Biochemical Pharmacology, Vol. 25, pp. 21–25. Pergamon Press, 1976. Printed in Great Britain.

MECHANISM OF QUINIDINE AND CHLORPROMAZINE INHIBITION OF SARCOTUBULAR ATPase ACTIVITY*

DAVID C. PANG and F. NORMAN BRIGGS
Department of Physiology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Va. 23298, U.S.A.

(*Received* 1 *February* 1975; *accepted* 21 *March* 1975)

Abstract—Quinidine and chlorpromazine in concentrations ranging from 0·25 to 1·2 mM inhibited sarcotubular ATPase activity by distinctly different mechanisms. The noncompetitive inhibition produced by quinidine was entirely due to its effects on hydrolysis of the phosphorylated intermediate. Formation of the phosphorylated intermediate from γ-AT^{32}P was unaffected by quinidine. In contrast to these results, chlorpromazine was found to have no effect on the hydrolysis of phosphorylated intermediate but to depress its levels. Using the β,γ-methylene analogue of ATP, it was possible to show that chlorpromazine lowers the phosphorylated intermediate levels by depressing the affinity of the enzyme for its substrate. Chlorpromazine inhibition of substrate binding was non-competitive.

The sarcotubular catalyzed hydrolysis of ATP involves lipid-independent and -dependent phases. It is the formation of the phosphorylated intermediate which can progress independently of lipid [1, 2]. The hydrolysis of the intermediate, on the other hand, requires phospholipid [1]. Although numerous drugs are known to depress sarcotubular ATPase activity [3], little attempt has been made to determine whether they act on lipid-dependent or -independent steps, although both the understanding of drug action and the mechanism of sarcotubular catalysis of ATP would be increased if drugs were found which would act on one class of steps or the other. An example of the use of a drug to study the mechanism of ATP hydrolysis, once the phase on which it acts is known, has been demonstrated by Pang *et al.* [4], who used their observation [5] that propranolol blocks ATP breakdown by interfering with the hydrolysis of the phosphorylated intermediate to demonstrate that an ATP-induced conformational change in sarcoplasmic reticulum membrane structure depends upon the hydrolysis of the phosphorylated intermediate rather than its formation. Since most, if not all, drug inhibitors of sarcotubular ATPase activity are amphoteric with partition coefficients favoring distribution into lipid, one might predict that the action of all would be limited to the lipid-dependent phase of ATP hydrolysis. The object of the present study was to find a drug which would disprove this prediction and provide a tool for probing the steps involved in the formation of the phosphorylated intermediate. The drugs chosen were quinidine and chlorpromazine. Quinidine was chosen because of its ability to depress the phosphorylated intermediate levels in the ($Na^+ + K^+$) ATPase [6]. Chlorpromazine was chosen because of its demonstrated ability to inhibit enzymes whose catalytic activities do not depend upon lipid. Glutamate dehydrogenase [7] and muscle aldolase [8] have been shown to be inhibited by chlorpromazine.

* A preliminary report has been presented at the Sixth Annual Meeting of the International Study Group for Research in Cardiac Metabolism in Freiburg, Germany, 1973.

METHODS AND MATERIALS

Cardiac sarcoplasmic reticulum was prepared from canine ventricular muscle as described by Pang and Briggs [9]. γ-AT^{32}P prepared by Amersham–Searle was used to measure ATPase activity and to form the ^{32}P intermediate of ATP hydrolysis [9]. The ^{32}P content of the phosphorylated intermediate was determined as described by Pang and Briggs [9]. Isolation of the phosphorylated intermediate from substrate and reaction products was also carried out with cardiac sarcoplasmic reticulum as described previously [9]. Inorganic ^{32}Pi was extracted by the method of Wahler and Wollenberger [10] and counted by liquid scintillation. Neither quinidine nor chlorpromazine interfered with the extraction. β,γ-Methylene [8-^{3}H]ATP was obtained from Amersham–Searle. The unlabeled analogue was obtained from Sigma. The free analogue was separated from the bound by a centrifugation technique similar to that described by Fuchs and Briggs [11]. Binding was measured from the loss of analogue in the supernatant.

RESULTS

Although quinidine [12, 13] and chlorpromazine [14] have been shown to be inhibitors of sarcotubular ATPase, the conditions used to demonstrate inhibition were quite different from those we use to analyze the formation and breakdown of the phosphorylated intermediate. Our first experiments (Fig. 1) were thus designed to determine if these drugs are inhibitors of ATPase activity under the conditions we use to study the formation and breakdown of the intermediate. The conditions employed are shown in the legend to Fig. 1. Both drugs were effective inhibitors at concentrations ranging from 0·3 to 2·4 mM.

The next series of studies was designed to determine if either drug affects intermediate level. Calcium (5 mM) was used to promote the formation of the phosphorylated intermediate and inhibit its breakdown [9]. Figure 2a shows that 1 mM quinidine had no effect on the phosphorylated intermediate level,

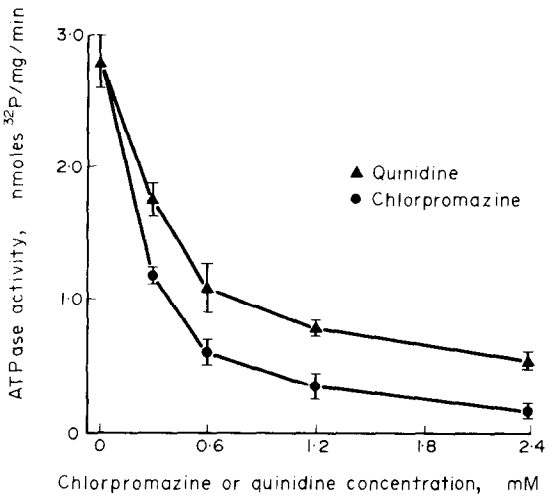

Fig. 1. Effect of chlorpromazine and quinidine on Ca-ATPase activity of cardiac sarcoplasmic reticulum. Quinidine (▲), chlorpromazine (●). Sarcoplasmic reticulum (0·8 to 1·0 mg/ml) was incubated with 2 μM γ-AT^{32}P in the presence of 5 mM $MgCl_2$, 10 mM imidazole, pH 7·0, 50 mM KCl and 10 mM sodium azide at 2°. The liberated ^{32}Pi at 0·2 min was extracted and measured by liquid scintillation. Values are expressed as mean ± S.E.M.

while Fig. 2b shows that chlorpromazine in concentrations ranging from 0·25 to 5 mM increasingly depressed the phosphorylated intermediate levels. The study presented in Fig. 2b also indicates that the effect of chlorpromazine was not due simply to a decrease in the rate of formation of the phosphorylated intermediate, for the intermediate levels did not change significantly between 0·2 and 1·8 min. This suggests that the reaction had virtually come to equilibrium by 0·2 min and that chlorpromazine shifts the equilibrium between substrates and products.

The results presented in Figs. 1 and 2 suggest that the inhibition of ATPase activity by quinidine is due to an effect on the breakdown of the phosphorylated intermediate, while that by chlorpromazine is due to some step involved in the formation of the intermediate. In order to test these conclusions, phosphorylated sarcoplasmic reticulum was isolated [9], and the effects of quinidine and chlorpromazine on the rate constants for its hydrolysis (K_d) were determined. The

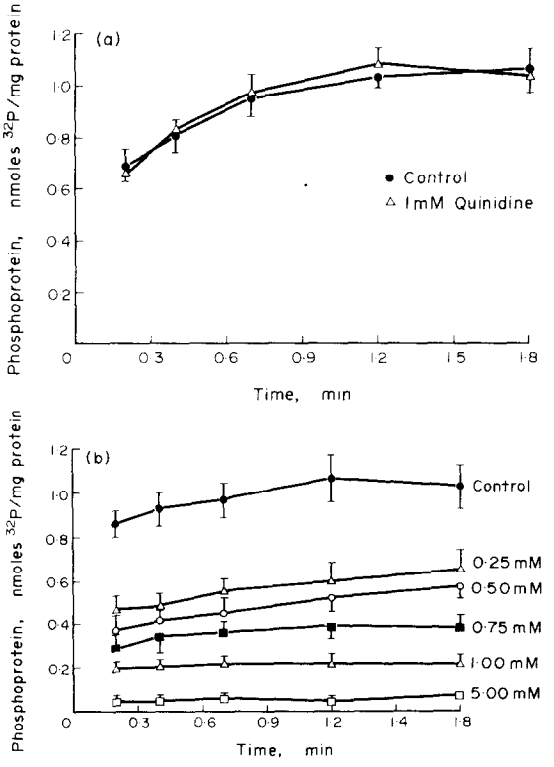

Fig. 2. Effect of quinidine (a) and chlorpromazine (b) on the level of phosphoprotein. (a) Control (●); quinidine, 1 mM (△). (b) Control (●); chlorpromazine, 0·25 mM (△); 0·50 mM (○); 0·75 mM (■); 1 mM (△); and 5 mM (□). Conditions same as in Fig. 1 except that 5 mM $CaCl_2$ was added instead of $MgCl_2$.

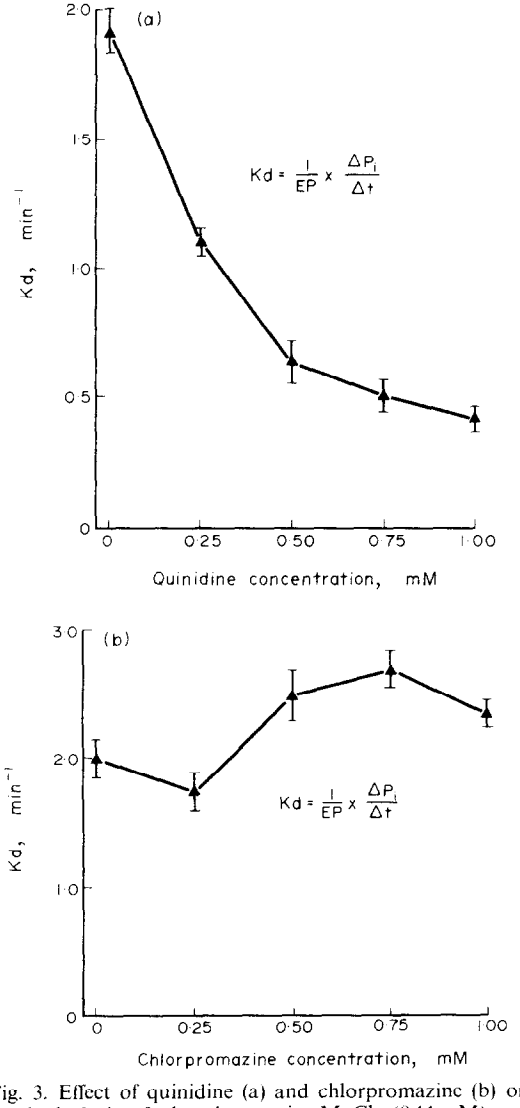

Fig. 3. Effect of quinidine (a) and chlorpromazine (b) on the hydrolysis of phosphoprotein. $MgCl_2$ (0·11 mM) and EGTA (2 mM) were added to the purified phosphorylated intermediate fraction (0·3 to 0·6 nmole ^{32}Pi/mg of protein and 0·5 mg protein/ml) in 1·0 mM $CaCl_2$, 10 mM imidazole, pH 7·0, at 2°. Samples were taken at 0, 0·2, 0·4, 0·6, 0·8 and 1·0 min after addition of $MgCl_2$ and EGTA. K_d was measured as described previously [9].

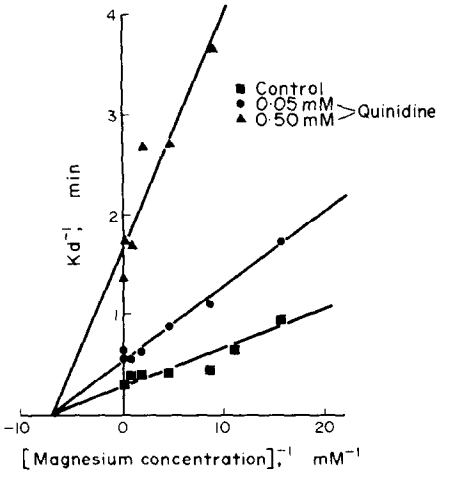

Fig. 4. Effect of quinidine on the magnesium-facilitated hydrolysis of phosphoprotein. Control (■); quinidine, 0·05 mM (●); 0·5 mM (▲). Conditions were the same as in Fig. 3.

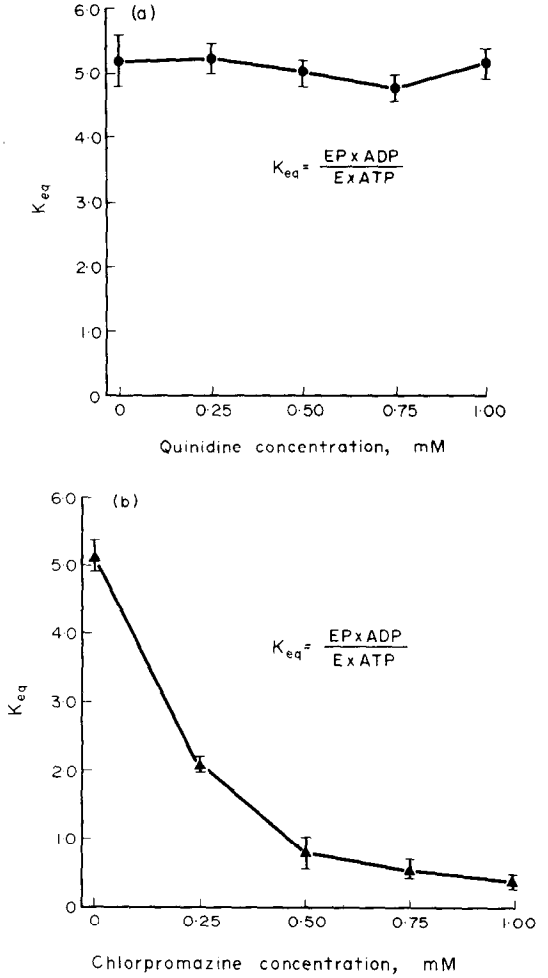

Fig. 5. Effect of quinidine (a) and chlorpromazine (b) on the apparent equilibrium constant (K_{eq}) for the phosphorylation reaction. Conditions were the same as in Fig. 3 except that ADP (5 μM) was added instead of $MgCl_2$ and EGTA. K_{eq} was calculated as described previously [9].

results of these studies, shown in Fig. 3b, indicate that chlorpromazine has no effect on the hydrolysis of the phosphorylated intermediate. On the other hand, quinidine, in the concentrations comparable to those inhibiting ATPase activity, produced comparable inhibitions of the hydrolysis of the intermediate (Fig. 3a). Since the hydrolysis of the phosphorylated intermediate is magnesium dependent, we carried out a series of experiments to determine if the action of quinidine involves competition between quinidine and magnesium. The data presented in Fig. 4 show that quinidine is indeed a noncompetitive inhibitor of the magnesium-facilitated hydrolysis of the phosphorylated intermediate. The dissociation constant of the enzyme–magnesium complex in this study was $1{\cdot}5 \times 10^{-4}$ M. The dissociation constant for the enzyme–quinidine complex was 9×10^{-5} M, and the $K_{d_{max}}$ was 3·72 min^{-1}.

The inhibition of ATPase activity by chlorpromazine appears due to a shift in the equilibrium between the substrate, ATP, and the product, ADP. Quinidine appeared not to influence this equilibrium. This conclusion was tested by isolating the phosphorylated intermediate and determining the effect of ATP on its level. The experiments were carried out under conditions (1 mM $CaCl_2$, 50 mM KCl and 10 mM imidazole, pH 7·0) which block the hydrolysis of the phosphorylated intermediate [9]. Figure 5 shows that the equilibrium constant was unaffected by quinidine and depressed by chlorpromazine.

The data presented in Fig. 5b suggest that chlorpromazine shifts the reaction between the intermediate and ADP toward E and ATP. One explanation for this shift would be a decrease in the affinity of E for ATP. To determine if this is the case, we studied the effect of chlorpromazine on the binding of the β,γ-methylene analogue of ATP to sarcoplasmic reticulum [15]. The effect of analogue concentration and 0·2 mM chlorpromazine on analogue binding is shown in Fig. 6. Binding saturated at an analogue

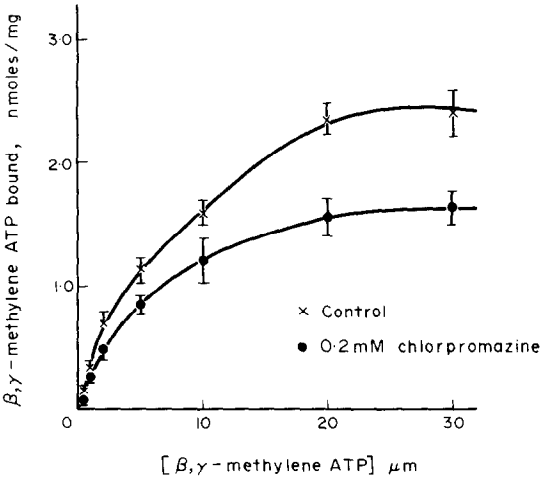

Fig. 6. Effect of chlorpromazine on the binding of β,γ-methylene ATP to cardiac sarcoplasmic reticulum. Control (x); chlorpromazine, 0·2 mM (●). β,γ-Methylene [8 . ^{3}H]ATP was added to the incubation medium containing 5 mM $CaCl_2$, 50 mM KCl, 10 mM imidazole, pH 7·0, and sarcoplasmic reticulum (2 mg/ml) at 2°. Bound analogue was separated from free by ultracentrifugation.

Table 1. Effect of chlorpromazine on β,γ-methylene ATP binding to cardiac sarcoplasmic reticulum*

Chlorpromazine concn (mM)	β,γ-Methylene ATP bound (nmoles/mg)
0	0·42 ± 0·03
0·25	0·24 ± 0·06
0·50	0·09 ± 0·02
0·75	0·02 ± 0·00
1·00	0·05 ± 0·01

* Cardiac sarcoplasmic reticulum (2 mg/ml) was incubated with 1 μM β,γ-methylene [8-^{3}H]ATP, 5 mM $CaCl_2$, 50 mM KCl, 10 mM imidazole, pH 7·0, and 10 mM azide at 2°. Bound ATP analogue was separated from free through centrifugation.

concentration of 20 μM. Chlorpromazine depressed binding in a fashion which appeared to be noncompetitive. A double reciprocal plot of these data (not shown) confirmed this conclusion. The dissociation constant for the enzyme complex with chlorpromazine was 6×10^{-4} M and for the complex with β,γ-methylene-ATP was $7{\cdot}4 \times 10^{-6}$ M. Although not shown, quinidine (1 mM) had no effect on analogue binding. Table 1 shows that chlorpromazine in concentrations ranging from 0·25 to 1·0 mM inhibited the binding of 1 μM β,γ-methylene-ATP from 40 to 88 per cent. It thus appears that the chlorpromazine inhibition of the sarcotubular ATPase activity and the shift in the equilibrium constant, K_{eq}, are due to the effect of chlorpromazine on the affinity of the sarcoplasmic reticulum for ATP.

DISCUSSION

Since the rate-limiting step in sarcotubular catalyzed hydrolysis of ATP is the magnesium-dependent hydrolysis of the phosphorylated intermediate [9], a substance acting at this step should produce equal inhibitions of the rate of the phosphorylated intermediate and ATP hydrolysis. This proved to be the case for quinidine in the present study. We have also found this to be the case for propranolol, another drug which blocks ATP hydrolysis [12, 13] by inhibiting the hydrolysis of the phosphorylated intermediate [5].

Since lipid is required for the hydrolysis of the phosphorylated intermediate [1] and quinidine is lipid soluble, it is tempting to speculate that its effects are related to interaction with the lipid portion of the membrane. Lipid solubility and the requirement of lipid for enzymatic activity do not however, provide compelling evidence for lipid as the site of action of lipid-soluble drugs. The lipid solubility of drugs simply indicates their preference for a hydrophobic environment. Such an environment can, however, be provided by proteins. This is particularly true for enzymes like the ATPase investigated in this study, which is a lipoprotein [16]. We think that it can be hypothesized that drugs like quinidine and propranolol, which inhibit a lipid-dependent phase of ATPase activity, do so by directly interacting with the protein portion of the enzyme. One argument in favor of this possibility is the virtual equality of the effects of quinidine and ouabain on ($Na^+ + K^+$) ATPase [6]. Since there is excellent evidence [17] that ouabain interacts with the protein portion of the ($Na^+ + K^+$) ATPase and quinidine and ouabain have similar effects, it is not unreasonable to hypothesize that quinidine is also acting directly on the protein. Further evidence that quinidine might act directly on protein rather than indirectly through effects on lipid is provided by studies by Pang *et al.* [4] with propranolol, a drug which has effects on sarcotubular ATPase activity identical to those of quinidine. Pang *et al.* [4], using fatty acid and SH group-directed spin labels, found that the concentration of propranolol required to produce a measurable change in the lipid environment was much greater than that required to inhibit the ATPase or to alter ATP-induced changes in the motility of the SH-directed spin label. It remains entirely possible, therefore, that drugs like quinidine and propranolol depress the hydrolysis of the phosphorylated intermediate by interacting with hydrophobic portions of the membrane proteins rather than by a direct effect on the membrane lipids.

On the basis of indirect evidence, Balzer *et al.*[14] have proposed that chlorpromazine inhibits calcium accumulation by blocking the hydrolysis of the phosphorylated intermediate, a conclusion entirely different from ours. They based this conclusion on their observations that 0·1 mM chlorpromazine had little effect on either the phosphorylated intermediate level or on the rate of ATP-$AD^{32}P$ exchange. Inspection of their data shows that chlorpromazine produced a 12 per cent decrease in the phosphorylated intermediate level and stimulated the rate of phosphate exchange. Extrapolation of our phosphorylated intermediate data to a 0·1 mM chlorpromazine concentration indicates that we would have expected a 10–20 per cent reduction in the level of the phosphorylated intermediate at that dose. The data of Balzer *et al.*, are thus consistent with ours. Since they failed to go to higher concentrations of chlorpromazine, they failed to observe the marked effect of chlorpromazine on the phosphorylated intermediate. Our data are not incompatible with an increased rate of phosphate exchange. In order to find a decreased level of substrate binding at equilibrium and an increased rate of exchange, the rate constant, k_2 in the following reaction sequence:

$$E + ATP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-}ATP \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EP + ADP$$

would, however, have to be increased. Studies by Holmes and Piette [18] may indicate how k_2 could be increased. This group found that the environment around one of the SH groups in the erythrocyte member is changed by chlorpromazine in such a way that the mobility of a spin label probing that environment is decreased. They concluded on the basis of this and other data that the drug buries the SH groups in the membrane. This might account for the decrease in the apparent number of substrate binding sites and the decrease in substrate affinity observed in our studies and might be related to the hypothesized increase in k_2.

Acknowledgements—This investigation was supported by research grants from the U.S. Public Health Service, HL 14538-03, to F. N. B. and from Richmond Area Heart Association and Virginia Heart Association to D. C. P.

REFERENCES

1. G. Meissner and S. Fleischer, *Biochim. biophys. Acta* **255**, 19 (1972).
2. A. Martonosi, *J. biol. Chem.* **244**, 613 (1969).
3. A. Martonosi, in *Biomembranes* (Ed. L. A. Manson), Vol. 1, p. 191. Plenum Press, New York (1971).
4. D. C. Pang, F. N. Briggs and R. S. Rogowski, *Archs Biochem. Biophys.* **164**, 332 (1974).
5. D. C. Pang and F. N. Briggs, *Biochem. Pharmac.* **22**, 1301 (1973).
6. K. Lowry, S. N. Roo, B. J. R. Pitts and A. Askari, *Biochem. Pharmac.* **22**, 1369 (1973).
7. O. A. Shemisa and L. A. Fabien, *Molec. Pharmac.* **7**, 8 (1971).
8. A. K. Chowdbury, H. Rogers, A. Skinner, R. G. Spector and D. C. Watts, *Br. J. Pharmac. Chemother.* **37**, 459 (1969).
9. D. C. Pang and F. N. Briggs, *Biochemistry* **12**, 4905 (1973).
10. B. E. Wahler and A. Wollenberger, *Biochem. Z.* **329**, 508 (1958).
11. F. Fuchs and F. N. Briggs, *J. gen. Physiol.* **51**, 655 (1968).
12. F. Fuchs, E. W. Gertz and F. N. Briggs, *J. gen. Physiol.* **52**, 955 (1968).
13. H. Balzer, *Naunyn-Schmiedebergs Arch. epp. Path. Pharmak.* **274**, 256 (1972).
14. H. Balzer, M. Makinose and W. Hasselbach, *Naunyn-Schmiedebergs Arch. epp. Path. Pharmak.* **260**, 444 (1968).
15. Y. Ogawa and S. Ebashi, in *Organization of Energy-Transducing Membranes* (Eds. M. Nakao and L. Packer), p. 127. University Park Press, Baltimore (1973).
16. D. H. MacLennan, *J. biol. Chem.* **245**, 4508 (1970).
17. K. Taniguchi and S. Iida, *Biochim. biophys. Acta* **233**, 831 (1971).
18. . E. Holmes and L. H. Piette, *J. Pharmac. exp. Ther.* **173**, 78 (1970).