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THE BINDING OF Ca^{2+} TO THE CELL MEMBRANE INCREASED BY VOLATILE ANESTHETICS (ALCOHOLS, ACETONE, ETHER) WHICH INDUCE SENSITIZATION OF NERVE OR MUSCLE

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SUMMARY

1. In order to test whether volatile anesthetics induce sensitization of tissues by altering the Ca^{2+} of the cell membrane, the effect of various anesthetics on the binding of $^{45}Ca^{2+}$ to erythrocyte ghost membranes was examined. The control uptake of Ca^{2+} by these EDTA-pre-washed membranes was between 0.03 and 0.08 mole Ca^{2+} per kg dry membrane at a free Ca^{2+} concentration of 0.8 mM.

2. Methanol, ethanol, propanol, butanol, pentanol and hexanol all increased the membrane Ca^{2+}, the first three drugs eliciting increases of as much as 2.5-fold at high concentrations. Ether, chloroform, acetone and benzyl alcohol increased the membrane Ca^{2+} by as much as 1.7-fold.

3. Ethyl acetate, stearic acid, carbon tetrachloride and veratrine sulfate all decreased the membrane Ca^{2+}.

4. Methoxyflurane, pentobarbital and fluothane had no effect. Heptanol, octanol and nonanol had no consistent effects.

5. About one-half the ghosts in each test tube were impermeable to inulin; this inulin-inaccessible space did not change in the presence of the drugs except at 3 M ethanol at which point lysis of the membrane permitted the entry of inulin.

6. The membrane Ca^{2+} was very sensitive to acetone and the lower alkanols, and rose by 6 ± 1 % at 0.09 % ethanol (20 mM) which is the intoxicating level of ethanol.

7. If these effects of volatile anesthetics on erythrocyte membranes also occur on electrically excitable membranes, including nerve endings, then this phenomenon could explain how methanol, ethanol, propanol and acetone increase the frequency of miniature end-plate potentials, or how they sensitize muscles to acetylcholine.

INTRODUCTION

This study indicates that the amount of Ca^{2+} which adsorbs to the erythrocyte membrane is markedly increased by volatile anesthetics which are known to sensitize nerve, muscle or other tissues. This increased level of membrane Ca^{2+} may explain the anesthetic-induced sensitization of these tissues.

ETTINGER *et al.*[1] observed that low concentrations of ethanol and ether potentiated the acetylcholine-induced contractions of frog rectus. Further work[2-6] extended

this phenomenon of muscle sensitization to include propanol, butanol, pentanol, ketones, chloroform and ether. It is also known that phenols can antagonize the action of curare at the neuro-muscular junction[7]. Methanol, ethanol, propanol and acetone increase the frequency of the miniature end-plate potentials as well as enhance the post-junctional potential produced by exogenous acetylcholine[8–10]. Phenols, on the other hand, only appear to increase the amount of transmitter produced without affecting the post-junctional sensitivity to applied acetylcholine[7]. Stretch receptors are also sensitized by low concentrations of volatile anesthetics[11].

One possible explanation for this anesthetic-induced sensitization is that these volatile compounds may increase the amount of Ca^{2+} which is associated with the membrane since membrane-associated Ca^{2+} is required for excitation–contraction coupling[12] and for stimulus–secretion coupling[13]. It is known that the common local anesthetics (*i.e.* tertiary amines, such as procaine) interfere with this coupling, presumably by displacing the membrane Ca^{2+}, as has been found for chlorpromazine[14]. The results reported in the present paper indicate that volatile anesthetics of low molecular weight (under about 90) increase the amount of membrane Ca^{2+}.

METHODS

(1) *Preparation of erythrocyte ghosts*

As described previously[14], acid–citrate–dextrose-stored human blood was washed, and erythrocyte ghosts were prepared by the method of DODGE *et al.*[15]. As shown by HARRISON AND LONG[16], EDTA virtually completely removes the Ca^{2+} bound to these membranes. The hemoglobin-free ghosts (white in appearance) were then washed 3 times in 15 mM Tris–HCl buffer (pH 7.0) and finally packed by centrifugation at 36900 × *g* for 40 min. The dry weight of each final ghost stock suspension was obtained by drying an aliquot at 80° for 16–20 h; after a correction for the dry weight of the buffer salts, the dry weight was usually between 1 and 2 % (g dry membrane per 100 ml ghost suspension).

(2) *The adsorption of Ca^{2+} to ghost membranes, and the effect of anesthetics on this adsorption*

$^{45}CaCl_2$ (Atomic Energy of Canada, Ltd.) was obtained at a specific activity of 12 mC/mole and diluted down to $8 \cdot 10^{-5}$ M Ca^{2+} (with 96 μC/l) using 15 mM Tris–HCl buffer (pH 7). This diluted solution was mixed with an equal aliquot of a $7.4 \cdot 10^{-3}$M Ca^{2+} solution to yield a final radioactive $^{45}Ca^{2+}$ stock solution of 3.74 mM Ca^{2+}, containing 48 μC/l; this final radioactive stock solution was used for all the experiments.

The following procedure was used for determining the effect of anesthetics on the adsorption of Ca^{2+} to the ghost membranes. All steps were done at 21°.

(a) An aliquot of 0.3 ml of the ghost suspension was weighed into a 10 mm × 75 mm glass test tube. A Stanton digital unimatic balance (Model CL5D) was used and each weight recorded to within 0.05 mg.

(b) An aliquot of 0.2 ml of the radioactive stock solution (containing about 80000 counts/min) was weighed into the test tube containing the ghosts. The contents were mixed for 2 sec by whirling, using a Vortex mixer.

(c) Approx. 10 min after adding the $^{45}Ca^{2+}$, an aliquot of 0.3 ml of the drug

solution was weighed into the test tube. The drugs had been dissolved in 15 mM Tris–HCl buffer (pH 7). The contents were again mixed, and after about 10 more min, the ghosts were centrifuged at 36900 × *g* for 20 min at 21° in a temperature-controlled centrifuge.

(d) The radioactivity in the supernatant was measured by weighing in 0.1 ml of the supernatant into a standard liquid scintillation vial. The vial was filled up with 10 ml of Bray's liquid scintillation medium[14] and counted in a Packard Tri-Carb liquid scintillation spectrometer (Model 3380) using an amplification of 9.5 % with the lower and upper windows set at 20 and 800 units, respectively. The high-tension voltage was set at 1467 V. Further details are given in ref. 14.

The adsorption or uptake of Ca^{2+} by the membrane may be expressed as a membrane concentration of Ca^{2+}, according to Eqn. 1.

$$Ca^{2+}_{\text{dry mem}} = \frac{(Ca^{2+}_{\text{stock}} \cdot V_{\text{stock}}) - (Ca^{2+}_{\text{free}} \cdot V_{\text{total}})}{W_{\text{dry mem}}} \text{ moles/kg dry membrane} \quad (1)$$

where $Ca^{2+}_{\text{dry mem}}$ is the dry membrane concentration of Ca^{2+}, Ca^{2+}_{stock} is the Ca^{2+} concentration of the radioactive stock solution, V_{stock} is the volume of the aliquot from the radioactive stock, Ca^{2+}_{free} is the Ca^{2+} concentration in the supernatant, V_{total} is the volume of the entire contents of the tube, and $W_{\text{dry mem}}$ is the dry weight (in kg) of the membranes added to the test tube. It was assumed that V_{stock} and V_{total} each had a specific gravity very close to unity and that the volumes, therefore, were virtually identical to the weights.

Ca^{2+}_{free} was determined by Eqn. 2.

$$Ca^{2+}_{\text{free}} = Ca^{2+}_{\text{stock}} \cdot \frac{(\text{disint./min per ml})_{\text{sup}}}{(\text{disint./min per ml})_{\text{stock}}} \quad (2)$$

where $(\text{disint./min per ml})_{\text{sup}}$ and $(\text{disint./min per ml})_{\text{stock}}$ represent the disint./min per ml of supernatant and of stock, respectively.

(3) Determination of the water space inaccessible to inulin

An assumption inherent in Eqn. 1 is that the Ca^{2+} distributes throughout the water volume in the test tube. This assumption is not entirely correct, as explained previously[14], and is considered in DISCUSSION of the present paper.

To determine the inulin-inaccessible water space, radioactive inulin (methoxy-[^{3}H]inulin, 89.2 mC/g, New England Nuclear, U.S.A.) was used. The inulin-inaccessible space was determined simultaneously with the $^{45}Ca^{2+}$ in the supernatant by the following procedure.

(a) A combined radioactive stock solution of both isotopes was prepared containing 3.7 mM Ca^{2+}, 48 μC/l for $^{45}Ca^{2+}$, and 800 μC/l for [^{3}H]inulin.

(b) The same procedure was followed as outlined in METHODS, Section 2, except that the radioactive aliquot was replaced by an aliquot of the [^{3}H]-inulin–$^{45}Ca^{2+}$ stock solution.

The volume of water inaccessible to inulin (V_{closed}) was expressed as a fraction of the total volume in the test tube (V_{total}) according to Eqn. 3.

$$\frac{V_{\text{closed}}}{V_{\text{total}}} = \frac{(^3\text{H/ml})_{\text{sup}} - (^3\text{H/ml})_{\text{control}}}{(^3\text{H/ml})_{\text{sup}}} \quad (3)$$

where $(^3H/ml)_{sup}$ and $(^3H/ml)_{control}$ are the ^3H inulin distin./min per ml in the experimental supernatant and control supernatant, respectively; the control test tube in this case means that which does not contain any ghost membranes.

The procedure for counting both isotopes simultaneously and making the appropriate separation of the ^3H and the $^{45}Ca^{2+}$ disintegrations has already been presented elsewhere[14].

MATERIALS

The drugs and chemical compounds were used without further purification and were obtained from the following companies. Ether, *n*-methanol, *n*-propanol, *n*-butanol, *n*-pentanol, *n*-octanol, benzyl alcohol, acetone, chloroform, ethyl acetate and carbon tetrachloride were from Fisher Scientific Co; *n*-ethanol from the Ontario Liquor Control Board; *n*-hexanol (in excess of 99% pure) was from Analabs., Inc., P. O. Box 501, North Haven Conn., U.S.A.; *n*-heptanol, *n*-nonanol and stearic acid were from Eastman Organic Chemicals; veratrine sulfate was from K & K Labs., New York; methoxyflurane was from Abbott Labs.; sodium pentobarbital was from Ingram & Bell; fluothane (halothane) was from Ayerst Labs., Montreal.

RESULTS

Effect of alkanols on the binding of Ca^{2+} to membranes

The binding of Ca^{2+} to erythrocyte membranes was increased by methanol, ethanol, propanol, butanol, pentanol and hexanol. This is shown in Fig. 1, where the results are expressed in terms of a ratio as follows: $\{(Ca^{2+}_{membrane})$ in presence of drug$\}/\{(Ca^{2+}_{membrane})$ in absence of drug$\}$. The value for $Ca^{2+}_{membrane}$ in the absence of any drug was always between 0.03 and 0.08 mole/kg dry membrane. As is usual with any adsorption process, the amount of ligand adsorbed depends on the free concentration of ligand. Since such a dependence of $Ca^{2+}_{membrane}$ on Ca^{2+}_{free} has been shown[14,17], it was necessary to normalize all the $Ca^{2+}_{membrane}$ values to the same Ca^{2+}_{free}. The value chosen for a common Ca^{2+}_{free} was 0.8 mM.

The results in Fig. 1 indicate that the longer molecules (*e.g.* hexanol) are less

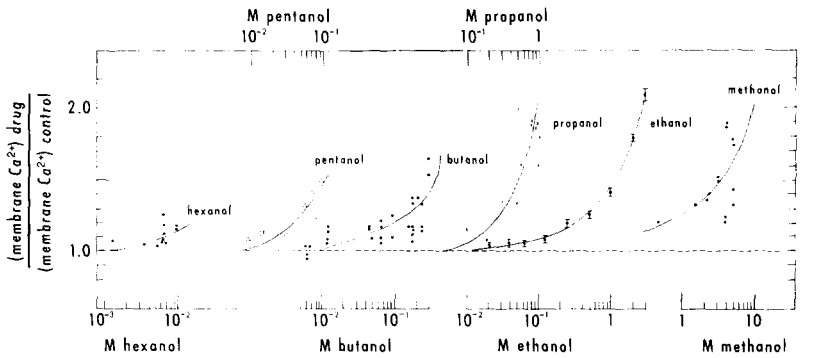

Fig. 1. Showing that the lower alkanols (methanol to hexanol) increase the binding of Ca^{2+} to erythrocyte ghost membranes. The amount of Ca^{2+} which binds to the membranes in the absence of any drugs is represented by the value 1.00 on the ordinate. All vertical bars indicate S. E. of 5–10 determinations.

effective in producing a large increase in the Ca^{2+} binding. Methanol, ethanol and propanol in high concentrations increased the Ca^{2+} by as much as 2.5–3-fold in some experiments; hexanol, on the other hand, never produced more than a 20 % increase in the binding of Ca^{2+}. The longer aliphatic alkanols, heptanol, octanol and nonanol did not produce any consistent changes in Ca^{2+} binding. These alkanols did, however, reduce the Ca^{2+} binding if they were present in solution in high concentrations as an emulsion; the membranes presumably were precipitated or denatured under these circumstances; no electron microscopy was carried out under these conditions.

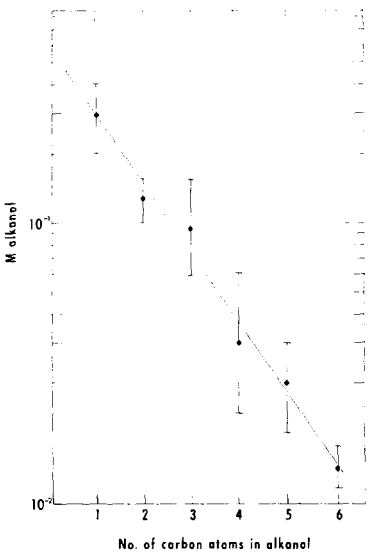

Fig. 2. The concentration of each alkanol which increases the Ca^{2+}-binding by 10 % is plotted on the ordinate. The potency of each alkanol goes up approximately by a factor of 3 with each additional methylene group (Traube's rule).

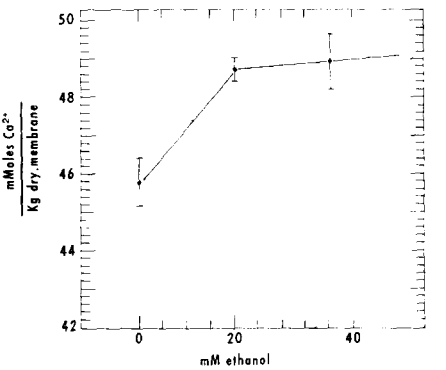

Fig. 3. The binding of Ca^{2+} to the membrane is very sensitive to low concentrations of ethanol. The control value in the absence of drug is 45–78 mmoles Ca^{2+} per kg dry membrane.

The concentrations of the alkanols which increase the binding of Ca^{2+} by 10 % are plotted in Fig. 2. It can be seen that the potency of each compound is enhanced approximately by a factor of 3 with each additional methylene group.

The binding of Ca^{2+} to the erythrocyte membranes is extremely sensitive to low concentrations of ethanol. A significant elevation in $Ca^{2+}_{membrane}$ could readily be detected at 20 mM ethanol (0.09 g/100 ml) and this is shown in Fig. 3 for erythrocyte ghosts.

The effect of volatile anesthetics on the binding of Ca^{2+} to membranes

The binding of Ca^{2+} to erythrocyte membranes was increased by ether, benzyl alcohol, acetone and chloroform, as illustrated in Figs. 4 and 5.

Methoxyflurane (up to 40 mM) and halothane (up to 18 mM) had no effect. Ethyl acetate and carbon tetrachloride depressed the binding of Ca^{2+} to the membranes (Figs. 4 and 5).

Effects of carbachol, veratrine, pentobarbital and stearic acid on membrane Ca^{2+}

At very high concentrations (*i.e.* between 1.10^{-3} M and $1 \cdot 10^{-2}$ M) both veratrine sulfate and carbachol lowered the binding of Ca^{2+}. Only the results for veratrine are shown (Fig. 5). The findings for carbachol were similar to those already reported for suxamethonium[18] and indicate that carbachol is no more potent than Na^+ in displacing Ca^{2+} from the membrane (see ref. 14).

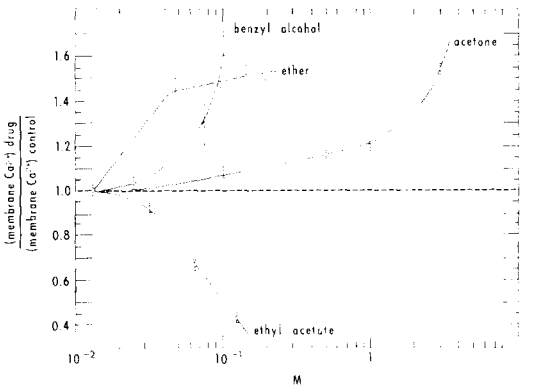

Fig. 4. Effects of acetone, benzyl alcohol and ethyl acetate on the membrane Ca^{2+}. The ratio of 1.00 (ordinate) represents the control membrane Ca^{2+} in the absence of any drugs and is between 0.03 and 0.08 mole Ca^{2+} per kg dry membrane.

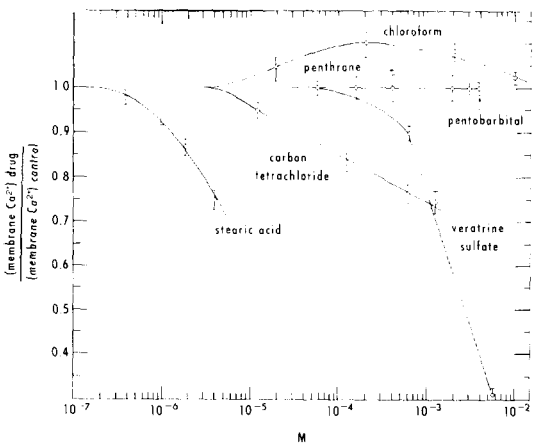

Fig. 5. Effects of chloroform and other drugs on the binding of Ca^{2+} to membranes. The control membrane Ca^{2+} of 1.00 (ordinate) represents between 0.03 and 0.08 mole Ca^{2+} per kg dry membrane. Methoxyflurane (penthrane) and pentobarbital had no effect, and the highest concentrations tested are indicated.

Stearic acid depressed the membrane Ca^{2+} while pentobarbital (up to 38 mM) had no effect. Since pentobarbital invariably raised the pH of the solution, it was necessary to use control solutions where the final medium had the same pH as that in the presence of pentobarbital; when this was done, no effect of pentobarbital was observed. (The alkanols did not alter the pH of the solutions.)

Effect of alkanols on the inulin-inaccessible water space

As illustrated in Figs. 1 and 6, the effects of methanol, ethanol and propanol were particularly marked, the $Ca^{2+}_{membrane}$ increasing by as much as 2.5-fold. It is known that erythrocyte ghost membranes reseal[19] and that not all the water in the test tube is available for the distribution of Ca^{2+} (ref. 14). It was possible, therefore, that the increase in membrane Ca^{2+} could have resulted from a gross disruption of the sealed ghost membranes, resulting in an increased surface area for adsorption.

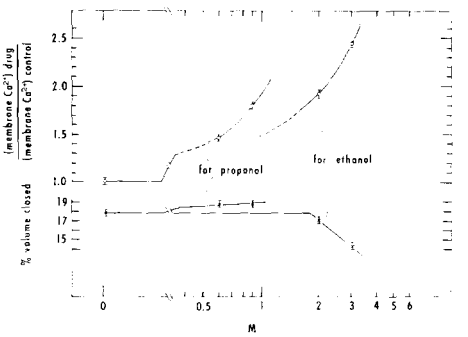

Fig. 6. The top curves illustrate that ethanol and propanol increase the amount of Ca^{2+} adsorbed to the erythrocyte ghost membranes. The control value of 1.00 represents exactly 0.030 mole Ca^{2+} per kg dry membrane. The data at the bottom indicate that the inulin-inaccessible space becomes reduced only at very high concentrations of the two alkanols.

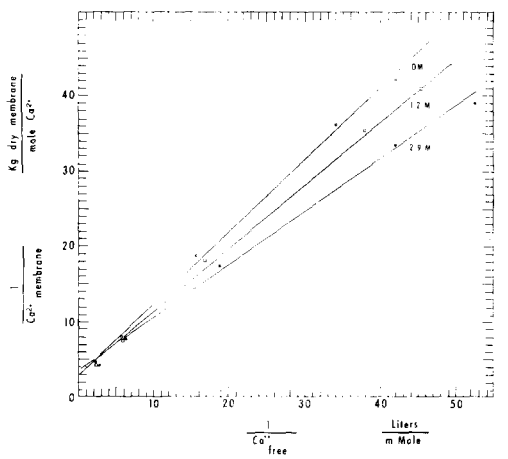

Fig. 7. Showing that ethanol increases the affinity of erythrocyte membranes for Ca^{2+}.

The inulin-inaccessible water space was measured, therefore, in the presence and absence of ethanol and propanol. These findings are shown at the bottom of Fig. 6. The inulin-inaccessible space was 17.7 $\pm$ 0.3 % of the total volume in the test tube. This amounts to a "closed volume" of about 0.14 ml, since the total volume in the test tube was always around 0.8 ml. This inulin-inaccessible volume of 0.14 ml comes from the 0.3-ml aliquot of ghosts and represents, therefore, about 50 % of the ghosts which were added. In other words, of all the ghosts in the test tube approxim-

ately one half are grossly open (and accessible to inulin) while the other half of the cells are inulin-inaccessible. According to Fig. 6, it can be readily calculated that 3 M ethanol could only expose an additional 20 % of inner membrane surface. The membrane Ca^{2+}, however, rises by 2.4-fold in the presence of 3 M ethanol.

Effect of ethanol on the affinity of the membrane for Ca^{2+}

The results just presented indicate that concentrations of ethanol above 2 M increase the inulin space, and must, therefore to a certain extent increase the number of binding sites.

In order to examine whether ethanol also alters the affinity of the membrane for Ca^{2+}, an adsorption isotherm for Ca^{2+} was done at different concentrations of ethanol. The results are plotted in Fig. 7 in the form of a double reciprocal isotherm (see ref. 14 for further references). The results in Fig. 7 indicate that ethanol increases the affinity of the membrane for Ca^{2+}.

Finally, serum has no effect on membrane Ca^{2+}, and there was no difference when the sequence of adding Ca^{2+} and drug was changed.

DISCUSSION

The possible relation between elevated $Ca^{2+}_{membrane}$ and tissue sensitization by anesthetics

The major finding in this study is that volatile anesthetics of low molecular weight increase the amount of Ca^{2+} which binds to the erythrocyte ghost membrane.

If these effects of volatile anesthetics on erythrocyte membranes also occur on electrically excitable membranes, then this could be the explanation of anesthetic-induced sensitization, since membrane-bound Ca^{2+} is needed for stimulus–response coupling[12,13]. It is interesting to note that all compounds hitherto tested and which are known to increase the frequency of miniature end-plate potentials, namely methanol, ethanol, propanol and acetone[8–10], also elevated the Ca^{2+} binding of the erythrocyte membrane. Furthermore, compounds which had no effect on the Ca^{2+} binding (*e.g.* halothane) do not have any potentiating characteristics at the neuromuscular junction[20].

There are of course, several other ways in which these sensitizing anesthetics could cause potentiation.

(1) The drugs might inhibit the active uptake of Ca^{2+} by the endoplasmic reticulum[21]. Nerve terminals, smooth muscle and frog ventricle muscle, however, have little endoplasmic reticulum.

(2) The drugs might inhibit the active uptake of Ca^{2+} by the mitochondria[22].

(3) The anesthetics might depolarize the cell membrane. INOUE AND FRANK[23] in a systematic study did not find any significant drop in the resting potential of the frog sartorius muscle fibers in concentrations of ethanol as high as 2 %. The work of HOUCK[24] has also shown that depolarization of the lobster axon membrane only occurs for ethanol above 1 M, for methanol above 2.4 M, for propanol above 0.25 M, for butanol above 0.1 M and for pentanol above 0.3 M. The data in Figs. 1 and 4, however, indicate that the membrane Ca^{2+} is increased by concentrations of these alkanols which are far below (by a factor of 50 in the instance for ethanol) than the depolarizing concentrations.

The anesthetics may change the conformation of the lipoprotein binding sites

to increase or decrease the number of exposed anionic groups. It is known that anesthetics can alter the conformation of proteins[26] and of membranes in general. This subject has been discussed previously[14]. The drugs may alter the state of membrane hydration[27] and thus alter the pK_a of the anionic groups or change the accessibiltiy of these anionic groups.

Erythrocyte membranes and excitable membranes

There is reason to believe that the effects described in this study on erythrocytes will also be found with excitable membranes, since EHRENPREIS[26] observed that isopropyl alcohol increased the binding of Ca^{2+} to an extract of sciatic nerve. If the binding of Ca^{2+} to excitable membranes is also increased by these drugs, more Ca^{2+} would be released from the membrane when the membrane's association constant for Ca^{2+} decreases in the excited state (see ref. 28 for a thorough discussion on this point).

Current experiments in progress with intact erythrocytes indicate that the drug-induced increase in membrane Ca^{2+} occurs at the inner (cytoplasmic) surface of the membrane.

ACKNOWLEDGMENTS

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REFERENCES

1 G. H. ETTINGER, A. B. BROWN AND A. H. MEGILL, *J. Pharmacol. Exptl. Therapy*, 73 (1941) 119.
2 J. P. FENG AND T. H. LI, *Chin. J. Physiol.*, 16 (1941) 317.
3 P. B. SABAWALA AND J. B. DILLON, *Anesthesiology*, 19 (1958) 587.
4 F. A. NELEMANS, *Acta physiol. Pharmacol. Neerl.*, 11 (1962) 76.
5 K. S. SACHDEV, M. H. PANJWANI AND A. D. JOSEPH, *Arch. Intern. Pharmacodyn.*, 145 (1963) 36.
6 F. INOUE AND G. B. FRANK, *Can. J. Physiol. Pharmacol.*, 43 (1965) 751.
7 M. OTSUKA AND Y. NONOMURA, *J. Pharmacol.*, 140 (1962) 41.
8 P. W. GAGE, *J. Pharmacol. Exptl. Therapy*, 150 (1965) 236.
9 K. OKADA, *Japan. J. Physiol.*, 17 (1967) 245.
10 K. OKADA, *Japan. J. Physiol.*, 20 (1970) 97.
11 A. S. PAINTAL, *Pharmacol. Rev.*, 16 (1964) 341.
12 A. SANDOW, *Pharmacol. Rev.*, 17 (1965) 265.
13 W. W. DOUGLAS, *Brit. J. Pharmacol.*, 34 (1968) 451.
14 W. O. KWANT AND P. SEEMAN, *Biochim. Biophys. Acta*, 193 (1969) 338.
15 J. T. DODGE, C. MITCHELL AND D. J. HANAHAN, *Arch. Biochem. Biophys.*, 100 (1963) 119.
16 D. G. HARRISON AND C. LONG, *J. Physiol.*, 199 (1968) 367.
17 W. L. GENT, J. R. TROUNCE AND M. WALSER, *Arch. Biochem. Biophys.*, 105 (1964) 582.
18 R. COLEMAN AND G. VRBOVA, *J. Physiol.*, 201 (1969) 40P.
19 P. SEEMAN, *J. Cell Biol.*, 32 (1967) 55.
20 A. J. GISSEN, J. H. KARIS AND W. L. NASTUK, *J. Am. Med. Assoc.*, 197 (1966) 770.
21 W. FIEHN AND W. HASSELBACH, *European J. Biochem.*, 9 (1969) 574.
22 L. MELA, *Biochemistry*, 8 (1969) 2481.
23 F. INOUE AND G. B. FRANK, *Brit. J. Pharmacol. Chemotherapy*, 30 (1967) 30.
24 D. J. HOUCK, *Am. J. Physiol.*, 216 (1969) 364.
25 D. BALASUBRAMANIAN AND D. B. WETLAUFER, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 762.
26 S. EHRENPREIS, *J. Cellular Comp. Physiol.*, 66 (1965) 159.
27 P. SEEMAN, R. I. SHA'AFI, W. R. GALEY AND A. K. SOLOMON, *Biochim. Biophys. Acta*, 211 (1970)365
28 C. P. BIANCHI AND N. LAKSHMINARAYANAIAH, *Federation Proc.*, 28 (1969) 1627.