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Interactions between Ions and the Axon Plasma Membrane: Effects of Cations and Anions on the Axonal Cholinergic Binding Macromolecule of Lobster Nerves

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Summary. It has been demonstrated that cations and ions interact directly with the axonal cholinergic binding macromolecule (ACBM) from lobster walking leg nerves. This interaction results in an increased affinity for binding of [^{3}H] nicotine. The action sequence for the enhancement effect is $Na^+ > K^+ > Li^+ > Rb^+ > Cs^+$ while the anion sequence is $I^- > Br^- > Cl^- > F^-$. These results have been interpreted in terms of Eisenman's theory of equilibrium cation specificity in an attempt to acquire information about the ion binding sites on the ACBM. The mechanism of the enhancement of nicotine binding to the ACBM may serve as a model for studying the conformational changes arising from binding of ions to axonal macromolecules.

As an alternative to the concept of separate Na^+ and K^+ gates or channels through which flow the cation currents that produce an axonal action potential, some models have been proposed (Tasaki, 1968; Weiss, 1969) which postulate a general increase in cation permeabilities caused by a cation exchange process generating a conformational change in the membrane.

One approach in attempting to distinguish between these various theories is to study the interaction between the cations and the axon plasma membrane. Direct binding studies of Na^+ and K^+ are not feasible because the relatively low affinity of their binding and low concentration of binding sites makes equilibrium dialysis difficult and the presumably rapid equilibrium between cation and membrane prevents isolation of cation-membrane complexes. It was observed that the binding of [^{3}H] nicotine to the axonal cholinergic binding macromolecule (ACBM), from the walking leg nerve

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bundle of the lobster *Homarus americanus*, was sensitive to the ionic composition of the solution. This phenomenon was studied in some detail because it has been postulated that cholinergic receptors in the axon play a major role in the conduction of an action potential (Nachmansohn, 1959). In addition, it offers an indirect method for measuring the interactions between ions and one possible functional component of the axon plasma membrane. These interactions were interpreted in terms of Eisenman's theory of cation equilibrium selectivity (Eisenman, 1961; Diamond & Wright, 1969, summary).

Materials and Methods

The axon plasma membrane preparation was isolated by differential centrifugation from a hypotonic homogenate of the nerve bundle from the walking legs of the lobster *Homarus americanus*. This membrane fraction contained 5% of the total protein, and 60%, 45%, and 39% of the total activities of the axonal cholinergic binding macromolecule, acetylcholinesterase, and Na^+, K^+-ATPase, respectively. A detailed account of the isolation and characteristics of the membrane preparation has been published (Denburg, 1972).

ACBM activity was measured as binding of [^{3}H] nicotine (Amersham, specific activity 355 mC/mmole) to the axon membranes by equilibrium dialysis. Aliquots of 0.35 ml of the membrane preparation, containing 1 to 2 mg protein/ml, were dialyzed for 16 hr at 4 °C against 100 ml of bath solution containing the particular salt to be studied along with the radioactive nicotine, which was usually at the nonsaturating concentration of 10^{-7} M. The volume of the contents of the bag was remeasured or the protein was redetermined (Lowry, Rosebrough, Farr & Randall, 1951), to correct for any errors caused by changes in volume of the sample resulting from the dialysis against solutions of different osmotic pressure. This was particularly significant in comparing the effect of different concentrations of salts on the binding. The difference in radioactivity of samples from inside the bag and the outer solution was measured and represented the amount of [^{3}H] nicotine bound. Further details on these procedures have been published (Eldefrawi, Eldefrawi & O'Brien, 1971).

ACBM was solubilized by adding lysolecithin (Sigma) in a 3:1 ratio by weight of lysolecithin to protein to the membrane suspension. Centrifugation at $100{,}000 \times g$ for 1 hr yielded all the binding activity in the supernatant. This binding activity could also be measured by equilibrium dialysis since the solubilized ACBM is nondialyzable and has a sedimentation coefficient of 7.8 S. More details on the properties of the soluble ACBM will be published elsewhere.

Results

The binding of nicotine to the ACBM was very sensitive to the concentration of NaCl present in the Ringer's solution used in the equilibrium dialysis assay. At 10^{-7} nicotine, the amount bound increased as the NaCl concentration was increased up to 2 M. These high salt concentrations did not solubilize any ACBM or acetylcholinesterase. Complete binding curves were determined in three different concentrations of NaCl (Fig. 1). It was

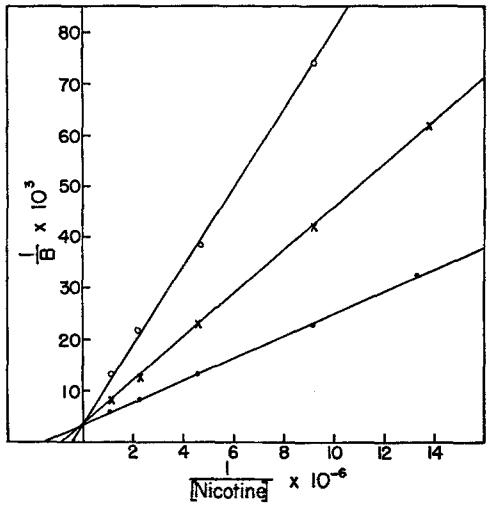

Fig. 1. The effect of concentration of NaCl on binding of [^{3}H] nicotine. Double reciprocal plot of binding of nicotine, B (pmole/mg protein) to axon membranes in the presence of 1.0 M NaCl (•—•—•), 0.45 M NaCl (×—×—×), and 0.1 M NaCl (○—○—○)

inferred from the common intercepts on the double reciprocal plot that the total number of nicotine binding sites remained unchanged. Experimental error was approximated to be $\pm 10\%$ so that only small changes in the total number of binding sites could not be detected by the assays. The increase in binding seen at the nonsaturating concentration of 10^{-7} M was caused by an increase in affinity for nicotine by the ACBM because in 0.1 M NaCl, $K_D = 1 \times 10^{-6}$ M; in 0.45 M NaCl, $K_D = 4.5 \times 10^{-7}$ M; and in 1 M NaCl, $K_D = 2.4 \times 10^{-7}$ M.

The nature of the alkali cation determined the ability of a salt to enhance the binding of 10^{-7} M nicotine. From Fig. 2, which presents data for five chlorides over a 20-fold concentration range, the sequence of cation selectivity is $Na^+ > K^+ > Li^+ > Rb^+ > Cs^+$. The binding assay is not accurate enough to distinguish differences between the cations at 0.1 M and 0.25 M. However, at higher concentrations, the standard deviations are 2 to 4% and the differences are truly significant. The enhancement of binding by the cations within the concentration range of nicotine of 10^{-8} M to 2×10^{-7} M all gave the same cation selectivity sequence. This sequence did not represent the cation permeabilities of the axon plasma membrane vesicles because 10^{-6} M valinomycin had no effect on the enhancement of nicotine binding by KCl. At this concentration, valinomycin greatly increased the rate of efflux of radioactive K^+ from the axon plasma membrane vesicles (T. Bratkowski, *unpublished results*) presumably by increasing the K^+ permeability.

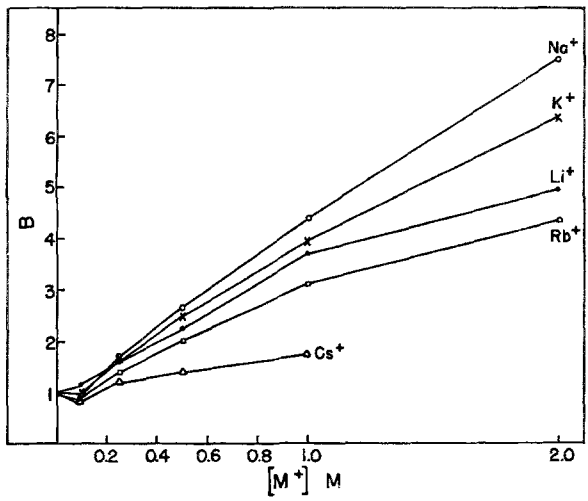

Fig. 2. Cation specificity of the enhancement by salts of binding of 10^{-7} M [^{3}H] nicotine to axon plasma membrane preparation. *B* is amount of nicotine bound relative to that with no salt present. The binding in the absence of salt has been normalized to 1.00

The ACBM, solubilized by lysolecithin, had the same cation specificity as the membrane-bound material although the magnitude of the enhancements was greater (Table 1).

The effects of pH on the binding of 10^{-7} M nicotine are shown in Fig. 3. The measurements were made in lobster Ringer's (457 mM NaCl, 15 mM KCl, 25 mM $CaCl_2$, 4 mM $MgCl_2$, 4 mM $MgSO_4$, 10 mM Tris). Although the pH range examined was beyond the buffering capacity of the Tris, the values reported are those measured after the 16-hr incubation required in the equilibrium dialysis assay. The decrease in binding at pH values below 6.2 is caused by an irreversible denaturation of the ACBM. At the higher pH's the decrease in binding is reversible because membranes thus treated recovered all their binding activity when assayed at pH 7.8.

Table 1. The cation specificity of the ACBM under various conditions

Chloride	Binding of 10^{-7} M nicotine[a]					
(1 M)	pH 6.4	pH 7.8	pH 9.4	0.1 M Ca^{++} pH 7.8	0.25 M Ca^{++} pH 7.8	Lysolecithin solubilized
Li^+	7.2	3.7	1.4	3.0	1.4	7.2
Na^+	15.6	4.4	1.7	3.6	2.3	9.1
K^+	7.6	3.9	1.5	3.3	2.0	7.6
Rb^+	6.7	3.1	1.1	2.3	1.6	5.8
Cs^+	4.7	1.5	0.6	1.2	0.6	1.4

[a] Binding is relative to that in the absence of salt which was normalized to 1.0.

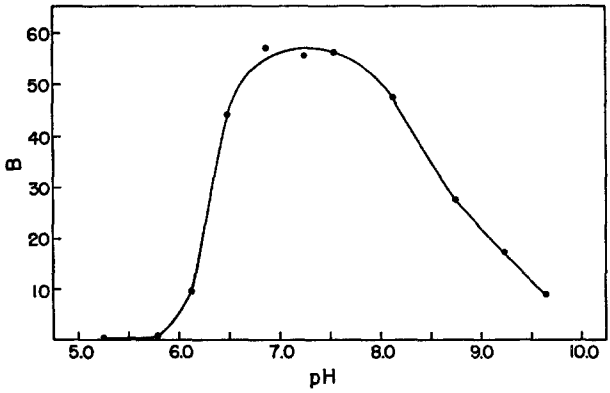

Fig. 3. The effect of pH on the binding of [^{3}H] nicotine to axon plasma membrane preparation. All points were done in lobster Ringer's with 10 mM Tris buffer. *B* is amount of nicotine bound in pmole/mg protein

In an attempt to alter the cation specificity sequence, cation effects were measured at various pH's and in the presence of Ca^{++}. These results are summarized in Table 1. In no case was the sequence changed although the relative magnitudes of the enhancements were greater at the lower pH and in the absence of Ca^{++}.

The divalent cations of the alkaline earth metals also enhanced binding of nicotine although there were only slight differences between them (Table 2). At 0.5 M, the results are complicated by the onset of possible inhibitory effects as seen for Ca^{++}.

Unexpectedly, the nature of the anion of the salt was also found to affect its ability to enhance the binding of nicotine to the ACBM (Fig. 4). The effectiveness of the halide anions followed the sequence $I^- > Br^- > Cl^- > F^-$. The decrease in effectiveness of NaI at concentrations greater than 0.25 M is caused by I^- binding since the presence of 1 M NaCl with the 0.25 M NaI caused no decrease in the enhancement of nicotine binding. The decrease in enhancement by salts as the pH is increased (Table 1) may

Table 2. Effect of divalent cations of alkaline earth metals on the binding of nicotine to ACBM

Concentration	Binding of 10^{-7} M [^{3}H] nicotine[a]			
	$MgCl_2$	$CaCl_2$	$BaCl_2$	$SrCl_2$
0.1 M	1.23	1.27	1.11	1.30
0.25 M	2.10	2.17	1.77	2.14
0.5 M	3.40	1.79	2.28	2.59

[a] Binding is relative to that in the absence of salt which was normalized to 1.0.

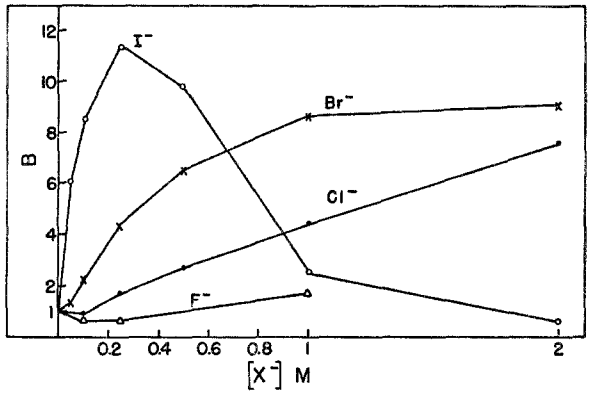

Fig. 4. Anion specificity of the enhancement by salts of binding of 10^{-7} M [^{3}H] nicotine to axon plasma membrane. *B* is amount of nicotine bound relative to that with no salt present. The binding in the absence of salt has been normalized to 1.00

be caused by the ionization of some cationic group in the ACBM which is responsible for the anion binding and its subsequent effect on nicotine binding. If this is the case, then the major part of the enhancement by salts is caused by the anion.

Discussion

Since most cholinergic ligands are positively charged, a significant past of the energy of their interaction with macromolecular binding sites comer from coulombic electrostatic interactions. This may also apply to the binding of nicotine to the lobster ACBM since the decline in binding above pH 7.5 may be caused by the progressive deprotonation of the pyrrolidine nitrogen of nicotine, whose pK_a is 8.1 (Fujita, Nakajima, Soeda & Yamamoto, 1971). However, the observed increase in affinity of the ACBM for nicotine with increasing salt concentration is contrary to the effect on simple electrostatic interactions that would be expected by increasing the ionic strength. Increasing the concentration of NaCl above 0.15 M causes a decrease in binding of cholinergic ligands to the acetylcholine receptor from housefly heads and *Torpedo* electroplax (M. Eldefrawi, *unpublished results*). Therefore, the interactions between the ACBM and ions are not a general salt effect and hopefully are characteristic of axonal macromolecules. This is further supported by the differential responses to the chlorides of the alkali metals and to the halides of sodium.

Experiments were done to determine the site at which the ions were acting. In a membrane preparation there are several possible direct or indirect ways in which an ion might enhance the binding properties of one

of the macromolecules in the membrane. Transport of the alkali cations into the membrane vesicles was not limiting since valinomycin, which acts as a K^+ carrier through membranes (Moore & Pressman, 1964), had no effect on the KCl enhancement of nicotine binding. Therefore, the cation and anion specificity sequences do not reflect their permeability into the axon plasma membrane vesicles. Preliminary electron-micrographs (*to be published*) indicated that the amount of membranes in vesicle form increased as the salt concentration increased until in lobster Ringer's about 80% of the membranes were in vesicular form. However, the observation of identical cation and anion specificity sequences with solubilized ACBM, which has a sedimentation coefficient of 7.8 S, indicates that supramolecular interactions are not causing the increase in affinity for nicotine. This identical specificity is strong evidence for the direct binding of the ions to sites on the phospholipoprotein ACBM so that the selectivity sequences are characteristic of sites on this macromolecule. However, the possibility exists that the ACBM is only a small component of the solubilized 7.8 S component. In this case, binding of ions to other components of the complex might indirectly affect ACBM activity.

The ability to extrapolate information about the ion binding sites on membranes from the ion binding selectivity sequences of these sites was made possible by the work of Eisenman, 1961. In general, his theory states that ion selectivity is determined by the electric field strength of the binding sites. The theory is based on the fact that the free energy differences between ion-membrane and ion-water interactions determine the equilibrium ion specificity, and that these interactions are primarily coulombic electrostatic forces. This theory successfully predicts the 11 out of a possible 120 alkali cation selectivity sequences that are observed in biological and physical systems. The alkali cation specificity of the ACBM, $Na^+ > K^+ > Li^+ > Rb^+ > Cs^+$, is identical to his sequence IX which is caused by a relatively strong negatively charged electric field. In the ACBM, such an electric field would be produced by anionic groups constituting the cation binding sites. A further prediction of Eisenman's theory is that the magnitude of selectivity among ions, that is the ratios of Na^+/K^+, K^+/Li^+, etc., within a particular sequence (at a constant field strength) will be dependent upon the amount of water near the binding site. This prediction was confirmed as seen in Fig. 2 where, as the concentration increases (concentration of water decreases) the differences between the enhancement of nicotine binding caused by the various alkali cations is magnified.

If indeed the negative electric field strength determines the cation selectivity sequence, then one should be able to alter the alkali cation specificity

of the binding site by modifying the electric field strength (Diamond & Wright, 1969). All attempts to do this with the binding of alkali cations to the ACBM were unsuccessful. The irreversible denaturation of the ACBM at values of pH less than 6.0 limited the range in pH in which one could attempt to reduce the negative field strength by protonation of anionic groups. The carboxylate and phosphate anions which are probably part of the cation binding site would have no change in their ionization state within the pH range examined. The addition of Ca^{++}, which under some conditions might be expected to have a stronger affinity for anionic sites, also failed to alter the alkali cation selectivity sequence.

The possibility that the extent of binding of nicotine to the ACBM would change the cation specificity of the binding site was examined. However, with 10^{-8} M nicotine, where about 4% of the binding sites were occupied, the same cation selectivity sequence was observed as with 10^{-7} M nicotine where 35% of the sites were occupied.

The observation that the binding of nicotine to the ACBM is sensitive to the nature of the anion as well as the alkali cation complicates the quantitative interpretation of the results. It is impossible to separate the anion effects from the cation effects. However, the order of anion selectivity on the ACBM, observed at salt concentrations up to 0.6 M, $I^- > Br^- > Cl^- > F^-$, is identical to sequence I predicted by Eisenman (1965) and corresponds to the sequence produced by a weak positive electric field. At higher concentrations of salts, sequences II and III predicted by Eisenman described the anion selectivity. The fact that both anions and cations can enhance the binding of nicotine might imply that both must be bound to get the effect. However, the observation of only slight differences in response to the divalent cations of the alkaline earth series may indicate that divalent cations are not binding to the alkali cation binding site on the ACBM. If this is the case, the observed enhancement of nicotine binding by divalent cations may be caused only by Cl^-. The distances between the cation binding sites determine the relative affinities for monovalent and divalent cations (Truesdell, 1964; Eisenman, 1965). Therefore, the apparent greater affinity for monovalent cations by the ACBM is indicative of widely spaced binding sites. This is consistent with the calculation that ACBM represents approximately 1% of the protein of the axon plasma membrane (Denburg, Eldefrawi & O'Brien, 1972).

The sequence of alkali cation binding to ACBM, $Na^+ > K^+ > Li^+ > Rb^+ > Cs^+$, is different from any of the known cation sequences for axonal physiological phenomena. The sequence for the permeability of cations through the Na^+ gate during an action potential in the squid giant axon is

$Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$ (Chandler & Meves, 1965) and that of the K^+ gate in the frog nerve is $K^+ > Rb^+ > Cs^+ > Li^+ > Na^+$ (Hille, 1972). The order in which internally perfused cations maintained the excitability of the squid giant axon is $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$ (Tasaki, Singer & Takenaka, 1965). Externally applied cations maintain the excitability according to the sequence $Li^+ > Na^+ > Cs^+ > Rb^+ > K^+$. The fact that the cation sequence for the ACBM does not coincide to that of any physiological effect, does not necessarily exclude the possibility that this macromolecule plays a role in any of these phenomena. We have previously postulated that ACBM is on the internal surface of the axon plasma membrane and is a component common to both the Na^+ and K^+ gates. It is possible that in the process of isolation of the membrane fraction the electric field strength around the cation binding site was sufficiently modified so as to alter the cation specificity of the site.

Although the concentrations of salts used in this study may appear to be unusually high, it must be remembered that lobster Ringer's contains 0.45 M NaCl. In addition, in the process of conducting an action potential, the macromolecules in the axon membrane are probably in contact with much higher localized concentrations of ions.

In conclusion, direct interactions between ions and the ACBM have been demonstrated. Since ACBM is a plausible candidate as a molecule of importance in axonal conduction, by virtue of its high affinity for cholinergic agents and local anesthetics (Denburg *et al.*, 1972), the sequence of cation and anion sensitivities reported here should aid in identifying its physiological role. In addition, the mechanism of the enhancement of nicotine binding to the ACBM may serve as a model for studying the conformational changes arising from binding of ions to axonal macromolecules. Such conformational changes are an integral part of Tasaki's (1968) and Weiss' (1969) theories of axonal conduction. The application of Eisenman's (1961) theory of equilibrium cation specificity to the data enables the acquisition of information about the ion binding sites. Such an approach would be most fruitful in further studies on the interaction of ions and axonal macromolecules.

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