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PRELIMINARY CHARACTERIZATION OF THE ACETYLCHOLINE RECEPTOR IN HUMAN ERYTHROCYTES

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The response of human erythrocytes to cholinergic ligands was studied with an electron spin resonance assay. The membrane response to carbamyl choline was found to be antagonized by atropine and, in the absence of calcium, by tetrodotoxin. Experiments with resealed ghosts showed that the membrane response to carbamyl choline required ATP and calcium. Reductive alkylation of intact cells eliminated the cholinergic response, but the presence of saturating amounts of carbamyl choline protected the putative receptor against inactivation. Affinity labeling was used to demonstrate an apparent molecular weight of 41,000 for the carbamyl choline-binding species. A lipid vesicle extraction technique was used to induce a specific cation permeability defect in intact cells. Preliminary investigation of this phenomenon is described.

INTRODUCTION

Cholinergic and adrenergic neurotransmitter responses have been observed in a variety of cells with no conventional communicative or contractile function (1–6). The impact of these activities on physical properties and metabolic processes of the cells has not yet been fully explored for any system, but is receiving increasing attention as a possible regulatory mechanism involved in diverse processes. The physiological significance aside, the presence of these receptor systems in small, discrete, easily manipulated cells such as erythrocytes permits use of some novel experimental approaches to study their biochemical characteristics. We describe here spectrometric and biochemical studies of the cholinergic response in human erythrocytes.

METHODS AND MATERIALS

Human erythrocytes were obtained from adult volunteers and used within 2 days of withdrawal. Electron spin resonance (ESR) experiments on washed cells spin-labeled with an N-oxyl-4′, 4′-dimethyl oxazolidine derivative of 5-keto palmitic acid were conducted as described previously (6). Resealed ghosts were prepared by a modification of published methods (7) and otherwise treated as intact cells for ESR. The ATP and/or $CaCl_2$ were omitted in some experiments, as described under Results. The ghosts were then washed

Abbreviations used: CbCh, carbamyl choline; TTX, tetrodotoxin; cytB, cytochalasin B; AChE, acetylcholinesterase; AChR, acetylcholine receptor

in the phosphate-buffered saline used in all other experiments. The resealing medium (i.e. the internal medium) of the ghosts contained 4.4 mM NaCl, 56 mM KCl, 0.75 mM Na_2HPO_4, 0.22 mM NaH_2PO_4, 0.05 mM ATP, 0.22 mM $MgCl_2$, and 0.066 mM $CaCl_2$.

Intact cells were reductively alkylated by published procedures (8), in both presence and absence of 10^{-6} M carbamyl choline or atropine. Atropine-protected cells were reductively alkylated a second time (in the absence of atropine) with ^{14}C-N-ethyl maleimide. The radioactively labeled cells were lysed and the washed stroma were subjected to SDS-polyacrylamide gel electrophoresis in 0.1 M phosphate, pH 7, and 1% SDS (other conditions as described in published procedures [9]). The gels were sliced into 1 mm segments, prepared by peroxide oxidation (10), and counted in a Packard liquid scintillation counter.

Phospholipid vesicles were prepared by sonication of dimyristol or dipalmitoyl lecithin in phosphate-buffered saline solution, pH 7.4. Erythrocytes were incubated with vesicles at 37°C. Phospholipid exchange and small molecule and ion uptake by cells were determined by radioactive tracers. Acetylcholinesterase and Na^+ -K^+ ATPase activities were measured by published procedures (11, 12). Protein extracts of erythrocytes were prepared by two other techniques: (1) incubation of cells in 1 M NaCl for 15 min at 25°C, and (2) incubation of cells in 0.2 M urea for 16 hr at 4°C. These extracts were dialyzed against distilled water and lyophilized, then added to phospholipid vesicle suspensions.

RESULTS

ESR Studies of Erythrocyte Response

The use of spin-labeled fatty acids to detect changes in lipid bilayer fluidity has been described for a number of natural and synthetic membranes (13–17). Human erythrocyte membranes labeled in this way were observed to become less fluid when the intact cells were treated with carbamyl choline. Figure 1 shows the effect of CbCh concenteration on the order parameter* of a 3,10 fatty acid spin label (N-oxyl-4′, 4′-dimethyl oxazolidine derivative of 5-keto palmitic acid) in erythrocyte membranes. Increasing values of S indicate increased rigidity of the membrane. Thus, CbCh produces an increase in membrane rigidity which is at a maximum at 10^{-6} M CbCh, and which is less at higher and lower concentrations. If the cells are treated first with d-tubocurarine before the addition of CbCh, the S increase is almost as great as in the control (Table I); however, prior treatment of the cells with atropine diminishes the CbCh effect to a greater extent.

Addition of the neurotoxin tetrodotoxin (TTX) to the cells also reduces the CbCh response substantially, but only in the presence of a calcium-chelating agent such as EGTA. Tetrodotoxin alone, or EGTA alone, does not block the CbCh effect, but both agents together reduce the response significantly (Table I).

Treatment of intact erythrocytes with cytochalasin B, (cyt B), a fungal metabolite which disrupts microfilament structures and interferes with a variety of cytological proc-

*The order parameter S is a quantitative measure of membrane flexibility (13, 14). It is determined experimentally from the peak positions of the ESR spectrum. The peak positions are a sensitive function of the anisotropic motion of the nitroxide group on the hydrocarbon chain.

esses mediated by contractile proteins (18–21), results in a small decrease in membrane rigidity (Table I), and subsequent addition of CbCh has little or no additional effect on S.

The effect of CbCh on membrane rigidity in resealed ghosts is shown in Table II. Ghosts prepared with normal cation gradients and supplied with internal ATP responded to CbCh treatment by an increase in S smaller than that observed in intact cells. As for whole cells, the ghost response could be blocked if TTX and EGTA were first added to the external solution. Moreover, ghosts prepared with no internal ATP showed no S change on CbCh treatment. Ghosts containing no internal calcium could not respond to CbCh either, unless 0.1 mM calcium was added to the external medium.

Table III shows the effect of reductive alkylation on intact cell response to CbCh. Cells treated first with dithiothreitol and then with N-ethylmaleimide (NEM) showed no response to CbCh. Cells treated identically but in the continuous presence of 10^{-6} M CbCh to protect the binding site, responded normally to CbCh after all reagents and protective species had been removed by washing.

The Molecular Weight of the Species Protected by CbCh

The result of an "affinity label" determination of the CbCh-binding species molecular weight is shown in Fig. 2. Cells protected during reductive alkylation with ^{12}C-NEM by 10^{-6} M atropine were washed thoroughly, then reduced again, and alkylated with ^{14}C-NEM. A second sample of cells was treated identically, except that no protecting agent was present during the first reduction and alkylation. Both sets of cells were lysed osmotically and the membranes were washed with distilled water. Aliquots of these membranes were subjected to electrophoresis in 0.1% SDS and β-mercaptoethanol on 10% polyacrylamide gels. The gels were sliced and counted. As shown in Fig. 2, the atropine-protected cells contained a radioactively labeled species at a position corresponding to ~ 41,000 daltons. No such radioactive labeling was observed in nonprotected cells.

Induction of Specific Sodium Leaks in Erythrocytes

Erythrocytes treated with suspensions of sonicated phospholipid vesicles undergo exchange of phospholipids with the vesicles. Figure 3 shows the time course of lipid uptake into red cells which were incubated with dipalmitoyl lecithin vesicles labeled with ^{14}C-lecithin. (Subsequent incubation of the ^{14}C-labeled cells with unlabeled vesicles results in equilibration of label between cell and vesicle fractions, indicating that exchange occurs.) The lipid-treated cells are eventually destroyed by this exposure to vesicles. Figure 3 also shows the time course of cell lysis, which develops somewhat later than the rapid lipid exchange.

That this cell lysis results from specific damage of small numbers of membrane constituents, rather than from a gross general alteration of lipid phase properties, is suggested by the following evidence. (1) Lipid treatment of erythrocytes in 50 mM sucrose (a nonpermeant species in sufficient concentration to compensate for the osmotic potential of hemoglobin) results in normal lipid exchange, but greatly diminished hemolysis. This is evidence that the cells are destroyed by osmotic lysis, rather than by direct disruption of the membrane by the foreign lipid. (2) The permeability of lipid-treated cells to many small molecules (^{14}C-sucrose, $^{86}Rb^{+}$, ^{14}C-choline, organic phosphates) is normal or even lower than normal. This shows that the osmotic lysis is not induced by a general small

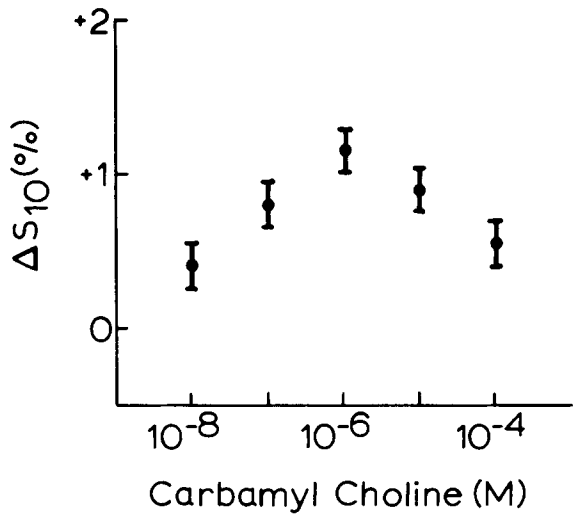

Fig. 1. Effect of CbCh on the order parameter S of the erythrocyte membrane, as a function of CbCh concentration. The value of S in resting cells at 37°C was 0.6550.

TABLE I. Effect of CbCh, Cholinergic Antagonists and TTX on the Order Parameter of Spin-Labeled Human Erythrocytes

Condition	% change in S
Control cells	0
Cells + 10^{-6} M CbCh	+ 1.6 ± 0.3
Cells + 10^{-5} M Curare	
+ 10^{-6} M CbCh	+ 1.3 ± 0.3
Cells + 10^{-6} M Atropine	+ 0.1 ± 0.3
+ 10^{-5} M CbCh	+ 0.5 ± 0.3
Cells + 10^{-8} M TTX	+ 1.1 ± 0.3
Cells + 10^{-8} M TTX	
+ 10^{-6} M CbCh	+ 1.6 ± 0.3
Cells + 10^{-4} M EGTA	− 0.6 ± 0.3
Cells + 10^{-4} M EGTA	
+ 10^{-6} M CbCh	+ 0.7 ± 0.3; $\lvert\Delta S\rvert$ = +1.3
Cells + 10^{-4} M EGTA	
+ 10^{-8} M TTX	
+ 10^{-6} M CbCh	− 0.2 ± 0.3; $\lvert\Delta S\rvert$ = +0.4

molecule permeability increase. The only small molecule tested that showed increased uptake into lipid-treated cells was $^{22}Na^+$, and for this ion the increase in permeability was dramatic. (3) Addition of tetrodotoxin (TTX) to lipid-treated cells produced a pronounced decrease in both $^{22}Na^+$ uptake and hemolysis. This effect (Fig. 4) was observed in a concentration range of TTX similar to the concentration required for neurotoxic activity, ca. 10^{-8} M. The ability of small numbers ($\sim 10^4$/cell) of TTX molecules to diminish $^{22}Na^+$ uptake and hemolysis suggests that the $^{22}Na^+$ flux involves a "sodium channel" pharmacologically similar to the channels in nerves. (4) Uptake of $^{22}Na^+$ into lipid-treated cells

TABLE II. Response of Resealed Ghosts to CbCh, With and Without ATP and Ca^{+2}

Condition	% change in S
Control ghosts	0
Ghosts + 10^{-6} M CbCh	+ 0.7 ± 0.3
Ghosts –no internal ATP	– 1.0 ± 0.3
Ghosts –no internal ATP + 10^{-6} M CbCh	– 1.3 ± 0.3
Ghosts –no internal Ca^{+2}	– 0.9 ± 0.3
Ghosts –no internal Ca^{+2} + 10^{-6} M CbCh	– 0.6 ± 0.3
Ghosts –no internal Ca^{+2} + 10^{-4} M external Ca^{+2} + 10^{-6} M CbCh	+ 0.5 ± 0.3

TABLE III. Effect of Reductive Alkylation on CbCh Response

Condition	% change in S
Control cells	0
Cells + 10^{-6} M CbCh	+ 1.4 ± 0.3
Cells, reductively alkylated	+ 0.1 ± 0.3
Cells, reductively alkylated + 10^{-6} M CbCh	+ 0.3 ± 0.3
Cells, protected by CbCh	+ 0.6 ± 0.3
+10^{-6} M CbCh	+ 1.5 ± 0.3

shows a marked increase as the pH is lowered (Fig. 5). In normal cells (Fig. 5) decreased pH decreases $^{22}Na^{+}$ uptake. The pH behavior of $^{22}Na^{+}$ uptake in the defective cells is thus more suggestive of a protein-related defect than of general increase in the lipid phase permeability.

These observations suggest that lipid vesicle treatment of erythrocytes specifically disrupts membrane components involved in control of $^{22}Na^{+}$ influx. The observations which follow are preliminary evidence that the membrane defect arises because one or more essential membrane proteins are removed during lipid vesicle treatment.

It was noted that lipid-treated erythrocytes exhibit decreased levels of acetylcholinesterase (AChE) activity. The lipid vesicles recovered from those experiments were found to contain up to 90% of the missing AChE activity, even after removal of soluble proteins by gel filtration or sucrose density gradient centrifugation. (Nonspecific trapping of soluble protein activity during these separations was less than 2% of total soluble protein, in the same concentration range.) Moreover, when such vesicles were subjected to polyacrylamide gel electrophoresis in SDS and β-mercaptoethanol, five protein bands were observed consistently (Fig. 6). The appearance of these extracted proteins paralleled development of the $^{22}Na^{+}$ leak and the hemolysis.

Of the five protein bands in these vesicle extracts, only the AChE(which would contribute to the band at ~91,000 daltons) has been identified. To examine the effect of added AChE on further AChE extraction and on development of the $^{22}Na^{+}$ leak,

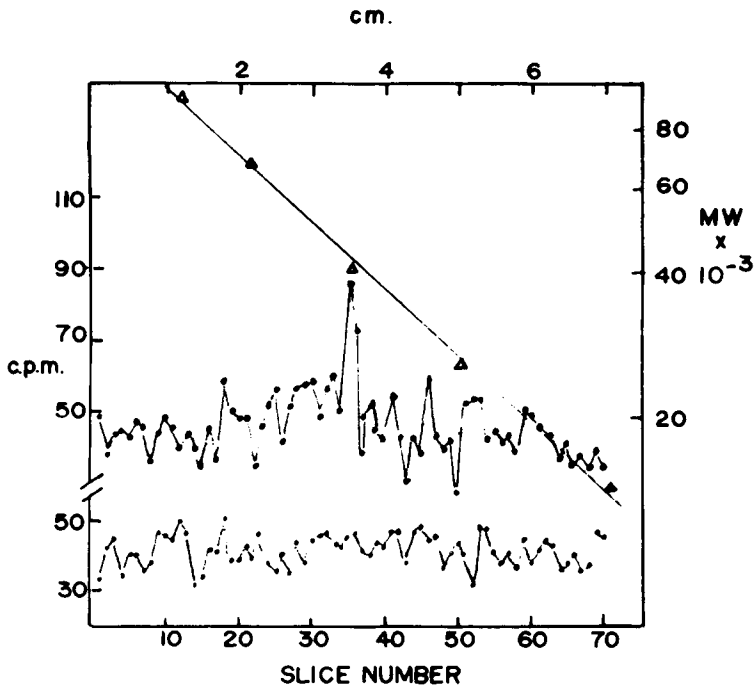

Fig. 2. Localization of ^{14}C-N-ethylmaleimide-labeled protein in a polyacrylamide gel. Upper trace: cpm in cells protected by atropine during preliminary alkylation with ^{12}C-NEM, then alkylated again with ^{14}C-NEM after removal of atropine. Lower trace: cpm in control cells not protected by atropine in preliminary alkylation with ^{12}C-NEM. Triangles above both traces indicate positions to which marker proteins migrated under these electrophoresis conditions.

erythrocytes were treated with vesicles previously "loaded" with impure AChE (obtained by 1 M NaCl extraction of erythrocytes). Lipid exchange between "loaded" vesicles and cells was essentially identical to exchange with control vesicles, and loss of AChE from the cells was the same in both cases (Fig. 7). However, the uptake of $^{22}Na^+$ into the cells was significantly reduced in the sample treated with protein-loaded vesicles (Fig. 7). Subsequent experiments showed that addition of commerical erythrocyte AChE, bovine serum albumin, or glycophorin (22) to vesicles did not diminish $^{22}Na^+$ uptake into the erythrocytes. However, a urea extract of intact cells (containing the components shown in Fig. 6) also showed some suppression of the $^{22}Na^+$ uptake in lipid-treated cells. No AChE was present in the urea extract. These observations suggested that (1) the $^{22}Na^+$ leak is not a direct consequence of loss of AChE from the cells, and (2) the mere presence of any protein species in the incubating solution does not suppress the $^{22}Na^+$ leak. The results do not exclude the possibility that another component of the extracted protein mixtures is directly responsible for the development and suppression of the leak. Experiments are now underway to separate the components of the extracted mixtures and to assay their activities in suppression of the sodium leak.

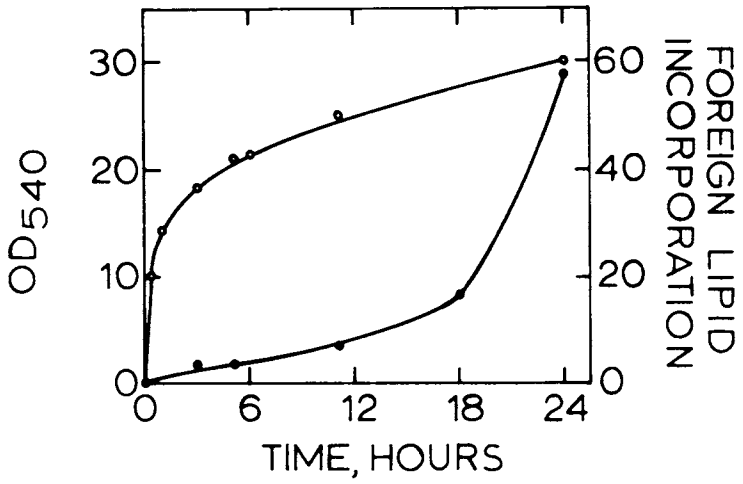

Fig. 3. —○— Incorporation of ^{14}C-lecithin into human erythrocytes from labeled dipalmitoyl lecithin vesicles at 37°C. —●—. Release of hemoglobin into supernatant by cell lysis.

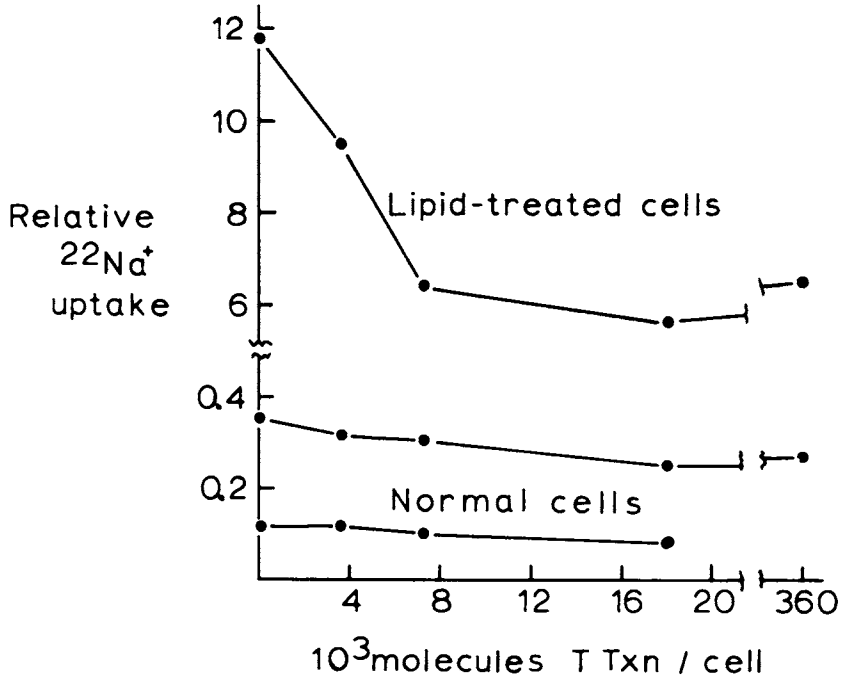

Fig. 4. Effect of TTX on $^{22}Na^{+}$ uptake into lipid-treated and normal cells (two samples of the latter show normal variations between cell samples). A TTX concentration of $\sim 10^{4}$ per cell corresponds to a molar concentration of 10 nM.

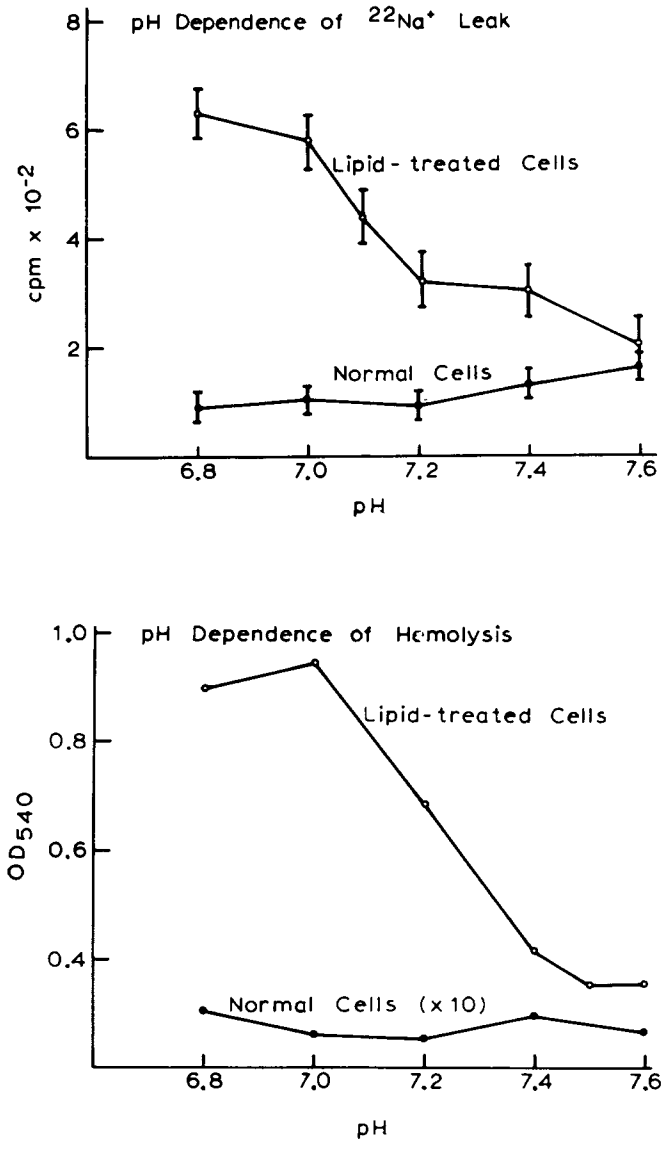

Fig. 5. Effect of pH on $^{22}Na^{+}$ uptake in normal – ○ – and lipid-treated – ● – cells.

DISCUSSION

An ESR assay has been used to demonstrate and partially characterize the response of human erythrocytes to cholinergic stimulation. The cell response, which can be detected as a change in fluidity of the membrane lipid phase, can be antagonized by the muscarinic blocking agent atropine. This blockage is consistent with the participation of a specific cholinergic receptor species in the cell response. The chemical similarity of this

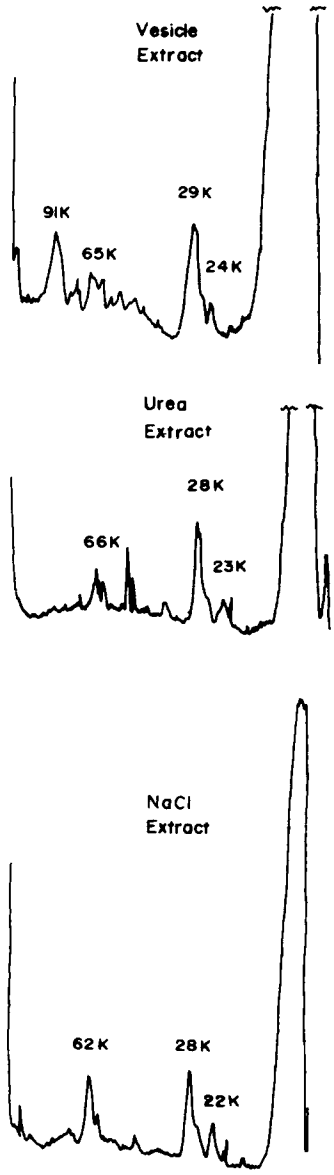

Fig. 6. Densitometer traces of polyacrylamide gels showing protein components extracted from intact erythrocytes by lipid vesicles, 0.1 M urea, and 1 M NaCl.

putative receptor to well-characterized ACh receptors in other excitable tissues is indicated by the observation that reductive alkylation destroys cell response, but that saturating concentrations of CbCh or atropine can protect the cell against this kind of inactivation. An experiment in which radioactive N-ethylmaleimide was used to label previously protected disulfide groups showed that the atropine-protected site is found in a protein of ~41,000 dalton molecular weight. This figure is similar to that found by Karlin and coworkers for the AChR from Electrophorus electricus tissue (8).

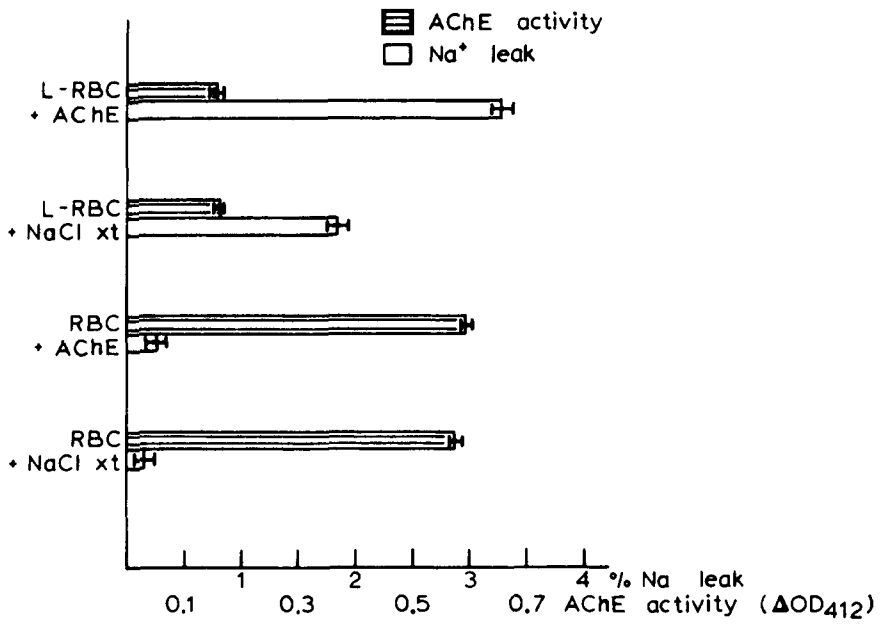

Fig. 7. Effect of protein-loaded vesicles (the NaCl extract, in this case) on $^{22}Na^{+}$ uptake and AChE loss of lipid-treated erythrocytes.

The erythrocyte response to CbCh also appears to involve ion fluxes of Na^{+} and/or Ca^{+2}, since, in the absence of extracellular Ca^{+2}, TTX prevents the response. In other excitable systems the particular cation channels most closely associated with AChR (for example, those which are involved in miniature end plate potentials and in initiation of the action potential) are not sensitive to TTX. At present, we have no evidence for or against the presence of non-TTX-sensitive channels in erythrocytes. The most reasonable inference to be drawn from our observations is that TTX-sensitive channels are required for the response we measure, whether or not a pharmacologically distinct class of channels is involved at some stage of the excitation. The fact that extracellular Ca^{+2} renders the cells excitable even in the presence of TTX may mean that (a) Ca^{+2} alters the TTX-ion channel association, or (b) Ca^{+2} influx occurs upon cholinergic stimulation in quantities adequate to supplement or substitute for the Na^{+} flux. The latter possibility seems more likely, since (a) "calcium-less" ghosts (which do not respond to CbCh even in the absence of TTX) can be rendered responsive by addition of extracellular Ca^{+2}, and (b) there is precedent in smooth muscle systems for part or all of the cation influx during excitation to be due to Ca^{+2} (23).

In these chemical and pharmacological details, the response of erythrocytes to cholinergic stimulation resembles well-characterized neuromuscular responses. The detailed molecular mechanism of erythrocyte response is not known; indeed, the nature of the subtle membrane stiffening detected by our ESR technique is not understood at present. Current investigations are aimed at eventual understanding of these questions.

The use of phospholipid vesicles to remove membrane proteins, resulting in specific disruption of sodium channel function, is described. Analysis of this phenomenon is still in preliminary stages, but the results reported here suggest that these experiments may yield new information on molecular species involved in regulation of ion flux in excitable membranes.

REFERENCES

1. Allen, J. E., and Rasmussen, H., Science 174:512 (1971).
2. Levitsky, A., Atlas, D., and Steer, M. L., Proc. Natl. Acad. Sci. U.S.A. 71:2773–2776 (1974).
3. Lefkowitz, R. J., Mukherjie, C., Coverstone, M., and Caron, M. G., Biochem. Biophys. Res. Commun. 60:703 (1974).
4. Strom, T. B., Sytkowski, A. M., Carpenter, C. B., and Merrill, J. P., Proc. Natl. Acad. Sci. U.S.A. 71:1330 (1974).
5. Yamashita, K., and Field, J. B., J. Biol. Chem. 247:7062 (1972).
6. Huestis, W. H., and McConnell, H. M., Biochem. Biophys. Res. Commun. 56:578 (1974).
7. Humphries, G. K., and McConnell, H. M., Proc. Natl. Acad. Sci. U.S.A. 71:1691 (1974).
8. Karlin, A., and Cowburn, D. A., Proc. Natl. Acad. Sci. U.S.A. 70:3636 (1973).
9. Weber, K., and Osborn, M., J. Biol. Chem. 244:4406 (1969).
10. Bakay, B., Anal. Biochem. 40:429 (1971).
11. Ellman, G. L., Courtney, K. D., Andres, V., Jr., and Featherstone, R. M., Biochem. Pharmacol. 7:88 (1961).
12. Bouting, S. L., Arch. Biochem. Biophys. 95:416 (1961).
13. Hubbell, W. L., and McConnell, H. M., J. Amer. Chem. Soc. 93:314 (1971).
14. McConnell, H. M., Wright, K. L., and McFarland, B. G., Biochem. Biophys. Res. Commun. 47:273 (1972).
15. Trudell, J. R., Hubbell, W. L., and Cohen, E. N., Biochem. Biophys. Acta 291:321 (1973).
16. Kury, P. G., Ramwell, P. W., and McConnell, H. M., Biochem. Biophys. Res. Commun. 47:273 (1974).
17. Dehlinger, P. J., Jost, P. C., and Griffith, O. H., Proc. Natl. Acad. Sci. U.S.A. 71:2280 (1974).
18. Carter, S. B., Nature 213:261 (1967).
19. Allison, A. C., Davies, P., and DePetris, S., Nat. New Biol. 232:153 (1971).
20. Wessells, N. K., Spooner, B. S., Ash, J., Ludena, M. A., and Wrenn, J., Science 173:358 (1971).
21. Schofield, J. G., Nat. New Biol. 234:215 (1971).
22. Marchesi, V. T., Tillack, T. W., Jackson, R. L., Segrest, J. P., and Scott, R. E., Proc. Natl. Acad. Sci. U.S.A. 69:1445 (1972).
23. Quastel, D. M. J., and Hackett, J. T., in "The Structure and Function of Muscle," G. H. Bourne (Ed.). Academic Press, New York, pp. 116–120 (1973).