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Properties of Energy-Dependent Calcium Transport by Rat Liver Microsomal Fraction as Revealed by Initial-Rate Measurements

By FYFE L. BYGRAVE
Department of Biochemistry, Faculty of Science, Australian National University, Canberra, A.C.T., 2600 Australia

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Measurements of the initial rate of Ca^{2+} transport by rat liver microsomal preparations, reveal the existence of two phases of transport activity. The first, a phase of rapid transport, is complete by 3–5 min, at which time the second (slower) phase begins; this remains linear for up to at least 40 min. The initial phase is minimal in the absence of MgATP. The initial rate of Ca^{2+} transport reaches values as high as 25 nmol/min per mg of protein; the K_m for Ca^{2+}_{total} is 1–2 μM and that for $MgATP_{total}$ about 500 μM. Ruthenium Red (3–5 nmol/mg of protein) has little effect on the initial rate of transport, whereas tributyltin (2 μM) inhibits equally in a KCl- or a KNO_3-containing medium. Compounds that collapse components of the proton electrochemical gradient in mitochondria (valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone) each inhibit by 70–80% the initial rate of microsomal Ca^{2+} transport.

Two aspects of cell calcium have been the subject of increasing interest. The first is the role of Ca^{2+} in cell function and the second the means by which the intracellular distribution of Ca^{2+} is regulated. Although mitochondria in a range of tissues and species are known to be involved in the regulation of cell Ca^{2+} (see Bygrave, 1977, for a review), it is recognized that in skeletal tissue in particular, Ca^{2+} transport by the sarcoplasmic reticulum plays a vital and possibly predominant role in such regulation (see Martonosi, 1972, for a review). Much less is known about the ability of the endoplasmic reticulum to control cell Ca^{2+} in non-skeletal tissue such as liver.

Various reports have described aspects of Ca^{2+} transport by vesicular fractions (microsomal) derived from the endoplasmic reticulum of liver (Moore *et al.*, 1975*a*, 1976; Farber *et al.*, 1977), kidney (Moore *et al.*, 1974), adipocyte (Bruns *et al.*, 1976), submandibular gland (Watson & Siegel, 1977) and vascular smooth muscle (Fitzpatrick *et al.*, 1972; Moore *et al.*, 1975*b*). However, little attempt to measure initial rates of Ca^{2+} transport was made in the majority of these studies. Accordingly the ability of the microsomal fraction to participate in cellular Ca^{2+} homoeostasis may have been considerably underestimated in some of the above reports.

The aims of the present work were, firstly, to determine the initial rates of Ca^{2+} transport in the microsomal fraction of rat liver, secondly, to determine if some properties of microsomal Ca^{2+} transport, already reported, differed significantly when initial-rate measurements are used, and also to use initial-rate measurements to glean further information about the mechanism of microsomal Ca^{2+} transport.

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; ATPase, adenosine triphosphatase.

Experimental

Microsomal fractions were prepared from the livers of Wistar-strain albino rats that had been deprived of food overnight. The livers were minced with scissors and washed with a medium containing 250 mM-sucrose, 2.5 mM-Hepes* and 0.5 mM-EGTA (pH 7.4). A homogenate (20%, w/v) in this medium was prepared by four or five passes in a Thomas size-C glass homogenizer fitted with a Teflon pestle. An equal volume of the medium was added to the homogenate and after thorough mixing the suspension was centrifuged at 2500*g* for 10 min in a Sorvall RC-2B refrigerated centrifuge and the resulting supernatant was centrifuged at 17000*g* for 20 min in the same centrifuge. The pellet was discarded and the supernatant centrifuged at 105000*g* for 60 min in a Spinco high-speed centrifuge. The resulting pellet was resuspended in 250 mM-sucrose/2.5 mM-Hepes, pH 6.8, to give a final protein concentration of about 10 mg/ml.

Ca^{2+} transport by the microsomal fraction was measured by using the Millipore-filtration technique with the medium described by Moore *et al.* (1975*a*). The medium consisted of 100 mM-KCl, 30 mM-imidazole/histidine buffer, pH 6.8, 5 mM-ammonium oxalate, 5 mM-$MgCl_2$ and 5 mM-ATP in a total

volume of 1.5ml and was left to temperature equilibrate at 37°C. The microsomal fraction (containing about 100μg of protein) was added and the mixture thoroughly agitated. After exactly 60s $CaCl_2$ (20μM, containing 0.1μCi of $^{45}Ca^{2+}$) was added and the mixture again quickly agitated. Samples (100μl), taken from the mixture at defined intervals, were placed on pre-wetted filter papers (Gelman 0.45μm pore size) and washed rapidly with about 2ml of 250mM-sucrose/2.5mM-Hepes, pH6.8. The filter papers were placed in vials, oven-dried and 10ml of scintillation fluid added (see Reed & Bygrave, 1974). They were counted for radioactivity in a Beckman Tri-Carb scintillation counter by using the ^{14}C channel.

Initial rates were calculated by drawing a tangent to the curve at zero time. The earliest time sample could be obtained by about 10s. Agreement between experiments, which were carried out at least twice and usually three times, was usually within 10–15%.

Results

Time course of microsomal Ca^{2+} transport and the affinity for Ca^{2+} and MgATP

Data in Fig. 1(*a*) are from two experiments in which the progress of Ca^{2+} transport by the microsomal fraction derived from rat liver was followed at relatively short time intervals. In each experiment it is seen that Ca^{2+} transport was not linear with time, but consisted of two components. Initially Ca^{2+} transport was rapid; this rapid phase progressively diminished so that by 3–5min it had merged into the second slower phase of transport, which continued

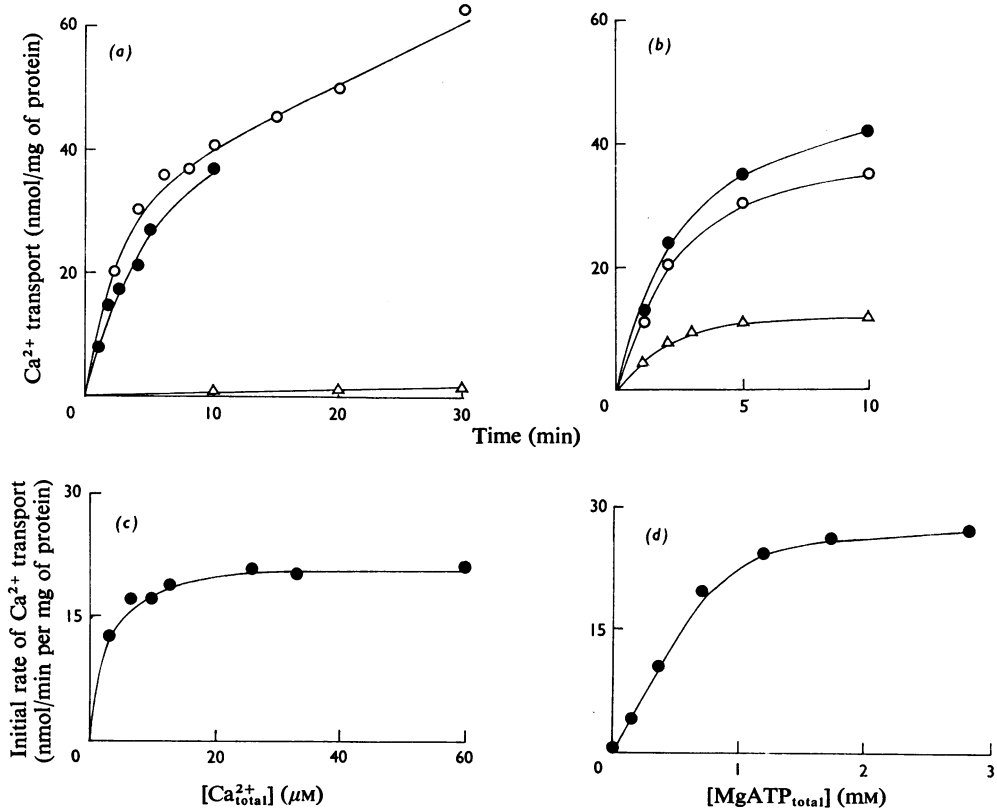

Fig. 1. *Time course of Ca^{2+} transport by rat liver microsomal fractions under various conditions and the affinity of the system for Ca^{2+} and MgATP*

Incubation conditions were as indicated in the Experimental section unless stated otherwise. (*a*), Time course for experiments 1 (○) and 2 (●); △, Mg^{2+} or ATP were omitted separately or together. (*b*) The medium contained 100mM-KCl (●) or 100mM-NaCl (○); △, oxalate was omitted from the medium containing KCl. (*c*) The initial rate was determined in the presence of various concentrations of Ca^{2+}_{total}. (*d*) The initial rate was determined in the presence of various concentrations of $MgATP_{total}$. In all experiments the temperature was 37°C.

unabated for the duration of the incubation. In some experiments the progress of this reaction was followed for up to 40min. Measurement of the initial rate and steady-state rates revealed that the former was some 4- to 5-fold greater, giving rates of about 25nmol of Ca^{2+} transported/min per mg of microsomal protein. These compare with rates of about 10nmol of Ca^{2+}/min

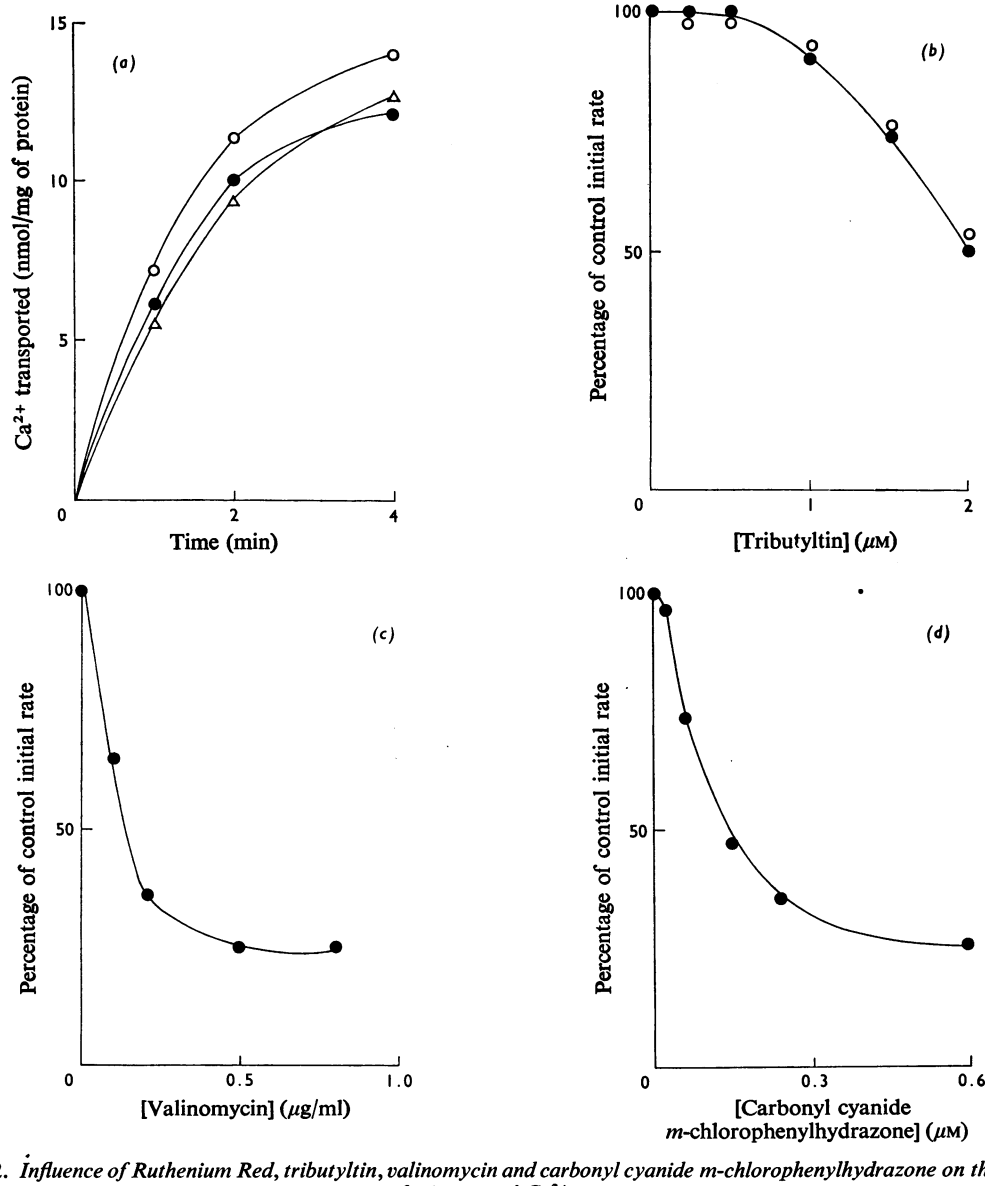

Fig. 2. *Influence of Ruthenium Red, tributyltin, valinomycin and carbonyl cyanide m-chlorophenylhydrazone on the initial rate of microsomal Ca^{2+} transport*

Incubation conditions were as indicated in the Experimental section unless stated otherwise. (*a*) Initial rate of Ca^{2+} transport in the absence (○) or presence of Ruthenium Red at 3nmol/mg of protein (●) or at 5nmol/mg of protein (△). (*b*) Initial rate of Ca^{2+} transport in the presence of various concentrations of tributyltin in a medium containing KCl (●) or KNO_3 (○). (*c*) and (*d*) Initial rates of Ca^{2+} transport in the presence of various concentrations of valinomycin or carbonyl cyanide *m*-chlorophenylhydrazone. In all experiments the temperature was 37°C.

per mg of protein obtained by Moore *et al.* (1974, 1975*a*) and Bruns *et al.* (1976).

Other data in Fig. 1(*a*) show that the initial rate of Ca^{2+} transport was negligible in the absence of Mg^{2+} or ATP or both together. These data confirm knowledge that transport of Ca^{2+} across the reticular membrane requires the energy of MgATP hydrolysis (Martonosi, 1972), and show that the contribution of any energy-independent binding component is minimal.

The experiment of Fig. 1(*b*) was undertaken in part to assess the potential contribution of plasma-membrane vesicles to Ca^{2+} transport by the microsomal fraction. Use was made of the observation that Ca^{2+} transport by plasma-membrane vesicles from rat kidney is greatly decreased in a NaCl medium (Moore *et al.*, 1974). Assuming this argument to be valid also for liver, data in Fig. 1(*b*) would indicate that there was minimal contribution from the plasma-membrane fraction. Work with specific marker enzymes for microsomal fraction and plasma membranes provides further evidence in support of this view (F. L. Bygrave & C. Tranter, unpublished work). Fig. 1(*b*) reveals also that in the absence of oxalate, the initial rate of transport was decreased some 60%, but that of the steady state was decreased to practically zero. Thus the maintenance of the steady-state rate, by contrast with the initial rate, has an obligatory requirement for a permeant Ca^{2+}-sequestering agent inside the vesicles (see also Moore *et al.*, 1975*a*).

Figs. 1(*c*) and 1(*d*) show how the initial rate of Ca^{2+} transport changed when the concentration of total Ca^{2+} or total MgATP in the incubation medium was increased; the values for the K_m are 1–2 μM for Ca^{2+} and about 500 μM for MgATP. The K_m for Ca^{2+}_{total} obtained from steady-state rate measurements ranges from 12 μM in adipocytes (Bruns *et al.*, 1976) to 23 μM in liver (Moore *et al.*, 1975*a*), kidney (Moore *et al.*, 1974) and aorta (Moore *et al.*, 1975*b*). These reports, except that of Bruns *et al.* (1976), indicated too that the K_m for MgATP was greater than 1 mM.

Influence on the initial rate of microsomal Ca^{2+} transport of substances that influence mitochondrial Ca^{2+} transport

These experiments were designed to provide information on the mechanism of microsomal Ca^{2+} transport by utilizing knowledge already gained about the mechanism of mitochondrial Ca^{2+} transport (reviewed by Bygrave, 1977).

Fig. 2(*a*) shows that the initial rate of microsomal Ca^{2+} transport was little affected by concentrations of Ruthenium Red as high as 3–5 nmol/mg of protein. This concentration is some 30-fold in excess of that which produces maximal inhibition of mitochondrial Ca^{2+} transport (Reed & Bygrave, 1974). Thus mitochondria were not considered a serious contaminant of the microsomal preparations used in this work. Further evidence for this stems from the finding that 0.5–1 μM-tributyltin, which promotes a Cl^-/OH^- exchange across the inner mitochondrial membrane (Selwyn *et al.*, 1970) and thereby stimulates by over 100% the initial rate of mitochondrial Ca^{2+} transport (F. L. Bygrave, C. Ramachandran & R. N. Robertson, unpublished work), had no stimulatory effect on microsomal Ca^{2+} transport (Fig. 2*b*). Rather, concentrations above 0.5 μM inhibited in both a Cl^-- and NO_3^--containing medium.

Valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone, ionophores for K^+ and H^+ respectively, both inhibit mitochondrial Ca^{2+} transport by collapsing the $\Delta\psi$ and $-Z\Delta pH$ components of the proton electrochemical gradient (Mitchell & Moyle, 1969). Figs. 2(*c*) and 2(*d*) show that these compounds, at similar low concentrations, also decreased by 70–80% the initial rate of microsomal Ca^{2+} transport.

Discussion

Experiments in the present paper provide new information on several aspects of Ca^{2+} transport by the microsomal fraction of liver that may have relevance also to other mammalian tissues. It is shown firstly that the time course of microsomal Ca^{2+} transport can be described in terms of a rapid initial phase of 3–5 min duration followed by a phase of considerably slower transport that remains linear for up to at least 40 min. It seems quite likely that the initial phase is one in which Ca^{2+} movement into the microsomal vesicles is the predominant direction of Ca^{2+} flux, whereas the subsequent slower phase is one in which a steady-state situation is approached wherein Ca^{2+} efflux takes place together with the continuing influx.

As mentioned in the introduction, few if any of the reports on microsomal Ca^{2+} transport in non-skeletal tissues have used initial-rate measurements in studying the properties of the system; most measurements have been carried out in the steady-state phase, where considerable cycling of the Ca^{2+} most probably occurs. Data in the present paper reveal that the apparent rates of Ca^{2+} transport and the apparent affinities for Ca^{2+} and for MgATP are significantly greater in liver microsomal preparations than previously appreciated, and as such strongly support the view (Moore *et al.*, 1975*a*; Bruns *et al.*, 1976) that the microsomal fraction possesses the ability to play an important role in Ca^{2+} homoeostasis in the cell. Moreover we would strongly argue that serious consideration be given to the routine use of initial-rate measurements in determining the properties of the microsomal Ca^{2+}-transport system in these tissues.

A second consideration emanating from this work relates to the choice of compound needed to quantify the degree to which contaminating mitochondria contribute to Ca^{2+} sequestration by the microsomal fraction. Some laboratories (see Moore *et al.*, 1974, 1975*a*,*b*; Bruns *et al.*, 1976) have used azide, an inhibitor of mitochondrial electron transport. However, the use of this compound is open to criticism, especially as it does not prevent mitochondrial Ca^{2+} transport supported by oligomycin-sensitive ATPase activity. I regard Ruthenium Red to be a better choice, since it has no significant effect on microsomal transport even when present at a concentration at least an order of magnitude higher than that required to maximally inhibit mitochondrial Ca^{2+} transport (Fig. 2*a*; Ash & Bygrave, 1977).

A third point relates to the comparative mechanisms of action of tributyltin on mitochondrial and microsomal Ca^{2+} transport. We have shown that tributyltin in the concentration range 0.5–4μM stimulates the initial rate of mitochondrial Ca^{2+} transport in a Cl^--containing medium by more than 100% (F. L. Bygrave, C. Ramachandran & R. N. Robertson, unpublished work). The present work reveals, on the other hand, that the same compound inhibits microsomal Ca^{2+} transport in both Cl^-- and NO_3^--containing media, a feature clearly reflecting a different mechanism of action. Although it is not possible from this study to assign a molecular basis to its inhibitory effect on microsomal Ca^{2+} transport, the observation furthers knowledge about the potential sites of action of trialkyltin compounds in biological systems (Aldridge, 1958, 1962).

Finally, and in relation to the mechanism of Ca^{2+} transport across the microsomal membrane, it seems significant that very low concentrations of the ionophore valinomycin and the protonophore carbonyl cyanide *m*-chlorophenylhydrazone inhibit by 70–80%, the initial rate of Ca^{2+} transport. The findings suggest that the establishment and maintenance of ion and proton gradients may be as fundamental to the mechanism of Ca^{2+} transport in microsomal fractions as they are to the mechanism of Ca^{2+} transport in mitochondria (Mitchell, 1966).

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References

Aldridge, W. N. (1958) *Biochem. J.* **69**, 367–376
Aldridge, W. N. (1962) *Biochem. J.* **83**, 527–533
Ash, G. R. & Bygrave, F. L. (1977) *FEBS Lett.* **78**, 166–168
Bruns, D. E., McDonald, J. M. & Jarett, L. (1976) *J. Biol. Chem.* **251**, 7191–7197
Bygrave, F. L. (1977) *Curr. Top. Bioenerg.* **6**, 259–318
Farber, J. L., El-Mofty, S. L., Schanne, F. A. X., Aleo, J. J. & Serroni, A. (1977) *Arch. Biochem. Biophys.* **178**, 617–624
Fitzpatrick, D. F., Landon, E. J., Debbas, G. & Hurwitz, L. (1972) *Science* **176**, 305–306
Martonosi, A. (1972) *Curr. Top. Memb. Transp.* **3**, 83–197
Mitchell, P. (1966) *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, Glynn Research, Bodmin
Mitchell, P. & Moyle, J. (1969) *Eur. J. Biochem.* **7**, 471–484
Moore, L., Fitzpatrick, D. F., Chen, T. S. & Landon, E. J. (1974) *Biochim. Biophys. Acta* **345**, 405–418
Moore, L., Chen, T., Knapp, H. R. & Landon, E. J. (1975*a*) *J. Biol. Chem.* **250**, 4562–4568
Moore, L., Hurwitz, L., Davenport, G. R. & Landon, E. J. (1975*b*) *Biochim. Biophys. Acta* **413**, 432–443
Moore, L., Davenport, G. R. & Landon, E. J. (1976) *J. Biol. Chem.* **251**, 1197–1201
Reed, K. C. & Bygrave, F. L. (1974) *Biochem. J.* **140**, 143–155
Selwyn, M. J., Dawson, A. P., Stockdale, M. & Gains, N. (1970) *Eur. J. Biochem.* **14**, 120–126
Watson, E. L. & Siegel, I. A. (1977) *Biochem. Pharmacol.* **26**, 125–127