Alterations in Muscarinic Receptors of Ventricular Muscle in Carbachol-Induced Short-Term Desensitization

Toshifumi KAGIYA*, Shuji UCHIDA, Atsushi MIZUSHIMA and Hiroshi YOSHIDA

Departments of Pharmacology I and *Internal Medicine I, Osaka University School of Medicine, Nakanoshima, Kita-ku, Osaka 530, Japan

Accepted May 8, 1986

Abstract—The inhibitory action of a muscarinic agonist on the contractile response of cardiac muscle is transient due to short-term desensitization of muscarinic cholinergic receptors. Studies were made on the binding of the muscarinic antagonist L-[3H]-quinuclidinyl benzilate ([3H]QNB) to the muscarinic receptor in the membrane fraction of ventricular muscle of guinea pigs desensitized by perfusion with carbachol for 10 min. Desensitization did not change the maximum binding or equilibrium dissociation constant (K_d) of [3H]QNB, but shifted the inhibition curves of [3H]QNB binding by carbachol to the right both in the presence and absence of 5'-guanylylimidodiphosphate (GppNHp). Analysis of these inhibition curves with a multiple site model suggested that superhigh and high affinity agonist binding sites were converted to low affinity sites in the desensitized state. GppNHp has additive effects to the prior exposure to carbachol, suggesting a different site of action from short-term exposure of agonist. We conclude that agonist-induced short-term desensitization of the muscarinic receptor of ventricular muscle is caused by reduction in the affinity of the receptor for agonist without reduction in its amount or affinity for antagonist.

Muscarinic agonists cause only transient inhibition of the contractile and electrophysiological response of cardiac muscle (1, 2). Martin et al. (3) noted a fading of cardiac responses during tonic vagal stimulation and postulated that this desensitization was mainly due to receptor-effector desensitization mechanisms. Mubagwa and Carmeliet (4) observed a biphasic response of electrophysiological properties of cardiac Purkinje fibers on short-term stimulation with acetylcholine, indicating a desensitization mechanism of muscarinic receptors. Although receptor desensitization has been generally thought to explain the gradual reduction, or fading, of the muscarinic response, there is little direct evidence for change of the receptor molecule (5).

Recently, use of radioactive ligands such as the antagonist [3H]QNB has made it possible to identify the muscarinic acetylcholine receptor (mACHr) directly (6, 7). Although [3H]QNB binding shows the characteristics of a single population of binding sites, experiments on competition of muscarinic agonist with [3H]QNB for binding to membranes from heart and brain revealed the presence of three populations of agonist binding sites, superhigh (SH), high (H) and low (L) affinity sites (for review, see 8). We have demonstrated the interconversion of these different types of agonist binding sites using guanine nucleotide and sulfhydryl reagent in cardiac membrane of guinea pig (9). However, the physiological significances of these three populations and of these interconversions are unknown.

Desensitization of the muscarinic response seems to occur in two phases, rapid desensitization within minutes (3) and then long-term desensitization within hours. Galper et al. (5) demonstrated that the
changes of muscarinic receptors of cultured heart cells exposed to agonist for 15 min and 3 hr are different.

In previous studies with guinea pig vas deferens cultured with acetylcholine for a long period, we observed decrease of mAChR on long-term desensitization. During long-term desensitization, degradation of mAChR through clustering and endocytosis are accelerated (10-12).

To clarify the molecular mechanism of agonist-induced short-term desensitization, we analyzed the changes of mAChR of guinea pig cardiac muscle perfused with carbachol by studies on [3H]QNB binding.

Materials and Methods

1. Chemicals: L-[3H]-quinuclidinyl benzilate (QNB) (33.0 Ci/mmol) was purchased from New England Nuclear, carbachol chloride from Sigma and 5'-guanylylimidodiphosphate (GppNHp) from Boehringer Mannheim. Other drugs and chemicals were standard commercial products.

2. Contraction of papillary muscle: Papillary muscles from the right ventricles of guinea pigs weighing 300 to 350 g were mounted in an organ bath containing Locke's solution that was continuously bubbled with 100% O2 at 37°C. The tissue was attached to an isotonic transducer for recording the contraction, and it was stimulated electrically with square wave pulses of 5 msec duration at a rate of 30 stimuli/min through bipolar electrodes. The contraction of the papillary muscle was activated by 10^-8 M isoproterenol.

3. Perfusion techniques: Hearts from male guinea pigs weighing 300 to 350 g were perfused retrogradely by Langendorff's technique with Locke's solution containing 156 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl2, 4.8 mM NaHCO3 and 5.6 mM glucose, bubbled with 100% O2 and maintained at 37°C. A pin was inserted into the apex of the heart and attached to an isotonic transducer for recording contraction of the ventricle. After perfusion with standard Locke's solution for 10 min, the heart was perfused with the same solution with or without 2 x 10^-4 M carbachol for 10 min.

4. Preparation of ventricular membranes: The left and right ventricular muscles were perfused for 20 min, and then they were minced with scissors and homogenized in 5 volumes of 0.32 M sucrose with a Polytron operated at a power setting of 6 for 2 min. The homogenate was centrifuged at 1,000 g for 10 min, and the supernatant was recentrifuged at 70,000 g for 30 min. All procedures were done at 0-4°C. The precipitate obtained on the second centrifugation was suspended in 25 mM Tris-HCl buffer (pH 7.4) and stored at -80°C until use. Freezing at -80°C for 4 weeks did not affect the Kd or Bmax of [3H]QNB binding of the fraction. Therefore, we used frozen preparations within 4 weeks.

5. Assay of QNB binding: Binding of [3H]-quinuclidinyl benzilate (QNB) was assayed by incubations at 37°C for 90 min. The composition of the reaction mixture was 100 mM NaCl, 1 mM MgCl2, 50 mM Tris-HCl (pH 7.4) and 300-400 µg membrane protein in 1 ml. For study of inhibition of [3H]QNB binding by carbachol, 380 pM [3H]QNB and various concentrations of carbachol were added to the mixture. To study the effect of guanine nucleotide, 50 µM GppNHp was added to the reaction medium. Nonspecific binding was defined as [3H]QNB binding in the presence of 10 µM atropine.

After incubation, membrane bound [3H]-QNB was separated from free radioligand by rapid filtration using a Whatman GF/F glass filter. The filters were washed with two 3 ml portions of ice-cold 50 mM Tris-HCl buffer, and trapped radioactivity was measured by liquid scintillation spectrophotometry in a Triton/toluene based scintillation cocktail.

Protein was determined by the method of Lowry et al. (13) with bovine serum albumin as a standard.

6. Data analysis: The equilibrium Kd value and maximum binding (Bmax) of [3H]QNB were determined by Scatchard analysis by linear regression of least squares.

The inhibition curves of [3H]QNB binding by carbachol were analyzed with a one-site model with a Hill coefficient, a two-site model and a three-site model by nonlinear least squares regression using a computer program (SIMPLEX) from the program library of the Computation Center of Osaka University. The
equation used for curve fitting was
\[
\frac{B_{\text{max}} - B}{B_{\text{max}}} = \alpha \cdot \frac{[C]}{[C + K_{i1}]} + \beta \cdot \frac{[C]}{[C + K_{i2}]} + \gamma \cdot \frac{[C]}{[C + K_{i3}]},
\]
where \(B\) represents [\(^3\)H]-QNB bound, \(C\) is the concentration of carbachol, \(K_{i}\) is the apparent dissociation constant of each site for carbachol in the presence of QNB, and \(\alpha, \beta, \gamma\) are the subpopulation ratios of each site.

Ki values were corrected by the equation of Cheng and Prusoff (14)
\[
K_{i\text{cor}} = \frac{K_i}{[C_{\text{QNB}}]/K_{d\text{QNB}} + 1},
\]
where \(C_{\text{QNB}}\) and \(K_{d\text{QNB}}\) represent the concentration and equilibrium dissociation constant of the radioligand, respectively.

Student's \(t\)-test was applied for comparison of the groups of means in inhibition curves and the subpopulation ratios of each site. Differences were considered significant when \(P < 0.05\).

Results

1. Effects of carbachol on the contraction of ventricular muscle: Carbachol had a negative inotropic action (IC50 \(5 \times 10^{-7}\) M) on the contraction of papillary muscle (Fig. 1). It also inhibited the contraction of perfused ventricle. Its inhibitory effects on the contraction of perfused heart was transient, as shown in Fig. 2. Its negative inotropic effect and its negative chronotropic effect disappeared within 10 min in all experiments. These results indicated that short-term desensitization of the muscarinic receptor occurred under our experimental conditions.

2. Scatchard analysis of [\(^3\)H]QNB binding: [\(^3\)H]QNB binding to guinea pig ventricular membrane showed a single affinity both in the control and in the desensitized state as judged by Scatchard analysis in the [\(^3\)H]QNB concentration range of 10 to 600 pM. The \(K_d\) value of [\(^3\)H]QNB binding was 46.4 ±6.2 pM (mean±S.D., \(n=4\)) for control membranes and 61.0±11.1 pM (\(n=4\)) for desensitized membranes. These values were not significantly different. The maximum binding of [\(^3\)H]QNB was 202.9±35.7 fmol/mg protein (\(n=4\)) with control membranes and 194.6±35.3 fmol/mg protein (\(n=4\)) with desensitized membranes. These values

![Fig. 1. Effects of carbachol on contraction of papillary muscle. Points are means for three experiments.](image)

![Fig. 2. Effects of carbachol on contraction of perfused ventricle. The perfusate was changed from standard Locke's solution to the same solution containing 2×10^{-4} M carbachol at the indicated point.](image)
were also not significantly different. Thus neither the total number nor the affinity of antagonist binding to muscarinic receptor was changed by short-term desensitization.

3. Inhibition of $[^3H]QNB$ binding by carbachol: Next we examined the affinity for agonist by studies on inhibition of $[^3H]QNB$ binding by carbachol. Figure 3 shows the inhibition curves of $[^3H]QNB$ binding by carbachol for control and desensitized ventricular membranes. The inhibition curve of control membrane was shifted to the right both by GppNHp and by the desensitization. The curves of desensitized membranes both in the presence and absence of GppNHp were shifted to the right of that for control membranes. The shifts caused by GppNHp and by the desensitization were additive.

For the inhibition curves in control membrane shown in Fig. 3, the three-site model gave a better fit than the one-site or two-site model, in agreement with the results obtained for rat brain by Birdsall et al. (15). Previous studies in our laboratory indicated the presence of three different affinity sites for agonist binding, superhigh (H), high (H) and low (L) affinity sites in control membranes (9). However, a very small population of SH sites was concluded to be present by applying the three-site model to the inhibition curves in desensitized or GppNHp treated membranes. In the other case, we obtained nearly equal $K_i$ values for two sites of three sites by applying the three-site model to the inhibition curves in desensitized or GppNHp-treated membrane. Furthermore, little or no improvement in the sum of squares of residuals were obtained by adopting the three-site model for these curves. Therefore, the three-site model was adopted for the control curves, and the two-site model was adopted for the curves of desensitized and GppNHp-treated membranes.

The results of nonlinear least square
Table 1. Subpopulation ratio and $-\log[K_i]$ of each site

| Treatment            | SH    | H     | L     | n  |
|----------------------|-------|-------|-------|----|
| Control, GppNHp(-)   | 20.3±3.0 | 50.3±8.5 | 25.6±4.6 | 4  |
| Control, GppNHp(+)   | 6.39±0.15 | 5.23±0.32 | 3.94±0.09 |    |
| Carbachol, GppNHp(-) | 47.0±6.5 | 5.55±0.42 | 48.4±6.8n | 4  |
| Carbachol, GppNHp(+) | 33.7±4.4n | 6.58±0.31 | 62.4±4.3n | 5  |

Inhibition curves obtained in each heart were fitted to the respective models independently. Values are means±S.D. Significant differences from the values: for the control-GppNHp(-), **P<0.02** and *P<0.01; for the control-GppNHp(+), ***P<0.01; for carbachol-GppNHp(-), **P<0.01; for carbachol-GppNHp(+), *P<0.01. SH, superhigh; H, high; L, low affinity site for agonist binding.

Discussion

In general, neurotransmitter receptors and linked reactions are thought to maintain their homeostasis by decrease in their response to excessive stimulation by nerves or exogenously added agonists. Two types of changes are considered to be responsible for desensitization of the receptor mediated response. One is decrease in the receptor number, a quantitative change that is known as "down regulation". When guinea pig vas deferens is exposed to acetylcholine for a long period (more than 6 hr), its response to acetylcholine is lost selectively and the number of mAChR is decreased. The decrease in mAChR is not easily restored by washing the tissue. This phenomenon is generally called "long-term homologous desensitization". We have reported that this type of desensitization seems to be due mainly to accelerated degradation of mACHR through its clustering and endocytosis (11). The other type of desensitization is change in reactivity, that is a qualitative change of the receptor. As shown in the present study, short-term desensitization caused by exposure to agonist for a short period seemed to result from configurational changes of the receptor in the plasma membrane without any reduction in its amount. Time-dependent change in the affinities for agonist have also been reported in $\beta$-adrenergic receptors on intact lymphoma cells and chick erythrocytes (16, 17).

Previously, we reported that mAChRs of guinea pig ventricular muscle consist of three different agonist binding sites. In the presence of guanine nucleotide, the population of the superhigh (SH) site is decreased and that of the low (L) site is increased, while in the presence of sulfhydryl reagent, the population of the L site is decreased (9). If there was residual carbachol in the membrane preparation of desensitized heart, the inhibition curves of $[^3H]$QNB binding by carbachol would be shifted to the left. So the rightward shift of the inhibition curves in our experiment are not due to the residual carbachol in the membrane preparation. Moreover, the $K_d$ values of $[^3H]$QNB binding are not affected by the desensitization. Thus the effects of residual carbachol would be negligible. Since the maximum number of $[^3H]$QNB binding sites is not decreased in desensitized membrane,
the disappearance of SH sites (Table 1) indicates that the SH site is converted to the L site or that the SH site is converted to the H site and the H site to the L site. The populations of H sites are decreased significantly in the desensitized membranes compared with the control membranes. So there is a possibility of the interconversion of the H site to the L site in the carbachol-induced desensitization. These processes may be due to configurational changes of mAChR. Guanine nucleotides are known to regulate the affinity of agonists, but not antagonists to mAChR (18) as well as to many other receptor systems (19, 20). In this work, the effect of guanine nucleotide was still observed in desensitized membranes (Fig. 3). Thus the conformational changes of the receptor in the short-term desensitized state seems to be independent of the guanine nucleotide binding site. A concentration of 50 μM GppNHp seemed sufficient to cause changes in configuration of the receptor through the GTP binding protein, because 50 μM and 100 μM GppNHp had the same effects on the inhibitory curve of [3H]QNB binding by carbachol (data not shown).

Burgoyne has shown that phosphorylating conditions reduce the affinity of mAChR of rat brain synaptic membranes for agonists (21). These processes may have some role in the conformational changes of receptors on short-term desensitization. Receptor phosphorylation correlated with attenuation of adenylate cyclase activity in desensitization were already known in β-adrenergic receptors of turkey erythrocytes (17). Another possible reason for the decrease in affinity for agonist is that mAChR may be separated from the GTP binding protein by endocytosis: The lipid-soluble ligand ([3H]QNB) may show reduced affinity for internalized mAChR.

Galper et al. (5) showed that the response of muscarinic receptors to agonists in cultured heart cells and homogenates is biphasic with respect to both time and agonist concentration. They demonstrated that brief (15 min) exposure to agonist resulted in reduction in affinity to agonist and about 25% decrease in the receptor number both in intact cells and in homogenates. The slower loss of [3H]QNB binding (70%) over 3 hr could only be demonstrated in intact cells incubated with agonist prior to homogenization. Our work provides further evidence that reduction in affinity of mAChR for its agonist is predominant to reduction in its affinity for antagonist or in its amount in short-term desensitization of the receptor. We showed GTP-independent reduction in the affinity of the receptor for agonist without decrease of the receptor number in the membrane of isolated working heart. We desensitized the ventricular muscle by perfusion with agonist for 10 min and recorded the contractile response simultaneously. Ten minutes was enough to cause desensitization of mAChR in our experimental conditions, and further perfusion with agonist may have resulted in decrease in receptor number.

The short-term desensitization shown in this study may be easily recovered by washing the ventricle as compared with the long-term desensitization which is not easily recovered by washing. The conformational change of the L site to the H and SH sites may explain the recovery from the short-term desensitization.

In conclusion, we demonstrate that short-term desensitization of muscarinic receptor of ventricular muscle is caused by reduction in the affinity of the receptor for agonist without reduction in its amount or affinity for antagonist. The interconversions of heterogeneous agonist binding sites mainly cause the reduction in affinity of agonist. These phenomena seem to have significant roles in the modulation of physiological responses on continuous exposure to agonist.

Acknowledgements: We thank Dr. M. Inoue (First Department of Medicine, Osaka University School of Medicine) for constant interest and encouragement in this investigation and thank Mrs. M. Nakamura for secretarial assistance. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and a Research Grant from the Ministry of Health and Welfare of Japan.

References
1 Gertjegerdes, W., Ravens, U. and Ziegler, A.: Time courses of carbachol-induced responses in guinea pig atria under the influence of ouabain.
calcium, and rate of stimulation. J. Cardiovasc. Pharmacol. 1, 235–243 (1979)

2 Jalife, J., Hamilton, A.J. and Moe, G.K.: Desensitization of the cholinergic receptor at the sinoatrial cell of the kitten. Am. J. Physiol. 238, H439–H446 (1980)

3 Martin, P., Levy, M.N. and Matsuda, Y.: Fade of cardiac responses during tonic vagal stimulation. Am. J. Physiol. 243, H219–H225 (1982)

4 Mubagwa, K. and Carmeliet, E.: Effects of acetylcholine on electrophysiological properties of rabbit cardiac Purkinje fibers. Circ. Res. 53, 740–751 (1983)

5 Galper, J.B., Dziekan, L.C., O’Hara, D.S. and Smith, T.W.: The biphasic response of muscarinic cholinergic receptors in cultured heart cells to agonist. J. Biol. Chem. 257, 10344–10356 (1982)

6 Hulme, E.C., Birdsall, N.J.M., Burgena, A.S.V. and Mehta, P.: The binding of agonists to brain muscarinic receptors. Mol. Pharmacol. 14, 737–750 (1978)

7 Snyder, S.H., Chang, K.J., Kuhar, M.J. and Yamamura, H.I.: Biochemical identification of the mammalian muscarinic cholinergic receptor. Fed. Proc. 34, 1915–1921 (1975)

8 Birdsall, N.J.M. and Hulme, H.C.: Muscarinic receptor subclasses. Trends Pharmacol. Sci. 4, 459–463 (1983)

9 Uchida, S., Matsumoto, K., Mizushima, A., Osugi, K., Higuchi, H. and Yoshida, H.: Effects of guanine nucleotide and sulfhydryl reagent on subpopulations of muscarinic acetylcholine receptors in adult rat heart: possible evidence for interconversion of super-high and low affinity agonist binding sites. Eur. J. Pharmacol. 100, 291–298 (1984)

10 Higuchi, H., Takeyasu, K., Uchida, S. and Yoshida, H.: Receptor activated and energy-dependent decrease of muscarinic cholinergic receptors in guinea-pig vas deferens. Eur. J. Pharmacol. 75, 305–311 (1981)

11 Higuchi, H., Takeyasu, K., Uchida, S. and Yoshida, H.: Mechanism of agonist induced degradation of muscarinic cholinergic receptor in cultured vas deferens of guinea-pig. Eur. J. Pharmacol. 79, 87–77 (1982)

12 Higuchi, H., Uchida, S., Matsumoto, K. and Yoshida, H.: Inhibition of agonist-induced degradation of muscarinic cholinergic receptors by quinacrine and tetracaine—possible involvement of phospholipase A2 in receptor degradation. Eur. J. Pharmacol. 94, 229–239 (1983)

13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275 (1951)

14 Cheng, Y.C. and Prusoff, W.H.: Relationship between the inhibition constant (K\text{I}) and the concentration of inhibitor which causes 50 per cent inhibition (I\text{50}) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108 (1973)

15 Birdsall, N.J.M., Burgen, A.S.V. and Hulme, E.C.: The binding of agonist to brain muscarinic receptors. Mol. Pharmacol. 14, 723–736 (1978)

16 Hoyer, D., Reynolds, E.E. and Molinoff, P.B.: Agonist-induced changes in the properties of beta-adrenergic receptors on intact S49 lymphoma cells. Mol. Pharmacol. 26, 209–218 (1984)

17 Sibley, D.R., Peters, J.R., Nambi, P., Caron, M.G. and Lefkowitz, R.J.: Desensitization of turkey erythrocyte adenylate cyclase. J. Biol. Chem. 259, 9742–9749 (1984)

18 Ehler, F.J., Roeske, W.R., Rosenberger, L.B. and Yamamura, H.I.: The influence of guanyl-5'-yl imidodiphosphate and sodium on muscarinic receptor binding in the rat brain and longitudinal muscle of the rat ileum. Life Sci. 26, 245–252 (1980)

19 Lefkowitz, R.J., Mullikin, D. and Caron, M.G.: Regulation of β-adrenergic receptors by guanyl-5'-yl imidodiphosphate and other purine nucleotides. J. Biol. Chem. 251, 4686–4692 (1976)

20 Lefkowitz, R.J., Mullikin, D., Wood, C.L., Gore, T.B. and Mukherjee, C.: Regulation of prostaglandin receptors by prostaglandins and guanine nucleotides in frog erythrocytes. J. Biol. Chem. 252, 5295–5303 (1977)

21 Burgoyne, R.D.: Regulation of the muscarinic acetylcholine receptor: effects of phosphorylating conditions on agonist and antagonist binding. J. Neurochem. 40, 324–331 (1983)