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THE EFFECTS OF IONS ON THE BINDING OF AGONISTS AND ANTAGONISTS TO MUSCARINIC RECEPTORS

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1 There are no selective effects of Na^+, K^+, Ca^{2+}, Mg^{2+} or Cl^- on the binding of antagonists or agonists to muscarinic receptors in rat brain. A decrease in affinity related to ionic strength is found for all these ions.

2 Larger effects were produced by Tl^+, La^{3+}, and some transition metal ions.

Introduction

In all cases in which the question has been examined, the actions of agonist drugs on structures to which they are sensitive has been shown to cause a change in the permeability of the cell membrane for one or more of the common ions Na^+, K^+, Ca^{2+}, Cl^- present in the cytoplasm and the extracellular fluid. The ionic permeability is selective and for instance at the motor endplate of skeletal muscle, acetylcholine increases the permeability for Na^+, K^+ and Ca^{2+} but not for Cl^- (Takeuchi & Takeuchi, 1960; Takeuchi, 1963). On the other hand, the inhibitory transmitter released by stimulating posterior root fibres from opposing muscles and acting on spinal motor neurones increases the permeability to Cl^- and other small anions and to K^+ but does not increase the permeability to Na^+ (Ito, Kostyuk & Oshima, 1962; Eccles, Eccles & Ito, 1964). Many other examples of such selectivity are known.

One can envisage such a permeability change arising in two possible ways: (a) an ionophore channel or carrier is directly coupled to the receptor; conformation changes in the receptor molecule induced by agonists result in conformation changes in the ionophore thereby altering its permeation characteristics, or (b) activation of the receptor does not cause a direct change in permeability but by activating some intracellular metabolic pathway (e.g. a nucleotide cyclase) causes a change in permeability through an indirect mechanism. This could also occur through a change of binding of an ion. For instance the primary action might be the release of bound Ca^{2+} into the cytoplasm followed by a metabolic sequence that then leads to the permeability change. A further variant of this mechanism is that a relatively minor change in permeability is produced directly by agonist action and a metabolic sequence plays the role of an amplifier.

Direct evidence that a receptor operates through one of these mechanisms is not easily obtained. However, there is evidence that the nicotinic receptor belongs to the first class based on the observation of a permeability change in isolated electroplax microsacs apparently free of metabolic substrates, the maintenance of responses under metabolically changed conditions (e.g. anaerobiosis), and the rapid response (≈ 1 ms) which is short compared with potential diffusion time of molecules within the cell. (Katz & Miledi, 1973; Hess & Andrews, 1977). On the other hand, for a number of receptors there is evidence of direct coupling to adenylate cyclase, that is, to a mechanism apparently not directly requiring an increase in ion permeability (Lefkowitz & Williams, 1978). For the most part, clear evidence of either sort of mechanism is not available and this is the situation for the muscarinic receptor.

In the case of a receptor directly coupled to an ionophore, simple theory requires that the ionophore can exist in (at least) two states, i.e. a resting or closed state and an active or open state which are coupled to corresponding ground and active states of the receptor. The closed and open states of the ionophore will interact with permeant ions to a different extent, in that the open state is able in some manner to surround the ion and provide a counterionic atmosphere, whereas in the closed state it either cannot interact at all or at any rate in a different manner. This kind of behaviour is well documented for a system such as valinomycin in which the interaction with K^+ occurs in a conformation of the ionophore which is different from that predominating in polar solvents in the absence of K^+, and thus the equilibrium between the two states of valinomycin is perturbed by the availability of K^+. This process is highly selec-

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tive for K^+ and is dependent on the optimal fit for this cation in the expanded form of the ionophore compared to other alkali metal cations. (Patel, 1973; Smith, Duax, Langs, De Titta, Edmunds, Rohrer & Weeks, 1975; Neupert-Lawes & Dobler, 1975). Furthermore, for acetylcholine receptors at the amphibian neuromuscular junction and on molluscan neurones, it has been demonstrated that differing permeant ions modify the equilibrium between open and shut channels to varying extents (Van Helden, Hamill & Gage, 1977; Ascher, Marty & Nield, 1978).

If the conformational state of the receptor is coupled to the conformational state of the ionophore it is reasonable to expect that this will be a bidirectional relationship, i.e. that changes in the conformational state of the ionophore will change the conformational equilibrium of the receptor. Evidence of such bidirectional conformational coupling is well known in multisubunit enzymes and there is evidence in two cases so far of changed drug binding caused by small inorganic ions (Pert & Snyder, 1974; Young & Snyder, 1974; Simon, Hiller, Groth & Edelman, 1975; Birdsall, Hulme, Bradbury, Smyth & Snell, 1976; Muller & Snyder, 1978) which appear to fit this proposition. It has also been shown that hormone binding to an adenylate cyclase can be modulated by metabolic intermediates (Birnbaumer, 1977).

The conformational equilibrium can be represented thus:

$$RX \overset{K_x}{\rightleftharpoons} R^*X^*$$

as an equilibrium between the ground state RX of the receptor-ionophore complex and its active state R* X*. It is predicted that the equilibrium constant for this interconversion, K_x, will be a function of the interaction with permeant ions, $M^\pm$, i.e. that $K_x = f(M^\pm)$.

If we now consider the interaction of the system with drugs, we have the familiar two-state model (Monod, Wyman & Changeux, 1965; Karlin, 1967; Colquhoun, 1975)

$$\begin{array}{ccc} D + RX & \overset{K_1}{\rightleftharpoons} & D.RX \\ {\scriptstyle K_x}\downarrow\uparrow & & \downarrow\uparrow{\scriptstyle K_\beta} \\ D + R^*X^* & \underset{K_2}{\rightleftharpoons} & D.R^*X^* \end{array}$$

The affinity constant for any drug binding to this system is

$$K = \frac{K_1 + K_2K_x}{1 + K_x}$$

where K_1 and K_2 represent the affinity constants for the binding of D to RX and R*X* respectively. To simplify our argument we will assume that in the absence of drugs the great proportion of the receptors will be in the ground state, i.e. $K_x \ll 1$ and we can therefore write

$$K \simeq K_1 + K_2K_x$$

Now an antagonist can be defined as a drug that combines with the receptor without causing a net increase in the active state i.e. $K_1 \geq K_2$; since $K_x \ll 1$ we can write for antagonists $K \approx K_1$.

On the other hand an agonist is a drug that combines with the receptor, resulting in a net increase of the active state, i.e. $K_2 > K_1$. However, the binding relationship will still be dominated by K_1 unless $K_2K_x \gg K_1$. As $K_x \ll 1$, this requires a high level of discrimination in favour of binding to the active state if the predominant bound species is to be D.R*X*. If this is achieved, we reach the situation in which we would predict that an ion that specifically interacts with the ionophore should perturb the binding of agonists to the receptor but should not perturb the binding of antagonists, that is, it affects K_x, K_β and K_2 but not K_1.

It is with these considerations in mind that we have examined the effects of ions on the binding of antagonists and agonists to the muscarinic receptor in rat brain.

Methods

(−)-N-[^{3}H]-methyl scopolamine ([^{3}H]-methylhyoscinium([^{3}H]-NMS) 3.33 Ci/mmol) and N-(2',3'-[^{3}H]-propyl)-N,N-dimethyl-2-aminoethyl benzilate ([^{3}H]-PrBCH, 40 Ci/mmol) were synthesized as previously described (Hulme, Birdsall, Burgen & Mehta, 1978). (±)-3-Quinuclidinylbenzilate (QNB) was a kind gift of Dr R.B. Barlow.

Binding studies

Binding experiments were conducted with a crude synaptosome preparation from rat cerebral cortex (Hulme *et al.*, 1978). Appropriate concentrations of membranes were suspended in a buffer consisting of 10 mM Na-HEPES, 0.32 M sucrose, pH 7.0, supplemented with salts as indicated. Preincubation was carried out for 2 h at 0°C. All binding measurements were made in quadruplicate.

Measurement of [^{3}H]-methylhyoscinium binding

Binding of [^{3}H]-NMS to the muscarinic receptor was determined by a filtration assay. In order to minimize depletion of the ^{3}H-ligand, very dilute membrane suspensions (0.04 mg protein/ml) were used. After preincubation, such suspensions were supplemented with 7.5×10^{-11} M[^{3}H]-NMS and incubated for 4 h at 0°C. Incubations were terminated by filtration of 3

to 5 ml aliquots through glass fibre filters (Whatman GF/F) under reduced pressure. Filters were rapidly washed with two 2 ml aliquots of ice-cold buffer, and retained radioactivity assayed by liquid scintillation counting. Bound radioactivity was corrected by subtraction of nonspecific binding, defined as residual binding measured after equilibration of the receptor with 2×10^{-6} M (±)-QNB. A small correction (Hulme *et al.*, 1978) was applied for the increase in free [^{3}H]-NMS occasioned by increasing concentrations of the unlabelled competitor. Under these conditions, non-specific binding of the ^{3}H-antagonist to the filters themselves was negligible. However, at higher [^{3}H]-NMS concentrations ($\geq 10^{-9}$ M), it was possible to detect QNB-displaceable binding of [^{3}H]-NMS to filters.

Measurement of [^{3}H]-N-propylbenzilylcholine binding

Binding of [^{3}H]-PrBCh to the muscarinic receptor was determined by a microcentrifugation assay (Hulme *et al.*, 1978). The concentration of crude synaptosome fraction used corresponded to 0.5 to 1.0 mg protein/ml; the [^{3}H]-PrBCh concentration was 10^{-9} M. Incubations were carried out at 0°C for 2 to 4 h, and terminated by centrifugation for 5 min at 14,000 *g* in a cooled microcentrifuge. Non specific binding was determined by inclusion of 2×10^{-6} M (±)-QNB in the incubation medium. This value was subtracted from total binding to yield estimates of specific binding.

Estimation of antagonist affinity constants

Binding of [^{3}H]-PrBCh and [^{3}H]-NMS to the muscarinic receptor obeys the simple Langmuir isotherm (Birdsall, Burgen, Hulme & Wells, 1977; Hulme *et al.*, 1978). Affinity constants were therefore estimated from single point measurements of antagonist binding by using the relationship

$$K = \frac{P}{1 - P} \frac{1}{[D]}$$

where P is the receptor occupancy measured at free antagonist concentration [D]. D was estimated by subtraction of the concentration of receptor/antagonist complex from the total antagonist concentration present in the incubation medium; P was calculated by division of specific binding by the total concentration of binding sites present in the assay. The latter was obtained by measuring specific binding of a saturating concentration (3×10^{-8} M) of [^{3}H]-NMS, using 6×10^{-6} M (±)-QNB to estimate non specific binding.

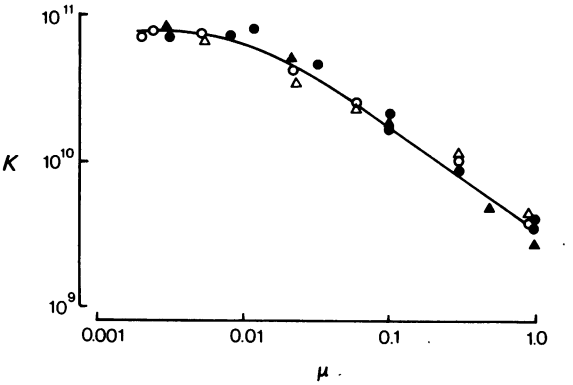

Figure 1 Affinity of N-methylscopolamine for muscarinic receptors in rat cerebral cortex membranes as a function of salt concentration: (●) NaCl; (○) $CaCl_2$; (▲) KCl; (△) $MgCl_2$. Ordinate scale: affinity constant M^{-1}; abscissa scale: ionic strength

Agonist binding curves

Carbachol binding curves were measured by competition with [^{3}H]-PrBCh (10^{-9} M) as described previously (Birdsall, Burgen & Hulme, 1978b). Binding curves were fitted to a two site model of agonist binding, which has previously been shown to provide a good description of agonist binding to the muscarinic receptor in the cerebral cortex (Birdsall, *et al.*, 1978b). This procedure yielded three parameters describing each agonist binding curve: K_H, the affinity constant of the agonist for the high affinity class of sites; K_L, the corresponding affinity constant for the low affinity class of sites and α, the proportion of high affinity sites, expressed as a percentage of the total concentration of high and low affinity sites.

The standard errors of the mean of quadruplicate determinations of bound radioactivity were generally in the range 0.5 to 2.0% and only rarely exceeded 3%. Independent estimates of affinity constants agreed to within ±0.05 log unit. Typical standard errors in K_H, K_L and α were 0.15, 0.06 log units and 0.03.

Results

Effects of ions on antagonist binding

The affinity of the antagonist N-methylscopolamine for the receptor at low ionic strengths was 7.6×10^{10} M^{-1}. It was decreased by increase in ionic strength due to NaCl, KCl, $CaCl_2$ or $MgCl_2$ (Figure 1) so that at $\mu = 1$ the binding constant was 3.3×10^9 M^{-1} i.e. reduced by a factor of 23. In no case was the binding capacity of the preparation changed by ionic strength. There was no discernible ion specific effect.

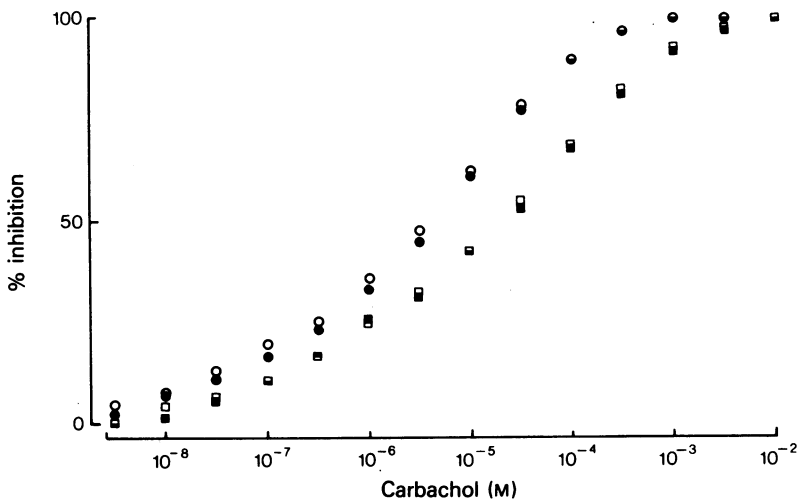

Figure 2 Displacement of specific [^{3}H]-N-propylbenzilylcholine binding by carbachol in NaCl and KCl: NaCl (○) = 0.02 M; (□) = 0.3 M; KCl (●) = 0.02 M; (■) = 0.3 M. Abscissa scale: concentration of carbachol; ordinate scale: % inhibition.

Very similar effects of ionic strength were obtained when the test antagonist was N-propylbenzilylcholine (PrBCh). For instance, in a test of the effect of NaCl and KCl at $\mu = 0.1$ the binding constants were $1.73 \pm 0.10 \times 10^8$ M^{-1} and $1.51 \pm 0.08 \times 10^8$ M^{-1} respectively, the differences not being significant. Tests were also carried out with other alkali metal chlorides (Table 1) and it can be seen that with the exception of Cs^+ and Tl^+ there was no specific effect of the ions. Both these latter ions had significantly greater inhibiting effect on antagonist binding. In a series of the sodium salts of univalent anions no significant differences were seen (Table 2).

Preliminary studies with a wide range of cations have shown that La^{3+} had greater effects on affinity than expected from ionic strength alone (Birdsall *et al.*, 1977). For instance La^{3+} at $\mu = 0.01$ reduced the binding of N-methyl scopolamine by a factor of 10 whereas this would require $\mu = 0.3$ to obtain the same effect with Na^+. The capacity for binding antagonists was reduced by millimolar concentrations of Hg^{2+}, Pb^{2+}, Cu^{2+}, Cd^{2+} and Sc^{3+}. In no case did the antagonist binding curves deviate from that predicted by a simple mass action relationship for a ligand binding to a uniform population of binding sites. This result was independent of whether antagonist binding was measured by filtration, microcentrifugation or equilibrium dialysis assay procedures.

Effects of ions on agonist binding

In Figure 2 are shown competition curves for the agonist carbachol with [^{3}H]-PrBCh measured in the presence of NaCl or KCl at concentrations $\mu = 0.02$ and 0.3. There is certainly no striking difference

Table 1 Effect of alkali metal cations on binding of propylbenzilylcholine (PrBCh)

	log k		
	$\mu = 0.02$	$\mu = 0.10$	$\mu = 0.30$
LiCl	8.58	8.23	7.99
NaCl	8.64	8.24	7.97
KCl	8.60	8.18	7.89
RbCl	8.61	8.11	7.89
CsCl	8.28	7.74	7.49
$TlNO_3$	8.14	7.54	—

Table 2 Effect of univalent anions on the binding of propylbenzilylcholine (PrBCh)

	log k	
	$\mu = 0.02$	$\mu = 0.10$
NaCl	8.63	8.21
NaF	—	8.22
NaBr	8.63	—
NaI	8.67	8.22
$NaNO_3$	8.68	8.20
$NaClO_4$	8.68	—
NaSCN	8.68	—
Na pyruvate	—	8.18
Na tosylate	—	8.24

Table 3 Effect of NaCl and KCl on carbachol binding*

	μ = 0.02		μ = 0.10		μ = 0.30	
	NaCl	KCl	NaCl	KCl	NaCl	KCl
log K_H	7.48	7.17†	6.95	6.64†	6.43	6.39
log K_L	5.06	4.97	4.47	4.39	3.99	3.95
log K_I	8.65	8.63	8.30	8.23	7.99	7.92
α	0.35	0.37	0.39	0.38	0.41	0.41

* K_H affinity constant for high affinity sites; K_L affinity constant for low affinity sites; K_I affinity for PrBCh; α fraction of total sites that are high affinity.
† Na/K difference significant: $P < 0.05$

between the effect of Na^+ and K^+ but it can be seen that carbachol displacement of [^{3}H]-PrBCh binding is less effective at higher salt concentrations.

Table 4 Effects of NaCl, $MgCl_2$ and $CaCl_2$ on carbachol binding

		% *displacement of* PrBCh *by carbachol*	
	μ	5×10^{-7} M	2×10^{-5} M
NaCl	0.003	24	69
$MgCl_2$	0.003	22	69
$CaCl_2$	0.003	27	69
NaCl	0.06	26	60
$MgCl_2$	0.06	29	70
$CaCl_2$	0.06	25	66

Binding studies of muscarinic agonists are complicated by the presence in the cortex of two major components, the high and low affinity sites (Birdsall *et al.*, 1978a, b) which can be resolved by suitable curve fitting programs. The results of such an analysis are seen in Table 3.

It can be seen that raising the salt concentration decreases the affinity at both the low and high affinity sites to a comparable extent, but to a greater extent than the affinity for the antagonists. The effects of K^+ are consistently greater than those of Na^+ on the high affinity site and these differences are significant for μ = 0.02, 0.10. On the other hand, there were no significant Na^+, K^+ differences on the low affinity site. However it can be seen from Figure 2 that the experimental differences from which these computed

Table 5 Effects of univalent cations and of anions on carbachol binding

		% *displacement of* PrBCh *by carbachol*		
	μ	5×10^{-7} M	2×10^{-5} M	6×10^{-5} M
LiCl	0.1	20	50	—
NaCl	0.1	23	51	—
KCl	0.1	20	51	—
RbCl	0.1	14	47	—
CsCl	0.1	22	50	—
$TlNO_3$	0.01	15	47	62
$TlNO_3$	0.1	9	17	32
NaCl	0.1	22	49	64
$NaNO_3$	0.1	20	56	72
$NaClO_4$	0.1	25	71	81
Na tosylate	0.1	27	69	82
Na acetate	0.1	14	50	70
Na isethionate	0.1	14	47	67
NaF	0.1	18	55	74
NaCl	0.1	26	54	69
NaBr	0.1	25	55	70
NaI	0.1	24	63	78

values were derived were small. There were also no significant changes in the fraction of the sites which were of the high affinity type.

The type of experiment illustrated in Figure 2 is unsuited for screening a large number of ions and for this purpose a competition between carbachol and PrBCh was measured at just two or three carbachol concentrations designed to test effects on (a) the high affinity site and (b) the combined high and low affinity sites. Results of this kind of test are shown in Tables 4 and 5. It can be seen first of all that no significant differences are seen between Na^+, Mg^{2+}, and Ca^{2+} on agonist binding. In the alkali metal series it is interesting that the higher activity of Cs^+ on antagonist binding is not present in agonist binding, but there is a very marked effect of Tl^+ which will be discussed in greater detail elsewhere. Amongst the anions there is evidence that ClO_4^- and tosylate have a selective effect on the low affinity site and that acetate and isethionate have a weaker activity on the high affinity site than Cl^- and most other anions. There is little indication of selectivity amongst the halide ions, minor modulations being produced by fluoride and iodide ions.

Discussion

The results obtained with the cations, Na^+, K^+, Ca^{2+} and Mg^{2+} show a small selective effect of K^+ on the binding of carbachol to the high affinity class of receptors. The effect is such as to reduce the affinity by a factor of two at the lower ionic strengths. There is possibly a very small selective effect of K^+ on antagonist binding. While the effect does meet the criteria discussed earlier in this paper, it is not sufficiently large to point at all conclusively to receptor coupling to a K^+ mechanism. It is interesting that it is selective for the high affinity receptor whereas other evidence has pointed to the low affinity receptor being the pharmacologically important form at least in smooth muscle.

It is also interesting that Tl^+, which is generally regarded as being very like K^+ in its properties, showed effects like K^+ but in much greater degree. At $\mu = 0.1$, K_H was reduced by a factor of 170 by Tl^+ with respect to Na, while the effect on K_L was a reduction by a factor of 22. Tl^+ also reduced the binding of the antagonist but to a lesser extent. By contrast Cs^+, which reduced the binding of the antagonist by a factor of 3 at $\mu = 0.1$, had no greater effect on the agonist binding than Na^+.

It is to be noted that in no cases were selective effects of Ca^{2+} seen, which is to be contrasted with the suggestive evidence that the nicotinic receptor includes a binding site for Ca^{2+} from which the ion is released by agonists (Chang & Neumann, 1976; Neumann & Chang 1976).

The effect of ions on the muscarinic receptor is disappointingly small compared to those found in opiate receptors where substitution of Na^+ for K^+ at $\mu = 0.1$ results in a reduction of affinity of morphine for one of the binding sites of approximately 100 fold whereas the affinity for the antagonist naloxone is increased by ~10% (Birdsall, *et al.*, 1976; Hulme 1977; Birdsall, Burgen & Hulme, unpublished results). The morphine receptor thus gives results that are in accord with the hypothesis presented in this paper.

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