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MECHANISM OF PROPRANOLOL INHIBITION OF THE CARDIAC SARCOTUBULE—γ-AT^{32}P REACTION

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Abstract—Analysis of the mechanism by which propranolol inhibits calcium-stimulated, magnesium-dependent, ATPase activity of a sarcotubule-enriched fraction (SR) prepared from canine ventricle was undertaken with the aid of γ-labeled AT^{32}P. When calcium was the dominant divalent cation during incubation, the level of an intermediate, a phosphoprotein (EP), in the hydrolytic reaction increased rapidly to 0·9 nmole/mg of SR protein and plateaued. However, when magnesium was the dominant cation, the level of EP was much lower and decreased as the incubation period increased. Propranolol (1 mM) had no influence on the levels of phosphoprotein formed in the presence of calcium but increased EP levels when excess magnesium was added. Since calcium is required for the formation of EP and magnesium for the hydrolysis of EP, these observations suggest that 1 mM propranolol is primarily limiting EP breakdown. This conclusion was confirmed in an experiment in which propranolol was shown directly to produce a 67 per cent inhibition of the breakdown of EP to inorganic phosphate. Since 5 mM propranolol was found to depress the level of EP formed during incubation of SR with calcium, it appears that higher doses of the drug may also interfere with EP formation.

PROPRANOLOL depression of sarcotubular (SR) ATP-dependent calcium transport[1] has been shown[2] to be associated with inhibition of ATPase activity. This inhibition could take place at any of a number of steps during the enzymatic hydrolysis of ATP. The steps involved are presented schematically below.

Reaction	Reference
(1) $E + Ca + Mg\text{-}ATP \rightleftharpoons E^{Ca}_{Mg\text{-}ATP}$	6
(2) $E^{Ca}_{Mg\text{-}ATP} \rightleftharpoons E^{Ca}_{ATP} + Mg$	6
(3) $E^{Ca}_{ATP} \rightleftharpoons E^{Ca}_{P} + ADP$	3–7
(4) $E^{Ca}_{P} + Mg \rightleftharpoons E^{Mg}_{P} + Ca$	3–8
(5) $E^{Mg}_{P} \xrightarrow{\text{phospholipid}} E + Mg + Pi$	3–8

The noteworthy points in the scheme are the following: (1) calcium is essential for the formation of EP, a phosphoprotein intermediate;[3–7] (2) although there is dispute about whether Mg-ATP is the substrate, it does appear to be a better substrate than

* Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetra-acetic acid; EDTA, ethylenediamine tetra-acetic acid; TCA, trichloroacetic acid.

Ca-ATP;[6] (3) magnesium is a cofactor for the hydrolysis of EP;[3-8] and (4) membrane phospholipid is essential for EP hydrolysis.[8]

The effects of propranolol on the formation or breakdown of EP can be examined, therefore, by studying its effects under conditions either favoring EP formation, presence of calcium ion, or EP breakdown, presence of magnesium ion.

MATERIALS AND METHODS

Preparation of sarcotubule-enriched fraction (SR)

Canine hearts removed from anesthetized dogs were used as starting material. Ventricular muscle was chilled in ice and washed free of blood with saline, cut into small pieces and homogenized in 4 vol. of 0·3 M sucrose, 10 mM imidazole, pH 7·0, for 40 sec with a Servall omnimixer. After centrifugation at 17,300 *g*-max for 20 min to remove myofibrils and mitochondria, SR pellets were collected by centrifugation at 34,800 *g*-max for 25 min. The SR pellet was washed with 0·6 M KCl, 10 mM imidazole, pH 7·0 (1·3 ml/g heart) to remove contaminant proteins.[9] The SR was collected by centrifugation at 144,000 *g*-max for 30 min and resuspended in a small volume of 0·3 M sucrose, 10 mM imidazole, pH 7·0. The protein concentration averaged 5 mg/ml.

Incubation of cardiac sarcoplasmic reticulum with γ-AT^{32}P

Incubation of SR (1 mg/ml) was carried out at 2° with γ-labeled AT^{32}P (Schwartz and Mann, spec. act. = 1992 mCi/m-mole). The reaction was started by the addition of 0·1 ml of 8×10^{-5} M ATP with 0·4 μCi of γ-labeled AT^{32}P to 3·9 ml of an incubation medium containing 51·5 mM KCl, 1·03 mg of SR/ml and either 10·3 mM imidazole (pH 7·0), or 10·3 mM Tris (pH 8·8). When added, the concentrations of the following were: $CaCl_2$, 0·2 to 5 mM; $MgCl_2$, 1·2 to 5 mM; propranolol, 1–5 mM; EGTA,* 1 mM; EDTA, 1 mM; ouabain, 0·1 mM; and sodium azide, 10 mM. The incubation mixture which was sampled (0·5 ml) at intervals between 12 sec and 2·5 min after the addition of γ-AT^{32}P was pipetted into 1 ml of ice-cold 1·2 M perchloric acid containing 80 g/liter of silicotungstic acid. Since addition of cold 10^{-4} M ATP and 10^{-3} M inorganic phosphate with the perchloric acid was found not to influence the apparent level of EP, these additions were abandoned. The denatured protein, after precipitation, was centrifuged at 10,000 *g*-max for 10 min. The resultant pellet was used for measurement of phosphoprotein and the supernatant for the measurement of liberation of inorganic phosphate from γ-AT^{32}P.

Measurement of phosphoprotein. For the measurement of phosphoprotein, the sediment was washed successively with 4 ml of ice-cold 5% TCA and 4 ml of ice-cold 2% TCA. The amount of radioactivity in the supernatant after the 2% TCA wash was found to be insignificant. The final protein pellet was resuspended in 2 ml of 2% Na_2CO_3 in 0·1 N NaOH and heated in a boiling water bath for 15 min. Aliquots were assayed for protein by the method of Lowry *et al.*[10] and for radioactivity by liquid scintillation.

Measurement of radioactive inorganic phosphate. The amount of radioactive inorganic phosphate in 1 ml of supernatant formed after centrifugation at 10,000 *g*-max for 10 min was extracted by the method of Wahler and Wollenberger[11] and counted by liquid scintillation. Quench curves for the butyl acetate used in this method were prepared by addition of butyl acetate to ^{32}P standards.

Calculations of ATP concentrations. The fraction of ATP remaining in the incubation medium was calculated with the following formula:

$$\text{Fraction of ATP remaining} = 1 - \frac{\dfrac{\text{phosphoprotein (nmoles/mg)} + \text{PO}_4\text{ (nmoles/mg)}}{\text{initial ATP (nmoles/ml)}}}{\text{SR (mg/ml)}}$$

Measurement of calcium in SR

SR was digested with 70% perchloric acid and assayed for calcium in the presence of 0·5% La^{3+} and 2·5% trichloroacetic acid by atomic absorption spectroscopy.

RESULTS AND DISCUSSION

Effect of propranolol on the ATPase activities of cardiac sarcoplasmic reticulum. Although it has been shown by Scales and McIntosh[2] that propranolol inhibits the ATPase activity of cardiac SR, the conditions under which they carried out their experiments are quite different from those usually used to study phosphoprotein (EP) formation and breakdown.[5–7] We have, therefore, reinvestigated the effect of propranolol on ATPase activity under such conditions i.e. at low temperature, 2°, and low ATP concentration, 2 μM. Since we were particularly interested in the effects of the drug on the calcium-stimulated ATPase of SR and since cardiac SR preparations are not highly pure,[12] experiments were also carried out which would allow distinction between the effects of propranolol on the calcium-stimulated ATPase and other ATPases in the SR fraction. The results of these studies are shown in Table 1. Column 1 of Table 1 shows that about 57 per cent of the total ATPase activity of the SR preparation is Mg dependent. The magnitude of the magnesium-dependent ATPase activity was obtained by adding 1 mM EGTA to complex any calcium in the incubation mixture. The calcium-dependent ATPase activity was then taken to be the

TABLE 1. EFFECT OF PROPRANOLOL ON THE ATPASE ACTIVITIES OF CARDIAC SARCOPLASMIC RETICULUM

	Propranolol concn (mM)		
Experiment	0	1	5
Control ATPase*	6·27 ± 0·20	3·68 ± 0·30	3·18 ± 0·21
Mg-ATPase†	3·60 ± 0·09	3·22 ± 0·05	2·89 ± 0·16
Ca-ATPase‡	2·67 ± 0·15	0·46 ± 0·17	0·29 ± 0·18

* Cardiac SR (0·8 to 1·0 mg/ml) which contained, on the average, 40 nmoles of calcium/mg of protein was incubated at 2° in the presence of 2 μM $AT^{32}P$, 50 mM KCl, 10 mM imidazole, pH 7·0, and 5 mM $MgCl_2$. Amount of radioactive inorganic phosphate liberated at 0·2 min was used to calculate the enzyme activity. ATPase activity is expressed as nanomoles of Pi liberated per milligram of protein per min.

† Mg-dependent ATPase activity was assayed by addition of 1 mM EGTA to the incubation medium.

‡ Ca-dependent ATPase activity equals control ATPase activity minus Mg-dependent ATPase activity.

difference between the total or control ATPase activity and the Mg-dependent ATPase activity. Column 2 shows that although propranolol inhibits the control ATPase activity (42 per cent), it has almost no (11 per cent inhibition) effect on the Mg-dependent ATPase activity. It is concluded from this observation that the inhibition of ATPase activity in the control preparation was almost entirely (83 per cent) due to inhibition of calcium ATPase activity. Column 3 shows that 5 mM propranolol produces almost identical effects. The Mg-ATPase activity was inhibited by 20 per cent and Ca-ATPase activity by 89 per cent.

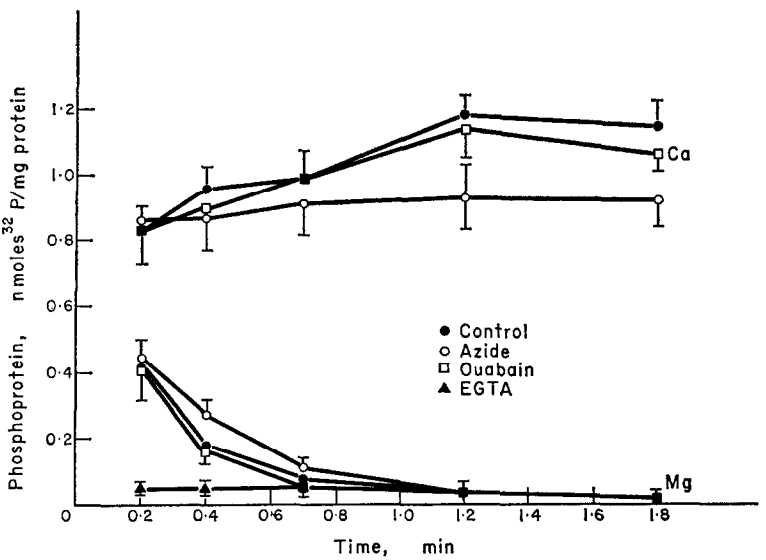

FIG. 1. Effect of EGTA, azide and ouabain on the phosphoprotein level in cardiac sarcoplasmic reticulum. SR (0·8 to 1·0 mg/ml) was incubated with 5 mM $CaCl_2$ or $MgCl_2$, 2 μM ATP with 0·1 μCi/ml of γ-AT^{32}P in the standard buffer (50 mM KCl and 10 mM imidazole, pH 7·0) at 2°; 10 mM azide, 1 mM EGTA or 0·1 mM ouabain was added as indicated. In this and all subsequent figures the values presented are expressed as mean ± S. E. M. and the number of experiments was three.

Phosphorylation of cardiac SR by γ-AT^{32}P. Before determining whether the block in Ca-ATPase activity is due to inhibition of the formation or the breakdown of the EP, it was necessary to determine if cardiac SR forms this intermediate and to be certain that the EP formed originated from sarcotubular material. γ-AT^{32}P was chosen as substrate for the formation of EP. A low level of ATP (2 μM) was used since it is economical and since it can be used to obtain substantial levels of EP in skeletal muscle.[3–8] A low temperature, 2°, was chosen for the incubation of the SR with ATP, since under these conditions temperature favors high levels of EP.[4–8] Figure 1 shows the levels of EP obtained at various intervals of time after addition of γ-AT^{32}P. In the presence of 5 mM calcium, the level was nearly maximal by 0·2 min and remained essentially constant up to 1·8 min. In the presence of 5 mM magnesium, on the other hand, the EP level was much lower at 0·2 min and decreased rapidly thereafter. Since, upon addition of 1 mM EGTA with $MgCl_2$, the phosphoprotein levels were insignificant, it is probable that the phosphoprotein formation

observed with magnesium was dependent on the presence of calcium in the isolated SR. The levels of calcium in the SR used in these studies averaged 40 nmoles/mg of protein. Addition of ouabain (10^{-4} M) and azide (10^{-2} M) had no effect on the levels of phosphoprotein. These results indicate that ($Na^+ + K^+$)ATPase[13] and mitochondrial[14] ATPase activities are not significantly effecting the levels of phosphoprotein.

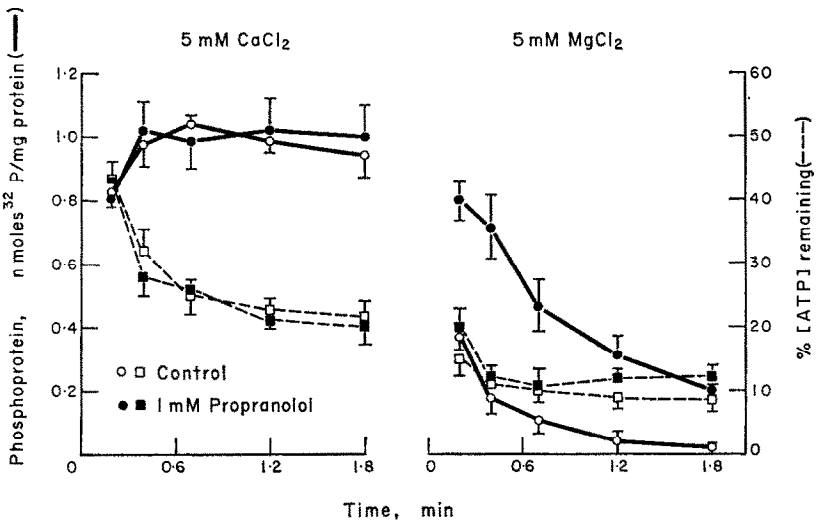

FIG. 2. Effect of propranolol on the phosphoprotein level in cardiac sarcoplasmic reticulum. Incubation conditions were the same as in Fig. 1. Propranolol was 1 mM.

Effect of propranolol on the phosphoprotein levels. Since 1 mM propranolol has been shown to effectively depress SR ATPase activity and calcium transport,[1,2] we studied the effect of this concentration of the drug on the phosphoprotein levels. Although propranolol had no effect on the levels in the presence of calcium, the levels were increased in the presence of magnesium (Fig. 2). It should be noted that the level of ATP available throughout the incubation with calcium or magnesium was not influenced by propranolol and, therefore, these differences were not related to different ATP concentrations. The effect of propranolol on EP levels in the presence of magnesium suggests, but does not prove, that propranolol can decrease the rate of breakdown. An increase in level could also be due to an increase in the rate of EP formation, which was too rapid to measure in the presence of 5 mM calcium (Figs. 1 and 2).

Effect of propranolol on the formation of phosphoprotein. Since it has been reported that calcium antagonizes the interaction between propranolol and SR phospholipids[14] and since the experiments described above (Fig. 2) were done at calcium concentrations five times higher than propranolol, it is possible that at more physiological levels of calcium propranolol might influence EP formation. We have studied, therefore, the effects of propranolol on EP levels at various propranolol:Ca ratios. The results of these experiments are shown in Fig. 3. Propranolol in ratios ranging from 5:1 to 1:5 had no effect on EP levels. The experiments carried out at 0·2 mM calcium were particularly informative because the rate of rise of the EP levels was

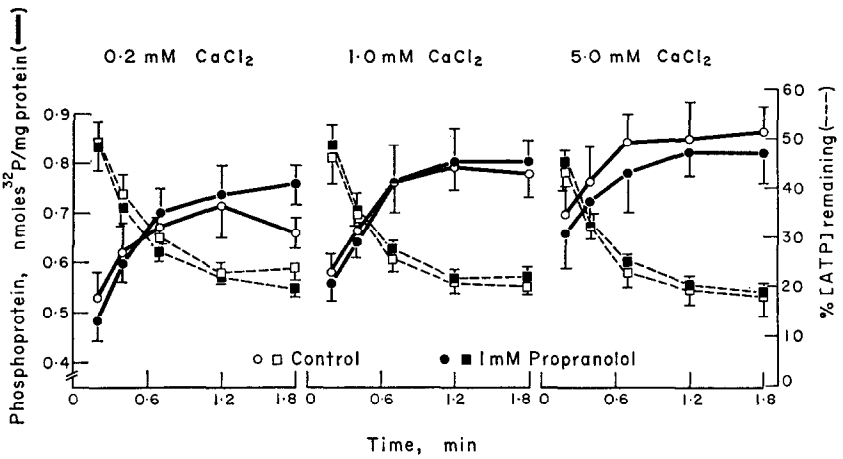

FIG. 3. Effect of calcium and propranolol on the phosphoprotein level in cardiac sarcoplasmic reticulum. SR (0·8 to 1·0 mg/ml) was incubated with 0·2 to 5·0 mM $CaCl_2$ and 2 μM $AT^{32}P$ in standard buffer at 2°. Propranolol was 1 mM.

sufficiently slow that it could be followed. The failure of propranolol to alter the rate of rise of EP suggests that the increased levels of EP observed in Fig. 2 when propranolol was added with magnesium were indeed due to decreased rates of EP breakdown. It appears, therefore, that 1 mM propranolol does not influence the formation of EP. We have, however, found that a higher dose of propranolol, 5 mM, will depress EP levels in the presence of calcium (Fig. 4). Thus, it appears that 5 mM propranolol can block EP formation.

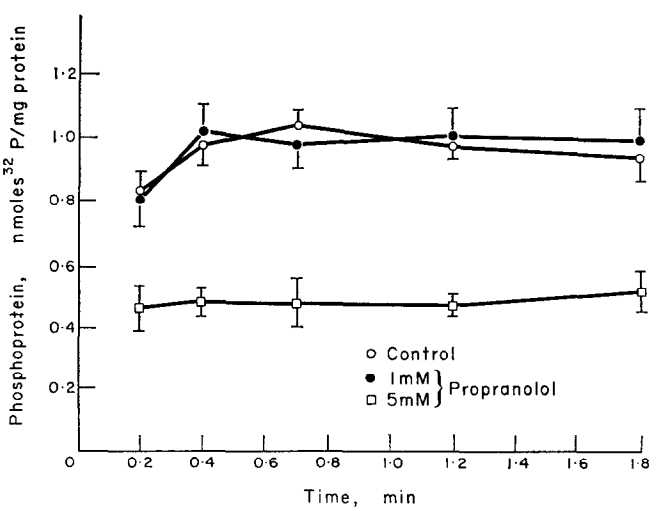

FIG. 4. Effect of propranolol concentration on the level of phosphoprotein in cardiac sarcoplasmic reticulum. SR (0·8 to 1·0 mg/ml) was incubated with 5 mM $CaCl_2$ and 2 μM $AT^{32}P$ in standard buffer at 2°. Propranolol (1–5 mM) was added as indicated.

Effect of propranolol on the hydrolysis of phosphoprotein. It has been argued, up to this point, that propranolol inhibits ATPase activity by blocking the breakdown of EP because propranolol did not influence the level of EP in the presence of calcium but did influence EP levels in the presence of magnesium. To establish the site of action more directly, an experiment was designed to study the effect of propranolol on the rate of EP breakdown to E + Pi. Kanazawa *et al.*[6] has shown that the reaction, EP + ADP → ATP + E, could be blocked if the calcium inside the SR were removed. Under this condition, a change in EP level can, therefore, only occur as a consequence of the hydrolysis of EP to inorganic phosphate. An experiment was, therefore, carried out under this condition and is shown in Fig. 5. In order to make the SR membrane permeable to the calcium chelators which are needed to remove intravesicular calcium, the experiment was carried out at pH 8·8.[16] Although not shown,

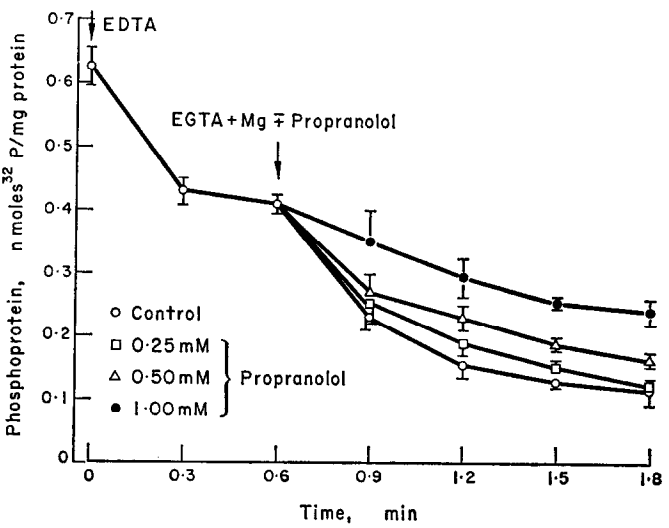

FIG. 5. Effect of propranolol and magnesium on the hydrolysis of phosphoprotein in cardiac sarcoplasmic reticulum. SR (0·8 to 1·0 mg/ml) was phosphorylated with 2 μM $AT^{32}P$ in the presence of 0·5 mM $CaCl_2$, 10 mM Tris, pH 8·8, 10 mM azide and 50 mM KCl at 2°; 0·7 min after the start of the reaction (zero time in the figure) EDTA was added to give a concentration of 1 mM; 0·6 min after the addition of EDTA, $MgCl_2$ (1·2 mM) and EGTA (1 mM), in the presence or absence of propranolol (0·25 to 1·00 mM), were added. Concentrations in brackets denote final concentrations in the incubation medium.

calcium was added during an initial period of incubation to promote the formation of EP. EDTA was then added to complex all the calcium and magnesium in the system. The drop in the EP level is due to the formation of ATP from EP before the calcium inside the SR was chelated.[6] (No increase in the level of inorganic phosphate was observed in the present experiment.) After chelation of calcium with EDTA, magnesium was added with EGTA to facilitate the breakdown of EP which, as shown in Fig. 5, was prompt. Note that the rate of breakdown was not proportional to the level of EP. This same observation has been made by other investigators[4,6,7] in skeletal SR. Addition of 0·25 to 1·0 mM propranolol produced a progressive decline in rate of EP breakdown under these conditions. One mM propranolol reduced the

initial rate of EP breakdown by 67 per cent. Although this degree of inhibition does not completely account for the 83 per cent inhibition of the calcium-stimulated ATPase, it does account for a major share of the inhibition. The difference in the degree of inhibition may be in part a consequence of the different conditions under which the ATPase activity and EP breakdown activity were measured.

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