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MEMBRANE EFFECTS OF BETA-ADRENERGIC BLOCKING AGENTS: INVESTIGATIONS WITH THE FLUORESCENCE PROBE 1-ANILINO-8-NAPHTHALENE SULFONATE (ANS) AND ANTIHEMOLYTIC ACTIVITIES*

GERHARD WIETHOLD, DIETER HELLENBRECHT, BJÖRN LEMMER and DIETER PALM

Pharmakologisches Institut der Universität Frankfurt, D-6 Frankfurt am Main, Theodor-Stern-Kai 7, West Germany

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Abstract—The unspecific membrane effects of eight therapeutically used β-receptor blocking drugs and several phenoxypropanolamine derivates of the Kö-series with β-adrenergic blocking potency were studied. As parameters of the membrane affinity of these compounds served: (1) their ability to increase the number of ANS-binding sites in isolated erythrocyte ghosts; (2) their ability to protect human erythrocytes against hypotonic hemolysis; and (3) their partition coefficients between $CHCl_3$/buffer. From the strong correlations between these three parameters it can clearly be demonstrated that compounds with high lipid solubility induce high ANS-binding and have strong antihemolytic potency. A decreasing potency with both parameters was found in the following order: propanolol > alprenolol = KL 255 > oxprenolol = pindolol = Kö 592 > practolol = sotalol. Furthermore, a significant correlation between the potency to increase the number of ANS-binding sites and the respective cardiodepressant potency† could be shown. Our results, discussed in connexion with the findings of other authors, lead to the assumption that the basic mechanism of the nonspecific membrane effects of beta-adrenergic blocking drugs is a conformational change of the cell membrane.

IT HAS BEEN the aim of many investigations recently to study the nonspecific, membrane stabilizing effects of beta-adrenergic blocking agents[1–4]. The well established use of these drugs in the therapy of angina pectoris made it necessary to have quantitative information of the so-called quinidine-like or cardiodepressant actions, which are independent from their specific actions on adrenergic receptors.[5] Experimental data from many laboratories suggest that the underlying mechanism of the nonspecific effects of beta-adrenergic blocking drugs is a change in membrane conformation leading to an impairment of the membrane permeability.

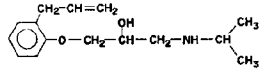
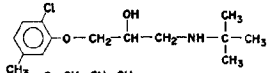
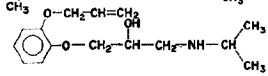
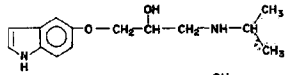
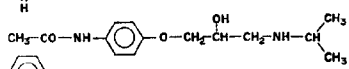
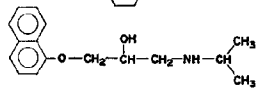
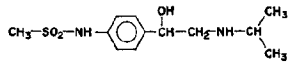
In recent years many investigators have used an anionic fluorophore, 1-anilino-8-naphthalene sulfonate (ANS) to study membrane conformational changes under different experimental conditions in various types of membrane,[6,7] e.g. erythrocyte membranes,[8–10] vesicles of the sarcoplasmic reticulum,[11–13] membranes of mitochondria and submitochondrial particles,[14–16] membranes of liver microsomes[17] and membranes of isolated nerves.[18,19] ANS is a very useful tool in these studies due to its characteristic fluorescent properties: ANS is practically nonfluorescent in water, but it is highly fluorescent when bound to membranes with a shift of the emission peak to shorter wavelength.

* This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

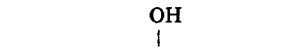
† Taken from the literature.

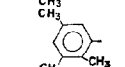
TABLE 1. BETA-ADRENERGIC BLOCKING DRUGS

A. THERAPEUTICALLY USED COMPOUNDS

Non-proprietary name	Abbreviation	Chemical structure	Company
Alprenolol	Alpr		(Hässle)
KL 255	KL		(Sanol)
Oxprenolol	Oxpr		(Ciba)
Pindolol	Pind		(Sandoz)
Practolol	Pract		(ICI)
Propranolol	Prop		(ICI)
Sotalol	Sot		(Mead u. Johnson)

B. COMPOUNDS OF THE KÖ-SERIES



Code-No.	R_1	R_2
Kö 1439		$-NH_2$
Kö 1561		$-NH-CH_3$
Kö 1560		$-NH-CH_2-CH_3$
Kö 1313		$-NH-CH(CH_3)_2$
Kö 1366		$-NH-C(CH_3)_2-CH_3$
Kö 1500		$-NH-CH(CH_3)-CH_2-CH_2-CH_3$
Kö 592		$-NH-CH(CH_3)_2$
Kö 707		$-NH-CH(CH_3)_2$
Kö 1010		$-NH-CH(CH_3)_2$
Kö 1030		$-NH-CH(CH_3)_2$
Kö 1124		$-NH-CH(CH_3)_2$

In the present experiments ANS was used to study the nonspecific membrane effects of β-receptor blocking drugs on human erythrocyte ghosts. As it is well known that lipid soluble drugs with local anesthetic activities are able to protect intact human erythrocytes against hypotonic hemolysis,[20–22] this stabilizing effect of the β-adrenergic blocking agents was also put under investigation. Furthermore, an attempt was made to determine the importance of lipid solubility, expressed by the respective partition coefficients of the drugs, for these two parameters. In order to find some structure–activity relationships, eight therapeutically used β-receptor blocking drugs and several phenoxypropanolamine derivates (Kö-substances) with β-adrenergic blocking potency were used.

MATERIALS AND METHODS

Beta-adrenergic blocking agents. Those investigated are summarized in Table 1; with the exception of pindolol (base) all substances were hydrochlorides and if not indicated otherwise, the racemates were used.

Human erythrocyte ghosts. These were prepared according to the method of Dodge *et al.*[23] The hemoglobin-free ghosts were lyophilized and stored at −20° until use. For the fluorometrical determinations, ghosts were suspended in isotonic phosphate buffer, pH 7·0 and sonified for 30 sec at 40 W (Ultra sonifier, Branson). Protein determination was carried out according to the fluorometrical method described by Resch *et al.*[24] The absolute protein content was measured by the method of Lowry,[25] with cristalline bovine serum albumin as a reference standard (Behring, Marburg).

Fluorometrical measurements. These were carried out at room temperature in an Aminco-Bowman spectrophotofluorometer. The assay mixture, with a total volume of 2·0 ml consisted of 1·0 ml ghost-suspension, 0·5 ml drug solution and 0·5 ml ANS (1-anilino-8-naphthalene sulfonate, as Mg-salt; Serva, Heidelberg). Isotonic phosphate buffer pH 7·0 served as solvent. The excitation wavelength was held constant at 380 nm (uncorrected), the emission wavelength was 470 nm (uncorrected). The following titrations were performed: (a) constant ghost and ANS concentrations varying the drug-concentration; (b) constant ANS- and drug concentrations varying the ghost concentration; (c) constant ghost and drug concentrations varying the ANS concentration. The values of the relative fluorescence intensity reported in text and figures were corrected by subtraction of the respective control values (fluorescence intensities of the assay mixtures, in which the varied constituent was substituted by isotonic phosphate buffer pH 7·0). The number of ANS binding sites (n) and the apparent dissociation constants (K_{app}) were calculated as described by Klotz.[26]

Stabilization of human erythrocytes. Stabilization against hypotonic hemolysis by β-receptor blocking drugs was investigated according to the method of Seeman[21] with slight modifications. All incubations were performed in one-way plastic tubes for 5 min at room temperature. The samples consisted of 0·5 ml drug solution dissolved in isotonic phosphate buffer pH 7·0, 4·0 ml hypotonic NaCl solution and 0·5 ml 5% erythrocyte suspension. After centrifugation, the hemoglobin concentration in the supernatant was determined by direct photometric methods at 540 nm (Zeiss PMQ II).

Partition coefficients. Partition coefficients of the drugs were determined in a system of isotonic phosphate buffer pH 7·0 and $CHCl_3$ by spectrophotometric measurement

TABLE 2. PARTITION COEFFICIENTS

Practolol	0·02	Kö 1313	0·36
Sotalol	0·01	Kö 1366	0·73
Oxprenolol	2·7	Kö 592	1·3
Pindolol	0·05	Kö 707	5·2
Alprenolol	10·1	Kö 1010	16·9
KL 255	10·7	Kö 1030	12·7
Propranolol	10·7	Kö 1124	30·0

($CHCl_3$/Phosphate buffer pH 7·0).

of the u.v.-absorbance. The values obtained, summarized in Table 2, are the mean of two to three experiments performed in duplicate.

RESULTS

ANS shows some fluorescence in a polar environment like water, but exhibits marked fluorescence in apolar milieus.[26] Thus, ANS (10^{-5} M) exhibits a stronger fluorescence (50–100 times) when added to a membrane suspension. This phenomenon is accompanied with a shift of the emission peak from 520 to 470 nm. The enhancement of the fluorescence intensity can be further increased by addition of β-receptor blocking drugs, whereas the emission peak remains unchanged. These results clearly support previous findings[8,9,28] with ions and local anesthetics.

The increase of the fluorescence intensity induced by the addition of β-adrenergic blocking agents to an ANS-containing ghost suspension is dose-dependent, but differs markedly among the β-receptor blocking drugs investigated (Figs. 1 and 2). Among the therapeutically used β-receptor blocking drugs (Fig. 1) three main groups could

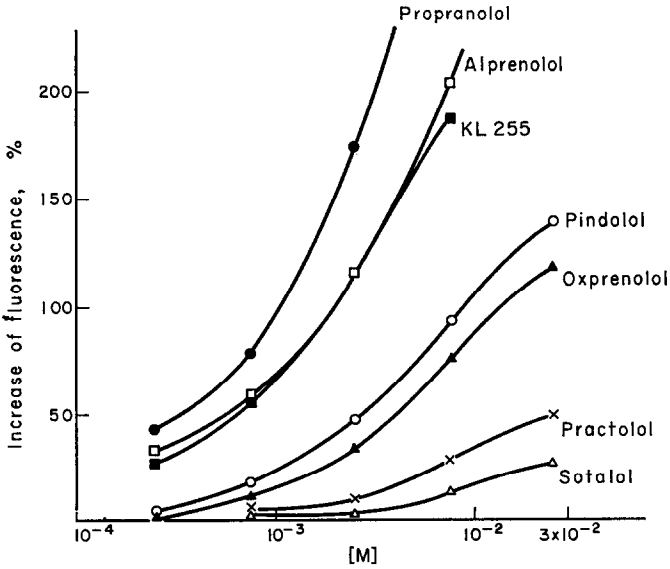


FIG. 1. Effect of beta-adrenergic blocking drugs (therapeutically used compounds) on ANS fluorescence in human erythrocyte ghosts. Experimental conditions: titration with drugs, ANS 10^{-5} M, ghosts 2 mg protein/ml, buffer pH 7·0. For further details see Materials and Methods.

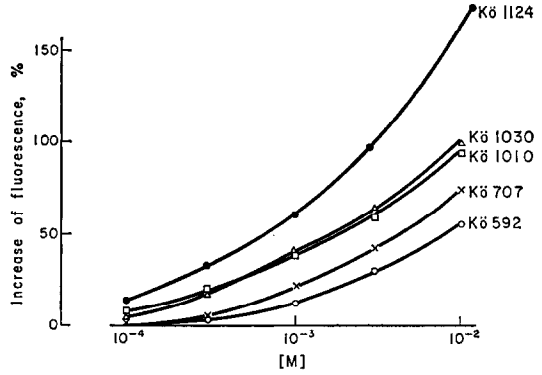


FIG. 2. Effect of beta-adrenergic blocking drugs (Kö compounds) on ANS fluorescence in human erythrocyte ghosts. Experimental conditions as in Fig. 1.

be distinguished: propranolol, alprenolol and KL 255, all inducing a very strong fluorescence intensity. Pindolol and oxprenolol were distinctly weaker, whereas practolol and sotalol cause only a small enhancement of the fluorescence intensity. The Kö-substances show the following order of potency: all compounds with a nitrilo-group at the phenylring and different substituents at the amino group (Kö 1439, Kö 1561, Kö 1560, Kö 1313, Kö 1366, Kö 1500; Fig. 1b) induce no enhancement of the fluorescence up to a concentration of 10^{-2} M; however, those Kö-compounds with an isopropyl group at the amino group and different alkylsubstituents at the aromatic ring (Kö 592, Kö 707, Kö 1010, Kö 1030, Kö 1124) cause a dose-dependent enhancement of the fluorescence, the intensity of which increases with the number of methyl groups at the aromatic ring (Fig. 2). The different fluorescence intensities induced by the β-adrenergic blocking agents of the ANS-containing ghost suspensions could be due either to an increase in the quantum yield of the ANS-molecules bound to the membrane in the presence of the added β-blocking agents, or alternatively, to an

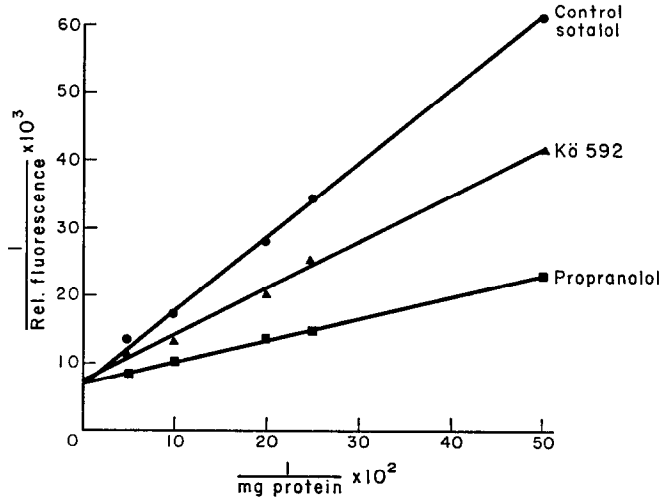


FIG. 3. A double reciprocal plot of the ANS-binding to erythrocyte ghosts as a function of the ghost-protein concentration. Drugs 5×10^{-3} M, ANS 10^{-5} M, buffer pH 7·0.

increase in the number of ANS-molecules bound to the membrane, the quantum yield remaining unchanged. These alternatives were studied experimentally with three β-receptor blocking drugs by titrating fixed concentrations of ANS and drugs with increasing amounts of ghost-protein. As can be seen from the double reciprocal plot in Fig. 3 the fluorescence intensities at infinite ghost concentrations, at which all dye molecules are bound, are essentially the same. This means, that β-receptor blocking drugs induce an increased ability of the membrane for ANS-binding. Furthermore, the hypothesis that β-receptor blocking drugs enhance the quantum yield of bound ANS can be excluded, as this would result in different maximum fluorescence intensities at infinite ghost concentration.

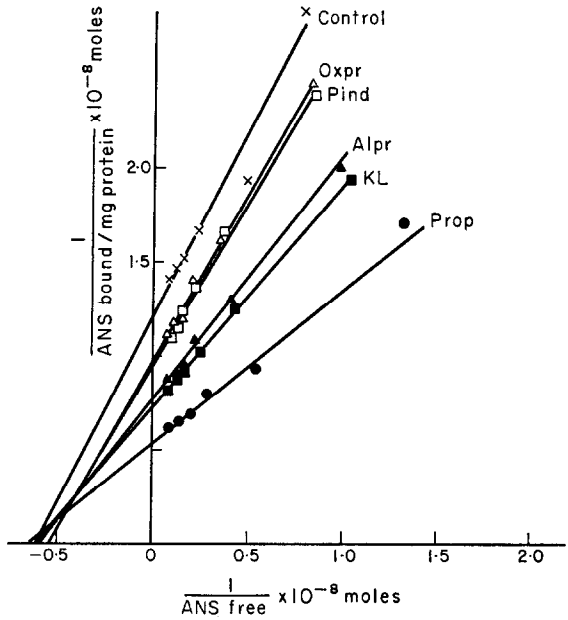


FIG. 4. The binding of ANS to erythrocyte ghosts as a function of the ANS concentration under the influence of therapeutically used β-receptor blocking drugs. Data plotted according to Klotz. Drugs 5×10^{-3} M, ghosts 2 mg protein/ml, buffer pH 7·0.

By titration of fixed drug amounts and ghost concentrations with ANS (2×10^{-5} M to $2{\cdot}8 \times 10^{-4}$ M) the type of increased ANS binding under the influence of beta-adrenergic blocking agents was determined. The data from these titrations were plotted according to Klotz[26] as shown in Figs. 4 and 5. The calculated parameters n and K_{app} are summarized in Tables 3 and 4. It is obvious that the increased ANS fluorescence induced by β-receptor blocking drugs in membrane suspensions is caused mainly by an increase of ANS-binding sites, not by an increase of the affinity of the membrane for ANS. Similarly Feinstein *et al.*,[8] Harris[16] and Rubalcava *et al.*[9] have shown that the enhancement of ANS fluorescence brought about by cations and butacain, respectively, is mainly due to an increase of n, not due to a decrease in K_{app}.

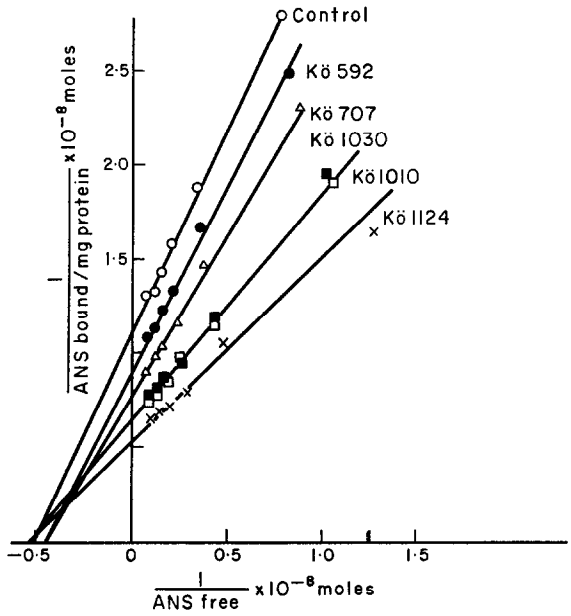


FIG. 5. The binding of ANS to erythrocyte ghosts as a function of the ANS concentration under the influence of Kö compounds. Data plotted according to Klotz. Drugs 5×10^{-3} M, ghosts 2 mg protein/ml, buffer pH 7·0.

The levo- and dextro-isomers of β-sympatholytics produce the same enhancement of the fluorescence intensity. This is shown in Fig. 6 for propranolol, KL 255 and Kö 592, where the increase of fluorescence intensity is determined by titration with ANS at constant drug and ghost-protein concentrations. This finding demonstrates that changes of ANS fluorescence are due to a nonspecific alteration of the erythrocyte membrane and are not the result of an interaction between β-receptor blocking drugs and specific receptor sites. It is well established that the levo-isomers are about 50–100 times more potent β-receptor blocking agents than the respective dextro-isomers.[1,2]

To characterize further the nonspecific membrane effects of the β-adrenergic blocking agents, the stabilizing effects of the respective drugs on human erythrocytes against hypotonic hemolysis were investigated. This effect can be demonstrated with all lipid

TABLE 3. EFFECT OF β-RECEPTOR BLOCKING DRUGS (5×10^{-3} M) ON THE ANS-BINDING SITES (n) AND THE APPARENT DISSOCIATION CONSTANT (K_{app})

	K_{app} ($\times 10^{-6}$ M)	n ($\times 10^{-9}$ moles/mg protein)
Control	8·2	8·3
Practolol	8·2	8·3
Sotalol	8·2	8·3
Oxprenolol	9·3	10·4
Pindolol	9·1	10·5
Alprenolol	8·6	13·1
KL 255	8·3	13·8
Propranolol	7·7	18·7

TABLE 4. EFFECT OF KÖ-COMPOUNDS (5×10^{-3} M) ON THE ANS-BINDING SITES (n) AND THE APPARENT DISSOCIATION CONSTANT (K_{app})

	K_{app} ($\times 10^{-6}$ M)	n ($\times 10^{-9}$ moles/mg protein)
Control	9·8	9·03
Kö 592	11·6	11·2
Kö 707	11·3	12·9
Kö 1030	9·5	15·5
Kö 1010	9·8	15·3
Kö 1124	9·5	19·2

soluble anesthetics as Seeman[20,29] has shown. In Figs. 7 and 8 the increase in osmotic resistance of erythrocytes against hypotonic NaCl-medium by equimolar concentrations of the β-blockers is depicted. The control lines without addition of drugs in both figures show the increased degree of hemolysis produced by decreasing NaCl concentrations in the incubation medium. A 50 per cent hemolysis was achieved at a final concentration of 0·46% NaCl, which was within the normal range. By addition of β-receptor blocking drugs an enhancement of the osmotic resistance was indicated by a shift of the lines towards lower NaCl concentrations. Within the group of therapeutically used β-blockers (Fig. 7) about the same order of potency can be seen as described in the investigations with ANS (Fig. 1). Propranolol, alprenolol and KL 255 induce the strongest increase in osmotic resistance (50 per cent hemolysis at 0·39% NaCl), followed by Kö 592 and oxprenolol, whereas sotalol and practolol induce only a slight increase in the osmotic resistance. It must be taken into account that the high drug concentrations used (3×10^{-3} M) may be osmotically active

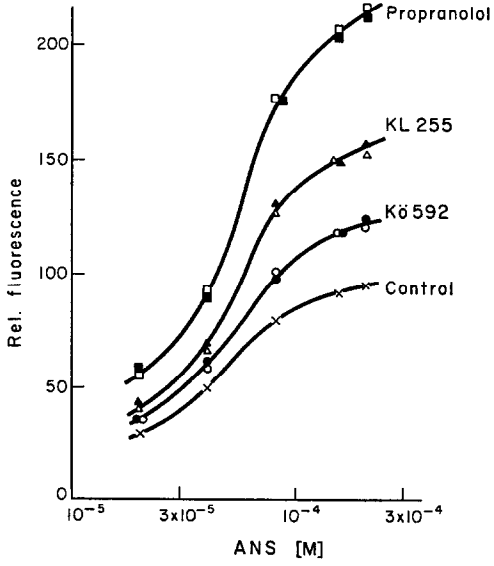


FIG. 6. The influence of optical isomers on the binding of ANS to erythrocyte ghosts as a function of the ANS concentration. Drugs 5×10^{-3} M, ghosts 2 mg protein/ml, buffer pH 7·0. Open symbols: dextro isomere, closed symbols: laevo isomere.

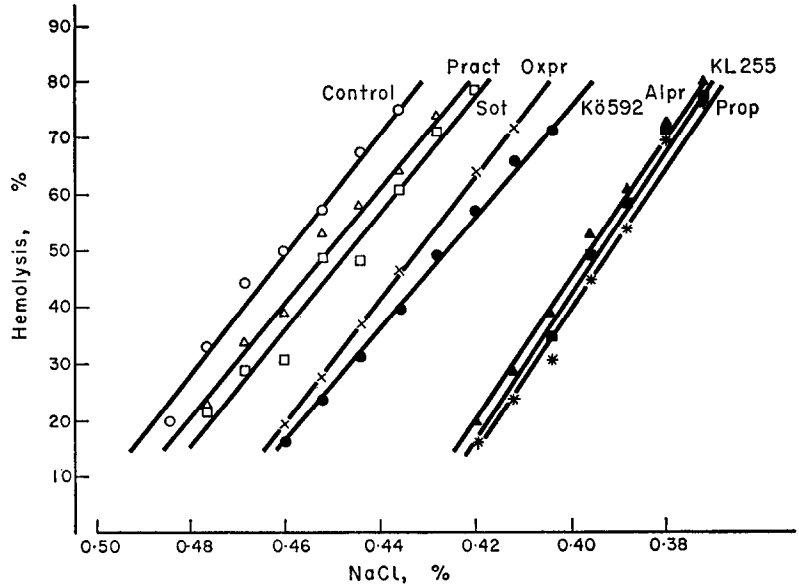


FIG. 7. The stabilization of human erythrocytes against hypotonic hemolysis by equimolar concentrations of therapeutically used β-receptor blocking drugs (3×10^{-3} M). For details see text.

separately, thus resulting in a small shift of the control curve to lower NaCl concentrations. However, for instance, propranolol and Kö 592 protect erythrocytes against hypotonic hemolysis in concentrations as low as 10^{-5} M and 10^{-4} M, respectively. Among the Kö-compounds (Fig. 8) a similar order of potency as in the experiments

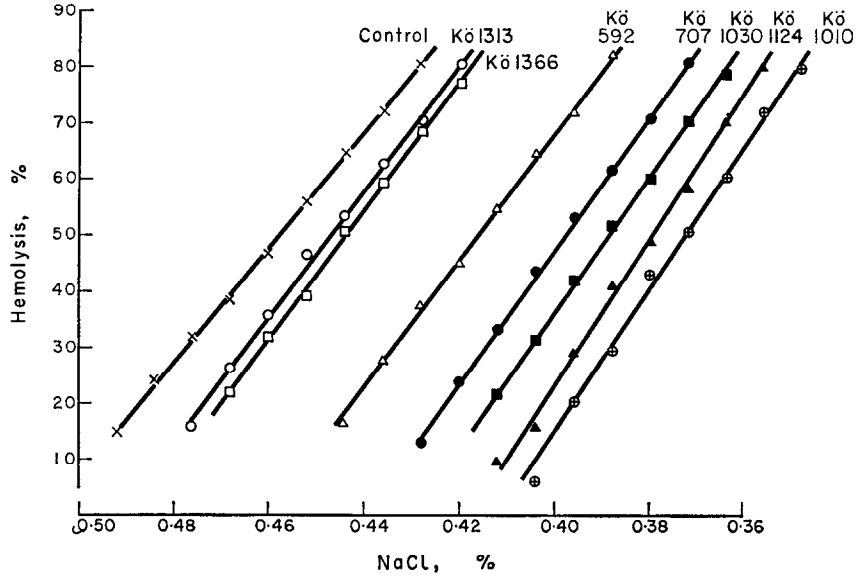


FIG. 8. The stabilization of human erythrocytes against hypotonic hemolysis by equimolar concentrations of Kö compounds (3×10^{-3} M). For details see text.

with ANS can be seen (Fig. 2) with the exception of Kö 1010, a structural isomer of Kö 1030. Kö 1010 caused the greatest shift of the osmotic resistance to the right, whereas it was expected in the range of Kö 1030. As a means of comparison, the nitrilo-compounds Kö 1313 and Kö 1366, which were ineffective in the ANS experiments, were investigated. These two compounds protect human erythrocytes against hypotonic hemolysis as has been described with practolol and sotalol, thus being quite ineffective.

DISCUSSION

The nonspecific, membrane stabilizing effects of β-adrenergic blocking agents, apart from their specific actions on adrenergic receptors, have been reflected by the findings of other investigators. These findings include electrophysiological investigations,[30] ion flux measurements in isolated hearts,[31–33] measurement of the Ca^{2+}-transport across an aqueous–lipid-solvent system[34] as well as the Ca^{2+}-transport in mitochondria,[35] and finally the effects on the active transport system of serotonin in blood platelets.[36,37]

From these findings it can be assumed that β-receptor blocking agents exert their nonspecific effects by changing the properties of the membranes of a given effector cell, resulting in an impaired membrane permeability.

Our results indicate that the membrane effects of β-receptor blocking drugs can easily be demonstrated in systems *in vitro*, i.e., in erythrocyte ghosts and intact erythrocytes. Moreover, our quantitative data might allow the prediction of the strength of the nonspecific, e.g. cardiodepressant effects of these drugs.* The enhancement of ANS fluorescence reflects a definite alteration of membrane structure. In our experiments the alteration is characterized by an increase of ANS binding sites. In this respect within the group of therapeutically used β-receptor blocking drugs, propranolol, alprenolol and KL 255 were most active, whereas practolol and sotalol were ineffective. The same order of potency was also found measuring the protective effect of these drugs against osmotically induced hemolysis. The significant correlation between both parameters is revealed by a $r = 0{\cdot}8945$ with a $P < 0{\cdot}001$. Concerning the order of potency, which results from both kinds of experiments, ANS-binding and erythrocyte stabilization, one can recognize the order in which the β-blocking drugs depress the cardiac contractility. For example, in Fig. 9 the linear correlation between per cent increase of ANS binding sites and the relative negative inotropic actions (from literature) can be seen.

The different degree of increase in ANS-binding sites produced by the various β-receptor blocking drugs characterizes not only the extent of the change of the membrane structure but could also indicate a different degree of accumulation of these drugs in the membrane due to their different lipid solubilities. This assumption is supported by the following results: a highly significant correlation between the increase of the number of ANS-binding sites and the partition coefficients of the respective drugs could be obtained ($r = 0{\cdot}8423$; $P < 0{\cdot}001$). Similarly a correlation was found between antihemolytic potency and lipid solubility ($r = 0{\cdot}8825$; $P < 0{\cdot}001$).

This is in accordance with the hypothesis of Hansch.[38] This author has demonstrated

* The term cardiodepression refers only to direct negative inotropic action of the drugs on cardiac muscle.

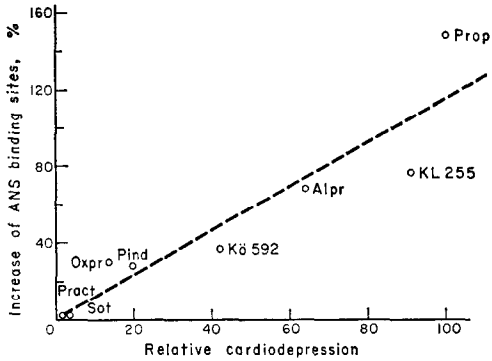


FIG. 9. Correlation between the potency of increasing the ANS-binding sites in human erythrocyte ghosts and the respective negative inotropic effects of some β-receptor blocking drugs. Values of relative cardiodepression are means related to propranolol = 100, taken from the literature: Morales-Aguilera and Vaughan Williams,[30] Nayler *et al.*,[34] Engelhardt and Traunecker,[41] Barret and Collum,[44] Grodzinska and Gryglewsky,[45] Koch-Weser,[46] Parmley and Braunwald,[47] Reuter *et al.*[48] Saameli,[49] Singh and Vaughan Williams,[50] Wagner *et al.*[51] $r = 0{\cdot}927$; $P < 0{\cdot}001$. Linearity of the regression line was tested by the F-distribution.

a relation between physico–chemical and pharmacological properties of many drugs in various experimental models, i.e., the biological activity depends on the respective lipid solubility. The existence of such a correlation, e.g. between physico–chemical properties and cardiodepressant actions, within the group of β-adrenergic blocking agents was first put forward by Levy.[39] Recently Hellenbrecht *et al.** found a strict correlation between myocardial depressant activity of β-receptor blocking drugs and their respective partition coefficients.

From all these correlations it is possible to derive some structure–activity relationships of β-blocking drugs relative to their cardiodepressant potency. The beta-blocking agents used have a phenoxypropanolamine or phenylethanolamine structure in common, but differ with respect to the substituents at the phenyl ring and at the amino group. Since all these drugs show p*K*-values between 8 and 9[39] due to the secondary amino group the cationic form is predominantly available in the physiological environment. The different affinities of these drugs to the membrane and the resulting ability to exert their nonspecific effects to a different degree must therefore mainly be due to the more or less lipophilic substituents at the aromatic ring. It is, therefore, not unexpected that drugs like propranolol, KL 255 and alprenolol with apolar groups at the ring have pronounced membrane stabilizing effects, e.g. negative inotropic activities, whereas on the other hand sotalol and practolol, with the hydrophylic acetanilide and the methansulfonanilide substituents, respectively, have little or no membrane effects. With the several Kö-compounds (Table 1B) we can support this hypothesis. All Kö-compounds with the hydrophilic nitrilo substituent at the ring (Kö 1439, Kö 1561, Kö 1560, Kö 1313, Kö 1366, Kö 1500) do not enhance the number of ANS-binding sites and have only a weak stabilizing effect on erythrocytes against hypotonic hemolysis, irrespective of the increasing alkyl substitution at the amino group; the negative inotropic effects of these compounds are weak and occur

* D. Hellenbrecht, B. Lemmer, G. Wiethold and H. Grobecker, *Naunyn-Schmiedeberg's Arch. Pharmak.* in press.

only at much higher concentrations than those required for β-receptor blockade.[40] In contrast, the second group of Kö-compounds with the isopropyl substituent at the amino group and increasing alkylsubstitution at the aromatic ring (Kö 592, Kö 707, Kö 1010, Kö 1030, Kö 1124) demonstrates that an increase of the degree of alkylation results in an increased potency to enhance the number of ANS-binding sites and to protect erythrocytes against hypotonic hemolysis. Engelhardt and Traunecker[41] on comparing different phenoxypropanolamine derivatives with β-adrenolytic activity found that compounds with double substitution at the ring had strong negative inotropic actions, in contrast to single substituted compounds. Furthermore Hellenbrecht *et al.* have shown that the nonspecific pharmacological action of β-receptor blocking drugs is determined by the number as well as by the length of the ring alkyl substituents.

Our investigations permit the following conclusion. The different lipid solubilities of beta-receptor blocking agents have a different affinity to cellular and subcellular membranes resulting in a varying degree of accumulation of the drugs within the membranes. According to Seeman[21] a pronounced accumulation is followed by an expansion of the cell membrane responsible, for instance, for the antihemolytic effect. The positively charged β-receptor blocking drugs can neutralize negative fixed charges within the membrane, leading to a change in the lipid–protein interaction of these membrane constituents. ANS is presumably bound at the lipid–protein interphase of the membrane,[42] although it is still uncertain whether the proteins[10,13] or the lipids[8,9] are responsible for the binding of ANS. The relative preponderance of positive charges within the membrane as a result of the accumulation of the β-receptor blocking drugs leads to an increased binding of the negatively charged anilino-naphthalene sulfonate ion. This assumption is strongly supported by experimental results,[17,43] in which an enhancement of ANS fluorescence was found in the presence of positively charged substances, e.g. the lipophilic anesthetic ketamine, tetraphenylphosphonium and tetraphenylarsonium. On the other hand negatively charged substances, e.g. oleate and tetraphenylboron depressed ANS fluorescence in ghosts and submitochondrial particles. In both cases changes in ANS binding sites were mainly responsible for changes in the fluorescence intensity. Therefore, the increase in the number of ANS-binding sites by beta-adrenergic blocking drugs can serve as a quantitative measure for the drug induced membrane conformational changes, which may be assumed to be a unique basis for their various membrane effects.

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