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Activation and Inhibition of ^{3}H-Strychnine Binding to the Glycine Receptor by Eccles' Anions: Modulatory Effects of Cations

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SUMMARY

The ammonium salts of Eccles' anions are inhibitors of ^{3}H-strychnine binding. By contrast, the binding is activated by the sodium salts of these anions. This activation is due to an increase in the affinity for ^{3}H-strychnine. Pretreatment of the membranes with acetic anhydride abolishes the effect of sodium salt, whereas ammonium salts remain unaffected. In the presence of chloride the inhibitory effect of ammonium predominates over the effect of sodium. These results suggest that the effect of Eccles' anions is profoundly modified by their counter-ions and that Na^+ and ammonium are acting through distinct sites on the glycine receptor.

Glycine is a major inhibitory neurotransmitter in the spinal cord and other regions of the vertebrate central nervous system (1–3). The antagonist strychnine has been widely used to study (4–8) and purify (9–11) the glycine receptor.

The IPSP generated by inhibitory neurotransmitters such as glycine appears to be mediated by an increase in chloride permeability (12–15). Certain anions cause a hyperpolarization of the neuronal membrane when injected iontophoretically into the neuronal soma, and subsequent stimulation of inhibitory inputs to the neuron results in a depolarization, i.e., a reversal, of the IPSP (12–15). These anions—the Eccles' anions—have hydration sizes similar to or smaller than those of chloride; thus, their ability to invert the IPSP has been attributed to their capacity to traverse the chloride channel upon receptor activation. In contrast, other anions with large hydration radii cause hyperpolarization when injected intracellularly, but stimulation of inhibitory pathways does not result in a change in the membrane potential.

The close correlation between the ability of Eccles' anions to invert the IPSP and their inhibitory effect on specific ^{3}H-strychnine binding has suggested that strychnine binding is associated with the ionic conductance mechanism of the glycine receptor (6). The same anions also appear to reduce the potency of glycine and other glycine receptor agonists as inhibitors of ^{3}H-strychnine binding (7).

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However, only the ammonium salts of Eccles' anions can inhibit ^{3}H-strychnine binding (6), whereas NaCl, KCl, and LiCl activated the binding (5). This activation was thought to be due to nonspecific effects of the increased ionic strength, i.e., the unmasking of "buried" receptor sites. The aim of this work was to further study the mechanism of activation of ^{3}H-strychnine binding to the glycine receptor by ions. Our results show that this effect is specific of Eccles' anions and that only the affinity for strychnine, but not the number of binding sites, is affected. The fact that these anions are able to both increase and decrease ^{3}H-strychnine binding also suggest a modulatory role for cations.

Materials and Methods

All the experimental procedures related to membrane preparation, binding assay, treatment of membranes with acetic anhydride, and protein determination are as described in Ref. 16.

Results

Several chloride salts, including NaCl, KCl, and Tris-HCl buffer, activate specific ^{3}H-strychnine binding to spinal cord membranes in a concentration-dependent manner (Fig. 1). In contrast, NH_4Cl inhibits specific ^{3}H-strychnine binding with an IC_{50} of about 0.3 M (Figs. 1 and 4). These findings confirm earlier results of Young and Snyder (5, 6).

Next, saturation experiments in the presence of increasing concentrations of NaCl were done. An analysis of variance revealed that the enhancement of ^{3}H-strychnine binding by NaCl was due to a significant increase in the affinity for the

ABBREVIATIONS: IPSP, inhibitory postsynaptic potential; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

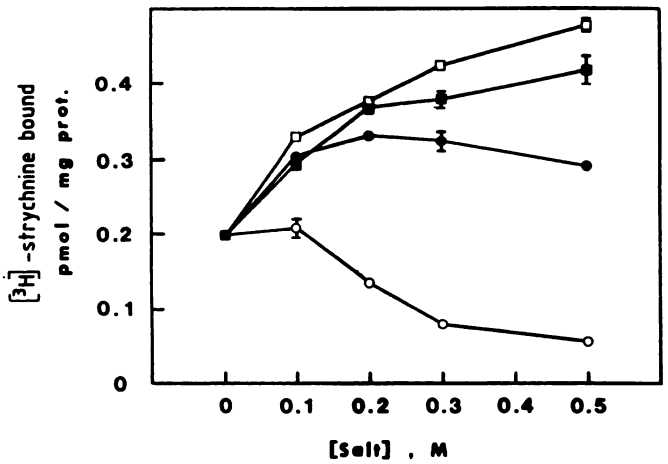

Fig. 1. Effect of several chloride salts on the specific binding of ^{3}H-strychnine. Spinal cord membranes were incubated with 2 nM ^{3}H-strychnine in 50 mM sodium-potassium phosphate buffer, pH 7.1 at 4°, and increasing concentrations of the following salts: □, NaCl; ■, KCl; ●, Tris-Cl; ○, NH_4Cl. The pH was corrected after the addition of each concentration of salt. Points are the mean ± standard error of three determinations. The experiment was replicated.

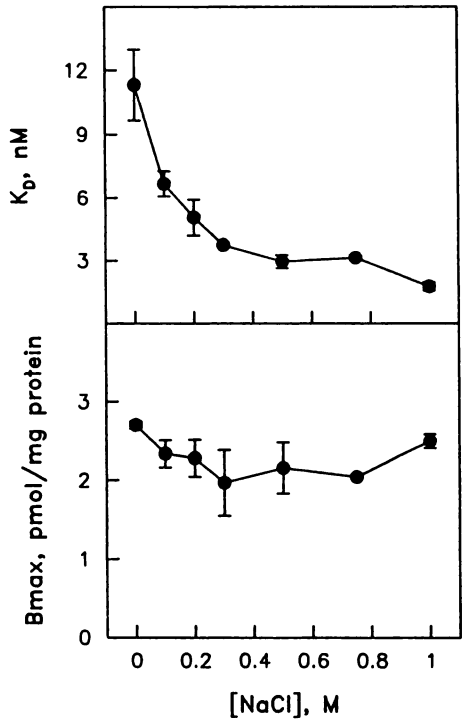

Fig. 2. Effect of NaCl on the saturation parameters of ^{3}H-strychnine binding. Spinal cord membranes were incubated in triplicate with different concentrations of ^{3}H-strychnine in 50 mM sodium-potassium phosphate buffer, pH 7.1, at 4°, alone or with the indicated concentrations of NaCl. K_D and B_{max} values in the figure are the mean ± standard error of three different saturation experiments done with six (0.5–10 nM), seven (1–20 nM), or eight (0.5–30 nM) concentrations of ^{3}H-strychnine, respectively. In each experiment K_D and B_{max} were calculated by least squares fitting of experimental data linearized by the method of Scatchard.

receptor ($F_{(4,9)}$=12, $p \leq 0.001$, Fig. 2), with no significant changes in B_{max} (Fig. 2, $F_{(4,9)}$=1.16, p>0.3). Thus, NaCl does not non-specifically "unmask" cryptic sites but must, instead, act specifically on the conformation of the glycine receptor.

The alkali metal salts of other Eccles' anions also increased specific ^{3}H-strychnine binding, whereas salts of other anions

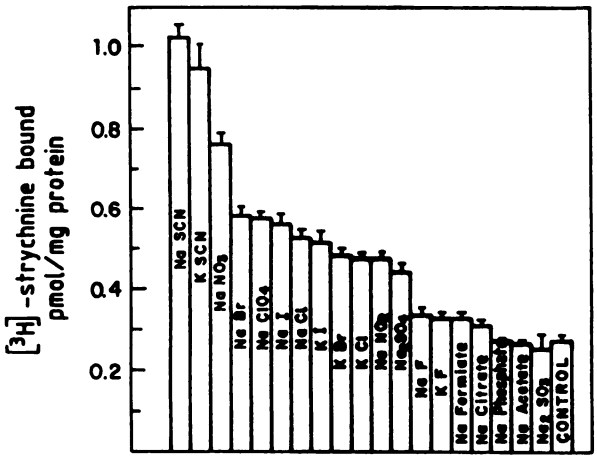

Fig. 3. Activation of specific ^{3}H-strychnine binding by several salts. Spinal cord membranes were incubated with 2 nM ^{3}H-strychnine in 50 mM sodium-potassium phosphate buffer, pH 7.1 at 4°, alone or with 0.2 M concentration of the salts indicated in the figure. The pH was corrected after the addition of each salt. Columns are the mean ± standard error of three determinations of specific ^{3}H-strychnine binding.

TABLE 1
Effect of anions on the glycine receptor

Anion	Hydration radii[a]	Effect on reversal of IPSP[a]	IC_{50}, NH_4^+ salts[b]	Activation of control, Na^+ salts[c]
			mM	%
Bromide	0.94	Active	235	212
Chloride	0.95	Active	260	191
Iodide	0.96	Active	300	205
Nitrate	1.03	Active	335	277
Perchlorate	1.09	Active	440	210
Thiocyanate	1.12	Active	620	371
Fluoride	1.33	Inactive		122
Formate	1.35	Active	160	120
Acetate	1.80	Inactive		96
Sulfate	1.85	Inactive		161
Sulfite	2.05	Inactive		92
Phosphate				
Monobasic	2.05	Inactive		100
Dibasic	2.56	Inactive		100

[a] Data from Ito *et al.* (14).
[b] Data from Young and Snyder (6).
[c] Values from Fig. 3.

were ineffective (Fig. 3). This activation was also due to an increase in the affinity for strychnine, without significant changes in the number of binding sites (data not shown). The effect of thiocyanate (0.2 M) and nitrate (0.2 M) was particularly strong, enhancing ^{3}H-strychnine binding 4- and 3-fold, respectively. A further increase of the binding was found in the presence of 0.5 M solutions of Eccles' anions, except in the case of thiocyanate and perchlorate, which enhanced ^{3}H-strychnine binding to the same extent at 0.2 M and 0.5 M (data not shown).

Sulfate, which is not an Eccles' anion, slightly increased ^{3}H-strychnine binding, probably because of a nonspecific, chaotropic effect (17, 18).

The opposite effects of Eccles' anions, depending on their accompanying cation, suggest that either NH_4^+ or Na^+ might interact with the glycine receptor (Table 1). In order to investigate this possibility, we studied the inhibition of ^{3}H-strychnine binding by NH_4Cl in the presence of increasing concentrations of Na^+ (Fig. 4) added in the form of sodium acetate,

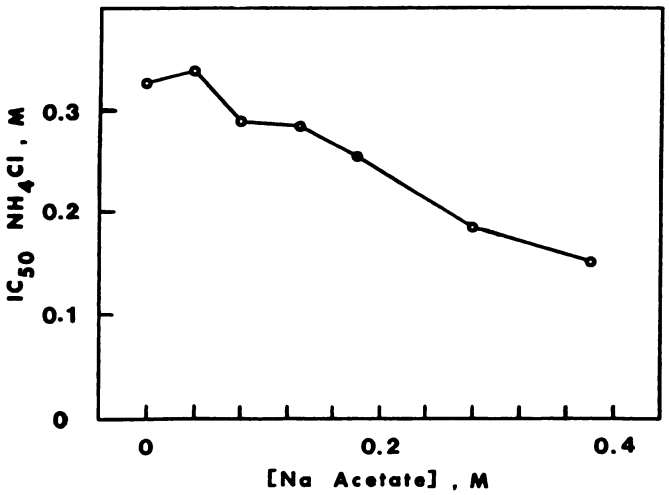

Fig. 4. Effect of Na^+ on the inhibition of specific ^{3}H-strychnine binding by NH_4Cl. Spinal cord membranes were incubated with 2 nM ^{3}H-strychnine in 50 mM sodium-potassium phosphate buffer, pH 7.1 at 4°, and different concentrations of sodium acetate and NH_4Cl. For each concentration of sodium acetate indicated in the figure, a displacement curve with seven different concentrations of NH_4Cl was done. IC_{50} values were then calculated by least squares fitting of Hill plots (correlation coefficient r >0.96). Control values in the absence of NH_4Cl did not change with increasing concentrations of sodium acetate (see also Fig. 7). Nonspecific binding values were determined for each concentration of salts.

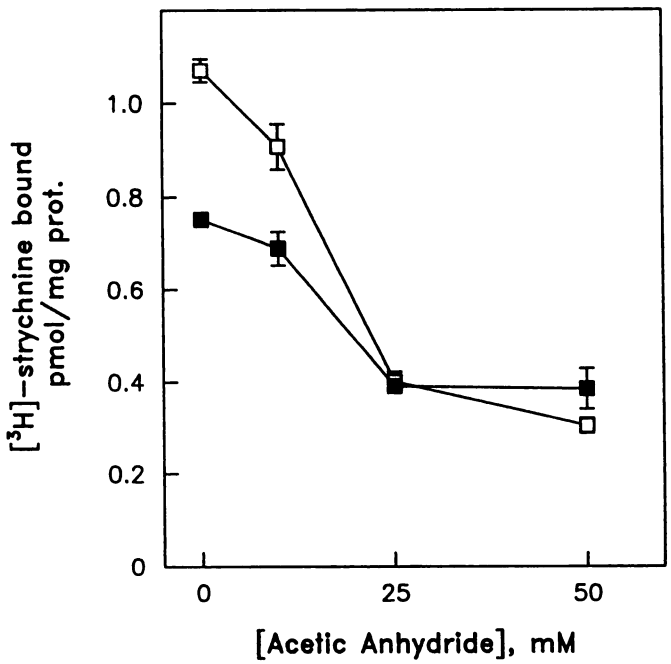

Fig. 5. Activation of specific ^{3}H-strychnine binding by NaCl in membranes treated with acetic anhydride. Spinal cord membrane fractions were treated with different concentrations of acetic anhydride as indicated in Materials and Methods, and then incubated with 2 nM ^{3}H-strychnine in 50 mM sodium-potassium phosphate, pH 7.1 at 4°, alone (■) or in the presence of 0.2 M NaCl (□). Points are the mean ± standard error of three determinations. ^{3}H-Strychnine binding values in the presence and absence of NaCl were significantly different only in control membranes ($p \leq 0.001$) and in membranes treated with 10 mM acetic anhydride ($p \leq 0.02$).

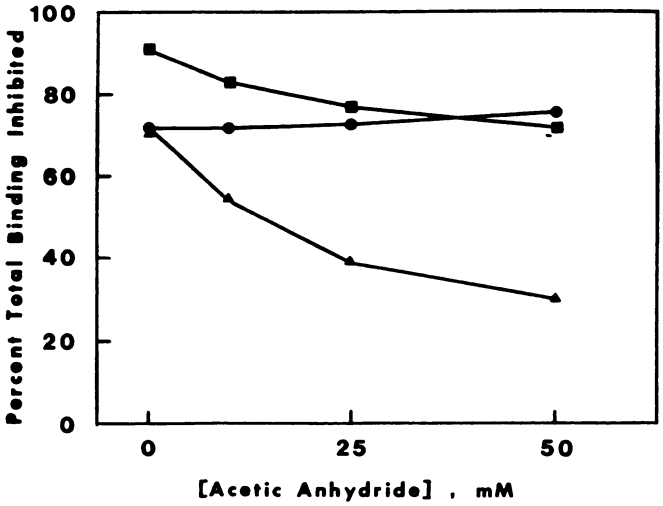

Fig. 6. Inhibition of total ^{3}H-strychnine binding by NH_4Cl, glycine, and strychnine in membranes treated with acetic anhydride. Spinal cord membrane fractions were treated with different concentrations of acetic anhydride and then incubated with 2 nM ^{3}H-strychnine in 50 mM sodium-potassium phosphate, pH 7.1 at 4°, alone or in the presence of 1 M NH_4Cl (●), 0.1 mM cold strychnine (■), or 10 mM glycine (▲). Points are the mean of three determinations, and were calculated as percentage of the total binding obtained in membranes treated with the same concentration of acetic anhydride. Standard errors were less than 10% of the experimental values.

the latter having no apparent effect per se on ^{3}H-strychnine binding (Fig. 3). In this experiment the simultaneous presence of Cl^- and Na^+ ions failed to activate ^{3}H-strychnine binding. Indeed, the inhibition of the binding by NH_4Cl was potentiated by Na^+, as could be seen by a decrease in its IC_{50}.

Similarly, NaCl up to 0.5 M failed to activate ^{3}H-strychnine binding in the presence of relatively low concentrations (~50 mM) of NH_4^+ (see Fig. 7B). No inhibition of ^{3}H-strychnine binding was found with this concentration of NH_4Cl.

Thus, both Na^+ and NH_4^+ seem to affect, in distinct but interacting ways, the glycine receptor, the inhibitory effect of NH_4^+ predominating over the activating effect of Na^+. Potassium, and probably other alkali metal cations like Li^+ (see Figs. 1 and 3, and also Ref. 5), have the same activating properties as Na^+. The effect of K^+, however, is somewhat weaker than the effect of Na^+ (Fig. 3).

The interactions of NH_4^+ and Na^+ with the glycine receptor in the presence of Eccles' anions are also affected differently by treatment with the protein-modifying agent, acetic anhydride. This reagent seems to have a selective effect on the glycine receptor, suppressing the inhibition by glycine of ^{3}H-strychnine binding at concentrations which do not affect the strychnine-binding site (5). The enhancement of ^{3}H-strychnine binding by NaCl was eliminated by treatment with acetic anhydride (Fig. 5), whereas the inhibition by 1 M NH_4Cl was not so affected (Fig. 6).

The buffer used in the assays can also affect ^{3}H-strychnine binding. In the experiment shown in Fig. 7, we studied the effect of NaCl and sodium acetate on ^{3}H-strychnine binding in the presence of different buffers. The activation by NaCl observed in sodium-potassium phosphate buffer (A) was moderately reduced in Tris-citrate (C), and completely abolished in ammonium phosphate (B) and MOPS (D). The effect of Tris may account, perhaps, for the difference between our results and those obtained by Müller and Snyder (7), who found a

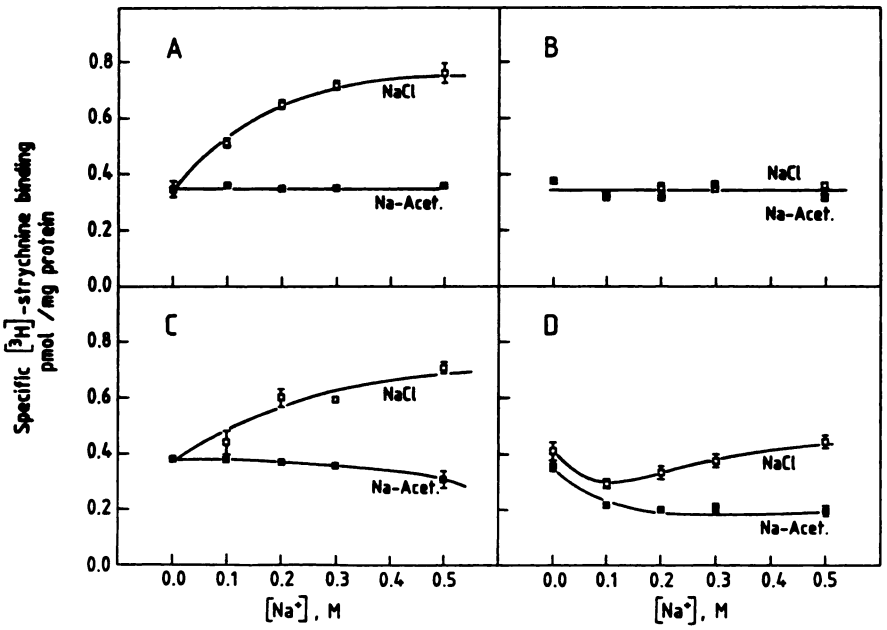

Fig. 7. Different effects of NaCl and sodium acetate on ^{3}H-strychnine binding in the presence of several buffers. Spinal cord membranes were incubated with 2 nM ^{3}H-strychnine in the following buffers: A, 50 mM sodium-potassium phosphate; B, 50 mM ammonium phosphate; C, 50 mM Tris-citrate; D, 50 mM sodium-MOPS (with NaCl, □) or 50 mM ammonium-MOPS (with sodium acetate, ■). Values on the abcissa indicate the concentration of Na^+ added to the buffer in the form of NaCl (□) or sodium acetate (■), not taking into account the amount of sodium present in some of the buffers. pH values were adjusted to 7.1 at 4° after the addition of the salts. Specific binding values (mean ± standard error) were calculated from total and nonspecific binding values determined in triplicate for each ionic condition.

biphasic effect of NaCl and KCl on ^{3}H-strychnine binding in the presence of this buffer. Such a biphasic effect is found in Fig. 7D, where inhibition by NaCl is seen at low concentrations and activation at higher concentrations, but this is probably due to some effect of MOPS on the glycine receptor. Although MOPS does not affect ^{3}H-strychnine binding at 50 mM, it does inhibit 60% of the binding at 175 mM (data not shown). Moreover, glycine inhibition of ^{3}H-strychnine binding is decreased in the presence of 50 mM MOPS (18). These facts suggest that MOPS can interact with the glycine receptor; interestingly, HEPES, another organic buffer, seems to have similar effects on the muscarinic acetylcholine receptor (19).

Discussion

The effect of Eccles' anions on specific ^{3}H-strychnine binding suggests that strychnine interacts with the chloride conductance mechanism associated with the glycine synaptic receptor. In a previous study Young and Snyder (5) characterized the inhibition of ^{3}H-strychnine binding by the ammonium salts of Eccles' anions. Here we show that Eccles' anions can also activate ^{3}H-strychnine binding if accompanied by other cations, particularly Na^+ and K^+, but also $Tris^+$ (Fig. 1).

The activation of ^{3}H-strychnine binding by alkali metal salts of Eccles' anions is probably due to specific interactions with the glycine receptor: (a) NaCl enhances the affinity of the receptor for strychnine without changing its B_{max}; (b) only the salts of Eccles' anions can activate the binding. The enhancement of ^{3}H-strychnine binding by NaCl, KCl, and LiCl was explained in previous works (5) as a nonspecific effect, such as the unmasking of "buried" receptors. This however, does not seem to be the case as this would result in an increased B_{max} with no changes in K_D.

Although formate can reverse the IPSP (13–15) and strongly inhibit ^{3}H-strychnine binding in the presence of NH_4^+ (6), it does not enhance the binding in the presence of Na^+. Indeed, the effect of this anion does seem to be quite anomalous since its hydration radius is larger than those of other Eccles' anions (14), and the mechanism by which it reverses the IPSP is not clear.

These opposite effects of Eccles' anions, depending on their accompanying cation, together with other experiments presented here (see Figs. 4–6), suggest that both NH_4^+ and the alkali metal cations have different modulatory effects on the glycine receptor. NaCl enhancement of ^{3}H-strychnine binding is abolished by treatment with acetic anhydride, whereas the inhibition by NH_4Cl is not. Although these data only provide indirect evidence, they suggest that these cations interact in different ways with the glycine receptor. Moreover, the effect of NH_4^+ seems to predominate over that of Na^+, since no enhancement by NaCl could be observed in the presence of 50 mM NH_4^+ (Fig. 7B). In contrast, rather than antagonizing the inhibition by NH_4Cl, Na^+ seems, in fact, to potentiate it (Fig. 4), further suggesting that both cations act through distinct mechanisms.

The effect of Na^+ and K^+ on ^{3}H-strychnine binding has been described elsewhere (7) as biphasic, inhibiting at low concentrations and not at higher concentrations. It should be noted, however, that the effects seen with such ions can differ markedly if organic buffers, and not the phosphate buffer utilized here, are used: Tris and, in particular, MOPS buffer seem to have direct effects on the glycine receptor. HEPES buffer has been described similarly to affect the muscarinic acetylcholine receptor (19).

Eccles' anions have been described to have several important effects on the γ-aminobutyric acid-barbiturate-central benzodiazepine receptor complex (20, 21) (see Refs. 22 and 23 for review). The effects reported here could therefore reflect a general phenomenon associated with receptors coupled to chloride ionic conductance mechanisms.

Acknowledgments

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