Guanosine 5'-Triphosphate Converts Some Populations of Propylbenzilylcholine Mustard-Sensitive Muscarinic Cholinoceptor Sites to Sites Resistant to the Drug in Intestinal Smooth Muscle

Youichi Kiuchi, Naomi Kumagai, Tetsuhiro Hisayama* and Issei Takayanagi*

Department of Chemical Pharmacology, Toho University School of Pharmaceutical Sciences, 2-2-1, Miyama, Funabashi, Chiba 274, Japan

Received September 12, 1990 Accepted December 21, 1990

ABSTRACT—From functional studies with propylbenzilylcholine mustard (PrBCM), we reported that there coexist PrBCM-sensitive and PrBCM-resistant muscarinic cholinoceptor mechanisms in guinea pig taenia caecum. We investigated the interrelationship between these two cholinoceptor mechanisms using an in vitro receptor binding assay with [3H]quinuclidinyl benzilate (QNB) and [3H]PrBCM. Pretreatment of the muscle strips with 300 nM PrBCM (in vivo alkylation) for 10–50 min resulted in progressive decreases of the number of the maximum [3H]QNB binding sites. However, a prolongation of the period of in vivo alkylation up to 90 min was accompanied with no further loss in the binding sites. Under these conditions, there is no significant change in the affinity of [3H]QNB for the binding sites. The concentration of carbachol required to displace 50% of the bound [3H]QNB was larger in membranes obtained from the tissues that had been alkylated in vivo with PrBCM for 50 min than that from control strips, but was not altered when the pretreatment with the drug was carried out after homogenization (in vitro alkylation). When GTP was added during in vitro alkylation, the affinity of carbachol was lower than that in control membranes, as observed when in vivo alkylation was carried out. In the presence of guanine nucleotide, PrBCM thus appears to recognize two distinct populations or states of muscarinic receptors.

Muscarinic cholinoceptors have been pharmacologically divided into three major subtypes, M₁, M₂ and M₃ subtypes, by differences in affinities for the selective competitive antagonists (1–5). By molecular cloning, researchers have recently identified the genes encoding a family of five distinct muscarinic receptors, m₁ to m₅, with seven predicted transmembrane domains (6, 7). Pharmacological analysis has shown, but not unambiguously proven, that the m₁, m₂ and m₃ receptors would correspond to the M₁, M₂ and M₃ receptors, respectively, but the pharmacological properties of m₄ and m₅ receptors have not been identified. These muscarinic cholinoceptors are supposed to interact, via a guanine nucleotide
binding regulatory protein (G protein)-regulated process, with multiple effector systems leading to inhibition of adenylate cyclase, opening of potassium channels and activation of polyphosphoinositide metabolism (7–15).

Differing from these subclassifications using the selective competitive antagonists, based on findings obtained from functional studies, we have tentatively proposed the possibility that the guinea pig taenia caecum possesses two types of muscarinic cholinceptor mechanisms, one being sensitive to and the other being resistant to propylbenzilycholine mustard (PrBCM, N-2'-chloroethyl-N-propyl-2-aminoethyl benzilate) (16, 17), an irreversible specific muscarinic cholinergic antagonist (18). In this communication, we investigated the mechanisms of PrBCM interactions with the two putative muscarinic cholinceptor mechanisms, and suggested that in the presence of guanine nucleotide, PrBCM appears to recognize two distinct populations or states of muscarinic receptor sites, the PrBCM-sensitive and PrBCM-resistant ones in guinea pig taenia caecum.

MATERIALS AND METHODS

Isolated guinea pig taenia caecum
Female Hartley-strain guinea pigs, weighing 300 to 500 g, were stunned by a blow on the head and exsanguinated. The taenia caecum was rapidly dissected, cleaned of adhering tissues, blotted dry on filter paper and weighed. The preparations were then immersed in a container filled with physiological saline solution (PSS) of the following composition: 154 mM NaCl, 5.6 mM KCl, 2.1 mM MgCl₂, 0.8 mM CaCl₂, 6.0 mM NaHCO₃ and 2.8 mM glucose, kept at 30°C and continuously bubbled with air for about 1 hr. If necessary, the muscle strips were further treated with PrBCM as described below.

Preparation of membrane fraction
After incubation in PSS, the taenia caecum was placed in chilled 0.32 M sucrose containing 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), the pH of which was adjusted to 7.4 at room temperature by titration with Tris. The following procedure was carried out at 0–4°C. The muscles were minced finely with scissors and homogenized with a Teflon-glass homogenizer in 50 volumes of the buffered sucrose solution, using 5 up-and-down strokes. They were further homogenized with a Polytron PT-10 (Kinematica GmbH, Switzerland) by means of two 10-sec bursts at a rheoset of 8 and then a one 5-sec burst at full speed. The homogenate was centrifuged at 1,000 × g for 10 min, and the supernatant was filtered through nylon mesh, followed by recentrifugation at 100,000 × g for 60 min. The resultant pellet was suspended with ice-cold phosphate-buffered solution (PBS) (1 mM ethylenediamine tetraacetic acid (EDTA), 11 mM MgCl₂ and 50 mM NaHPO₄/KH₂PO₄, pH 7.4) and used as a membrane fraction.

Treatment of intact tissue with PrBCM (in vivo alkylation)
PrBCM was cyclized or activated according to the method of Young et al. (18) by incubation in 50 mM phosphate buffer (pH 7.4) at 30°C for 20 min. Generally, the muscular preparations were treated for 50 min with activated PrBCM (300 nM) in PSS, and this drug was renewed every 10 min, to avoid its inactivation in aqueous fluid. The taenia caecum was then washed out for at least 60 min with PSS and the membrane fraction was prepared as described above.

Treatment of membrane fraction with PrBCM (in vitro alkylation)
The membrane fraction prepared from the PrBCM-nontreated tissues was preincubated in PBS with and without 3 mM GTP plus a GTP-regenerating system (20 mM creatine phosphate and 100 µg/ml creatine phosphokinase) at 30°C for 3 min. The preincubated membrane was then washed twice by centrifugation at 100,000 × g for 60 min. The pellets obtained
by the first and second centrifugation were re-suspended in ice-cold MgCl₂-omitted PBS and PBS, respectively. The former solution was used to remove GTP from G protein.

**Measurement of [³H]-quinuclidinyl benzilate (QNB) binding**

Thirty to forty μg (PrBCM-nontreated preparation) or 100 to 300 μg (PrBCM-treated preparation) of membrane protein was incubated at 30°C for 90 min in 1 ml of 50 mM PBS containing 0.02 to 0.3 nM [³H]QNB in the absence or presence of various concentrations of the unlabeled ligands. The amount of membranes added was appropriately selected so that in each preparation, the count of the specific binding was about 500 cpm at 0.1 nM [³H]QNB and the membrane concentration used was within the range where the bound radioligand was linearly related to the concentration of membrane protein. Under all conditions, the nonspecific binding was less than 10% of the total binding at 0.3 nM [³H]QNB. The reaction was terminated by rapid filtration over a Whatman GF/F glass fiber filter. The filters were then washed twice with 5 ml of ice-cold PBS. They were dried in liquid scintillation vials by an infrared lamp, 10 ml of a toluene-based scintillator was added and the radioactivity was counted by an ALOKA LSC-900 liquid scintillation spectrometer. Specific [³H]-QNB binding was determined from the difference between the radioligand bindings in the absence and presence of 5 μM atropine.

**Measurement of [³H]-PrBCM binding**

Labeling of the membranes with [³H]-PrBCM was carried out essentially by the method of Liang et al. (19). The membranes (0.4 mg) were initially incubated with and without 5 μM atropine at 25°C for 30 min. Ten minutes after addition of 30 μM guanosine 5’-O-(3-thiotriphosphate) (GTPγS), if necessary, the receptor sites were labeled with varying concentrations of precyclized [³H]-PrBCM in a final volume of 1 ml for 30 min. The labeling was terminated by filtration through a glass fiber filter. The specific [³H]PrBCM binding was determined from the difference between the radioactivities in the absence and presence of atropine. The specific binding was about 30% of the total binding at 30 nM [³H]PrBCM. Since reliable data could not be obtained, the experiment was not carried out at higher concentrations than 30 nM.

**Estimation of the protein concentration**

Protein was measured by the method of Lowry et al. (20) using bovine serum albumin as the standard.

**Statistical analyses**

Binding data were analyzed by nonlinear least squares regression using a program compiled for a PC-9800 (NEC, Tokyo, Japan) personal computer by Drs. S. Uchida and A. Mizushima (Osaka University), a version of the SIMPLEX program from the library of Osaka University. Results are expressed as means ± S.E., and statistical significance was calculated using Student’s t-test or Duncan’s new multiple range test. A P value less than 0.05 was considered to be statistically significant.

**Drug used**

The following materials were obtained from commercial sources: Carbachol chloride, atropine sulfate, Tris, creatine phosphate, creatine phosphokinase, GTP and GTPγS (Sigma Chemical Co., St. Louis, MO); PrBCM and [³H]PrBCM (1.22 TBq/mmol) (New England Nuclear, Boston, MA); [³H]QNB (1.44 TBq/mmol) (Amersham Japan, Co., Ltd., Tokyo, Japan); HEPES and EDTA (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

**RESULTS**

In *vivo* alkylation of [³H]QNB binding sites with PrBCM

In washed membranes prepared from the muscle strips of guinea pig taenia caecum which had been pretreated with PrBCM (300
nM), Scatchard analyses of saturation isotherms of \[^3H\]QNB bindings show that PrBCM decreased the maximum binding sites for the radioligand without a significant change in the $K_d$ values (Fig. 1 and Table 1). When 0.1 $\mu M$ atropine was used as the pretreatment drug (the concentration of atropine was calculated to occupy about 99% of the \[^3H\]QNB binding sites), the receptor site density and the affinity of \[^3H\]QNB toward the binding sites in the washed membranes from the untreated muscles were the same as those in the membranes from atropine-treated muscles.

**Fig. 1.** Scatchard plots of saturation isotherms of (A) and progressive decreases in receptor density (B) of \[^3H\]QNB bindings to the washed membranes prepared from in vivo alkylated muscular strips of guinea pig taenia caecum. In A, the preparations were pretreated with PrBCM (300 nM) for 0 (○), 10 (●), 50 (▲) and 90 min (▲). One representative datum out of 4 separate experiments is shown. In B, the abscissa and ordinate represent the periods of the PrBCM-pretreatment and the receptor density as estimated from Scatchard analyses, respectively. Symbols and bars represent means and S.E., respectively, and the number of experiments is 4.

**Table 1.** The $B_{\text{max}}$ and $K_d$ values for bindings of \[^3H\]QNB to the control and in vivo alkylated membranes from guinea pig taenia caecum

|                | $B_{\text{max}}$ (fmol/mg protein) | $K_d$ (pM)  |
|----------------|----------------------------------|-------------|
| Control        | 1568 ± 263                       | 68.6 ± 8.1  |
| In vivo alkylation for |                                 |             |
| 10 min         | 265 ± 47a                        | 78.8 ± 10.8 |
| 50 min         | 128 ± 18b,ab                     | 98.0 ± 14.9 |
| 90 min         | 98 ± 11a,ab                      | 112.6 ± 18.5|

For in vivo alkylation, the intact muscular strips were pretreated with 300 nM PrBCM for 10, 50 and 90 min, followed by thorough washes of the tissues and their homogenization. Binding assay was carried out using 100,000 × g particulate membranes. Figures represent the mean ± S.E. of 4 separate experiments. *Significant difference from the control value at $P < 0.05$. **Not significantly different from each other at $P > 0.05$. 

When the period of the PrBCM-pretreatment was between 10 and 50 min, the amount of maximum \([{}^3\text{H}]\text{QNB}\) binding sites progressively decreased as the pretreatment of the tissues with PrBCM was prolonged. However, prolongation of the period of in vivo alkylation up to 90 min was accompanied with no further loss in the binding sites (Fig. 1B and Table 1). As shown in Table 1, a small amount of \([{}^3\text{H}]\text{QNB}\) binding sites (6-8\% of the control value) always persisted even after the 90-min pretreatment with PrBCM. These results indicate that there coexist two distinct populations of muscarinic receptor sites or states that can be discriminated by PrBCM, but not by \([{}^3\text{H}]\text{QNB}\), in guinea pig taenia caecum: One is PrBCM-sensitive and occupies most of the \([{}^3\text{H}]\text{QNB}\) binding sites, and the other is PrBCM-resistant although it is minimal in quantity. Thus, we studied the binding characteristics of the PrBCM-sensitive and -resistant sites using membranes prepared from unpretreated muscle strips and strips pretreated with 300 nM PrBCM for 50 min.

As shown in Fig. 2, in vivo alkylation with PrBCM induced an approximately 6-fold rightward shift of the displacement curve for carbachol. The pIC$_{50}$ values for carbachol in inhibiting \([{}^3\text{H}]\text{QNB}\) binding towards the PrBCM-sensitive and -resistant sites were $5.84 \pm 0.08$ and $5.01 \pm 0.06$, respectively (Table 2). Nonlinear regression multiple sites analysis revealed that there are three heterogeneous populations of carbachol binding substates, super-high (SH), high (H) and low (L) affinity substates, on both PrBCM-sensitive and -resistant binding sites. There was no significant difference between these two binding sites in the affinity of carbachol to each subsite [pK$_i$ values for carbachol were 7.29 (the SH sub/site), 5.12 (H) and 3.26 (L) in the unpretreated membranes and 7.23 (SH), 5.08 (H) and 3.08 (L) in the PrBCM-pretreated membranes] (Table 2). The lower pIC$_{50}$ value for carbachol in the pretreated membranes was predominantly derived from the abundance in the H and L substates and the lesser amount in the SH substate. Addition of the nonhydrolyzable GTP analogue GTP$\gamma$S (10 \(\mu\text{M}\)) in the binding assay mixtures decreased the pIC50 values in both unpretreated and pretreated membranes, which would be mainly due to conversion of the SH substate to the H and/or L substate by the nucleotide (Table 2).

In contrast, the displacement curve for atropine fit a mass action curve, suggesting a single population of binding sites in both membranes from unpretreated muscles and muscles pretreated with PrBCM (data not shown).

**In vitro alkylation of \([{}^3\text{H}]\text{QNB}\) binding sites with PrBCM**

We next investigated the binding characteristics of the muscarinic receptor sites using the membranes which had been pretreated with PrBCM followed by 2 washings by centrifugation. Differing from in vivo alkylation, the affinity of carbachol to the \([{}^3\text{H}]\text{QNB}\) binding sites was not changed by in vitro alkylation [$5.84 \pm 0.08$ and $5.78 \pm 0.05$ in the control and in vitro alkylated membranes, respectively (Fig. 3 and Table 3)]. However, the presence of GTP during in vitro alkylation produced a loss in affinity of carbachol to $5.12 \pm 0.02$.
Table 2. The pIC₅₀ values and the pKᵢ values for carbachol at super-high (SH), high (H) and low (L) affinity subsites with their relative densities in the washed membranes prepared from control and in vivo alkylated guinea pig taenia caecum and the effects of GTPγS (10 μM) on these parameters

|                      | pIC₅₀     | SH           | H           | L           |
|----------------------|-----------|--------------|-------------|-------------|
|                      | pKᵢ       | %            | pKᵢ         | %           | pKᵢ         | %           |
| Control membranes    |           |              |             |             |             |             |
| no addition          | 5.84 ± 0.08 | 7.29 ± 0.19  | 49.2 ± 4.0  | 5.12 ± 0.21 | 39.5 ± 5.3  | 3.26 ± 0.35 | 11.4 ± 2.2  | (6)         |
| + 10 μM GTPγS        | 4.96 ± 0.11ᵃ | 7.21 ± 0.31  | 10.2 ± 1.1ᵈ | 5.34 ± 0.37 | 68.8 ± 1.0ᵈ | 3.98 ± 0.56 | 17.9 ± 6.8  | (3)         |
| In vivo alkylated     |           |              |             |             |             |             |             |             |
| membranes             | 5.01 ± 0.06ᵇ | 7.23 ± 0.19  | 23.1 ± 4.0ᵈ | 5.08 ± 0.26 | 57.3 ± 3.2ᵈ | 3.08 ± 0.16 | 15.9 ± 5.3  | (5)         |
| + 10 μM GTPγS        | 4.29 ± 0.09ᶜ |  —           |  —           | 5.49 ± 0.08 | 33.8 ± 3.4ᵉ | 3.14 ± 0.11 | 57.6 ± 2.9ᵉ | (3)         |

In vivo alkylation was carried out by incubation of the muscle strips with 300 nM PrBCM for 50 min. Each figure represents the mean ± S.E., and the number of experiments (n) are indicated in parentheses. ⁺ᵇ Significantly different from the value in control membranes assayed in the absence of GTPγS at P < 0.05. ᵈSignificantly different from the value of the corresponding subsite in the control membranes assayed in the absence of GTPγS at P < 0.05. ᵉSignificantly different from the value of the corresponding subsite in vivo alkylated membranes assayed in the absence of GTPγS at P < 0.05. ᶠDisplacement of [³H]QNB binding by carbachol was fitted to the 2-sites model, so the parameters with respect to the SH subsite was not determined.

Table 3. The pIC₅₀ values and the pKᵢ values for carbachol at super-high (SH), high (H) and low (L) affinity subsites with their relative densities in the washed membranes after in vitro alkylation, and the effect of GTP (3 mM) during the alkylation on these parameters

|                      | pIC₅₀     | SH           | H           | L           |
|----------------------|-----------|--------------|-------------|-------------|
|                      | pKᵢ       | %            | pKᵢ         | %           | pKᵢ         | %           |
| Controlᵇ             | 5.84 ± 0.08 | 7.29 ± 0.19  | 49.2 ± 4.0  | 5.12 ± 0.21 | 39.5 ± 5.3  | 3.26 ± 0.35 | 11.4 ± 2.2  | (6)         |
| In vitro alkylated    |           |              |             |             |             |             |             |             |
| in the absence of GTP | 5.78 ± 0.05 | 7.46 ± 0.23  | 41.3 ± 4.3  | 5.67 ± 0.16 | 33.2 ± 7.5  | 3.36 ± 0.10 | 15.6 ± 2.8  | (6)         |
| in the presence of GTP | 5.12 ± 0.02ᵇ | 6.98 ± 0.22  | 30.1 ± 4.1ᵇ | 5.09 ± 0.26 | 56.7 ± 11.5ᵇ | 3.35 ± 0.24 | 13.2 ± 3.4  | (3)         |

In vitro alkylation was carried out by incubation of the membranes with 10 mM PrBCM for 30 min. Each figure represents the mean ± S.E., and the number of experiments (n) are indicated in parentheses. ᵇThe data are the same as those of the control membranes assayed in the absence of GTPγS in Table 2. ᵇSignificantly different from the corresponding values in the control and the in vitro alkylated (with GTP) membranes at P < 0.05.
Fig. 3. Displacement of [3H]QNB bindings by carbachol in the control (●) and in vitro alkylated membranes (○). In vitro alkylation was carried out by incubation of membranes with 10 nM PrBCM for 30 min, followed by 2-centrifugal washes. One representative datum out of 6 separate experiments is shown. Ordinate: [3H]QNB binding (%), abscissa: log of concentration (M) of carbachol.

Fig. 4. Displacement of [3H]QNB bindings by carbachol in the membranes which had been in vitro alkylated in the absence (●) and presence (○) of the 3 mM GTP plus GTP-regenerating system (20 mM creatine phosphate and 100 μg/ml creatine phosphokinase). In vitro alkylation was carried out by incubation of membranes with 10 nM PrBCM for 30 min, followed by 2-centrifugal washes. One representative datum out of 3 separate experiments is shown. Ordinate: [3H]QNB binding (%), abscissa: log of concentration (M) of carbachol.

(Fig. 4 and Table 3). This value was significantly different from that in the untreated membranes (P < 0.05) (Table 3) and was not different from that in the membranes obtained from the in vivo alkylated muscle strips (P > 0.05) (see Table 2). When membranes were preincubated with GTP alone followed by centrifugal washing, the affinity of carbachol was not altered from the control value (data not shown).

In vitro alkylation of membranes with [3H]PrBCM

Table 4 shows the direct bindings of [3H]PrBCM. The data were not analyzed by Scatchard plot, since the mode of bindings of [3H]PrBCM to the receptor sites is irreversible in nature. The presence of GTPγS (30 μM) decreased the amount of the specifically bound [3H]PrBCM by about 15% at the [3H]PrBCM concentrations examined.

Table 4. Effects of GTPγS (30 μM) on the specific [3H]PrBCM bindings to membranes prepared from guinea pig taenia caecum

| Concentration of [3H]PrBCM (nM) | Control [3H]PrBCM bound (fmol/mg protein) ± S.E. | GTPγS-treated [3H]PrBCM bound (fmol/mg protein) ± S.E. |
|---------------------------------|---------------------------------|---------------------------------|
| 1 nM                            | 40.3 ± 1.8                      | 35.7 ± 0.9                      |
| 3 nM                            | 221.9 ± 3.2                     | 188.6 ± 0.4*                   |
| 10 nM                           | 308.1 ± 7.3                     | 250.2 ± 5.3*                   |
| 30 nM                           | 792.8 ± 19.0                    | 649.4 ± 34.3*                  |

Each figure is presented as a mean ± S.E. of 3 separate experiments. *Significantly different from the corresponding control values at P < 0.05.
DISCUSSION

Using a functional technique, we have reported the possibility that two muscarinic cholinergic mechanisms coexist (16, 17). Progressive pretreatment (up to 50 min) with PrBCM of the guinea pig taenia caecum inhibited irreversibly the responses to muscarinic full agonists such as carbachol and displaced the concentration-response curves for the cholinomimetics to the right by about 15-fold with a slight reduction in the maximum response. However, more prolonged (90 min) pretreatment with PrBCM had no further significant inhibitory effect on the concentration-response curves, suggesting PrBCM-sensitive and -resistant muscarinic receptor mechanisms that are not discriminated by pirenzepine or AF-DX 116 (16, 17). Furthermore, the activation of PrBCM-sensitive receptors was found to utilize cytoplasmic Ca\(^{2+}\) more effectively for contraction in the intestinal smooth muscle of guinea pig than the activation of PrBCM-resistant ones (21).

In the present study, using direct receptor binding techniques with \[^{3}H\]QNB and \[^{3}H\]PrBCM, we have shown and confirmed that in vivo alkylation with PrBCM for 50 min reduced the density of \[^{3}H\]QNB binding sites to about 1/15 the control value, but more prolonged alkylation was not accompanied with further reduction in the maximum \[^{3}H\]QNB binding sites. These results agreed well with those of our previous functional experiments (16, 17).

Nonlinear regression analyses revealed that carbachol binds to three different affinity sites, that is, the SH, H and L subsites in membranes obtained from guinea pig taenia caecum, as reported in a variety of tissues (22–26). The pIC\(_{50}\) value for carbachol was decreased, but not changed for atropine, by in vivo alkylation of the muscle strips with 300 nM PrBCM for 50 min, under which condition only the PrBCM-resistant sites remained. The loss in the affinity of carbachol in the PrBCM-pretreated membranes could not be explained by a selective inactivation of the SH and/or H subsites by the in vivo alkylation, because if such higher affinity subsites which accounted for about 14/15 of the total receptor sites were destroyed by PrBCM, the PrBCM-resistant receptor would be essentially occupied only by the L subsite. Actually, there also coexist SH, H and L subsites in the PrBCM-resistant sites; and consequently, the apparent decreased affinity of carbachol to the receptor sites was revealed to be due to both the relative abundance of the H and L subsites and the relative scarcity of the SH subsite in the PrBCM-resistant receptors. The difference in the relative densities of each subsite between the PrBCM-sensitive and -resistant sites seems to further support the distinct entities or states of both receptor sites.

It is well-known that the guanine nucleotides lowered the affinity of agonists but not antagonists to many types of receptor systems including muscarinic receptors via interaction of the receptor with G proteins. The decreased affinity of carbachol, but not of atropine, by in vivo alkylation is apparently not due to a mechanism by which PrBCM might act on the receptor systems to convert them to a state similar to that in the presence of the guanine nucleotide, since GTP\(_{\gamma}\)S was able to further decrease the affinity of carbachol in membranes from the PrBCM-pretreated muscles, as in the control membranes. The shift of the pIC\(_{50}\) value by GTP\(_{\gamma}\)S in the PrBCM-resistant receptor sites suggest that these receptor sites may couple with G proteins to induce a PrBCM-resistant contraction.

To investigate the mode of interaction of PrBCM with both receptor sites, the binding characteristics were studied in the membranes which had been pretreated with PrBCM followed by centrifugal washes to remove the alkylating drug. Interestingly, by this in vitro alkylation method, the pIC\(_{50}\) values for carbachol was not changed. Taking into account the results obtained with the in vivo alkylation method, some missing cytoplasmic factor would be responsible for the selective inactivation by PrBCM of the receptor sites for the drug.
Our study showed that GTP may be one of the candidates for such a cytoplasmic factor and that GTPYS makes some population of the receptor sites resistant to PrBCM. First, the guanine nucleotide reduced the amount of $[^{3}H]$PrBCM specifically bound to the membranes by about 15%. Although somewhat larger, the value was comparable in order to the relative density of the PrBCM-resistant sites revealed by Scatchard analyses of $[^{3}H]$QNB binding sites in in vivo alkylated membranes.

Secondly, simultaneous incubation of membranes with GTP during the in vitro alkylation by PrBCM decreased the pIC$_{50}$ value for carbachol, as observed in in vivo alkylated membranes (GTPYS was not used in this experiment, since the binding of the nucleotide to G proteins are almost irreversible). When membranes were preincubated with GTP alone followed by centrifugal washes, the affinity of carbachol was not altered compared with the control value. Thus, the decrease in the affinity of carbachol by in vitro alkylation in the presence of GTP is considered not to be induced via a so-called GTP-shift by the contaminating GTP in the washed membranes due to an incomplete removal of the nucleotide.

Taken together, the present results suggest the possibility that in guinea pig taenia caecum, there coexist two types of muscarinic receptor mechanisms, although it is now unknown whether the difference in sensitivity of the two sites to PrBCM is due to the distinct entities of the receptor molecules, the same receptor with different modifications or as yet undefined mechanisms. PrBCM can differentiate between the two mechanisms, PrBCM-sensitive and -resistant ones, in the presence of the guanine nucleotide, but in its absence, PrBCM cannot discern the two. In other words, one mechanism is PrBCM-sensitive, irrespective of the presence or absence of the guanine nucleotide, and the other is PrBCM-resistant only when GTP is present.

Generally, in an unstimulated state, a G protein-linked receptor and a G protein are considered to be present separately; and once the receptor is activated by its binding with an agonist, the receptor and the G proteins will associate to elicit a final response. In the light of such a scheme, it is not known how the guanine nucleotide would make the "PrBCM-sensitive" PrBCM-resistant receptor mechanism actually PrBCM-resistant. Since under this condition agonists are absent and the receptor is not activated, we need to determine why the interaction of PrBCM, an antagonist, with the receptor is regulated by the guanine nucleotide. In cardiac M$_2$ receptors, however, guanine nucleotides have been reported to modulate the receptor affinity for the competitive antagonists (27, 28). Our present results might provide clues to help find unknown muscarinic receptor subtypes or different modified states of the receptors and their novel mode of interaction with G proteins, although further experimental work is necessary.

Acknowledgments

We thank Drs. S. Uchida and A. Mizushima (Department of Pharmacology, Osaka University School of Medicine) for generously giving us the program for nonlinear multiple sites analysis, SYMPLEX.

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