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The mechanism of calcium uptake by liver microsomes: effect of anions and ionophores

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The mechanism of calcium uptake by liver microsomes was investigated using various anions and ionophores. Calcium uptake was shown to be specific to microsomes and unlikely to be due to contamination by plasma membranes by correlation of calcium uptake to the marker enzymes specific for these two fractions. Under the conditions employed, phosphates, sulfate, chloride, acetate, nitrate, thiocyanate, maleate, succinate and oxalate all stimulated calcium uptake by microsomes, but to different degrees. The greatest effect (4–6-fold) was observed with phosphate. On the contrary, phosphate is the only anion that stimulates the plasma membrane calcium uptake to any significant degree. Treatment of isolated microsomes with 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS) resulted in inhibition of ATP- and anion-dependent calcium uptake. A lipid-permeable organic acid such as maleate retained its ability to promote calcium uptake in DIDS-treated microsomes. However, a lipophilic anion, such as nitrate, stimulated calcium uptake only in the presence of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). In addition, 2 μM valinomycin, when added in the absence or presence of 10 to 100 mM K^+, had no stimulatory effect on calcium uptake. These results appear to be consistent with a model in which the active uptake of calcium into microsomes involves electroneutral Ca^{2+}-nH^+ exchange.

Introduction

ATP-dependent calcium pumps have been demonstrated in a variety of tissues [1,2]. In many tissues, such as the liver, the maintenance of the low intracellular calcium concentration is thought to be controlled through the concerted effort of the calcium pumps located in plasma membranes [3,4], mitochondria [5], and microsomes [6–8]. The kinetics and effects of various hormone manipulations on the activity of the plasma membrane and microsomal pumps have been described [3,5,9–12]. To date, relatively little is known regarding the primary mechanism by which these pumps operate.

Evidence has been accumulated thus far on the mechanism of the more extensively studied mammalian erythrocyte pump [13–16]. On the basis of the available data, it appears that the pump acts electroneutrally [15] or electrogenically [16] as a Ca^{2+}/nH^+ antiporter involving the band III protein [17].

As a first step towards understanding the means by which the activity of the plasma membrane or microsomal pumps of liver can be modulated, we have explored the effects of various anions and ionophores on the activity of these calcium pumps.

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Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Pipes, 1,4-piperazinediethanesulfonic acid.

The results indicate that calcium uptake by plasma membrane vesicles is stimulated primarily by phosphate, whereas the microsomal pump is supported by a number of anions in a concentration-dependent fashion. Using microsomes that had previously been incubated with DIDS and a combination of various ions and ionophores, we have accumulated evidence to suggest that the microsomal calcium uptake system operates by means of a electroneutral Ca^{2+}-nH^+ exchange.

Materials and Methods

Materials. ^{45}Ca (10–40 mCi/g) was obtained from Amersham. Ultrapure sucrose was obtained from Schwarz-Mann. All other chemicals were reagent grade obtained from either Fisher Scientific Co. or Sigma Chemical Co. Whenever calcium hydroxide of magnesium hydroxide were used, they were adjusted to the desired pH with imidazole or Pipes.

Animals. Male Sprague-Dawley rats weighing 200–250 g were obtained from Eldridge Laboratory Animals, Barnhart, MO. They were fed Purina Lab Chow ad libitum and given free access to water.

Preparation of subcellular fractions. Liver plasma membranes were prepared according to Touster et al. [18]. Microsomes were prepared on the method described by Touster et al. [18] and Moore [19]. For the preparation of K^+- or Na^+-loaded microsomes, fractionation was performed in the presence of 10 or 130 mM potassium or sodium Pipes. Both plasma membranes and microsomes were resuspended in 0.25 M sucrose and frozen at −70°C until needed. All preparative steps were carried out at 0–4°C. Purity of the fractions was assayed by measuring 5′-nucleotidase [20] and glucose 6-phosphate [21]. Protein was determined by the method of Lowry et al. [22].

Calcium uptake. The standard calcium uptake assay for plasma membrane was performed at pH 8.0 as previously described [9] and in the presence of 2.5 μM ruthenium red. Calcium uptake by microsomes were assayed at pH 6.8 at 37°C according to Moore [19]. When the effect of anions was studied, sodium salts were used and all anions other than the specific anion addition were replaced with either Pipes, hydroxide or gluconate.

Determination of total and free calcium concentrations. The total calcium was determined by atomic absorption spectroscopy. Free calcium concentrations were estimated using the MUSIC/FORTRAN version of the HALTAFALL program as described in detail by Ingri et al. [23]. The association constants used in the calculation were essentially identical to those described previously [3].

Results

Calcium uptake by plasma membranes and microsomes

Before the effects of anions on the microsomal calcium uptake system could be studied, it was necessary to differentiate the latter from the plasma membrane calcium uptake system. We correlated calcium uptake with the activity of the marker enzymes for plasma membranes and microsomes at different stages of their fractionation. Results (Table I) indicate that plasma membrane calcium uptake correlated with the marker enzyme of 5′-nucleotidase, with the activities of both being the highest in plasma membrane fraction and low in the $1000 \times g$ low speed supernatant and microsomes. The calcium uptake and 5′-nucleotidase activities were approx. 5- and 35-fold of that in the homogenate, respectively. Likewise similar correlation existed between the microsomal calcium uptake and glucose-6-phosphatase activities. The calcium uptake and glucose-6-phosphatase activities were about 2- and 5-fold higher, respectively, than those in the homogenate.

Effect of anion concentration on calcium uptake

Calcium uptake by plasma membrane was not significantly affected by most of the anions tested, at concentrations as high as 4 mM for oxalate (Fig. 1C) or 40 mM for all others (Figs. 1A, 1B). The only exception was sodium phosphate which, when present at a concentration of 40 mM increased calcium uptake by 2-fold (Fig. 1A). The effect of phosphate could be demonstrated at a concentration as low as 5 or 10 mM, and started to level off between 30 and 40 mM. Conversely, microsomal calcium uptake was enhanced by every anion tested. The effect of sodium phosphate, sulfate and chloride was linear up to at least 40

TABLE I

INTRACELLULAR DISTRIBUTION OF THE 5'-NUCLEOTIDASE, GLUCOSE-6-PHOSPHATASE, AND CALCIUM UPTAKE ACTIVITIES IN RAT LIVER

Plasma membranes, microsomes and the various intracellular fractions were isolated from rat liver according to Touster et al. [18]. 5'-Nucleotidase and glucose-6-phosphatase activities were assayed according to Avruch and Wallach [20] and Aronson and Touster [21], respectively. Calcium uptake was measured at 37°C for 30 min under the conditions for either plasma membranes [9] or microsomes [19]. Results represent the mean ± S.E. of 3–14 separate experiments. The number in parenthesis represents the activity expressed as a percentage of that for the homogenate.

Fraction	5'-Nucleotidase (nmol P/mg per min)	Glucose-6-phosphatase (μg P/mg per min)	ATP-dependent calcium uptake using	
			plasma membranes conditions [9] (nmol Ca/mg)	microsomal conditions [19] (nmol Ca/mg)
Homogenate	20.4 ± 6.5 (100%)	1.3 ± 0.2 (100%)	0.49 ± 0.07 (100%)	35.32 ± 6.28 (100%)
1000 × g low speed pellet	50.5 ± 12.2 (247%)	0.4 ± 0.1 (34%)	0.30 ± 0.04 (62%)	12.80 ± 2.10 (70%)
Plasma membranes	713.6 ± 75.9 (3493%)	0.7 ± 0.1 (54%)	2.28 ± 0.24 (467%)	8.28 ± 0.71 (24%)
1000 × g low speed supernatant	11.3 ± 2.1 (55%)	1.5 ± 0.1 (119%)	0.36 ± 0.13 (73%)	40.04 ± 6.99 (113%)
Microsomes	31.8 ± 3.7 (155%)	6.3 ± 0.1 (490%)	0.13 ± 0.07 (26%)	77.32 ± 8.26 (219%)

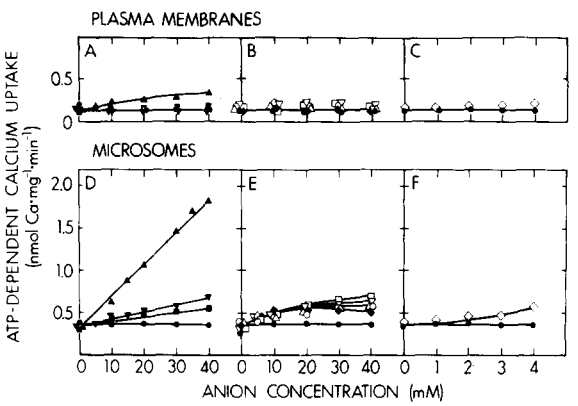

Fig. 1. Effect of anion concentration on plasma membrane and microsomal uptake of rat liver. Calcium uptake into plasma membranes and microsomes were performed at 37°C for 5 min in a 500 μl reaction mixture containing 60–100 μg protein and either 50 mM Tris-Pipes (pH 8.0), 0.25 M sucrose, 10 mM $Mg(OH)_2$, 0 or 10 mM Tris-ATP, 0.1 mM EGTA, 0.1 mM $^{45}Ca(OH)_2$ and 2.5 μM ruthenium red or 30 mM imidazole-Pipes (pH 6.8), 0.25 M sucrose, 5 mM $Mg(OH)_2$, 0 or 5 mM Tris-ATP, 20 μM $^{45}Ca(OH)_2$ and 2.5 μM ruthenium red, respectively. Reaction was initiated by the addition of $^{45}Ca(OH)_2$ after a 5 min preincubation in the absence (●) or presence of varying concentrations of phosphate (▲), sulfate (▼), chloride (■), thiocyanate (◆), acetate (○), nitrate (△), maleate (▽), succinate (□) or oxalate (◇). Each point represents the mean of 3–5 experiments.

mM, with sodium phosphate having the greatest effect, followed by sulfate and chloride. At 40 mM, phosphate, sulfate and chloride stimulated the calcium uptake by 6-, 2- and approx. 1-fold, respectively.

The effects of the other anions (acetate, nitrate, thiocyanate, maleate and succinate) reached the maximum at 20–40 mM with approx. 1–2-fold increase in calcium uptake. Oxalate had optimal effect at 4 mM with little or no effect at 1–2 mM. Concentrations higher than 5 mM resulted in an increase in background to unacceptable level as a result of calcium oxalate precipitation.

Time-course of stimulation of microsomal calcium uptake by anions

The effect of anions on ATP-dependent calcium uptake as a function of time is shown in Fig. 2. Calcium uptake in the absence of anions reached a steady-state level in approx. 5 min. The presence of 40 mM phosphate resulted in an increase in calcium uptake which is linear for approx. 10 min (Fig. 2A). Oxalate, at a concentration of 4 mM, promoted calcium uptake at a faster rate for the initial 3 min followed by a slower but still steady

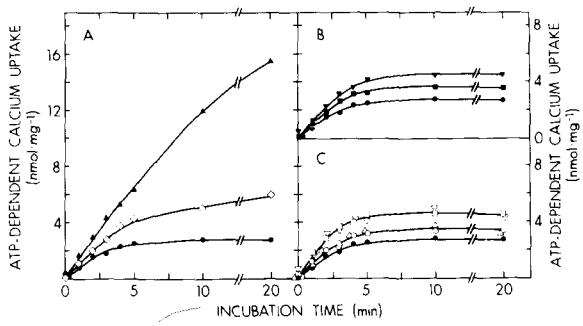

Fig. 2. Time-course of stimulation of microsomal calcium uptake by anions. calcium uptake by microsomes was measured as described in the legend to Fig. 1 in a reaction volume of 3500 μl and in the absence (●) or presence of (A) 40 mM phosphate (▲), 4 mM oxalate (◇), (B) 40 mM sulfate (▼), chloride (■), thiocyanate (◆) or (C) 40 mM acetate (○), nitrate (△), maleate (▽) or succinate (□). At the appropriate times, 450 μl aliquots were removed and filtered through 0.45 μm Millipore filters and ^{45}Ca uptake was measured as previously described [3]. Each point represents the mean of 2–6 experiments.

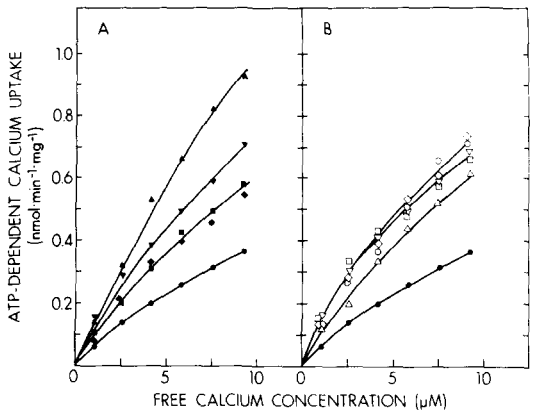

Fig. 3. Effect of anions on the kinetics of microsomal calcium uptake. Calcium uptake was measured in 5-min assays at 37°C for 2 min as described in Materials and Methods and the legends to Fig. 1, and in the absence (●) or presence of: (A) 40 mM phosphate (▲), sulfate (▼), chloride (■) or thiocyanate (◆); (B) 40 mM acetate (○), nitrate (△) maleate (▽), succinate (□) or 4 mM oxalate (◇). Free calcium concentrations were varied between 1 and 10 μM. Data represent mean of 2–6 experiments.

increase over the remaining incubation period (Fig. 2A). The time-course of effect of all the other anions resembled that of control, reaching a steady state after 5 min (Figs. 2B, 2C).

Effect of anions on the kinetics of microsomal calcium uptake

The kinetics of calcium uptake in the presence or absence of anions was measured over the free calcium concentration range of 1 to 10 μM (Fig. 3). Calcium uptake was measured after only a 2 min incubation at 37°C to ensure an accurate estimation of the initial rate. The rate of calcium uptake by microsomes was found to be markedly different with the various anions (Fig. 3). Based on the double-reciprocal analysis of the data, the apparent K_m values of the uptake process for calcium were similar in the absence and presence of the different anions, with a mean ± S.D. of 10.3 ± 3.5 μM. The various anions affected only the V_{max} of the calcium uptake system. At 40 mM, phosphate increased was the V_{max} by 5.8-fold from 903.1 to 5335.6 pmol $Ca \cdot min^{-1} \cdot mg^{-1}$ (Table II). At 40 mM, sulfate increased the V_{max} by 1.5-fold to 1346.6 pmol $Ca \cdot min^{-1} \cdot mg^{-1}$ whereas chloride had only a small effect. The effects of the same concentration (40 mM) of nitrate, thiocyanate, acetate, maleate and succinate varied with a V_{max} of 1.1–1.6-fold over the control. The presence of oxalate at 4 mM increased the V_{max} by 2-fold to 1726.4 pmol $Ca \cdot min^{-1} \cdot mg^{-1}$.

TABLE II

DETERMINATION OF APPARENT V_{A3x} OF ANION STIMULATED CALCIUM TRANSPORT

Calcium uptake was performed in the presence of 1–10 μM of free Ca^{2+} as described in the legend to Fig. 3. Apparent V_{max} for the calcium uptake was calculated from double reciprocal analysis of the data presented in Fig. 3.

Addition	Apparent V_{max} (pmol·min^{-1}·mg^{-1})
None	909.1
Phosphate	5235.0
Sulfate	1346.6
Chloride	1117.8
Nitrate	944.5
Thiocyanate	1401.5
Acetate	1426.5
Maleate	999.6
Succinate	1029.8
Oxalate	1726.4

Effect of maleate, nitrate on calcium uptake in DIDS-treated microsomes

To assess the mechanism of calcium uptake by microsomes, we utilized microsomes that had been

TABLE III

EFFECT OF ORGANIC ACIDS, LIPOPHILIC ANIONS AND pH PROBES ON CALCIUM UPTAKE IN DIDS-TREATED MICROSOMES

Calcium uptake was measured in a 15-min assay at 37°C (described previously in Materials and Methods and legend to Fig. 1) using microsomes that had been pretreated with 20 μM DIDS at 37°C for 30 min. When indicated, 10 μl of 500 μM CCCP in 20% ethanol was added to the medium to a final concentration of 10 μM. Results represent the mean ± S.E. of 3–5 experiments.

Additions	ATP-dependent calcium uptake (pmol $Ca \cdot mg^{-1}$)
None	151.35 ± 16.65
CCCP	137.34 ± 16.21
40 mM maleate	360.29 ± 30.71
40 mM maleate/CCCP	356.00 ± 28.12
40 mM nitrate	212.55 ± 27.71
40 mM nitrate/CCCP	331.52 ± 24.40

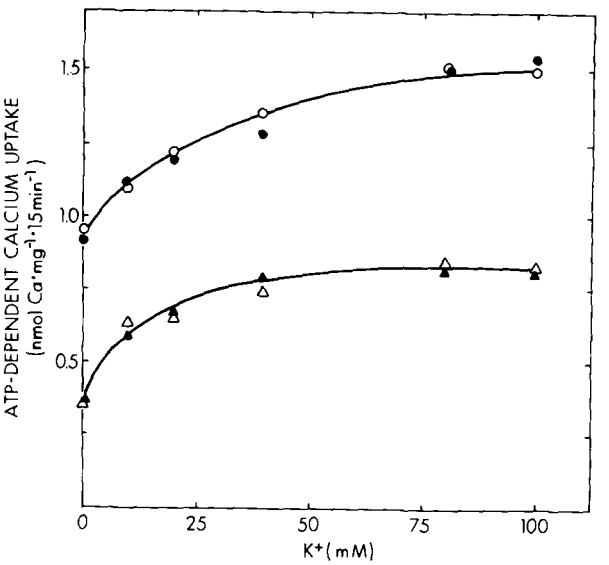

Fig .4. Effect of valinomycin on microsomal calcium uptake. Microsomes were prepared in the presence of 10 mM K^+, and pretreated for 30 min at 37°C in the absence (○, ●) or presence (△, ▲) of 20 μM DIDS. Calcium uptake was assayed as described in Materials and Methods in the presence of either varying concentrations of potassium gluconate alone (open symbols) or together with 2 μM valinomycin (solid symbols).

preincubated with DIDS for 30 min at 37°C. In the presence of 40 mM maleate, an anion that could penetrate membranes as neutral acid, the calcium uptake by the DIDS-treated microsomes increased by more than 2-fold from 151.35 to 360.29 pmol $Ca \cdot mg^{-1}$ (Table III). Presence of 10 μM CCCP had no affect on the basal or any additional effect on the maleate-stimulated calcium uptake activity. On the other hand, 40 mM nitrate, a lipophilic anion, resulted in only a slight stimulation of calcium uptake into the DIDS-treated microsomes above that observed in the absence of nitrate. Simultaneous addition of 10 μM CCCP with nitrate led to a substantial stimulation of calcium uptake. Such results appear to be consistent with the model of Ca^{2+}-nH^+ exchange that is described for the erythrocyte calcium pump [16].

Effect of valinomycin on calcium uptake, in the absence and presence of DIDS

To further test if the microsomes could also operate electrogenically, we prepared microsomes in a medium containing 10 mM potassium and assayed the calcium uptake in the presence of 2 μM valinomycin and varying concentrations of potassium. If the microsomes operate electrogenically the presence of valinomycin would increase the permeability of the microsomal membrane to potassium and is expected to stimulate the calcium uptake by decreasing the membrane potential created by it. In the presence of potassium alone calcium uptake activity increased with increasing concentration of potassium. Similar effects of potassium have also been reported with the (Ca^{2+} + Mg^{2+})-ATPase and/or calcium uptake activities in adipocyte endoplasmic reticulum [24], sarcoplasmic reticulum [25] or cardiac membrane vesicles [26]. Although the stimulatory effect of potassium on calcium uptake has been described to be related to the relaxation process of muscle [25], a more plausible explanation for our observation may be on the dephosphorylation of the phosphorylated (Ca^{2+} + Mg^{2+})-ATPase as postulated for the cardiac membrane vesicles [26]. Moreover, addition of 2 μM valinomycin resulted in no additional stimulation in calcium uptake regardless of the concentration of potassium that was added to the medium (Fig. 4). Valinomycin also did not have any effect on calcium uptake when added to microsomes prepared in the presence of higher

concentrations (130 mM) of K^+ or Na^+ and assayed in the presence of either K^+ or Na^+ (data not shown).

Since the simultaneous uptake of anions by the microsomes could dissipate any potential regarded by the calcium uptake thus rendering a presumably electrogenic transport of calcium electroneutral, we also investigated the effect of valinomycin on the calcium uptake in the presence of an anion transport inhibitor DIDS. Assuming that the uptake of anions by microsomes also occurs via a DIDS-sensitive anion transport protein, the presence of the inhibitor DIDS should be able to eliminate charge neutralization due to the influx of anions and reveal an electrogenic transport, if present. Results in Fig. 4 showed that the presence of 20 μM DIDS inhibited the calcium uptake by approx. 50%, but preserved the potassium-stimulated calcium uptake activity. The inhibitory effects of DIDS on calcium uptake as well as Ca^{2+}-ATPase activity have also been reported on the erythrocyte system [15]. Under these conditions, valinomycin was equally ineffective in stimulating the calcium uptake activity.

Discussion

Using the standard published procedures for measuring calcium uptake in plasma membranes [3,9] and microsomes [19], we compared the calcium uptake activities at various stages of the fractionation with the marker enzyme activities for plasma membrane and microsomes. Our results indicated a good correlation between plasma membrane or microsomal calcium uptake and their respective marker enzymes. Results thus suggested that the assays were measuring preferentially either plasma membrane or microsomal calcium uptake. The plasma membrane and microsomal calcium uptake activities could further be differentiated based on their differences in pH optimum and oxalate dependency. The microsomal calcium uptake activity has a pH optimum of approx. 6.5–6.8 and can be stimulated substantially in the presence of oxalate whereas the plasma membrane system has a higher pH optimum (approx. 7.2) (unpublished observation) and shows less dependency on oxalate. Moreover, results imply that the assay conditions that are described here adequately distinguish the plasma membrane from microsomal calcium uptake systems.

The present results also showed that the transport of calcium across the plasma membranes and microsomes could be distinguished from their response to the different anionic composition in the incubation medium, suggesting a different mechanism by which these systems operate. Why the plasma membrane and microsomal systems respond differently to the anions is not known. One can postulate that perhaps in addition to being involved in regulating the intracellular calcium concentration, they may also play a role in modulating cellular pH values. Moreover, the similarity in the effects of the various anions on the present microsomal system and erythrocyte pump [16] implies that the same mechanism could operate in both systems. The following findings are taken as evidence that the microsomal calcium uptake system operates by means of Ca^{2+}-nH^+ exchange and does not create a membrane potential. 40 mM maleate, an anion which can penetrate membranes in neutral protonated form and donate proton to the inside of the vesicles, was equally effective in stimulating calcium uptake in DIDS-treated (Table III) and untreated (Table II) microsomes. Simultaneous addition of 10 μM CCCP and maleate did not result in any additional of calcium uptake. However, addition of an unprotonated lipid-soluble anion, such as nitrate, did not support substantial calcium uptake unless CCCP was also present (Table III). Second, the ionophore valinomycin would be expected to stimulate the electrogenic calcium uptake by increasing the passive permeability of the membrane for potassium, resulting in charge equilibration across the membrane and subsequent stimulation of calcium uptake. However, it had no effect on the calcium uptake by microsomes in both DIDS-treated and untreated vesicles (Fig. 4). Furthermore, valinomycin also did not have any significant effect on calcium uptake regardless of the concentration of K^+ or whether Na^+ or K^+ was used in the preparation of microsomes, and whether the assay was performed in the presence of K^+ or Na^+ (data not shown). This could imply that the formation of any transmembrane electrochemical potential (either negative or positive) as a result of the preformed K^+ gradient had no effect on calcium uptake.

The findings presented here appear to be consistent with the model for Ca^{2+}-nH^+ exchange presented by Smallwood et al. [16] and do not suggest that calcium uptake in microsomes occurs via a electrogenic mechanism. This could also explain the differential effects of anions like phosphate, sulfate and chloride on calcium uptake. Because of its ability to donate up to two protons to the vesicle interior, phosphate is the most effective promoter of calcium uptake in normal vesicles. Sulfate, on the other hand, is presumed to be capable of exchanging only one H^+ for each monovalent anion as it is transported inward. Thus it is less effective than phosphate in stimulating calcium uptake. As chloride can only be exchanged for one monovalent anion, it is least effective in stimulating calcium uptake. However, several questions remain. The use of the anion transport inhibitor DIDS is based on the assumption that an anion transport system similar to the one described for the erythrocytes is also present in the microsomes. Cheng and Levy [27] reported the identification in the plasma membranes of hepatocytes of 54000 and 43000 dalton proteins that may be the components of a transmembrane anion transport system and the inhibition by DIDS of sulfate uptake by hepatocytes. Identification of a similar system is yet to be described for the microsomes. In addition, at least in isolated sarcoplasmic reticulum vesicles, the membrane has been reported to be permeable to anions and small cations [28,29]. Such a permeability would make it difficult to evaluate the electrical properties of the calcium uptake system. Whether the liver microsomal fraction may also be permeable to anions by means of mechanism independent of any DIDS-sensitive system should be considered. Efforts are underway to reconstitute the Ca^{2+}-ATPase from the microsomes into a relatively impermeable membrane for further investigation.

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