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INTERACTIONS OF ADRENERGIC COMPOUNDS WITH BRAIN MEMBRANE CONSTITUENTS

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Abstract—The specific activity of the Na^+, K^+-ATPase of rat brain synaptosomes was modulated by a series of adrenergic compounds in a manner related to each compound's partition between aqueous and organic solvents. The more organic-soluble compounds, the β-2 adrenergic blockers propranolol, pronethalol and butoxamine, inhibited the enzyme between 13 and 30 per cent. The more aqueous-soluble compounds, the agonists, epinephrine, norepinephrine and isoproterenol, and the β-1 blockers, practolol and acebutolol, stimulated the enzymatic activity by 30 per cent. These effects may be due to non-specific membrane interactions rather than to specific receptor effects. Optical measurements with pure protein and phospholipid indicated that the aqueous-soluble compounds bound to protein while the organic-soluble compounds interacted with acidic phospholipid phosphatidyl serine. The possible consequences of the compounds binding with acidic phospholipids and the resulting effect on membrane properties are discussed.

Several β-adrenergic blocking agents have been shown to have a 'non specific' stabilizing or local anesthetic effect on biological membranes [1–3]. The stabilizing effect, originally used to show that local anesthetics protected against hypotonic lysis in erythrocytes, has been observed at concentrations several orders of magnitude higher (10^{-5} to 10^{-3} M) than that necessary for optimum drug-specific receptor interactions [1, 2]. These studies indicated that drugs with greater solubility in non-polar solvents generally had greater anesthetic potency. The adrenergic agents chosen for this study were considered as a group of compounds that had structural homology (Fig. 1), although from a pharmacological point of view, they represented both β-1 and β-2 blockers as well as agonists. The approach was initially to examine the interaction of these compounds with membrane components, at concentrations similar to those used to elicit anesthetic effects. The Na^+, K^+-ATPase, an important intrinsic membrane constituent, was chosen for study since this enzyme requires phospholipid for full activity [4–6] and its activity may reflect modifications to either the protein or lipid region of the membrane. A goal was to determine which membrane components might mediate drug effects on the enzyme and which structural features of the drugs were essential for these effects.

METHODS

Adult male Sprague–Dawley rats were decapitated and the brains removed and rapidly cooled in 0.32 M sucrose, pH 7.4. Synaptosomes were prepared by differential centrifugation, essentially as described by Gray and Whittaker [7]. The resulting material was stored at −80° in sucrose. The Na^+, K^+-ATPase was assayed ± Na^+, using AT[^{32}P] as substrate [8]. For determining the effects of the adrenergic compounds on Na^+, K^+-ATPase activity, the synaptosomes were preincubated in a buffered solution containing the desired concentration of the compound at 37° for 10 min. The subsequent enzyme assay solutions also

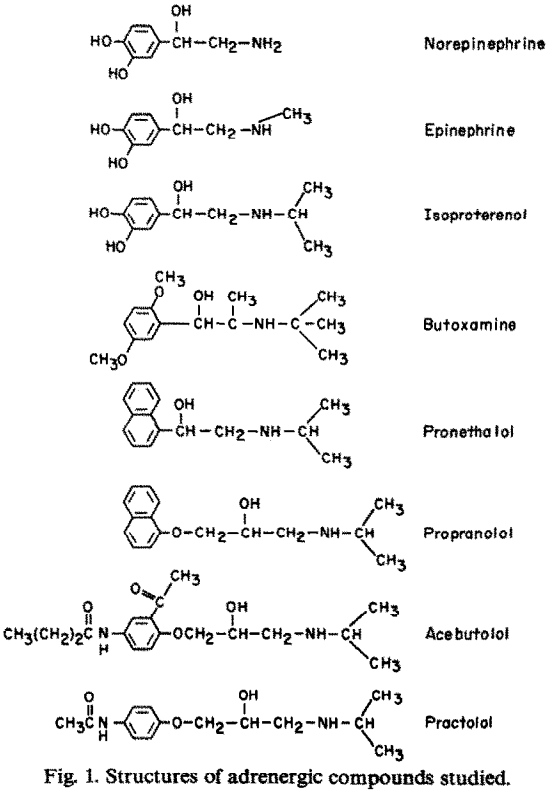

Fig. 1. Structures of adrenergic compounds studied.

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contained an identical concentration of the compound under study. All reported values were obtained from a minimum of three separate determinations, each done in duplicate.

The partitioning of the compounds was performed as described by Wiethold *et al.* [3]. A 0.5 mM drug solution in 0.1 M phosphate buffer, pH 7.0, was mechanically shaken with an equal volume (1 ml) of chloroform for 20 min. The phases were separated by low speed centrifugation and the optical density of the aqueous phase was determined before and after extraction, to determine the concentration of drug in each phase.

The techniques of solvent perturbation difference spectroscopy, as they apply to protein study, have been reviewed by Herskovits [9]. Two double-compartment cuvettes were used. The compartments were in tandem so that initially the compound under study was separated from the protein or phospholipid in each cuvette and a baseline spectrum was run. The compartments of the sample cuvette were mixed to allow interaction between the drug and protein or lipid. About 5 min was allowed for equilibrium before the second spectrum was scanned. The total concentration and optical density were the same in both cuvettes before and after mixing, so the difference between the spectra was due to interaction between the constituents in the sample cuvette. A difference of less than 0.01 O.D. in a solution with a total O.D. of greater than 1 could be easily detected. The adrenergic compounds were used at concentrations of 0.1–0.5 mM in 0.1 M phosphate buffer, pH 7.0; the bovine serum albumin (BSA) concentration was 1.75 mg/ml, and phosphatidyl serine (PS) in phosphate buffer was between 75 and 80 μM, as noted in the legends.

The D and L isomers of propranolol, pronethalol and practolol were products of Ayerst Laboratories, New York, N.Y. and were a gift of Mr. W. D. Northcroft. Acebutolol (Ives Laboratory, New York, N.Y.) and butoxamine (Burroughs Wellcome Co., Research Triangle Park, N.C.) were gifts from Dr. J. Brown. Norepinephrine, epinephrine and isoproterenol were obtained from the Sigma Chemical Co. (St. Louis, MO). Each compound in this collection was made as a stock solution of 50–100 mM in 1 mM HCl. Dilutions with buffer were made prior to the introduction of membrane material.

RESULTS

Effects of drugs on Na^+, K^+*-ATPase activity.* The Na^+,K^+-ATPase within rat brain synaptosomes appeared to be affected by all of the adrenergic compounds examined (Fig. 2). All the β-2 blocking agents, propranolol, pronethalol, and butoxamine, inhibited enzyme activity. The D-optical isomer of propranolol was as effective as the L isomer in producing inhibition and this was taken as evidence that the inhibition was produced by a general membrane phenomenon rather than by a specific interaction at a drug receptor site. In the latter case the L isomer would be 100–500 times more effective than the D isomer [2, 10]. Pronethalol, a close structural homolog of propranolol (Fig. 1), was not as potent an inhibitor as propanolol. Butoxamine, the third β-2 blocker used, was equal to pronethalol in its ability to inhibit the Na^+,K^+-ATPase.

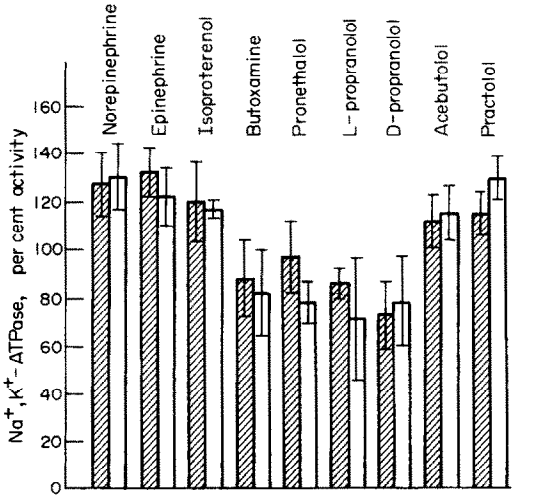

Fig. 2. Effects of adrenergic compounds on the specific activity of the Na^+, K^+-ATPase of synaptosomes. Hatched bars are 250 μM and the open bars, 1 mM, for the compounds examined $\pm$ S.D. for at least three experiments.

The β-1 blockers, acebutolol and practolol, on the other hand caused a stimulation of the Na^+,K^+-ATPase. Practolol (1 mM) caused a 30 per cent stimulation of enzyme activity over controls and was as effective in the stimulation of the agonists used below.

The β-agonists, epinephrine, norepinephrine and isoproterenol, stimulated Na^+,K^+-ATPase activity between 20 and 35 per cent. Stimulations of between 200 and 250 per cent have been reported by others [11–13]. The synaptosome preparation used in this study was isolated from whole rat brain. One previous study used synaptosomes from the hypothalamic region of the brain, which is rich in adrenergic neurons [11] and might be expected to exhibit greater adrenergic sensitivity than whole brain synaptosomes.

In order to correlate the effects of the drugs on enzyme activity with their solubility in the nonpolar phase of the synaptosome membrane, partition coefficients were examined. The partitioning of compounds, with local anesthetic action, between aqueous and organic phases has been correlated previously with membrane-stabilizing capability [1]. The procedure described by Wiethold *et al.* [3] was used to determine the partition coefficients of the adrenergic compounds used in the present study (Table 1). It is evident that all the β-blockers, propranolol, pronethalol, and butoxamine, are more soluble in the organic phase ($CHCl_3$) than in the phosphate buffer. The β-1 blockers, acebutolol and practolol, have solubility characteristics similar to the agonists; they are all more water soluble than the β-2 blockers.

This pattern is similar to the effect each compound exerted on the Na^+, K^+-ATPase (Fig. 2). Table 1 shows that there is a clear correlation between the partition coefficient of each compound and its effect on the Na^+, K^+-ATPase. Those compounds with higher solubilities in the organic phase inhibited activity while the more water-soluble compounds stimulated the enzyme.

Table 1. Partition of drugs between aqueous and organic phases and effects on Na^+, K^+-ATPase*

Drug	Partition coefficient		Na^+, K^+-ATPase
	Present work	Literature value†	(% control activity)
Propranolol	10.8	10.7	71
Pronethalol	4.00		78
Butoxamine	4.55		83
Acebutolol	0.078		115
Practolol	0.028	0.02	130
Isoproterenol	0.025		118
Epinephrine	0.034		122
Norepinephrine	0.007		131

*To establish the partition coefficient (Drug concn. in $CHCl_3$)/(drug concn. in phosphate buffer), 1 ml of 0.10 M phosphate buffer containing 0.5 mM drug and 1 ml $CHCl_3$ were shaken for 20 min and centrifuged into separate phases. The optical density at the drug absorbance maximum of the aqueous phase after extraction was compared to that before extraction. For assessing effects on specific activity of the Na^+,K^+-ATPase, drugs were present at 1 mM in the enzyme assay (data from Fig. 2).

†Ref. 3.

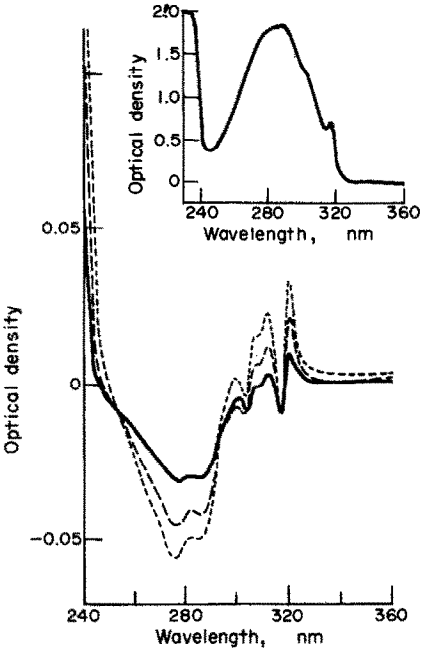

Fig. 3. Difference spectra of propranolol mixed with phosphatidyl serine. The cuvette contained initially: 0.1 M phosphate buffer, pH 7.0, 0.5 mM propanolol and PS concentrations of 90, 180 and 270 μM. Inset: absorbance spectrum of 1 mM propanolol in the same buffer.

The Na^+,K^+-ATPase enzyme requires phospholipid for optimum activity [4–6]. The question arose as to whether the organic-soluble compounds exerted their influence by interacting with the lipid domain of the membrane while the aqueous-soluble compounds interact at polar locations on the membrane surface. This was approached by using the technique of solvent perturbation using difference spectroscopy as developed by Herskovits [9] for use with water-soluble proteins. Difference spectroscopy allows accurate determination of very small optical density changes in a system of high optical density. The procedure followed is described in Methods.

The difference spectra of synaptosomes (90–360 μg protein/ml) separated from and later mixed in double-compartment cuvettes and a 1 mM propanolol solution were analyzed. Several positive peaks in the 320–310 nm region and a lesser 'negative' peak near 280 nm were observed (data not shown). Unfortunately the light scattering of the synaptosomal suspension posed technical problems. The scatter factor for 90 μg protein/ml in a 0.5-cm path was not identical to 45 μg protein/ml in a 1-cm path. A computer method was employed to correct the scattering of the individual components from the mixed spectra, but with the equipment available a poor signal-to-noise ratio existed. Although quantitation of the changes was imprecise, the qualitative changes in the spectrum were quite evident. Because of these problems we turned to phospholipids for further study. Phosphatidyl serine was used because it has been shown to reactivate the Na^+, K^+-ATPase enzyme in lipid-depleted membranes [6]. Bovine serum albumin was chosen as a model protein, and even though it is water soluble, it has been shown to bind many organic compounds. Its role in the blood stream has been postulated as a nonspecific transport protein for diverse compounds. Thus, it has the potential of binding or interacting with the compounds studied.

Figure 3 shows the difference spectra of 0.5 mM propranolol mixed with three concentrations of phosphatidyl serine. When mixed with BSA there was no change in optical properties observed. The interaction between propranolol and PS was obvious in the difference spectra, and the change was dependent upon the amount of phospholipid present. The difference spectra show maxima in optical density at 300, 309, 312 and 320 nm with minima at 318, 285 and 275 nm. The insert presents the absorbance spectra of propranolol in the same buffer system. Since PS does not absorb light in this wavelength region, it can be concluded that the spectral changes seen were due to adrenergic compound. It would have been desirable to utilize a purified intrinsic protein, such as the Na^+, K^+-ATPase, here as well; however, no convenient preparation of this enzyme devoid of bound phospholipid is presently available.

Similar spectral scans were obtained when pronethalol was mixed with three concentrations of PS (data not shown). Pronethalol did appear to cause a small spectral change with BSA but there was a more pronounced change when it was mixed with PS. The pattern of peaks and valleys was very similar to that of propranolol and PS, but with pronethalol these were shifted to lower wavelengths. Peaks were at 287 and 278 nm with valleys at 271 and 261 nm. The changes were dependent upon the amount of phospholipid present.

The difference spectra of isoproterenol, a water-soluble agonist, are shown in Fig. 4. There was no change evident when it was mixed with PS, but in the

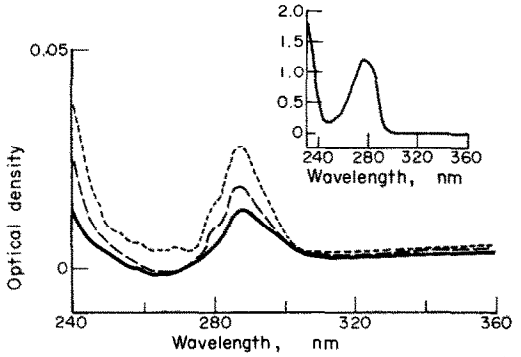

Fig. 4. Difference spectra of isoproterenol mixed with bovine serum albumin in 0.10 M phosphate buffer, pH 7.0. The BSA concentrations were 0.875, 1.75 and 2.6 mg/ml. Inset: absorbance spectrum of isoproterenol in 0.1 M phosphate.

presence of BSA, there was a small peak at 286 nm. The absorbance maximum of the drug was 277 nm and that of the protein 280 nm. Both of these maxima overlap the wavelength where the change was observed. It is difficult therefore, to determine which structure is contributing to the difference spectrum.

The difference spectra data for the compounds examined are summarized in Table 2. These data are consistent with the partition coefficient of each compound in that those that are more soluble in organic solvents interact with the phosphatidyl serine while those with lower partition coefficients do not. The three agonists, epinephrine, norepinephrine and isoproterenol, did cause a change in the absorbance in the ultraviolet region when mixed with BSA. Acebutolol and practolol did not produce discernible absorbance changes when mixed with either the phospholipid or the protein. The absorbance changes of the drugs reflect changes in their ring structure, and changes associated with the protein are due to the aromatic amino acids of the compound. The interaction or binding may occur without causing an absorbance change. Therefore, the lack of optical density change with any of these compounds cannot be taken as evidence for lack of interaction. The presence of a change does indicate that an interaction between the two species exists.

DISCUSSION

In the present study we have examined some of the interactions of a series of adrenergic compounds with an intrinsic membrane enzyme (Na^+, K^+-ATPase) as well as with a phospholipid (PS) and a soluble protein (BSA). A general hypothesis for anesthetic action has been that more lipid-soluble compounds exhibit local anesthetic potency [1] although the exact nature of the hydrophobic site involved remains uncertain. This could involve action with the bulk phase of lipid, increasing the bilayer thickness [14], or be a direct action on protein hydrophobic sites, or both. The spectral studies reported here show that drugs with high solubility in non-polar solvents definitely interact with PS micelles. For technical reasons, particularly overlap of absorbance maxima, interaction of the drugs with BSA is less clear. However, drugs like propranolol and pronethalol, having high partition coefficients, interacted with PS and not with BSA. On the other hand, drugs like isoproterenol and norepinephrine, with low partition coefficients, interacted only weakly with PS. Relatively high drug concentrations were used on synaptosome membranes so that all specific sites as well as many non-specific sites should have been occupied. Under these conditions the Na^+, K^+-ATPase was found to respond to all of the adrenergic agents tested, in a way which seemed to be related to the lipid solubility

Table 2. Detection of interaction of drugs with protein and phospholipid by difference spectrophotometry*

	PS (180 μM)		BSA (1.75 mg/ml)	
Drug (0.5–1 mM)	Spectral change	Wavelength (nm)	Spectral changes	Wavelength (nm)
Propranolol	Yes	+320 +310 +285	No	
Pronethalol	Yes	+287 +278 −271 −260	Small	+284
Butoxamine	Yes	−288 +305	No	
Acebutolol	No		Yes	+315 −271 +255
Practolol	No		Yes	+283 −250
Isoproterenol	No		Yes	+286
Epinephrine	No		Yes	+286
Norepinephrine	No		Yes	+284

*Changes in absorbance spectra caused by drug interaction with PS or BSA. Each sample was scanned from 370 to 230 nm to establish a baseline. The sample cuvette was mixed to allow interaction to occur. The samples were scanned to determine any spectral changes. The molar ratio of drug to PS was 5:1.

of the compounds and not to their adrenergic efficacy. That is, the β-2 blockers propranolol, pronethalol and butoxamine, had high partition coefficients and inhibited enzyme activity, while the β-1 blockers, practolol and acebutolol, and the β-agonists, epinephrine, norepinephrine and isoproterenol, had low partition coefficients and tended to stimulate enzymatic activity. The Na^+, K^+-ATPase is known to require phospholipid for normal functioning [6]. The observed inhibitory effects might be due to either a direct interaction of drugs with specific phospholipids in the microenvironment of the enzyme or more generally to phospholipids in the bulk phase of the membrane, since either could affect activity of the enzyme.

Lipid-soluble compounds should partition and concentrate in the lipid–phospholipid region of the membranes. The drug's presence in this region could affect several membrane constituents. For example, calcium is displayed by propranolol [15]. Displacement of calcium could fluidize the phospholipid fatty acid tail near sites of binding. Recent studies by Onishi and Ito [16], Lansman and Haynes [17], and Papahadjopolous *et al.* [18] have shown that calcium can cause a clustering of acidic phospholipids. When the drug displaces calcium, the character of the membrane would be altered from one with patches of rigid hydrocarbon in an area of more fluid hydrocarbon to one more uniform and with a less fluid nature. Since the Na^+, K^+-ATPase requires phospholipid for activity, modification of the phospholipid fluidity by the drug could inhibit the enzyme.

A positive correlation has been made between the potency of a local anesthetic and ability to cause a volume expansion of the membrane [1, 15]. This may be due to the randomization of the fatty acid tails of the membrane rather than to the physical inclusion of the compound into the membrane; more likely, a combination of both effects is observed. A partial explanation of this lies in different solubility characteristics which would direct the drugs to either a polar or non-polar binding site. The optical measurements with the model protein and phospholipid give some indication of this. A difficulty is encountered if this hypothesis is applied to specific receptor mechanisms. Blocking agents by definition prevent the action of the agonists. If these sites were located at different regions of the membrane, it is hard to envision the competitive inhibition that has been observed between agonists and antagonists. If, however, the receptor site possessed both polar and non-polar regions as well as the recognition area, then a compound bound within non-polar region, whether making the hydrocarbon region more or less fluid, could determine the overall effect of the compound.

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