Effects of Depolarizing Agents and Na^+-Channel Inhibitors on Ligand Binding to Muscarinic Receptors from Rat Cerebral Cortex

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In a vesicle preparation from rat cerebral cortex, carbachol recognizes a high-affinity and a low-affinity muscarinic agonist binding site. A number of agents, including veratridine (10 μM), gramicidin (10 μM) and valinomycin (10 μM), which depolarize the vesicles also appear to block the high-affinity muscarinic agonist binding site. Lowering $[Na^+]$ (from 137 to 80 mM) or raising $[K^+]$ (from 5 to 50 mM) produced effects similar to those of the depolarizing agents. Agents such as tetrodotoxin (1 μM), which block the Na^+-channel, also appear to block the high-affinity agonist binding site or to convert it into a low-affinity agonist binding form.

Occupancy of muscarinic receptors with agonists depolarizes the Renshaw cells[1,2] and hyperpolarizes the heart.[3] These opposite effects on the membrane potential may be mediated via opening of different ion-channels.

Muscarinic receptor-mediated potassium fluxes in smooth muscle,[4] and sodium fluxes in acinar cells[5] and in the parotid gland[6] have been reported. One could expect that the agents and ions which affect the channel which is "coupled" to the muscarinic receptor would influence the ligand-binding properties of the receptor.

Sodium ions, which have been found to have no specific effects on antagonist binding to muscarinic receptors,[7] can alter agonist binding in membranes from the heart[7] and medulla pons.[8] The presence of sodium ions (instead of $Tris^+$) in the buffer causes a conversion or blockade of the high-affinity agonist binding site, leaving the low-affinity agonist binding site intact.[7] In view of this very specific effect of sodium ions on muscarinic agonist binding it was of interest to study the effects of several drugs which activate or block activation of sodium channels. The present study provides data which suggest that interactions between the high-affinity muscarinic agonist binding site and a voltage-sensitive sodium channel may take place in a synaptic membrane preparation from rat cerebral cortex.

Materials and methods

[4,4']-[^{3}H]3-quinuclidinyl benzilate ([^{3}H]-3-QNB) (29.4 Ci mmol $^{-1}$ or 40.2 Ci mmol^{-1}) (an hallucinogenic substance) and [^{14}C]-tetraphenylphosphonium bromide ([^{14}C]-TPP) (19.2 mCi mmol^{-1}) were purchased from New England Nuclear Co., Boston, Mass. Veratridine was purchased from Aldrich, Belgium. All other chemicals were of reagent grade and were purchased from Sigma.

Cerebral cortex from the brains of male Sprague-Dawley rats (200–225 g) was excised and homogenized in 0.32 M sucrose at 695 rpm (15 up and down strokes with a loose-fitting glass–Teflon homogenizer). The resulting homogenate was centrifuged at 1000 × *g* for 5 min and the supernatant thus obtained was further centrifuged at 20000 × *g* for 40 min. The resulting pellet was resuspended in Krebs-Ringers buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM Na-HEPES, 10 μM phenylmethylsulfonyl fluoride, 5.6 mM glucose, pH = 7.4).

Equilibrium binding studies were carried out as described by Hedlund and Bartfai.[9] Specific binding was determined as the difference in binding of the labelled ligand in the absence and presence of atropine (10 μM). The ratio specific/nonspecific binding was about 6:1. Agonist binding was determined by measuring the competition between an unlabelled agonist (carbachol) (1–10 mM) and a labelled antagonist ([^{3}H]-3-QNB) (at a constant non-saturating concentration). Together with carbachol, *d*-tubocurarine (10 μM) was added to block the nicotinic actions of the agonist. This concentration of *d*-tubocurarine had no effect on [^{3}H]-3-QNB binding (data not shown). All incubations were carried out at room temperature for 60 min for [^{3}H]-3-QNB concentrations higher than 1 nM, and for 90 minutes for concentrations below 1 nM. These incubation times were sufficient to ensure saturation of the binding.

Antibiotics were dissolved in a small volume of ethanol and the solutions diluted with Krebs-Ringer's buffer. Ethanol was present in the incubation mixture at a final concentration of less than 0.1 %, which did not influence the results obtained.

Accumulation of [^{14}C]-TPP was measured in Krebs-Ringer's buffer at room temperature by incubating the membranes (0.6–0.8 mg protein) together with approximately 0.15 nmol ml^{-1} of [^{14}C]-TPP for 30–40 min. [^{14}C]-TPP did not affect [^{3}H]-3-QNB binding (data not shown). The reaction was stopped within seconds by filtration through Whatman GF/B glassfiber filters without further washing of the filters. The filters were dried and counted the next day at an efficiency of 30 %. The values obtained were compared by a two-tailed Student's t-test.

Fitting to the binding data was carried out on an IBM 360/75 computer using the non-linear regression program (BMDP3R, University of California, Los Angeles) described earlier.[10] Inhibition constants were calculated according to the definition of competitive inhibition [eqn. (1)] given by Cleland,[11] where Q = specifically bound ligand, V is the maximal binding capacity for ligand Q with concentration [Q], K_Q is the dissociation constant for the receptor-Q complex and [I] is the concentration of the inhibitor I which binds with the inhibition constant K_I.

$$v = \frac{V\,[Q]}{K_Q\,(1 + [I]/K_I) + [Q]} \qquad (1)$$

Results

Experiments with [^{14}C]-TPP indicated that this lipophilic anion accumulated in the membrane fraction which was used in the binding experiments. The accumulation was linearly dependent on the [^{14}C]-TPP concentration (data now shown) and on protein concentration, and was saturated after 40 min of incubation at room temperature (Fig. 1a).

Synaptosomes or vesicles from synaptic membranes have been shown to behave as K^+-electrodes.[12] It was therefore tested whether changes in [K^+] evoked changes in the accumulation of [^{14}C]-TPP (which is assumed to reflect changes in the membrane potential[13]). Fig. 1b shows that the amount of [^{14}C]-TPP which is accumulated by the membrane fraction decreases with increasing [K^+]. This indicates that a number of such vesicles are present which can change their membrane potential in response to changes in the ratio $[K^+]_{in}/[K^+]_{out}$.

Table 1 shows that several other agents which can influence the membrane potential in nerve cells also influence [^{14}C]-TPP accumulation in our preparation. Incubation with veratridine (10 μM), which activates sodium channels,[14] and with gramicidin (10 μM), a sodium ion ionophore,[15] or with valinomycin (10 μM), a potassium ion ionophore, led to a decrease in the amount of [^{14}C]-TPP which was accumulated by the membrane fraction. Tetrodotoxin (TTX) (1 μM), which itself had no effect on the accumulation of [^{14}C]-TPP, blocked the changes produced by veratridine, but not those caused by gramicidin or valinomycin.

The next step was to relate the results obtained with [^{14}C]-TPP to effects on muscarinic ligand binding. Fig. 2a shows that tetrodotoxin and veratridine both act as competitive inhibitors of the specific binding of [^{3}H]-3-QNB. The K_I values defined by eqn. (1) (cf. Ref. 11) were 0.25 μM and 0.17 μM, respectively. Binding of [^{3}H]-3-QNB was inhibited to a lesser extent by veratridine if TTX was present (Fig. 2a). Gramicidin and valinomycin also acted as competitive inhibitors with K_I values of 24 μM and 0.8 μM, respectively (Fig. 2b). Inhibition of [^{3}H]-3-QNB binding by these drugs was not affected by TTX (Fig. 2b).

The effects of veratridine and TTX on agonist binding are illustrated in Fig. 3a. This figure, to-

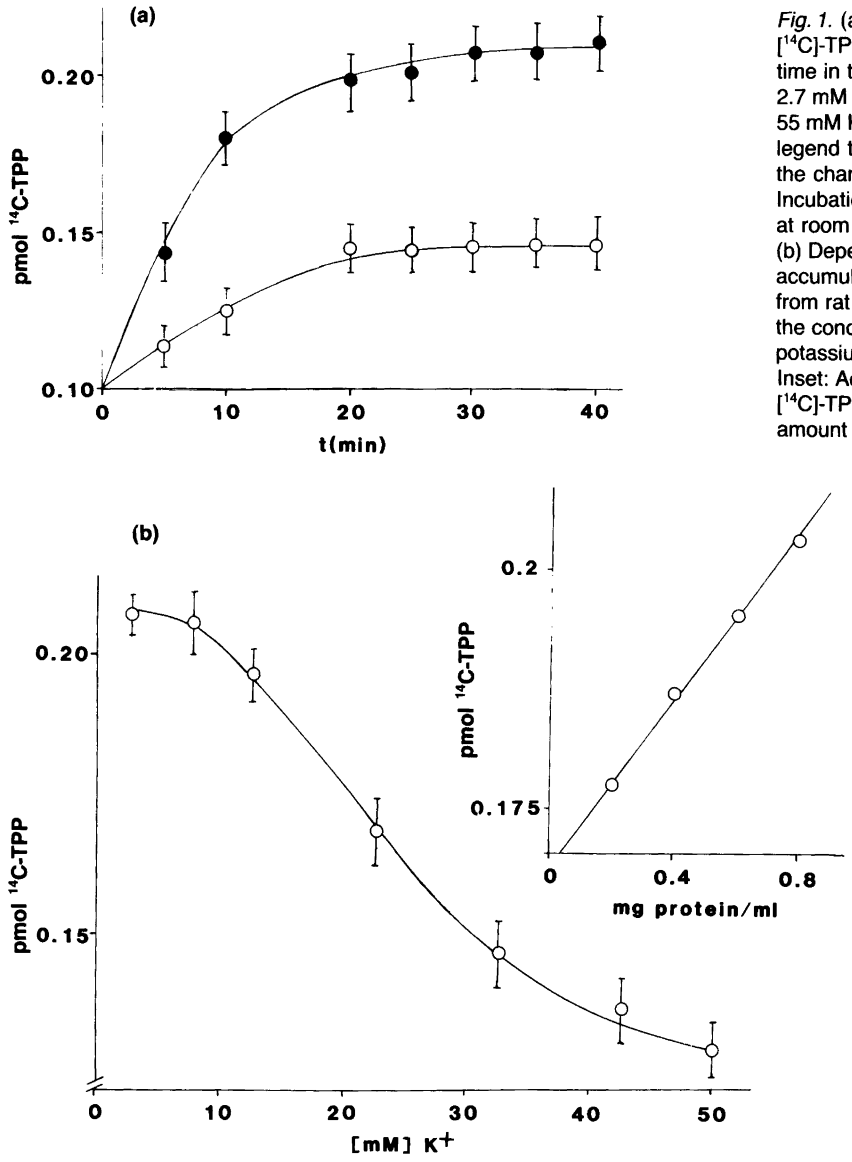

Fig. 1. (a) Accumulation of [^{14}C]-TPP as a function of time in the presence of 2.7 mM K^+ (●–●) and 55 mM K^+ (○–○) (see legend to Fig. 1b concerning the change in ionic strength). Incubation time was 40 min at room temperature.
(b) Dependence of [^{14}C]-TPP accumulation by membranes from rat cerebral cortex on the concentration of potassium in the medium. Inset: Accumulation of [^{14}C]-TPP as a function of the amount of protein present.

gether with the non-linear regression analysis of the binding data (Table 2), indicates that in the absence of these drugs, agonist (carbachol) binding can be described by assuming the presence of two binding sites with affinities of 2.8 μM and 75.8 μM, respectively. In the presence of these substances the agonist binding data fit equally well to a single binding isotherm which roughly corresponds to binding to the "low"-affinity agonist binding site. When the data were fitted to a model with two binding sites, either there was no improvement in the residual sum of squares, as compared by an F-test,[10] or parameter values were obtained which were negative or whose standard deviation was too high.[10] Thus, the data in Table 2 suggest that the high-affinity agonist binding site is blocked, abolished or converted to the low-affinity site in the presence of these

Table 1. The effects of drugs affecting the membrane potential on [^{14}C]-TPP accumulation to membranes from rat cerebral cortex.

Treatment	% [^{14}C]-TPP accumulated[a]
Control	100±3 (n=15)
Veratridine (10 μM)	40±2[b] (n=15)
Tetrodotoxin (1 μM)	103±3 (n=15)
Veratridine (10 μM) + tetrodotoxin (1 μM)	102±2 (n=10)
Gramicidin (10 μM)	72±2[b] (n=10)
Gramicidin (10 μM) + tetrodotoxin (1 μM)	70±2[b] (n=10)
Valinomycin (10 μM)	42±5[b] (n=5)
Valinomycin (10 μM) + tetrodotoxin (1 μM)	42±6[b] (n=5)

[a]100 % [^{14}C]-TPP accumulated corresponds to 9800±470 cpm per mg protein. [b]$p < 0.001$ as compared to control.

Table 2. Effects of tetrodotoxin, veratridine, gramicidin and valinomycin on carbachol binding to membranes from rat cerebral cortex.

Conditions	Equation	K_1/μM	K_2/μM	V_1/%[a]	V_2/%	No. of data points	Residual sum of squares/10^{-6}
Control	B	2.8±0.6	75.8±15.6	41.1±4.2	59.9±3.2	42	0.10
	A	40.8±6.9		100±4.1		42	0.51
+ TTX	B	11.4±12.7[b]	80.7±17.5	39.5±3.8	60.5±4.2	40	0.60
(1 μM)	A	73.2±12.7		100±4.1		40	0.64
+ Veratridine	B	0.3±0.7[b]	102.9±73.5	7.3±4.0	92.7±6.4	40	0.46
(10 μM)	A	70.9±9.3		100±4.2		40	0.43
Gramicidin	B	−0.9±0.4[c]	192.0±71.5	0.5±1.5[a]	99.5±1.5	36	2.12
(10 μM)	A	1000±177		100±5.0		36	2.12
Valinomycin	A	−0.1±0.1[c]	1028±166	4.0±4.0[a]	96.0±4.5	38	4.48
(10 μM)	B	908±207		100±5.0		38	4.48

[a]Total binding ($V_1 + V_2$) as measured with [^{3}H]-3-QNB at 0.2 nM concentration is slightly different from the control in the presence of the different agents because the affinity of receptors is changed. V_1 and V_2 are therefore expressed as a percentage of the actual [^{3}H]-3-QNB binding. [b]Parameter value rejected because of large standard deviation. [c]Parameter rejected because of negative parameter value. The binding was measured by competition between [^{3}H]-3-QNB (0.2 nM) and carbachol (1 nM−10 mM). Eqns. A and B were fitted to the data, where V_1 and V_2 are the binding capacities of the agonist binding sites with dissociation constants K_1 and K_2, respectively, and v equals the amount bound at carbachol concentration [L]:

Eqn. (A): $$v = \frac{V_1\,[L]}{K_1 + [L]}$$

Eqn. (B): $$v = \frac{V_1\,[L]}{K_1 + [L]} + \frac{V_2\,[L]}{K_2 + [L]}.$$

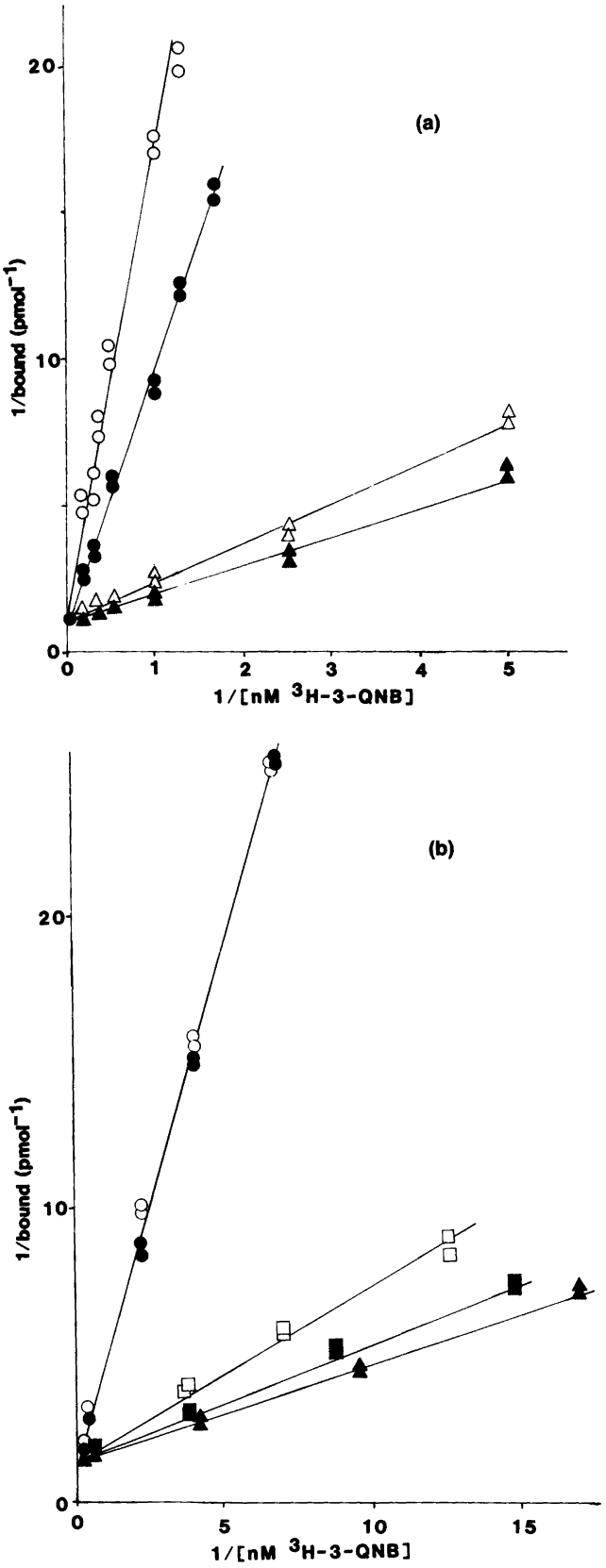

Fig. 2. (a) Inhibition of [^{3}H]-3-QNB binding to membranes from rat cerebral cortex by tetrodotoxin (0.1 μM) (△–△), veratridine (10 μM) (○–○) and tetrodotoxin+veratridine (●–●). (▲–▲) Control. (b) Inhibition of [^{3}H]-3-QNB binding to membranes from rat cerebral cortex by valinomycin (10 μM) (●–●), gramicidin (10 μM) (■–■), valinomycin+tetrodotoxin (0.1 μM) (○–○) and gramicidin+tetrodotoxin (0.1 μM) (□–□). (▲–▲) Control.

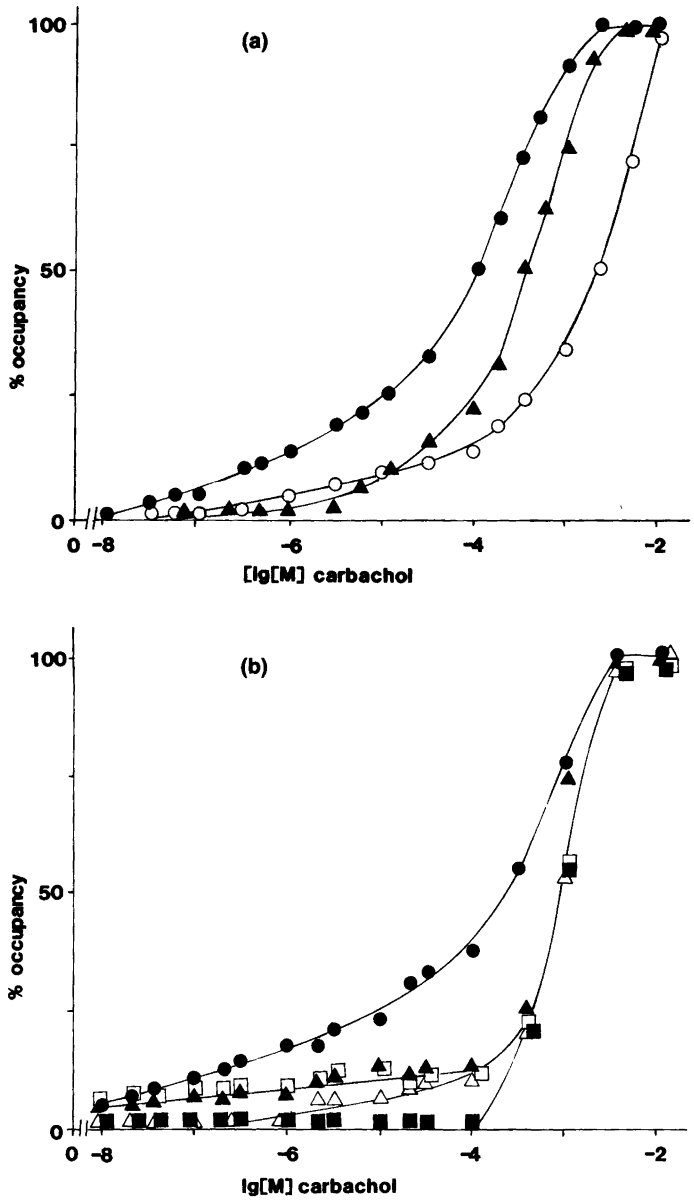

Fig. 3. (a) The effects of veratridine (10 μM) (○–○) and tetrodotoxin (0.1 μM) (▲–▲) on the ability of carbachol to compete with [^{3}H]-3-QNB (0.2 nM) for binding to membranes from rat cerebral cortex. 100 % occupancy corresponds to 0.10, 0.097 and 0.06 pmol bound ligand per mg protein for the control (●–●), tetrodotoxin and veratridine curves, respectively. (b) Influence of gramicidin (10 μM) (▲–▲), valinomycin (10 μM) (■–■), gramicidin+tetrodotoxin (0.1 μM) (△–△) and valinomycin+ tetrodotoxin (0.1 μM) (□–□) on the ability of carbachol to compete with [^{3}H]-3-QNB (0.2 nM) binding. 100 % occupancy corresponds to 0.10, 0.05, 0.08, 0.06 and 0.07 pmol ligand bound per mg protein for the control curve (●–●), valinomycin curve±tetrodotoxin and gramicidin curve±tetrodotoxin, respectively.

drugs. Studies with gramicidin and valinomycin indicated a similar loss of the high-affinity agonist binding site (Fig. 3b, Table 2), as with TTX and veratridine. TTX itself did not influence the effects of gramicidin and valinomycin.

Control experiments were performed to examine two aspects: Firstly, the possibility that the agents used affected residual [^{14}C]-TPP bound to filters, and via such interactions caused an apparent change in the amounts of [^{14}C]-TPP detected had to be ruled out. This was found not to be the case, i.e. the agents were acting on the membrane preparation to alter the [^{14}C]-TPP accumulation (data not shown). Secondly, if the agents were acting on [^{3}H]-3-QNB binding sites directly, effects would still be present in ruptured cell

preparations. Membranes were therefore frozen and thawed, and possible effects of the drugs on [^{3}H]-3-QNB binding were explored. None of the substances had any significant effect on [^{3}H]-3-QNB binding in this type of preparation, suggesting that vesicles are needed to obtain an effect on muscarinic receptor binding (data not shown).

The intracellular volume of synaptosomes has been estimated by Deutsch *et al.*[12] to be approximately 4 µl per mg protein. Using this value an equilibrium potential as measured with [^{14}C]-TPP was calculated. The extracellular concentration of [^{14}C]-TPP was 0.15 nmol ml^{-1}. Addition of KCl depolarizes the synaptosomes and causes a change in [^{14}C]-TPP accumulation of 0.1 pmol per mg protein (Fig. 1b). Using the Nernst equation[12,13] this leads to an estimated membrane potential of −104 mV. This is below the equilibrium potential for potassium, estimated to be around −70 mV.[12] As a consequence of this a potassium ionophore such as valinomycin should also be expected to depolarize the synaptosomes. This is, in fact, what is observed (Table 1). The volume of mitochondria in synaptosomal preparations has been estimated to be between 3 and 9%, and the maximum possible mitochondrial membrane potential to be −59 mV.[13] Even if one takes into account the fact that a membrane preparation is 2–3-fold less pure than a synaptosomal fraction,[14] the mitochondria do not play a major role in the total membrane potential measured with [^{14}C]-TPP.

Discussion

These data suggest that there is an interaction between the high-affinity muscarinic receptor form and sodium channels in the membrane, and that this receptor form senses the membrane potential. In the synaptic membrane preparation used here a sufficient number of vesicles are present with a given membrane potential. Agents such as veratridine, which activates sodium channels,[15] and ionophones[16] such as valinomycin and gramicidin, which can change this membrane potential, therefore caused a change in the accumulation of the [^{14}C]-TPP (Table 1); [^{14}C]-TPP has previously been used as a marker of membrane potential changes.[13] The membrane potential change induced by carbachol lasts for seconds,[17] and it is therefore difficult to make an accurate study of carbachol-induced changes in the accumulation of [^{14}C]-TPP. Some conclusions concerning whether there is an interaction between sodium channels and muscarinic receptors, and whether the receptors can sense the membrane potential across the membrane can, however, be drawn on the basis of ligand binding studies.

Veratridine, TTX, valinomycin and gramicidin are all also able to inhibit binding of the muscarinic antagonist [^{3}H]-3-QNB in a competitive manner (Figs. 2a and 2b). This indicates that a direct interaction of these drugs with the muscarinic receptor is possible. However, an inhibition of [^{3}H]-3-QNB binding based on interaction with the receptor or on general disruption of membranes should influence agonist binding to [^{3}H]-3-QNB binding sites uniformly. Thus, the ratio of high- and low-affinity binding sites to [^{3}H]-3-QNB binding sites should not change even though the total number of [^{3}H]-3-QNB binding sites decreases, since [^{3}H]-3-QNB itself does not distinguish between high- and low-affinity agonist binding sites.[9] The high- and low-affinity muscarinic agonist binding sites are, however, differentially inhibited by these agents (Figs. 3a and 3b). Non-linear regression analysis of the binding data indicated "disappearance" of the high-affinity agonist binding site (Table 2). Such observations on disappearance of high-affinity agonist binding sites have been reported using membranes from medulla pons[18] and heart.[19] In these instances GTP or Na^+ were suggested to cause an interconversion of the high- to the low-affinity agonist binding site. The molecular mechanism of this interconversion is, however, not clear. Again, another study reported no effects of Na^+ on agonist binding,[20] possibly as a result of the low binding assay temperature (0 °C) used. This may have prevented actions of proteins in the plane of the membrane. Yet another study[21] showed that the binding of phencyclidines was similarly influenced by monovalent cations; a shift towards lower-affinity binding also occurred in this case.[21]

Thus, it appears that the high-affinity muscarinic agonist binding site senses the membrane potential across the membrane as well as the activity state of sodium channels in the membrane, since depolarizing agents as well as sodium channel blocking agents (TTX) abolish binding to high-affinity agonist binding sites. This assumes some coupling between the high-affinity

site and a sodium channel in the membrane. It is reasonable to assume that sodium channels on vesicles not containing muscarinic receptors are also affected by veratridine and TTX, and that gramicidin and valinomycin affect the membrane potential of these vesicles also. This could, however, not be detected in the ligand binding studies. It could only be established that these drugs do not have a general membrane-disrupting effect on the vesicles containing muscarinic receptors. Whether the GTP-effect on muscarinic agonist binding observed in other brain regions and in the heart has a similar mechanisms of action could not be determined with our preparation, in which GTP does not affect agonist binding studied in competition with [^{3}H]-3-QNB.

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