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INHIBITION OF MUSCARINIC CHOLINERGIC RECEPTORS BY DISULFIDE REDUCING AGENTS AND ARSENICALS

DIFFERENTIAL EFFECT ON LOCUST AND RAT

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Abstract—Muscarinic receptors are altered by sulfhydryl reagents. Arsenic compounds, which have been used as insecticides, exert their toxic effects by combining with sulfhydryl groups. We compared the action of arsenicals and other sulfhydryl reagents on the muscarinic receptor from invertebrate and vertebrate species (locust and rat). Disulfide-reducing reagents dithiothreitol (DTT) and British Anti-Lewisite (BAL), but not arsenicals, inhibited [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding. However, after disulfide reduction, arsenicals caused a further inhibition of muscarinic binding. The effect of DTT + arsenicals was largely irreversible. The locust receptors were more sensitive to the action of both disulfide reagents either in the absence or presence of arsenicals than the rat receptors. The sulfhydryl reagent *p*-chloromercuric benzoate (PCMB) was more effective at inhibiting the locust receptors than the rat receptors, but addition of arsenicals did not cause further inhibition in either the locust or rat receptors. In locust, DTT + cacodylate and DTT + arsenite caused a reduction in the number of sites without modifying the affinity of [^{3}H]QNB binding. In rat, DTT + arsenite caused a decrease in the affinity, while DTT + cacodylate caused a decrease in the affinity of [^{3}H]QNB binding and its number of sites. Competition experiments after DTT + cacodylate showed that the IC_{50} and the Hill coefficient (n_H) remained unchanged in the locust. In the rat, the IC_{50} for atropine was increased without alteration in the n_H, and both parameters were increased for carbachol. These results are explained assuming that the binding site of the locust receptor has a disulfide group similar to that of the mammalian receptor, but that the hydrophobic interactions within the binding site are weaker in the locust receptor. The higher sensitivity of the insect receptor to sulfhydryl reagents could be of interest for developing methods of pest control.

Muscarinic cholinergic receptors (mAChRs) are a group of at least five homologous proteins [1–5] that belong to a family of structurally related receptors coupled to GTP-binding proteins [5]. mAChRs mediate a diversity of cellular responses, such as stimulation of phospholipid turnover [6–8], synthesis of cyclic GMP [9], ionic conductances [10, 11] and attenuation of adenylate cyclase [12]. These receptors are present in a variety of tissues in vertebrates [13], as well as in invertebrate species [14–16].

Vertebrate and invertebrate mAChRs have different properties. We have characterized previously the muscarinic binding sites of neural membranes from the locust *(Schistocerca gregaria)* [14]. We found that the locust mAChRs differ from the rat mAChRs in pharmacological properties, thermal stability and hydrophobic properties [14, 17].

Here we compare the effect of sulfhydryl compounds on the locust and rat mAChRs. These receptors are very sensitive to the action of sulfhydryl reagents both in insects [18] and vertebrates [19–22]. Arsenic compounds, which have been used as insecticides, exert their high toxicity by combining with sulfhydryl groups [23, 24]. However, their effects on the mAChR have not been reported. To further define the differences between invertebrate and vertebrate mAChRs, we have studied the effects of arsenic and other sulfhydryl reagents on the locust and rat mAChRs.

Our results show that disulfide reagent treatment inhibited [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding. Arsenicals alone did not modify the muscarinic binding properties, but they caused a further inhibition of [^{3}H]QNB binding after disulfide reduction. The locust receptors were more sensitive to the action of disulfide reagents, either in the absence or presence of arsenicals, than the rat receptors. This greater sensitivity may result from the weaker hydrophobic interactions in the locust mAChR [17], which makes disulfide links more important for keeping its active state.

MATERIALS AND METHODS

Membrane preparations

Locust. A total membrane fraction from the locust supraoesophageal ganglion was prepared as previously described [14]. Ganglia were removed from 100 locusts and homogenized in 4 mL of 1 mM EDTA, 2 mM benzamidine chloride, 50 mM Na^+,K^+ PO_4H_3 buffer, pH 7.4, at 0°. The homogenate was filtered through a nylon bolting cloth (160 μm mesh), and the filtrate was centrifuged at 120,000 *g* for 45 min. The pellet was resuspended in the buffer and stored at −80° until used.

Rat. Rats were killed by cervical dislocation and

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the gray matter of the cerebral cortex was separated, placed in cold 0.32 M sucrose, and homogenized. A crude synaptosomal membrane fraction was prepared according to De Robertis *et al.* [25]. This fraction was submitted to an osmotic shock and centrifuged at 20,000 *g* for 30 min. The pellet was resuspended in buffer and stored as above.

Protein content in the membrane fractions was determined by the method of Lowry *et al.* [26].

Binding assays

The binding of [^{3}H]QNB (30 Ci/mmol, Amersham International) to the locust membranes was carried out as previously described [14]. Aliquots (1 mL) of membranes containing 0.25 mg of protein were incubated at 25° for 60 min in the presence of different ligands. After incubation, samples were placed on ice for 5 min, filtered under vacuum through GF/B Whatman filters, and washed with 3 × 5 mL of ice-cold buffer. Filters were placed in scintillation vials, dried, and counted by scintillation spectroscopy. Non-specific binding was measured under the same conditions in the presence of 100 μM atropine. The binding to rat synaptosomal membranes was carried out as in Aguilar *et al.* [27] at 37° for 60 min in 4-mL aliquots containing 40 μg of protein. Samples were filtered and counted as above. Non-specific binding was measured in the presence of 25 μM atropine.

For the different treatments, membranes were incubated either at 25° (insect) or at 37° (mammalian) with the reagents and for the time indicated in each case (see Results). Controls were samples incubated in parallel under the same conditions without reagents. [^{3}H]QNB binding was then carried out as described.

Data analysis

In the case of single population of sites, data were analyzed by linear regression after Scatchard or Logit transformation [28]. When heterogeneity of sites was apparent, data were fitted by computer using a non-linear regression program [29] and the appropriate models as previously described in more detail [14]. The goodness of fit between models was compared with an F-test as described by Munson and Rodbard [30].

RESULTS

To assess the pharmacological and toxicological significance of the action of sulfhydryl reagents, we treated rat and locust membranes at different temperatures. With rat membranes, the treatment was carried out at physiological temperature (37°). Since the locust is a cold-blooded animal, we carried out membrane treatment at room temperature (25°).

Effect of arsenic compounds on [^{3}H]QNB binding

Initially, we tested the action of arsenical compounds on the muscarinic binding properties of locust and rat membranes. In both species [^{3}H]QNB binding was insensitive to membrane treatment with the inorganic arsenicals arsenite and arsenate, as well as to the organic derivative cacodylate (data not shown). Membrane treatment with these compounds caused no alteration in the displacement of this binding by the agonist oxotremorine (data not shown). Thus, arsenic compounds do not react with those sulfhydryl groups whose modification alters the binding of muscarinic ligands [20].

Effects of arsenic compounds after disulfide reduction on [^{3}H]QNB binding

We then analyzed the effects of arsenic compounds on [^{3}H]QNB binding after disulfide reduction. Since arsenicals have higher affinity for vicinal sulfhydryl groups [23, 24], reduced disulfide groups could be targets for arsenicals. For this reason, we studied the effects of arsenicals after disulfide reduction by dithiothreitol (DTT) and British Anti-Lewisite (BAL) treatment.

The locust receptor was more sensitive to both DTT and BAL treatment than the rat receptor. In locust, DTT inhibited [^{3}H]QNB binding in a concentration-dependent manner and a 50% inhibition was produced at 2.9 ± 0.4 mM (Fig. 1A). In contrast, only a 20% inhibition was observed on the rat receptor at 10 mM DTT (Fig. 1B). BAL caused 50% inhibition of the locust receptor at 50 ± 4 μM (not shown), but in the rat there was only 20% inhibition at 1 mM BAL (not shown).

Addition of arsenical compounds after disulfide reduction caused a further inhibition of the binding. Arsenite, arsenate and sodium cacodylate at 10 mM enhanced the inhibition of [^{3}H]QNB binding by DTT in locust and rat. In both species, cacodylate was the most effective and arsenate the least effective (Fig. 1, A and B). Cacodylate at 10 mM also increased the inhibitory effect of BAL in insect and in rat (not shown).

The action of DTT + arsenical treatment was partially irreversible. The inhibition of [^{3}H]QNB binding caused by DTT was reversed completely after its removal (Table 1). However, when membranes were treated with DTT and either arsenite or cacodylate, partial inhibition remained after washing. While the effect of arsenite was more irreversible than the effect of cacodylate in the locust, the contrary was true for the rat.

Effects of arsenicals after membrane treatment with non-disulfide reducing agents

To determine if the action of arsenical compounds took place after modification of disulfide groups or in association with other alterations of the receptor molecule, we studied their effects after sulfhydryl blockade and hydrophobic perturbations. The sulfhydryl reagent *p*-chloromercuric benzoate (PCMB) inhibited both locust and rat receptors, but its effect was greater in the locust. While PCMB caused 50% inhibition of [^{3}H]QNB binding to the locust receptor at 3.3 ± 0.5 μM, about 10-fold higher concentrations (32 ± 7 μM) were needed to cause the same degree of inhibition in the rat receptor (Fig. 2). This inhibition was not increased significantly by 100 mM cacodylate in either the locust or the rat receptors (IC_{50} 3.0 ± 0.4 μM in locust, and 27 ± 5 μM in rat). Ethanol, which alters hydrophobic interactions, inhibits both the locust and rat receptors [17]. When locust and rat membranes were treated with 10–100 mM cacodylate after treatment with

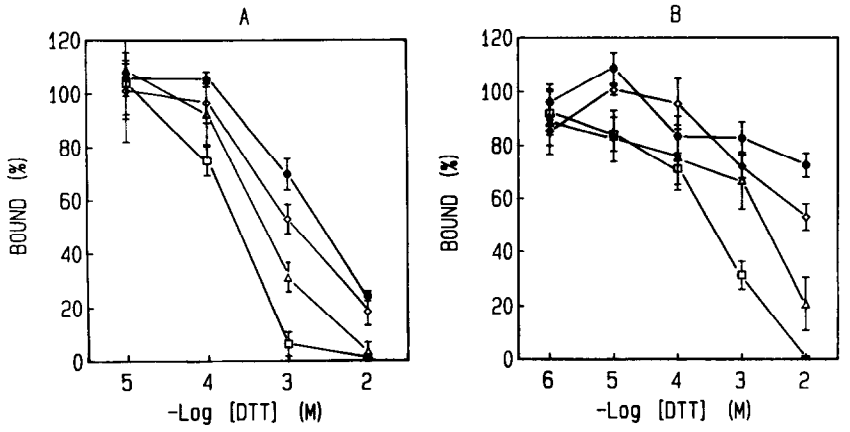

Fig. 1. Inhibition of [^{3}H]QNB binding by DTT and DTT–arsenical compound. (A) Locust head ganglion membranes were preincubated with different concentrations of DTT at 25° for 15 min and for a further 15 min in the absence (●) or in the presence of 10^{-2} M sodium arsenate (◇), 10^{-2} M sodium arsenite (△) or 10^{-2} M sodium cacodylate (□). Then the incubation with 1.5 nM [^{3}H]QNB was carried out for 1 hr. The 100% value was 115 ± 5 fmol/mg protein. (B) Rat cortex membranes were preincubated with different concentrations of DTT at 37° for 15 min and for a further 15 min in the absence (●) or in the presence of 10^{-2} M sodium arsenate (◇), 10^{-2} M sodium arsenite (△) or 10^{-2} M sodium cacodylate (□). Then [^{3}H]QNB was added to a final concentration of 0.08 nM, and the incubation was continued for 1 hr. The 100% value was 450 ± 9 fmol/mg protein. Data points are the means ± SEM of three separate experiments in duplicate.

Table 1. Reversibility of the inhibition of [^{3}H]QNB binding by DTT or DTT + arsenical compound in locust or rat membranes

	[^{3}H]QNB bound (%)			
	Insect		Mammal	
Treatment	Non-washed	Washed	Non-washed	Washed
DTT	24 ± 2	100 ± 1	73 ± 5	91 ± 6
DTT + sodium arsenite	4 ± 3	39 ± 4	20 ± 9	80 ± 1
DTT + sodium cacodylate	0 ± 1	59 ± 1	0 ± 1	36 ± 9

Crude membranes from insects or synaptosomal membranes from rats were preincubated with 10 mM DTT during 15 min and for a further 15 min in the presence or absence of a 10 mM concentration of the arsenical compound at 25° or 37° in locust and rat respectively. Controls were incubated simultaneously. After this treatment, part of the membranes was centrifuged and pellets were resuspended and washed three times with phosphate buffer, pH 7.4. Using aliquots of 1 mL (0.25 mg protein/mL) or 2 mL (0.01 to 0.02 mg protein/mL), binding assays were carried out with 1.5 or 0.08 nM [^{3}H]QNB in insects and mammals, respectively, for 1 hr. Values are the means ± SD of three separate experiments in duplicate.

400–500 mM ethanol, there was no significant (<7%) potentiation (data not shown). These results indicate that the effect of arsenicals takes place only after disulfide reduction.

Effect of DTT–arsenical treatment on the binding properties of [^{3}H]*QNB*

To determine if the inhibition of [^{3}H]QNB binding by DTT + arsenical treatment caused a reduction in the number of sites or decreased the affinity of the binding, we carried out saturation binding experiments. These experiments were done at concentrations of DDT + arsenical causing a 50% inhibition of [^{3}H]QNB binding. In the locust, [^{3}H]QNB binds to a heterogeneous population of sites [14]. We used [^{3}H]QNB concentrations (0.1 to 2.0 nM) that bind mostly to the high-affinity sites. At these concentrations the data did not fit a two-site model. Thus, equilibrium binding parameters were estimated for a single population of sites (Fig. 3).

DTT + arsenical treatment caused a different alteration of [^{3}H]QNB binding properties in the locust and the rat. In the locust, the number of sites was reduced to a similar extent when the membranes were treated with either DTT + arsenite or DTT + cacodylate, but the affinity was unaltered (Fig. 3). However, in the rat, treatment with DTT + arsenite

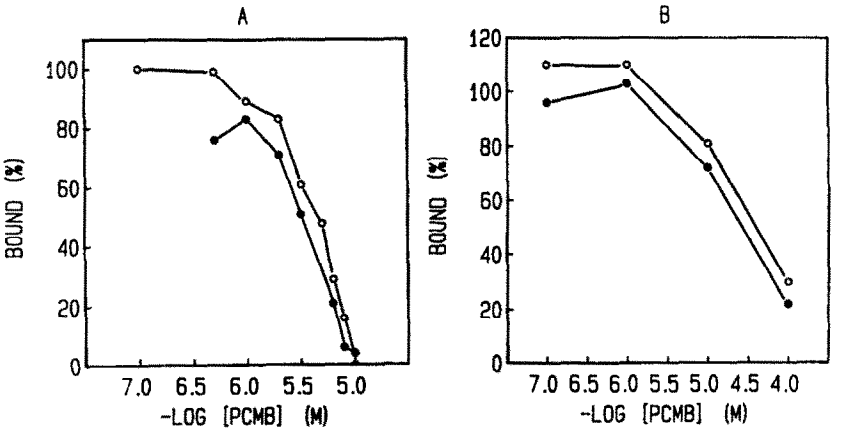

Fig. 2. (A) Inhibition of [^{3}H]QNB binding to locust head ganglion membranes by PCMB and PCMB + cacodylate. Membranes were preincubated with different concentrations of PCMB at 25° for 15 min and for a further 15 min in the absence (○) or in the presence (●) of 10 mM sodium cacodylate. The incubation with 1.5 nM [^{3}H]QNB was then carried out for 1 hr. The 100% value was 112 ± 6 fmol/mg protein. (B) Inhibition of [^{3}H]QNB binding to rat cortex membranes by PCMB and PCMB + cacodylate. Membranes were preincubated with different concentrations of PCMB at 37° for 15 min and for a further 15 min in the absence (○) or in the presence (●) of 10 mM sodium cacodylate. Then the incubation with 0.08 nM [^{3}H]QNB was carried out for 1 hr. The 100% value was 445 ± 8 fmol/mg protein. Data points are the means of three separate experiments in duplicate.

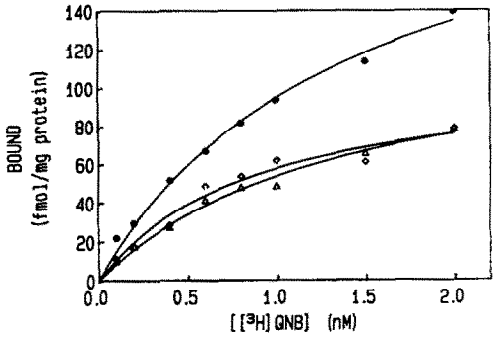

Fig. 3. Equilibrium binding isotherm of [^{3}H]QNB to locust head ganglion membranes. Membranes were preincubated at 25° for 15 min with 5×10^{-4} M DTT and for a further 15 min with 10^{-2} M sodium arsenite (◇), or with 2×10^{-4} M DTT and a further 15 min with 10^{-2} M sodium cacodylate (△). Control membranes were preincubated simultaneously in the absence of DTT and arsenicals in both cases (●). After this treatment different concentrations of [^{3}H]QNB from 0.1 to 2.0 nM were added. The incubation was carried out for 1 hr. Data points are the means of three separate experiments in duplicate. Parameters express the mean ± SD of three separate experiments in duplicate. B_{max} (fmol/mg protein): control = 180 ± 20, DTT + arsenite = 113 ± 15*, and DTT + cacodylate = 108 ± 20*. K_d (nM): control = 0.9 ± 0.3, DTT + arsenite = 1.0 ± 0.4, and DTT + cacodylate = 1.0 ± 0.4. Key: *$P < 0.01$.

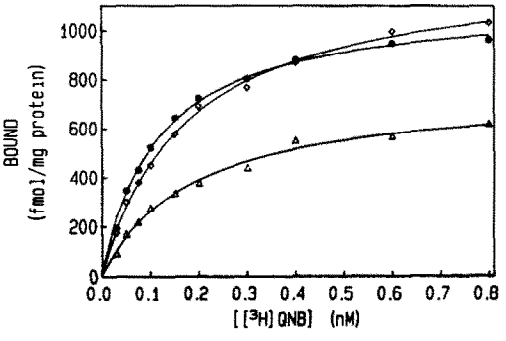

Fig. 4. Equilibrium binding isotherm of [^{3}H]QNB to rat cortex membranes. Membranes were preincubated at 37° for 15 min with 2×10^{-3} M DTT and for a further 15 min with 10^{-2} M sodium arsenite (◇), or with 2×10^{-4} M DTT and for a further 15 min with 10^{-2} M sodium cacodylate (△). Control membranes were preincubated simultaneously in the absence of DTT and arsenicals in both cases (●). After this treatment different concentrations of [^{3}H]QNB from 0.1 to 0.8 nM were added. The incubation was carried out for 1 hr. Data points are the means of three separate experiments in duplicate. Parameters express the mean ± SD of three separate experiments in duplicate. B_{max} (fmol/mg protein): control = 1200 ± 100, DTT + arsenite = 1250 ± 50, and DTT + cacodylate = 800 ± 100*. K_d (nM): control = 0.12 ± 0.02, DTT + arsenite = 0.18 ± 0.01**, DTT + cacodylate = 0.20 ± 0.02**. Key: *$P < 0.01$, and **$P < 0.005$.

reduced only the affinity of [^{3}H]QNB sites, whereas treatment with DTT + cacodylate affected both the affinity and the number of sites (Fig. 4).

Effect of DTT + cacodylate treatment on the binding properties of other muscarinic ligands

We further analyzed the effect of DTT + arsenicals on the muscarinic binding properties by competition experiments. The binding of [^{3}H]QNB was displaced by the antagonist atropine and the agonist carbamylcholine in control membranes and in membranes treated with DTT + cacodylate from both locust and rat. In agreement with saturation

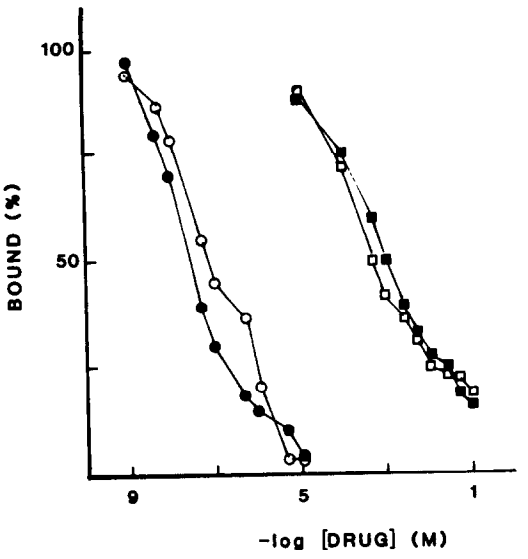

Fig. 5. Competition experiments of [^{3}H]QNB vs atropine (●, ○) or carbachol (■, □) in locust head ganglion membranes. Membranes were preincubated at 25° for 15 min with 2×10^{-4} M DTT and for a further 15 min with 10^{-2} M sodium cacodylate (○, □) or 30 min without both of them (●, ■). Then 1.5 nM [^{3}H]QNB and different concentrations of the competitors were added. The incubation was continued for 1 hr. The 100% value was 117 ± 6 fmol/mg protein. Data points are the means of three separate experiments in duplicate.

experiments, competition showed alteration in the binding affinity only in the rat mAChR.

In the locust, there was no alteration in the binding properties of atropine and carbachol (Fig. 5, Table 2). There was no significant variation in the IC_{50} for either ligand. As previously reported [14], both atropine and carbachol displaced [^{3}H]QNB binding to locust membranes with $n_H < 1$, indicating heterogeneity of sites. The n_H for atropine and carbachol was also unaffected by DTT + cacodylate treatment. Thus, there was no variation either in the proportion of sites with different affinities or in their corresponding affinities.

In contrast, the binding properties of both atropine and carbachol were altered in rat membranes (Fig. 6, Table 2). Treatment with DTT + cacodylate caused a decrease in the apparent inhibition constant (K_i) for both ligands. In vertebrates, the n_H is close to unity for atropine and near 0.5 for carbachol, indicating the presence of a single and a heterogeneous population of sites respectively [32]. After DTT + cacodylate treatment there was no change in the n_H atropine, but it increased for carbachol. Fitting the carbachol data for a two-site model showed a reduction in the number and in the affinity of the high-affinity sites (Table 3).

DISCUSSION

The results in this paper show that the locust mAChR was markedly more sensitive to the action of disulfide reagents, either in the absence or presence of arsenicals, than the rat mAChR. The locust receptor was also more sensitive than the rat receptor to the sulfhydryl reagent PCMB. These observations agree with our previous speculation that the binding site of the locust mAChR has a disulfide bond similar to that in the mammalian mAChR but it has weaker hydrophobic interactions [17].

To our knowledge, this is the first report of the action of arsenicals on mAChR binding to either insect or vertebrate. However, the action of sulfhydryl reagents has been studied in vertebrate receptors. The combination of mercury with sulfhydryl reagents causes inhibition of muscarinic binding. Methyl mercury and mercuric chloride inhibit antagonist binding [19]. Treatment of synaptosomal membranes with PCMB also causes an irreversible inhibition of antagonist binding [20]. In the present work, we also observed a complete inhibition of [^{3}H]QNB binding by PCMB in both locust and rat membranes. This suggests a conservation of the sulfhydryl group(s) whose modification may affect the binding properties.

Our results on the effect of treatment with disulfide reagents on muscarinic binding are in agreement with other reports. Shaker and Eldefrawi [18] also observed that DTT caused a greater inhibition of [^{3}H]QNB binding to insect (housefly) than to vertebrate (rat) membranes. Similarly, the membrane-bound receptor in vertebrates was very little affected by DTT treatment, and a reduction of agonist affinity was observed only at high pH and long incubation times [21]. However, the porcine receptor solubilized and purified is more sensitive to DTT [33]. In this case, low DTT concentrations caused a reduction in the affinity of antagonists and agonists, as well as in the number of high-affinity agonist sites. At higher concentrations, DTT also caused a reduction in the number of antagonist sites.

The greater sensitivity of locust mAChRs to disulfide reagents may result from their weaker hydrophobic interactions. The muscarinic receptor in mammals contains seven hydrophobic domains [1–4]. The onium group of muscarinic ligands interacts with an aspartic residue localized in hydrophobic domain III, that is brought close to hydrophobic domain IV by a disulfide bridge [34]. Since this disulfide bridge is very conserved in all mammalian mAChR subtypes, we may assume that it is also present in the insect receptors. Given the hydrophobicity of amino acid residues in domains III and IV [1], other interactions besides the disulfide link which maintain the configuration of the binding locus are very likely of the hydrophobic type. Thus, the cleavage of the disulfide bridge causes an almost complete inactivation of the locust receptors and only a reduction of the affinity in the rat receptors, possibly because the hydrophobic interactions are weaker in the locust. In explanation, we previously speculated that the locust binding site has a similar disulfide bond, but weaker hydrophobic interactions than the mammalian site [17].

Several observations support this speculation. The detergent solubilized mammalian receptor is more sensitive to DTT than the membrane bound receptor [33]. In the solubilized receptor, the detergent may

Table 2. Competition parameters for carbachol and atropine obtained by displacement of [^{3}H]QNB binding in insect and mammalian membranes treated with DTT + cacodylate (DDT + C)

(A) Locust	IC_{50} (M)		n_H
Carbachol			
Control	$(1.0 \pm 0.2) \times 10^{-3}$		0.40 ± 0.04
DTT + C	$(0.9 \pm 0.3) \times 10^{-3}$		0.40 ± 0.06
Atropine			
Control	$(4.1 \pm 1.3) \times 10^{-8}$		0.60 ± 0.09
DTT + C	$(8.8 \pm 1.7) \times 10^{-8}$		0.60 ± 0.07
(B) Rat	IC_{50} (M)	K_i app (M)	n_H
Carbachol			
Control	$(5.7 \pm 1.9) \times 10^{-4}$	$(3.4 \pm 1.1) \times 10^{-4}$	0.51 ± 0.04
DTT + C	$(9.6 \pm 1.3) \times 10^{-4}$*	$(6.9 \pm 0.9) \times 10^{-4}$*	0.71 ± 0.02*
Atropine			
Control	$(0.77 \pm 0.09) \times 10^{-8}$	$(0.46 \pm 0.05) \times 10^{-8}$	1.18 ± 0.04
DTT + C	$(1.56 \pm 0.11) \times 10^{-8}$†	$(1.11 \pm 0.08) \times 10^{-8}$†	1.11×0.07

For experimental details see Figs. 5 and 6. The K_i values were calculated from the following equation: $K_i = [(IC_{50})/(1 + [^3H]QNB/K_d)]$ [31], where IC_{50} = concentration of the drug displacing 50% of the specific binding of [^{3}H]QNB; and K_d = dissociation constant of [^{3}H]QNB. Results are the means ± SD of three separate experiments in duplicate.

* $P < 0.01$.

† $P < 0.001$.

reduce the hydrophobic interactions and the cleavage of the disulfide bond has a greater effect. These interactions may be weaker in the locust receptor

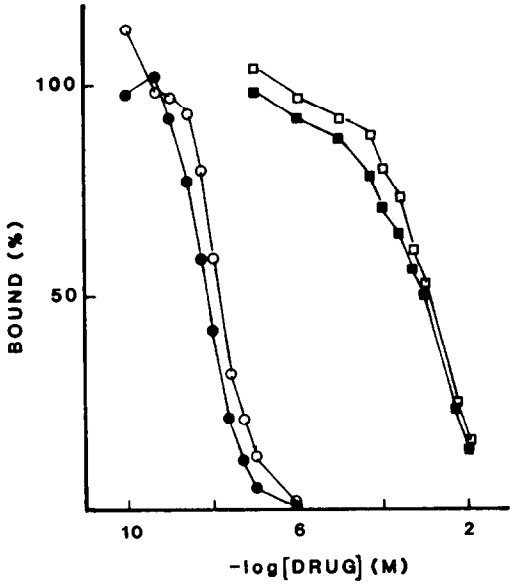

Fig. 6. Competition experiments of [^{3}H]QNB vs atropine (●, ○) or carbachol (■, □) in rat cortex membranes. Membranes were preincubated at 37° for 15 min with 2×10^{-4} M DTT and for a further 15 min with 10^{-2} M sodium cacodylate (○, □), or 30 min with neither DTT nor cacodylate (●, ■). Then 0.08 nM [^{3}H]QNB and different concentrations of the competitors were added and the incubation was continued for 1 hr. The 100% value was 463 ± 7 fmol/mg protein. Data points are the means of three separate experiments in duplicate.

Table 3. Computerized parameters for carbachol binding to mammalian membranes

	K_H (μM)	K_L (μM)	B_H (%)
Control	1.04	586	17.5
DTT + cacodylate	2.40	786	10.4

Values were obtained by computerized analysis of the data from the competition experiments of carbachol vs [^{3}H]QNB in rat membranes shown in Fig. 6. K_H = high-affinity dissociation constant; K_L = low-affinity dissociation constant; and B_H = percentage of sites with high-affinity dissociation constant.

because it is more sensitive than the rat receptor to the action of ethanol, which is known to alter the hydrophobic interactions of membrane proteins. In addition, the disulfide reagent BAL, which is more effective than DTT in inactivating the locust mAChR, is also more hydrophobic. Thus, besides cleaving the disulfide bridge, BAL may alter the hydrophobic interactions of the binding site. Finally, weaker hydrophobic interactions in the binding site of the locust receptor can also explain its greater inhibition by PCMB. The site of action of this compound may be one of the cysteine residue located in a hydrophobic domain, possibly near the binding site [1]. The covalent modification of this group by PCMB may alter both the hydrophobic interactions and the packaging of the binding locus.

The enhancement of muscarinic binding inhibition by arsenicals after disulfide reduction may be caused by a further alteration of the configuration of the binding site by these compounds. Once the disulfide bridge is reduced, arsenicals can react with the

resultant vicinal sulfhydryls. Accordingly, there is no potentiation of the action of PCMB or ethanol by arsenicals. The covalent binding of arsenicals may alter both the package and the interactions of the amino acid residues in the binding site. Thus, cacodylate, which is both the more hydrophobic and the more bulky arsenical, causes the greatest alteration. The covalent modification of the sulfhydryls may also cause the irreversible inhibition of [^{3}H]QNB binding after DTT–arsenical treatment. However, part of the inhibition is reversed after washing. This can be explained by assuming that some free arsenical molecules or their complex with DDT [23, 24] may have been interposed in the binding locus.

A binding site environment containing a disulfide bond similar to that found in mammals but with weaker hydrophobic interactions may be a general feature of insect mAChRs. Recently, the sequence of a putative mAChR gene from the *Drosophila* has been reported [15]. This sequence is similar to that of the mammalian receptors with seven putative transmembrane domains and conservation of the cysteine residues that can form the disulfide bond joining transmembrane domains III and IV. The aspartic acid residues that are the potential interaction site of the amino group of muscarinic ligands [34, 35] are also conserved in the transmembrane domain III. Interestingly, the transmembrane domain IV in the *Drosophila* receptor is less hydrophobic than in the mammalian receptor. Thus, it is likely that the mAChR binding site in several insect species includes transmembrane domains III and IV joined by a disulfide bond, but with weaker hydrophobic interactions.

Our results suggest that there is conservation of disulfide groups between mAChRs in vertebrate and invertebrate species. However, there may be significant differences between both species in the hydrophobic interactions involved in the formation of the binding locus. These differences may be of interest for the development of methods for insect control. Given the vital role of cholinergic systems, muscarinic receptors are the target of many insecticides [36]. Because these insecticides also have high toxicity for other species, a better knowledge of the differences between insect and vertebrate receptors may help in finding substances with more selective toxicity.

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REFERENCES

1. Bonner TI, The molecular basis of muscarinic receptor diversity. *Trends Neurosci* **12**: 148–151, 1989.
2. Liao C-F, Themen APN, Joho R, Barberis C, Birnbaumer M and Birnbaumer L, Molecular cloning and expression of a fifth muscarinic acetylcholine receptor. *J Biol Chem* **264**: 7328–7337, 1989.
3. Kubo T, Fukuda K, Mikami A, Maeda A, Takahashi H, Mishina M, Haga T, Haga K, Ichiyama A, Kangawa K, Kojima M, Matsuo H, Hirose T and Numa S, Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. *Nature* **323**: 411–416, 1986.
4. Peralta EG, Winslow JW, Peterson GL, Smith DH, Ashkenazi A, Ramachandran J, Schimerlik MI and Capon DJ, Primary structure and biochemical properties of an M2 muscarinic receptor. *Science* **236**: 600–605, 1987.
5. O'Dowd BF, Lefkowitz RJ and Caron MG, Structure of the adrenergic and related receptors. *Annu Rev Neurosci* **12**: 67–83, 1989.
6. Claro E, Wallace MA, Lee H-M and Fain JN, Carbachol in the presence of guanosine 5′-*O*-(3-thiotriphosphate) stimulates the breakdown of exogenous phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 4-phosphate, and phosphatidylinositol by rat brain membranes. *J Biol Chem* **264**: 18288–18295, 1989.
7. Martinson EA, Goldstein D and Brown JH, Muscarinic receptor activation of phosphatidylcholine hydrolysis. Relationship to phosphoinositide hydrolysis and diacylglycerol metabolism. *J Biol Chem* **264**: 14748–14754, 1989.
8. Diaz-Meco MT, Larrodera P, Lopez-Barahona M, Cornet ME, Barreno PG and Moscat J, Phospholipase C-mediated hydrolysis of phosphatidylcholine is activated by muscarinic agonists. *Biochem J* **263**: 115–120, 1989.
9. El-Fakahani E and Richelson E, Involvement of calcium channels in short-term desensitization of muscarinic receptor-mediated cyclic GMP formation in mouse neuroblastoma cells. *Proc Natl Acad Sci USA* **77**: 6897–6901, 1980.
10. Yatani A, Codina J, Brown M and Birnbaumer L, Direct activation of mammalian atrial muscarinic potassium channels by GTP regulatory protein G_k. *Science* **235**: 207–211, 1987.
11. Fukuda K, Higashida H, Kubo T, Maeda A, Akiba I, Bujo H, Mishina M and Numa S, Selective coupling with K^+ currents of muscarinic acetylcholine receptor subtypes in NG108-15 cells. *Nature* **335**: 355–358, 1988.
12. Ehlert FJ, Delen FM, Yun SH, Friedman DJ and Self DW, Coupling of subtypes of the muscarinic receptor to adenylate cyclase in the corpus striatum and heart. *J Pharmacol Exp Ther* **251**: 661–671, 1989.
13. Peters NL, Snipping the thread of life. Antimuscarinic side effects of medication in the elderly. *Arch Intern Med* **149**: 2412–2420, 1989.
14. Aguilar JS and Lunt GG, Cholinergic binding sites with muscarinic properties on membranes from the supraoesophageal ganglion of the locust *(Schistocerca gregaria) Neurochem Int* **6**: 501–507, 1984.
15. Onai T, Fitzgerald MG, Arakawa S, Gocayne JD, Urquhart DA, Hall LM, Fraser CM, McCombie WR and Venter JC, Cloning sequence analysis and chromosome localization of a *Drosophila* muscarinic acetylcholine receptor. *FEBS Lett* **225**: 219–225, 1989.
16. Shapiro RA, Wakimoto BT, Subers EM and Nathanson NM, Characterization and functional expression in mammalian cells of genomic and cDNA clones encoding a *Drosophila* muscarinic acetylcholine receptor. *Proc Natl Acad Sci USA* **86**: 9039–9043, 1989.
17. Aguilar JS, Fonseca MI and Lunt GG, Differential effect of ethanol on muscarinic cholinergic binding to rat and locust neural membranes. *Neurochem Res* **14**: 765–770, 1989.
18. Shaker N and Eldefrawi A, Muscarinic receptor in house fly brain and its interactions with chlorobenzilate. *Pestic Biochem Physiol* **15**: 14–20, 1981.
19. Abd-Elfattah ASA and Shamoo AE, Regeneration of a functionally active rat brain muscarinic receptor by D-penicillamine after inhibition with methylmercury and mercuric chloride: Evidence for essential sulfhydryl

groups in muscarinic receptor binding sites. *Mol Pharmacol* **20**: 492–497, 1981.
20. Aronstam RS, Abood LG and Hoss W, Influence of sulfhydryl reagents and heavy metals on the functional state of the muscarinic acetylcholine receptor in rat brain. *Mol Pharmacol* **14**: 575–586, 1978.
21. Aronstam RS and Eldefrawi ME, Reversible conversion between affinity states for agonists of the muscarinic acetylcholine receptor from rat brain. *Biochem Pharmacol* **28**: 701–703, 1979.
22. Birdsall NJM, Burgen ASV, Hulme EC and Wong WHF, The effects of *p*-chloromercuribenzoate on muscarinic receptors in the cerebral cortex. *Br J Pharmacol* **80**: 187–196, 1983.
23. Knowles FC, The enzyme inhibitory forms of inorganic arsenic. *Biochem Int* **4**: 647–653, 1982.
24. Knowles FC and Benson AB, The biochemistry of arsenic. *Trends Biol Sci* **8**: 178–180, 1983.
25. De Robertis E, Alberici M, Rodriguez de Lores Arnaiz G and Azcurra JM, Isolation of different types of synaptic membranes from the brain cortex. *Life Sci* **5**: 577–582, 1966.
26. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
27. Aguilar JS, Salas PJI and De Robertis E, Cholinergic muscarinic receptor sites in synaptosomal membranes. *Mol Pharmacol* **22**: 304–309, 1982.
28. Aguilar JS, Ochoa ELM and De Robertis E, Reconstitution of solubilized atrial cholinergic muscarinic receptors into liposomes. *Neurochem Res* **12**: 83–91, 1986.
29. Gill PE and Murray W, Algorithms for the solution of the non-linear least squares problem. *SIAM J Numerical Analysis* **15**: 977–992, 1978.
30. Munson PJ and Rodbard D, Ligand: A versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **107**: 220–239, 1980.
31. Cheng Y-C and Prusoff WH, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099–3108, 1973.
32. Birdsall NJM, Burgen ASV and Hulme EC, The binding of agonists to brain muscarinic receptors. *Mol Pharmacol* **14**: 723–736, 1978.
33. Berstein G, Haga K, Haga T and Ichiyama A, Agonist and antagonist binding of muscarinic acetylcholine receptor purified from porcine brain: Interconversion of high- and low-affinity sites by sulfhydryl reagents. *J Neurochem* **50**: 1687–1694, 1988.
34. Curtis CM, Wheatley M, Bansal S, Birdsall NJ, Eveleigh P, Padder EK, Poyner D and Hulme EC, Propylbenzilylcholine mustard labels and acidic residue in the transmembrane helix 3 of the muscarinic receptor. *J Biol Chem* **254**: 489–495, 1989.
35. Fraser CM, Wang C-D, Robinson DA, Gocayne JD and Venter JC, Site-directed mutagenesis of m_1 muscarinic acetylcholine receptors: Conserve aspartic acids play important roles in receptor function. *Mol Pharmacol* **36**: 840–847, 1989.
36. Eldefrawi ME and Eldefrawi AT, Neurotransmitter receptors as targets for pesticides. *J Environ Sci Health* **B18**: 65–88, 1983.