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EFFECTS OF DRUGS ON PIGEON ERYTHROCYTE MEMBRANE AND ASYMMETRIC CONTROL OF ADENYLATE CYCLASE BY THE LIPID BILAYER

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Summary

In pigeon erythrocyte membrane, the β-adrenergic receptor and the enzyme adenylate cyclase can be uncoupled in two different ways depending on the type of drug used.

Cationic drugs: chlorpromazine, methochlorpromazine, tetracaine, *n*-octylamine and a neutral alcohol, octanol, abolished alprenolol receptor binding ability and in the same range of concentration of the drug, sensitized adenylate cyclase to fluoride or Gpp(NH)p stimulation. Anionic drugs: di- and trinitrophenols, indomethacin and octanoic acid did not affect the total number of β-adrenergic receptor sites and, with the exception of trinitrophenol, did not change the association constant for alprenolol but they abolished the stimulation of adenylate cyclase by isoproterenol, fluoride or Gpp(NH)p. These modifications of the adenylate cyclase system occurred in a range of drug concentration where cell shape and protection against hemolysis were also affected.

As chemical composition varies widely from one drug to another, it is suggested that these effects are largely nonspecific and mediated by the lipid bilayer. They are probably related to a preferential sidedness of action of the drugs in the lipid bilayer, displaying the role of an asymmetric control of the adenylate cyclase system in the membrane by the two halves of this bilayer.

Introduction

The incubation of biological membranes with various drugs is known to modify both their mechanical and physiological properties [1]. A first class of drugs, most of them amphipathic, cationic molecules, including local anesthet-

ics, phenothiazines, tranquilizers, antihistamines, colchicine, vinblastine and reserpine induce discocytic erythrocytes to invaginate into a stomatocytic form. A second class of drugs, most of them emphipathic, anionic compounds, induce erythrocytes to crenate into an echynocytic form; these include nitrophenols, free fatty acids, phloretin and phlorizin, barbiturates, salycilates and indomethacin [2].

As emphasized by Sheetz and Singer [3] in the 'bilayer couple' theory, this differential effect of the two classes of drugs may be related to the asymmetric structure of the biological membrane. If this asymmetric structure is related to the asymmetric function of the biological membrane one would anticipate a differential action of stomatocytogenic and echynocytogenic drugs on some important membrane function. The hormone-stimulated adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) was considered in this regard since hormone recognition and cyclic AMP production are facing two different sides of the membrane. Preliminary data on pigeon erythrocyte adenylate cyclase were recently reported [4] suggesting that the two groups of drugs do in fact act very differently on the function of the enzyme system. Additional data are now provided here to include a parallel of the binding of the drugs to the membrane with their effect on protection against hemolysis, adenylate cyclase stimulation and β-adrenergic receptor binding.

The drugs studied were chosen both for their important pharmacological or biochemical functions and their difference in chemical structure.

Experimental procedures

Materials. Chlorpromazine-HCl and its quaternary ammonium derivative methochlorpromazine iodide were a generous gift of Rhône-Poulenc, their concentrations were determined in 0.1 M phosphate buffer (pH 7.4) from absorption coefficients of 33 000 at 254 nm [5] and 39 000 at 254 nm, respectively; 2,4-dinitrophenol was from Merck and 2,4,6-trinitrophenol from Prolabo and their concentrations measured in 0.1 N NaOH from their absorption coefficients of 14 800 at 359 nm [6] and 14 100 at 355 nm [7], respectively. Tetracaine and indomethacin were from Sigma and concentration determinations made with absorption coefficients of 21 340 at 308 nm in 0.1 M bicarbonate (pH 9.5) [8] and 17 480 at 265 nm in 0.1 M phosphate buffer (pH 7.4) [9], respectively.

Octanol, *n*-octylamine and octanoic acid were from Sigma and were determined by weight. Tetracaine, indomethacin, octanol, *n*-octylamine and octanoic acid suspensions in aqueous solution were sonicated for 10 min with a 25 W Bransom sonicator prior to use.

Cyclic AMP, guanosine triphosphate (disodium salt), theophylline, creatine kinase, creatine phosphate (disodium salt) and isoproterenol were supplied by Sigma. Adenosine triphosphate (disodium salt) was purchased from Calbiochem and 5′-guanylylimidodiphosphate (Gpp(NH)p) from Boehringer.

[α-^{32}P]ATP (15 Ci/mM) and (—)-[^{3}H]alprenolol (40 Ci/mM) were from the Radiochemical Center (Amersham) and cyclic [^{3}H]AMP from CEN (Saclay). Neutral-activated alumina was from Prolabo. 7-Amino-1,3-naphthalene disulfonic acid monosodium salt (Eastman) was used for ^{32}P liquid scintillation

counting in water. Scintillating media for ^3H counting were from Lumac. All other chemicals were reagent grade.

Preparation of erythrocytes and ghosts. Strasser-breed pigeons were bled by puncture through the alar vein, 5—10 ml of blood were collected in 1 ml of anticoagulant (0.72% citric acid, 1.98% trisodium citrate, 2.2% D-glucose). Red blood cells were washed three times in cold isotonic buffer, pH 7.4 (146 mM NaCl, 20 mM Tris-HCl 20 mM D-glucose) by centrifugation (3000 × *g*, 5 min), aspiration of supernatant, removing the white cells and buffy coat. Finally, the washed erythrocytes were suspended in a volume of isotonic buffer equal to the initial volume of blood and stored at 4° C for a maximum of 48—72 h.

Ghosts were prepared by hemolysis of 1 vol. of this suspension in 100 vols. of cold lysis buffer, pH 7.4, (30 mM phosphate, 4 mM $MgCl_2$). This buffer was reported to cause complete hemolysis of turkey erythrocytes without nuclear vacuolation [10]. After a first centrifugation (16 000 × *g*, 10 min), the pellet was washed twice in 10 vols. of hypotonic buffer, pH 7.4 (10 mM Tris-HCl, 4 mM $MgCl_2$) and centrifuged (12 000 × *g*, 10 min). The sticky button at the bottom of the pellet of ghosts was discarded. The remaining pellet was suspended in 1 vol. of hypotonic buffer and used immediately. Visual inspection by light microscopy detected minor nucleus contamination. Centrifugation on 50% sucrose (w/w) for 15 min, 1000 × *g*, separated this minor contamination leaving a top layer having similar lipid and protein contents than the original ghost suspension with the same hormone-stimulated adenylate cyclase activity/mol of phospholipid, so that this fractionation was no longer used.

Numeration and analysis of membrane constituents. The number of red blood cells was determined by 200 fold dilution of the suspension or initial blood in Marcano liquid (5% Na_2SO_4, 1% of 4% formol in water) and counted in a Thoma cell. The number of erythrocytes/μl was usually between 3 and $3.7 \cdot 10^6$.

Lipids were extracted according to Folch et al. [11], with 24 ml of chloroform/methanol (2/1) added to 200—500 μl of ghost suspension previously made up to 1 ml with water. Phosphorus was determined spectrophotometrically according to Bartlett [12]. Phospholipids were estimated as phosphorus × 25. Cholesterol was estimated using the reaction of Liebermann [13]. Membrane phospholipids were usually found to be in the range of 1.5—2 g/l of initial whole blood, and cholesterol in the range of 0.35—0.6 g/l. These values are similar to those from pigeons [14], hens [15] and humans [16,17].

On the other hand, estimates of proteins by the method of Lowry et al. [18] gave results (13—15 g of proteins/l of ghost suspension) 5—10 times higher than human ghosts [19] in spite of extensive washings. For this reason, adenylate cyclase activities were referred to phospholipids instead of proteins. High protein to lipid ratios of 3—5 have also been reported for pigeons [14] and hens [15].

Drug binding assays. 180 μl of ghost suspension was added to 180 μl of drug solution in hypotonic buffer (pH 7.4) and incubated 30 min at 37° C. The tubes were centrifuged (11 000 × *g*, 2 min in an Eppendorf microcentrifuge) and concentration of drugs in the supernatant was determined spectrophotometrically after subtraction of the absorbance from a control without drug.

Protection against hemolysis and shape change. 180 μl of erythrocyte sus-

pension in isotonic buffer (pH 7.4) were added to 180 µl of drug solution in distilled water to give a final osmolarity of 150 mosM. After 30 min at room temperature, absorbance of the supernatant (11 000 × *g*, 2 min) was read at 540 nm and expressed as percentage of control hemolysis performed with only distilled water.

Shape changes were observed by light microscopy under glass cover-slips immediately after mixing at room temperature 0.1 ml of drug solution in isotonic buffer (pH 7.4) to 0.1 ml of erythrocyte suspension previously diluted to 1/4 in isotonic buffer (pH 7.4). Temperature, time of incubation (except for methochlorpromazine which is first echynocytogenic, then with time of incubation, stomatocytogenic), use of glass cover-slips or fixation with 2% glutaraldehyde did not affect shape changes.

Adenylate cyclase assays. Adenylate cyclase was assayed following the methods of Birnbaumer et al. [20] and Ramachandran et al. [21]. They were carried out with a final concentration of 2 mM ATP, 4 mM theophylline, 10 mM phosphocreatine, 0.5 g/l creatine kinase, 7.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4) in a total volume of 75 µl containing 1 µCi [α-^{32}P]ATP. Depending on the experiment, 0.1 mM GTP (when no Gpp(NH)p added), 10 mM NaF, 0.05 mM isoproterenol, and the drug to the desired final concentration was added. When present, Gpp(NH)p (0.1 mM) was added to the ghost suspension during the 20 min of drug preincubation at 37° C and the 10 min of enzyme assay.

Unless otherwise stated, 1 vol. of the ghost suspension (13—15 mg protein/ml) was preincubated 20 min at 37° C with 1 vol. of the drug solution in hypotonic buffer (pH 7.4). Then the enzyme assay was initiated by adding 50 µl (about $8 \cdot 10^7$ cells) of this preincubated suspension to 25 µl of concentrated adenylate cyclase assay medium containing the drug at the same concentration and stopped after 10 min at 37° C by addition of 300 µl of 0.5 N HCl and 3 min boiling [22]. Fro drug incubation times shorter than 30 min, i.e. 15, 10 and 5 min, the enzyme assays lasted 5 min and drug preincubation lasted 10, 5 or 0 min, respectively. The solution was then neutralized with 300 µl of 1.65 N imidazole and centrifuged 10 min at 4000 × *g*. 400 µl of the supernatant were then poured into a column of neutral-activated alumina [21], and eluted with 3 ml of 10 mM imidazole (pH 7.5). Yields from alumina columns for cyclic AMP were measured with cyclic [^{3}H]AMP and found to be 95%. Samples were counted in an Intertechnique SL-32 scintillation counter using Cerenkov effect after addition of 10 ml of 1 g/l aqueous solution of 7-amino-1,3-naphthalene disulfonic acid.

Data are given as the median of triplicate assays in pmol cyclic AMP $\cdot$ min^{-1} $\cdot$ mg^{-1} phospholipids. Each set of experiments with one drug was repeated 3—4 times.

(—)-[^{3}H]Alprenolol binding assays. The method was adapted from Lefkowitz [23]. 50 µl of preincubated ghost suspension with the drug (see above) were added to 25 µl of (—)-[^{3}H]alprenolol in hypotonic buffer. After 10 min at 37° C, 50 µl of this medium were poured into 1.5 ml of ice-cold hypotonic buffer immediately filtered under vacuum on a Whatman GF/C filter and rinsed once with 8.5 ml of ice-cold hypotonic buffer. Filters were then dried (65° C) in the scintillation bottle. 4 ml of scintillation mixture (96% Lipoluma, 3% Lumasolve, 1% water) were added, and samples were counted after one night

of digestion at room temperature. When experiments were performed in the same medium as adenylate cyclase assays (see above) no differences in binding were found.

Nonspecific binding was determined in the presence of 10 μM of unlabelled (—)-alprenolol. Measurements were made in triplicate for each concentration of the labelled antagonist with the range of 0—100 nM to establish the saturation curves. The number of binding sites and the dissociation constant were obtained from least-squares fittings of the saturation curves performed with a Hewlett-Packard 9830 A desk computer.

Results

Drug binding, protection against hemolysis and shape change

Some cationic drugs bound to a greater extent to the membrane preparations than some anionic drugs (Fig. 1). For instance, at 10^{-4} M, 1 mol of chlorpromazine was incorporated/25 mol of membrane phospholipids, instead of 1 mol of dinitrophenol/200 mol of membrane phospholipids. However, as their hydrophobic parts are also different, no definite conclusion can be drawn about the contribution of the charge to the binding.

On the other hand, 50% of relative hemolysis was reached at close values of bound drug to phospholipid ratios: 1/35 for chlorpromazine, 1/20 for methochlorpromazine, 1/15 for tetracaine, 1/15 for dinitrophenol, 1/20 for trinitrophenol. Unique was the case of indomethacin, with a ratio of 1/1. It is worth noticing that, at such a level of incorporation within the membrane, the drugs may affect the lipid annulus [24] around integral proteins, inasmuch as adenylate cyclase has been shown to require a selected environment of phospholipids to function correctly [25]. At higher drug concentrations ($>5 \cdot 10^{-3}$ M), only anionic drugs maintained their antihemolytic effect.

A different concentration range for hemolysis protection by chlorpromazine was observed between pigeon erythrocytes (Fig. 1) and human erythrocytes, for which a range from 2 to $5 \cdot 10^{-5}$ M has been reported [3,26,27]. No such difference was found for methochlorpromazine [3,27] or dinitrophenol (personal observation). This can be tentatively related to the observation of a shift towards higher concentrations of the dose vs. response curve of chlorpromazine protection of human erythrocytes induced by human serum albumin [26]. As pigeon erythrocyte membrane preparations contain more proteins than human ones, chlorpromazine, aside from binding to the membrane, might adsorb to protein components present in the nucleated pigeon erythrocyte which are absent in the human erythrocyte.

Sheetz and Singer [3] and Elferink [27] proposed that the positively charged chlorpromazine would preferentially bind to the inner leaflet of the membrane in human erythrocytes because of the net negative charge brought by the preferential inclusion of phosphatidylserine in this leaflet [28], while dinitrophenol would rather bind to the outer leaflet. If only traces of phosphatidylserine were found in avian erythrocytes from hens, negatively charged phosphatidylinositol was present [29] but the asymmetric composition in regards to the bilayer is not yet determined. However, pigeon erythrocytes underwent the same drug-induced shape changes as human erythrocytes with

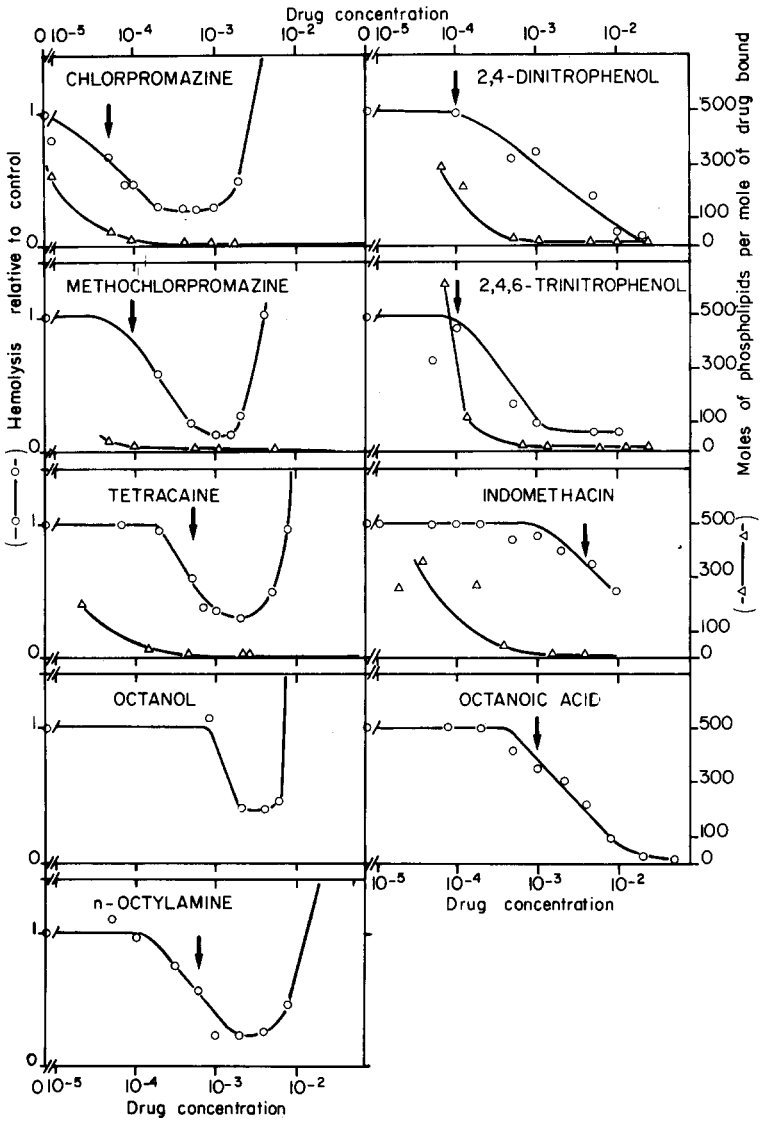

Fig. 1. Protection against hemolysis and drug bound as a function of drug concentration. Left: cationic or neutral drugs. Right: anionic drugs. ○———○, hemolysis of erythrocytes relative to control; △———△, ratio of mol membrane phospholipids/mol of drug bound to the membrane. Arrows indicate the minimum concentration of the drug necessary to induce changes in erythrocyte shape.

the same drug classification although the overall shape of the pigeon cells and their echynocytic and stomatocytic forms were different [4].

Octanol, up to a concentration of 0.1 M did not induce shape change. For induction of shape change, the ratios of drug to phospholipid were scattered: it occurred at 1 mol of chlorpromazine/60 mol of phospholipids, 1/20 for methochlorpromazine, 1/15 for tetracaine, 1/200 for dinitrophenol, 1/300 for trinitrophenol, 1/5 for indomethacin. Note that shape changes were observed by light microscopy (arrows in Fig. 1) at the threshold of protection against hemolysis.

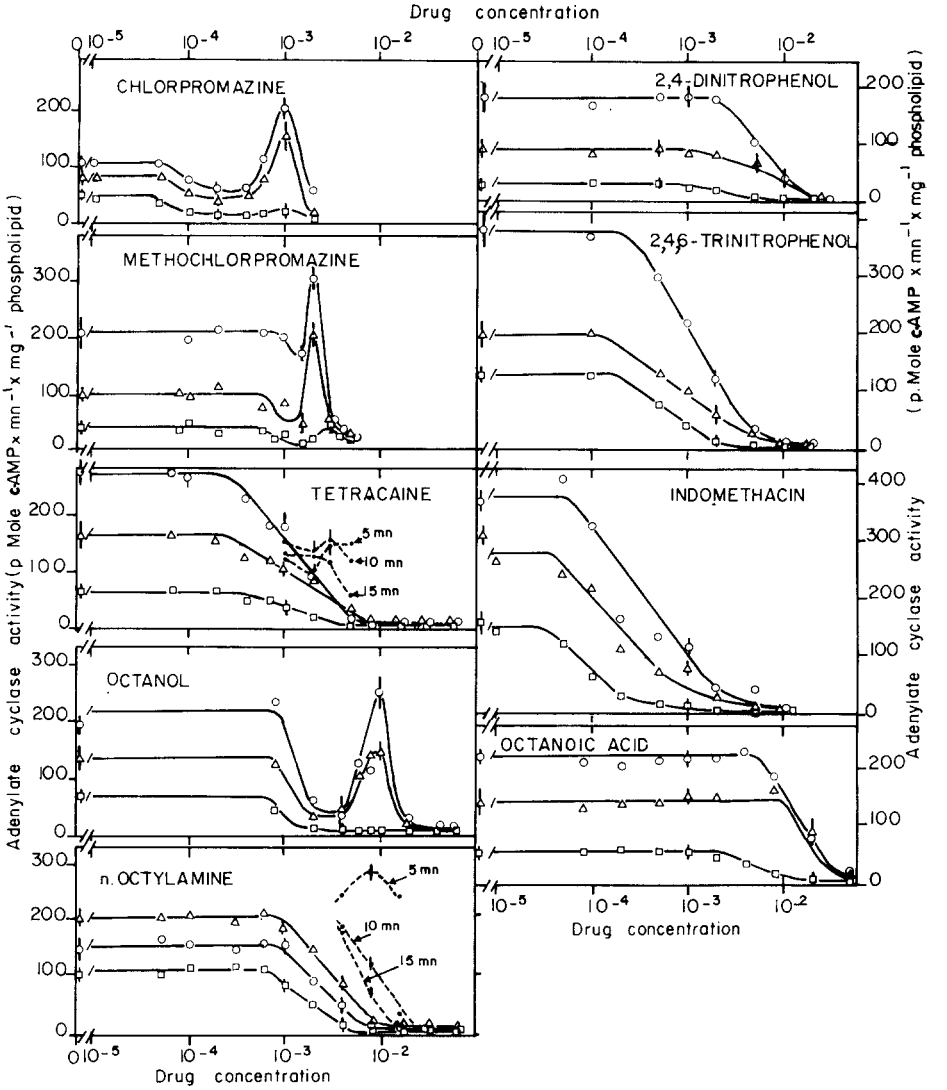

Fig. 2. Influence of the drug on adenylate cyclase activity. The activity of the enzyme is referred to the amount of membrane phospholipids in the assay tube. Left: cationic or neutral drugs. Right: anionic drugs. □———□, isoproterenol stimulation; △———△, fluoride stimulation; ○———○, Gpp(NH)p stimulation; ●- - - - -●, fluoride-stimulated adenylate cyclase activity at shorter incubation times with the drug. Heights of vertical bars represent two S.D. of the median of three experiments.

Drug effect on adenylate cyclase

An other interesting differential effect was found between negatively and positively charged drugs.

All drugs at high concentration decreased or abolished the stimulation of adenylate cyclase (Fig. 2). However, at certain concentrations, octanol and positively charged drugs increased the stimulation of adenylate cyclase by fluoride, Gpp(NH)p or, and to a remarkably lesser degree isoproterenol. This suggests that these compounds by interacting with the inner layer may create a state of the enzyme which is more sensitive to stimulation. This was also ob-

served by Dipple and Houslay [30], who found that benzyl alcohol enhanced fluoride and glucagon stimulation of adenylate cyclase in rat liver plasma membrane. They showed that the energy of activation diminishes when the enzyme is maximally sensitized by benzyl alcohol, thus increasing the rate of cyclic AMP production.

A sensitization of fluoride-stimulated adenylate cyclase by chlorpromazine was also observed in various membrane preparations [31], by mellitin, a cationic amphipatic polypeptide in pigeon erythrocyte [14] and by halothane in rat uterine homogenate [32]. On the other hand chlorpromazine was also found to decrease adenylate cyclase stimulation by other hormones or neurotransmitters, adrenocorticotropin, glucagon or adrenalin [31] and dopamine [33].

Both methochlorpromazine and chlorpromazine sensitized adenylate cyclase when assayed in open ghosts. It is interesting to note that the concentration ranges were the same for maximum hemolysis protection and enzyme sensitization, whatever the drug used, and that the peaks of maximum sensitization were close to the recovery of normal sensibility to hemolysis (Figs. 1 and 2). *n*-Octylamine and tetracaine seemed to be an exception, but shorter incubation times with ghost suspension did increase the fluoride stimulation of adenylate cyclase (Fig. 2).

In contrast, negatively charged drugs never showed this sensitization of the enzyme activity in the concentration ranges studied (Fig. 2) even with shorter incubation times down to 5 min as above. A similar inhibition was also reported for various fatty acids [34,35].

Drug effect on hormone binding

An antagonist of high affinity, alprenolol, was used to characterize the β-adrenergic receptors of pigeon erythrocytes. The dissociation constant for (—)-[^{3}H]alprenolol was found to be between 4 and 12 nM with 1000—3000 sites/erythrocyte depending on the individual (Fig. 3).

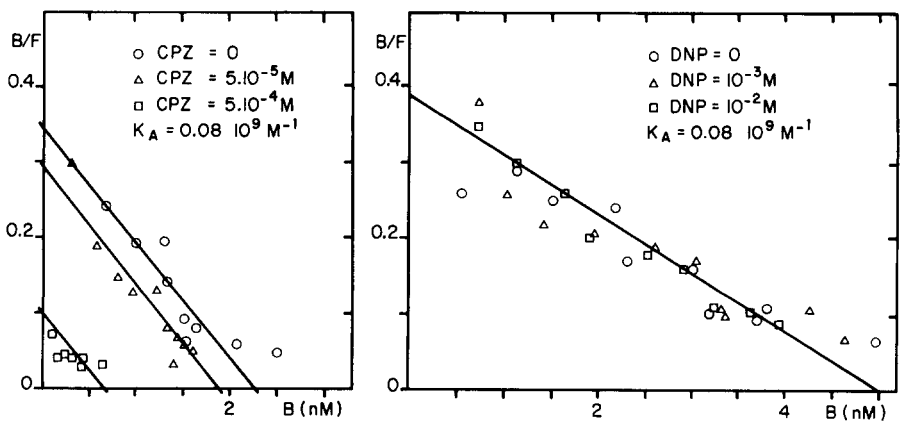

Fig. 3. Scatchard plots of (—)-[^{3}H]alprenolol binding in the presence of increasing chlorpromazine (left) and dinitrophenol (right) concentrations. B, concentration of specifically bound (—)-[^{3}H]alprenolol in the assay tube; F, the concentration of free (—)-[^{3}H]alprenolol.

In terms of antagonist binding, the drugs can also be divided into two groups as follows:

Positively charged drugs nd octanol decreased the total number of binding sites without affecting the dissociation constant. Scatchard plots were parallel for all these drugs, indicative of a non-competitive type of inhibition. An example of such a case is presented in Fig. 3 for chlorpromazine. Inhibition of isoproterenol-stimulated adenylate cyclase can be interpreted as the loss of binding sites for the hormone (Fig. 4), and the small bump observed at a high

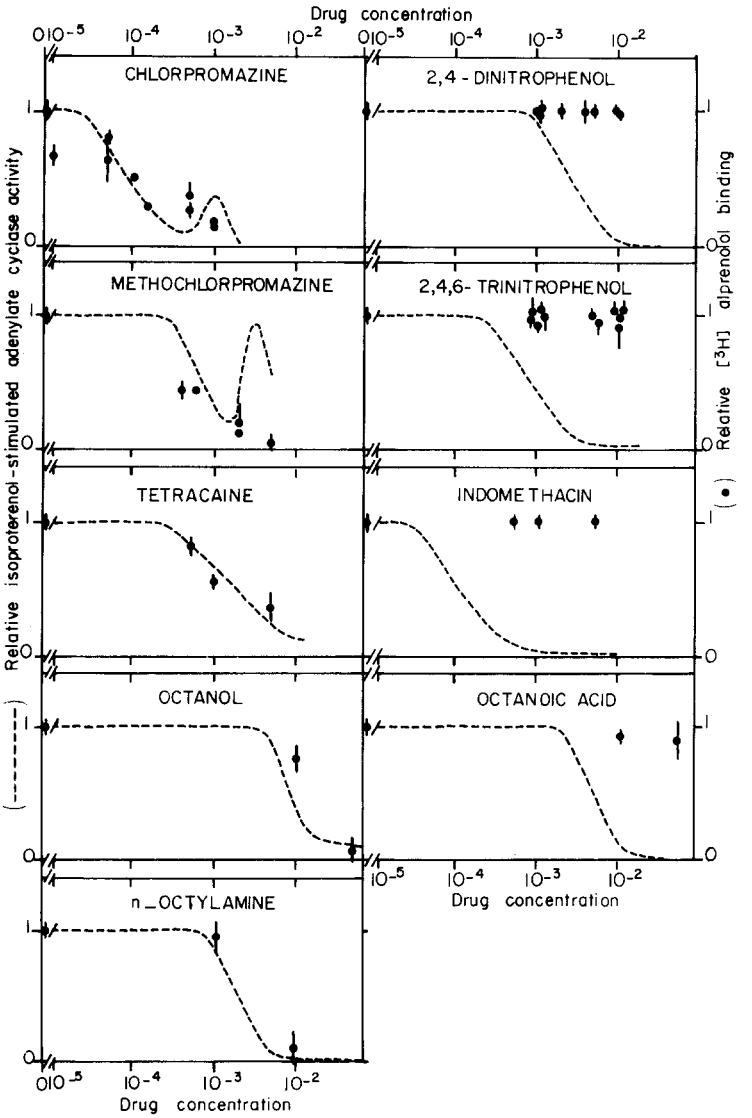

Fig. 4. Comparison of relative (—)-[^{3}H]alprenolol binding and relative isoproterenol-stimulated adenylate cyclase activity as a function of drug concentration. Left: cationic or neutral drugs. Right: anionic drugs. •, (—)-[^{3}H]alprenolol binding relative to control; ------, relative isoproterenol-stimulated adenylate cyclase activity. Both assays were done on the same batch of erythrocyte membrane preparation. Heights of vertical bars represent two S.D. of the median of three experiments.

concentration of chlorpromazine or methochlorpromazine for hormone stimulation (Fig. 2) can be attributed to the sensitization of adenylate cyclase rather than to the recovery of binding sites (Fig. 4). Washing of the ghost suspension by hypotonic buffer for up to 2 h after chlorpromazine treatment failed to restore isoproterenol stimulation, while fluoride stimulation reached approximately control values; in contrast, hormone and fluoride inhibition with di- and tri-nitrophenol can be reversed almost entirely after washing out the drug. The irreversible loss of receptor sites by chlorpromazine could be caused by the release of the receptor into the medium by the drug acting as a detergent. A detergent effect has been proposed by Leterrier et al. [36] for chlorpromazine. After 30 min incubation of ghosts in presence of $3 \cdot 10^{-4}$ M chlorpromazine and (—)-[^{3}H]alprenolol, the supernatant was analyzed on a Sephadex G-50 column, but no radioactivity was detected in the void volume. This suggests that no receptor was released from the membrane, or alternatively, a release and a parallel loss of binding activity for the radiolabelled antagonist. With regard to the high partition coefficient of chlorpromazine of 1600 for erythrocyte membrane [1] or 1400 for octanol/buffer solution [27], another exaplanation for the apparent irreversible inhibition is the incomplete release of the drug during the washing.

Negatively charged drugs did not affect either the number of receptor sites or the association constant of the antagonist. An example of such a behaviour is given in Fig. 2 for 2,4-dinitrophenol. One exception is trinitrophenol which decreased the association constant for alprenolol without modifying the total number of sites, but this effect is reversible upon washing. From this it can be inferred that the inhibitory effect of negatively charged drugs occurred on the pathway to enzyme stimulation but not directly on the receptor. As fluoride stimulation was also inhibited, their effect seems likely to be directly on the enzyme itself.

Discussion

The time-dependent sensitization of adenylate cyclase observed with tetracaine and *n*-octylamine could result of two processes, occurring at different rates but for these specific drugs in the same range of concentration: a quick sensitization process and a slower enzyme inactivation, possibly a detergent-like effect. This can be related to the observation that for chlorpromazine concentrations greater than 1 mM, an inhibition of fluoride stimulation is observed after 30 min incubation period, whereas at smaller incubation times, less than 15 min at 37° C, a sensitization of the enzyme can be detected as high as is observed at drug concentrations less than 1 mM. Nevertheless for chlorpromazine concentrations less than 1 mM, sensitization of adenylate cyclase is independent of incubation time at 37° C from 5 min up to 2 h (unpublished results).

As chemical structure and charge of all these drugs are different, one may suggest that their effect on the adenylate cyclase system (in the same range as maximum protection against hemolysis) is mostly indirect, acting very likely through modification of the lipid environment of the proteins. Indeed, most if not all of these drugs have been reported to promote an increase of the lipid bilayer fluidity: local anesthetics [37,38], alcohols [39,40], chlorpromazine

([41—43] and reviews, see Refs. 1 and 44). As Borochov and Shinitzky [45] reported, modulation of the fluidity of the lipid bilayer may control the degree of embedding of an integral protein in the membrane with concomitant variation of conformation and/or inability to bind to other proteins during lateral diffusion in the plane of the membrane. That such a transverse exclusion can occur is shown by the observation of Chevalier [46], who found by freeze-etching electron microscopy that echynocytogenic drugs increased the number of particles within the cytoplasmic half-layer at the summit of erythrocyte evaginations. As the active adenylate cyclase system is proposed to be composed of four functional subunits [47], it is likely that the interactions of the subunits together and/or with a substrate or an hormone may be altered even by slight perturbations of the bilayer fluidity.

As the two classes of amphipathic drugs differently affect cell shape, they do affect differently the adenylate cyclase system. This differential effect may be due to a preferential localization of these drugs into one of the two halves of the bilayer [3,27]. Houslay et al. [48] reported that stimulation of adenylate cyclase is asymmetrically controlled in rat liver plasma membranes: when coupled, the enzyme-glucagon receptor complex appeared sensitive to modification of the lipid environment in the outer half of the bilayer, while the enzyme alone appeared insensitive, when stimulated by fluoride or Gpp(NH)p.

The action of the two classes of drugs may be also related to a differential sensitivity of the components of the adenylate cyclase system: anionic drugs may affect directly the enzyme and perhaps its lipid annulus, with no perturbation of the adrenergic receptor. On the other hand, cationic and neutral drugs may affect not only the lipid annulus of the receptor unit, but also the fluidity of the whole bilayer, thus leading to the activation of the enzyme as was found with benzyl alcohol [30]. This later explanation offers an alternative to the hypothesis of a preferential localization and perturbation of one half-layer without excluding it.

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