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Synthesis of Adenosine Triphosphate during Release of Intravesicular and Membrane-Bound Calcium Ions from Passively Loaded Sarcoplasmic Reticulum

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Sarcoplasmic reticulum isolated from rabbit skeletal muscle and incubated in a medium containing Ca^{2+} in the absence of ATP retains intravesicular and/or membrane-bound Ca^{2+}. The synthesis of ATP coupled with the release of intravesicular Ca^{2+} is totally inhibited by the ionophore X-537A. Release of the membrane-bound Ca^{2+}, retained after short periods of incubation (10min) or after release of the intravesicular Ca^{2+} by ionophore X-537A, still supports some synthesis of ATP. The ratios of Ca^{2+} released to ATP synthesized are 2.5–3.2, when bound and intravesicular Ca^{2+} are released simultaneously, and 3.1–4.0, when only bound Ca^{2+} is released. The results show that the synthesis of ATP by sarcoplasmic reticulum during release of passively accumulated Ca^{2+} by EGTA [ethanedioxybis(ethylamine)tetra-acetic acid] is accompanied by a loss of membrane-bound Ca^{2+}.

In a previous paper (Vale & Carvalho, 1975) we distinguished between intravesicular and membrane-bound Ca^{2+} in sarcoplasmic-reticulum vesicles passively loaded with Ca^{2+} by utilizing the ionophore X-537A to discharge the intravesicular Ca^{2+} (Scarpa *et al.*, 1972). We showed that a large fraction of the Ca^{2+} retained by sarcoplasmic reticulum under conditions of passive uptake is bound to the external side of the membrane, and we detected only a small capacity on the internal side of the membrane to bind Ca^{2+}.

It has been reported that reversal of the Ca^{2+} pump (Inesi *et al.*, 1973) and subsequent ATP synthesis (Makinose, 1972; Yamada *et al.*, 1972; Yamada & Tonomura, 1973) may occur during the release of passively loaded Ca^{2+} from sarcoplasmic reticulum provided that ADP and P_i exist in the medium. However, the Ca^{2+} fraction released from sarcoplasmic-reticulum vesicles that is responsible for ATP synthesis has not been determined, although it has been implied that it is the efflux of the intravesicular Ca^{2+} that is responsible for ATP synthesis (Makinose, 1972; Yamada *et al.*, 1972). The ratio of Ca^{2+} released to ATP synthesized by sarcoplasmic-reticulum vesicles passively loaded with Ca^{2+} was reported to be 2 (Yamada *et al.*, 1972), as for ATP synthesis by sarcoplasmic reticulum actively loaded (Makinose & Hasselbach, 1971; Makinose, 1972; Panet & Selinger, 1972).

In the present paper we present data showing that the ionophore X-537A or ADP+P_i liberate the same intravesicular fraction of Ca^{2+} from sarcoplasmic-reticulum vesicles passively loaded with Ca^{2+}, but that ATP synthesis coupled to Ca^{2+} efflux does not occur if compound X-537A is present. Further, the release of externally bound Ca^{2+}, retained after short periods of incubation or after release of intravesicular Ca^{2+} by compound X-537A, may also be associated with the synthesis of ATP.

Methods and Materials

Isolation of sarcoplasmic reticulum

Sarcoplasmic reticulum was isolated from rabbit skeletal muscle as described in an earlier paper (Vale & Carvalho, 1975) and stored at 3°C.

Ca^{2+} release from isolated sarcoplasmic-reticulum vesicles

Sarcoplasmic-reticulum vesicles were incubated as described in the legends of the Figures. The release of Ca^{2+} from the loaded sarcoplasmic-reticulum vesicles was promoted by the addition to the dilution medium of either EGTA* (4mM), EGTA (4mM)/ADP (1mM)/P_i (1mM) or compound X-537A (20μM). The protein (0.5mg) was removed by Millipore filtration (Martonosi & Feretos, 1964) at several time-intervals during Ca^{2+} efflux.

After filtration, the protein-containing filters (Millipore HA, 0.45μm) were washed twice by filtering each time with 1ml of 0.25M-sucrose. They were finally immersed in 2.5ml of a solution containing 2% trichloroacetic acid and 0.5% La^{3+} (as the chloride), which, after vigorous agitation, was analysed for Ca^{2+} and Mg^{2+} by atomic absorp-

* Abbreviations: EGTA, ethanedioxybis(ethylamine)-tetra-acetate; ATPase, adenosine triphosphatase.

tion spectroscopy in a Perkin–Elmer spectrophotometer, model 305 (Carvalho & Leo, 1967).

Coupling between Ca^{2+} release and ATP synthesis in passively loaded sarcoplasmic-reticulum vesicles

Sarcoplasmic reticulum was passively loaded with Ca^{2+} as described in the legends of the Figures. The reaction for ATP synthesis was started by adding either EGTA (4mM) plus ADP (1mM), or compound X-537A (20μM) plus ADP (1mM) followed by EGTA (4mM) in the presence of 0.04mg of hexokinase/ml (Figs. 3 and 4). After 30 and 60s, the reaction was stopped by transferring 1ml portion to 0.1ml of 1.0M-$BaCl_2$. The γ-phosphate of ATP synthesized was trapped as glucose 6-phosphate, which was determined as described by Deamer & Baskin (1972). The radioactivity was measured in a gas-flow Tracerlab counter. Samples (1ml) were also taken from the reaction medium at 30 and 60s for Ca^{2+} analysis.

The protein was determined by the biuret method (Layne, 1957), with bovine plasma albumin as a standard.

Materials

All chemical reagents were of analytical grade. $^{32}P_i$ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and purified by chromatography on a column (1cm × 10cm) of Dowex AG1 resin (X10; 200–400 mesh) previously treated with 1M-NaOH. $^{32}P_i$ was eluted with 40mM-HCl after the column had been washed with deionized water. The ionophore X-537A was kindly supplied by Dr. Julius Berger, Hoffmann–La Roche, Nutley, NJ 07110, U.S.A.

Results

Release of Ca^{2+} from passively loaded sarcoplasmic-reticulum vesicles

Fig. 1 shows that of the 85nmol of Ca^{2+}/mg of protein, corresponding to the total Ca^{2+} retained by sarcoplasmic reticulum after loading overnight in a medium containing 20mM-$CaCl_2$, about 35nmol/mg of protein are released by the addition of EGTA, and this Ca^{2+} release can be increased to 55nmol/mg of protein by the presence of ADP plus P_i in the dilution medium.

Subsequent addition of the ionophore X-537A, after the addition of EGTA alone, releases an additional fraction of Ca^{2+} of about 25nmol/mg of protein. This fraction of Ca^{2+} released by compound X-537A is similar to that released by the ionophore under the control conditions, in which compound X-537A was added before the EGTA. On the other hand, if compound X-537A is added after Ca^{2+} efflux induced by EGTA/ADP/P_i, no more Ca^{2+} is released (Fig. 1).

Thus ADP and P_i in the presence of EGTA liberate the fraction of Ca^{2+} mobilized by compound X-537A

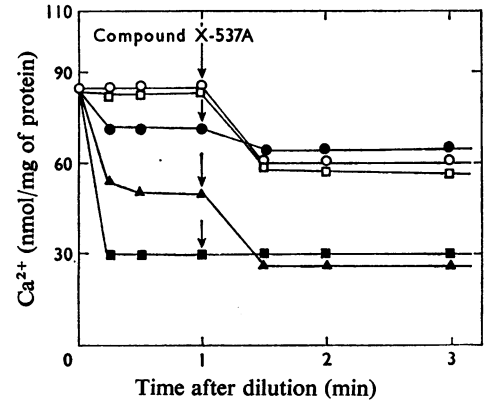

Fig. 1. *Distinction between the fractions of Ca^{2+} released by reversal of the Ca^{2+} pump and by dissociation from the sarcoplasmic-reticulum membrane*

Sarcoplasmic reticulum (10mg/ml) was incubated overnight at 3°C in a medium containing 20mM-Tris/maleate (pH6.9), 100mM-KCl, 2mM-$MgCl_2$ and 20mM-$CaCl_2$. After the incubation period, the mixture was diluted 20-fold with the Ca^{2+}-free reaction medium containing ADP (1mM), ADP (1mM)+P_i (1mM), EGTA (4mM) or EGTA (4mM)+ADP (1mM)+P_i (1mM) against a control without any of these compounds. The protein (0.5mg) was removed by Millipore filtration at 15, 30 or 60s after dilution. Then compound X-537A (20μM) was added to the medium, and portions containing 0.5mg of protein were filtered at 30, 60 and 120s. ○, Simple dilution medium; □, dilution medium containing ADP; ●, dilution medium containing ADP+P_i; ▲, dilution medium containing EGTA; ■, dilution medium containing EGTA+ADP+P_i.

that probably corresponds to the intravesicular Ca^{2+}. However, it is noteworthy that the EGTA also releases the membrane-bound Ca^{2+}, so that after EGTA/ADP/P_i addition the only Ca^{2+} retained by sarcoplasmic-reticulum membranes is the intrinsic Ca^{2+}.

Fig. 2 shows that ADP activates the efflux of intravesicular Ca^{2+} from the passively loaded sarcoplasmic-reticulum vesicles only when P_i is present in the dilution medium, as was also reported for the release of actively transported Ca^{2+} (Barlogie *et al.*, 1971; Masuda & Meis, 1974).

These results indicate that all compounds required for ATP synthesis during release of Ca^{2+} from actively loaded sarcoplasmic reticulum (Barlogie *et al.*, 1971; Masuda & Meis, 1974) are also necessary for the efflux of Ca^{2+} from passively loaded vesicles.

Coupling between Ca^{2+} release and ATP synthesis in passively loaded sarcoplasmic-reticulum vesicles

The results presented in Figs. 1 and 2 indicate that fractions of externally bound and intravesicular Ca^{2+} retained by sarcoplasmic reticulum passively loaded with Ca^{2+} can be released from the vesicles

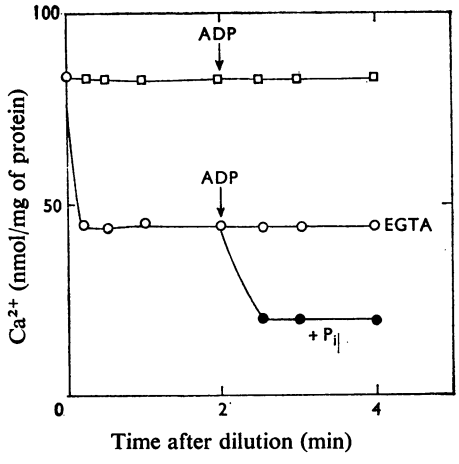

Fig. 2. *Potentiating effect of ADP in the Ca^{2+} release from passively loaded sarcoplasmic reticulum in the presence of phosphate only*

Sarcoplasmic reticulum was incubated as described in Fig. 1. After the incubation period, the mixture was diluted 20-fold with the Ca^{2+}-free reaction medium with and without EGTA or P_i. The protein (0.5mg) was removed by Millipore filtration at 15, 30, 60 and 120s after dilution. ADP (1mM) was added to the diluted medium where indicated, and portions containing 0.5mg of protein were filtered at 30, 60 and 120s. □, Absence of EGTA and P_i; ○, 4mM-EGTA in the absence of P_i; ●, 4mM-EGTA and 1mM-P_i.

by addition of ADP plus P_i in the presence of EGTA. Therefore it is of interest to determine whether both fractions of Ca^{2+} can promote the synthesis of ATP, which has been reported to occur as a result of the apparent reversal of the pump mechanism coupled to the efflux of Ca^{2+} from the loaded vesicles (Makinose & Hasselbach, 1971; Makinose, 1972; Panet & Selinger, 1972; Deamer & Baskin, 1972; Yamada *et al.*, 1972).

Fig. 3(*a*) represents the synthesis of ATP coupled to Ca^{2+} release by EGTA+ADP+P_i from sarcoplasmic reticulum incubated for 10min or 14h in a medium containing 20mM-$CaCl_2$. The amount of ATP synthesized by sarcoplasmic reticulum incubated for 14h is higher (about 15nmol of ATP/mg of protein) than that obtained by sarcoplasmic reticulum incubated for 10min (about 6nmol of ATP/mg of protein).

We have shown that 10min is insufficient for Ca^{2+} penetration into the sarcoplasmic-reticulum vesicles, and that essentially all Ca^{2+} retained after this period is bound to the external side of the vesicles (Vale & Carvalho, 1975). This conclusion is supported by the observation that no more Ca^{2+} is released from the vesicles by compound X-537A added after EGTA, as is observed in sarcoplasmic-reticulum vesicles loaded during 14h of incubation (Fig. 3*b*). However, we observe some synthesis of ATP even when the incubation period is only 10min (Fig. 3*a*), which suggests that simple dissociation of bound Ca^{2+} promotes net synthesis of ATP.

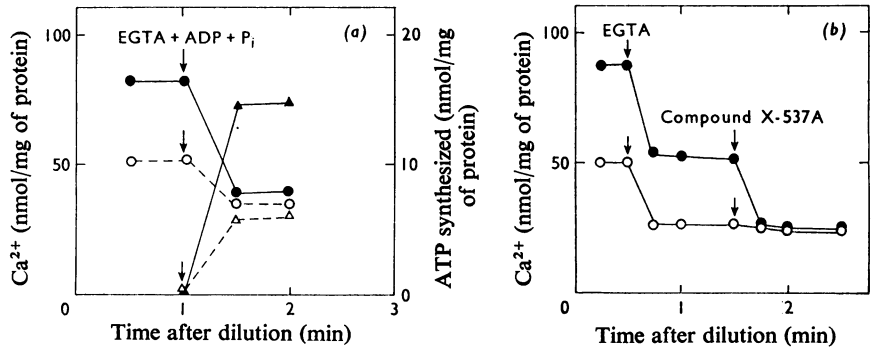

Fig. 3. *Coupling between ATP synthesis and Ca^{2+} release from passively loaded sarcoplasmic reticulum*

(*a*) Sarcoplasmic reticulum was incubated as described in Fig. 1. After 10min or 14h incubation for passive Ca^{2+} uptake, the mixture was diluted 20-fold with Ca^{2+}-free reaction medium containing 100mM-glucose and 1mM-$^{32}P_i$. At 30 and 60s after dilution, 0.5mg of protein was removed by Millipore filtration. Then EGTA (4mM) plus ADP (1mM) in the presence of 0.04mg of hexokinase/ml were added to the reaction medium, and portions containing 0.5mg of protein were filtered at 30 and 60s. Simultaneously, a portion (1ml) was transferred to 0.1ml of 1.0M-$BaCl_2$. After separation of the unchanged $^{32}P_i$, samples were taken for radioactivity measurements. ○, Ca^{2+} retained when sarcoplasmic reticulum was incubated for 10min; ●, Ca^{2+} retained when sarcoplasmic reticulum was incubated for 14h; △, ATP synthesis by sarcoplasmic reticulum incubated for 10min; ▲, ATP synthesis by sarcoplasmic reticulum incubated for 14h. (*b*) After the incubations for 10min and 14h, sarcoplasmic reticulum was diluted 20-fold with Ca^{2+}-free reaction medium, and the protein (0.5mg) was removed by the filtration method. Then EGTA (4mM) was added to the reaction medium, and portions containing 0.5mg of protein were filtered at 15, 30 and 60s. Finally, compound X-537A (20μm) was added to the remaining medium, and 0.5mg of protein was filtered at 15, 30 and 60s. ○, Ca^{2+} retained when sarcoplasmic reticulum was incubated for 10min; ●, Ca^{2+} retained when sarcoplasmic reticulum was incubated for 14h.

Table 1. *Coupling between Ca^{2+} release and ATP synthesis in passively loaded sarcoplasmic-reticulum vesicles*

Sarcoplasmic reticulum was passively incubated for 10min and 14h in a medium as described in Fig. 1. Measurement of the synthesis of ATP coupled with the Ca^{2+} release from the vesicles was carried out as described in Fig. 3.

Expt.	Period of incubation	Total Ca^{2+} retained (nmol/mg)	Ca^{2+} released (nmol/mg)	ATP synthesized (nmol/mg)	Ca^{2+}/ATP ratio
1	10min	55	25	6	3.1
	14h	75	35	14	2.5
2	10min	70	20	5	4.0
	14h	90	40	12	3.2
3	10min	60	20	6	3.3
	14h	80	40	14	2.8

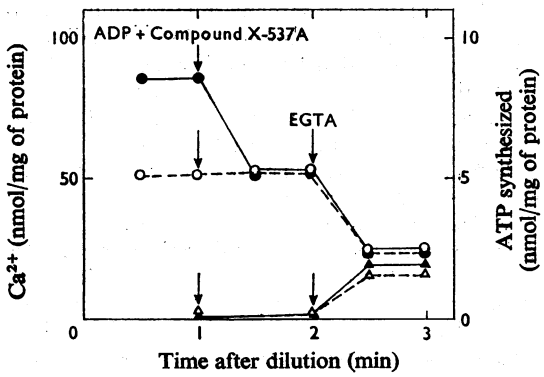

Fig. 4. *Effect of compound X-537A in promoting ATP synthesis by Ca^{2+} discharge from passively loaded sarcoplasmic reticulum*

The reaction was followed as described in Fig. 3. After taking 0.5mg of protein to determine the total amount of Ca^{2+} retained by sarcoplasmic reticulum, ADP (1mM) and compound X-537A (20μM) were added to the medium. At 30 and 60s, samples (1ml) were taken for Ca^{2+} analysis and determination of ATP synthesis by the methods described in Fig. 3. Finally EGTA (3mM) was added to the medium, and the Ca^{2+} released and ATP synthesized were measured after 30 and 60s. ○, Ca^{2+} retained by sarcoplasmic reticulum when incubated for 10min; ●, Ca^{2+} retained by sarcoplasmic reticulum when incubated for 14h; △, ATP synthesis by sarcoplasmic reticulum incubated for 10min; ▲, ATP synthesis by sarcoplasmic reticulum incubated for 14h.

The ratio of Ca^{2+} released to ATP synthesized is summarized in Table 1 for three different experiments in which ATP synthesis and Ca^{2+} released were measured after sarcoplasmic-reticulum vesicles had been loaded with 20mM-Ca^{2+} for 10min or 14h. The ratio of Ca^{2+} released/ATP synthesized varies between 3.1 and 4.0 for sarcoplasmic reticulum equilibrated with 20mM-Ca^{2+} for 10min. A larger amount of ATP is synthesized by sarcoplasmic reticulum loaded with Ca^{2+} for long periods (14h) concurrently with a higher release of Ca^{2+}, which corresponds to intravesicular and membrane-bound Ca^{2+} accumulated after 14h of incubation (Vale & Carvalho, 1975). We found that 2.5–3.2mol of Ca^{2+} are released for each mol of ATP synthesized under these conditions in which both intravesicular and membrane-bound Ca^{2+} are released.

Synthesis of ATP coupled with the dissociation of bound Ca^{2+} is also observed in the results summarized in Fig. 4. We studied here the effect of the ionophore X-537A on the process of ATP synthesis by sarcoplasmic-reticulum vesicles passively loaded with Ca^{2+} during 10min or 14h of incubation in a medium containing 20mM-$CaCl_2$. The movement of Ca^{2+} from inside to outside the vesicles caused by compound X-537A does not promote ATP synthesis, even though intravesicular Ca^{2+} is released from sarcoplasmic reticulum loaded for 14h (Fig. 4). On the other hand when EGTA is added after the ionophore, a small net synthesis of ATP is observed (Fig. 4). We believe that most Ca^{2+} released by EGTA added after compound X-537A represents membrane-bound Ca^{2+}, since the intravesicular Ca^{2+} was liberated previously by the ionophore.

Discussion

The results of the present study confirm that the efflux of Ca^{2+} retained by sarcoplasmic-reticulum vesicles after passive loading can be utilized to synthesize ATP when ADP and P_i are present. Further, it is shown that the fraction of Ca^{2+} bound to the membrane may also result in net synthesis of ATP (Figs. 3 and 4).

The intravesicular Ca^{2+} can be released either by ADP+P_i or compound X-537A, but ATP synthesis, which occurs when ADP+P_i are present, does not take place if compound X-537A is also present (Fig. 4). We suggested earlier that when compound X-537A is present the efflux of the intravesicular Ca^{2+} by-passes the Ca^{2+}-stimulated ATPase system through the ionophore-mediated transport route

(Carvalho & Madeira, 1974). On the other hand, during reversal of the pump, the formation of the high-energy phosphoprotein (E–P) depends on the Ca^{2+} concentration gradient (Makinose, 1972; Meis & Carvalho, 1974), which is destroyed by the ionophore X-537A.

The results summarized in Figs. 3 and 4 clearly show that, under conditions in which only externally bound Ca^{2+} exists, either because the sarcoplasmic reticulum vesicles were incubated for only 10min in a medium of Ca^{2+} (Fig. 3) or because the intravesicular Ca^{2+} was first liberated by compound X-537A (Fig. 4), some ATP synthesis is still observed as the bound Ca^{2+} is released by addition of EGTA. Table 1 gives the results of three different experiments performed with sarcoplasmic-reticulum vesicles containing only externally bound Ca^{2+} (10min incubation) or externally bound plus intravesicular Ca^{2+} (14h incubation). The ratio of Ca^{2+} released to ATP synthesized (Ca^{2+}/ATP) varies between 3.1 and 4.0 when only bound Ca^{2+} is released and between 2.5 and 3.2 when bound and intravesicular Ca^{2+} are released simultaneously. The efflux of actively or passively accumulated Ca^{2+} coupled to ATP synthesis gives a value for the Ca^{2+}/ATP of about 2.0 under conditions in which most Ca^{2+} liberated is intravesicular (Makinose & Hasselbach, 1971; Makinose, 1972; Panet & Selinger, 1972; Yamada *et al.*, 1972). Thus, although the release of bound Ca^{2+} can induce the synthesis of ATP, the efficiency of this Ca^{2+} fraction in promoting ATP synthesis is lower than that of the fraction that comes exclusively from the intravesicular region, as is the case with Ca^{2+} accumulated actively.

The mechanism for the synthesis of ATP during the release of bound Ca^{2+} is not clear. Meis and co-workers (Masuda & Meis, 1973; Meis & Masuda, 1974) reported that $^{32}P_i$ interacts with the sarcoplasmic-reticulum membrane forming a high-energy phosphoprotein (E–P) in the absence of a Ca^{2+} gradient. Further, we also observed (Carvalho *et al.*, 1975) that the addition of EGTA increases the amount of E–P observed in the presence of compound X-537A, probably because the type of E–P (Masuda & Meis, 1973) that is inhibited by Ca^{2+} and does not depend on the Ca^{2+} gradient is formed. The fraction of ATP synthesis that we observed under conditions in which only externally bound Ca^{2+} exists (Fig. 3) may be the result of an interaction of P_i with an external phosphorylating site, forming a phosphoprotein whose energy is derived from a membrane conformational change induced by removal of Ca^{2+} by EGTA addition. Thus, if ADP exists in the medium, net synthesis of ATP is observed by this mechanism which does not depend on the Ca^{2+} gradient across the membrane, as is observed when intravesicular Ca^{2+} passes through the Ca^{2+} pump from inside to outside the vesicles. A mechanism for ATP synthesis depending on the ion–protein interaction has been suggested by Knowles & Racker (1975). They observed net synthesis of ATP by the purified Ca^{2+}-stimulated ATPase, which forms leaky vesicles unable to accumulate Ca^{2+}, and therefore without formation of an ion gradient across the membrane (Knowles & Racker, 1975). The experiments of Fig. 4 do not rule out the possibility that, after the loss of the intravesicular Ca^{2+} in the presence of compound X-537A (Fig. 4), the addition of EGTA to the external medium would create a new Ca^{2+} gradient. However, in preliminary experiments, we have observed that the synthesis of ATP derived exclusively from the reversal of the Ca^{2+} pump (actively loaded sarcoplasmic reticulum) is completely inhibited by compound X-537A even after addition of EGTA, which indicates that the new Ca^{2+} gradient created by EGTA is not enough to promote synthesis of ATP by reversal of the Ca^{2+} pump. Further, the experiments of Fig. 3 show conclusively that ATP is synthesized by the liberation of exclusively membrane-bound Ca^{2+} (sarcoplasmic reticulum incubated for 10min).

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References

Barlogie, B., Hasselbach, W. & Makinose, M. (1971) *FEBS Lett.* **12**, 267–268

Carvalho, A. P. & Leo, B. (1967) *J. Gen. Physiol.* **50**, 1327–1352

Carvalho, A. P. & Madeira, V. M. C. (1974) in *Biomembranes—Lipids, Proteins and Receptors* (Burton, R. & Packer, L., eds.), pp. 347–367, BI-Science Publication Division, Webster Groves, MO

Carvalho, A. P., Vale, M. G. P. & Osório e Castro, V. R. (1975) in *Calcium Transport in Contraction and Secretion* (Carafoli, E., Clementi, F., Drabikowski, W. & Margreth, A., eds.), pp. 349–358, North-Holland Publishing Co., Amsterdam and Oxford

Deamer, D. W. & Baskin, R. J. (1972) *Arch. Biochem. Biophys.* **153**, 47–54

Inesi, G., Millman, M. & Eletr, S. (1973) *J. Mol. Biol.* **81**, 483–504

Knowles, A. F. & Racker, E. (1975) *J. Biol. Chem.* **250**, 1949–1951

Layne, E. (1957) *Methods Enzymol.* **3**, 447–454

Makinose, M. (1972) *FEBS Lett.* **25**, 113–115

Makinose, M. & Hasselbach, W. (1971) *FEBS Lett.* **12**, 271–272

Martonosi, A. & Feretos, R. (1964) *J. Biol. Chem.* **239**, 648–658

Masuda, H. & Meis, L. (1973) *Biochemistry* **12**, 4581–4585

Masuda, H. & Meis, L. (1974) *Biochim. Biophys. Acta* **332**, 313–315

Meis, L. & Carvalho, M. G. C. (1974) *Biochemistry* **13**, 5032–5038

Meis, L. & Masuda, H. (1974) *Biochemistry* **13**, 2057–2062
Panet, R. & Selinger, Z. (1972) *Biochim. Biophys. Acta* **255**, 34–42
Scarpa, A., Baldassare, J. & Inesi, G. (1972) *J. Gen. Physiol.* **60**, 735–749
Vale, M. G. P. & Carvalho, A. P. (1975) *Biochim. Biophys. Acta* **413**, 202–212
Yamada, S. & Tonomura, Y. (1973) *J. Biochem.* (*Tokyo*) **74**, 1091–1096
Yamada, S., Sumida, M. & Tonomura, Y. (1972) *J. Biochem.* (*Tokyo*) **72**, 1537–1548