Selectivity of Cations and Nonelectrolytes for Acetylcholine-Activated Channels in Cultured Muscle Cells

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ABSTRACT The selectivity of acetylcholine (ACh)-activated channels for alkali cations, organic cations, and nonelectrolytes in cultured muscle cells has been studied. To test the effect of size, charge, and hydrogen-binding capacity of permeant molecules on their permeability, we have obtained the selectivity sequences of alkali cations, compared the permeability of pairs of permeant molecules with similar size and shape but differing in charge, and studied the permeability of amines of different hydrogen bonding capacity. ACh-activated channels transport alkali cations of small hydration radii and high mobility. The molecules with positive charge and (or) a hydrogen-bond donating moiety are more permeable than the ones without. On the other hand, several nonelectrolytes, i.e., ethylene glycol, formamide, and urea, do have a small, but measurable, permeability through the channels. These results are consistent with a model that ACh-activated channel is a water-filled pore containing dipoles or hydrogen bond accepting groups and a negative charged site with a pK of 4.8.

INTRODUCTION

The binding of acetylcholine (ACh) to its receptor in the muscle endplate induces an increase in the permeability of the membrane to small ions (Fatt and Katz, 1951). From tracer-flux studies in denervated rat diaphragm muscle, Jenkinson and Nicholls (1961) observed that ACh increases both sodium and potassium permeability. Studying the reversal potential at frog neuromuscular junction by voltage clamp technique, Takeuchi and Takeuchi (1960) and Takeuchi (1963) found that E_{rev} varies significantly with changes of external concentration of sodium and potassium ions, whereas chloride ions have no effect on E_{rev}. Recent evidence obtained from the studies of equilibrium potential (Kordas, 1969), the observation of monotonic decay of endplate current (Magleby and Stevens, 1972), and the measurement of end plate current variance from noise analysis (Dionne and Ruff, 1977) strongly suggests that both

sodium and potassium ions use one channel for their permeation.

Besides sodium and potassium ions, various organic cations are also permeable to the ACh-activated channel (Furukawa and Furukawa, 1959; Koketsu and Nishi, 1959; Maeno et al., 1977; Guy et al., 1977). ACh-activated channels are much less selective than the Na^+ channels responsible for the action potential in nerve and muscle. Large organic cations such as biguanide and bis (2-hydroxyethyl)dimethylammonium can pass through the ACh-activated channels. The single ACh-activated channel conductance derived from fluctuation analysis is approximately 25 pS (Anderson and Stevens, 1973; Colquhoun et al., 1975; Neher and Sakmann, 1976).

Muscle cells cultured in vitro form multinucleate myotubes which have a high density of acetylcholine receptors distributed over the entire cell surface (Fischbach, 1972; Vogel et al., 1972). These cells respond to acetylcholine or other cholinergic agonists with a large increase in permeability to small cations that can be measured electrophysiologically (Fischbach, 1972) or by isotopic flux methods (Catterall, 1975). Like Ach-activated channels at the endplate, Ach-activated channels in cultured muscle cells are permeable to both Na^+ and K^+ (Ritchie and Fambrough, 1975). Their single-channel conductance is 31–39 pS at 25°C (Sachs and Lecar, 1977; Lass and Fischbach, 1976).

To understand the factors important for the postsynaptic membrane selectivity, we have studied the effect of charge of the transported molecules on the rate of their permeation. The specific questions considered here are whether the charge on the molecules plays an essential role in their permeability and whether the hydrogen binding ability of test molecules affects their permeation. We have approached these questions by studying different series of compounds which differ in size, charge, or chemical properties. The selectivity ratios for both cations and nonelectrolytes were determined by isotopic flux methods in cultured muscle cells, using a modification of the methods described previously (Catterall, 1975).

MATERIALS AND METHODS

Materials

The chemicals and media used were purchased from the following sources: Dulbecco-Vogt modification of Eagle's minimal essential medium (DMEM) and Dulbecco's phosphate-buffered saline from the Media Unit of National Institutes of Health, horse serum from Microbiological Associates, Walkersville, Md.; fetal calf serum from Grand Island Biological Co., Grand Island, N. Y.; recrystallized trypsin from Worthington Biochemical Corp., Freehold, N. J.; carbamylcholine, D-arabinofuranosylcytosine, ouabain, and *d*-tubocurarine from Sigma Chemical Co., Saint Louis, Mo.; calf tail collagen from Calbiochem, San Diego, Calf.; $^{22}NaCl$, [1,2-^{14}C]ethanolamine, [1,2-^{14}C]ethylene glycol, [1,3-^{14}C]glycerol, [1-^{14}C]-mannitol, [^{14}C]urea, [^{14}C]methylamine, and [1-^{14}C]ethylamine from New England Nuclear, Boston, Mass.; labelled [^{14}C]Tris and [U-^{14}C]ethylenediamine from Amersham/Searle Corp., Arlington Heights, Ill.; and [^{14}C]guandine, [^{14}C]formamide, and [1-^{14}C]acetamide from ICN Corp., Irvine, CA. Chick embryo extract was prepared as described by Cahn et al. (1967). α-Bungarotoxin was purified from the venom of *Bungarus multicinctus* (Miami Serpentarium, Miami, Fla.) according to the method of Mebs et al. (1971).

Muscle Cultures

Suspension of single muscle cells from thigh muscle of 10 or 11-day-old chick embryos was prepared as described by Fischbach (1972). Cells were then seeded at a density of 75,000 cells/cm^2 in collagen coated multi-well plates (16-mm diameter, Costar Co. Mass.). The growth medium contained 91% DMEM, 2% fetal calf serum, 5% horse serum, 2% chick embryo extract, 50 U/ml penicillin, and 10 μg/ml streptomycin. The cells were grown in a humidified atmosphere of 10% CO_2-90% air, and the growth medium was changed every 2–3 days. 10 μM D-arabinofuranosylcytosine was added in the medium on day 3 or day 4 to reduce the number of fibroblasts in the culture (Fischbach, 1972).[^{3}H]leucine (0.2 μCi/ml) was added with growth medium 24 h before the experiment to allow the protein recovery to be measured from the ^{3}H counts per minute.

Measurement of Influx Rates

Cell cultures were removed from the incubator and the growth medium was replaced with a medium consisting of 135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM glucose, and 50 mM HEPES (adjusted to pH 7.4 with Tris base). Cells were equilibrated with this buffer medium at 36°C (15–30 min), and assays for influx were then carried out. In some experiments the acetylcholine receptors of the muscle cells were inhibited by incubation with 10 nM α-bungarotoxin in the same medium for 60 min at 37°C before assay. This treatment is sufficient to inhibit all ACh receptors (Catterall, 1975).

Influx rates were measured at 2°C, because desensitization of the receptor is slower at low temperature (Catterall, 1975). Cell cultures were transferred to an ice bath. Isotopic influx measurements were initiated by addition of assay medium consisting of 142.5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM glucose, and a buffer mixture of 10 mM *N*-hydroxyethylpiperazine-*N'*-ethane sulfonic acid (HEPES, $pK_a = 7.5$), 10 mM cyclohexylaminopropane sulfonic acid (CAPS, $pK_a = 10.4$), 10 mM 2(*N*-Morpholino) ethane sulfonic acid (MES, $pK_a = 6.2$), 10 mM Tris (hydroxymethyl)methylaminopropane sulfonic acid (TAPS, $pK_a = 8.4$), and 10 mM glycylglycine ($pK_{a_1} = 3.1$, $pK_{a_2} = 8.1$). 5 mM ouabain was added to inhibit active extrusion of Na^+ (Catterall, 1975). The assay media contained test permeant molecules at concentrations from 2 to 1 mM (5 μCi/ml) as indicated in the figure legends. Carbamylcholine, at a final concentration of 10 mM, was included in the medium to activate ACh receptors in the muscle cells. The mixture of buffers used was chosen to provide adequate buffer capacity over the range from pH 4 to pH 11. The desired pH was obtained by titration with KOH. Uptake measurements were terminated after 15-s incubation at 2°C by aspirating the assay medium and washing the cells four times at 2°C with 3 ml of wash medium consisting of 142.5 mM KCl, 10 mM choline Cl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM HEPES (adjusted to pH 7.4 with Tris base), and 1 mM *d*-tubocurarine to inhibit the activation of ACh-activated channels. Cells were suspended in 0.6 ml of 0.4N NaOH or 1% sodium lauryl sulphate and transferred to scintillation vials containing 1 ml 1 M Tris HCl, pH 7.4, and 10 ml scintillation fluid consisting of 5.5% (vol/vol) RPI scintillation cocktail, 61.4% toluene, and 33.1% Triton X-100. Protein concentration was determined by the method of Lowry et al. (1951) on representative cultures and the protein content of the remaining samples was calculated from the recovered [^{3}H]leucine counts per minute. The results are expressed as nanomoles of permeant compound influx per minute per milligram cell protein.

In each experiment, the rate of influx of each permeant compound in cells not treated with carbamylcholine or in cells treated with α-bungarotoxin plus carbamylcholine was determined and subtracted from the rate of influx in cells treated with carbamylcholine alone. This difference represents the increase in permeability caused by activation of the acetycholine receptors (Catterall, 1975).

RESULTS

Selectivity for Alkali Metal Cations

Previous results showed (Catterall, 1975) that carbamylcholine and other cholinergic agonists cause a 20-fold increase in initial rates of $^{22}Na^+$ uptake into cultured muscle cells which is blocked specifically by nicotinic acetylcholine receptor antagonists. Those results demonstrated the feasibility of using ion flux procedures to measure changes in permeability of cultured muscle cells due to activation of nicotinic acetylcholine receptors. The flux depends on external ion concentration, membrane potential, and permeability. In these experiments, we have modified the conditions by using a medium with potassium concentration (142.5 mM K^+) identical to the intracellular K^+ concentration in these cells (Catterall, 1975; Ritchie and Fambrough, 1976). Under these conditions, the membrane potential is zero and the large increase in Na^+ and K^+ permeability caused by cholinergic agonists has no effect on membrane potential. Thus, according to the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949), the measured flux (J_i) is simply proportional to the concentration of the permeant cation and the permeability (P_i). Permeabilities were determined by measuring the initial rate of influx of permeant ions or nonelectrolytes at six different concentrations and calculating P_i from the slope of the plot of influx vs. concentration. $^{22}Na^+$ influx was measured in each experiment and the data are presented as the ratio (R) of the permeability of the test cation or nonelectrolyte to that of Na^+. In all cases the rate of influx of the test cations and nonelectrolytes in the absence of carbamylcholine has been subtracted from the data.

The rate of densensitization of ACh receptors depends on carbamylcholine concentration as well as cations in the assay medium (Nastuk and Parsons, 1970; Manthey, 1972). To test whether the estimated permeability is affected by the densensitization of the ACh receptor, we preincubated the cells in medium containing various test molecules and 10 mM or 1 mM carbamylcholine for various times, and then measured the rate of $^{22}Na^+$ uptake. The $^{22}Na^+$ uptake remained constant for at least 25 s of preincubation time. Desensitization does not obscure the permeability measurement with the experimental procedures employed.

An experiment illustrating results for the alkali metal cations Na^+, K^+, Rb^+, and Cs^+ is presented in Fig. 1. In each case the dependence of flux on concentration is linear verifying that the membrane potential remains constant and that flux coupling can be ignored. To study the permeability of K^+ at low ionic concentration, and relatively constant membrane potential, the uptake of alkali cation in some experiments is measured in medium containing 285 mM sucrose instead of KCl (Fig. 1). Replacement of KCl by sucrose changes the membrane potential of cells during the assay. Inasmuch as the membrane potential of cells is no longer maintained at 0 mV in this case, uptake of test molecule vs. its concentration depends not only on permeability but also on the membrane potential. When the concentration of the test ions is below 12 mM, the change in membrane potential caused by replacing KCl by sucrose is about the same for all test ions. Therefore, even though the individual permeabilities

might be affected by the change in membrane potential, the permeability ratio $R = P_x/P_{Na}$ should not be affected. We have tested this assumption by repeating the same kind of experiments in media containing either high K^+ or high Na^+. The results for R obtained under these various experimental conditions agree within experimental error. The mean experimental $R \pm$ SEM of these ions are given in Table I. The selectivity sequence we observe in 285 mM sucrose is Cs > Rb > K > Na (1.91: 1.52: 1.47:1.00). The same selectivity sequence for the alkali ions is obtained in medium containing 142.5 mM KCl. The experiments illustrated below, unless specified, were all done in high K^+ medium.

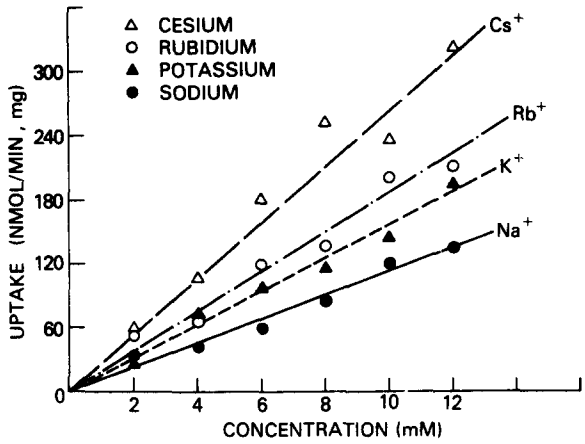


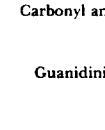
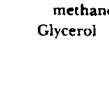
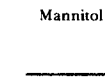
FIGURE 1. Effect of concentration on alkali ion uptake. Two identical sets of muscle cells were incubated for 60 min at 36°C in preincubation medium containing either 0 or 10 nM α-bungarotoxin. The cells were transferred to an ice bath and rinsed to remove excess Na^+. The uptake of Na^+, K^+, Rb^+, and Cs^+ in this specific experiment was measured for 15 s at 2°C in assay medium containing 285 M sucrose, instead of KCl, and various indicated concentrations of alkali cations. The uptake of alkali ion in the presence of α-bungarotoxin was then subtracted from uptake data obtained from cultures without toxin treatment.

The Effect of pH

As part of our study of the effect of charge on permeability, we planned to study charged and uncharged species of the same permeant molecules by altering pH. In preparation for those experiments, we studied the effect of pH on the acetylcholine receptor and the ACh channel using Na^+ as the permeant ion. We find that the Na^+ permeability increase due to carbamylcholine is markedly reduced as the pH is reduced below 5.5, but is little affected by increasing pH above 7 (Fig. 2). At pH 3.0, the permeability increase is completely blocked. The data are fit well by a theoretical dissociation curve with a pK of 4.8 (Fig. 2). The inhibition is reversible at all pH's studied. The rate of Na^+ influx in the absence of agonist or in the presence of both agonist and α-bungarotoxin is unaffected by pH. These results indicate that external protons

TABLE I

PERMEABILITY RATIO OF ALKALI IONS AND ORGANIC MOLECULES

Transporting molecules	Molecular formula	Molecular dimension*	pK_a	$R = \frac{\text{permeability of test molecules}}{\text{permeability of } Na^+}$	
				Uncharged form	Charged form
		Å		(n)	(n)
Alkali ion					
Sodium	Na^+	$r = 0.90$			1.0
Potassium	K^+	$r = 1.33$			1.47 ± 0.26 (5)‡
Rubidium	Rb^+	$r = 1.48$			1.52 ± 0.41 (6)
Cesium	Cs^+	$r = 1.69$			1.91 ± 0.65 (7)
Calcium	Ca^{++}	$r = 0.99$			0.22 ± 0.08 (7)
One- or two-carbon compound					
Ethanolamine	$NH_2—(CH_2)_2—OH$	3.84 × 4.11 × 7.93	9.5	≈0	0.72 ± 0.17 (9)
Ethylenediamine	$NH_2—(CH_2)_2—NH_2$	3.84 × 4.11 × 6.82	9.98, 7.52	≈0	0.63 ± 0.20 (15)
					0.57 ± 0.14§ (5)
Ethylamine	$NH_2—(CH_2)_2—H$	3.84 × 4.11 × 5.98	10.63	≈0	0.45 ± 0.16 (7)
Methylamine	$NH_2—CH_3$	3.79 × 3.87 × 4.32	10.62	≈0	0.82 ± 0.30 (6)
Ethylene glycol	$OH—(CH_2—OH$	3.84 × 4.11 × 6.50	14.77	0.19 ± 0.05 (9)‡	
Carbonyl and related compound					
Guanidinium		3.00 × 5.12 × 5.49			0.92 ± 0.21 (3)
Formamide		3.00 × 4.38 × 5.35		0.17 ± 0.05 (4)	
Urea		3.00 × 4.97 × 5.34		0.12 ± 0.05 (4)	
Acetamide		3.76 × 5.12 × 5.30		≈0	
Thiourea		3.77 × 5.77 × 5.90		0.04 ± 0.016 (5)	
Other compounds					
Tris (hydroxy-methyl) amino methane	$(CH_2—OH)_3—C—NH_3^+$	6.03 × 6.89 × 7.71	8.1	≈0	0.11 ± 0.04 (4)
Glycerol		4.69 × 5.99 × 6.90		0.05 ± 0.02 (3)	
Mannitol		7.38 × 8.11 × 11.92		≈0	

* r – radius of the crystal size of alkali ion. Molecular dimensions of organic molecules were estimated from CPK model.

‡ Number in parentheses represents the number of experiments done for the specific molecules.

§ Doubly-charged form.

specifically inhibit the transport of Na^+ through ACh-activated channels in tissue cultured muscle cells.

There are at least two interpretations of this observation. (*a*) There may be charged group(s) near or in the channels. The ACh-activated channels are blocked by the protonation of the charged group(s). (*b*) Charged group(s) may exist on the acetylcholine receptors. The protonation of these groups prevents opening the ACh-activated channels, perhaps by inactivation of the receptor. To test the possibility that protonation prevents carbamylcholine binding, we have studied the titration curves of the activation by carbamylcholine at pH 4.75 and 7.4 (Fig. 3). The apparent K_D changes very little with pH, whereas the

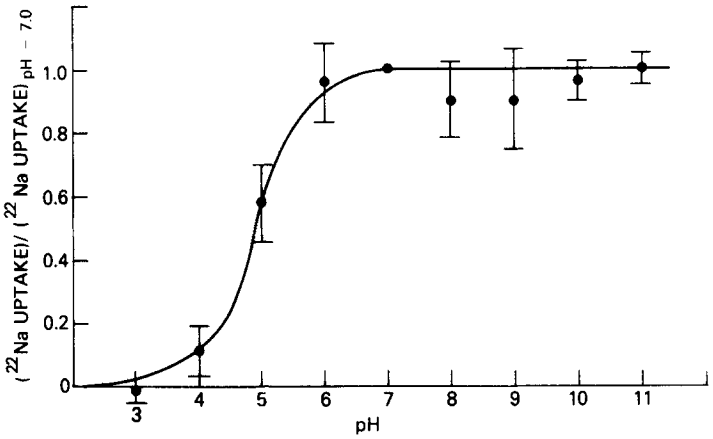


FIGURE 2. Effect of pH on $^{22}Na^+$ uptake. Muscle cells were first incubated in preincubation medium at 36°C for 15 min and then transferred to an ice bath. The preincubation medium subsequently was removed and replaced by a medium containing 142.5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM glucose and good buffers (described in Materials and Methods). Cells were equilibrated in this medium which was buffered at the indicated pH, for 2–3 min. The initial rate of Na^+ uptake is measured at the same pH in the assay medium for 15 s. Each point represents the average of 7–12 separate measurements. The vertical bars indicate the experimental variations. Solid line is the least-squares fit to the theoretical dissociation equation with a pK at 4.8.

maximum influx rate is lower at pH 4.75. Thus, the inhibition by hydrogen ions is noncompetitive with respect to carbamylcholine binding, indicating hydrogen ions bind at a site separate from the carbamylcholine binding site. The protonation site could be located elsewhere on the receptor or within the channel itself.

Selectivity for Organic Cations and Nonelectrolytes

Organic molecules, particularly amines, replacing the sodium in assay medium may have a pharmacological effect on ACh channels. To test this, $^{22}Na^+$ uptake was measured in assay medium containing different concentrations of the organic molecules studied. We found that a low concentration of organic molecules (<20 mM) does not change the sodium uptake. Unless specified, all

data presented below were obtained in the presence of a low concentration of test molecules.

TWO-CARBON COMPOUNDS Three two-carbon amines (ethylamine, ethanolamine, and ethylenediamine) were examined. All these compounds have a pK_a above 9.5 (Table I), 99% of each amine is monovalent at pH 7.4. The uptake of these organic cations is linearly proportional to their external concentration (Fig. 4). The permeability sequence for the monovalent ions is ethanolamine > ethylenediamine > ethylamine.

At pH 11.5, where more than 90% of each of the above compounds is

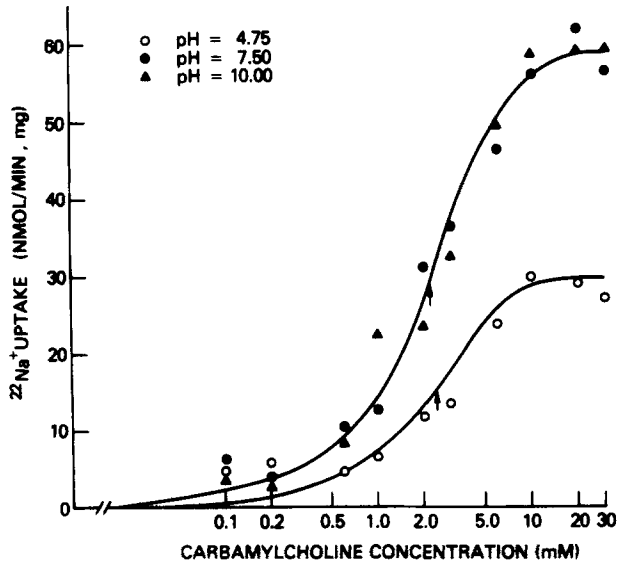


FIGURE 3. Activation of $^{22}Na^+$ uptake by carbamylcholine at different pH. The cells were incubated in preincubation medium for 15 min at 36°C, and were subsequently incubated in buffer medium (content was described previously) of pH 4.75, 7.50, and 10.00 for 3 min at 2°C. $^{22}Na^+$ uptake were then measured at the same pH, 2°C for 15 s in assay medium containing the indicated concentration of carbamylcholine. $^{22}Na^+$ uptake in the absence of carbamylcholine has been subtracted. The arrow (↑) indicates the apparent K_D at the specific pH.

uncharged, the rate of uptake of all the amines drops substantially. The permeability ratio R ($= P_X/P_{Na}$) for ethanolamine, ethylenediamine, and ethylamine is <0.05. Thus, we observe large differences between the permeability of charged and uncharged forms of the molecules demonstrating the importance of electric forces in determining the selectivity of organic molecules in ACh-activated channels.

At pH 6, where most of the ethylenediamine (>95%) is divalent, the ethylenediamine uptake changes very little (Table I). Thus, ethylenediamine with two positive charges has approximately the same permeability ratio as its singly charged species.

Ethylene glycol, an uncharged molecule with a size comparable to ethanolamine has a permeability ratio R of 0.15–0.25 at both pH = 7 and 11. Thus, its

permeability is larger than that of the uncharged forms of all three amines but is smaller than the permeability of monovalent charged amines tested (Table I).

FORMAMIDE AND RELATED COMPOUNDS We studied formamide and four compounds of similar size and shape (urea, thiourea, acetamide, and guanidinium). Each of these molecules is approximately planar due to the sp^2 hybridization of the carbon atom, and the partial double-bond character of the CN bonds. Fig. 5 presents the uptake measurements for this group of compounds. Guanidinium ion, which is positively charged, has a permeability ratio 0.92 and is the most permeable of the organic molecules tested here. The other four compounds are uncharged. The permeability of formamide is much lower than

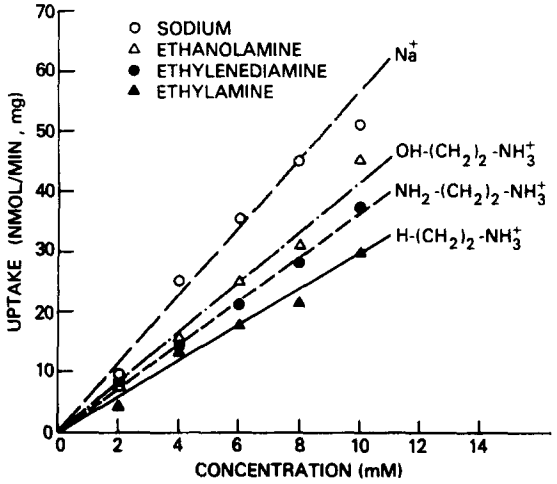


FIGURE 4. Effect on concentration on the uptake of two-carbon amines. Two duplicate sets of cells were incubated for 60 min at 36°C in preincubation medium with or without 10 nM α-bungarotoxin, and washed to remove excess Na^+. Uptake of ^{14}C-labelled ethanolamine, ethylenediamine, ethylamine, and $^{22}Na^+$ were measured for 15 s at 2°C in assay medium containing indicated concentration of test molecules. The difference of the test molecules uptake in the presence and in the absence of α-bungarotoxin was then plotted.

that of guanidinium despite the small molecular size of formamide (Table I). Urea is very similar in size and shape to guanidinium, but is uncharged. The difference in permeability of these molecules is striking. Urea has a low permeability (R = 0.12) in contrast to high permeability for guanidinium. The larger molecules, thiourea and acetamide, are essentially impermeant.

OTHER COMPOUNDS Besides the above compounds, Ca^{++}, methylamine, tris-(hydroxymethyl) aminomethane, glycerol, and mannitol were studied (Table I). Contrary to other findings (Maeno et al., 1977; Ritchie and Fambrough, 1975), Tris is not very permeable in our system (R = 0.11). The permeabilities of glycerol and mannitol are very small.

DISCUSSION

The major objective of this work is to consider the role of charge, hydrogen bonding capacity, and size of permeant cations and nonelectrolytes on their

permeability through ACh-activated channels. To examine each of these properties separately, we have compared: (*a*) the permeability of organic molecules of similar size and shape but different charges, e.g., charged and uncharged ethanolamine; (*b*) the permeability of two-carbon amines with different functional groups, e.g., ethanolamine vs. ethylenediamine vs. ethylamine; and (*c*) the permeability of monovalent cations and nonelectrolytes of different sizes, e.g., alkali cations, two-carbon vs. three-carbon alcohols.

We consider first the role of charge. The permeability of several amines, namely ethylamine, methylamine, ethanolamine, and ethylenediamine were each compared at two different pH values. These amines are positively charged at pH 7.4, and become mostly uncharged when the pH medium is changed to

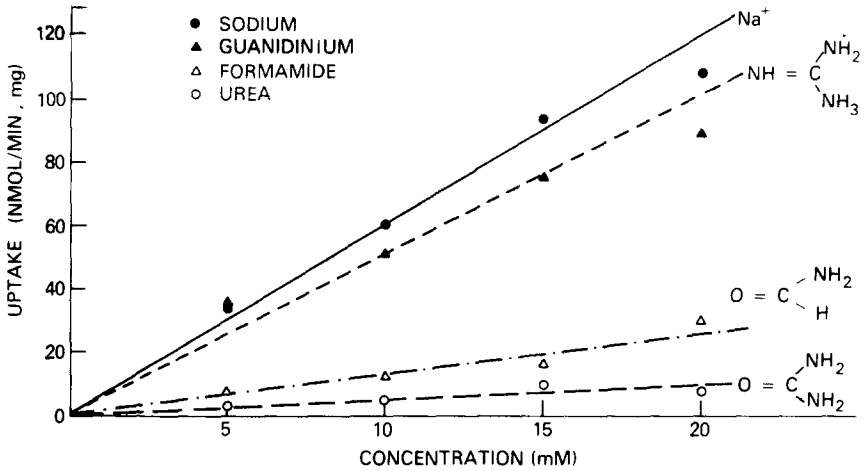


FIGURE 5. Dependence of $^{22}Na^+$, ^{14}C-labelled guanidinium, formamide, and urea uptake on their concentrations. Two sets of cultures were incubated for 60 min at 36°C in preincubation medium containing either 0 or 10 nM α-bungarotoxin. After rinsing the cells to remove excess Na^+, the uptakes were measured for 15 s at 2°C in assay medium containing indicated concentration of the test molecules. The uptake of these test molecules is the difference between cultures treated with and without α-bungarotoxin.

11.5. As shown in Table I, the charged amines have much higher permeability than their uncharged counterparts, even though the charged and uncharged forms of these amines have the same shape and are very similar in size. Furthermore, charged ethanolamine, ethylamine, and ethylenediamine are more permeable than uncharged ethylene glycol, although they differ very little in size and shape (Table I). Guanidinium and urea have similar molecular structure and size, but cationic guanidinium is far more permeant than neutral urea (Table I). All these results clearly indicate that positive charges on the molecules increase their permeability through ACh-activated channels. On the other hand, because several nonelectrolytes do have measurable permeability, as shown in Table I, positive charge is not an absolute requirement for a molecule to permeate the channels.

At neutral pH, ethanolamine, ethylenediamine, and ethylamine are similar in

charge and size. However, their permeabilities through ACh-activated channels are in the ratio of ethanolamine : ethylenediamine : ethylamine = 0.72 : 0.63 : 0.45. These three molecules differ by having different groups, namely —OH, —NH_2, —H, attached on the carbon 2 of the amines. The substitution of the different groups increases the hydrogen bonding capacity in the order of OH > NH_2 > H. Inasmuch as this difference in the hydrogen bonding ability of these three molecules may alter the interaction with ACh-activated channels during their transport, it is likely that the permeability to ethanolamine and ethylenediamine is enhanced because they are better hydrogen bond donors than ethylamine. Further evidence for this is provided by some of the uncharged molecules tested. Ethylene glycol, formamide, and urea are measurably permeant and have some hydrogen bonding capacity. Ethylene glycol, in having two —OH groups, is much more permeant than uncharged ethanolamine, ethylenediamine, and ethylamine (Table I). In all these cases, there is a positive correlation between permeability and the hydrogen-bonding ability of the molecules. Formamide and urea have moderate dipole moment due to electronegative character of the oxygen atom in carbonyl groups. Thus, in addition to hydrogen-bonding, the dipole moment of these two compounds can result in favorable interactions with the channel structure, increasing their permeability.

Steric restriction and mobility also play important roles in determining the permeability of molecules through ACh-activated channels. We observed a selectivity sequence for alkali cations to be $Cs^+ > Rb^+ > K^+ > Na^+$ (Fig. 1). In our system, K^+ is more permeant than Na^+, which is opposite to Takeuchi's (1963) results, but is in agreement with the findings of Lassignal and Martin (1977).[1] Na^+ has the smallest crystal radius and the lowest mobility of our series; Cs^+, the largest ion, has the highest mobility. Thus, ACh-activated channels are selective for the cations of highest mobility in free solution (Fig. 6). From the selectivity studies of ACh-activated channels for large organic cations in postsynaptic membranes, Maeno et al. (1977) estimated the minimum internal diameter of ACh-activated channels to be 6.4Å. This channel diameter is large enough to allow cations to permeate in their hydrated forms. Our observation that the selectivity sequences are in the order of the mobilities of the hydrated alkali cations is consistent with this estimate of channel diameter.

Our analysis suggests that the ACh-activated channel is a relatively large water-filled pore containing hydrogen-accepting groups and at least one negative-charged site. This charged site may be a carboxyl group with a pK of 4.8 inasmuch as protonation of a group with pK = 4.8 inhibits Na^+ transport without changing the affinity of carbamylcholine for its receptor. Hydrogen bond-accepting groups are likely present in the channel because hydrogen bond-donating moieties enhance the permeability of transported molecules. The hydrogen bond acceptors may interact with the water of hydration of cations or form hydrogen bonds with the cations, themselves, or with nonelectrolytes during their transport.

Among the ion channels studied in biological membranes, the most detailed information on selectivity is available for Na^+ channels of nerve axons (Hille,

[1] Lewis, C., and C. F. Stevens. 1977. Personal communication.

1972). Both ACh-activated and Na channels are cation-selective. Both channels are also somewhat permeable to nonelectrolytes, but much less so than to comparable molecules with positive charge. Several examples of this for ACh-activated channels are shown in Table I. We have also examined the selectivity of nonelectrolytes in batrachotoxin-activated Na^+ channels of neuroblastoma cells, and found that formamide and urea have small but measurable permeability. However, these uncharged molecules are much less permeant than charged guanidinium (Huang et al., unpublished result). The most striking differences between ACh-activated channels and Na^+ channels are that the latter channels are much more selective, and have a selectivity sequence for alkali cations of $Na^+ > K^+ > Rb^+ > Cs^+$ (Chandler and Meves, 1965; Hille, 1972;

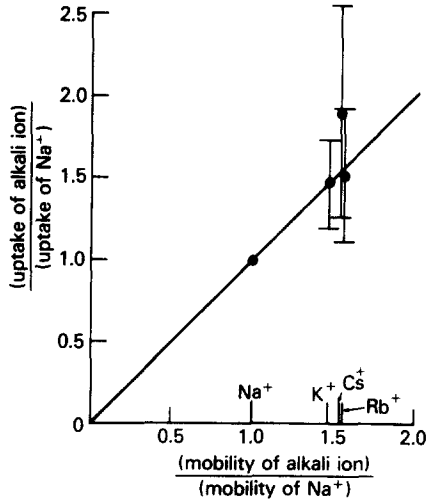


FIGURE 6. Permeability ratio (R) for alkali ions as a function of their relative mobility (relative to Na^+) in aqueous solution. The R values are taken from Table I; the relative mobility values are from Robinson and Stokes (1959). The vertical bars represent the experimental variations. The solid line has a slope of 1 representing the perfect correlation between R and relative mobility.

Cahalan and Begenisich, 1976; Ebert and Goldman, 1976). These differences are consistent with the relatively smaller (3 × 5Å) cross section estimated for Na^+ channels (Hille, 1972).

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REFERENCES

ANDERSON, C. R., and C. F. STEVENS. 1973. Voltage clamp analysis of acetylcholine produced end-plate current fluctuation at frog neuromuscular junction. *J. Physiol. (Lond.).* **235**:655–691.

CAHALAN, M., and T. BEGENISICH. 1976. Sodium channel selectivity. Dependence on internal permeant ion concentration. *J. Gen. Physiol.* **68**:111–125.

CAHN, R. D., H. G. COON, and M. B. CAHN. 1967. *In* Methods in Developmental Biology. F. H. Wilt and N. K. Wellses, editors. Thomas Y. Crowell Co. Inc., New York. 525 pp.

CATTERALL, W. A. 1975. Sodium transport by the acetylcholine receptor of cultured muscle cells. *J. Biol. Chem.* **250**:1775–1781.

CHANDLER, W. K., and H. MEVES. 1965. Voltage clamp experiments on internally perfused giant axons. *J. Physiol. (Lond.).* **180**:788–820.

COLQUHOUN, D., V. E. DIONNE, J. H. STEINBACH, and C. F. STEVENS. 1975. Conductance of channels opened by acetylcholine-like drug in muscle end-plate. *Nature (Lond.).* **253**:204–206.

DIONNE, V. E., and R. L. RUFF. 1977. Endplate current fluctuations reveal only one channel type at the frog neuromuscular junction. *Nature (Lond.).* **266**:263–265.

EBERT, G. A., and L. GOLDMAN. 1976. The permeability of the sodium channel in *Myxicola* to alkali cations. *J. Gen. Physiol.* **68**:327–340.

FATT, P., and B. Katz. 1951. An analysis of the end-plate potential recorded with an intra-cellular electrode. *J. Physiol. (Lond.).* **115**:320–370.

FISCHBACH, G. D. 1972. Synapse formation between dissociated nerve and muscle cells in low density cell culture. *Dev. Biol.* **28**:407–429.

FURUKAWA, T., and A. FURUKAWA. 1959. Effects of methyl and ethyl-derivatives of NH_4^+ on the neuromuscular junctions. *Jpn. J. Physiol.* **9**:130–142.

GOLDMAN, D. E. 1943. Potential impedance and rectification in membranes. *J. Gen. Physiol.* **27**:37–60.

GUY, H. R., M. S. DEKIN, and R. MORELLO. 1977. Effect of denervation on the permeability of acetylcholine-activated channels to organic cations. *J. Neurobiol.* **8**:491–506.

HILLE, B. 1972. The permeability of sodium channel to metal cations in myelinated nerves. *J. Gen. Physiol.* **59**:637–658.

HODGKIN, A. L., and B. KATZ. 1949. The effect of sodium ions on the electrical activity of giant axons of squid. *J. Physiol. (Lond.).* **27**:37–77.

JENKINSON, D. H., and J. G. NICHOLLS. 1961. Contractures and permeability changes produced by acetylcholine in depolarized denervated muscle. *J. Physiol. (Lond.).* **159**:111–127.

KOKETSU, K., and S. NISHI. 1959. Restoration of neuromuscular transmission in Na^+ free hydrazinium solution. *J. Physiol. (Lond.).* **147**:239–252.

KORDAS, M. 1969. The effect of membrane polarization on the time course of the end plate current in frog sartorius muscle. *J. Physiol. (Lond.).* **204**:493–502.

LASS, T., and C. D. FISCHBACH. 1976. A discontinuous relationship between acetylcholine activated channel conductance and temperature. *Nature (Lond.).* **263**:150–151.

LASSIGNAL, N. L., and A. R. MARTIN. 1977. Effect of acetylcholine on post-junctional membrane permeability in eel electroplaque. *J. Gen. Physiol.* **70**:23–36.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.

MAENO, T., C. EDWARDS, and M. ANRAKU. 1977. Permeability of the end-plate membrane activated by acetylcholine to some organic cations. *J. Neurobiol.* **8**:173–184.

MAGLEBY, K. L., and C. F. STEVENS. 1972. A quantitative description of end-plate currents. *J. Physiol. (Lond.).* **223**:173–197.

MANTHEY, A. A. 1972. The antagonistic effects of calcium and potassium on the time

course of action of carbamylcholine at the neuromuscular junction. *J. Membr. Biol.* **9**:319–340.

MEBS, D., K. NARITA, S. IWANAGA, Y. SAMEJIMA, and C. Y. LEE. 1971. Amino acid sequences of α-bungarotoxin from the venom of Bungarus Muticinctus. *Biochem. Biophys. Res. Commun.* **44**:711–716.

NASTUK, W. L., and R. L. PARSONS. 1970. Factors in the inactivation of post-junctional membrane receptors of frog skeletal muscle. *J. Gen. Physiol.* **56**:218–248.

NEHER, E., and B. SAKMANN. 1976. Single channel currents recorded from membrane of denervated frog muscle fibers. *Nature (Lond.)*. **260**:799–802.

RITCHIE, A., and D. M. FAMBROUGH. 1975. Ionic properties of the acetylcholine receptor in cultured rat myotubes. *J. Gen. Physiol.* **65**:751–767.

ROBINSON, R. A., and R. H. STOKES. 1959. Electrolyte solutions. Butterworth and Co. Ltd., London, 463 pp.

SACHS, F., and H. LECAR. 1973. Acetylcholine noise in tissue-cultured muscle cells. *Nat. New. Biol.* **246**:214–216.

SACHS, F., and H. LECAR. 1977. Acetylcholine-induced current fluctuations in tissue-cultured muscle cells under voltage clamp. *Biophys. J.* **17**:129–143.

TAKEUCHI, A., and N. TAKEUCHI. 1960. On the permeability of the end-plate membrane during the action of transmitter. *J. Physiol. (Lond.)* **154**:52–67.

TAKEUCHI, N. 1963. Some properties of conductance changes at the end-plate membrane during the action of acetylcholine. *J. Physiol. (Lond.)*. **167**:128–140.

VOGEL, Z., A. J. SYTKOWSKI, and M. W. NIRENBERG. 1972. Acetylcholine receptors of muscle grown in vitro. *Proc. Natl. Acad. Sci. U. S. A.* **69**:3180–3184.