Biochem. J. (1990) 272, 549–552 (Printed in Great Britain)

MgATP-dependent accumulation of calcium ions and inorganic phosphate in a liver reticular pool

Rosella FULCERI,* Giorgio BELLOMO,† Alessandra GAMBERUCCI* and Angelo BENEDETTI*‡
* Istituto di Patologia Generale, University of Siena, 53100 Siena, and † Clinica Medica I, University of Pavia, 27100 Pavia, Italy

1. MgATP-dependent Ca^{2+} uptake by rat liver microsomal preparations and permeabilized hepatocytes was measured in the presence or absence of P_i. 2. Monitoring of free Ca^{2+} in incubation systems with a Ca^{2+} electrode in the presence of P_i (2–7 mM) revealed a biphasic Ca^{2+} uptake, with the onset of a second, P_i-dependent, Ca^{2+} accumulation. 3. Increasing P_i concentrations (up to 10 mM) caused a progressive enlargement of $^{45}Ca^{2+}$-loading capacity of microsomal fractions. 4. As a result of P_i stimulation of active Ca^{2+} uptake, [^{32}P]P_i and $^{45}Ca^{2+}$ were co-accumulated. 5. Experiments with permeabilized hepatocytes revealed that the amount of Ca^{2+} releasable by *myo*-inositol 1,4,5-trisphosphate is unaffected by P_i.

INTRODUCTION

Increasing evidence strongly suggests that the endoplasmic reticulum (ER) plays a key role in the regulation of cytosolic free Ca^{2+} concentrations in a variety of non-muscle cells. The liver ER *in situ* appears to be a major Ca^{2+} storage site [1], and the MgATP-dependent Ca^{2+} accumulation system is present in (liver) microsomal fractions as well as in the intact ER of permeabilized cells (hepatocytes) [2,3]. On the other hand, the second messenger *myo*-inositol 1,4,5-trisphosphate (IP_3) releases Ca^{2+} from ER, or a specialized part of it, in the cytosol [4–6].

In many Ca^{2+}-translocating systems Ca^{2+} movements are affected by P_i at cellular and subcellular levels [7–14]. In the case of ER (or sarcoplasmic reticulum) the effect of P_i on Ca^{2+} fluxes has seldom been investigated under cytosol-like incubation conditions (e.g. [15,16]); in most instances, in fact, high P_i concentrations have been merely employed to enlarge the reticular Ca^{2+}-loading capacity (see [8,12]), assuming that the anion penetrates the microsomal membranes and precipitates the transported Ca^{2+}.

In the present study the effect of cytosol-like concentrations of P_i (2–4mM in the liver [17,18]) on the ER Ca^{2+} fluxes has been studied in rat liver microsomal fractions as well as in digitonin-permeabilized hepatocytes.

EXPERIMENTAL

Materials

ATP, phosphocreatine, creatine phosphokinase (Sigma Type III) and digitonin were from Sigma, St. Louis, MO, U.S.A. $^{45}CaCl_2$ (1650 Ci/mol) and $Na_2H^{32}PO_4$ (1100 Ci/mol) were from du Pont–New England Nuclear, Dreieich, Germany. IP_3 and collagenase were from Boehringer Mannheim G.m.b.H., Mannheim, Germany. All other chemicals were of analytical grade. Ca^{2+} electrodes were constructed as reported [19] or purchased from Ionetics Inc., Palo Alto, CA, U.S.A.

Preparation of liver microsomal fractions and digitonin-permeabilized hepatocytes

Male Sprague–Dawley rats (weighing 180–230 g) were used. 'Total' liver microsomes (referred to as 'microsomes' in the text below) were prepared as reported [20]. Two microsomal subfractions, one mainly corresponding to rough ER ('rough microsomes') and the other to smooth ER ('smooth microsomes'), were prepared by centrifugation on discontinuous sucrose gradients [21] as previously described [5]. The microsomal fractions were resuspended (approx. 100 mg of protein/ml) in a medium which had the following composition (mM): KCl, 100; NaCl, 20; $MgCl_2$, 5; Mops, 20, pH 7.2. The suspensions were frozen and maintained under liquid N_2 until work-up.

Hepatocytes were isolated by collagenase perfusion as reported [22]. Cells were permeabilized by incubation with digitonin (80 μg/ml) for 5 min at 37 °C in the KCl/Mops medium as above, containing 5 mM-NaN_3. After permeabilization, the cells were washed (three times) and resuspended (approx. 300 mg of protein/ml) in the KCl/Mops medium containing NaN_3. Permeabilized cells were maintained at 0–4 °C and used within 3 h.

Measurement of Ca^{2+} uptake with Ca^{2+} electrodes

Microsomes or permeabilized hepatocytes were incubated in a thermostatically controlled (37 °C) Plexiglas vessel in which a Ca^{2+} electrode and a reference electrode (Radiometer K4040) were immersed. The incubation medium (1 ml) was as follows (mM): KCl, 100; NaCl, 20; $MgCl_2$ 5; Mops, 20, pH 7.2; ATP, 3; phosphocreatine, 10; NaN_3 (as mitochondrial inhibitor), 5. Creatine phosphokinase (10 μM units/ml) was also present. The Ca^{2+} electrodes were calibrated as described by others [23].

Measurement of $^{45}Ca^{2+}$ accumulation by liver microsomal fractions

The microsomal fractions were incubated at 37 °C in a medium which had the following composition (mM): KCl, 100; NaCl, 20; $MgCl_2$, 5; Mops, 20, pH 7.2; ATP, 3; phosphocreatine, 10; NaN_3, 5. Creatine phosphokinase (10 μM units/ml) was also present. $CaCl_2$ (20 μM final concn.) with $^{45}CaCl_2$ (0.1 μCi/ml) was added to the medium. Total Ca^{2+} present in the incubation medium (Ca^{2+} added plus Ca^{2+} already present as contaminant in reagents) was measured by atomic-absorption spectroscopy in each experiment, and ranged from 29 to 36 nmol/ml. The amount of Ca^{2+} accumulated by microsomes was calculated on the basis of the actual total Ca^{2+} content of each incubation mixture. Ca^{2+} accumulated by microsomes was measured by using a rapid-filtration technique [24]. Each Ca^{2+} uptake assay was corrected for non-specifically bound Ca^{2+} , i.e. Ca^{2+} already associated with microsomes in the absence of ATP from the assay medium.

Abbreviations used: ER, endoplasmic reticulum; IP_3, *myo*-inositol 1,4,5-trisphosphate.

‡ To whom correspondence should be addressed.

Measurement of ^{32}P-labelled phosphates accumulated by liver microsomes

The amount of P_i co-accumulated with Ca^{2+} by microsomes was measured by adding ^{32}P-labelled P_i to incubation systems identical with that used for measuring $^{45}Ca^{2+}$ accumulation, but in the absence of $^{45}Ca^{2+}$. ^{32}P associated with (or accumulated by) microsomes was determined by the rapid-filtration technique used to determine microsomal $^{45}Ca^{2+}$ (see above).

Other assays

Protein determination was performed as reported by others [25], using BSA as standard. P_i was measured as reported [25a].

RESULTS

In the first set of experiments, Ca^{2+} uptake by liver 'total' microsomes or the non-mitrochondrial compartment of permeabilized hepatocytes was continuously monitored by measuring the free Ca^{2+} concentration in the incubation systems with a Ca^{2+}-selective electrode. In the presence of MgATP (Fig. 1*a*, lower curve), liver microsomes exhibited a monophasic Ca^{2+} uptake, until a steady-state level of approx. 15 nmol/mg of protein was achieved; this value is in the range of those previously reported for the maximal Ca^{2+}-loading capacity of liver microsomes in the absence of Ca^{2+}-trapping agents [3]. On the other hand, in the presence of P_i the MgATP-dependent Ca^{2+} uptake by liver microsomes was biphasic: a rapid initial uptake (lasting approx. 3.5 min), similar to that observed in the absence of P_i, was followed by an additional Ca^{2+}-uptake phase, which was more and more rapid in the presence of increasing P_i concentrations (2–7 mM added). Owing to microsomal ATP hydrolysis, a linear continuous P_i supply to the incubation systems was also present (approx. 0.06 μmol/min per mg of protein in all incubation mixtures.) However, such P_i supply was ineffective by itself to stimulate Ca^{2+} uptake (Fig. 1*a*, lower curve). Ca^{2+} accumulated by microsomes with or without P_i was rapidly released by the Ca^{2+} ionophore A23187. In the absence of MgATP, no Ca^{2+} uptake was effected by microsomes, either with or without P_i (results not shown). The second P_i-dependent Ca^{2+}-uptake phase was observed also when P_i (3.5 mM) was added at various times after the start of the MgATP-dependent Ca^{2+} uptake (results not shown); however, in all cases P_i-stimulated Ca^{2+} uptake was evident only after the steady-state levels of MgATP-dependent Ca^{2+} accumulation had been attained. Other cytosolic, bivalent anions (i.e. sulphate, acetate, up to 10 mM) failed to stimulate active microsomal Ca^{2+} accumulation.

Fig. 1(*a*) also shows that, in the presence of 4 mM-oxalate (as intravesicular Ca^{2+}-trapping agent), a monophasic pattern of stimulated microsomal Ca^{2+} uptake was observed, with an initial rate higher than that of P_i-stimulated Ca^{2+} uptake. Analogously, in parallel experiments with isolated digitonin-permeabilized rat hepatocytes, it was shown, in the presence of mitochondrial inhibitors, that a second Ca^{2+} uptake phase is elicited by P_i in a non-mitochondrial hepatocyte Ca^{2+} pool (Fig. 1*b*).

The effect of P_i stimulation on the maximal Ca^{2+}-loading capacity of different microsomal preparations ('total', 'rough' and 'smooth' microsomes) was studied with $^{45}Ca^{2+}$. In these experiments, the low amount of microsomal protein used (< 0.1 mg/ml) minimized P_i supply, owing to microsomal ATP hydrolysis, thus allowing prolonged incubation (60 min) at rather constant P_i concentration. As reported in Fig. 2, in the presence of increasing P_i concentrations (1–10 mM), correspondingly increasing Ca^{2+}-loading capacities were observed in all the microsomal fractions employed, although the stimulatory effect of P_i was much more marked in the 'smooth' as compared with the 'rough' microsomal subfraction. This difference is particularly evident at the lower P_i concentrations employed (1–3 mM; see the insert to Fig. 2). In parallel experiments run with a Ca^{2+} electrode, both smooth and rough microsomal subfractions exhibited the same biphasic Ca^{2+} uptake as that observed with total microsomes, although the second P_i-stimulated uptake was more pronounced in smooth microsomes (results not shown).

In further experiments the mechanism of P_i stimulation was investigated. To this aim, $^{45}Ca^{2+}$ and [^{32}P]P_i were employed (Fig. 3). As expected, in the presence of MgATP, a steady-state level of approx. 17 nmol of $^{45}Ca^{2+}$/mg of protein was reached by microsomes within 7 min, and was maintained up to 1 h of

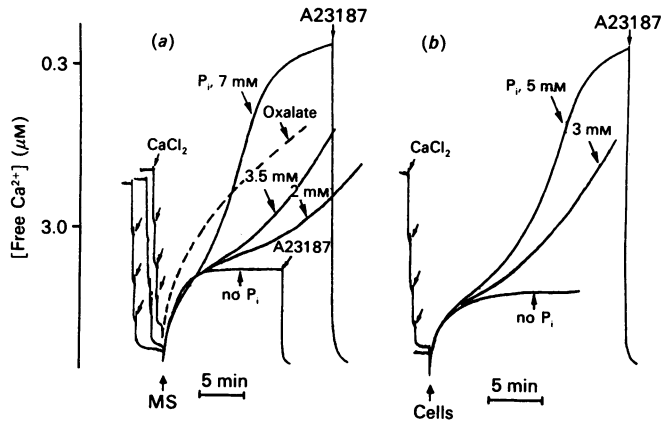

Fig. 1. Time course of MgATP-dependent Ca^{2+} uptake by rat liver microsomes (*a*) and digitonin-permeabilized hepatocytes (*b*) in the presence or absence of P_i

Ca^{2+} uptake was monitored by measuring free Ca^{2+} in incubation mixtures with a Ca^{2+} electrode. Where indicated, P_i (as potassium phosphate buffer, pH 7.2) or ammonium oxalate (4 mM) were included in the medium for MgATP-dependent Ca^{2+} uptake (see the Experimental section). Each $CaCl_2$ addition was 10 nmol (arrows). Incubation was started by adding 30–50 μl of microsomal (MS) or cell suspension to give 2 or 3.5 mg of protein/ml respectively. A23187 was at 1 μM (final concn.). A typical set of experiments out of three to six is reported.

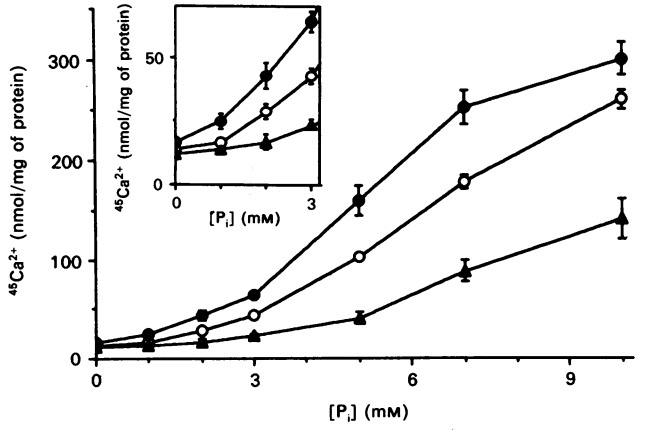

Fig. 2. Stimulatory effect of P_i on MgATP-dependent $^{45}Ca^{2+}$-loading capacity of 'total' (○), 'smooth' (●) and 'rough' (▲) liver microsomes

Microsomes (0.08–0.1 mg of protein/ml) were incubated in the medium for MgATP-dependent $^{45}Ca^{2+}$ uptake (see the Experimental section), including P_i (as potassium phosphate, pH 7.2) at the indicated concentrations, for 1 h. Results are means ± S.E.M. for four to six experiments.

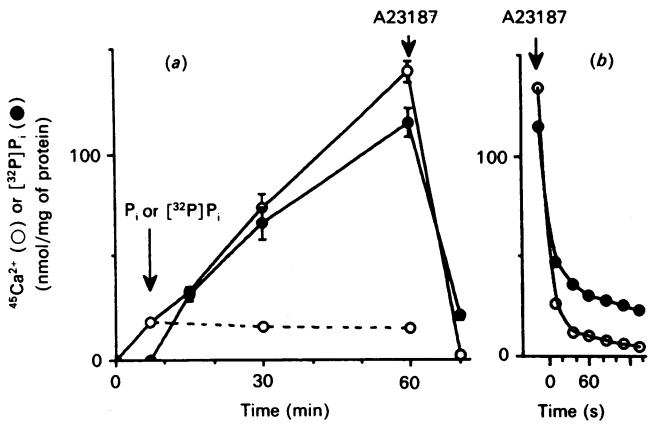

Fig. 3. Co-accumulation (*a*) and simultaneous A23187-induced release (*b*) of Ca^{2+} and P_i from liver microsomes

Microsomes (0.08–0.1 mg of protein/ml) were incubated in the medium for MgATP-dependent $^{45}Ca^{2+}$ uptake (see the Experimental section). As indicated, 7 mM-P_i (as potassium phosphate, pH 7.2) was added to $^{45}Ca^{2+}$ samples (○——○); alternatively, 7 mM-$[^{32}P]P_i$ was added to samples including non-labelled Ca^{2+} only (●——●). A23187 was at 1 μM (final concn.). Results are means ± S.E.M. for four to six experiments (panel *a*); a typical experiment is shown in panel *b*. ○----○, $^{45}Ca^{2+}$ accumulation in the absence of P_i.

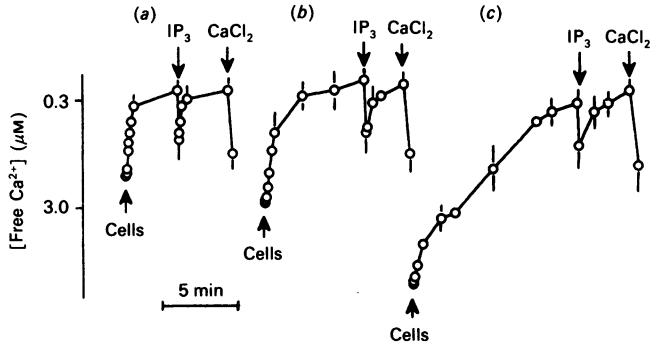

Fig. 4. Releasing effect of IP_3 on Ca^{2+} accumulated by digitonin-permeabilized hepatocytes in the presence of MgATP (*a*) or MgATP plus P_i (*b*,*c*)

Ca^{2+} uptake was monitored by measuring free Ca^{2+} in incubations with a Ca^{2+} electrode; curves shown represent three separate experiments (means ± S.E.M.). Hepatocytes (5 mg of protein/ml) were incubated in the medium for Ca^{2+}-electrode measurements (see the Experimental section), containing 15 μM (*a*), 35 μM (*b*) or 60 μM total Ca^{2+} (*c*); 3 mM-P_i (as potassium phosphate, pH 7.2) was also present in (*b*) and (*c*). IP_3 (arrows) was at 5 μM. Each $CaCl_2$ addition (arrows) was 10 nmol.

incubation (Fig. 3*a*, broken line). Upon addition of P_i (7 mM), $^{45}Ca^{2+}$ uptake was markedly stimulated. In parallel samples including non-radiolabelled Ca^{2+} the addition of $[^{32}P]P_i$ resulted in a progressive increase of the amount of ^{32}P becoming associated with microsomes, and such amounts were similar to the amounts of $^{45}Ca^{2+}$ accumulated at the corresponding incubation times. A major portion of $[^{32}P]P_i$ associated with microsomal vesicles was likely accumulated intravesicularly, as a consequence of the attendant Ca^{2+} accumulation; the Ca^{2+} ionophore A23187 caused in fact a rapid, simultaneous release of both accumulated $^{45}Ca^{2+}$ and $[^{32}P]P_i$, as shown in detail in Fig. 3(*b*).

The low amount (about 20 nmol/mg of protein) of $[^{32}P]P_i$ still associated with microsomes after the A23187-induced release likely represents a non-specific binding of P_i to the microsomal membrane. Indeed, in complete incubation systems to which 1 mM-EGTA or 500 μM-vanadate were also added, thus suppressing active Ca^{2+} accumulation, the amount of $[^{32}P]P_i$ associated with microsomes was about 20 nmol/mg of protein, at any time of incubation (results not shown). By comparing the net P_i-dependent $^{45}Ca^{2+}$ accumulations (P_i-stimulated minus MgATP-dependent $^{45}Ca^{2+}$ uptake) with the corresponding net $[^{32}P]P_i$ accumulations (i.e. subtracted of the non-specific P_i microsomal binding), Ca^{2+}/P_i molar ratios of 1.4 (15 min), 1.24 (30 min) and 1.29 (1 h) could be calculated.

The possibility that P_i stimulation of reticular Ca^{2+} accumulation might result in an enlargement of the IP_3-releasable Ca^{2+} pool was verified in additional experiments with digitonin-permeabilized hepatocytes. In the presence of MgATP only, hepatocytes rapidly accumulate Ca^{2+} down to free Ca^{2+} external concentrations lower than 0.3 μM; the subsequent addition of IP_3 causes a rapid Ca^{2+} release (Fig. 4*a*). By increasing Ca^{2+} concentrations in the incubation medium, in the presence of 3 mM-P_i, Ca^{2+}-loading capacity of cells was enlarged, and the second P_i-dependent Ca^{2+}-uptake phase becomes apparent (Figs. 4*b* and 4*c*); however, the extent of Ca^{2+} release elicited by IP_3 is unaffected. This suggests that the P_i-stimulated Ca^{2+} pool is unrelated with the IP_3-releasable Ca^{2+} pool, at least under the experimental conditions used.

DISCUSSION

A major finding of the present study is that low concentrations of P_i, in the range of those reported to occur in hepatocellular cytosol [17,18], are able to stimulate MgATP-dependent Ca^{2+} uptake by different liver reticular preparations. The effect of P_i was in fact evident both in isolated liver microsomal fractions and in a non-mitochondrial compartment of permeabilized hepatocytes which allegedly corresponds to ER *in situ*. A stimulation of liver microsomal Ca^{2+} accumulation has been reported to occur in the presence of high (10–40 mM) P_i concentrations [24,26]. With low P_i concentrations we actually showed a minor (5 mM-P_i) or even a lack of (2 mM-P_i) effect of P_i on liver microsomes obtained in the presence of 3 mM-EDTA [24], although the discrepancy can be explained by differences in the procedures used for preparation of microsomal fractions. In the present study liver microsomes were in fact prepared in the absence of EDTA [5,20], since we observed that the inclusion of EDTA (< 1 mM) or EGTA (< 0.3 mM) in the preparative media results in a marked attenuation of P_i stimulation on Ca^{2+} uptake, despite MgATP-dependent Ca^{2+} uptake not being modified (R. Fulceri, G. Bellomo, A. Gamberucci & A. Benedetti, unpublished work).

The biphasic pattern of P_i-stimulated Ca^{2+} accumulation observed both with isolated microsomes and with permeabilized hepatocytes (in the presence of mitochondrial inhibitors) suggests that a distinct P_i-dependent Ca^{2+} pool is involved. A possible explanation for this finding is that liver ER preparations comprise distinct populations of vesicles, provided with different P_i sensitivity. The stimulatory effect of P_i was actually found to be predominant in smooth vesicles, although the latter cannot be viewed as a pure P_i-sensitive compartment, since P_i-stimulated Ca^{2+} uptake by smooth microsomes was also biphasic. In all cases the accomplishment of the first MgATP-dependent, P_i-sensitive, Ca^{2+}-uptake phase appeared to be a prerequisite for the onset of the P_i-stimulated uptake.

The observed biphasic pattern of Ca^{2+} uptake appears to be a specific effect of P_i stimulation. The currently employed intravesicular Ca^{2+}-trapping agent, oxalate, appeared in fact to stimulate both the initial rate and the maximal Ca^{2+}-loading capacity of liver microsomes with a monophasic pattern, and

other anions tested (i.e. sulphate, acetate) had no effect at all under our experimental conditions.

As far as the mechanisms involved in P_i stimulation of Ca^{2+} uptake are concerned, data obtained with [^{32}P]P_i indicate that a co-transport and a co-accumulation of Ca^{2+} and P_i occur in liver microsomes. Co-accumulation of Ca^{2+} and P_i is likely to result in lowering intravesicular concentrations of free Ca^{2+} ions, which have been shown to exert an inhibitory effect on further active Ca^{2+} transport [27], thus allowing unhampered Ca^{2+} accumulation. With respect to the route of P_i entry into microsomal vesicles, a phosphate translocase protein has been purified from rat liver microsomes [28] that might be involved in the observed co-accumulation of P_i and Ca^{2+}.

As shown in Fig. 4, the enlargement of the reticular Ca^{2+}-loading capacity effected by cytosol-like P_i concentrations did not result in any alteration of the IP_3-releasable Ca^{2+} pool. Actually, low P_i concentrations appear to exert only a minor stimulation of Ca^{2+} uptake by liver rough microsomes (Fig. 2), and indeed the latter have been shown to be highly sensitive to the Ca^{2+}-releasing effect of IP_3 [5,29]. The possibility that IP_3 acts on organelles other than ER [30,31] should also be considered. On the other hand, stimulation of reticular Ca^{2+} uptake by P_i has been described to result in an enlargement of the IP_3-releasable pool of other cell types [15,20,32], and further studies are required to elucidate the role that P_i appears to play at the reticular level in different cell types.

In conclusion, cytosol-like concentrations of P_i appear to allow a considerable enlargement of the liver ER Ca^{2+}-loading capacity, by stimulating Ca^{2+} uptake in a distinct, IP_3-insensitive, Ca^{2+} pool, which could play an important physiological role in the intact hepatocyte.

We thank Dr Tony Scarpa, Case Western Reserve University, Cleveland, OH, U.S.A., for advice.

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Received 20 August 1990/24 September 1990; accepted 5 October 1990