biol. Chem. **260**, 11635–11642
Moore, L., Chen, T., Knapp, H. R. & Landon, E. J. (1975) J. Biol. Chem. **250**, 4562–4568
Parker, J. C. & Barritt, G. J. (1981) Biochem. J. **200**, 109–114
Penniston, J. P. (1983) in Calcium and Cell Function (Cheung, W. Y., ed.), pp. 99–149, Academic Press, New York
Poggioli, J., Mauger, J.-P., Guesdon, F. & Claret, M. (1985) J. Biol. Chem. **260**, 3289–3294
Poggioli, J., Mauger, J.-P. & Claret, M. (1986) Biochem. J. **235**, 663–669
Prpic, V., Green, K. C., Blackmore, P. F. & Exton, J. (1984) J. Biol. Chem. **259**, 1382–1385
Putney, J. W. (1987) Trends Pharmacol. Sci. **8**, 481–486

Sastrasinh, M., Knauss, T. C., Weinberg, J. M. & Humes, H. D. (1982) J. Pharmacol. Exp. Ther. **222**, 350–358
Schanne, F. A. X. & Moore, L. (1986) J. Biol. Chem. **261**, 9886–9889
Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) J. Cell Biol. **94**, 325–334
Uhing, R. J., Prpic, V., Jiang, H. & Exton, J. H. (1986) J. Biol. Chem. **261**, 2140–2146
von Tscharner, V., Prod'hom, B., Baggiolini, M. & Reuter, H. (1986) Nature (London) **324**, 369–372
Whipps, D. E., Armston, A. E., Pryor, H. J. & Halestrap, A. P. (1987) Biochem. J. **241**, 835–845

Received 26 January 1988/17 June 1988; accepted 28 June 1988