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COMPARATIVE STUDY OF THE EFFECTS OF PROPRANOLOL AND TETRACAINE ON CATION MOVEMENTS IN RESEALED HUMAN RED CELL GHOSTS

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SUMMARY

1. The effects of two positively charged local anaesthetic amines, tetracaine and propranolol, on cation permeability were studied in resealed human red cell ghosts prepared from metabolically depleted erythrocytes.

2. The K permeability was reduced by tetracaine but increased by propranolol. The effect of tetracaine was independent of the extracellular Ca concentration but was abolished when the intracellular free Ca concentration was raised to $2–5 \times 10^{-7}$ M. The effect of propranolol, which was enhanced when the external Ca concentration was raised, could be completely inhibited by lowering the internal free Ca to less than 10^{-7} M.

3. Propranolol, but not tetracaine, increased the intracellular Ca ion concentration by releasing up to 20 % of the membrane-bound Ca to the cell interior. This increase in intracellular Ca was sufficient to mediate the observed change in K permeability.

4. Tetracaine and propranolol reduced the Ca binding capacity of the ghost membrane by about 20 and 40 % respectively. The Ca permeability was increased by propranolol and was slightly reduced by tetracaine.

5. In high concentrations (2–7 mM) propranolol by itself moderately increased K and Na permeability, but suppressed completely the Ca-induced increase in K permeability. Tetracaine in concentrations up to 4 mM enhanced the Ca-induced increase in K permeability. Higher concentrations of the drug caused lysis of the cells.

6. Maximally effective concentrations of tetracaine and propranolol inhibited the ATP-dependent Ca outward transport by 30 and 70 % respectively.

7. The effects of tetracaine on K permeability were shared by the local anaesthetics prilocaine and lidocaine, those of propranolol were shared by practolol, a β-adrenergic antagonist and tetraethylammonium, a ganglionic blocking agent.

8. It is suggested that the differences in the effects of tetracaine and propranolol on cation permeability reflect qualitatively different interactions of the two drugs with Ca binding sites on the inner surface of the membrane.

INTRODUCTION

Propranolol, an adrenergic β-receptor antagonist, induces human red cells to lose K. The increase in K outflow is not accompanied by an equivalent increase in Na uptake. This effect is certainly not due to an interaction of the drug with adrenergic receptors which do not exist in mature erythrocytes, but may be related to the drug's well known unspecific, local anaesthetic and antiarrhythmic properties (Ekman, Manninen & Salminen, 1969; Manninen, 1970).

As a local anaesthetic propranolol should be comparable best to the chemically related class of local anaesthetic amines like procaine or tetracaine. These drugs, which are positively charged at physiological pH values, interfere with the cation permeability of the membrane (cf. Seeman, 1972). However, in excitable tissues at anaesthetizing concentrations their prevailing and pharmacologically relevant effect appears to be an inhibition rather than an augmentation of the passive transmembrane movements of Na, K, and Ca (cf. Shanes, 1958; Feinstein, 1966; Ritchie & Greengard, 1966). The available evidence suggests that these compounds decrease passive Na and K permeability in red cells at least under some of the conditions in which propranolol is reported to increase K outflow (Andersen, 1968; Seeman, 1972). The nature of the mechanism by which positively charged anaesthetics change the cation permeability of the membrane is unknown. However, some of them have been shown to compete with Ca for negatively charged fixed sites in cell membranes (Feinstein, 1964, 1966; Blaustein & Goldman, 1966; Feinstein & Paimre, 1969). On the other hand, Ca plays an important role in the regulation of transmembrane cation movements (Romero & Whittam, 1971; Bolingbroke & Maizels, 1959).

Therefore, experiments were designed to test whether differences in the effects of individual amine anaesthetics on cation permeability might reflect differences in their interactions with membrane Ca. Resealed red cell ghosts were chosen as a model system because with this preparation it is possible to incorporate the local anaesthetics into the cell. Their effects under these conditions will be independent of their rate of penetration across the membrane.

The results suggest that the effects of propranolol on red cell K permeability unlike those of typical local anaesthetic amines are partially Ca-mediated and may be related to the drug's antiarrhythmic activity.

METHODS

Fresh human red blood cells were supplied by the Swiss Red Cross Blood Transfusion Service in Berne and were used within 7 days after withdrawal of the blood.

The washed cells were incubated 15–20 hr in a substrate-free solution (soln. I or I*a* in Table 1) at 37° C in order to deplete them of endogenous energy stores. In control experiments the cells were starved instead for 3 hr in a solution which contained iodoacetate and inosine (soln. II in Table 1). The two methods yielded cells with similar properties. At the end of the starvation period the cells were washed 4 times in isotonic NaCl solution and resuspended either in isotonic Tris-buffer solution (soln. III in Table 1) or in KCl-HEPES solution (soln. IX in Table 1) depending on whether HEPES or Tris-buffer was used in the final incubation medium (see below). The haematocrit of this cell suspension was adjusted to 50 %.

Resealed red cell ghosts were then prepared by reversal of osmotic haemolysis at 0° C according to the method of Passow (1969). Isotonicity was usually restored by the addition of 2 M-KCl solution or of a mixture of 2 M-KCl and 2 M-HEPES solution buffered to pH 7·2.

At the end of the equilibration period at 37° C, which always followed the formation of ghosts the cells were washed 3 times in Tris-buffer solution (soln. III) and were suspended in the experimental medium at 30° C. The standard incubation media, NaCl-Tris and NaCl-HEPES solution, are listed as soln. VII and VIII in Table 1. HEPES-buffered solutions were used whenever Ca-EGTA buffers were to be incorporated into the ghosts. Compared to Tris-buffer HEPES is characterized by a smaller temperature-induced shift of its pK value and a larger buffering capacity around pH 7·2 (Good, Winget, Winter, Conolly, Izawa & Singh, 1966).

During the 2 hr incubation period samples were taken at suitable intervals for the analysis of cellular Na, K and Ca concentrations. The procedure essentially followed the method of Passow (1969): 3 ml. samples of the ghost suspension used in the experiment were washed once at 0° C in 30 ml. isotonic Tris-Cl solution (soln. IV). The sample sediment was rehaemolysed overnight in double distilled water and finally diluted with double distilled water to a concentration suitable for the measuring procedure. All cations were measured by atomic absorption spectrophotometry using an Instrumentation Laboratory 353 instrument. The method for the determination of Ca has been described earlier (Porzig, 1972). The cellular concentrations are expressed in m-mole/l. ghosts and were always calculated on the basis of the haematocrit value at the beginning of the experiment.

All cellular concentrations were corrected for the amounts of extracellular Na, K and Ca carried over from the incubation medium into the washing solution by means of the Li method suggested by Passow (1969). It should be noted that all flow measurements of cations described in this paper refer to net movements and not to unidirectional fluxes.

The haematocrit of the experimental cell suspension was controlled at the beginning and at the end of each experiment using van Allen haematocrit tubes as well as conventional microhaematocrit capillaries. The details of the method were described by Porzig (1972). In most experiments the initial haematocrit values varied between 4 and 8 %.

The free Ca concentrations in the Ca-EGTA buffer solutions were calculated according to Portzehl, Caldwell & Rüegg (1964) using a pK′ (log of apparent formation constant) of 7·02 at pH 7·2 (personal communication of Professor Anderegg, ETH Zürich, to Dr P. Bally, Department of Pharmacology, University of Berne).

Rehaemolysis of ghosts during the incubation period was usually less than 5 %.

In experiments designed to study the interaction of propranolol and tetracaine

with the ATP-dependent Ca extrusion mechanism the experimental protocol given above was not applicable. Therefore, a method of Schatzmann (1966, 1973) was adopted and somewhat modified: red cells were haemolysed at 22° C in a medium which contained 3 mM-ATP and 4 mM-$MgCl_2$ (soln. X in Table 1). Two minutes after haemolysis sufficient 2 M-KCl solution was added to reestablish a tonicity of about 320 m-osmole. The ghost cells were then rapidly sedimented in a refrigerated centrifuge, washed once at 0° C and resuspended in the same medium at 25° C. The haematocrit was adjusted to 10–14%. 5 ml. samples were taken and centrifuged in small tubes. After careful separation of sediment and supernatant the sediment was deproteinized with 10% trichloroacetic acid. Both sediment and supernatant were then analysed for K, Na and Ca as described above.

The chemicals used for the preparation of solutions were of the highest purity which was commercially available. D,L-propranolol and practolol were gifts, of I.C.I., Macclesfield, U.K. (Practolol being kindly supplied by Dr J. D. Fitzgerald c/o I.C.I., Macclesfield). Prilocaine was a gift of Astra, Södertalje, Sweden. Tetracaine and lidocaine were the Ph. Helv. VI preparations. All solutions were prepared with deionized, double quartz-distilled water. They are generally identified by their respective numbers in Table 1.

Abbreviations used:

ATP: adenosine-5′-triphosphate.
EGTA: ethyleneglycol-bis-(β-aminoethylether)-N,N′-tetraacetic acid.
HEPES: 2- 4-(2-hydroxyethyl) piperazinyl-(1)-ethane-sulphonic acid.
Tris: Tris (hydroxymetyl)-aminomethane.
Propranolol is 1-isopropylamino-3-(1-naphtyloxy)-2- propanol hydrochloride.
Tetracaine is *p*-butylaminobenzoyl-2-dimetylaminoethanol hydrochloride.

RESULTS

Ca and the effect of propranolol on K and Na fluxes

Manninen (1970) has demonstrated that the increase in K permeability caused by propranolol in human erythrocytes is dependent on the presence of Ca in the external medium. On the other hand, it is known from a number of studies that an increase in the intracellular Ca concentration makes the human red cell highly permeable to K (Hoffman, 1962; Lew, 1970; Romero & Whittam, 1971). Therefore, propranolol may affect the K permeability of red cells indirectly by primarily increasing the Ca permeability of the membrane and hence raising the cellular Ca concentration. A first set of experiments was designed to test this hypothesis.

The action of propranolol in Ca-free medium. Red cell ghosts were prepared in Ca-free haemolysing solutions. The cells were incubated in isotonic NaCl-Tris solution (soln. VI) containing 10^{-6}–10^{-3} M propranolol. In most experiments 0·2–2 mM of the Ca-complexing agent EGTA were added to the Ca-free media to ensure an external Ca concentration of less than 10^{-8} M. Extracellular propranolol penetrates rapidly into red cells (Manninen, 1970). Moreover, in control experiments no difference in the effects of propranolol was detected when the drug was incorporated into the cell during reversal of haemolysis in addition to being present

TABLE 1. Composition of solutions (m-mole/l.)

Solution no.	Na	K	Ca	Mg	Tris	Cl	HEPES	EGTA	Special additives	pH at 22° C	Predominant use
I	131	5	—	—	27	163	—	—	chloramphenicol 2 μg/ml.	8·0	Starving of red cells
I*a*	138	—	—	—	18	156	—	—	chloramphenicol 1 μg/ml.	7·6	
II	—	140	—	—	—	130	20	—	10 inosine 2 Na iodoacetate	7·6	
III	—	—	—	—	175	175	—	—	—	7·4	Washing of reconstituted ghosts
IV	—	—	—	—	170	175	—	—	5 Li	7·4	Washing of samples from the experimental cell suspension
V	10	—	—	—	169	169	—	5	—	7·4	Experimental incubation
VI	140	—	—	—	20	160	—	—	—	7·4	
VII	139	5	—	—	20	164	—	2	—	7·4	
VIII	144	5	—	—	—	140	20	2	—	7·2	
IX	—	145	—	—	—	140	20	—	—	7·2	
X	10	2	2	4	—	12	5	1·8	3 ATP	7·2	Haemolysing solution in active transport experiments

The varying composition of the haemolysing solutions in experiments designed to study passive ion movements are given in the text.

in the incubation medium. Therefore, in the present study the drug was added only to the external medium.

The data were evaluated by calculating the fraction of the initial cellular K content which was lost into the medium during a given time interval. Other evaluations of the data in terms of initial velocities or first order rate constants could not be used in all conditions because of the

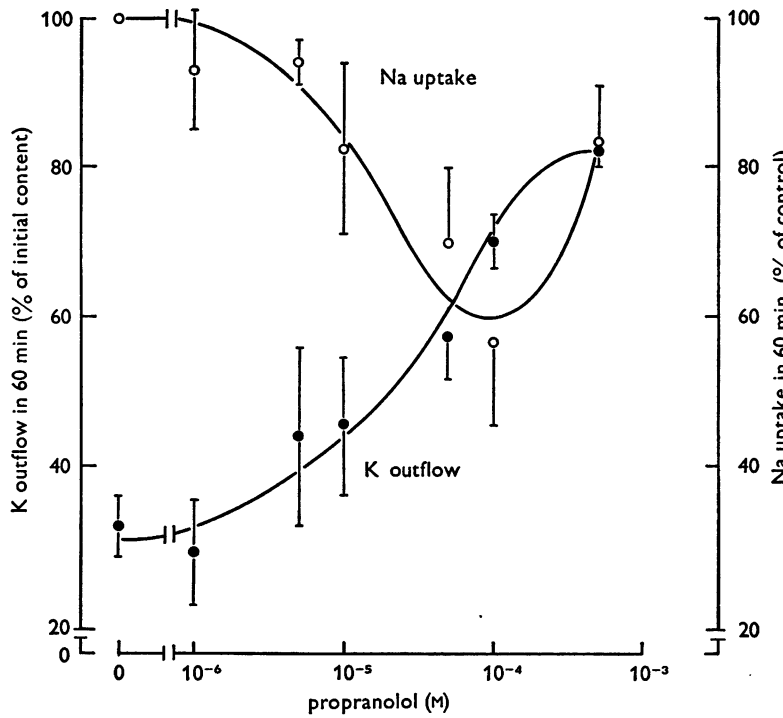

Fig. 1. The effect of propranolol on K and Na movements in red cell ghosts in the absence of extracellular Ca. Starved red cells were haemolysed in the presence of 4 mM-$MgCl_2$ and 8 mM Tris-Cl, reconstituted with 2 M-KCl and incubated at 30° C in isotonic NaCl solution (soln. VI) containing 0–10^{-3} M propranolol (abscissa, log scale). The total cellular K loss at the end of 60 min incubation (●–●) is given in % of the cellular K content at the beginning of the experiment (left ordinate). The total Na uptake in 60 min (○–○) is given in % of the Na uptake in the absence of propranolol (right ordinate). Haematocrit 4·3–9·3%. Data from sixteen experiments. The individual points represent three to sixteen estimations ± S.E. of mean.

following observations. (1) The initial cellular K content and hence the initial outward concentration gradient varied considerably. In different ghost preparations it ranged from 80 to 120 m-mole/l. cells. (2) Reversal of haemolysis did not yield a homogeneously sealed ghost population. Thus

the initial velocities were partially determined by a small fraction of 'leaky' ghosts which were less completely sealed to cations than the rest of the cells (see Bodemann & Passow (1972), for a discussion of this problem). (3) The cation movements which were induced by local anaesthetics or by variations of the intracellular free Ca concentration in many cases did not follow single exponential curves. For example, complex

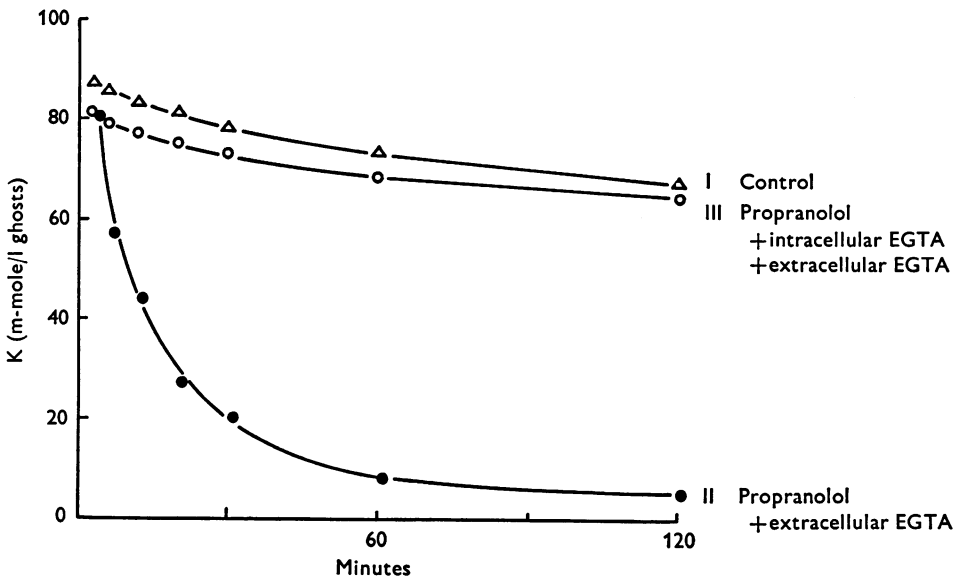

Fig. 2. Effect of the Ca-complexing agent EGTA on the propranolol-induced K outflow from red cell ghosts. The time course of net K outflow was measured under three different conditions (I–III). I: haemolysis in the presence of 2 mM-EGTA, incubation in isotonic NaCl (soln. VII). II: haemolysis in the presence of 4 mM-$MgCl_2$, incubation in solution VII containing in addition 1 mM propranolol and 2 mM-EGTA. III: same cell preparation as I, incubation medium identical to II. Reversal of haemolysis always with KCl. Temperature of incubation 30° C. Hematocrit 7·2–8·3 %. One of nine similar experiments.

kinetics were observed under conditions where a continuous change of the intracellular free Ca concentration might have occurred during the course of the experiment.

In Fig. 1 the fraction of the initial cellular K content lost within 60 min is plotted as a function of the propranolol concentration in the medium. When this concentration was raised stepwise from 10^{-6} to 5×10^{-4} M the K outflow increased from $28{\cdot}6 \pm 7{\cdot}3$ to $81{\cdot}9 \pm 2{\cdot}0$ % of the cellular content at the start of the experiment. No further increase was observed with higher concentrations of the drug.

The propranolol-induced K loss was not compensated by an equimolar inward movement of Na. Fig. 1 demonstrates on the contrary that the Na uptake, though rather variable, was reduced at propranolol concentrations above 5×10^{-6} M and reached a minimum at about 10^{-4} M. Due to the asymmetry in the propranolol-induced cation fluxes the cells shrank to about one third of their original volume during an incubation period of 1 hr in the presence of a maximally effective drug concentration.

Permeability changes in EGTA-containing ghosts. The time course of the K outflow from ghosts into a Ca-free medium (soln. VII) containing 2 mM-EGTA and 1 mM propranolol is illustrated by curve II in Fig. 2. Within 60 min the K outflow proceeded to almost complete equilibration between intra- and extracellular concentrations. However, the same high concentration of propranolol was completely ineffective in cells whose internal free Ca concentration was kept below 10^{-8} M. This is shown by the two curves labelled I and III in Fig. 2. They show the time course of the K outflow from Ca-free ghosts, containing 2 mM-EGTA, which were incubated either in the presence (curve III) or absence (curve I) of 1 mM propranolol. Similarly, the Na uptake was not reduced under these conditions.

These observations suggest that the effect of propranolol is mediated by an increase in the intracellular free Ca ion concentration, irrespective of whether or not the medium contained Ca. In the absence of extracellular Ca the only possible Ca source is the membrane which contains about $1{\cdot}6 \times 10^{-5}$ M bound Ca (Harrison & Long, 1968; Lichtman & Weed, 1972). Hence, it might be concluded that propranolol is able to displace Ca from membrane sites, thereby increasing the level of intracellular free Ca. The proposal of such a mechanism implies the assumptions: (1) that an intracellular Ca ion concentration of less than 2×10^{-5} M is sufficient to account for the observed increase in K permeability and (2) that the amount of Ca which can be released by propranolol meets the threshold requirements of the Ca-induced K outflow. The following experiments were designed to test the validity of these assumptions.

The relation between intracellular Ca ion concentration and K outflow. Ghost cells were prepared to contain K as the main osmotic constituent as well as Ca and EGTA in proportions varying between 1:100 and 95:100. The intracellular free Ca ion concentrations which were defined by these Ca/EGTA ratios ranged from about 10^{-9} to about 2×10^{-6} M. In some of the experiments the haemolysing solution contained in addition 4 mM-$MgCl_2$. The ghosts were incubated in an isotonic NaCl-medium (soln. VIII). The results are shown in Fig. 3. In ghost cells which were prepared in a Mg-free haemolysing solution the Ca-dependent increase in K outflow developed between 10^{-7} and 6×10^{-7} mole free Ca ions/l. cells (curve I). Curve II in Fig. 3 was obtained in the presence of 2·5–3 mM cellular Mg (resulting from

a concentration of 4 mM in the haemolysing medium). Mg acts as a Ca antagonist. It shifts the threshold for the increase in K permeability towards higher intracellular Ca ion concentrations. Nevertheless, the release of less than 10 % of the total membrane-bound Ca into the intracellular space would induce an increase in K outflow, even in the presence of Mg.

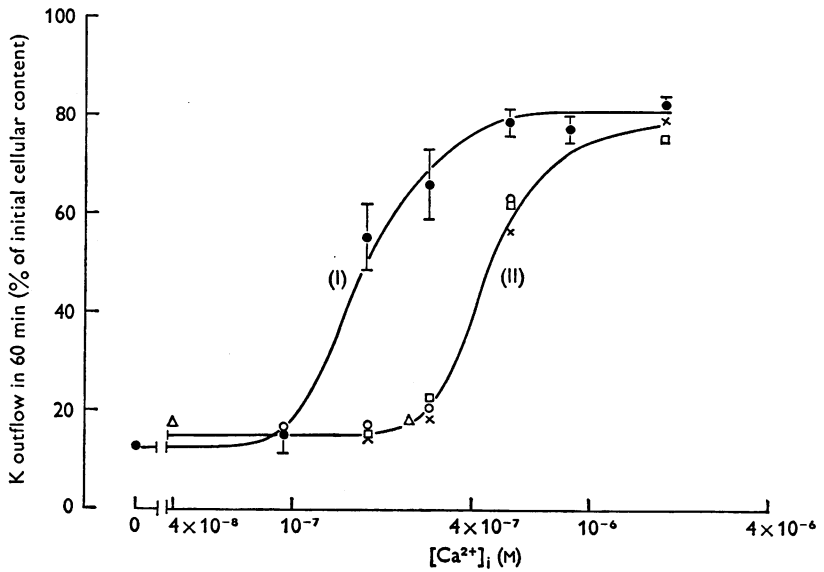

Fig. 3. Relationship between intracellular free Ca ion concentration (abscissa, log scale) and K outflow (ordinate: total K outflow in 60 min in % of the initial cellular K content). Curve I: haemolysis in the presence of 5 mM-HEPES, 2 mM-EGTA and increasing Ca concentrations to establish Ca/EGTA ratios ranging from about 0·001 (no Ca added) to 0·95 (1·9 mM-Ca added). The resulting free Ca ion concentrations ranged from $< 10^{-9}$ to $1{\cdot}8 \times 10^{-6}$. Reversal of haemolysis with KCl. Incubation at 30° C in isotonic NaCl solution (soln. VIII). Data from ten experiments, the individual points represent four to nine measurements ± S.E. of mean. Curve II: conditions similar to curve I except that the haemolysing medium contained, in addition, 4 mM-$MgCl_2$. The different symbols correspond to four individual experiments. Haematocrit 5·2–10·8 %.

The amount of Ca released by propranolol. The propranolol-induced change in the cellular Ca concentration was estimated by determining the minimal concentration of EGTA which was able to suppress the effect of a maximally effective concentration of the drug (Fig. 4). K-rich ghost cells were prepared in the presence of 0–20 μM-EGTA and 4 mM-$MgCl_2$. EGTA-loaded ghosts were then incubated in the presence of 0·5–1 mM propranolol. In Fig. 4, K outflow and Na uptake are plotted against the EGTA concentration in the haemolysing medium. It can be estimated that the

cellular EGTA concentration after reversal of haemolysis is directly proportional to and about 20 % lower than the concentration in the haemolysing solution. This estimate is based on measurements of the incorporation of Ca-EGTA into ghosts. In the concentration range used in the present experiments the cellular Ca-EGTA concentration reached 77·2 ± 0·9 % of the concentration in the haemolysing medium (mean ± S.E. of mean of thirty-eight measurements in seven experiments). The propranolol-induced K outflow decreased as the cellular EGTA concentration was increased. 5×10^{-6} M-EGTA was sufficient to inhibit the effect of

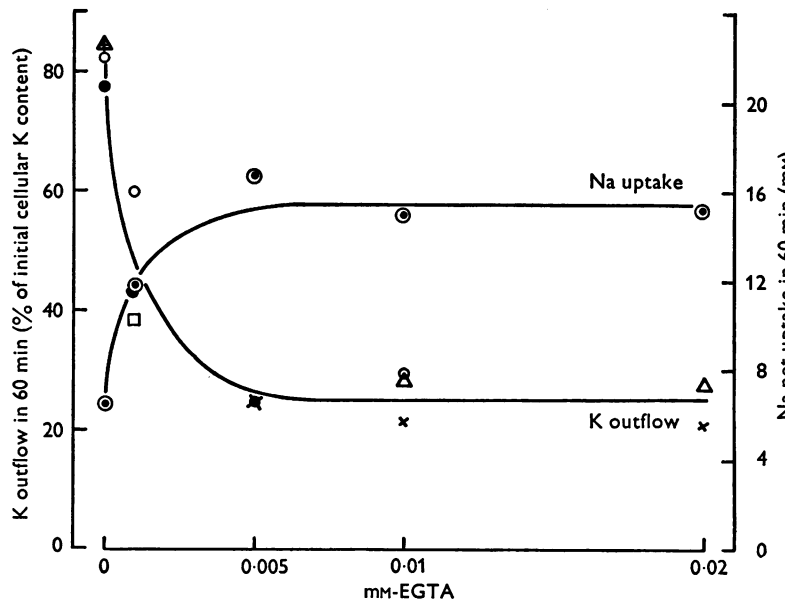

Fig. 4. Relationship between intracellular EGTA concentration and propranolol-induced K and Na movements. Haemolysis in the presence of 4 mM-$MgCl_2$ and 0–20 μM-EGTA. The cells were reconstituted with KCl and incubated at 30° C in isotonic NaCl solution (soln. VI) containing 0·5–1 mM propranolol. Left ordinate: Total K outflow in 60 min in % of the initial cellular K content. Right ordinate: Na net uptake in 60 min. Abscissa: EGTA concentration in the haemolysing solution. The different symbols in the K outflow curve correspond to five individual experiments. The experimental points on the Na uptake curve are mean values of two to four estimations. Haematocrit: 6·5–10 %.

1 mM propranolol completely. From these data the intracellular Ca ion concentration which was made available by propranolol was calculated to be between 7×10^{-7} and 3×10^{-6} mole/l.cells. This concentration corresponds to 5–20 % of the total membrane-bound Ca. Hence, from a

quantitative point of view the mechanism of propranolol action as proposed above would be possible.

The increase in Na uptake with increasing cellular EGTA concentrations which is shown in Fig. 4, suggests that the Na permeability is also regulated by the intracellular Ca ion concentration. Therefore, the effect of propranolol on Na uptake (Fig. 1) may be due to a change in the intracellular Ca ion concentration rather than to a direct membrane action of the drug.

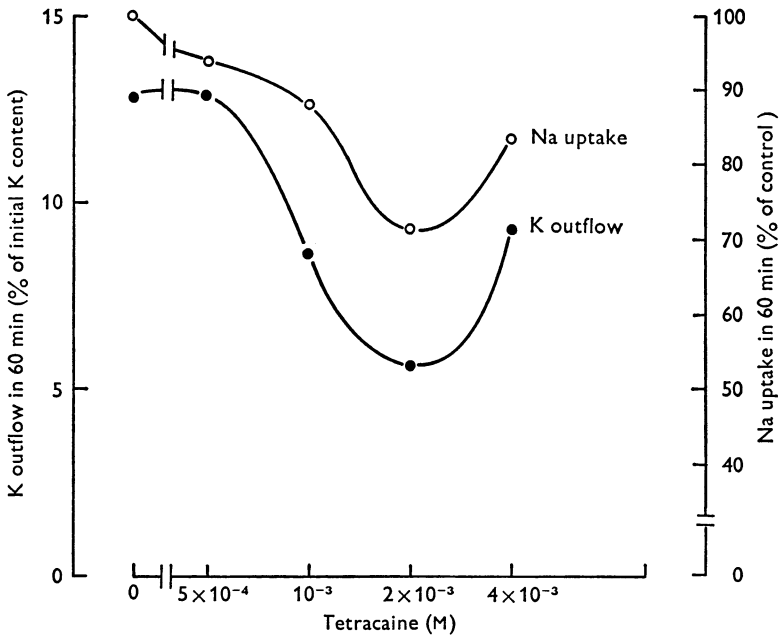

Fig. 5. The effect of tetracaine on Na and K movements in red cell ghosts. Haemolysis in the presence of 2 mM-EGTA and 0–4 mM tetracaine. Reconstitution of ghost cells with KCl. Incubation at 30° C in isotonic NaCl solution (soln. VI) containing 0–4 mM tetracaine and 2 mM-$CaCl_2$. Same ordinates as in Fig. 1. Haematocrit 11·6–13·4 %. One of four similar experiments.

Tetracaine induced changes in K and Na movements

The effects of tetracaine differed from those reported above for propranolol in several respects. Extracellular application of 0·1–4 mM tetracaine at pH 7·3 and 8·0 barely affected the K and Na permeability of resealed ghosts. However, when the drug was present in similar concentration in the haemolysing medium the yield of ghost cells, sealed to Na and K after reversal of haemolysis, was markedly increased. Upon incubation in a NaCl-medium these tetracaine-containing cells proved to be less permeable

to K and Na than the tetracaine-free controls. Therefore, in all experiments designed to evaluate the action of tetracaine, the drug was incorporated into the cells during haemolysis. The incubation media were always prepared to contain the same concentration of the drug as the haemolysing medium. In the experiment shown in Fig. 5, K and Na movements in tetracaine-loaded, Ca-free ghosts were plotted versus the tetracaine concentration. As the drug concentration on either side of the membrane was raised, both the inflow of Na and the outflow of K decreased to a minimum at about 2 mM. At concentrations above 4 mM the cation permeability increased exponentially and unspecifically, irrespective of whether the drug was present on both sides of the membrane or merely on the outside. The incorporation of EGTA into Ca-free ghosts left the effects of tetracaine unchanged. The propranolol-induced increase in K permeability was partially inhibited in cells which contained 1–2 mM tetracaine.

These observations suggest a direct interaction of tetracaine with membrane sites as possible basis for its effects on cation permeability rather than an indirect Ca-mediated effect.

Effects of propranolol and tetracaine on Ca uptake

During a 3-hr incubation period in a medium which contained 2 mM-Ca the total cellular Ca content of red cell ghosts increased from about 2×10^{-5} to 6×10^{-4} M. The maximally effective concentrations of the two drugs, 0·5 mM for propranolol and 2 mM for tetracaine, inhibited the total Ca uptake by $19{\cdot}6 \pm 5{\cdot}6$ and $44{\cdot}1 \pm 9{\cdot}2$ % respectively (mean ± S.E. of mean from four experiments). These drug concentrations were similar to those needed for maximal effects on Na and K permeabilities. However, this type of experiment provides no information to what extent the decrease in total Ca uptake reflects a reduced flow into the intracellular space or a decrease in membrane Ca binding. In order to separate the effects of the two drugs on transmembrane Ca flow from those on Ca binding, advantage was taken of the fact that in low concentrations intracellular but not extracellular Ca affects the K permeability. Thus the Ca-induced increase in K permeability can be used as a biological indicator for monitoring small changes in the intracellular free Ca concentration. The actual increase in the intracellular free Ca concentration was estimated from the particular intracellular EGTA concentration which was just insufficient to block the Ca-induced change in K outflow.

Fig. 6 shows the results of a representative experiment. All experimental points were obtained from the same batch of cells. The cells were loaded with EGTA concentrations ranging from 0 to 10^{-4} M and then exposed to media containing either 2 mM-Ca alone (curve I), 2 mM-Ca together with 1 mM propranolol (curve II) or together with 1 mM tetracaine (curve

III). The net K outflow in 60 min was measured and plotted on a relative scale versus the EGTA concentration. The intracellular Ca uptake estimated from the shift of the curves along the abscissa in Fig. 6 was changed significantly only by propranolol but not by tetracaine. In four similar experiments a five to tenfold increase in Ca influx was noted in the presence of 1 mM propranolol.

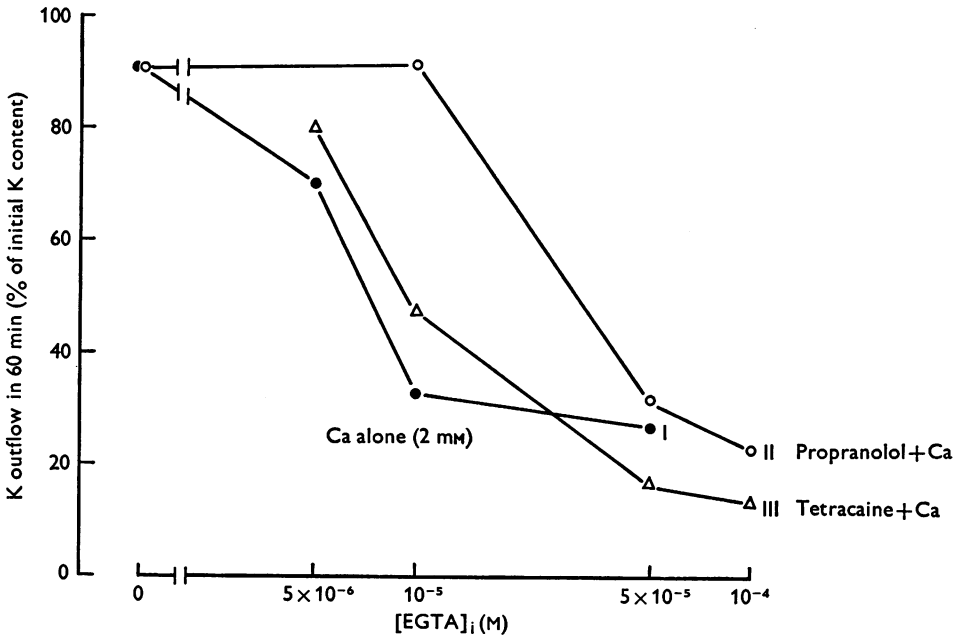

Fig. 6. Titration of net intracellular Ca uptake in the presence of tetracaine and propranolol by intracellular EGTA. The total K outflow in 60 min given in % of the initial cellular K content (ordinate), is plotted against the EGTA concentrations of the haemolysing solutions. Haemolysis in the presence of 0–10^{-4} M-EGTA. Reconstitution of ghosts with KCl. Incubation at 30° C in isotonic NaCl solution (soln. VII), containing 2 mM-$CaCl_2$ (I), 2 mM-$CaCl_2$ together with 1 mM propranolol (II) or 2 mM-$CaCl_2$ together with 1 mM tetracaine (III) haematocrit 4·4–7·4 %. One of four similar experiments.

In the concentration range between 0 and 10^{-4} M, intracellular EGTA alone had neither a significant effect on total Ca uptake nor did it change the percentage inhibition of total Ca uptake by propranolol or tetracaine. Since the transmembrane Ca flow under all conditions was small compared to the total Ca uptake and appeared not to be inhibited by either of the two drugs, the reduction in total Ca uptake must be attributed almost entirely to a decrease in the amount of membrane-bound Ca.

Ca-independent effects of propranolol

The previous results provided evidence that the effects of tetracaine on Na and K movements are the result of a direct interaction of the drug with membrane sites involved in the control of cation permeability. On the other hand, the effects of propranolol on cation permeability appeared to be a consequence of the drug-induced increase in the intracellular free Ca concentration. However, direct effects of propranolol became obvious in concentrations higher than those necessary to release Ca into the cell. In cells whose intracellular Ca concentration was maintained below 10^{-8} M by means of EGTA-buffering propranolol in concentrations between 2 and 10 mM caused a considerable increase in the flow of K and Na without concomitant loss of haemoglobin. The simultaneous increase in Na and K permeability with high concentrations of propranolol is clearly analogous to the effects described above for equimolar concentrations of tetracaine (see Fig. 5). Moreover, in earlier experiments a similar increase in K and Na permeability was observed in ghost cells which were prepared to contain more than 4 mM-Ca (Porzig, 1972). A possible explanation for the similarity of these effects is a common mechanism of action, emerging only when the intracellular concentration of one of the three substances has reached a threshold value of about 3 mM.

Effects of propranolol and tetracaine on the Ca-induced K outflow

The Ca-dependent increase in K permeability was chosen as a sensitive test system for possible competitive interactions between Ca and local anaesthetic amines at specific permeability-controlling Ca binding sites at the inner surface of the ghost membrane. The experimental results are illustrated by Fig. 7. The net K outflow under different experimental conditions is plotted against incubation time. In control cells the free Ca concentration on both sides of the membrane was kept below 10^{-8} M. The corresponding low rate of K loss is shown by curve I. Curve II describes the K outflow from cells containing 2×10^{-6} M free Ca. Curves III to V were obtained in Ca-free media containing 7 mM propranolol. The intracellular free Ca concentration was $< 10^{-8}$ (curve III), 3×10^{-7} (curve IV) and 2×10^{-6} M (curve V). Although propranolol *per se* at this high concentration increased K outflow (curve III), it inhibited the Ca-induced increase in the rate of K outward movement (compare curves II and V). Except for the initial portion, the K efflux in the presence of propranolol followed zero-order kinetics despite the continuous decrease of the concentration gradient. Therefore, the maximal flux velocity, given by the slope of the straight line relating intracellular K concentration to incubation time, had a mean value of 0·8 μmoles/min.ml ghosts and was inde-

pendent of the intracellular free Ca ion concentration. The parallel shift of curve V with respect to curve IV may be caused by a time lag of the onset of the blocking action of propranolol.

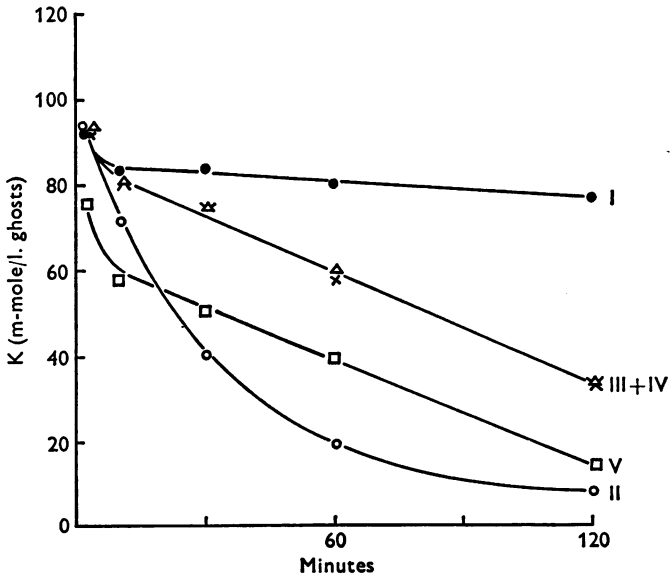

Fig. 7. Inhibition of Ca-induced K outflow by propranolol. The time course of net cellular K loss is plotted under different experimental conditions (I–V). I: haemolysis in the presence of 2 mM-EGTA. Incubation in isotonic NaCl solution (soln. VIII) containing 2 mM-EGTA, (●–●). II: haemolysis in the presence of 2×10^{-6} M-free Ca ions. Incubation as in I (○–○). III: haemolysis as in I. Incubation in isotonic NaCl solution (soln. VIII) containing 2 mM-EGTA and 7 mM propranolol (△–△). IV: haemolysis in the presence of 3×10^{-7} M-free Ca ions. Same incubation medium as in III (×–×). V: same haemolysing solution as in II, same incubation medium as in III (□–□). Reversal of haemolysis always with KCl. Note that the rate of K efflux under conditions III–V is of similar magnitude and linear in time. Haematocrit 7·8–10·4%. One of five similar experiments.

Fig. 8 shows the K outflow in the presence of either propranolol or tetracaine as well as in untreated controls as a function of the intracellular free Ca ion concentration. All data presented in this graph were collected from a single experiment. The control curve resembles the curve in Fig. 3. Half maximal activation of K outflow was achieved with $3{\cdot}7 \times 10^{-7}$ M-Ca. In the presence of 7 mM propranolol cellular Ca ion concentrations up to 10^{-5} M had little effect on K outflow.

Quite unexpectedly, tetracaine (2 mM) did not inhibit the Ca-induced K outward movement. In the presence of tetracaine, the intracellular Ca concentration required to induce a half maximal increase in K outflow

was reduced to about half the concentration in the control. This parallel shift was confirmed in two other experiments, where the K outflow in the presence of tetracaine was measured also at lower cellular Ca ion concentrations. Hence, in the presence of tetracaine the Ca influx driven by an inward concentration gradient will be overestimated if measured by the Ca-induced change in K permeability. In view of this synergistic action of Ca and tetracaine on K efflux the lack of effect of tetracaine on Ca permeability which was reported above (Fig. 6) in fact indicates a decrease in Ca permeability in the presence of the drug.

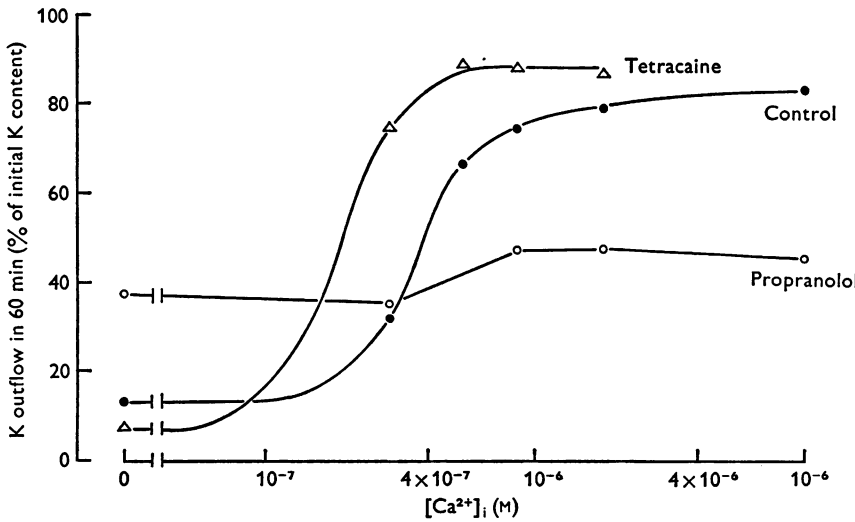

Fig. 8. Effect of tetracaine and propranolol on K outflow as a function of the intracellular free Ca ion concentration. The net K loss in 60 min is given in % of the initial cellular K content. Haemolysis in the presence of free Ca ion concentrations ranging from $< 10^{-9}$ to 10^{-5} M. Reconstitution of ghosts with KCl. Incubation at 30° C in isotonic NaCl solution (soln. VIII) containing 2 mM-EGTA (●–●), 2 mM-EGTA together with 2 mM tetracaine (△–△) or 2 mM-EGTA together with 7 mM propranolol (○–○). Haematocrit 7·9–11·1 %. One of six similar but less complete experiments.

The effect of tetracaine and propranolol on ATP-activated Ca efflux

The human red cell membrane is known to possess a specific ATP-dependent transport system which mediates a net outward movement of Ca against the existing electrochemical gradient of this ion (Schatzmann & Vincenzi, 1969; Schatzmann, 1973). If, in contrast to tetracaine, propranolol is indeed capable of interacting with *Ca-specific* sites at the inside of the membrane it might also interfere with the Ca transport mechanism. To test this, red cell ghosts were loaded with Ca, Mg, ATP according to the method of Schatzmann (1973). In addition, tetracaine was incor-

porated if the effect of this compound was to be tested. The ghosts were then incubated in an isotonic KCl medium (soln. IX) containing 0·2 mM-$CaCl_2$ and either propranolol or tetracaine. The incubation temperature was lowered to 25° C to slow down the active Ca outward movement. Moreover, enough EGTA was incorporated in the ghosts to establish an intracellular Ca: EGTA ratio of about 0·9. Thus, the rate of Ca outflow will progressively decrease as the intracellular free Ca concentration approaches the limiting concentration necessary to activate the pump

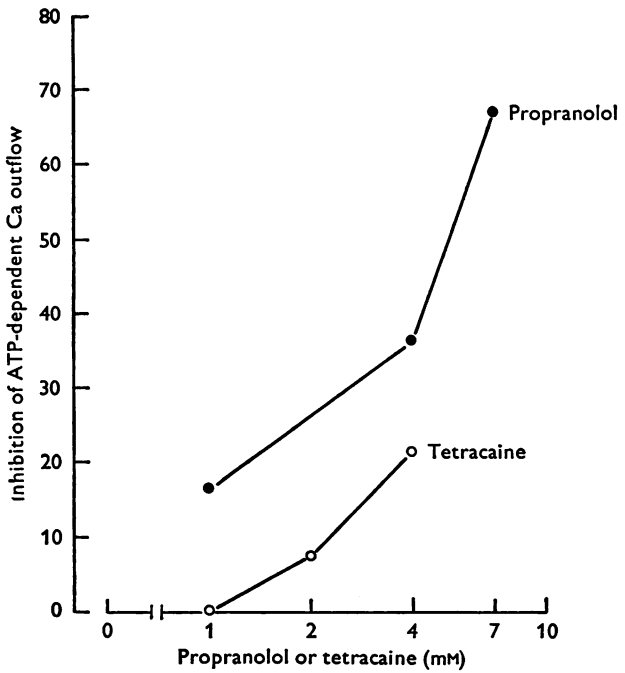

Fig. 9. Relationship between the inhibition of ATP-dependent Ca outward transport and the concentration of tetracaine or propranolol (abscissa, log scale). The total Ca outward transport in 60 min in % of the initial cellular Ca concentration in the absence of the drugs was compared to the respective numbers in the presence of the two compounds. Data from three experiments. The individual points are mean values of two to three measurements.

mechanism (Schatzmann, 1973). The Ca concentration of the external medium was made approximately equal to the initial internal free Ca concentration. Under those conditions the process of active Ca extrusion was extended to a period of about 60 min. In Fig. 9 the percentage inhibition of the total cellular Ca extrusion in 60 min is plotted against the concentration of propranolol or tetracaine. The inhibition increased with

increasing drug concentration. At 4 mM, tetracaine was about half as effective as propranolol (21·6 and 36·3 % inhibition respectively). However, the maximally possible inhibition which could be obtained with propranolol (7 mM) without destroying the integrity of the cell membrane was nearly 70 %. Tetracaine in concentrations above 4 mM caused rehaemolysis of the ghosts. In one experiment, where the cells did not lyse in the presence of 7 mM tetracaine the Ca transport was inhibited by 31 %.

Propranolol- or tetracaine-analogous effects of chemically or pharmacologically related amines

The present study could not answer satisfactorily the question of which structural or physico-chemical properties of the drug molecules are responsible for the differences in the interactions of propranolol and tetracaine with membrane Ca binding sites. However, in preliminary experiments the effects of a number of local anaesthetic amines on red cell K permeability were compared. The capability to induce a Ca-mediated increase in K permeability in a Ca-free medium was classified as 'propranolol-like' action, whereas a decrease in Na and K permeability under similar conditions was classified as 'tetracaine-like' action. No group-specific chemical configuration could be identified with the limited number of substances tested. Thus, a tetracaine-like action is not restricted to tertiary amines because it is shared also by prilocaine, a secondary amine. Similarly a tetracaine-like action does not require an ester linkage between the intermediate group and the aromatic residue in the local anaesthetic molecule. Lidocaine, which has an amide bond at this particular place equals tetracaine in its effect on red cell K permeability. A propranolol-like action was exerted by practolol and the ganglionic blocking agent tetraethylammonium (TEA). This effect of practolol is not very surprising since the drug is chemically closely related to propranolol. It possesses adrenergic β-receptor blocking activity and a weak 'quinidine-like' action (*see* Karow, Riley & Ahlquist, 1971). In our experimental system the concentrations of the drug necessary to increase the ghost cell K permeability were at least 10 times higher than equally effective concentrations of propranolol. Similarly, TEA (0·2–10 mM) was almost 10 times less potent than propranolol if incorporated into the ghost cells during osmotic haemolysis. Extracellular TEA was ineffective. The activity of this simple quaternary ammonium compound suggests that the typical amphiphilic structure of most local anaesthetic amines is not essential for an interaction with Ca at those specific sites in the membrane which are sensitive to propranolol.

In the same series of experiments three catecholamines, adrenaline,

noradrenaline and isoproterenol in concentrations up to 5×10^{-4} M neither had a significant effect on the K permeability of red cell ghosts nor did they increase the inflow of Ca.

DISCUSSION

The present study shows that propranolol and tetracaine cause in human red cells a distinctly different pattern of cation permeability changes. This is an unexpected result because the two drugs are both amphiphilic, positively charged amines and most existing theories would predict a common mode of action (*see* Seeman, 1972; Hellenbrecht, Lemmer, Wiethold & Grobecker, 1973; Metcalfe, 1970).

In excitable tissues, amine anaesthetics including tetracaine act primarily in the charged form on the inside of the cell membrane (Ritchie & Greegard, 1966; Narahashi, Frazier & Yamada, 1970; Ritchie, 1971; Seeman, 1972). Possibly they modify the cation fluxes by competing for Ca binding sites which are somehow involved in the control of membrane permeability. A preferential interaction of local anaesthetics with Ca binding sites has been demonstrated by Feinstein (1963, 1964, 1966) and Blaustein & Goldman (1966). Indeed, some effects of propranolol and tetracaine in red cells are in keeping with these results: (1) propranolol releases Ca into the intracellular space, (2) the potency of tetracaine as an inhibitor of Na and K permeability is increased if it is incorporated into the cells, (3) both drugs reduce the membrane-bound fraction of the total cellular Ca uptake, (4) the effects of the two compounds were observed at an experimental pH of 7·2 where they exist predominantly in their charged form.

However, if both drugs interact with Ca binding sites, what causes their opposite effects on K and Ca permeability? The present experiments suggest that the various types of binding sites in the red cell membrane differ strongly in their affinities for propranolol and tetracaine. In contrast to tetracaine, propranolol in low concentration can displace part of the endogenous, membrane-associated Ca (*see* Harrison & Long, 1972). Tetracaine seems to inhibit preferably the binding of additional extracellular Ca at sites which have a low association constant. Moreover, because of the lack of a direct Ca-antagonistic action of tetracaine on the inner surface of the membrane the inhibitory effect of the drug on the propranolol-dependent K outflow in Ca-free solution suggests that tetracaine impedes the intracellular release of membrane-bound Ca.

Recent studies on the influence of alkaline earth cations on the red cell cation permeability showed that two classes of Ca-sensitive intracellular Ca binding sites may be involved in the control of monovalent cation permeability. The large *increase* in potassium permeability which is

induced by small intracellular Ca concentrations (see Fig. 3) requires the interaction of Ca with sites at which only Sr can replace Ca (Blum & Hoffman, 1971). A second type of site was identified at which all alkaline earth cations tested (Sr, Ba, Ca, Mg) cause a *decrease* in Na, K and probably Ca permeability (Porzig, 1973). Propranolol in low concentrations promotes the Ca-activated K efflux by its Ca releasing properties. However, at high concentrations it turns gradually into a strong inhibitor of the Ca-dependent increase in K permeability and hence is probably capable of interacting with the Ca-specific type of binding sites (see Fig. 8). Tetracaine may reduce the cation permeability by interacting directly with the unspecific type of sites as a kind of weak Ca substitute. A high affinity of propranolol for Ca-specific sites is also demonstrated by its inhibitory action on the ATP-dependent transport mechanism. The Ca pump of the skeletal and cardiac muscle sarcoplasmic reticulum membrane, which shares important features with the active Ca transport system of red cells, was also shown to be inhibited by propranolol (Scales & McIntosh, 1968; Pang & Briggs, 1973; Temple, Hasselbach & Makinose, 1974). In equimolar concentrations propranolol was about twice as effective as tetracaine as a Ca transport ATPase inhibitor of the sarcoplasmic reticulum (Balzer, 1972). In intact red cells the partial inhibition of the Ca pump seems to contribute little to the effect of propranolol on K permeability. In Ca-containing solutions the K efflux is enhanced by low concentrations of propranolol which do not inhibit active Ca movements. Probably such drug concentrations are already sufficient to raise the Ca permeability to the extent where it cannot be compensated for even by an unimpaired pump.

Some evidence suggests that this inhibitory action on the Ca-Mg-activated transport ATPase is a function of the undissociated free base form of the amine anaesthetics (Feinstein & Paimre, 1969; Townsend, 1967). With a pK value for propranolol of 9·45 (Manninen, 1970) less than 0·6 % of the total concentration will be present in the free base form at the experimental pH of 7·2. A total concentration of 7 mM would correspond to 0·04 mM of free base. Even though the concentration of the free base form of tetracaine (pK value 8·48 (Büchi & Perlia, 1971)) under comparable conditions will be 10 times higher than that of propranolol, tetracaine is a much weaker and less specific inhibitor of the Ca pump. The small proportion of free base present at physiological pH values might explain the comparatively high concentrations of propranolol necessary to block the ATP dependent Ca movements. The very similar concentration requirements for the blocking action on the Ca-induced increase in K efflux suggest that also the latter effect of propranolol is a function of the free base rather than of the ionized form of the drug.

By comparison with tetracaine and other amine anaesthetics it became evident that the propranolol-type of interaction with membrane Ca binding and permeability of red cells is neither essential nor specific for drugs with local anaesthetic activity. Some recent electrophysiological studies suggest a possible relation of these findings to conductance changes which are caused by propranolol but not by tetracaine-type anaesthetics in some excitable tissues. Thus, in cardiac Purkinje fibres propranolol in concentrations comparable to those increasing red cell K permeability consistently accelerates the repolarization phase of the action potential (Davis & Temte, 1968; Giotti, Ledda & Mannoioni, 1973; Harrison, Wittig & Wallace, 1973). Anaesthetics of the tetracaine type, on the other hand, are known to decrease the K conductance or to leave this parameter unchanged (cf. Hille, 1970; Seeman, 1972). It is conceivable that an increase in the free Ca concentration near the inner surface of the membrane is the underlying mechanism for the propranolol-induced changes in K conductance. The experiments of Meech (1972, 1974), as well as of Krnjević & Lisiewicz (1972) have clearly demonstrated, that at least in certain nerve membranes, as in human erythrocytes, a rise in the intracellular free Ca concentration is followed by an increase in K permeability.

In discussing the arguments in favour of a causal relationship between propranolol-induced increase in K conductance and the drug's anti-arrhythmic action, Glynn & Warner (1972) and Harrison *et al.* (1973) conclude that the local anaesthetic and the antiarrhythmic activity of propranolol result from two clearly separable mechanisms of action. The results presented in this paper strongly support this view in showing that the 'pure' local anaesthetics and propranolol cause different patterns of membrane actions in erythrocyte ghosts.

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