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INFLUENCE OF *N*-ETHYLMALEIMIDE ON CHOLINOCEPTORS AND RESPONSES IN LONGITUDINAL MUSCLES FROM GUINEA-PIG ILEUM

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1 The binding of carbamylcholine to membranes prepared from the longitudinal muscle of guinea-pig ileum was determined from its inhibition of the binding of [^{3}H]-3-quinuclidinyl benzilate. Carbamylcholine binding was resolved into high and low affinity components with apparent dissociation constants of 0.11 ± 0.02 and 11 ± 1 μM; 42% of the receptors displayed high affinity carbamylcholine binding.

2 Alkylation of longitudinal muscle membranes with *N*-ethylmaleimide increased muscarinic receptor affinity for carbamylcholine in a manner consistent with a conversion of low affinity to high affinity receptors. After exposure the muscle membrane fragments to 1 mM *N*-ethylmaleimide for 20 min at 35°C, carbamylcholine binding was resolved into two components with apparent dissociation constants of 0.11 ± 0.01 and 9 ± 2 μM, with 74% of the receptors displaying the higher affinity.

3 Exposure of longitudinal membranes mounted in an organ chamber to 1 mM *N*-ethylmaleimide for 30 s depressed isometric contractions in response to acetylcholine by 80%, while contractions induced by K^+ and Ba^{2+} were reduced by less than 20% and 10%, respectively. Acetylcholine dose-response curves were shifted to the right while Ba^{2+} curves were unaffected.

4 It is suggested that *N*-ethylmaleimide has a selective effect on muscarinic responses in the longitudinal muscle by disrupting processes occurring after receptor occupancy but before the induction of phospholipid turnover or calcium influx in the postsynaptic membrane.

Introduction

Muscarinic acetylcholine receptors in several tissues exist in multiple states which are most clearly distinguished on the basis of their binding of receptors agonists (Ward & Young, 1977; Birdsall, Burgen & Hulme, 1978), although under appropriate conditions a heterogeneity of antagonist binding is also apparent (Ellis & Hoss, 1980; Hulme, Berrie, Birdsall & Burgen, 1981). Agonist binding to brain and ileum receptors determined by their inhibition of radiolabelled antagonist binding can be resolved into high and low affinity components; a smaller, superhigh, affinity component is apparent when agonist binding is measured directly (Birdsall, Hulme & Burgen, 1980b). While the functional significance of the various receptor populations is not entirely clear, it has been suggested that postsynaptic responses to muscarinic stimulation are associated with agonist occupancy of receptors with lower affinities for agonists (Birdsall, Berrie, Burgen & Hulme, 1980a). Regulation of muscarinic receptor binding, including the distribution of receptors among the various states of agonist affinity and the binding properties of receptors in each state, has been reported using sulphydryl modifying reagents (Aronstam, Hoss & Abood, 1977; Aronstam, Abood & Hoss, 1978), guanine nucleotides (Rosenberger, Roeske & Yamamura, 1979; Berrie, Birdsall, Burgen & Hulme, 1979; Wei & Sulake, 1979), ions (Birdsall, Burgen, Hulme & Wells, 1979; Rosenberger, Yamamura & Roeske, 1980), and dopamine receptor agonists (Ehlert, Roeske & Yamamura, 1981a). Alkylation of neural membranes with *N*-ethylmaleimide (NEM) alters agonist binding properties in a manner consistent with the conversion of low to high agonist affinity receptors, without affecting antagonist binding (Aronstam *et al.*, 1977; 1978; Ikeda, Aronstam & Eldefrawi, 1980). The density of superhigh affinity receptors in brain is also increased by NEM treatment (Ehlert, Roeske & Yamamura, 1981b). In the present paper, the influence of NEM on the binding of carbamylcholine to the muscarinic receptor in the longitudinal muscle from guinea-pig ileum is described. The induced alterations in receptor binding properties are compared to alterations in the ability of various agents to induce muscle contractions. A selective effect of NEM on muscarinic responses which is unrelated to any interference with the formation of receptor-agonist complexes is de-

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scribed, and possible sites of action are considered.

Methods

Male guinea-pigs weighing 300–400 g were stunned by a blow to the head and bled from the carotid artery. The ileum was rapidly removed to a modified Krebs-Henseleit solution, where a 2 cm segment was placed over a glass rod and the longitudinal muscle separated from the circular muscle. Strips of the longitudinal muscle were attached at opposite ends to glass rods and Grass FT04 force-displacement transducers. The tissues were suspended in organ chambers in a modified Krebs-Henseleit medium containing (mM): NaCl 122, KCl 4.72, $CaCl_2$ 2.50, $NaHCO_3$ 15.50, KH_2PO_4 1.19, $MgCl_2$ 1.19, dextrose 11.50, and $CaNa_2$-EDTA 0.026 at pH 7.2. The bathing solution was continuously oxygenated (97% O_2: 3% CO_2) and maintained at 37°C. The longitudinal muscles were equilibrated for 90 min under a resting tension of 250 mg before being exposed to any drug. During the equilibration period, the Krebs solution was changed every 30 min to prevent the accumulation of metabolic products. Isometric contractions were recorded with a Beckman R411 Dynograph recorder.

At the end of the initial equilibration period, multiple (2 or 3) cumulative concentration-response relationships to acetylcholine (ACh) or barium chloride were obtained before and after exposure to NEM. The tissues were exposed to 1 mM NEM for 30 s and then washed several times and allowed to reequilibrate for 90 min before repeating the concentration-response measurements. All concentration-response relationships were completed within 90 s and successive determinations were separated by at least 40 min. In a separate series of experiments, the responses of the tissue to single applications of ACh, $BaCl_2$ or KCl were determined before and after NEM treatment. Following these protocols, persistent desensitization of the muscle to ACh was not obtained. Successive dose-response curves, separated by washing and a 40 min re-equilibration period, were superimposable. A degree of desensitization was obtained within a given cumulative concentration-response relationship determination in so far as the response to the final concentration of ACh (1 mM) was about 15% less than the response to a single application of 1 mM ACh of a suitably equilibrated muscle.

Muscarinic acetylcholine receptors were labelled using the active, (−)-isomer of tritiated 3-quinuclidinyl benzilate ([^{3}H]-QNB, 44 Ci/mmol, Amersham) by the technique described by Yamamura & Synder (1974a). Isolated longitudinal muscles were minced and then homogenized in 20 volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.4) using a Tekmar tissue homogenizer (setting 50, two 15 s bursts). The suspension was filtered through 4 layers of cheese cloth to remove connective tissue and large fragments and used without further treatment. Protein content was measured by a modification of the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as the standard.

Carbamylcholine binding was inferred from its ability to inhibit the binding of 32 pM [^{3}H]-QNB to muscle membranes suspended at a concentration of 8–10 μg/ml protein in 10 ml of 50 mM Tris-HCl, pH 7.4. Binding data were fitted by iterative, non-linear regression analyses to a mass action expression for the case of two receptor populations which bind carbamylcholine with different affinities as follows: $B = B_H^*[C]/([C] + K_H) + B_L^*[C]/([C] + K_L)$, where B is binding, [C] is the concentration of carbamylcholine, and B_H, and B_L are the concentrations of receptors having dissociation constants of K_H and K_L, respectively. The concentration of [^{3}H]-QNB binding sites was 1.2 pmol/mg protein and the apparent [^{3}H]-QNB dissociation constant was 80 pM. Under the assay conditions used, the maximum fraction of receptors occupied by [^{3}H]-QNB in the carbamylcholine competition studies was about 28% and maximum tissue binding (specific and non-specific) was 2,270 d/min which represented less than 8% of the added radioactivity. While the concentrations of receptors and [^{3}H]-QNB were selected to optimize the determination of carbamylcholine binding properties, the ratio of receptor concentration to [^{3}H]-QNB dissociation constant (about 0.28) and any depletion of free [^{3}H]-QNB concentrations shift binding curves to higher concentrations, obscuring the highest affinity binding (Chang, Jacobs & Cuatrecasas, 1975; Wells, Birdsall, Burgen & Hulme, 1980).

Carbamylcholine was used in the equilibrium binding studies as a representative cholinoceptor agonist because the rapid hydrolysis of ACh would have necessitated the use of cholinesterase inhibitors. Carbamylcholine is closely related to ACh and appears to differentiate between the same populations of agonist binding sites in so far as the proportions of high and low affinity agonist sites are similar when measured using either ligand (Birdsall *et al.*, 1980b).

Results

Longitudinal muscle contraction

The influence of a brief (30 s) exposure of longitudinal muscle to 1 mM NEM on ACh-, KCl- and $BaCl_2$-induced contractions is illustrated in Figure 1. The response to ACh was markedly reduced by NEM while responses to Ba^{2+} and K^+ were only slightly attenuated. After NEM treatment, responses to

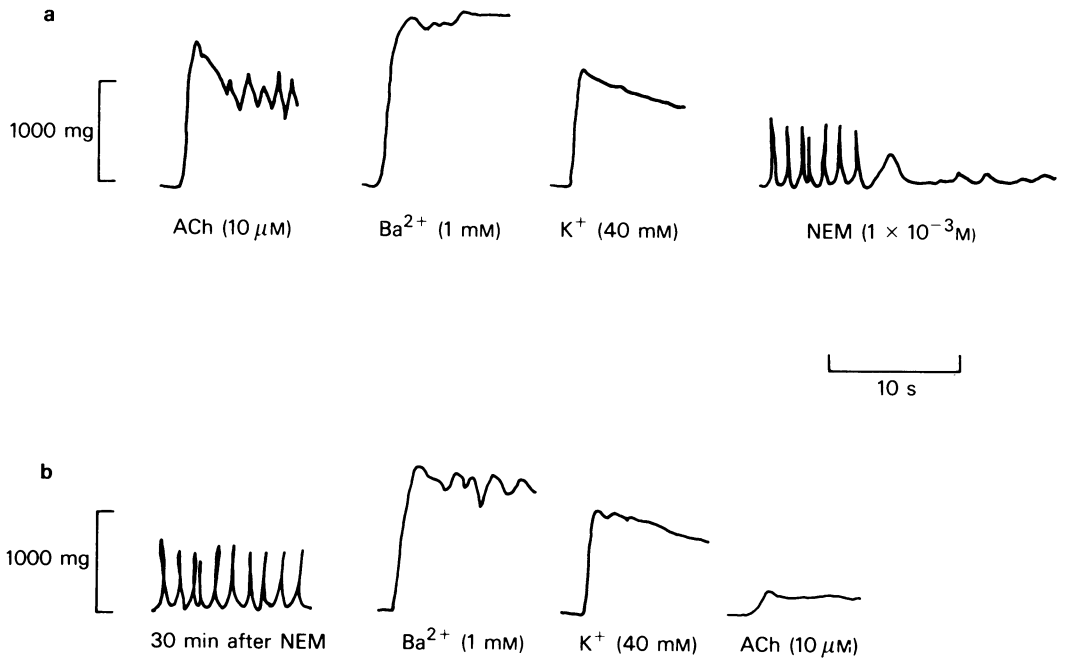


Figure 1 Influence of *N*-ethylmaleimide (NEM) on this contractile response of longitudinal smooth muscle of guinea-pig ileum. Isometric contractions to 10 μM acetylcholine (ACh), 1 mM barium chloride (Ba^{2+}) and 40 mM potassium chloride (K^+) are depicted before (a) and after (b) exposure to 1 mM NEM for 30 s. NEM depressed both the frequency and amplitude of the spontaneous activity of the muscle; however, 30 min after NEM removal spontaneous activity recovered substantially.

10 μM ACh were reduced by 80 ± 2% ($n = 12$), while responses to 1 mM Ba^{2+} were reduced by only 19.2% ($n = 14$) and responses to 40 mM K^+ were reduced by less than 10% in all cases ($n = 6$). These reductions were persistent over the course of 3 h.

ACh-induced contraction of the longitudinal muscle was measured over the concentration range of 0.1 to 1000 μM (Figure 2). Exposure of the muscle to 1 mM NEM for 30 s shifted the concentration-response curve to the right in a parallel manner, such that the EC_{50} concentration was increased from 3.0 ± 0.1 to 16 ± 2 μM ($n = 6$). In contrast, NEM treatment did not alter the EC_{50} value (0.6 mM) for Ba^{2+}-induced longitudinal muscle contractions.

Longitudinal muscle muscarinic receptor binding

[^{3}H]-QNB bound to a finite number of high affinity sites in longitudinal muscle membrane fragments. Scatchard analyses of specific (i.e., 1 μM atropine-sensitive) [^{3}H]-QNB binding indicated a single population of sites at a density of 1.2 pmol per mg protein. The apparent dissociation constant (80 ± 5 pM) did not vary systematically with tissue content of the assay media (from 5 to 15 μg/ml) and 80 pM was

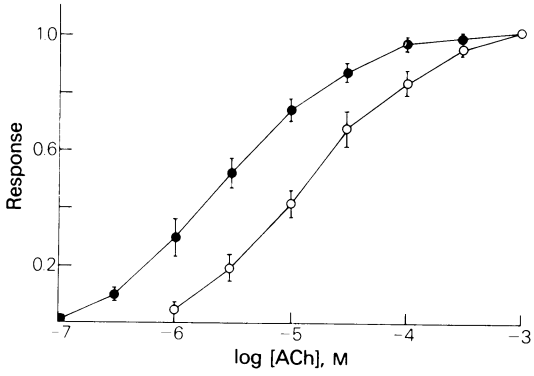


Figure 2 Influence of *N*-ethylmaleimidie (NEM) on contractile responses of guinea-pig ileum longitudinal muscles to acetylcholine (ACh). Isometric contractions were measured after addition of ACh to the indicated concentration before (○) and after (●) exposure of the muscle to 1 mM NEM for 30 s. Response is indicated as fraction of the response produced by 1 mM ACh, which evoked maximal responses in both cases. However the absolute magnitude of the maximum response was decreased 80% by NEM (see text). The points represent the mean and vertical lines the s.e.mean of the response from 6 muscles.

accepted as the true K_D, although this value is about twice that reported by others for neural and ileum muscle muscarinic receptors (Yamamura & Snyder 1974a, b).

Carbamylcholine binding curves were resolved into high and low affinity components with dissociation constants of 0.11 ± 0.02 and $11 \pm 1\ \mu M$ (Figure 3). Forty-two per cent of the receptors displayed high affinity binding. After exposure of the longitudinal muscle suspension (1 mg protein/ml) to 1 mM NEM for 20 min at 35°C, carbamylcholine binding curves were shifted to the right (Figure 3), while [^{3}H]-QNB binding was not altered (not shown). Nonlinear regression analyses indicated two populations of binding sites with dissociation constants of 0.11 ± 0.01 and $9 \pm 2\ \mu M$ with $74 \pm 2\%$ of the receptors displaying high affinity binding.

Longidtudinal muscle strips were mounted in tissue baths under the same conditions used to measure isometric contractions. After equilibration for 90 min, NEM (1 mM was added to the bath and then washed away after 30 s. The muscle strips were removed, homogenized and processed for binding studies as described above. Under these conditions, NEM had relatively little influence on receptor binding. The concentration of [^{3}H]-QNB binding sites was unaltered (1.2 ± 0.1 pmol/mg protein (untreated), $n = 10$, *vs* 1.2 ± 0.2 pmol/mg protein (NEM-treated), $n = 12$). The ability of carbamylcholine to inhibit the binding of a relatively large (320 pM) concentration of [^{3}H]-QNB was measured at 3 carbamylcholine concentrations (0.1, 1.0 and 10 μM Figure 4). The increase in the ability of carbamylcholine to inhibit [^{3}H]-QNB binding was statistically significant only at 1 μM ($38 \pm 7\%$ *vs* $48 \pm 3\%$, $n = 9-12$, $P < 20.01$, Student's t test).

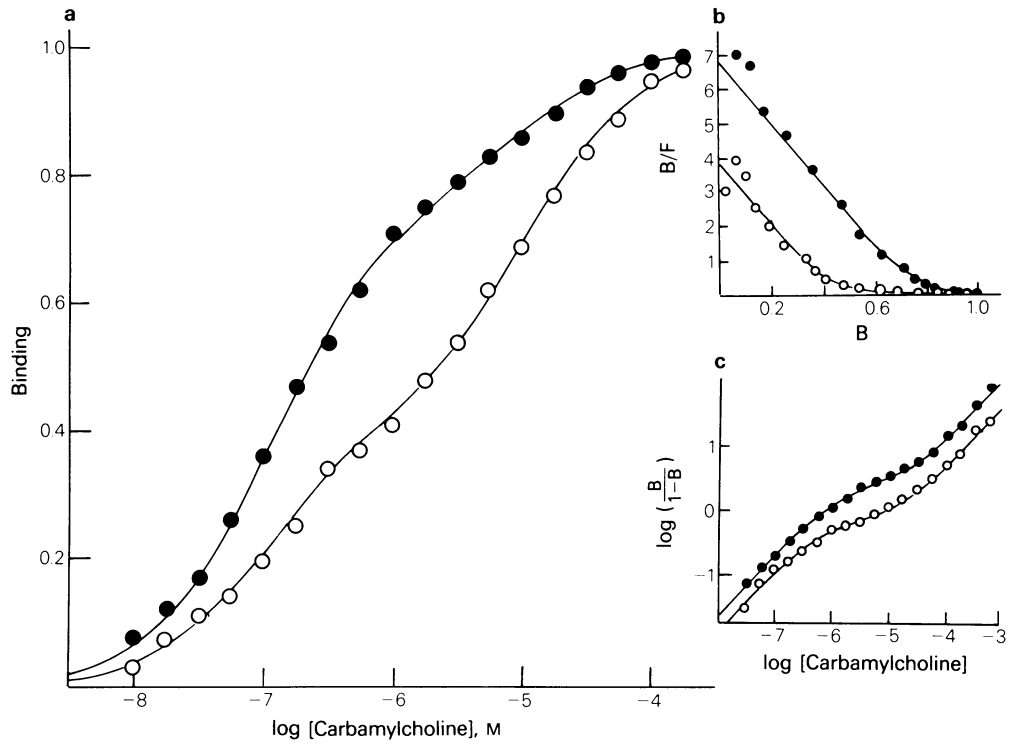


Figure 3 (a) Influence of *N*-ethylmaleimide (NEM) on carbamylcholine binding to muscarinic receptors in membranes prepared from ileum longitudinal muscle. Carbamylcholine binding, expressed as fraction of maximum binding, was inferred from its inhibition of specific [^{3}H]-3-quinuclidinyl benzilate ([^{3}H]-QNB) binding. Binding was measured after exposure of the membranes to 1 mM NEM for 20 min at 35°C (●), while membranes held at 35°C for 20 min in the absence of NEM (○) served as a control. Each point represents the average value from experiments using 4 different tissues, each performed in duplicate, which varied by less than 8%. The lines are drawn according to nonlinear regression fit to a two receptor population model as described in the text. Scatchard and Hill analyses of the same data are presented in (b) and (c). Again, lines are drawn according to parameters derived from a nonlinear regression fit to a two receptor model. B, fractional binding; F, concentration of carbamylcholine in μM.

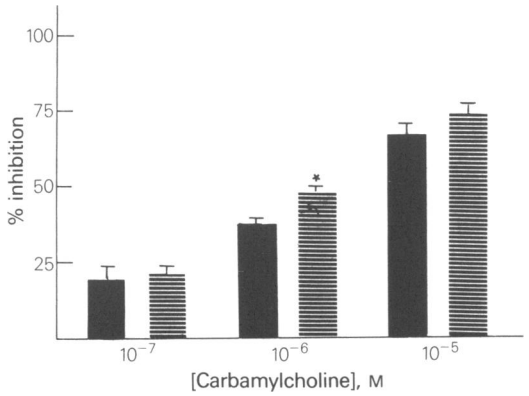


Figure 4 Influence of *N*-ethylmaleimide (NEM) treatment of intact longitudinal muscles on carbamylcholine binding to the muscarinic receptors. Longitudinal muscles were mounted in an organ chamber and exposed to 1 mM NEM for 30 s. The muscles were then homogenized and muscarinic receptor binding to the membrane fragments measured. The specific binding of 50 pM [^{3}H]-3-quinuclidinyl benzilate ([^{3}H]-QNB) to control (solid columns) and NEM-treated (striped columns) membranes was measured in the presence of the indicated concentration of carbamylcholine. The means from 10–14 tissues are shown; vertical lines indicate s.e.mean. $^*P < 0.01$, two tailed Students *t* test.

Discussion

The present results indicate that muscarinic acetylcholine receptors in the longitudinal muscle of guinea-pig ileum exist in multiple states which can be distinguished on the basis of their affinity for a receptor agonist, carbamylcholine, in agreement with the work of others (Ward & Young, 1977). About 42% of the receptors in untreated tissue display low affinity agonist binding. Alkylation of longitudinal muscle membranes with NEM increases muscarinic receptor affinity for carbamylcholine in a manner consistent with an increase in the proportion of receptors in the high affinity state to 74%. NEM has little influence on antagonist binding or the affinity for carbamylcholine of receptors in either the high or low affinity state. In these responses to NEM treatment, longitudinal muscle receptors are similar to those of rat brain (Aronstam *et al.*, 1977; 1978).

While ileum carbamylcholine binding is well described by a mass action relationship for two separate receptor populations which bind the same ligand with different affinities, the interpretation of these data is complicated by factors often encountered when determining the binding of an unlabelled ligand from its inhibition of a radiolabelled probe: the decrease in the concentration of free radiolabelled ligand due to tissue binding (up to 8% in the present experiments) and the substantial ratio of receptor concentration to dissociation constant for the labelled ligand (about 0.28) both shift the displacement curves to higher concentrations and obscure high affinity binding interactions (Chang *et al.*, Wells *et al.*, 1980). Other approaches, including the direct use of radiolabelled agonists, might be used to measure very high affinity interactions.

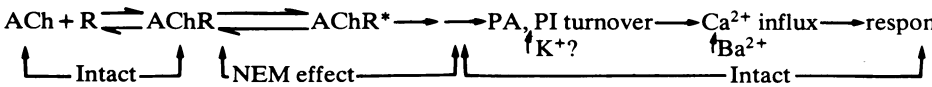
Shifts in ACh-induced contraction dose-response curves caused by alkylating agents may reflect decreases in the spare receptor population, such that a larger fraction of the remaining active receptor pool must be occupied in order to initiate the biological response. A decrease in the number of functionally active spare receptors may account for the shift seen in Figure 2. Longitudinal muscle spare receptor populations have been measured using receptor-directed alkylating agents and by comparing biological responses with direct measurements of receptor occupancy (e.g. Aronstam, Triggle & Eldefrawi, 1979). Such studies indicate that maximum contractile responses can be obtained when less than 1% of the receptors are occupied by ACh. Treatment of longitudinal muscles mounted in an organ chamber with 1 mM NEM for 30 s at 37°C results in an almost complete suppression of muscarinic responses, even though the observed alteration of muscarinic binding properties (i.e., the increase in agonist affinity) is much less than that which can be obtained using muscle membrane homogenates (compare Figures 3 and 4). This may reflect the presence of diffusional barriers which limit the accessibility of ACh and NEM to only the most superficial muscarinic receptors and sulphydryl groups in intact muscles.

NEM alkylates sulphydryl groups in solutions above pH 5.0, as well as amine groups starting at pH 7.0 (Means & Feeney, 1971). Groups which are highly reactive with NEM are present in numerous cellular components of smooth muscle, including those involved in cholinergic ligand recognition, cholinergic stimulus transduction, and the muscle contractile processes. A number of sulphydryl groups have been identified in muscarinic receptors from rat brain and the sulphydryl groups involved in the alteration of muscarinic agonist binding affinity are situated outside the ligand recognition site (Aronstam *et al.*, 1977; 1978). In the present experiments, a nonspecific alkylating reagent which has quite specific and interesting effects on muscarinic receptor binding properties was used in an attempt to assess the functional significance of the heterogeneity observed in ligand binding to muscarinic receptors.

Under mild treatment conditions NEM decreased the maximum extent of ACh-induced contractions by 80% without permanently affecting the frequency or amplitude of spontaneous motor activity. At the same time, the responses to K^+ and Ba^{2+} were only

slightly reduced. These findings suggest that the major influence of NEM is not on the contractile mechanism itself. Moreover, direct binding measurements indicate that the number of receptors available for interaction with receptor ligands (angatonists or agonists) is unchanged; in fact, because of the increase in affinity, the number of receptors occupied by ACh at any given concentration is greater after NEM treatment. Thus, the deficit in muscarinic responsiveness induced by NEM resides in a mechanism located between the initial receptor ligand recognition and interaction stage and the contraction process itself.

The sequence of events involved in the generation of smooth muscle contraction in response to muscarinic stimulation is incompletely understood (Boulton, 1981). A possible sequence of events initiated by ACh based on the work and suggestions of others, including possible sites of action of NEM, is as follows:



Muscle contraction is immediately controlled by the internal concentration of calcium ion. Ba^{2+} presumably mimics or stimulates Ca^{2+} influx. Evidence has been presented which supports the notion that acidic phospholipids are intimately involved in the calcium-gating process (Putney, Weiss, van De Wall & Haddas, 1980). Phospholipid turnover in ileum muscle is also stimulated by a number of non-muscarinic agents, including high potassium solutions (Jafferji & Michell, 1976), although it is not clear whether this is a direct effect or due to depolarization-induced release of endogenous substances (see Putney, 1981). Moreover, Salmon & Honeyman (1980) reported no increase in phospholipid turnover in response to high K^+ in dispersed smooth muscle cells from frog stomach. NEM may in fact uncouple muscarinic receptor macromolecules from the structures involved in the elaboration of the postsynaptic cholinergic responses (i.e., obviate the development of an activated receptor complex (AChR*)). As Burgen (1981) has pointed out, if a portion of an agonist's intrinsic binding energy is used to translate conformational changes to associated effector structures, a disruption of this coupling could increase the binding energy measured in direct binding assays, contributing to the measured increase in agonist affinity.

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