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PROPRANOLOL INHIBITION OF THE NEUTRAL PHOSPHOLIPASES A OF RAT HEART MITOCHONDRIA, SARCOPLASMIC RETICULUM AND CYTOSOL

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Abstract—Membrane damage caused by phospholipase A action is thought to be an important factor in ischemic myocardial injury. Propranolol has been shown previously to have beneficial effects in both animal experiments and clinical trials, and it has membrane-stabilizing properties *in vitro*. To investigate the possibility that these effects might be due, in part, to effects on phospholipases, we determined the effects of propranolol on rat heart phospholipases A at physiological pH using small unilamellar liposomes of di[1-^{14}C]oleoylphosphatidylcholine as substrate. Propranolol inhibited heart phospholipases A *in vitro*. The concentration required to give 50% inhibition was 0.2 mM for the mitochondrial and cytosolic phospholipases A and 2.9 mM for sarcoplasmic reticulum phospholipase A. The binding of [4-^{3}H]propranolol to fresh membrane preparations was studied using an ultracentrifugation method. Propranolol bound readily to both membrane fractions *in vitro* with no significant difference in the saturation number (0.20 to 0.28 mol drug per mol phospholipid) but the association constant, K_A, was higher for mitochondrial membranes (3760 ± 350) than for the sarcoplasmic reticulum (2190 ± 390). Our results show that propranolol inhibited heart phospholipases A *in vitro* at physiological pH. The mitochondrial and cytosolic phospholipases A were more susceptible to inhibition than the phospholipase A of sarcoplasmic reticulum. Propranolol bound to mitochondria and sarcoplasmic reticulum *in vitro*, suggesting the possibility that propranolol binding to heart membranes *in vivo* could result in drug concentrations in these membranes high enough to inhibit phospholipase A. This could represent an additional mechanism by which propranolol exerts beneficial effects in myocardial ischemia.

Previous studies from our laboratory have shown that propranolol [1] and other beta adrenoceptor blockers inhibit lysosomal phospholipase A *in vitro* [2]. Of six beta adrenergic blockers, propranolol was the most potent inhibitor, due in part to its high lipid solubility as reflected by its high octanol/water partition coefficient [2]. Inhibition of lysosomal phospholipase A is also dependent on the substrate. With phosphatidylethanolamine as substrate, the enzyme is inhibited by 50% at 0.32 mM propranolol. With phosphatidylinositol as substrate, concentrations of propranolol ten times higher are required, whereas phosphatidylcholine gives intermediate results [3]. In addition to its effects on lysosomal phospholipase, propranolol has also been shown to bind to small unilamellar liposomes of phosphatidylcholine [4, 5] and to phospholipid-stabilized triolein particles [6] which represent the substrates for phospholipase A and lipoprotein lipase respectively. In the case of lysosomal phospholipase A, binding of drug to substrate liposomes is not thought to be the principal cause of inhibition [5], while with propranolol inhibition of lipoprotein lipase [6] the role of drug binding to the substrate surface is presently not well-defined.

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Phospholipases A have been proposed to play an important role in myocardial ischemic injury since the products of phospholipase A action are free fatty acids (FFA) and lysophospholipids, each of which may be deleterious to the cell function [7, 8]. In the normoxic heart the concentration of these components is low, and it has been proposed that high levels of FFA and lysophospholipids in myocardial tissue could lead to arrhythmias [9–11]. During ischemia, degradation of myocardial phospholipids has been demonstrated [12–14], and levels of FFA [13, 15–17] and lysophosphatidylcholine [17–20] increase as do the concentrations of acyl-CoA and acylcarnitines [21, 22]. High levels of acyl-CoA may inhibit enzyme systems, for example, the exchange of ATP/ADP between mitochondria and cytoplasm by the adenine nucleotide transferase reaction.

After acute myocardial infarction, propranolol reduces the signs of ischemic injury which may be due, in part, to a membrane-stabilizing effect [23, 24] and, in clinical trials, propranolol treatment was beneficial with regard to physiological parameters [25], enzyme leakage [26], and general mortality rate [27–29]. Reduction of the infarct size by propranolol treatment has been documented in animal experiments [30–33].

In principle, the elevated levels of FFA and lysophospholipids in ischemic myocardial tissue could be prevented by inhibition of phospholipase A

activity. Therefore, it is of interest to examine and define the effect of propranolol on heart phospholipases A. In this paper, propranolol effects on the neutral phospholipases A of mitochondria, sarcoplasmic reticulum and cytosol from rat heart were studied using sonicated di[1-^{14}C]oleoylphosphatidylcholine small unilamellar liposomes as substrate, and the binding of [4-^{3}H]propranolol to mitochondrial and sarcoplasmic reticulum membrane fractions was examined *in vitro*.

METHODS

Preparation and characterization of subcellular fractions. To obtain the respective membrane preparations, thirty to seventy male rats of the Sprague–Dawley strain were killed by decapitation, and the hearts were quickly removed and placed in ice-cold buffer A [220 mM mannitol, 70 mM sucrose, 5 mM morpholinopropanesulfonic acid (MOPS), pH 7.4]. The subcellular fractions were isolated by the method of Palmer *et al.* as described earlier except that the fractions resulting from treatment with the bacterial protease, Nagarse, were not isolated [34, 35]. Briefly, 20–50 g of ventricular tissue was cut into small pieces and homogenized in buffer B [buffer A containing 2 mM ethylenglycolbis(amino-ethylether)tetra-acetate (EGTA)]. The ratio was about 10 g tissue/100 ml buffer. The heart tissue was homogenized with a Polytron tissue processor (PT-10 generator) (Brinkmann Instruments) at a rheostat setting of 6 for 6 sec, and 24 sec cooling in ice. This was performed using three to six aliquots of heart tissue dependent on the amount of tissue. All further operations were carried out at 4°. The homogenate was centrifuged at 500 *g* for 10 min. The pellet was resuspended in buffer A with a Potter–Elvehjem homogenizer and again centrifuged at 500 *g* for 10 min. The supernatant fractions were combined and centrifuged at 3000 *g* for 10 min to obtain the subsarcolemmal mitochondria as the pellet, which was washed twice by centrifugation as above with buffer B. The mitochondrial pellet was reserved at 4° and used for binding studies. Small aliquots of this fraction were stored at −70° and used for phospholipase A measurements. The supernatant fractions were combined and centrifuged at 17,000 *g* for 10 min to sediment the light mitochondrial pellet. The supernatant fraction was centrifuged at 100,000 *g* for 1 hr to obtain the pellet of sarcoplasmic reticulum and the supernatant (cytosol). The mitochondrial and sarcoplasmic reticulum membrane preparations was resuspended in buffer A containing 0.5 mM EGTA and stored at −70° until use. The cytosolic phospholipase A was obtained by treatment of the supernatant fraction with 50% ammonium sulfate, and the precipitate was collected by centrifugation as previously described [36], taken up in buffer A and stored at −70°. For the propranolol binding studies fresh membrane preparations were used without prior freezing. The purity of the mitochondria and sarcoplasmic reticulum fractions was similar to that previously reported in great detail by Palmer *et al.* [35] and by Nalbone and Hostetler [34].

Protein and lipid phosphorus assays, and separation of individual phospholipids. The protein content of the membrane fractions was determined by the method of Lowry *et al.* [37] using bovine serum albumin as standard, and the lipid phosphorus was determined according to Rouser *et al.* [38]. The individual phospholipids were separated by two-dimensional thin-layer chromatography using silica gel G containing 0.4 M boric acid as previously described [39]. Acid solvents were omitted in order to protect the plasmalogens. Briefly, 200–300 nmol of lipid phosphorus was applied to the silica gel plate and separated with chloroform–methanol–water–ammonia (66:34:3:2, by vol.). After drying under nitrogen for 20 min the plates were developed in the second dimension with chloroform–methanol–water (65:35:5, by vol.). The spots were visualized with iodine vapor, marked, and scraped into glass tubes for lipid phosphorus determination. The data were calculated as percent of recovered lipid phosphorus and as nmol lipid phosphorus per mg protein.

Phospholipase A assays. Phospholipase A was assayed as previously described by measuring the release of [1-^{14}C]oleic acid from di[1-^{14}C]oleoylphosphatidylcholine ([1-^{14}C]DOPC) (Amersham, Arlington Heights, IL) [40]. Incubation mixtures contained 0.05 mM [1-^{14}C]DOPC (cytosol) or 0.2 mM [1-^{14}C]DOPC (mitochondria and sarcoplasmic reticulum), with a specific activity of 112 mCi/mmol, 50 mM Tris–HCl (pH 7.1), and 44 μg protein (mitochondria and sarcoplasmic reticulum) or 60 μg protein (cytosol) in a final volume of 0.2 ml. Propranolol, in final concentrations of 0.1 to 1.0 and 2.0 mM for cytosol and mitochondria and 0.2 to 10 mM for sarcoplasmic reticulum membranes, was preincubated with the fractions for 30 min at 4°, and the reaction was started by adding sonicated substrate. The mitochondrial phospholipase A was assayed in the presence of 6 mM Ca^{2+} which was added immediately before the [1-^{14}C]DOPC. The cytosolic and sarcoplasmic reticulum phospholipases were assayed without added calcium. Control and propranolol-containing mixtures were incubated at 37° for 20 min. The reaction was stopped and [1-^{14}C]oleic acid was isolated by the modified Dole extraction [41] and counted as previously described [40]. All results were corrected for controls incubated without added enzyme. Propranolol (*d,l*) was provided by the Ayerst Laboratories Inc., New York.

Binding of propranolol to mitochondrial and sarcoplasmic reticulum membranes. Membrane fractions representing 2 mg protein were used to study the binding of [4-^{3}H]propranolol (New England Nuclear, Cambridge, MA). The phospholipid content of the assay was 1.2 ± 0.1 mM for mitochondria and 1.4 ± 0.1 mM for sarcoplasmic reticulum. Incubations were carried out at pH 7.1 (Tris buffer) at room temperature with [4-^{3}H]propranolol at concentrations of 0.01 to 3.16 mM for 30 min. The binding was measured as reported previously using an ultracentrifugation method [4, 5]. Assay mixtures of 600 μl were prepared, and duplicate 50-μl aliquots were removed and assayed for radioactivity. After the incubation of 30 min, the remaining mixture was centrifuged at 15° in a Beckman 42.2 Ti rotor at 209,000 *g* for 16 hr. Controls containing propranolol at each concentration without added membrane fractions were also treated in a similar fashion. After

centrifugation, duplicate 50-μl aliquots of the supernatant fraction were taken and counted as a measure of the amount of free drug remaining in the supernatant. Propranolol controls centrifuged without membranes showed a decline in ^3H in the supernatant of 14 ± 2%. All values were corrected for this non-phospholipid-dependent factor which may represent binding of propranolol to the wall of the tube or sedimentation of drug micelles. The weight of the tubes decreased by 4–5% after the 16-hr centrifugation, and the results were corrected accordingly. Finally, experiments done with a shorter centrifugation time (209,000 *g* for 2 hr) gave similar results.

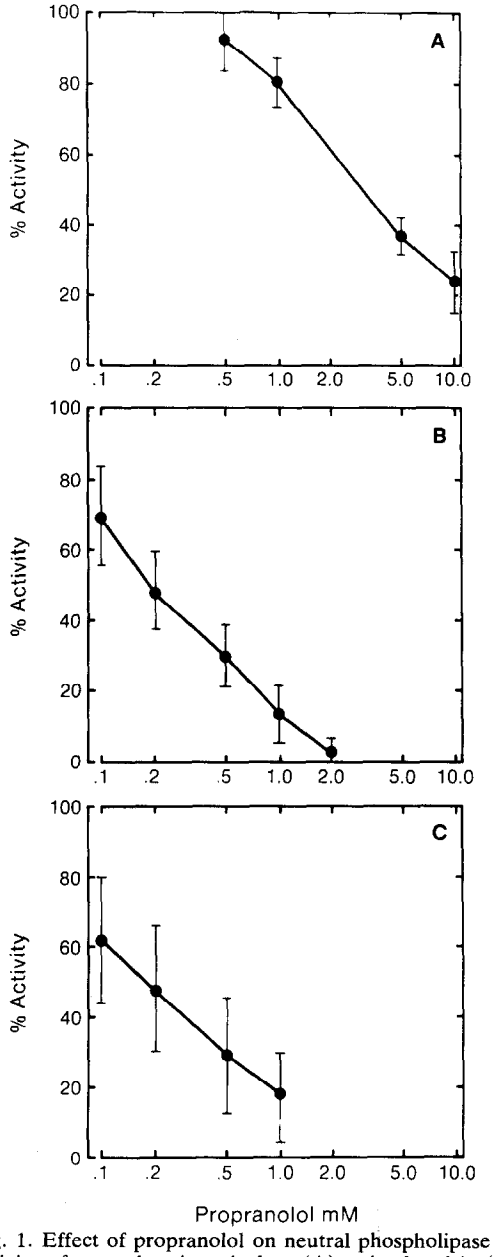

Fig. 1. Effect of propranolol on neutral phospholipase A activity of sarcoplasmic reticulum (A), mitochondria (B) and cytosol (C) of rat heat *in vitro*. Results are expressed as a percentage of the control activity obtained without inhibitor and represent the mean ± SD of separate enzyme preparations. The concentration of sonicated di[1-^{14}C]oleoylphosphatidylcholine in the incubation was 0.2 mM (mitochondria and sarcoplasmic reticulum) or 0.05 mM (cytosol). The control specific activities of the respective phospholipases A at pH 7.1 were: mitochondria, 7.1 ± 1.0 nmol · mg^{-1} · hr^{-1} (N = 4); sarcoplasmic reticulum, 7.8 ± 2.9 nmol · mg^{-1} · hr^{-1} (N = 3); and cytosol after ammonium sulfate precipitation, 3.3 ± 2.2 nmol^{-1} · hr^{-1} (N = 4).

RESULTS

To evaluate the effects of propranolol on rat heart phospholipases A, we isolated mitochondrial, sarcoplasmic reticulum and cytosol fractions as previously described [34]. The respective phospholipases A were assayed at physiological pH rather than at their optima, pH 8–9 [34], in order to partially simulate *in vivo* conditions. The results are shown in Fig. 1 where the data are expressed as a percentage of the phospholipase A activity obtained without propranolol. Propranolol inhibited the sarcoplasmic reticulum phospholipase A by 50% (IC_{50}) at 2.9 ± 0.8 mM and at 10 mM propranolol the enzyme was inhibited by 80% as shown in panel A. With both the mitochondrial and cytosolic phospholipases A (panels B and C, respectively), the IC_{50} for propranolol was 0.2 ± 0.1 mM, one-tenth of that observed for sarcoplasmic reticulum enzyme. At a propranolol concentration of 1 mM, mitochondrial and cytosolic phospholipase A activities were 15–19% of control and at 2 mM propranolol mitochondrial phospholipase A was blocked completely.

Inhibition of neutral phospholipases from heart *in vitro* requires levels of propanolol that are much higher than the plasma levels of propanolol in patients treated with this drug [42, 43]. However, the tissue distribution of propranolol is substantial, and this drug may concentrate in phospholipid membranes since we have shown previously that propanolol binds readily to small unilamellar phospholipid liposomes at pH 4.4 [4, 5]. To analyze propanolol binding to heart mitochondria and sarcoplasmic reticulum under conditions of the phospholipase assay, we studied the association of [4-^{3}H]propranolol with these membrane fractions at pH 7.1, and the results are shown in Fig. 2. At low drug concentrations (0.01 mM), 46% of the propranolol was bound to the respective membranes. The percentage of bound drug declined gradually to 19 and 23% for mitochondria and sarcoplasmic reticulum, respectively, at 1 mM propranolol. Drug binding was analyzed using Langmuir absorption isotherms as previously described [4, 5]. We determined the association constant, K_A, for propranolol from the data shown in Fig. 2 which represent the values obtained with three different membrane preparations of heart mitochondria and sarcoplasmic reticulum. The K_A was higher with mitochondria (3760 ± 350) than with sarcoplasmic reticulum (2190 ± 390). However, the saturation number (N) was not significantly different in mitochondria and sarcoplasmic reticulum, 0.20 ± 0.09 versus 0.28 ± 0.09 mol drug/mol phospholipid respectively.

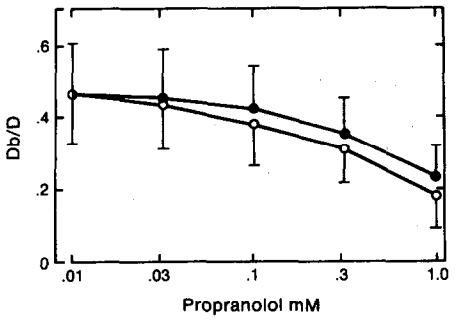

Fig. 2. Binding of [4-^{3}H]propranolol to mitochondria and sarcoplasmic reticulum of rat heart *in vitro*. Results are expressed as the fraction of drug bound (D_b/D) and represent the mean ± SD of three separate membrane preparations where each data point is the average of duplicate determinations. The phospholipid content of the binding assay was 1.2 ± 0.1 mM (mitochondria) and 1.4 ± 0.1 mM (sarcoplasmic reticulum), representing 2 mg of membrane protein. Key: (○) mitochondria; and (●) sarcoplasmic reticulum.

The phospholipid composition of mitochondria and sarcoplasmic reticulum are shown in Table 1. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the principal phospholipids of both membrane fractions together representing 73–74% of the total phospholipid. However, in mitochondria the percentages of PC and PE were roughly equal, whereas in the sarcoplasmic reticulum the percentage of PC was nearly twice that of PE. Mitochondria contained 12% cardiolipin versus 4% in the sarcoplasmic reticulum; conversely, the percentage of phosphatidylserine and phosphatidylinositol was two to five times higher in the sarcoplasmic reticulum than in the mitochondria. The content of lysophosphatidylcholine was low in both membrane fractions.

DISCUSSION

Except for a study on cardiac sarcolemmal phospholipase A which showed that propranolol blocks isoproterenol stimulation of phospholipase A activity [44], studies of the effects of propranolol on the phospholipase A of heart have not been carried out previously. In liver, inhibition of phospholipases by propranolol has been shown for lysosomal phospholipase A. Propranolol inhibits lysosomal phospholipase A by 50% at a concentration of 0.25 mM at pH 4.4 [1] which is similar to inhibition observed in the present study with heart cytosolic and mitochondrial phospholipases A. In contrast, the phospholipase A of heart sarcoplasmic reticulum required 2.9 mM propranolol to reach the IC_{50}, as shown in Fig. 1.

The differential inhibitory effects of propranolol on the various phospholipases A of heart are probably due to differences in the enzymes themselves. The properties of the phospholipases A of heart cytosol, mitochondria and sarcoplasmic reticulum are very different with regard to positional specificity, kinetic properties, and calcium dependency as we have shown earlier [34]. The cytosolic enzyme is not calcium dependent and is mainly a phospholipase A_1, while the mitochondrial enzyme hydrolyzes the *sn*-2 fatty acid ester of the substrate and requires calcium for activity. The positional specificity of sarcoplasmic reticulum phospholipase A has not yet been determined because this membrane contains a highly active lysophospholipase which rapidly removes the lysophosphatidylcholine product [34]. The sarcoplasmic reticulum phospholipase A is not calcium dependent. The apparent K_m values for exogenous sonicated DOPC are much lower for the cytosolic phospholipase A (0.07 mM) than for the membrane-associated phospholipases A of mitochondria and sarcoplasmic reticulum (0.3 mM) [34].

In previous studies we showed that propranolol bound to unilamellar phospholipid liposomes at pH 4.4 to a high affinity, low capacity site presumably at the surface of the liposome and to a low affinity, high capacity site which is independent of surface charge [4]. *In vitro* studies have shown that the naphthyl moiety of propranolol interacts with the

Table 1. Phospholipid composition of mitochondria and sarcoplasmic reticulum (SR) of rat ventricle

	Percentage of total lipid phosphorus		Lipid phosphorus (nmol/mg protein)	
Phospholipid	Mitochondria	SR	Mitochondria	SR
PC	39 ± 1	48 ± 4	129 ± 10	191 ± 24
PE	34 ± 5	26 ± 3	112 ± 15	103 ± 8
SM	6 ± 4	9 ± 3	20 ± 14	38 ± 12
PS	1 ± 0.4	5 ± 1	4 ± 1	20 ± 4
PI	2 ± 0.3	4 ± 1	8 ± 1	15 ± 3
CL	12 ± 1	4 ± 2	40 ± 4	16 ± 6
LPC	2 ± 1	3 ± 1	5 ± 3	10 ± 5
PG	3 ± 2	< 1	8 ± 5	1 ± 1
U	1 ± 0.3	2 ± 1	4 ± 1	6 ± 4

Values are mean ± SD of three membrane preparations that were used in the binding study. Total lipid phosphorus: mitochondria, 331 ± 15 nmol/mg protein; and sarcoplasmic reticulum, 405 ± 7 nmol/mg protein. Abbreviation: PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; CL, cardiolipin; LPC, lysophosphatidylcholine; PG, phosphatidylglycerol; and U, unidentified.

phospholipid acyl chains whereas the propranolol amino group is located near the polar head groups of the phospholipid bilayer [45]. When a negative charge is present because of the presence in the liposomes of negatively charged phospholipids such as phosphatidylinositol or phosphatidylglycerol, K_A is greater for the high affinity site [4]. Differences in binding affinity of propranolol to mitochondria and sarcoplasmic reticulum may be explained, in part, by different ratios of acidic and neutral phospholipids in these membranes. From the data in Table 1, we calculated the ratio of acidic phospholipids to neutral phospholipids which was 0.23 for mitochondria and 0.15 for sarcoplasmic reticulum. It seems unlikely that the difference in K_A can be explained on this basis alone. Propranolol binding to protein components of the respective membrane fractions may also account for differences in the observed K_A.

The phospholipid composition of the rat heart mitochondrial fraction in Table 1 is in general agreement with previous data for mitochondria as reported by Reddy *et al.* [46] and Vasdev *et al.* [12]. Our results with mitochondria differ from those of Victor *et al.* [47] in that these authors reported much lower percentages of cardiolipin, 2.6% versus 11.3 to 18.5% in other reports [12, 46] and 12% in this study. The phospholipid content of mitochondria in nmol lipid phosphorus per mg protein ranges from 304 to 380 ([12, 46], this report) while Victor *et al.* [47] reported lower values of 235. The phospholipid composition presented in Table 1 for rat heart sarcoplasmic reticulum agrees well with the reported data of Chamberlain *et al.* [48], Reddy *et al.* [46] and Ganguly *et al.* [49]. We cannot confirm the earlier report of Comte *et al.* [50] suggesting the presence of 11.7% cardiolipin in sarcoplasmic reticulum. If sarcoplasmic reticulum membranes are washed with 0.6 M KCl and reisolated from gradients, very low levels of cardiolipin (0 to 0.4%) can be achieved [48, 51]. The phospholipid content of sarcoplasmic reticulum varies widely from 330 nmol lipid phosphorus/mg protein [46] to as high as 1434 nmol/mg [51], apparently as a function of the method of membrane isolation and purification. When the membranes are prepared and washed with 0.6 M KCl and reisolated from gradients, protein is removed causing the ratio of phospholipid to protein to increase accordingly. In some cases this procedure also appears to alter the phospholipid composition [51].

The propranolol concentrations used in this *in vitro* study are higher than the blood levels measured after propranolol treatment in humans (0.1 to 0.8 μM) [42, 43, 52, 53]. However, tissue levels of propranolol may greatly exceed levels in serum based on studies in animals using [^{14}C]propranolol [54]. The intracellular binding of propranolol to mitochondrial and sarcoplasmic reticulum membranes as shown in this study *in vitro* could increase the concentration of propranolol in the immediate microenvironment of the membrane-bound phospholipases A. This could conceivably result in concentrations high enough to inhibit phospholipase A, especially in the mitochondria where 0.2 mM propranolol caused a 50% inhibition of the enzyme. Sensitive methods for the direct measurement of the propranolol concentrations in the individual heart membranes after drug treatment of animals have not yet been developed, but this is an extremely important area for future research.

In conclusion, our results show that propranolol inhibited the phospholipases A of heart mitochondria, sarcoplasmic reticulum and cytosol *in vitro* measured with [1-^{14}C]DOPC as substrate. The mitochondrial and cytosolic phospholipases A were more susceptible to inhibition than the phospholipase A of sarcoplasmic reticulum. Propranolol also bound to mitochondrial and sarcoplasmic reticulum membranes *in vitro* at physiological pH. These findings may be of importance during ischemia since some of the membrane-stabilizing effects of propranolol may occur as a result of drug binding and inhibition of the endogenous phospholipases A. This could explain, at least in part, some of the beneficial effects of propranolol reported during myocardial ischemia and infarction.

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