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# REDUCTION IN THE NUMBERS OF MUSCARINIC RECEPTORS BY AN ENDOGENOUS PROTEIN

YANG-IL FANG,* MICHIKO IIJIMA, MICHIKO OGAWA, TOSHIHIKO SUZUKI and KAZUTAKA MOMOSE

Department of Pharmacology, School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan

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**Abstract**—The soluble fraction from the ileal longitudinal muscle of guinea pigs was examined for the presence of an endogenous modulator of muscarinic receptors. In the presence of the soluble fraction, the binding of [$^3$H]quinuclidinyl benzilate to the membranes from the tissue was inhibited in a concentration-dependent manner. The inhibitory activity in the soluble fraction was heat stable, but was inactivated by trypsin treatment. Several protease inhibitors had no effect on the inhibitory activity. These results suggest the existence of an endogenous protein that inhibits the binding of the muscarinic ligand to the receptor. Ultrafiltration demonstrated that the protein factor had a molecular mass of more than 10,000 Da. Saturation binding and dissociation kinetic experiments indicate neither a competitive nor allosteric mode of inhibitory action and suggest that an irreversible block or internalization of muscarinic receptors is induced by the endogenous protein.

Modulation of muscarinic receptors has been demonstrated in a variety of cell types. In intact cells, agonist-induced desensitization of the receptors is accompanied by a significant reduction in the number of receptors [see 1 for review]. In cell-free systems, however, exposure to a high concentration of agonists fails to change the binding properties of the receptors. Thus, a variety of cellular events can probably mediate this agonist-induced desensitization and receptor loss. It has been reported that phosphorylation of the receptors at the serine and threonine residues may be involved in the desensitization of muscarinic receptors as well as other G-protein-linked receptors [2–5]. However, phosphorylation of chick heart muscarinic receptors decreases the affinity of muscarinic agonists without significant changes in the total number of receptors [6]. Moreover, for the $\beta_2$-adrenergic receptor, deletion of the phosphorylation sites by specific mutation does not abolish the agonist-induced receptor loss [7]. Therefore, even if receptor phosphorylation is involved in the desensitization process, some intracellular factors other than kinases are probably involved in the agonist-induced receptor loss.

Allosteric modulation of radioligand binding by gallamine and other drugs suggests the existence of an allosteric regulatory site on the muscarinic receptors [see 8 for review]. Although the physiological relevance is not clear, such an allosteric site might be available for interaction with endogenous substances. These results suggest the possibility of the presence of novel modulatory substances.

In this report we describe evidence that the soluble factor from smooth muscle reduces the muscarinic receptor number without changing the affinity for radioligands. Preliminary characterization of this factor showed that it was a heat-stable protein with a molecular mass of more than 10,000 Da.

## MATERIALS AND METHODS

*Chemicals.* [$^3$H]Quinuclidinyl benzilate ([$^3$H]-QNB†; 45.4 Ci/mmol) and [$^3$H]*N*-methyl scopolamine ([$^3$H]NMS; 79.5 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Trypsin (type III), soybean trypsin inhibitor (type I-S), atropine sulfate, aprotinin, pepstatin A and phenylmethylsulfonyl fluoride were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Antipain, benzamidine hydrochloride and leupeptin hemisulfate were from Wako Pure Chemical Industries (Osaka, Japan). *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) was from Dojindo Laboratories (Kumamoto, Japan). Other chemicals used were of analytical grade.

*Preparation of soluble fractions and membranes.* Ileal longitudinal strips from guinea pigs were minced and homogenized (1.0 g tissue/mL of buffer) in HEPES-buffered Tyrode solution (TH solution; pH 7.4) at 4° with a Polytron homogenizer (three 10-sec bursts at 18,000 rpm). The homogenate was centrifuged at 1000 *g* for 10 min, followed by another centrifugation of the resulting supernatant at 100,000 *g* for 60 min. The supernatant from the second spin was designated as the soluble fraction (SFr) and was incubated in a boiling water bath for 5 min. Denatured protein was removed by centrifugation (25,000 *g* for 20 min) and the supernatant obtained was designated as the heat-treated

* Corresponding author.

† Abbreviations: [$^3$H]QNB, [$^3$H]quinuclidinyl benzilate; [$^3$H]NMS, [$^3$H]*N*-methyl scopolamine; SFr, soluble fraction; HSFr, heat-treated soluble fraction; $B_{max}$, maximal ligand binding; $K_d$, equilibrium dissociation constant; $IC_{50}$, 50% inhibitory concentration; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; TH solution, HEPES-buffered Tyrode solution (pH 7.4).

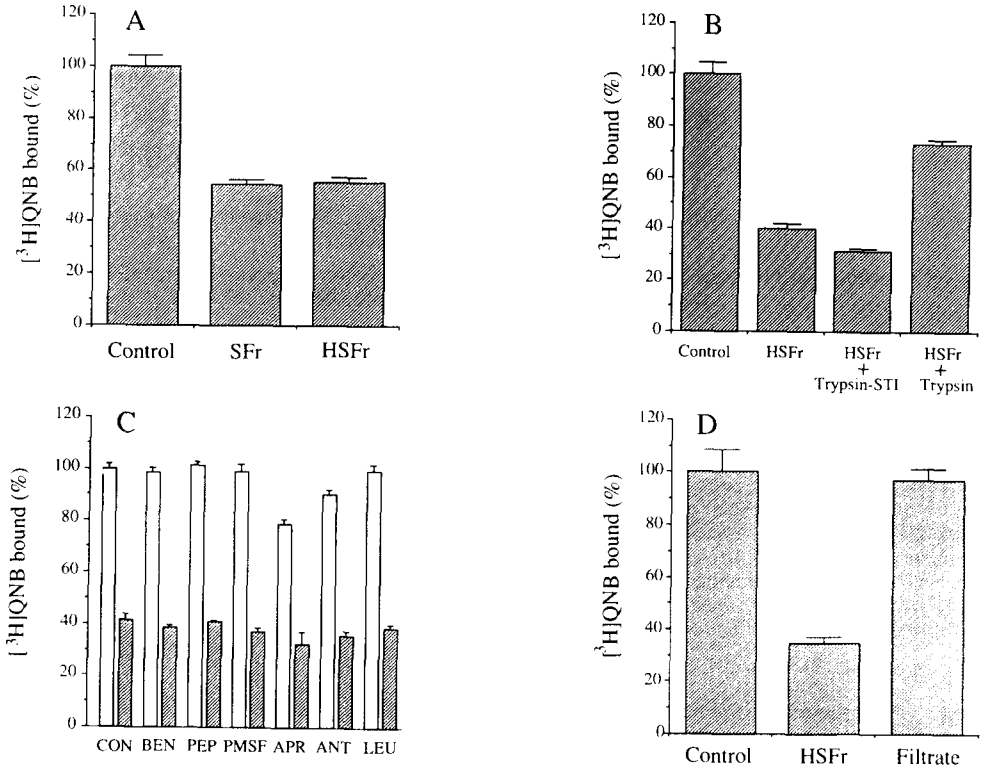


Fig. 1. Characteristics of inhibitory activity in the soluble fraction. Membranes were incubated (23°, 90 min) with 1 nM of [$^3$H]QNB in the presence and absence of soluble fractions. Data are means ± SEM of four experiments and are presented as percentages of specific binding obtained in the absence of the soluble fractions. (A) Effect of heat treatment (100°, 5 min) of the soluble fraction on the inhibitory activity. SFr, soluble fraction (930 μg protein/mL); HSFr, heat-treated soluble fraction (88 μg protein/mL which corresponds to equivolume of the SFr). (B) Effect of trypsin treatment of HSFr. HSFr was treated with $10^4$ U/mL of trypsin, at 37° for 3.5 hr. The reaction of trypsin was stopped by adding 2 mg/mL of soybean trypsin inhibitor (STI). As a control, both trypsin and STI were added to the HSFr. (C) Effect of protease inhibitors on inhibitory activity in the HSFr. Open bars represent incubation with the inhibitor only; hatched bars represent incubation with the inhibitor plus HSFr. CON, buffer control; BEN, 50 μM benzamidine; PEP, 10 μM pepstatin A; PMSF, 100 μM phenylmethylsulfonyl fluoride; APR, 10 μM aprotinin; ANT, 50 μM antipain; LEU, 10 μM leupeptin. (D) Ultrafiltration (10,000 Da cut-off) of HSFr.

soluble fraction (HSFr). The HSFr was concentrated about 10-fold by ultrafiltration (CentriCell 20; 10,000 Da cut-off; Polysciences, Warrington, PA, U.S.A.).

The membrane fraction was prepared from ileal longitudinal strips by homogenization and differential centrifugation in a similar manner except that 0.32 M sucrose containing 5 mM HEPES (pH 7.4) was used as the homogenization buffer in place of TH solution. The resultant 100,000-*g* pellet was washed and resuspended in the homogenization buffer and used as the membrane fraction.

Protein was measured by a Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, U.S.A.) using bovine serum albumin as a standard [9].

*Equilibrium binding assay and dissociation kinetics.* A membrane fraction (10 μg/mL) was incubated in TH solution in the absence and presence of several-fold diluted SFr or HSFr with [$^3$H]QNB at 23° for 90 min. The binding reaction was terminated by filtration under a vacuum through Whatman GF/B glass fiber filters (Whatman International, Maidstone, U.K.). The filters were then washed twice with 5 mL ice-cold TH solution. After drying the filters, radioactivity on the filters was counted by liquid scintillation spectrometry in 4 mL of toluene scintillator.

For saturation binding assays, the concentration of [$^3$H]ligands varied between 0.01 and 0.80 nM, and the ligands were incubated with the membrane at 23° for 90 min in the absence and presence of HSFr (20 μg protein/mL).

In order to evaluate the effect of the HSFr on [$^3$H]NMS dissociation, the radioligand (1 nM) was first equilibrated with the membranes at 23° for 90 min and then its dissociation was initiated by the addition of a final concentration of 2 μM atropine with or without HSFr (final 20 μg protein/mL). Aliquots were removed at various times and filtered as above.

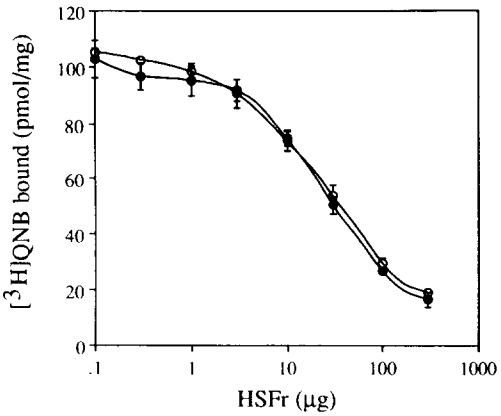


Fig. 2. Concentration-dependent inhibition of [$^3$H]QNB binding by HSFr. Membranes were incubated (23°, 90 min) with 1 nM (○) or 5 nM (●) of [$^3$H]QNB in the absence and presence of several-fold diluted HSFr. Data are means ± SEM from three experiments performed in duplicate, and are presented as percentages of specific binding obtained in the absence of the HSFr.

In all the experiments, specific binding was defined as the difference between the binding of [$^3$H]ligands in the absence and presence of 10 μM atropine.

## RESULTS

The inhibition of [$^3$H]QNB binding by soluble fractions and some characteristics of the inhibitory activity are shown in Fig. 1. In the presence of the SFr, [$^3$H]QNB binding to ileal longitudinal membranes was reduced to 54% of the control (Fig. 1A). Heating the SFr at 100° for 5 min (HSFr) produced no changes in the inhibitory activity (Fig. 1A) and, therefore, HSFr was used for further experiments. The inhibitory activity in the HSFr was inactivated by trypsin treatment (Fig. 1B). Several protease inhibitors had no effect on the inhibitory activity (Fig. 1C). These results suggest the presence of an endogenous protein other than proteases interfered with antagonist binding to the muscarinic receptors. Ultrafiltration demonstrated that this factor had a molecular mass of more than 10,000 Da (Fig. 1D).

Figure 2 shows that HSFr inhibited the specific binding of 1 nM [$^3$H]QNB in a concentration-dependent manner. The 50% inhibitory concentration ($IC_{50}$) of HSFr was 30.6 μg protein/mL. Increasing the concentration of [$^3$H]QNB (5 nM) did not affect inhibition. The Hill coefficient for inhibition at 1 and 5 nM [$^3$H]QNB was 0.73 and 0.85, respectively. Figure 3 shows the Scatchard plots of the saturation binding isotherms for [$^3$H]-ligand binding in the absence and presence of HSFr (20 μg protein/mL). Membranes incubated with [$^3$H]QNB in the presence of the HSFr exhibited 35% less maximal [$^3$H]QNB binding ($B_{max}$) than membranes incubated with buffer alone, while the equilibrium dissociation constant ($K_d$) for [$^3$H]QNB was not significantly changed (Fig. 3A). A similar result was obtained when [$^3$H]NMS was used as the radiolabeled ligand (Fig. 3B). Thus, the effect of the HSFr was not a specific property of [$^3$H]QNB, rather a property of the muscarinic receptor in general. Figure 4 shows that HSFr (20 μg protein/mL) had no effect on the dissociation of [$^3$H]NMS from muscarinic receptors induced by the high concentration of atropine. These results indicate neither competitive nor allosteric inhibition of [$^3$H]-ligand binding by the endogenous protein.

## DISCUSSION

In the present paper, we report the presence of an endogenous factor that inhibits the binding of muscarinic ligands to the receptor. Although the endogenous factor is heat stable (Fig. 1A), it is a protein since the activity was inactivated by trypsin treatment (Fig. 1B). The factor is not a protease because several protease inhibitors had no effect on the inhibitory activity (Fig. 1C). Ultrafiltration demonstrated that the factor had a molecular mass of more than 10,000 Da (Fig. 1D).

There are several reports of competitive and allosteric inhibition of radioligand binding to muscarinic receptors [see 8 and 10 for review], and their kinetics are well defined mathematically [11, 12]. Since the interaction of competitive or allosteric inhibitors with the receptors is reversible, increasing the radioligand concentration reverses their action and, therefore, results in a characteristic shift of their inhibition curves. For a similar reason, competitive and allosteric inhibitors only result in a change in $K_d$ and not $B_{max}$ of radioligands in a saturation experiment. However, inhibition of [$^3$H]-QNB binding by HSFr did not change with an increase in the concentration of [$^3$H]QNB (Fig. 2). Moreover, HSFr reduced the $B_{max}$ of [$^3$H]ligands without significant changes in the $K_d$ values (Fig. 3). Also, while allosteric interactions result in a change in the rate of radioligand dissociation from receptors, HSFr at the $IC_{50}$ concentration did not change the dissociation rate of [$^3$H]NMS (Fig. 4). These results indicate that the inhibitory action of the protein was neither of a competitive nor of an allosteric nature. Interestingly, our data strongly suggest an apparently irreversible action of the protein, since its action is independent of the radioligand concentration; this might be due to an irreversible block or an internalization of the muscarinic receptor induced by the protein.

Although the physiological significance of the action of the protein is not clear, a mechanism consistent with our data suggests a link to the phenomenon reported in cultured cells that agonist-induced desensitization of muscarinic receptors is accompanied by a significant reduction in the number of radioligand binding sites without changes in the affinity (refers to the $K_d$ value) [see 1 for review]. Reduction in receptor number induced by short-term exposure to the agonist is partially reversible, while long-term exposure leads to an irreversible loss of receptors. Thus, the protein could play a role in agonist-induced desensitization and down-regulation of the receptor.

Recent studies have shown that ileal longitudinal

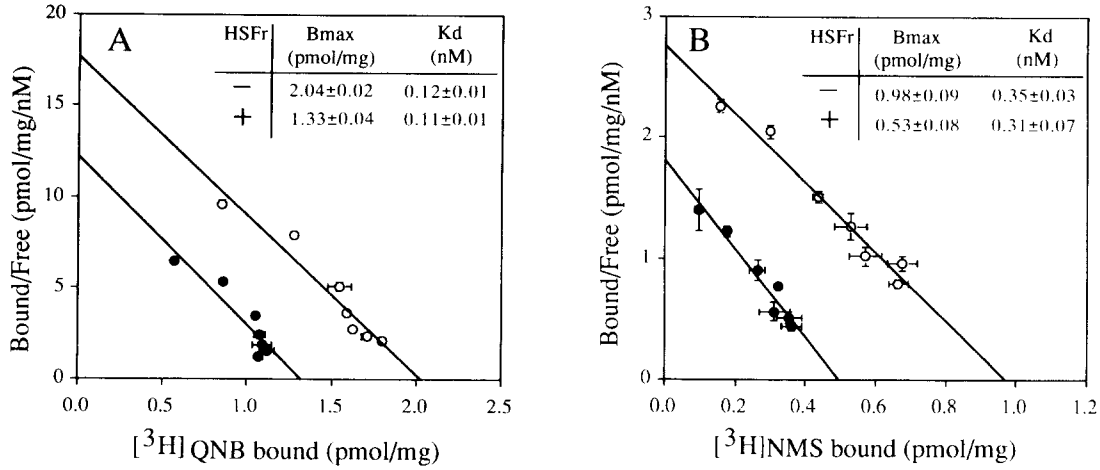


Fig. 3. Saturation isotherms (Scatchard plots) of [$^3$H]QNB (A) and [$^3$H]NMS (B) binding in ileal longitudinal membranes in the absence (○) and presence (●) of HSFr (20 μg protein/mL). Data are means ± SEM from three experiments performed in duplicate. The $K_d$ and $B_{max}$ values were derived from least-squares linear regression of the data ($r > 0.85$ in all cases).

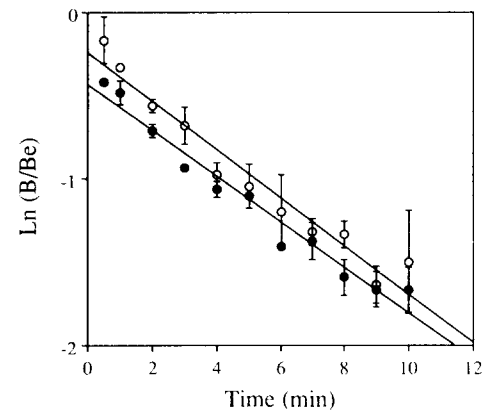


Fig. 4. [$^3$H]NMS dissociation from ileal longitudinal membranes in the absence (○) and presence (●) of HSFr (20 μg protein/mL). Results are expressed relative to the control level of specific binding measured before initiating dissociation (Be). Data are means ± SEM from three experiments. Lines represent least-squares linear regression of the data ($r > 0.95$ for all lines).

smooth muscle membranes contain both $M_2$ and $M_3$ muscarinic receptor subtypes [13, 14]. The action of the protein seems to be subtype selective, since the Hill coefficient of inhibition was slightly less than unity. However, further studies with purified factor are needed for elucidation of its subtype selectivity.

There are other reports stating that in embryonic chick heart and brain [15], and in calf thymus [16] a cellular factor inhibits the binding of [$^3$H]ligands to muscarinic receptors; the factor's heat-stable nature and mode of inhibition are similar to our present data. Although the identity of these factors is not clear, these endogenous factors may play an important role in the modulation of muscarinic receptor function in several tissues.

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