Biochemical Pharmacology, Vol. 34, No. 24, pp. 4245–4249, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

EFFECTS OF PHENOTHIAZINE DRUGS ON THE ACTIVE Ca^{2+} TRANSPORT BY SARCOPLASMIC RETICULUM

M. G. P. VALE
Center for Cell Biology, Department of Zoology, University of Coimbra, 3049 Coimbra, Portugal

(*Received* 19 *April* 1985; *accepted* 24 *June* 1985)

Abstract—The effect of phenothiazines (trifluoperazine and chlorpromazine) on the activity of the sarcoplasmic reticulum Ca^{2+} pump was investigated. These drugs have a biphasic action on the ATPase activity. They inhibit the enzyme at high concentrations, but below 150 μM, they promote a significant stimulation of the ATP hydrolysis, which is accompanied by a slight increase of the Ca^{2+} transport. Leaking of the membrane occurs only at drug concentrations above 150 μM. The phenothiazine stimulatory effect was not found in the isolated enzyme whose activity was inhibited at all concentrations studied. These results indicate that low concentrations of phenothiazines uncouple the sarcoplasmic reticulum Ca^{2+} pump without disrupting the membrane and that the drug stimulatory effect on the ATPase is not due to a direct interaction with the enzyme. It is suggested that a coupling factor or a specific microenvironment surrounding the enzyme regulates the association between the Ca^{2+} transport and the ATP hydrolysis by sarcoplasmic reticulum of the skeletal muscle cell.

Phenothiazine drugs have been extensively used as indicators of calmodulin-mediated processes [1]. However, several observations indicate that these drugs may act independently of any inhibition of calmodulin function [2–6].

Luthra [7] showed that trifluoperazine inhibits calmodulin-sensitive Ca^{2+}-ATPase and calmodulin-insensitive ($Na^+ + K^+$)-ATPase of erythrocyte membranes, suggesting that phenothiazines cannot be assumed as selective inhibitors of calmodulin interactions with its effectors.

The effect of phenothiazines on the Ca^{2+} uptake by sarcoplasmic reticulum isolated from skeletal muscle has been studied by several investigators [8–14], but the conclusions are still controversial.

Volpe *et al.* [13] have recently suggested that phenothiazines inhibit sarcoplasmic reticulum Ca^{2+} uptake by direct interaction with the pump, whereas Ho *et al.* [12] reported that the effect of trifluoperazine is principally due to structural perturbations produced by partitioning of the drug into the lipid phase of the sarcoplasmic reticulum membrane. On the other hand, Chiesi and Carafoli [10] have attributed the inhibition of Ca^{2+} uptake and ($Ca^{2+} + Mg^{2+}$)-ATPase activity by trifluoperazine to its interaction with an intrinsic glycoprotein of the sarcoplasmic reticulum. Finally, Campbell and MacLennan [11] reported that trifluoperazine reduces the efficiency of the Ca^{2+} pump by inhibiting the calmodulin-dependent phosphorylation of a minor protein component (~ 60,000 Daltons) of the sarcoplasmic reticulum membrane.

In this paper, it is reported that, under certain conditions, trifluoperazine and chlorpromazine uncouple the Ca^{2+} uptake and the ATPase activity of the sarcoplasmic reticulum without disrupting the membrane. It is suggested that phenothiazines alter the regulatory mechanism of the skeletal sarcoplasmic reticulum Ca^{2+} pump.

MATERIALS AND METHODS

Isolation of sarcoplasmic reticulum. Sarcoplasmic reticulum was isolated from rabbit white skeletal muscle as previously described [15].

The protein was determined by the biuret method using bovine serum albumin as standard [16].

Purification of sarcoplasmic reticulum ($Ca^{2+} + Mg^{2+}$)*-ATPase.* The ($Ca^{2+} + Mg^{2+}$)-ATPase was isolated from sarcoplasmic reticulum membranes, essentially, by using the method of Warren *et al.* [17].

Sarcoplasmic reticulum vesicles were previously washed with 1 mM EDTA and resuspended in a medium containing 0.25 M sucrose, 50 mM KH_2PO_4 (pH 7.3), 1 M KCl and 2.5 mM dithiothreitol. The EDTA treatment removed most of the extrinsic proteins from the membranes [18] which were further incubated 5 min with sodium deoxycholate (0.4 mg/mg protein). After centrifuging the mixture at 18,000 *g* for 1 hr at 4°, 3 ml aliquots of the supernatant were layered on a 20 ml sucrose gradient (20–60%, p/v) in 50 mM KH_2PO_4 (pH 7.3), 1 M KCl and 2.5 mM dithiothreitol. This system was centrifuged at 60,000 *g* for 24 hr at 4° and the purified ATPase was obtained from the upper part of a high molecular weight band. A small amount of low molecular weight proteins, which were not completely removed by EDTA treatment, remained in the detergent layer.

Measurement of active Ca^{2+} *uptake by sarcoplasmic reticulum vesicles.* Active Ca^{2+} uptake by sarcoplasmic reticulum was measured by the isotopic method [19]. The reticulum vesicles (2 mg) were incubated at 25° in a medium containing 5 mM Tris–HCl, 40 mM KCl, 5 mM $MgCl_2$, 70 μM [Ca^{45}]-$CaCl_2$, 1 mM ATP and various concentrations of trifluoperazine or chlorpromazine (if present) in a total volume of 3 ml at the pH value of 7.0. At different

time intervals after addition of ATP, 0.14 mg of protein were removed from the medium by filtering through glass microfiber filters (Whatman GF/B) prewashed with 4 ml of 0.32 M sucrose buffered with 10 mM Tris–HCl, pH 7.4. After filtration of the samples in a vacuum pump apparatus, the filters were washed again with 4 ml of the same solution. Blanks without protein were treated the same way as the samples.

The dried filters were placed in vials containing 6 ml of scintillation fluid composition per l. of toluene: 7.3 g 2,5-diphenyloxazole (PPO), 176 mg *p*-bis-[2-(5-phenyloxazolyl)] benzene (POPOP) and 250 ml Triton X-100 and the radioactivity was counted in a Packard TriCarb liquid scintillation spectrophotometer, model 460-CD. The quenching of radioactivity in the samples was corrected by using the external standard technique described in the manufacturer's instructions.

Measurement of $(Ca^{2+} + Mg^{2+})$*-ATPase activity of sarcoplasmic reticulum.* The ATPase activity was assayed by following the liberation of H^+ associated with the ATP hydrolysis [20–22]. Sarcoplasmic reticulum membranes (500 μg) or purified ATPase (100 μg) were incubated in a medium containing 5 mM Tris–HCl, 40 mM KCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM EGTA and various concentrations of trifluoperazine or chlorpromazine (if present) in a total volume of 3 ml at the pH of 7.0.

The reaction was started by adding 0.5 mM ATP previously adjusted at pH 7.0. At this pH, the hydrolysis of 1 mol ATP corresponds to the liberation of 0.75 mol of H^+.

In all cases, the reaction was carried out in a constantly stirred reaction mixture thermostatted at 25°.

Reagents. All reagents were analytical grade. Trifluoperazine was obtained from Smith Kline & French, Philadelphia, PA, U.S.A. Chlorpromazine was supplied by Sigma Chemical Company, St. Louis, MO, U.S.A.

RESULTS

Effect of trifluoperazine and chlorpromazine on the $(Ca^{2+} + Mg^{2+})$*-ATPase activity of sarcoplasmic reticulum membranes*

The phenothiazine drugs trifluoperazine and chlorpromazine, which have been described as calmodulin antagonists [1], have a biphasic action on the activity of the $(Ca^{2+} + Mg^{2+})$-ATPase of the sarcoplasmic reticulum.

Figure 1 shows that the activity of the $(Ca^{2+} + Mg^{2+})$-ATPase is stimulated as the concentration of trifluoperazine or chlorpromazine increases up to about 150 μM. Above these concentrations, both drugs inhibit the ATP hydrolysis. However, the inhibitory effect of trifluoperazine is more potent than that of chlorpromazine. At 300 μM of trifluoperazine, the $(Ca^{2+} + Mg^{2+})$-ATPase is completely inhibited, whereas 750 μM of chlorpromazine is required for maximal inhibition of the enzyme activity.

At low concentrations of phenothiazines (< 150 μM), which stimulate the $(Ca^{2+} + Mg^{2+})$-ATPase, the rate of ATP hydrolysis increases from about 485 nmol of ATP split per mg of protein per minute (absence of drugs) to a maximal value of about 850 or 1200 nmol of ATP split per mg of protein per min in the presence of chlorpromazine or trifluoperazine, respectively. Furthermore, the concentration of phenothiazines, which activates the $(Ca^{2+} + Mg^{2+})$-ATPase activity by 50% of the maximum, is about 95 μM in the case of chlorpromazine and 75 μM in the case of trifluoperazine.

These results indicate that both drugs have similar qualitative effects on the activity of the ATPase, but chlorpromazine is less effective than trifluoperazine to induce either stimulation or inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase activity of the sarcoplasmic reticulum. These effects are observed at concentrations of free Ca^{2+} in the medium up to 100 μM (results not shown).

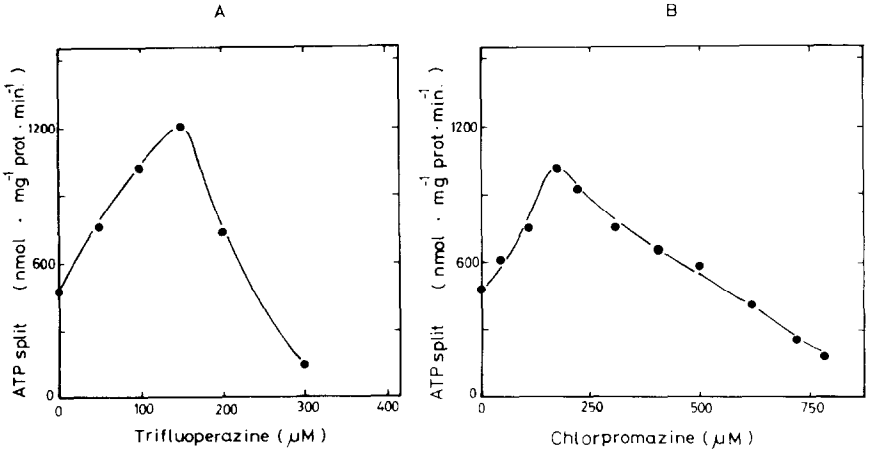

Fig. 1. Effect of trifluoperazine and chlorpromazine on the $(Ca^{2+} + Mg^{2+})$-ATPase activity by sarcoplasmic reticulum. The experiments were carried out in a medium as described in Materials and Methods. The reaction was started by adding 1 mM ATP and the H^+ release associated with the ATP hydrolysis was followed by using a pH meter connected to a Perkin–Elmer recorder. (A) ATPase activity in the presence of various concentrations of trifluoperazine. (B) ATPase activity in the presence of various concentrations of chlorpromazine.

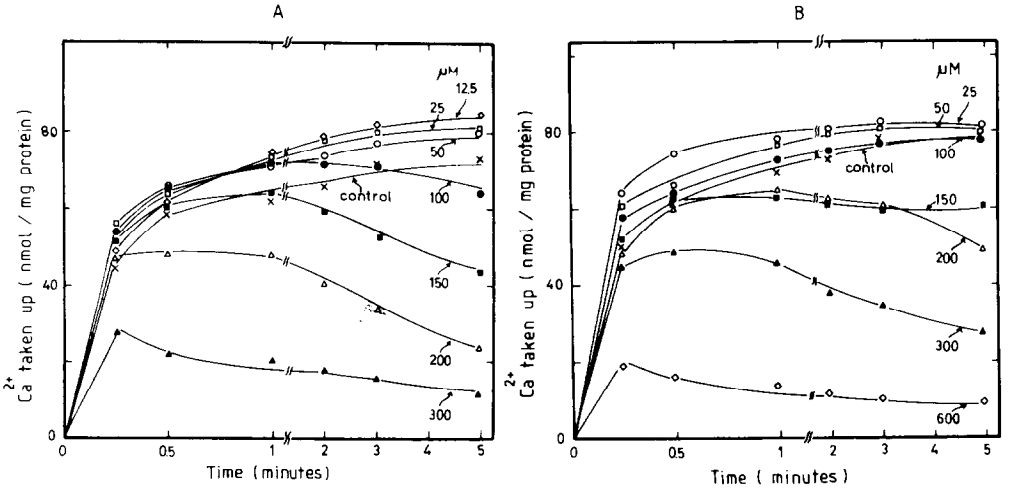

Fig. 2. Effect of trifluoperazine and chlorpromazine on the active Ca^{2+} accumulation by sarcoplasmic reticulum. The experiments were carried out as described in Materials and Methods. The reaction was started by adding 1 mM ATP and at several time intervals, it was stopped by the filtration method. (A) Time course of Ca^{2+} accumulation in the presence of various concentrations (12.5–300 μM) of trifluoperazine. (B) Time course of Ca^{2+} accumulation in the presence of various concentrations (25–600 μM) of chlorpromazine.

Effect of trifluoperazine and chlorpromazine on the active Ca^{2+} uptake by sarcoplasmic reticulum vesicles

Since chlorpromazine and trifluoperazine alter the ATPase activity of sarcoplasmic reticulum, the present work studied whether these drugs have similar effects on the Ca^{2+} accumulation within the sarcoplasmic reticulum vesicles.

Figure 2 shows that concentrations of phenothiazines, which stimulate the ATPase activity, slightly increase the Ca^{2+} transport rate by sarcoplasmic reticulum and the total amount of Ca^{2+} accumulated within the vesicles. Under these conditions, the Ca^{2+} taken up (~ 80 nmol/mg protein) is retained after long periods of incubation with the drugs (5 min), which indicates that sarcoplasmic reticulum membranes remains impermeable to Ca^{2+} ions. Ca^{2+} release is observed only at concentrations of phenothiazines above 100–150 μM, which, probably, induce disruption of the membrane. At the highest drug concentration studied (300 μM trifluoperazine or 600 μM chlorpromazine), the amount of Ca^{2+} accumulated by sarcoplasmic reticulum vesicles is significantly reduced (~ 10–20 nmol/mg protein). This effect reflects leaking of the membrane and also inhibition of the Ca^{2+} pump activity, according to the inhibition of the ATPase activity observed in Fig. 1.

These results show that both phenothiazines have similar effects on the activity of the sarcoplasmic reticulum Ca^{2+} pump, but trifluoperazine is effective at lower concentrations than those of chlorpromazine. Accurate values for Ca^{2+}/ATP ratio could not be calculated because the filtration technology is not rapid enough to detect the initial rate of Ca^{2+} uptake by sarcoplasmic reticulum in the absence of precipitating agents such as oxalate [20]. However, it appears that phenothiazine-stimulated ATP hydrolysis greatly exceeds the phenothiazine-stimulated Ca^{2+} uptake, which indicates that the Ca^{2+} pump system is uncoupled by the drugs.

Effect of phenothiazines on the activity of the (Ca^{2+} + Mg^{2+})-ATPase isolated from sarcoplasmic reticulum membranes

The activity of the purified ATPase is significantly higher than that observed in native membranes (Fig. 3).

In the absence of phenothiazines, about 3125 nmol of ATP are hydrolyzed per mg of purified ATPase per min, whereas in membranes, values of about 606 nmol of ATP split per mg of ATPase per minute were observed. This value, which is expressed per mg of ATPase, was corrected from the experimental value (485 nmol) expressed per mg of membrane protein, assuming that 80% of the total protein in sarcoplasmic reticulum is ATPase [23].

In native membranes, the (Ca^{2+} + Mg^{2+})-ATPase is not fully active probably because it is regulated by several factors such as Ca^{2+} concentration [24, 25], lipid–protein interactions [26, 27] and regulatory proteins [10, 11].

The stimulatory effect of phenothiazines on the ATPase activity of native membranes increases significantly the values of ATP hydrolyzed per mg protein per minute (Fig. 1), but the full activity (~ 3125 nmol/mg protein/min) is not reached (Fig. 3). Under these conditions, concentrations of phenothiazines, which are stimulatory of the ATPase in native membranes, inhibit the activity of the purified ATPase, in agreement with the results of other investigators who found that trifluoperazine inhibits Ca^{2+}-ATPase systems under conditions not mediated by calmodulin [28].

These results indicate that the drug stimulatory effect on the ATPase of sarcoplasmic reticulum is not due to a direct interaction with the enzyme.

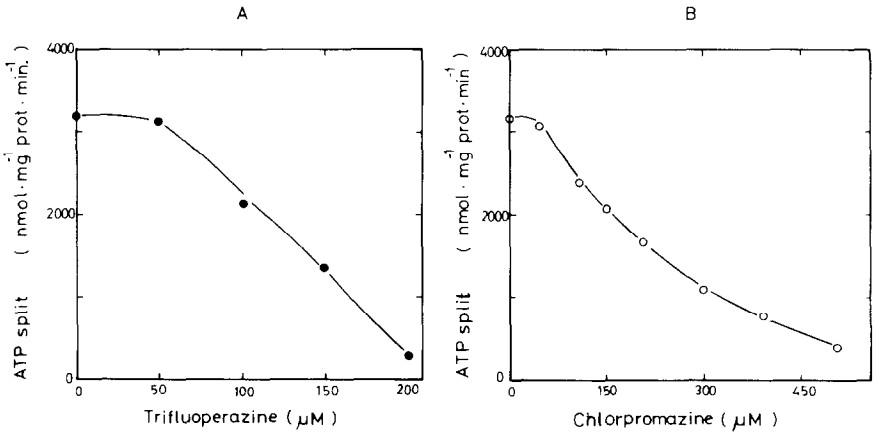

Fig. 3. Effect of trifluoperazine and chlorpromazine on the $(Ca^{2+} + Mg^{2+})$-ATPase activity isolated from sarcoplasmic reticulum membranes. The experiments were carried out as described in Fig. 1 except that 100 μg of protein was used instead 500 μg. (A) ATPase activity in the presence of various concentrations of trifluoperazine. (B) ATPase activity in the presence of various concentrations of chlorpromazine.

DISCUSSION

This work describes how trifluoperazine and chlorpromazine have a biphasic action on the activity of the sarcoplasmic reticulum $(Ca^{2+} + Mg^{2+})$-ATPase. They inhibit the enzyme at high concentrations, but below 150 μM, they promote a significant stimulation of the ATP hydrolysis.

The phenothiazines' stimulatory effect described here has not been detected in experiments recently reported by several investigators [10–14]. Some of them observed that low concentrations of trifluoperazine strongly inhibit the activity of the sarcoplasmic reticulum Ca^{2+} pump in a Ca^{2+}-dependent way [10, 12]. The inhibition is prevented by increasing the Ca^{2+} concentration in the medium, which indicates that the main effect of the drug is a shift of the $(Ca^{2+} + Mg^{2+})$-ATPase from a high to a low affinity form [10, 12].

In the experiments reported here, concentrations of Ca^{2+} which saturate the ATPase system were used, so that the inhibitory effect promoted by low concentrations of phenothiazines (< 100 μM) was not observed. These concentrations of phenothiazines were even stimulatory for the ATPase enzyme, in agreement with previous observations reported by Duggan [8, 9]. He found that phenothiazines stimulate the active Ca^{2+} transport, but this effect is not observed in the presence of 100 mM KCl [8]. Therefore, it appears that potassium concentration is another factor which influences the effect of phenothiazines on the activity of the sarcoplasmic reticulum Ca^{2+} pump [8]. On the other hand, Ho *et al.* [12] found that, at a given concentration of phenothiazines, the magnitude of the effect depends on the concentration of the sarcoplasmic reticulum protein. All these factors are, probably, the main reason for the apparent discrepant results reported by several investigators [10–14].

The stimulatory effect of phenothiazines on the sarcoplasmic reticulum $(Ca^{2+} + Mg^{2+})$-ATPase was suspected to be due to drug-induced leaking of the vesicles, so that Ca^{2+} should flow out through the membrane, stimulating continuously the ATPase system [29]. However, this hypothesis was ruled out since concentrations of phenothiazines, which stimulate the ATPase activity, do not promote release of the Ca^{2+} accumulated within the vesicles (Fig. 2). Instead, the total amount of Ca^{2+} accumulated is slightly increased (Fig. 2), according to the findings of Seeman [30], who suggested that low concentrations of phenothiazines have a 'stabilizing effect' on the biological membranes.

The results reported here also show that concentrations of phenothiazines up to about 100 μM stimulate the rate of Ca^{2+} uptake by sarcoplasmic reticulum. However, the magnitude of the ATPase stimulation is much higher than that of the Ca^{2+} uptake, which indicates that, under these conditions, the Ca^{2+} pump is uncoupled without leaking of the membrane. It was observed that only concentrations of phenothiazines above 100–150 μM disrupt the membranes liberating the Ca^{2+} accumulated (Fig. 2).

The stimulatory effect of low concentrations of phenothiazines was not found in the isolated enzyme whose activity was inhibited at all concentrations studied. This indicates that the drug stimulatory effect on the sarcoplasmic reticulum ATPase is not due to a direct interaction with the enzyme.

In native membranes, the Ca^{2+}-stimulated ATPase is not fully active as it is in the purified state. Trifluoperazine and chlorpromazine increase significantly the rate of ATP hydrolysis, but the values obtained for maximal stimulation do not reach those obtained with the purified enzyme. It appears, therefore, that the activity of the sarcoplasmic reticulum Ca^{2+}-ATPase is membrane controlled.

Results obtained by several investigators indicate that various factors are, probably, involved in the regulation of the enzyme: (1) it is well established that the $(Ca^{2+} + Mg^{2+})$-ATPase activity is 'back inhibited' by high intravesicular Ca^{2+} concentration [24, 25]; (2) membrane fluidity also modulates the sarcoplasmic reticulum Ca^{2+} pump, as it was sug-

gested by Almeida *et al.* [27]; and (3) regulatory proteins present in the sarcoplasmic reticulum membrane have been recently reported as important Ca^{2+} pump modulators [10, 11].

It is generally accepted that exogenous calmodulin does not stimulate the $(Ca^{2+} + Mg^{2+})$-ATPase activity of the skeletal sarcoplasmic reticulum [10, 31, 32]. Nevertheless, significant amounts of endogenous calmodulin have been found by various investigators, but its function in the sarcoplasmic reticulum membrane is unknown [10, 32].

The inhibitory effect of trifluoperazine and chlorpromazine reported here is not indicative of calmodulin involvement, since it is observed only at concentrations of drugs above 100 μM, which are characteristic of nonspecific enzyme inhibition by phenothiazines [33].

On the other hand, the stimulatory effect of low concentrations of phenothiazines on the $(Ca^{2+} + Mg^{2+})$-ATPase activity may reflect structural perturbations of the membrane lipid phase or it indicates an indirect effect of the drugs on a regulatory factor of the coupling between the Ca^{2+} transport and the ATPase activity of sarcoplasmic reticulum. We are currently exploring whether this regulatory factor exists and whether it corresponds to the phenothiazine-sensitive glycoprotein described by Chiesi and Carafoli [10].

The glycoprotein-mediated effect of phenothiazines on the sarcoplasmic reticulum Ca^{2+} pump is compatible with previous observations that positively charged drugs such as chlorpromazine interact strongly with the negatively charged sialic acid moieties of glycoproteins on membrane surfaces [34].

Acknowledgements—This work was supported by grants from the National Institute of Scientific Research (INIC) of the Portuguese Ministry of Education. Part of these results were presented at the 16th Federation of European Biochemical Societies Meeting, Moscow, U.S.S.R, 1984 [35].

REFERENCES

1. B. Weiss and T. L. Wallace, in *Calcium and Cell Function* (Ed. W. Y. Cheung), Vol. 1, p. 329. Academic Press, New York (1980).
2. Y. Landry, M. Amellal and M. Ruckstuhl, *Biochem. Pharmac.* **30**, 2031 (1981).
3. A. N. Corps, T. R. Hesketh and J. C. Metcalfe, *Fedn. Eur. Biochem. Soc. Lett.* **138**, 280 (1982).
4. M. Hirata, E. Suematsu and T. Koga, *Biochem. biophys. Res. Commun.* **105**, 1176 (1982).
5. M. G. P. Vale, A. J. M. Moreno and A. P. Carvalho, *Biochem. J.* **214**, 929 (1983).
6. F. F. Vincenzi, E. S. Adunyah, V. Niggli and E. Carafoli, *Cell Calcium* **3**, 545 (1982).
7. M. G. Luthra, *Biochim. biophys. Acta* **692**, 271 (1982).
8. P. F. Duggan, *Eur. J. Pharmac.* **13**, 381 (1971).
9. M. A. O'Callaghan and P. F. Duggan, *Biochem. Pharmac.* **24**, 563 (1975).
10. M. Chiesi and E. Carafoli, *J. biol. Chem.* **257**, 984 (1982).
11. K. P. Campbell and D. H. MacLennan, *J. biol. Chem.* **257**, 1238 (1982).
12. M. M. Ho, D. J. Scales and G. Inesi, *Biochim. biophys. Acta* **730**, 64 (1983).
13. P. Volpe, B. Costello, A. Chu and S. Fleischer, *Archs. Biochem. Biophys.* **233**, 174 (1984).
14. K. W. Anderson, R. J. Coll and A. J. Murphy, *J. biol. Chem.* **259**, 11487 (1984).
15. M. G. P. Vale and A. P. Carvalho, *Biochim. biophys. Acta* **413**, 202 (1975).
16. E. Layne, *Meth. Enzym.* **3**, 447 (1957).
17. G. B. Warren, P. A. Toon, N. J. M. Birdsall, A. G. Lee and J. C. Metcalfe, *Proc. natn. Acad. Sci. U.S.A* **71**, 622 (1974).
18. P. F. Duggan and A. Martonosi, *J. gen Physiol.* **56**, 147 (1970).
19. A. Martonosi and R. Feretos, *J. biol. Chem.* **239**, 648 (1964).
20. A. Martonosi and R. Feretos, *J. biol. Chem.* **239**, 659 (1964).
21. D. W. Deamer, *J. biol. Chem.* **248**, 5477 (1973).
22. V. M. C. Madeira, M. C. Antunes-Madeira and A. P. Carvalho, *Biochem. biophys. Res. Commun.* **58**, 897 (1974).
23. G. Meissner, G. E. Conner and S. Fleischer, *Biochim. biophys. Acta* **298**, 246 (1973).
24. M. Makinose and W. Hasselbach, *Biochem. Z.* **343**, 360 (1965).
25. A. Weber, *J. gen. Physiol.* **57**, 50 (1971).
26. J. P. Bennett, K. A. McGill and G. B. Warren, *Curr. Top. Membr. Transp.* **14**, 127 (1980).
27. L. M. Almeida, W. L. C. Vaz, K. A. Zachariasse and V. M. C. Madeira, *Biochemistry* **23**, 4714 (1984).
28. F. F. Vincenzi, E. S. Adunyah, V. Niggli and E. Carafoli, *Cell Calcium* **3**, 545 (1982).
29. A. P. Carvalho and V. M. C. Madeira, in *Biomembranes—Lipids, Proteins and Receptors* (Eds. R. M. Burton and L. Packer), p. 347, Webster Groves, Missouri (1974).
30. P. Seeman, *Pharmac. Rev.* **24**, 583 (1972).
31. E. Carafoli, *Cell Calcium* **2**, 353 (1981).
32. B. Eibschutz, A. P. G. Wong, G. D. Lopaschuk and S. Katz, *Cell Calcium* **5**, 391 (1984).
33. B. Weiss, *Meth. Enzym.* **102**, 171 (1983).
34. G. V. R. Born and G. M. Housley, *Br. J. Pharmac.* **79**, 481 (1983).
35. M. G. P. Vale, *16th Federation European Biochemical Societies Meeting*, p. 310 (1984).