Muscarinic Antagonist Binding to Intact Rat Thymocytes

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The presence and properties of muscarinic receptors on intact rat thymocytes have been studied by the use of the muscarinic antagonists [^{3}H]-3-quinuclidinyl benzilate (^{3}H-QNB), [^{3}H]-4-*N*-methylpiperidinyl benzilate ([^{3}H]-NMPB) and [^{3}H]-*N*-methylscopolamine (^{3}H-NMS). The course of binding of ^{3}H-QNB reveals a maximum at 5 min, and shows a subsequent decrease of bound radioactivity, suggesting internalization of the receptor ^{3}H-3-QNB complex. This phenomenon has also been studied by the use of another muscarinic antagonist, ^{3}H-NMPB, which has a faster on and off rate than ^{3}H-QNB, and which may be rapidly displaced by an excess of unlabeled muscarinic antagonist, atropine. Bound ^{3}H-NMPB is highly susceptible to atropine displacement only within the first two minutes of incubation with thymocytes at 37 °C. The kinetics of binding of the less lipophilic muscarinic antagonist ^{3}H-NMS to thymocytes, show no maximum as a function of incubation time and ^{3}H-NMS is susceptible to displacement by atropine up to 30 min incubation time with the thymocytes. These data suggest that binding of lipophilic benzilate type antagonists (^{3}H-QNB and ^{3}H-NMPB) may be followed by internalization and/or isomerization of the receptor–benzilate antagonist complex while the ^{3}H-NMS–receptor complex is not subject to these processes.

Acetylcholine and cholinergic agonists are capable of modulating several functions of the immune system such as cytotoxicity,[1–4] migration[5] and proliferation of lymphocytes[6,7] and antibody production.[8] Most of these cholinergic responses are blocked or significantly reduced in the presence of the specific muscarinic antagonist atropine, suggesting the existence of functional muscarinic cholinergic receptors on lymphocytes.

For the study of the presence and equilibrium binding properties of muscarinic receptors on lymphocytes and thymocytes, the tritiated muscarinic antagonist [^{3}H]-3-quinuclidinyl benzilate (^{3}H-QNB) has been used.[10–14] These binding studies have confirmed the existence of muscarinic receptors on mouse,[14] rat[10,11,13] and human[14] lymphocytes, and revealed that their expression depends on such factors as the method of cell isolation,[14] composition[11] and temperature[12,15] of the incubation medium and the time after activation of the cells by mitogens.[14]

Unusual dependence of ^{2}H-QNB binding to lymphocytes and thymocytes on the incubation time was found, showing a maximum at 5 min and subsequent decrease of specifically bound radioactivity.[11,12] This course of binding with time was suggested to be the result of two simultaneously ongoing, opposite processes: (a) an increase of specifically bound radioactivity involved in saturation of all receptor sites, since binding equilibrium is not reached within 5 min; (b) a decrease in bound radioactivity resulting from the disappearance of receptor–ligand complexes from lymphocytes.[11] However, our further studies, revealing a 'normalization' of binding kinetics at a lower temperature (4 °C), suggest that after surface binding, the receptor–ligand complex may be internalized, degraded and the ^{3}H-label released from lymphocytes. The receptor–ligand complex was susceptible to protease digestion only within the first 2–5 minutes of incubation which also seemed to corroborate this assumption.[15]

Our present studies were carried out by the use of two additional muscarinic antagonists: [^{3}H]-*N*-methylpiperidyl benzilate (^{3}H-NMPB), another benzilate with a more rapid on and off rate than ^{3}H-QNB, which could be rapidly displaced by atropine, and the less lipophilic [^{3}H]-*N*-methylscopolamine (^{3}H-NMS). Previous studies have shown that the receptor–benzilate antagonist complex can undergo isomerization[16] while this has not been reported for the receptor–NMS complex. The results below suggest that receptor–benzilate antagonist complexes may internalize subsequent to binding of the ligand at the cell surface.

Experimental

Chemicals. Atropine sulfate was obtained from Calbiochem (Los Angeles, CA), [^{3}H]-*N*-methylscopolamine methyl chloride (79.6 Ci mmol^{-1}), [^{3}H]quinuclidinyl benzilate (33.2 Ci mmol^{-1}), and Nuclear Chicago Solubilizer (NCS) were from Amersham International (Bucks, GB), [^{3}H]-*N*-methylpiperidyl benzilate (73.7 Ci mmol^{-1}) was a gift from Professor M. Skolovsky, Tel Aviv University, Israel, RPMI 1640 medium was from Flow Laboratories (Irvine, Scotland).

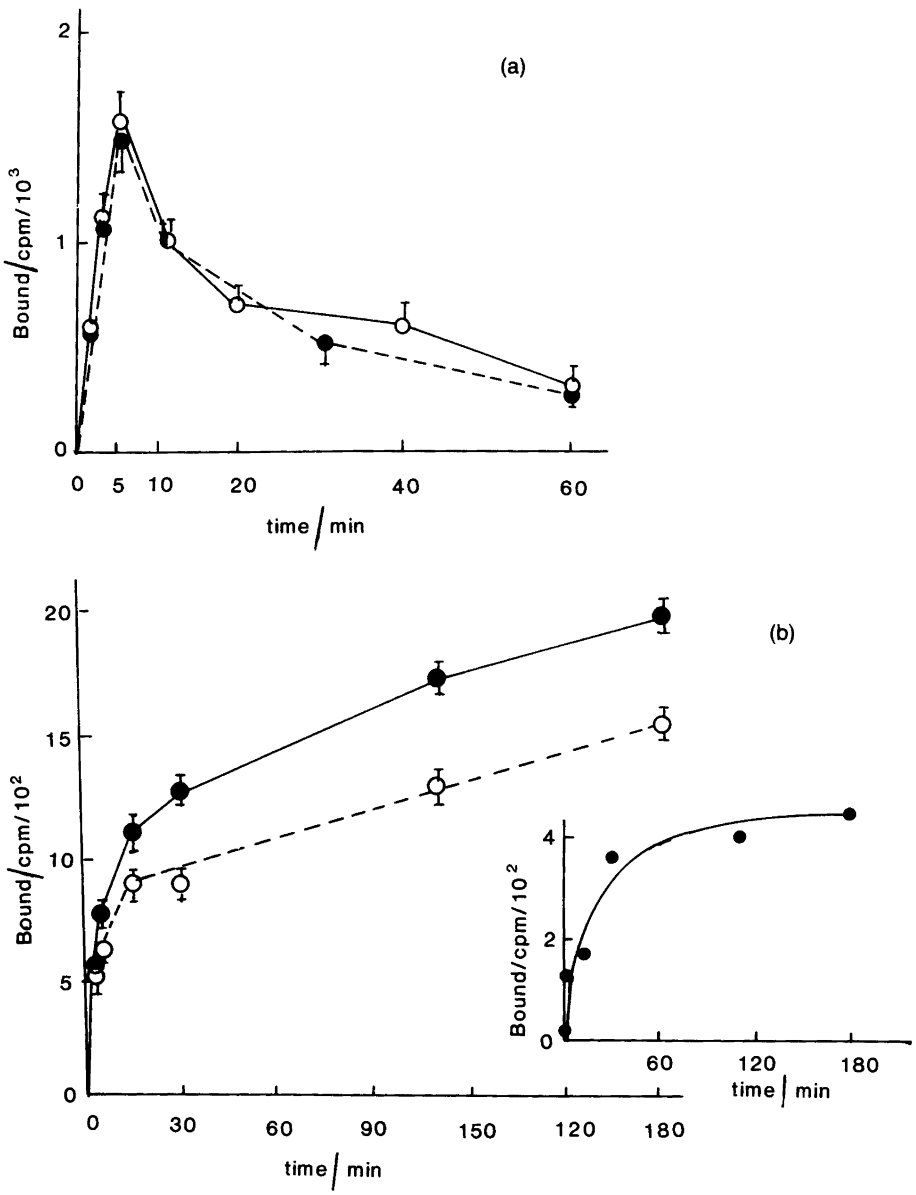

Fig. 1 (a) Kinetics of specific binding of ^{3}H-QNB (○) and ^{3}H-NMPB (●) to thymocytes. Thymocytes were incubated under standard assay conditions with 30 nM of either ^{3}H-QNB or ^{3}H-NMPB in a total volume of 0.2 ml of RPMI 1640 medium. At subsequent time intervals reaction was stopped by the addition of 2 ml cold RPMI and immediate centrifugation at 300 *g* for 5 min. Radioactivity of the pellets was counted. The points represent means of triplicate determinations carried out in the absence of atropine. Bars indicate ± SD of the means; (b) Kinetics of ^{3}H-NMS binding to rate thymocytes. Thymocytes (2×10^6 cells per 0.2 ml RPMI medium) equilibrated for 30 min at 37 °C were incubated with 20 µl of either 10^{-4} M atropine ['unspecific' (○)] or PBS ['total' (●)] followed by the addition of ^{3}H-NMS (final concentration 30 nM). The reaction was stopped by the addition of 2 ml of cold RPMI 1640 medium and immediate centrifugation. Radioactivity of pellets was measured (see the Experimental). The insert represents 'specific' (●) binding calculated as a difference between 'total' and 'unspecific' binding.

Animals. Male Sprague–Dawley rats, 2 months old (200 g) and inbred Wistar rats (200 g) were used, obtained from ALAB, Södertälje.

Cells. The rats were killed by decapitation and the thymuses removed. Thymocytes were obtained by teasing the tissue in serum-free RPMI 1640 culture medium. The cells were incubated for 30 min at 37 °C in RPMI medium in 5 % v/v CO_2 atmosphere before being cooled to 4 °C for the binding study. The cells were resuspended at a concentration of 20×10^6 cells ml^{-1} for the radioligand binding studies.

Radioligand binding studies. The thymocyte cell suspension (4×10^6 cells per 0.2 ml RPMI 1640 medium or 10×10^6 cells per 0.2 ml RPMI 1640) was first equilibrated in an atmosphere of 5 % CO_2, 100 % humidity, at 37 °C for 30 min and the incubation was then started by the addition of 20 µl of either 10^{-4} M (final concentration 10^{-5} M) atropine sulfate (unspecific binding) or PBS (total binding), followed by addition of 20 µl of ^{3}H-NMS (final concentration 30 nM), ^{3}H-4-NMPB (0–30 nM) or ^{3}H-QNB (30 nM). The binding reaction was stopped at different time intervals by the addition of 2 ml cold RPMI 1640 medium followed by immediate centrifugation ($2000\times g$, 30 s). Radioactivity of pellets was measured in toluene scintillation mixture at an efficiency of 35 % after solubilization with 0.5 ml of NCS (16 h, 37 °C).

Specific binding was calculated as the difference between total and unspecific binding and studied as the function of incubation time and ligand concentration.

Displacement experiments. The thymocyte cell suspension (4×10^6 cells per 0.2 ml RPMI 1640 medium) was incubated

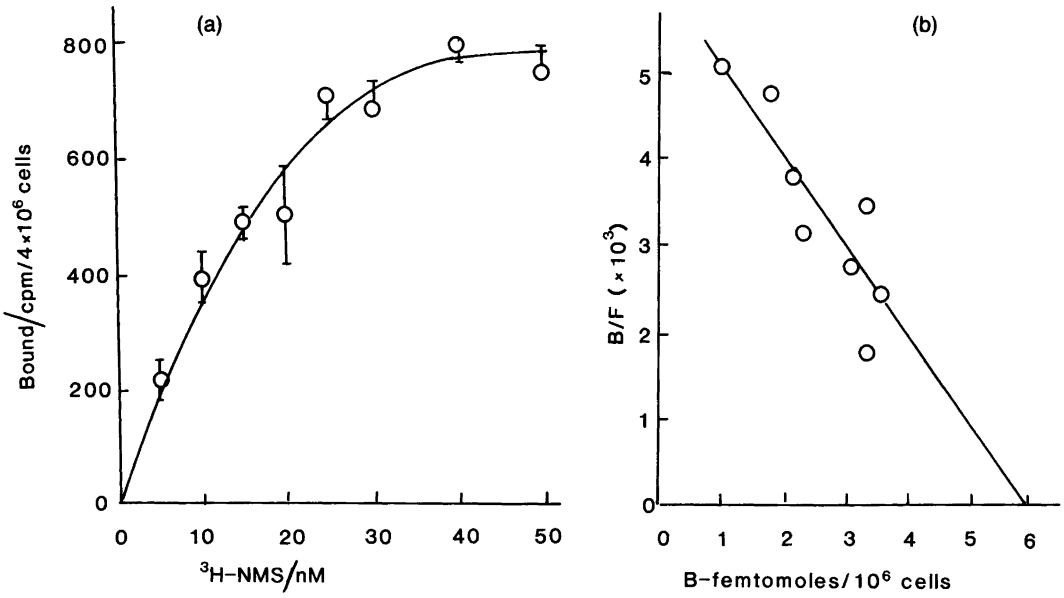

Fig. 2. Equilibrium binding of ^{3}H-NMS specific binding to rat thymocytes (a) and Scatchard plot of these data (b). Thymocytes (4×10^6 cells per 0.2 ml RPMI 1640 medium) were incubated with ^{3}H-NMS (0–50 nM) for 60 min at 37 °C in the presence or absence of atropine (5.1–5 nM) followed by addition of cold medium and centrifugation. Radioactivity of pellets was measured and specific binding calculated as described in the Experimental.

at 4 °C for 60 min in the presence of ^{3}H-NMPB (30 nM) without or with 10^{-4} M atropine sulfate, in order to saturate all surface binding sites. The supernatant was aspirated off following a brief centrifugation ($300\times g$, 30 s). To allow different time periods for internalization or isomerization of the receptor–^{3}H-NMPB complex, prewarmed RPMI 1640 medium (200 µl) was added and the suspended cells were further incubated for different time intervals at 37 °C. The displacement of bound ligand (^{3}H-NMPB) was started by the addition of 20 µl of either PBS (total binding) or atropine sulfate (final concentration 5×10^{-4} M) followed by incubation for 1 min. The reaction was stopped by rapid centrifugation ($3000\times g$, 15 s) and aspiration of the supernatant. Radioactivity of pellets was measured as described in radioligand binding studies.

'Specific total' binding was calculated as the difference between total binding (without any atropine added) and 'unspecific' binding (in the presence of atropine for the entire experiment). 'Inside' hidden radioactivity represents the difference between binding obtained in the presence of atropine present only for 1 min (displacement) and/or for entire period of incubation ('unspecific binding'). 'Surface' binding was calculated as the difference between total and 'inside' hidden radioactivity.

All experiments were carried out in quadruplicate and repeated at least twice with thymocytes from different groups of animals. Data are presented as the means ± SEM.

Results

The time course of the binding of ^{3}H-QNB and ^{3}H-NMPB to intact thymocytes is shown in Fig. 1(a), with a maximum of binding reached within 5 min, followed by a decline in total bound radioactivity within the subsequent 55 min.

The time course of the binding of the less lipophilic muscarinic antagonist [^{3}H]-*N*-methylscopolamine (^{3}H-NMS), to thymocytes at 37 °C reveals 'normal' saturation kinetics with no intermittent maximum but a plateau after 60 min and with half saturation occurring within 30 min of incubation [Fig. 1(b)].

Specific binding of ^{3}H-NMS to thymocytes is saturable [Fig. 2(a)] and Scatchard plot of these equilibrium binding data yields a B_{max} of 6 fmol per 10^6 cells [Fig. 2(b)], which, assuming equal numbers of receptor sites per cell, corresponds to about 3600 binding sites per cell.

^{3}H-NMPB that is specifically bound to muscarinic receptors of rat thymocytes is highly susceptible to atropine (10^{-4} M) displacement only within the first two minutes of incubation at 37 °C [Fig. 3(a)]. Addition of excess atropine at later time points does not displace thymocyte-bound ^{3}H-NMPB. The fraction which is resistant to atropine displacement represents about 20 % ^{3}H-NMPB bound within 1 min of incubation which at 37 °C follows the 50 min preincubation at 4 °C. In the case of ^{3}H-NMS as the labeled antagonist all specifically bound ^{3}H-NMS molcules are susceptible to displacement by excess atropine added within 1 min of incubation at 37 °C [Fig. 3(b)]. Full displacement is reached even when atropine is added at later time points than 1 min (data not shown).

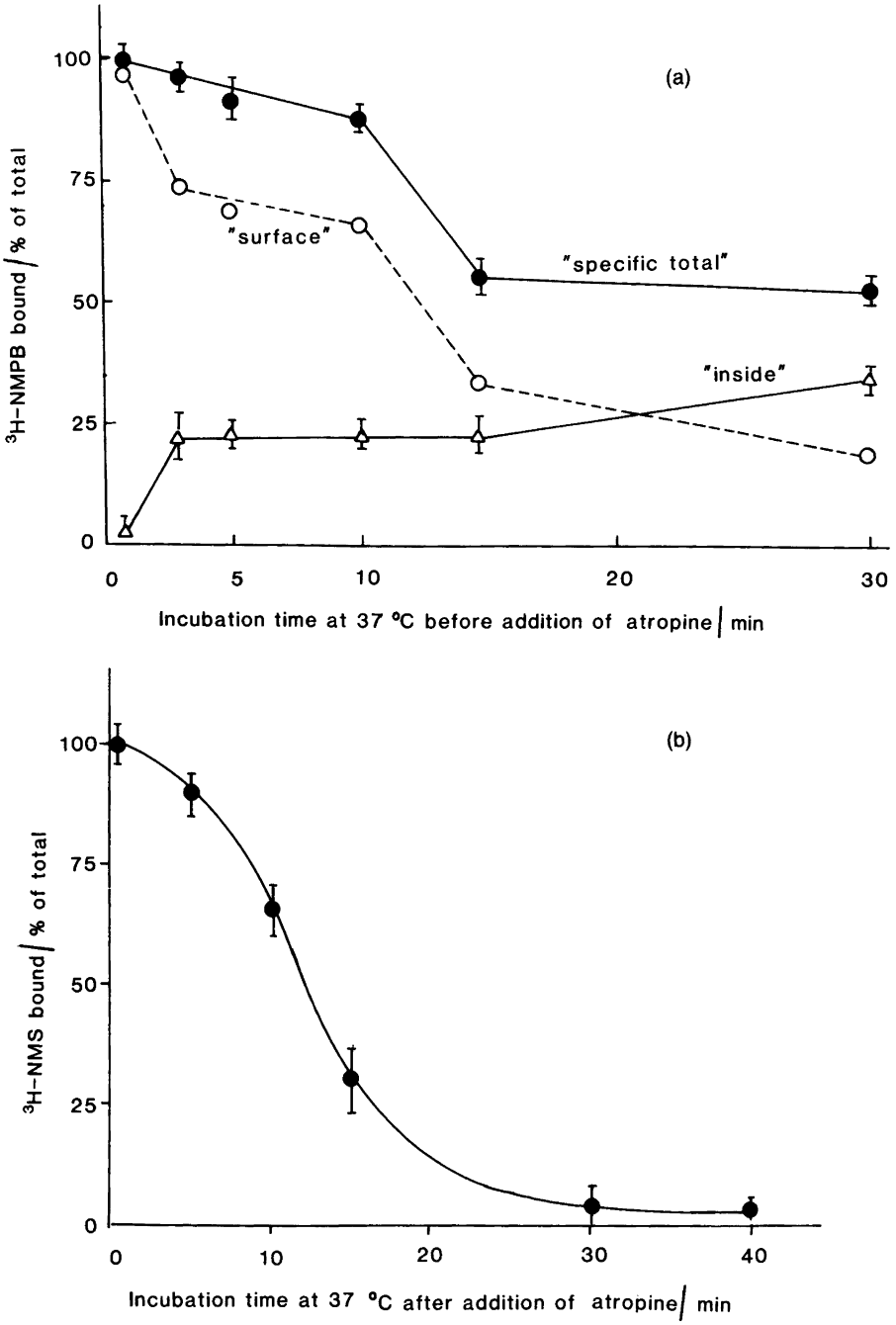

Fig. 3 (a) Displacement of specifically bound ^{3}H-NMPB by atropine at 37 °C. 'Specific total' binding (●) was calculated as the difference between 'unspecific' binding [carried out in the presence of atropine (10^{-5} M) for the entire experiment] and 'total' binding (without any atropine added) after 60 min incubation at 4 °C. 'Inside' hidden radioactivity (△) represents the difference between binding in the presence of atropine for 1 min at 37 °C (displacement) and/or for the entire period of incubation at 37 °C ('unspecific'). 'Surface' binding (○) was calculated as the difference between 'total' and 'inside' hidden radioactivity. For details see the Experimental; (b) Displacement of specifically bound ^{3}H-NMS by atropine at 37 °C. The methods as described for Fig. 3 (a).

Discussion

Binding of ligands to receptors on intact living cells is more rarely studied than binding to membranes prepared from these cells. In the present study anomalous kinetics of binding of the benzilate-type antagonists ^{3}H-3-QNB and ^{3}H-4-NMPB is reported when intact rat thymocytes were studied. Binding of a third antagonist ^{3}H-NMS showed saturation of binding as a function of incubation time.

Several possible explanations can be given for a 'transient' maximum of binding time curve for the [^{3}H]benzilate binding and for the partial loss of reversibility of their binding in atropine displacement experiments [Fig. 3(a)]. One possibility is that the receptor–[^{3}H]benzilate complex internalizes and will become inaccessible to replacement of the [^{3}H]benzilate by excess atropine. This model assumes that only a portion of the receptor–[^{3}H]benzilate complexes internalizes. This portion either represents a subclass of receptors, which is possible in view of the fact at least five muscarinic receptor subtypes have been reported up to now and that all of these are known to bind all of the three ^{3}H-antagonists ^{3}H-NMPB, ^{3}H-QNB and ^{3}H-NMS and atropine studied here.[17–19] Further experiments using specific oligonucleotide probes will be required to decide whether

or not several subtypes of the muscarinic receptor are expressed by rat thymocytes.

Another explanation for the fact that only a portion of receptor–[^{3}H]benzilate complex is accessible to displacement by excess atropine might be that the complex undergoes isomerization as reported by several groups.[16,20] The isomerization equilibrium between the first formed receptor–ligand (RL) complex and the isomerized complex RL* may be reached rapidly benzilate ligands.[16,20] If the isomerization reaction is fast in comparison with the rate of internalization of RL* then during the relatively short observation time employed here only a portion of RL* will be internalized, leaving those complexes still on the surface subject to dissociation and displacement by atropine.

Isomerization of the [^{3}H]-benzilate–receptor complex may explain the failure to displace all bound [^{3}H]-benzilates by excess atropine if the isomerization yields a very slowly dissociating complex which hence appears to be resistant to displacement by excess atropine. The isomerization of receptor–[^{3}H]benzilate complex alone, however, does not account for the maximum in the binding vs. time curve, since the radioactivity bound by the isomerized and not isomerized receptor–[^{3}H]benzilate complexes is equal. For this reason we find that one has to assume a change in the number of surface receptors during incubation of [^{3}H]benzilates with live thymocytes. This observation at first glance is surprising since most reports deal with agonist- rather than antagonist-induced changes in receptor numbers during the process of desensitization and internalization of receptors, while we report here that this may take place when the ligand is a lipophilic antagonist like the benzilates NMPB and QNB.

Our explanation for the lack of similar results with ^{3}H-NMS is that receptor–^{3}H-NMS complexes do not isomerize[16] which we assume is also a prerequisite for the internalization. The importance of lipophilic properties of the ligand in antagonist binding to the muscarinic receptor has also been shown in earlier equilibrium binding studies with a series of benzilate type ligands.[21]

Whether or not the internalization of receptor–benzilate complexes by thymocytes bears any resemblance to the internalization of receptors caused by persistent exposure to cholinergic agonists will be studied.

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