A FUNCTIONAL ACETYLCHOLINE RECEPTOR IN THE HUMAN ERYTHROCYTE

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Received February 14, 1974

SUMMARY: The effects of carbamyl choline and epinephrine on membrane rigidity of human erythrocytes have been studied using spin-labeled fatty acids. Treatment of the cells with neurotransmitters increases the cation permeability of the membrane, resulting in an increase in rigidity which apparently involves fibrous proteins in the cell.

In the course of a study of the effects of prostaglandins and other hormones on the human erythrocyte, Allen and Rasmussen (1) observed that erythrocyte flexibility changes in the presence of the adrenergic neurotransmitter adrenaline. Little is known about the mechanism of this effect, and no evidence has been presented to indicate whether red blood cells respond to cholinergic stimulation as well. This communication reports studies of the effect of neurotransmitters on erythrocyte membrane flexibility, using a spin label technique which permits detection of subtle changes in fluidity of the membrane hydrocarbon region. Treatment of erythrocytes with agents which block specific stages of the excitation process in nerve or muscle cells permits an analysis of some of the molecular processes involved in the membrane flexibility changes.

MATERIALS AND METHODS

Erythrocytes: Blood was obtained from adult volunteers and used within three days of withdrawal. Red cells were washed with buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 3.5 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 0.1 mM $CaCl_2$ and 1 mM glucose (pH 7.38). All experiments

Abbreviations used are: CbCh, carbamyl choline; TTxn, tetrodotoxin; d-TC, curare; cytB, cytochalasin B; AChE, acetylcholinesterase; S, order parameter

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were conducted in this medium, with the addition of 0.5 mM EGTA as indicated.

Reagents: The 10,3 fatty acid spin label (N-oxyl - 4', 4' - dimethyl oxazolidine derivative of 5 - keto-palmitic acid) was a gift of Dr. Betty Jean Gaffney. Carbamyl choline was obtained from Sigma Chemical Co. L-epinephrine, d-tubocurarine chloride and dimyristoyl lecithin were obtained from Calbiochem. Atropine sulfate was the product of Mann Research Laboratories, textrodotoxin was obtained from Sankyo Co., Ltd. and cytochalasin B was the product of Imperial Chemical Industries, Ltd., distributed by Aldrich Chemical Co. $^{22}Na^{+}$ was obtained from New England Nuclear.

Acetylcholinesterase was assayed by the spectrophotometric method of Ellman et al. (3).

Esr studies were conducted using a Varian E4 spectrometer with variable temperature accessory. Aliquots (5 μl) of a solution of 10, 3 - fatty acid spin label (3.1 mM in ethanol) were placed in 3 ml test tubes and the ethanol was evaporated with a stream of argon. A suspension of erythrocytes (150 μl of 67% hematocrit in phosphate-saline buffer) was added and the mixture incubated at 37°for ten minutes. CbCh, TTxn, curare and other effectors were then added in 5 μl of buffer and the mixture was left at 37° for five minutes. A sample was withdrawn and placed in a 50 μl sample tube. Spectra were recorded at 37°.

The order parameter, S, is a quantitative measure of membrane flexibility (4, 5). It is determined experimentally from the peak positions of the esr spectrum, which are a sensitive function of the anisotropic motion of the nitroxide group on the hydrocarbon chain.

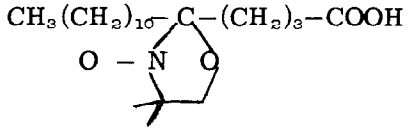
Radioactive isotopes were counted in a Nuclear Chicago Unilex liquid scintillation counter. After incubation with $^{22}Na^{+}$, aliquots of washed cells were lysed and suspended in 1 ml of water, to which 10 ml of Aquasol was added.

RESULTS

Studies involving treatment of erythrocytes with phospholipid vesicles (Huestis and McConnell, in preparation) showed that the erythrocyte membrane contains sodium-specific ion channels which can be blocked by the nerve poison tetrodotoxin (TTxn) at concentrations of 10^{-8} M. These "chan-

nels", which in their TTxn sensitivity resemble sodium channels involved in action potential propagation in nerve and muscle, appeared to be associated directly or indirectly with the membrane acetylcholinesterase (AChE). The presence of these functions in erythrocytes suggested to us that an acetylcholine receptor might also be found in the erythrocyte.

The work of Kury et al.(6) on prostaglandins showed that it is possible to detect very small changes in membrane fluidity of red cells by measuring the order parameter of the nitroxide group introduced in the fatty acid,



Reports (2) that adrenaline increases the rigidity of erythrocytes indicated that the order parameter probably would be sensitive to adrenaline association, and might permit detection of membrane changes associated with cholinergic stimulation.

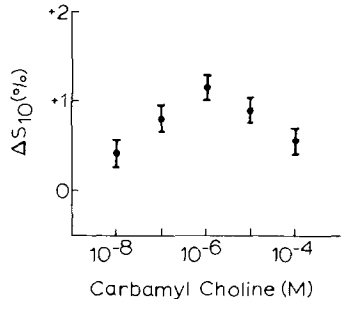


Figure 1. Effect of CbCh on the order parameter S_{10} of the erythrocyte membrane, as a function of CbCh concentration. The absolute value of S in control cells at 37° is 0.6550.

Figure 1 shows the response of the red cell order parameter to carbamylcholine (CbCh, an acetylcholine analog which is not hydrolysed by the membrane AChE). An increase in S, indicating an increase in the rigidity of the membrane, is observed. The effect is maximal at a CbCh concentration of 10^{-6}M. If this increase in membrane rigidity is due to specific association of the CbCh with a receptor molecule, it should be possible to prevent the effect by pretreatment of the membrane with a cholinergic antagonist,

either of the nicotinic (curare) or muscarinic (atropine) type.

Figure 2 shows the results of pretreating the cells with antagonists. Curare (10^{-5} M) has little effect on the CbCh induced increase in S, while atropine (10^{-5}M) reduces the effect 75%. This result suggests that the CbCh-induced membrane stiffening is due to specific interaction of CbCh with a muscarinic cholinergic receptor. If, moreover, this interaction produces a change in cation permeability of the membrane such as that accompanying nerve and muscle excitability, pretreatment of erythrocytes with TTxn should prevent the CbCh effect. As is shown in Figure 2, 10^{-8}M TTxn substantially reduces

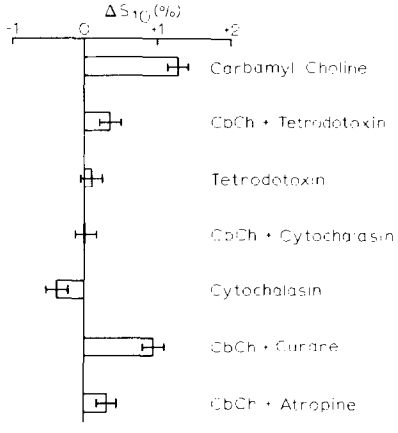


Figure 2. Effect of CbCh and some specific neurotoxins on the order parameter of erythrocytes. CbCh, 10^{-6}M; TTxn, 10^{-8}M; cytB, 10^{-5}M; curare, 10^{-5}M; atropine, 10^{-5}M.

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the CbCh effect, but only in the presence of a Ca^{+2} chelating agent such as EGTA. If free Ca^{+2} is present in the external medium, TTxn does not block the membrane stiffening.

The possibility that the erythrocyte stiffening is analogous to smooth muscle contraction is reinforced by the finding (7) that erythrocytes contain an "actomyosin-like" microfilamentous protein associated with the inner surface of the membrane. The suggestion has been made (8) that this protein (spectrin) aids in maintaining the shape and flexible strength of the cell. Carter (9) observed that a class of fungal metabolites, the cytochalasins, disrupt microfilament structure and interfere with a diverse assortment of

cytological processes mediated by contractile mechanisms (e.g. secretion, phagocytosis, cell movement).(10-12) Although disagreement exists concerning the specificity of action of the cytochalasins (specifically, cytB disrupts glucose transport in fat cells by binding to a membrane component described as the glucose receptor (13), considerable evidence has been presented to indicate that cytochalasins specifically disrupt actomyosin-like systems in vitro (14, 15) and probably in vivo (16) as well.

The addition of cytB to spin-labeled erythrocytes produces a decrease in S (Figure 2), and subsequent addition of CbCh produces no increase in S for cytB treated cells. This result is consistent with the participation of a contraction process, involving actomyosin-like proteins, in the stiffening of the erythrocyte membrane.

The response of erythrocytes to adrenergic stimulation is similar in most respects to their response to CbCh. As Figure 3 shows, adrenaline in-

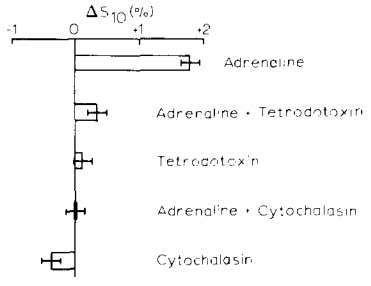

Figure 3. Effect of adrenaline and neurotoxins on the order paramenter of erythrocytes. Concentrations as in Figure 2.

duces a membrane-stiffening effect greater than that of CbCh, which could be largely prevented if cells were treated with TTxn or cytB before addition of adrenaline.

DISCUSSION

The response of red blood cells to chemical neurotransmitters parallels the smooth muscle contraction process to a striking degree. As with smooth muscle, the cells respond to adrenaline and to carbamyl choline, the latter response being of the muscarinic variety (i.e. mimicked by muscarine, not nicotine, and antagonized by atropine, not curare). Treatment of erythro-

cytes with these neurotransmitters renders their membranes more permeable to Na^+ and/or Ca^{+2}, and the consequent influx of these cations results in stiffening of the membrane. The increase in membrane rigidity appears to be related to contractile protein interactions, an aspect which is under further investigation.

The functional significance of this apparent neurotransmitter-contractile response in the human erythrocyte is not known. This response could contribute to the restriction of circulation in non-vital capillary beds when an organism is under stress. The flexibility of erythrocytes is critical to their circulatory efficiency since they must pass through capillaries smaller than their diameter. Under generalized adrenergic stimulation, capillary walls in liver and skeletal muscle dilate, while capillaries in other organs contract and reduce blood flow. A small decrease in cell flexibility should contribute significantly to diminishing blood flow in restricted capillary beds, while contraction of a protein network could well strengthen the membrane to prevent cell lysis induced by increased stress.

It is also possible that the observed phenomena have a role in metabolic regulation. The energy expenditure of the erythrocyte is determined in part by the activity of cation-dependent ATPase pumps, which are regulated by ion concentrations on both sides of the membrane. Moreover, the contractile process apparent from the present findings may require ATP hydrolysis, as do other contractile protein dependent phenomena. The excitation process may also be mediated by changes in cyclic nucleotide levels, as appears to be true in adrenaline-treated cardiac muscle (17), which response would have varied metabolic consequences. The effect of the neurotransmitters on glucose transport is another problem under investigation.

These metabolic consequences, if any, may be peripheral to the contractile process, or they may be a central feature of the mechanism. The question of their involvement is a fundamental problem in muscle physiology and one for which studies of the mammalian erythrocyte, as a simple and available model system, may be of unique value.

ACKNOWLEDGEMENTS

This research has been sponsored by the National Science Foundation (Grant GB 33501X1). W.H.H. is a postdoctoral fellow of the National

Institutes of Health. We thank Dr. C. Harbury of the Hematology Department, Stanford Medical School, for supplying the cells used in this research.

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