Exaprolol as a Modulator of Heart Sarcolemmal ($Na^+ + K^+$)-ATPase. Evidence for Interaction with an Essential Sulfhydryl Group in the Catalytic Centre of the Enzyme

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Abstract. Beta-adrenoceptor blocking agents may have, in addition to their primary action, also ancillary effects on the cell membrane. In the present paper the non-specific interaction of exaprolol with the ATPase systems in isolated rat heart sarcolemmal membranes was investigated. When preincubated with sarcolemmal membranes in vitro, exaprolol in concentrations below 10^{-4} mol.l^{-1} had no significant effect on sarcolemmal Mg^{2+}-, Ca^{2+}- and ($Na^+ + K^+$)-ATPase activities. At exaprolol concentration of 10^{-4} mol.l^{-1} the Mg^{2+}- and Ca^{2+}-ATPase activities became inhibited whereas the ($Na^+ + K^+$)-ATPase activity was markedly stimulated. A kinetic analysis of these interactions revealed a non-competitive inhibition of Mg^{2+}- and Ca^{2+}-ATPase. In the case of ($Na^+ + K^+$)-ATPase a synergistic type of stimulation characterized by an exaprolol-induced conversion of an essential sulfhydryl group in the active site of the enzyme to the more reactive [S^-] form has been observed thus increasing the affinity of the enzyme to ATP. Exaprolol concentrations exceeding 5×10^{-4} mol.l^{-1} induced an overall depression of the investigated enzyme activities.

Key words: Exaprolol — Heart sarcolemma — ($Na^+ + K^+$)-ATPase

Introduction

Beta-adrenoceptor blocking agents are usually distinguished by their preferential affinity to $beta_1$ or $beta_2$ adrenoceptors. In the former case they belong to the group

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of cardioselective agents and may further be characterized by their intrinsic sympathomimetic activity as well as by other, less-specific membrane effects (Clark 1982). The latter effects cannot be considered as unimportant since they are responsible for membrane stabilizing and/or local anesthetic actions of these drugs modulating the membrane permeability for different mono- and divalent cations (Van Zwieten and Timmermans 1983).

The present study deals with the influence of exaprolol, 1-(2-cyclohexyl fenoxy)-3-isopropylamino-2-propanol (Carrissimi et al. 1976; Jendrichovský et al. 1978), on the activity of sarcolemmal $(Na^+ + K^+)$-ATPase in partially purified membrane preparations from the rat heart.

Materials and Methods

Membrane preparation. Male Wistar albino rats (180—240 g) kept on standard laboratory pellet diet with free access to water were killed by decapitation. Hearts were rapidly excised, chilled in ice-cold saline and stripped of great vessels and valve structures. After preliminary mincing with scissors the tissue was homogenized twice for 30 s in 10 mmol.l^{-1} TRIS-HCl buffer (pH = 7.4) using a Polytron PT-20 homogenizer (Switzerland) setted in position 8. The ratio between the tissue and the homogenization medium was kept at 1 : 10. The further isolation procedure was essentially similar to that described by McNamara et al. (1974) with following modifications: LiBr treatment was replaced by treatment with 0.6 mol.l^{-1} NaI for 10 min and the subsequent extraction with KCl was omitted. The resulting membrane fraction was repeatedly washed and centrifuged at $1000 \times g$ and eventually resuspended in 10 mmol.l^{-1} TRIS-HCl buffer (pH = 7.4) to a final concentration of 1 mg protein per ml. Our modification of the isolation technique was similar to that described elsewhere (Kostka et al. 1981; Ziegelhöffer et al. 1983).

The purity of the obtained membrane fraction, its enzyme characteristics from the point of view of ATPases and the orientation of sarcolemmal vesicles were similar to those described in our previous papers (Ziegelhöffer et al. 1983; Vrbjar et al. 1984).

Estimation of ATPase activities. Specific activities of sarcolemmal $(Na^+ + K^+)$-ATPase, Mg^{2+}-ATPase as well as Ca^{2+}-ATPase were investigated under optimal conditions for their activation by determining the amount of P_i liberated from ATP splitting during 10 min reaction at 37 °C in the presence and absence of different amounts of exaprolol. Further details concerning the measurement of enzyme activities have been described previously (Ziegelhöffer et al. 1983; Vrbjar et al. 1984). Protein concentration was determined by the method of Lowry et al. (1951). The content of inorganic phosphorus was determined according to Taussky and Shorr (1953). $(Na^+ + K^+)$-ATPase activity was expressed as the difference between P_i liberated in the presence of Na^+, K^+ and Mg^{2+} ions, and that in the presence of Mg^{2+} ions only. Mg^{2+}-ATPase and Ca^{2+}-ATPase activities were determined in the presence of 4 mmol.l^{-1} $MgCl_2$ and $CaCl_2$, respectively.

Exaprolol assays. Various concentrations ($10^{-7} - 10^{-3}$ mmol.l^{-1}) of exaprolol were added to the incubation medium prior to starting the enzyme reaction by adding ATP. The conditions of preincubation were similar to those for enzyme reaction and the preincubation time was kept constant (15 min).

All chemicals were purchased from Boehringer and Lachema and they were of analytical grade. Exaprolol was kindly provided by Dr. Mahrla from the Drug Research Institute, Modra, Czechoslovakia.

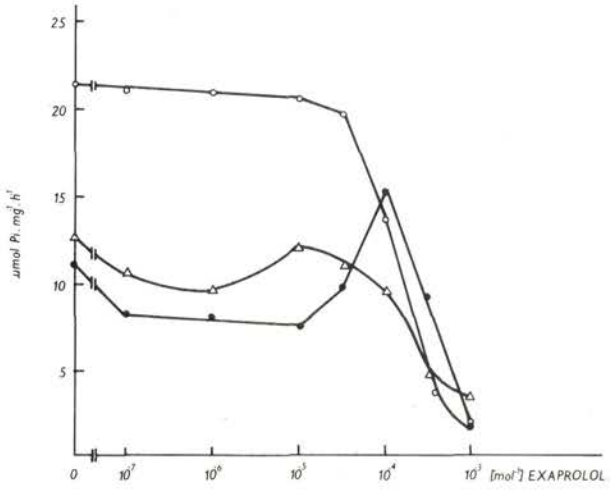

Fig. 1. Influence of exaprolol on activities of Mg^{2+}-ATPase, Ca^{2+}-ATPase and $(Na^+ + K^+)$-ATPase from isolated rat heart sarcolemma. Points represent means from 10 different measurements in the presence of exaprolol in concentrations as indicated. For details concerning the preparation and experimental technique see Materials and Methods. ○-Mg^{2+}-ATPase; ●-$(Na^+ + K^+)$-ATPase; △- Ca^{2+}-ATPase.

Results and Discussion

In the presence of optimal stimulatory concentrations of substrate and metallic ligands, increasing concentrations of exaprolol up to 5×10^{-5} mol.l^{-1} did not significantly influence the activities of sarcolemmal Mg^{2+}-, Ca^{2+}- and $(Na^+ + K^+)$-ATPase, (Fig. 1). The above finding is not very suprising, since other beta-adrenoceptor blocking agents such as propranolol have been shown to inhibit various membrane associated functions and particularly the activities of $(Na^+ + K^+)$-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase in isolated sarcolemmal membranes from myocytes only in concentrations exceeding 2.5×10^{-5} mmol.l^{-1} (Dhalla et al. 1977; Ziegelhöffer 1980). Nevertheless, inhibition with such high concentrations of the above drugs is generally considered as manifestation of their non-specific membrane deteriorating effect. In agreement with the above data, concentrations of exaprolol exceeding 5×10^{-5} mol.l^{-1} induced a significant inhibition of Mg^{2+}-ATPase and Ca^{2+}-ATPase activities ($p < 0.01$ at 5×10^{-4} mol.l^{-1}). Inhibition of Mg^{2+}-ATPase proved to be non-competitive (Fig. 2), yielding an approximately 30 per cent decrease in the V_{max} value (Table 1). A similar effect has been observed with the Ca^{2+}-ATPase activity (not shown). In contrast to ATPases activated by divalent cations, $(Na^+ + K^+)$-ATPase was found to be significantly stimulated by 10^{-4} mol.l^{-1} of exaprolol (Fig. 1). Kinetic parameters of

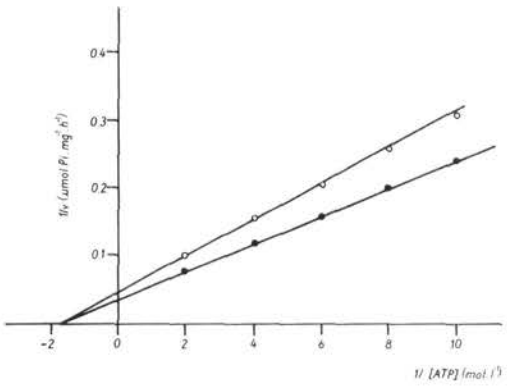

Fig. 2. Inhibition of heart sarcolemmal Mg^{2+}-ATPase by exaprolol. Points are means from 5 different measurements. Straight lines were obtained by linear regression. For further details concerning the preparation and experimental technique see Materials and Methods. Specific activities of Mg^{2+}-ATPase in the presence (○) and in the absence (●) of 5×10^{-4} mmol.l^{-1} exaprolol.

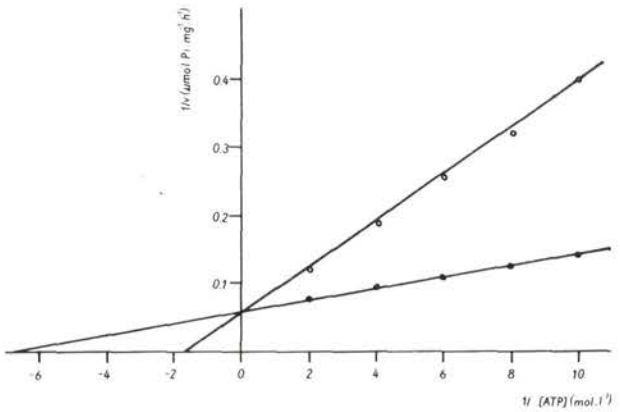

Fig. 3. Activation of heart sarcolemmal ($Na^+ + K^+$)-ATPase by increasing concentrations of adenosine triphosphate in the presence and absence of exaprolol. Single points are means from 5 different measurements. Straight lines were obtained by linear regression. For further details concerning the preparation and experimental technique see Materials and Methods. Specific activities of ($Na^+ + K^+$)-ATPase in the presence (●) and in the absence (○) of 10^{-4} mol.l^{-1} exaprolol.

this stimulation revealed that the observed enhancement in ($Na^+ + K^+$)-ATPase activity was not accompanied by any change in the V_{max} value for ATP (Fig. 3). However, it involved a considerable increase in affinity of the catalytic center of the enzyme to ATP as demonstrated by a decrease in the K_m value for the substrate (Table 1). An evaluation of allosteric properties of ($Na^+ + K^+$)-ATPase using the

Table 1. Kinetic parameters of heart sarcolemmal $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase in the presence and absence of exaprolol

KINETIC PARAMETERS	$(Na^+ + K^+)$-ATPase	Mg^{2+}-ATPase
V_{max}	17.85	29.37
$*V_{max}$	17.85	21.27
K_mATP	0.607	0.617
$*K_m$ATP	0.156	0.560
$S_{50}Na^+$	2.879	
$*S_{50}Na^+$	2.800	
$S_{50}K^+$	0.720	
$*S^{50}K^+$	0.710	
ouabain sensitivity	82	
*ouabain sensitivity	80	

V_{max} values are given in μmol $P_i.mg^{-1}.h^{-1}$; K_m and S_{50} values in $mmol.l^{-1}$; and the sensitivity to ouabain (1 $mmol.l^{-1}$) in per cent of the total activity. Data obtained in the presence of 10^{-4} $mol.l^{-1}$ exaprolol for $(Na^+ + K^+)$-ATPase and 5×10^{-4} $mol.l^{-1}$ exaprolol for Mg^{2+}-ATPase are indicated by asterisks.

Hill-plotting system (not shown) disclosed that either the activation of the enzyme by Na^+ and K^+ ions or its sensitivity to ouabain were involved in the mechanism of exaprolol-induced stimulation (Table 1).

Arguments against the physiological significance of beta-blocking agents-mediated membrane perturbations based on much lower effective therapeutic doses of these drugs ($10^{-7} - 10^{-6}$ $mol.l^{-1}$) may be counterbalanced by the knowledge of the ability these drugs to accumulate in the membranes. After a prolonged treatment such an accumulation may yield even membrane concentrations of drugs exceeding by 2—3 orders those found in the plasma (Kramer et al. 1983).

For enzymologic reasons, the isolated membrane fraction in our experiments was in contact with the exaprolol-containing medium only for 25 min including 15 min preincubation in the absence of ATP. Preliminary investigation of the sorption kinetics of exaprolol onto sarcolemmal membranes under similar conditions in vitro using a method developed by ourselves (Breier et al. 1984) revealed that the process of sorption was far from being completed within the applied time interval of 25 min. Hence, it seems reasonable to assume that a longer preincubation or a prolonged pretreatment with exaprolol might further enhance the amount of drug adsorbed on the membranes, making thus the modulation of membrane-bound ATPases more expressed.

Further elucidation of the particular molecular mechanism by which exaprolol may interact with $(Na^+ + K^+)$-ATPase was based on our previous finding that the active site of the enzyme contains an essential sulfhydryl (SH) group recognizing ATP (Ziegelhöffer et al. 1983). As for the mechanism of this recognition, it

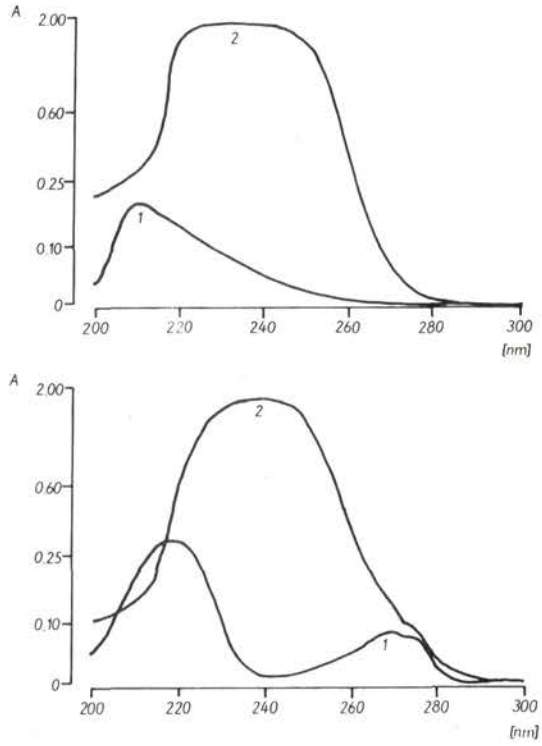

Fig. 4. Ultraviolet absorption spectra of 2-mercaptoethanol with partially dissociated and predominantly dissociated sulfhydryl groups (upper panel), and of 2-mercaptoethanol in the presence of exaprolol (lower panel). Upper panel: 1-UV spectra of mercaptoethanol at pH = 7.0 with partially dissociated sulfhydryl groups; 2-UV spectra of 2-mercaptoethanol at pH = 10.5 with sulfhydryl groups predominantly in the [S^-] form. Measurements were made at a 2-mercaptoethanol concentration of 10^{-3} mol.l^{-1}. Lower panel: 1-UV spectra of 2.5×10^{-5} mol.l^{-1} exaprolol; 2-UV spectra of 10^{-3} mol.l^{-1} 2-mercaptoethanol in the presence of 2.5×10^{-5} mol.l^{-1} exaprolol at pH = 7.0. Tracings were made by a Beckman DB-G spectrophotometer with a linear recorder. A — absorbance.

proceeds via formation of a hydrogen bound between the respective SH group and the 6-amino group of the purine moiety of ATP as it has been shown by Patzelt-Wenczler and Schoner (1981) on purified ($Na^+ + K^+$)-ATPase from the kidney and by ourselves (Ziegelhöffer et al. 1983) on heart sarcolemmal ($Na^+ + K^+$)-ATPase. Owing to a pair of free electrons on the tertiary amino group of the propanolamine side chain of the exaprolol molecule which may react with the proton on the SH group in the ATP-binding site of the enzyme, we supposed that similarly to ATP, such an interaction might represent the active principle of exaprolol action. To check this assumption a model experiment was designed in which membranes were replaced by 2-mercaptoethanol (2-ME) as well defined

representative of SH group-bearing compounds having distinct spectral characteristics. A comparison of the UV spectra of 2-ME at pH = 7 and pH = 10.5, when the SH groups of the compound will be in partially dissociated (mixed) and dissociated [S^-] form, respectively (Fig. 4, upper panel) with the same spectra of exaprolol in absence and presence of excess 2-ME (Fig. 4, lower panel) revealed that exaprolol induced a shift in the dissociation state of SH groups on 2-ME towards the more reactive [S^-] form. Hence, such a type of acid-base catalysis may be assumed to act also in the case of $(Na^+ + K^+)$-ATPase and exaprolol, increasing the reactivity, i.e. the affinity of the enzyme for ATP. The plausibility of such a reaction mechanism for exaprolol was conclusively verified in a special series of experiments where the stimulatory action of the drug (10^{-4} mol.l^{-1}) on $(Na^+ + K^+)$-ATPase activity could be abolished to 70 per cent in the presence of 5×10^{-4} mol.l^{-1} of cysteine added to the incubation medium. Similar results could also be obtained using 5×10^{-5} mol.l^{-1} glutathion.

The above results show that in addition to a specific $beta_1$-adrenoceptor blocking action exaprolol also has a stimulatory effect on heart sarcolemmal $(Na^+ + K^+)$-ATPase activity. The latter ancillary property of the drug may be involved in its membrane stabilizing effect.

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References

Breier A., Gemeiner P., Ziegelhöffer A. (1984): Simple estimation of carrier binding capacity using sorption kinetics curve fitting. J. Biochem. Biophys. Methods **9,** 267—275

Clark B. (1982): Beta-adrenoceptor-blocking agents: Are pharmacologic differences relevant? Am. Heart J. **104,** 334-346

Dhalla N. S., Lee S. L., Anand M. B., Chauhan M. S. (1977): Effects of acetobutolol, practolol and propranolol on the rat heart sarcolemma. Biochem. Pharmacol. **26,** 2055—2060

Jendrichovský J., Rybár A., Štibrányi L., Dřímal J., Jendrichovská E. (1978): Beta-amino-1-cyclohexyl aryloxy-2-propranols. Collect. Chem. Commun. **43,** 1100—1107

Kostka P., Ziegelhöffer A., Džurba A., Vrbjar N. (1981): A comparative study of the enzyme characteristics of different sarcolemmal preparations from the rat heart. Physiol. Bohemoslov. **30,** 173

Kramer J. H., Owens K., Weglicki W. B. (1983): Cardiac sarcolemmal $Na^+ - K^+$-ATPase: combined effects of propranolol and arachidonyl CoA. Am. J. Physiol. **244** (Heart Circ. Physiol. **13**), 4738—4742

Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J. (1951): Protein measurement with the folin phenol reagent. J. Biol. Chem. **193,** 265—275

McNamara D. B., Sulakhe P. V., Singh J. N., Dhalla N. S. (1974): Properties of heart sarcolemmal $(Na^+ + K^+)$-ATPase. J. Biochem. **75,** 795—803

Patzelt-Wenczler R., Schoner W. (1981): Evidence for two different reactive sulfhydryl groups in the ATP-binding sites of $(Na^+ + K^+)$-ATPase. Eur. J. Biochem. **114,** 79—87

Taussky H. H., Shorr E. (1953): A microcolorimetric method for the determination of inorganic phosphorus. J. Biol. Chem. **202,** 675—685

Van Zwieten P. A., Timmermans P.B.M.W.M. (1983): Differential pharmacological properties of beta-adrenoceptor blocking drugs. J. Cardiovasc. Pharmacol. **5** (Suppl. 1), S1—S7

Vrbjar N., Soós J., Ziegelhöffer A. (1984): Secondary structure of heart sarcolemmal proteins during interaction with metallic cofactors of $(Na^+ + K^+)$-ATPase. Gen. Physiol. Biophys. **3,** 317—325

Ziegelhöffer A. (1980): Antianginal and antiarrhythmic agents and cellular components. In: Pharmacological control of Heart Circulation (Eds. T. Tardos, L. Szekres and J. Gy. Papp), pp. 17—19, Akadémiai Kiadó, Budapest

Ziegelhöffer A., Breier A., Džurba A., Vrbjar N. (1983): Selective and reversible inhibition of heart sarcolemmal $(Na^+ + K^+)$-ATPase by p-bromophenyl isothiocyanate. Evidence for a sulfhydryl group in the ATP-binding site of the enzyme. Gen. Physiol. Biophys. **2,** 447-456

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