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MECHANISM OF AGONIST-INDUCED DEGRADATION OF MUSCARINIC CHOLINERGIC RECEPTOR IN CULTURED VAS DEFERENS OF GUINEA-PIG

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The mechanism of agonist-induced degradation of muscarinic cholinergic receptors (mAChRs) was examined by means of [^{3}H]QNB binding by organ-cultured guinea-pig vas deferens. Long-term exposure to ACh decreased the surface mAChR. This so-called 'down-regulation' of mAChR was significantly inhibited by anti-endocytotic drugs. Anti-microtubular agents also strongly inhibited the decrease of mAChR remaining on the cell surface. Ammonium chloride and protease inhibitors, which are known to inhibit lysosomal enzymes, had little effect on the decrease and no increase in intracellular mAChR could be detected under these conditions. Chloroquine blocked the decrease in mAChR remaining on the cell surface. Based on these findings, the involvement of clustering and endocytosis in mAChR degradation were considered as possibilities. Furthermore, contraction of the smooth muscle to ACh in long-term desensitization was also examined in relation to the number and nature of the receptors. When the muscles were cultured with ACh and chloroquine or vinblastine, there were indications that the surface mAChR and the contractile system were uncoupled.

Muscarinic cholinergic receptor Receptor-mediated endocytosis Smooth muscle Tissue culture Desensitization Microtubules

1. Introduction

The biosynthesis and degradation of neurotransmitter receptors have been much studied. With regard to nicotinic cholinergic receptors (nAChR) the following concepts have been proposed: nAChR is synthesized in ribosomes, transferred to the Golgi apparatus then incorporated into the surface membrane (Fambrough, 1979). The nAChR in the surface membrane is internalized by endocytosis and degraded in lysosomes (Devreotes and Fambrough, 1975). There have been fewer studies on the metabolism of the muscarinic cholinergic receptor (mAChR) than on that of nAChR. However, it has recently been reported that the number of mAChR is decreased by muscarinic agonists (Klein et al., 1979; Siman and Klein, 1979; Shifrin and Klein, 1980; Galper and Smith, 1980), and that the decrease is mainly dependent on the acceleration of degradation (Klein et al., 1979). In an organ culture system of guinea-pig vas deferens we also found that muscarinic agonists induced a decrease in mAChR with a simultaneous decrease of contractions to ACh (Takeyasu et al., 1981; Higuchi et al., 1981). This decrease was mainly due to increased degradation through activation of the receptor by the agonist, although contraction of the muscle also seemed to be important for decrease of the receptor. The decrease in mAChR is considered to be related to so-called long-term desensitization (Taylor et al., 1979; Takeyasu et al., 1981; Higuchi et al., 1981). In the present work, we examined the effects of several agents on the ACh-induced decrease in mAChR to determine the mechanism of degradation of mAChR in smooth muscle in relation with changes in ACh-induced contraction in organ cultures of guinea-pig vas deferens.

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2. Materials and methods

2.1. Organ culture of guinea-pig vas deferens

Guinea-pig (300–350 g) vas deferens was isolated aseptically and washed with Hanks' balanced salt solution containing penicillin (200 units/100 ml) and streptomycin (100 mg/100 ml). The whole vas deferens was mounted under a loading tension (320 mg/muscle) in a glass chamber filled with Eagle's MEM with 5% calf serum. Incubations were carried out at 37°C under 5% CO_2 in O_2, as reported before (Takeyasu et al., 1981). All experiments involving organ cultures with ACh were done in the presence of 10^{-5} M neostigmine.

2.2. Contractile responses of vas deferens

Whole vas deferens was mounted in a 10 ml organ bath in aerated Locke solution at 37°C. The resting tension was adjusted to 80 mg/muscle and the muscle was washed for over 30 min to remove the so-called short-term desensitization caused by ACh. Isometric contractions induced by the drugs were then recorded with a force-displacement transducer (SB1T, Nihon Koden Co. Japan) and recorders (Multipurpose polygraph RM-150, Nihon Koden Co., Japan).

2.3. [^{3}H]QNB binding to homogenate of vas deferens by the filtration method

After the contractile responses had been recorded, the muscles were weighed and homogenized in 100 vol of a solution of 100 mM NaCl, 50 mM-HCl (pH 7.4) and 1 mM $CaCl_2$ in a Polytron and then sonicated in a Kontes sonicator at a power setting of 5 for 30 s. Connective tissue was removed by filtration through nylon cloth (70 μm mesh). Samples of 400 μl of whole tissue homogenate were incubated with [^{3}H]QNB (12 Ci/mmol, RCC Amersham) in 2 ml of medium consisting of 100 mM NaCl, 50 mM Tris-HCl (pH 7.4) and 1 mM $CaCl_2$ at 37°C for 20 min, because the specific binding of [^{3}H]QNB to the whole homogenate reached equilibrium for 20 min at 37°C. [^{3}H]QNB bound to the tissue homogenate was trapped on a glass filter (Whatman GF/F) and its radioactivity was counted in 5 ml of Triton X-100 toluene-based scintillation fluid in a liquid scintillation counter (Packard 3385). Specific binding was calculated by subtracting non-specific binding in the presence of 10 μM atropine from total binding in the absence of atropine.

2.4. Determination of mAChR on the surface membrane by [^{3}H]QNB binding to tissue blocks

[^{3}H]QNB was used as a specific marker of muscarinic cholinergic receptors. Whole guinea-pig vasa deferentia were isolated and weighed. The muscles were stripped off and chopped into 8 blocks. These 8 blocks of whole muscles were then incubated with [^{3}H]QNB at 37°C for various times in 5 ml of isotonic solution consisting of 100 mM NaCl, 50 mM Tris-HCl (pH 7.4) and 1 mM $CaCl_2$. After incubation, the blocks were washed 3 times with 5 ml of ice-cold isotonic solution and solubilized in 10 ml of Triton X-100 (25%) toluene-based scintillation fluid for 24 h. Radioactivity was counted in a liquid scintillation counter (Packard 3385). For estimation of non-specific binding, radioactivity that was bound to the blocks in the presence of 10^{-5} M atropine was measured and the specific binding was calculated by subtracting non-specific binding from total binding.

2.5. Determination of total mAChR by equilibrium dialysis

As previously (Uchida et al., 1978), binding of [^{3}H]QNB with the homogenate was measured for determining the total mAChR (membrane-bound and cytosolic mAChR); this was done by equilibrium dialysis for 12 h at 4°C in an apparatus in which pairs of 0.5-ml chambers were separated by a cellophane membrane. The apparatus was shaken during the experiment. Samples of 20 mg (original wet weight) of homogenized tissue were put into incubation medium (100 mM NaCl, 50 mM Tris-HCl buffer (pH 7.4) and 1 mM $CaCl_2$) in the chambers on one side and various concentrations of [^{3}H]QNB into chambers on the other side. For estimation of non-specific binding, the medium contained 10^{-5} M atropine. This method allowed cytosolic mAChR to be measured in addition to

membrane bound mAChR. To estimate total mAChR including that in intracellular vesicles, we carried out the binding experiments in the presence of 0.05% digitonin by equilibrium dialysis for 12 h at 4°C, because concentrations of digitonin higher than 0.05% inhibited [^{3}H]QNB binding (data not shown).

2.6. Other methods

[^{3}H]WB 4101 binding to whole homogenate of guinea-pig vas deferens was measured as an index of α-adrenergic receptor by the filtration method of U'Prichard et al. (1977).

2.7. Drugs

[^{3}H]QNB (12 Ci/mmol) and [^{3}H]WB4101 (24.7 Ci/mmol) were obtained from the Radiochemical Centre, Amersham England. The following drugs; acetylcholine chloride, physostigmine salicylate, atropine sulfate, mono-dansylcadaverine, colchicine, vinblastine sulfate, cytochalasin B, chloroquine diphosphate salt and diisopropyl-fluorophosphate (DFP) were obtained from Sigma. Protease inhibitors; leupeptin, antipain, chymostatin and pepstatin A were purchased from Peptide Institute Protein Research Foundation Japan. Other drugs were methylamine hydrochloride, ethylamine hydrochloride, ammonium chloride and dimethyl sulfoxide, Wako Pure Chem. Ind.; paraformaldehyde and digitonin, Merck; neostigmine methylsulfate (Shionogi).i

3. Results

3.1. Determination of surface mAChR and localization of mAChR in guinea-pig vas deferens

The time course of binding of [^{3}H]QNB (0.8 nM) to blocks of guinea-pig vas deferens in isotonic medium is shown in fig. 1A. The specific [^{3}H]QNB binding was saturated in about 1 h and complete dissociation of [^{3}H]QNB was observed after addition of atropine (10^{-5} M). The equilibrium period for binding to the blocks was longer than that for binding to the homogenate. The cause of this prolongation was unclear. By this

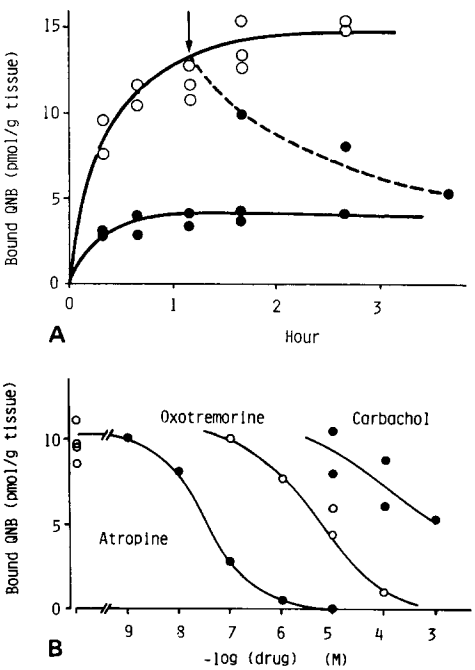

Fig. 1A. Time course of [^{3}H]QNB binding to intact tissue blocks. Eight blocks of whole vas deferens were incubated in isotonic medium for various times at 37°C with 0.8 nM [^{3}H]QNB. The blocks were then washed and solubilized and radioactivity was determined. Nonspecific binding was measured in the presence of 10^{-5} M atropine. At 70 min, 10^{-5} M atropine (arrow) was added to investigate the dissociation of [^{3}H]QNB from surface mAChR. Points are means of values for 8 blocks of whole vas deferens. ○ Without atropine; ● with 10^{-5} M atropine; ------ dissociation of bound [^{3}H]QNB by addition of 10^{-5} M atropine.

Fig. 1B. Inhibition of [^{3}H]QNB binding to intact tissue blocks of whole vas deferens by muscarinic agonists and antagonist. The concentration of [^{3}H]QNB was 0.8 nM. Points are means of values for 8 blocks of whole vas deferens.

method, the K_D value obtained from the Scatchard plot was 3.64×10^{-10} M and was of the same order as that of [^{3}H]QNB binding to the homogenate. The maximum number of sites of [^{3}H]QNB binding to the blocks as determined at 37°C for 1 h was equal to that obtained after incubation for 24 h at 0°C (data not shown), suggesting that uptake of [^{3}H]QNB was absent in this system. The curves for inhibition of [^{3}H]QNB (0.8 nM) binding to tissue blocks by muscarinic agonists and antagonist obtained by this procedure were similar to those for the tissue homogenate (fig. 1B). These

results indicate that mAChR on the surface membrane of the smooth muscle could be estimated by this procedure, though addition of glucose (1 g/l) to the isotonic medium caused a fairly large amount of transport of [^{3}H]QNB into the cells (data not shown).

The total mAChR in the homogenate was measured by equilibrium dialysis with or without digitonin as described under Materials and Methods. The specific binding was saturable and the K_D value was $3.90 \pm 0.88 \times 10^{-10}$ M. As shown in table 1, the numbers of surface mAChR, total mAChR and mAChR determined by filtration with a GF/F filter were almost equal. This indicates that the GF/F glass filter (diameter 0.7 μm) trapped almost all fragments of the surface membrane in the homogenate. Thus, it is concluded that in guinea-pig vas deferens almost all the mAChR in the smooth muscle, which could be detected by [^{3}H]QNB binding, exist on the surface membrane and that there is little intracellular soluble or particulate mAChR.

3.2. Effects of anti-endocytotic drugs on the accelerated degradation of mAChR by ACh

On organ culture of guinea-pig vas deferens with muscarinic agonists, the amount of mAChR in the smooth muscle decreased with a simultaneous decrease in the contraction to ACh (Takeyasu et al., 1981; Higuchi et al., 1981). This suggests that muscarinic agonists decreased the functional mAChR on the surface membrane. In fact, as shown in fig. 2, on culture of smooth muscle with 27 μM ACh for 24 h, the surface mAChR decreased in parallel with the number of mAChR determined in homogenates by the filtration method. At the same time, the number of α-receptors determined with [^{3}H]WB4101 did not decrease, as previously reported (Takeyasu et al.,

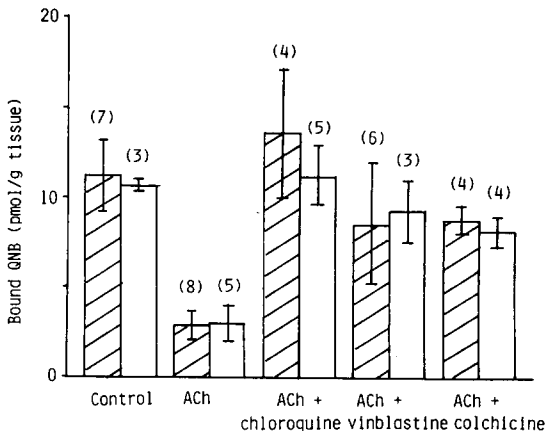

Fig. 2. Comparison of binding sites of [^{3}H]QNB to the homogenate and tissue blocks. [^{3}H]QNB binding to the homogenate and tissue blocks was measured by the filtration method as described in Methods, after culture with ACh and several agents. The amounts of binding sites of [^{3}H]QNB (pmol/g tissue) were determined at a concentration of 4 nM. The concentrations were as follows: ACh (27 μM), chloroquine (2×10^{-4} M), vinblastine (10^{-4} M), colchicine (5×10^{-3} M). Vertical bars indicate standard deviations (n = 3–8). ▨ mAChR in the homogenate; □ surface mAChR in tissue blocks.

TABLE 1

Localization of mAChR in the smooth muscle of guinea-pig vas deferens. The amounts of mAChR obtained by the 3 methods described under Materials and methods are shown. Data are given as means ± S.D. pmol/g tissue (n), obtained from Scatchard plot analyses (n = 3–5). In each experiment 4–10 vasa deferentia were combined for binding studies. The concentrations were as follows: ACh (27 μM), NH_4Cl (10 mM), leupeptin (2.9×10^{-4} M), digitonin (0.05%).

	Treatment with digitonin	Control (preculture)	ACh + NH_4Cl (9 h)	ACh + leupeptin (9 h)
Binding to tissue block (surface mAChR)	−	13.6 ± 1.7 (4) *		
Filtration method (membrane-bound mAChR)	−	13.5 ± 3.0 (5)	3.7 ± 0.8 (8)	4.0 ** (2)
Equilibrium dialysis method (total mAChR)	−	12.2 ± 1.3 (3)	3.2 ± 0.7 (3)	3.7 ** (2)
	+	11.2 ** (2)	3.8 ± 0.6 (3)	2.9 ** (2)

* The amount of mAChR was obtained as the specific [^{3}H]QNB binding measured at 4 nM. ** Values are averages for 2 separate experiments in which 10 vasa deferentia were combined for binding studies. Details of procedures are described in the text.

TABLE 2

Effects of anti-endocytotic agents on the accelerated degradation of mAChR by ACh (27 μM). All experiments were done in the presence of 10^{-5} M neostigmine for 24 h. Monodansylcadaverine (D.C.), methylamine hydrochloride and ethylamine hydrochloride were added with ACh to the culture medium after preincubation at 37°C for 5 h. Pretreatment with paraformaldehyde (1%) was at 37°C for 10 min. The amount of mAChR was measured as specific binding of [^{3}H]QNB (4 nM) by the filtration method. Details of procedures are given in the text. Data are given as means ± S.D., pmol/g tissue (n).

	mAChR	% of decrease
Control	11.2 ± 2.0 (7)	
ACh	2.2 ± 0.6 (8)	100
ACh + DMSO (1%)	2.6 ± 0.8 (8)	95.6 ± 9.9
ACh + D.C. (100 μM) + DMSO	3.3 ± 0.8 (4)	87.8 ± 9.9
ACh + D.C. (500 μM) + DMSO	6.0 ± 1.8 (7)	57.8 ± 20.0 *
ACh + methylamine (20 mM)	7.7 ± 2.2 (4)	38.9 ± 24.4 *
ACh + ethylamine (20 mM)	7.5 ± 2.6 (4)	41.1 ± 28.9 *
Dansylcadaverine (500 μM) + DMSO	11.1 ± 1.5 (4)	1.1 ± 16.7
Methylamine (20 mM)	11.1 ± 0.6 (4)	1.1 ± 6.7
Ethylamine (20 mM)	8.4 ± 1.4 (4)	31.1 ± 15.6
1% Paraformaldehyde (10 min 37°C)	12.1 ± 1.1 (4)	− 10.0 ± 12.2
ACh + 1% paraformaldehyde	8.5 ± 2.8 (8)	30.0 ± 31.1 *

* Significantly different from the value with ACh: $P < 0.001$.

1981), showing that the decrease was specific to surface mAChR. To determine whether receptor-mediated endocytosis participates in the accelerated degradation of the surface mAChR, we investigated the effects of mono-dansylcadaverine, methylamine hydrochloride, ethylamine hydrochloride and paraformaldehyde, which are known to prevent clustering through inhibition of transglutaminase and subsequent internalization. As shown in table 2, mono-dansylcadaverine had little effect at 100 μM, but at 500 μM it caused significant inhibition (43%). Methylamine hydrochloride and ethylamine hydrochloride at 20 mM caused 62 and 86% inhibition, respectively, and pretreatment with 1% paraformaldehyde (for 10 min at 37°C) caused 62% inhibition of the decrease in mAChR by ACh.

3.3. Role of microtubular systems in the accelerated degradation of mAChR

The effect of vinblastine, which depolymerizes cytoplasmic microtubules, on the accelerated degradation by ACh was examined. Results showed that 10^{-4} M vinblastine had a significant inhibitory effect (fig. 3) as observed previously with colchicine (Takeyasu et al., 1981), though vinblastine and colchicine showed no significant effect on the number of mAChR in the absence of ACh.

We next measured mAChR on the surface membrane. mAChR remained on the surface membrane in the presence of vinblastine and colchicine (fig. 2). These results indicated that micro-

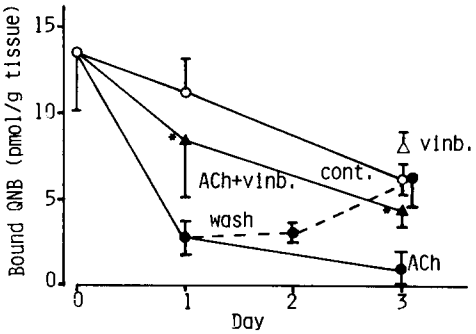

Fig. 3. Effect of vinblastine on the accelerated degradation of mAChR by ACh (27 μM) in culture medium. Neostigmine (10^{-5} M) was combined with ACh. Vinblastine 10 μM was used. The number of mAChR (pmol/g tissue) was measured as specific binding of [^{3}H]QNB (4 nM) by the filtration method. Points and vertical lines show means ± S.D. from 4 to 7 observations. Procedures are detailed in the text. * Significantly different from the value with ACh: $P < 0.001$; cont. control; vinb. vinblastine; ------ recovery.

TABLE 3

Effect of cytochalasin B on the accelerated degradation of mAChR by ACh (27 μM). Guinea-pig vas deferens was cultured with ACh in the presence of 20 μM cytochalasin B. Cytochalasin B was dissolved in dimethyl sulfoxide (DMSO). All experiments were done in the presence of 10^{-5} M neostigmine. After culture for 24 h, vasa deferentia were washed, homogenized and used for assay of [^{3}H]QNB binding by the filtration method. The number of mAChR was measured as the specific binding of [^{3}H]QNB (4 nM). Values are means ± S.D. for the number of experiments shown.

	B_{max} of [^{3}H]QNB (pmol/g tissue)
ACh (+1% DMSO)	4.0±0.7 (3)
ACh+cytochalasin B (20 μM) (+1% DMSO)	3.7±1.2 (4)

tubules around or inside the plasma membrane as a cytoskeleton participate in the accelerated degradation of mAChR and that anti-microtubule agents block the degradation of mAChR which remains on the surface membrane.

On the contrary, cytochalasin B, which disrupts oriented bundles of microfilaments (Wessells et al., 1971; Axline and Reaven, 1974) had no effect on the accelerated degradation of mAChR (table 3).

3.4. Effects of lysosomotropic agents and protease inhibitors on the accelerated degradation

We investigated the effects of chloroquine and ammonium chloride, which are weakly basic substances that accumulate in lysosomes, on lysosomal functions of mammalian cells (De Duve et al., 1974; Poole et al., 1977; Seglen et al., 1979). The effects of protease inhibitors that are known to affect lysosomal enzymes were also examined (Grinde and Seglen, 1980; Libby et al., 1980). As shown in fig. 4, 2×10^{-4} M chloroquine completely blocked not only the accelerated degradation of mAChR by ACh, but also the spontaneous degradation of mAChR observed in the control. However ammonium chloride and protease inhibitors did not affect the accelerated degradation of mAChR determined by the filtration method (tables 4, 5).

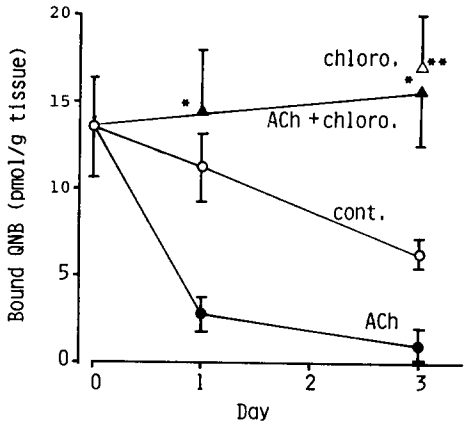

Fig. 4. Effect of chloroquine on the accelerated degradation of mAChR by ACh (27 μM) in culture medium. 2×10^{-4} M chloroquine was used. Experimental procedures and conditions were as described for fig. 3 and in the text. * Significantly different from the value with ACh: $P<0.001$. ** Significantly different from control: $P<0.001$; cont. control; chloro. chloroquine.

We also measured the total number of mAChR by equilibrium dialysis in the presence or absence of digitonin. The number of total mAChR thus measured decreased in the same way as that determined by the filtration method and no intracellular accumulation of mAChR could be detected even in presence of ACh plus ammonium chloride or leupeptin (table 1). When cultured with ACh and chloroquine, almost all the mAChR in the

TABLE 4

Effect of ammonium chloride on the accelerated degradation of mAChR by ACh (27 μM). Ammonium chloride 10 mM was used. All experiments were done in the presence of 10^{-5} M neostigmine. After culture, the amount of mAChR was measured as specific binding of [^{3}H]QNB (4 nM) by the filtration method. Values are means ± S.D. for the number of experiments shown. Details of procedures are given in the text.

	B_{max} of [^{3}H]QNB (pmol/g tissue)
ACh (8 h)	5.7±1.4 (4)
ACh+NH_4Cl (10 mM)	5.3±0.4 (4)
ACh (24 h)	2.8±0.9 (7)
ACh+NH_4Cl (10 mM)	2.8±0.8 (3)
Control (24 h)	11.2±2.0 (7)

TABLE 5

Effects of protease inhibitors on the accelerated degradation of mAChR by ACh (27 μM). Protease inhibitors were added at various concentrations with ACh to culture medium. Values are means ±S.D. for the number of experiments shown. Experimental procedures and conditions were as described for table 4 and in the text.

	B_{max} of [^{3}H]QNB (pmol/g tissue)
ACh (9 h)	3.79±1.08 (8)
ACh+leupeptin (4.8×10^{-5} M)	3.62±0.37 (4)
ACh+antipain (1.7×10^{-5} M)	5.48±1.11 (8) *
Chymostatin (1.7×10^{-5} M)	
Pepstatin A (1.8×10^{-5} M)	
ACh (8 h)	5.7 ±1.4 (4)
ACh+pepstatin A (3.6×10^{-4} M)	5.8 ±1.6 (6)
ACh+chymostatin (4.1×10^{-4} M)	4.7 ±0.6 (4)
ACh (24 h)	2.5 ±0.5 (4)
ACh+DFP (1 mM)	1.7 ±0.7 (4)
Control (24 h)	11.2 ±2.0 (7)

* Significantly different from the value with ACh: $P<0.01$.

smooth muscle remained on the surface membrane (fig. 2).

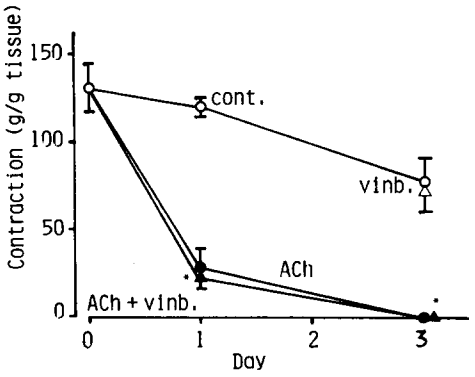

Fig. 5. Change of contraction to ACh of guinea-pig vas deferens when cultured with ACh (27 μM) and vinblastine (10^{-4} M). The responses to 5×10^{-5} M ACh after organ culture of the same muscle as used for fig. 3 were recorded isometrically at 37°C as described under Materials and methods. Culture conditions were as for fig. 3. Vertical bars represent standard errors of means (n=4–7). * Significantly different from contraction of the control: $P<0.001$; cont. control; vinb. vinblastine.

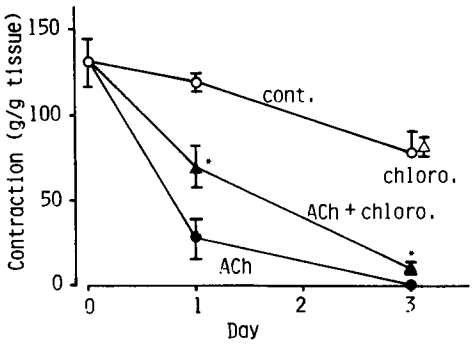

Fig. 6. Change of contraction to ACh of guinea-pig vas deferens when cultured with ACh (27 μM) and chloroquine (2×10^{-4} M). The responses to 5×10^{-5} M ACh of the same muscle as used for fig. 4 were recorded. Culture conditions were as for fig. 4. Experimental procedures and conditions were as described for fig. 5 and in the text. * Significantly different from contraction of the control: $P<0.01$; cont. control; chloro. chloroquine.

3.5. Impairment of coupling of receptor with the contractile system of muscle cultured with ACh and vinblastine or chloroquine

Chloroquine or vinblastine added to vasa deferentia being cultured with ACh did not prevent

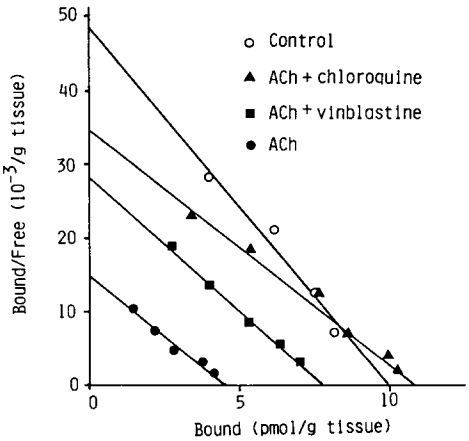

Fig. 7. Scatchard plot analysis of [^{3}H]QNB binding, when the muscle was cultured with ACh (27 μM) and vinblastine (10^{-4} M) or chloroquine (2×10^{-4} M). After organ culture for 24 h, [^{3}H]QNB binding to the homogenate was measured by the filtration method as described under Materials and methods. Points are the means of values in 2 separate experiments in which 4 vasa deferentia were combined for binding studies. Details of procedures are given in the text.

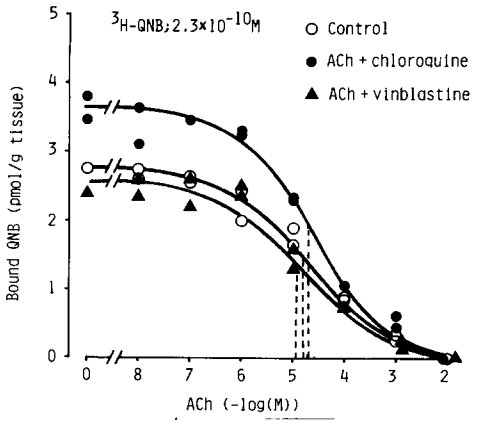

Fig. 8. Displacement of [^{3}H]QNB binding by various concentrations of ACh in the presence of 10^{-5} M physostigmine, when the tissue was cultured with ACh (27 μM) and vinblastine (10^{-4} M) or chloroquine (2×10^{-4} M). The concentration of [^{3}H]QNB was 0.23 nM. Values were obtained by the filtration method, with the same tissue homogenates as used for fig. 7. Details of procedures are given in the text.

the ACh-induced decrease in contractility to ACh (figs. 5 and 6), though they prevented the decrease in mAChR on the surface membrane (fig. 2). Under these conditions the contractions to norepinephrine and high K^+ were not affected (data not shown). Moreover culture with chloroquine or vinblastine alone did not affect the contractions of the smooth muscle to ACh or to NE and high K^+. These results indicate that the decreased contractions to ACh were not due to non-specific effects of chloroquine or vinblastine on the contraction. The K_D values for [^{3}H]QNB binding of the mAChR, as obtained from Scatchard plots (from 2.0 to 3.3×10^{-10} M), for vas deferens cultured with ACh and chloroquine or vinblastine were not significantly different from that (2.08×10^{-10} M) of the control (fig. 7). Furthermore the IC_{50} values of ACh for the inhibition of [^{3}H]QNB (2.3×10^{-10} M) binding in the presence of 10^{-5} M physostigmine were about 10^{-5} M, which was also similar to that of the control (fig. 8).

4. Discussion

Receptor-mediated endocytosis has recently been recognized as a general mechanism by which receptor-ligand complexes to polypeptide hormones are internalized (Goldstein et al., 1979; Stahl et al., 1980; Krupp and Lane, 1981; Hazum et al., 1980; Willingham and Pastan, 1980). Clustering of receptor-ligand complexes in clathrin-containing coated pits precedes internalization of the complexes in receptor-mediated endocytosis, and transglutaminase is essential for the clustering (Davies et al., 1980; Maxfield et al., 1979; Baldwin et al., 1980; Haigler et al., 1980a; Van Leuven et al., 1980; Lorand et al., 1979). It has also been reported that paraformaldehyde inhibited clustering and endocytosis of α-macroglobulin-protease complexes of macrophages (Kaplan and Nielsen, 1979a,b) and that methylamine and monodansylcadaverine blocked clustering during receptor-mediated endocytosis of α_2-macroglobulin receptors by inhibiting transglutaminase. To elucidate how degradation of surface mAChR is accelerated, we examined the effects of these anti-endocytotic agents. As shown in table 2, anti-endocytotic agents significantly inhibited the decrease of mAChR. Therefore, the accelerated degradation of mAChR seems to be achieved mainly through receptor-mediated clustering with endocytosis of agonist-mAChR complexes, though no intracellular mAChR could be detected under various experimental conditions. However, we cannot exclude the possibility that the accelerated degradation of mAChR may also be partly mediated by another mechanism, because it was not completely blocked by mono-dansylcadaverine 500 μM. The accelerated degradation of α_2-macroglobulin receptors was completely blocked by 500 μM dansylcadaverine (Davies et al., 1980; Maxfield et al., 1979; Van Leuven et al., 1980). A different mechanism not involving clustering through transglutaminase was reported for insulin receptors of lymphocytes (Baldwin et al., 1980), and EGF receptors (Haigler et al., 1980b).

It is thought that microtubular systems are involved in capping and patching, clustering of immunoglobulin receptors and receptor-mediated endocytosis. Our previous report showed that an anti-microtubule agent, colchicine, blocked the accelerated degradation of mAChR (Takeyasu et al., 1981). In the present experiments we used another anti-microtubule agent, vinblastine, and

found that it also blocked the accelerated degradation of mAChR, whereas an anti-microfilament agent, cytochalasin B, had no effect on the accelerated degradation (table 3). Thus microtubular systems seemed to be involved in the accelerated degradation of mAChR in smooth muscle. Furthermore, as shown in fig. 2, mAChR remaining after vinblastine and colchicine treatment was present on the surface membrane, indicating that the microtubular system present inside or around the plasma membrane as a cytoskeleton may participate in the accelerated degradation of mAChR.

We next investigated the effects of the lysosomotropic agents chloroquine, ammonium chloride and protease inhibitors on the accelerated degradation of mAChR to determine how lysosomes participate in the accelerated degradation of mAChR (fig. 4, tables 4 and 5). Ammonium chloride and protease inhibitors, which are thought to be mainly lysosomotropic inhibitors (Seglen et al., 1979; Grinde and Seglen, 1980; Libby et al., 1980), had little effect on the accelerated degradation of mAChR. Furthermore it is interesting that even under these conditions we could not detect any accumulation of intracellular mAChR by equilibrium dialysis in the presence and absence of detergent. These results suggest that lysosomes are not involved, or at least are not the rate limiting step, in the accelerated degradation of mAChR, as measured by [^{3}H]QNB binding. Chloroquine was also reported to be effective on the membrane and to inhibit receptor-mediated endocytosis of asialofetuin receptors and mannose-glycoconjugate receptors with dependence on both concentration and time (Berg and Tollesang, 1980; Tietze et al., 1980). Accordingly chloroquine seemed to inhibit the processes of clustering and endocytosis of mAChR.

All the above findings led us to speculate that the initial degradation of mAChR by agonists occurs in the plasma membrane and prevents [^{3}H]QNB binding because we could not detect any intracellular mAChR under our experimental conditions. Assuming that clustering, endocytosis and breakdown in lysosomes is a general pathway of receptor breakdown, the ability of mAChR to bind [^{3}H]QNB may be lost during endocytosis after clustering, as happens for example due to the action of phospholipases or neutral membrane-bound proteases so that protein molecules of the receptor that have lost their binding activity may be internalized and broken down in the lysosomes. It was reported that an acidic phospholipid is important for QNB binding and that unsaturated fatty acids inhibited the binding (Aronstam et al., 1977). These findings support the idea presented above. But with regard to this phenomenon, we could not exclude a possibility that we could not detect intracellular mAChR, because of the low sensitivity of our method or because the receptor was enclosed in vesicles in spite of the treatment with digitonin or hypotonic shock (data not shown).

Guinea-pig vas deferens has few spare mAChR, and so the maximum contraction to ACh is related to the number of mAChR (Takeyasu et al., 1981; Higuchi et al., 1981). There was also fairly good agreement between the number of mAChR measured with [^{3}H]QNB and the maximum contraction to ACh under various experimental conditions in the long-term desensitization experiments (Higuchi et al., 1981). However when accelerated degradation of mAChR was inhibited by adding chloroquine or vinblastine to the medium with ACh, the maximum contractions to ACh decreased after washing for 30 min whereas the level of mAChR on the surface membrane remained unchanged. Moreover under these conditions the contractile system was not impaired, and the decreased contractions to ACh were not due to non-specific effects of these drugs. These findings indicate that there is a another mechanism for long-term desensitization of the smooth muscle to ACh besides loss of mAChR, as previously assumed by Taylor et al. The mechanism might involve an undefined conformational change of the receptor or a change of some factors involved in 'coupling' between the muscarinic receptor on the surface membrane and the Ca^{2+}-channel. The undefined comformational change of the receptor described above may be the first event in receptor loss. These problems remain to be clarified.

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