Dual regulation by G proteins of agonist-dependent phosphorylation of muscarinic acetylcholine receptors

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Muscarinic acetylcholine receptors purified from porcine atrium were phosphorylated, depending on the presence of agonists, by a protein kinase partially purified from porcine brain, which had similar properties to the β-adrenergic receptor kinase. GTP-binding regulatory proteins (Go) had dual effects on the phosphorylation of muscarinic receptors, i.e. stimulation at lower concentrations and inhibition at higher concentrations. The stimulatory effect was reproduced with the βγ subunit of Go and the inhibitory effect with the combination of the α and βγ subunits.

Muscarinic receptor; Acetylcholine receptor; GTP-binding regulatory protein; Receptor phosphorylation; β-Adrenergic receptor kinase; Rhodopsin kinase

1. INTRODUCTION

Muscarinic acetylcholine receptors have been shown to be members of the receptor family which is characterized by the seven putative transmembrane segments [1–6] and the interaction with G proteins [7–10]. On the other hand, knowledge on the regulation of receptor functions is still limited [5,6,11]. Kwatra et al. reported that agonist-dependent phosphorylation of muscarinic receptors in chick heart may result in their desensitization [12,13] and that purified cardiac receptors are phosphorylated [14] by β-adrenergic receptor kinase, which is suggested to be involved in the homologous desensitization of β-adrenergic receptors [15]. We have also found that both cerebral and atrial muscarinic receptors are phosphorylated, depending on the presence of agonists, by a protein kinase partially purified from porcine cerebrum, and that the phosphorylation is inhibited by excess G proteins (Go) in the absence of a guanine nucleotide but not in the presence of either GTP or GDP [16]. In the present report, we provide evidence that the phosphorylation is dually regulated by G proteins.

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Abbreviations: G protein, GTP-binding regulatory protein; Go, a G protein with α subunit of apparent molecular mass of 39 kDa; [^{3}H]QNB, [^{3}H]L-quinuclidinyl benzylate; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol

2. MATERIALS AND METHODS

The protein kinase was partially purified from porcine cerbrum by a modification of the method used previously for the purification of β-adrenergic receptor kinase [17], as described [16]. The specific activity of the preparation was estimated to be 13 pmol/min/mg protein with 50 μM ATP and 1.1 nM muscarinic receptors as substrates in the presence of 1 mM carbamylcholine, 15 nM Go and 10 μM GTPγS. The K_m for muscarinic receptors of the present preparation has not been determined. If we assume that the K_m is the same as those for rhodopsin (6 μM) or β-adrenergic receptors (0.25 μM) of the β-adrenergic receptor kinase [17], the specific activity (V_{max}) is estimated to be 71 or 3 nmol/min/mg protein, respectively, which is comparable to the value (7.2 nmol/min/mg protein) reported for the β-adrenergic receptor kinase preparation (purity 10%) [17]. Contaminations of protein kinase C, cAMP-dependent protein kinase, phosphatases and ATPases in the present preparation were negligible (data not shown).

Muscarinic receptors were purified from porcine atrium by a single step of affinity chromatography [18,19] (sp. act. 2 nmol/mg protein, as assessed from [^{3}H]QNB binding activity), and Go from porcine cerebrum as described [9] (sp. act. 8 nmol/mg protein, as assessed from [^{35}S]GTPγS binding activity). The α subunit of Go was prepared as described [20] and kindly donated by Dr T. Asano. The βγ subunit of Go was isolated from a Go preparation by means of DEAE-Toyopearl column chromatography, following incubation of the Go with 50 μM GTPγS and 50 mM $MgCl_2$ [21]. The concentrations of Go and α subunits were estimated from the [^{35}S]GTPγS binding activity, and that of the βγ subunit was estimated by comparison of the densities of β bands stained with Coomassie brilliant blue following SDS-PAGE of the Go and βγ subunit preparations.

Muscarinic receptors in 0.1% digitonin/0.5 M K-phosphate buffer (pH 7)/10 mM carbamylcholine (30 pmol, 50 μl) were mixed with G proteins or G protein subunits in a buffer solution containing 0.3% sodium cholate (480 pmol in standard experiments, 25 μl), lipid in a Hepes buffer solution (HEN; 20 mM Hepes buffer (pH 8)/1 mM EDTA/160 mM NaCl) containing 0.04% sodium cholate and 0.18% sodium deoxycholate (0.15 mg each of a total lipid extract of porcine brain and Folch fraction I (Sigma), 100 μl), and HEN (total volume, 200 μl). The mixture was passed through a small column of Sephadex

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G 50 fine (2 ml) preequilibrated with HEN and the void volume fraction (400 μl) was collected. The recoveries of the [^{3}H]QNB and [^{35}S]GTPγS binding activities in the void volume fraction were 24% and 62%, respectively. The void volume fraction (50 μl) was incubated with the kinase preparation (3.7 μg of protein), 1 μM [^{32}P]ATP (5×10^5 cpm per tube), 10 μM GTPγS and muscarinic ligands in a solution comprising 20 mM Tris buffer (pH 7.5)/2 mM EDTA/5 mM $MgCl_2$/0.5 mM EGTA (total volume, 300 μl). After incubation for 60 min at 30°C, 2 ml of HEN was added and then the mixture was centrifuged for 30 min at 190 000×g. The pellet was suspended in a solution containing 2.5% SDS (60 μl), and then a portion (40 μl) of the suspension was subjected to SDS-PAGE (acrylamide concentration, 10%) followed by autoradiography. The band of muscarinic receptors was cut out and counted by the use of Cerenkov's effect. The recoveries of the [^{3}H]QNB and [^{35}S]GTPγS binding activities after the centrifugation were estimated to be 43 and 34%, respectively.

3. RESULTS

Muscarinic receptors were purified from porcine atrium [18], reconstituted with G proteins in lipid vesicles [9] and then subjected to phosphorylation with a protein kinase. Fig. 1 shows the effects of the concentrations of carbamylcholine and atropine on the phosphorylation. Muscarinic receptors were phosphorylated in the presence of carbamylcholine but not in its absence and were the only major proteins phosphorylated, no phosphorylation of G proteins being detected. The concentration of carbamylcholine giving the half-maximum effect was 13 μM and the concentration of atropine decreasing the effect of 1 mM carbamylcholine to 50% was 0.5 μM, which corresponds to an apparent dissociation constant of 6.4 nM on the assumption of a mass action. Thus, the effective concentrations of carbamylcholine and atropine are essentially the same as those in the absence of G proteins [14,16]. These apparent K_d values are comparable to those estimated from the [^{3}H]QNB binding experiments [9,10], confirming that the effects of carbamylcholine and atropine are due to their binding to muscarinic receptors. The agonist-dependent phosphorylation of muscarinic receptors by the present kinase preparation was not affected by cAMP or cGMP, did not require the presence of Ca and was inhibited by 0.15 M NaCl or KCl [16], which are properties common to the β-adrenergic receptor kinase [17]. In addition, the activity was found to be inhibited by 1 μM heparin (data not shown), as was reported for the

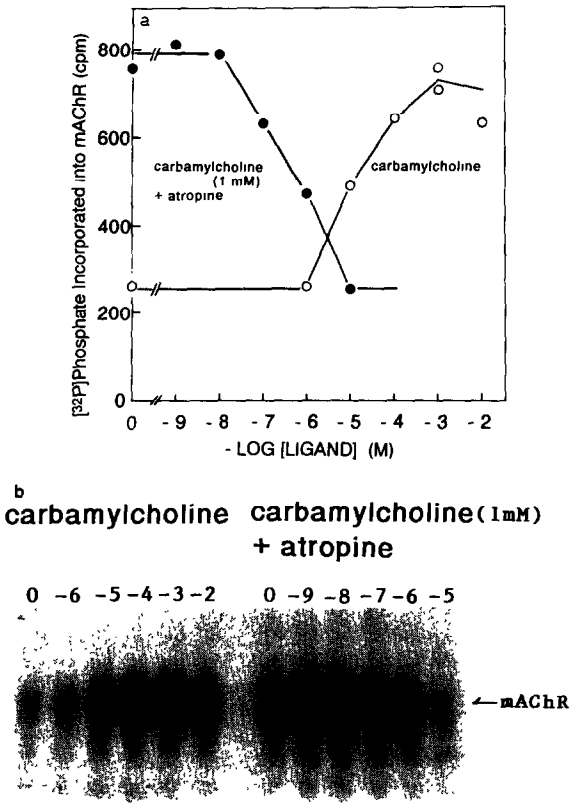

Fig. 1. Effects of the concentrations of carbamylcholine and atropine. Muscarinic receptors purified from porcine atrium and then reconstituted with Go were phosphorylated with a protein kinase, which was partially purified from porcine cerebrum, in the presence of different concentrations of carbamylcholine or 1 mM carbamylcholine and different concentrations of atropine, followed by SDS-PAGE, autoradiography (b) and counting of the receptor band (a).

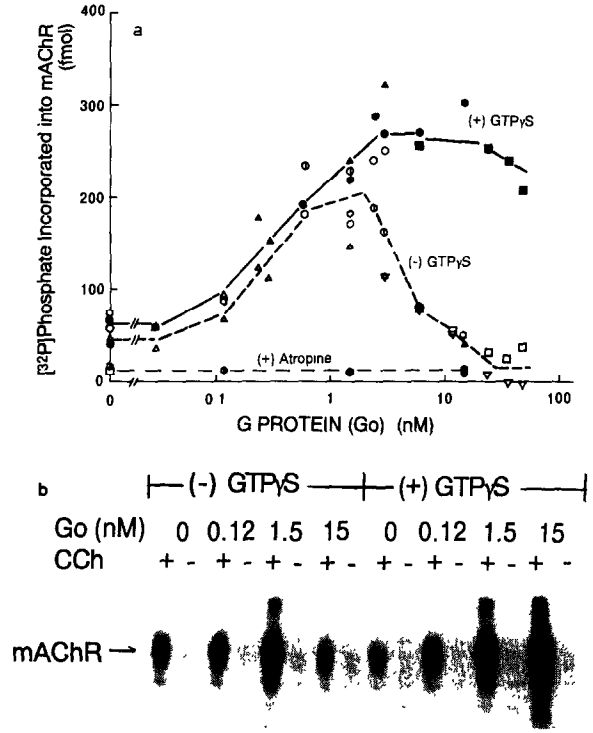

Fig. 2. Effect of the concentration of Go. Atrial muscarinic receptors reconstituted with different concentrations of Go were phosphorylated with the protein kinase in the presence (closed symbols) or absence (open symbols) of 10 μM GTPγS in the presence of 1 mM carbamylcholine or 10 μM atropine (broken line), followed by SDS-PAGE, autoradiography (b) and counting of the receptor band (a). The results of five independent experiments are plotted, using different symbols (a). Reconstitution and phosphorylation were carried out as described in the text, except that various concentrations of Go were used. Concentrations of Go in the phosphorylation reaction mixture are shown in the abscissa and that of receptors is 1.1 nM.

phosphorylation of β-adrenergic receptors by β-adrenergic receptor kinase [22]. These results indicate that the relevant kinase is very similar to, if not identical with, the β-adrenergic receptor kinase.

The effects of various concentrations of Go on the phosphorylation of atrial receptors (1.1 nM) are summarized in Fig. 2. The phosphorylation of receptors in the absence of a guanine nucleotide increased with Go concentrations up to approximately 2 nM and then markedly decreased with a further increase in the Go concentration, no phosphorylation being observed in the presence of more than 30 nM Go. On the other hand, the decreasing phase was not observed in the presence of GTPγS, the phosphorylation increasing up to approximately 3 nM Go and then remaining essentially the same, or only slightly decreasing from 3 to 50 nM Go. Essentially the same results were obtained with Gi ($\alpha_{41}\beta\gamma$) or Gn ($\alpha_{40}\beta\gamma$), as well as with Go, and also with cerebral muscarinic receptors and one of the three G proteins (data not shown).

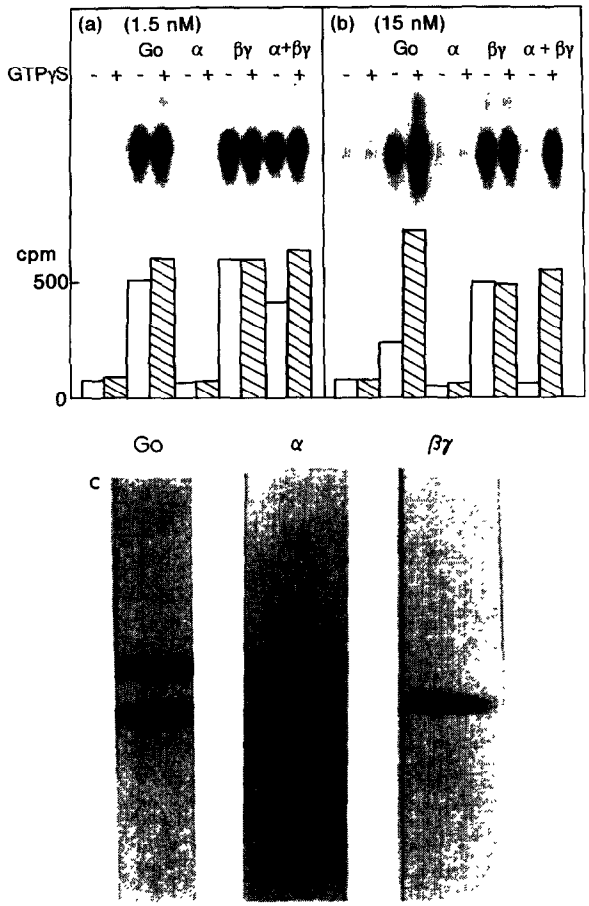

Fig. 3. Effects of the α and $\beta\gamma$ subunits of Go. Atrial muscarinic receptors were reconstituted with Go, the α subunit of Go, the $\beta\gamma$ subunit of Go or a mixture of the α and $\beta\gamma$ subunits and then subjected to phosphorylation in the presence of 1 mM carbamylcholine in the presence or absence of 10 μM GTPγS (concentrations of Go and Go subunits in the phosphorylation medium, 1.5 (a) and 15 nM (b)). The stained bands of α and β subunits on SDS-PAGE are shown in (c).

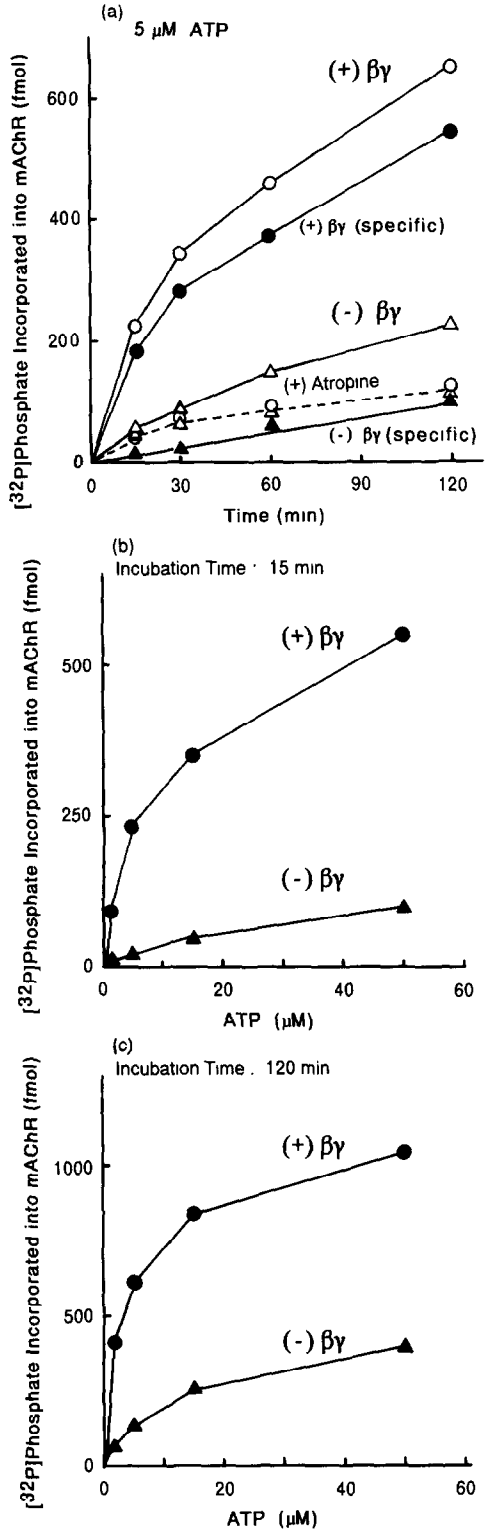

Fig. 4. Effects of incubation time and concentrations of ATP. Atrial muscarinic receptors were reconstituted with or without the $\beta\gamma$ subunit and then subjected to phosphorylation in the presence of 10 μM GTPγS and 1 mM carbamylcholine or 10 μM atropine (broken line) with (a) 5 μM or (b, c) various concentrations of ATP for (a) varied periods, (b) 15 min or (c) 120 min (concentrations of receptors, $\beta\gamma$ subunits and the kinase are 1.1 nM, 15 nM, and 4.9 μg protein per tube, respectively). The differences in [^{32}P]phosphates incorporated in the presence of carbamylcholine and those incorporated in the presence of atropine were shown as closed symbols in (a), (b) and (c).

G proteins are known to be composed of α, β and γ subunits, the trimer being split into α and $\beta\gamma$ subunits on the addition of GTPγS [23]. To determine which of the α and $\beta\gamma$ subunits is responsible for the stimulation and inhibition of the agonist-dependent phosphorylation of muscarinic receptors, they were separated from each other and their effects on the phosphorylation were examined, Fig. 3 summarizes their effects at two concentrations, 1.5 and 15 nM. The stimulation of the phosphorylation was observed by the $\beta\gamma$ subunits but not by the α subunits. The stimulation by $\beta\gamma$ subunits was also dependent on the presence of an agonist and was blocked on the addition of atropine (data not shown). The $\beta\gamma$ subunit stimulated the phosphorylation as much as Go did, the effective concentration of $\beta\gamma$ subunits being similar to those of Go. The difference between the effects of Go and $\beta\gamma$ subunits is that the stimulation by $\beta\gamma$ subunits was independent of GTPγS at both 1.5 and 15 nM, whereas there was marked inhibition of the phosphorylation at 15 nM Go in the absence of GTPγS, but not in its presence.

The α subunit at 1.5 nM showed neither a stimulatory nor an inhibitory effect, and did not affect the stimulation by $\beta\gamma$ subunits. The α subunit at 15 nM, however, suppressed the phosphorylation in the presence of 15 nM $\beta\gamma$ subunits in the absence of GTPγS, but not in its presence. Thus the stimulatory and inhibitory effects of Go were reproduced by the $\beta\gamma$ subunit, and the mixture of α and $\beta\gamma$ subunits, respectively.

Fig. 4 demonstrates the time course of phosphorylation in the absence and presence of $\beta\gamma$ subunits at 5 μM ATP (Fig. 4a), and the effects of concentrations of ATP on the phosphorylation at the incubation time of 15 min (Fig. 4b) and 120 min (Fig.4c). The stimulatory effect of $\beta\gamma$ subunit was greater on the initial rate (V_{max}) rather than on the K_m for ATP or the extent of phosphorylation. The apparent K_m for ATP were estimated to be 8 and 16 μM, and V_{max} to be 9.1 and 1.4 pmol/min/mg protein at the receptor concentration of 1.1 nM in the presence and absence of $\beta\gamma$ subunits, respectively. The amounts of phosphate incorporated into receptors after incubation of 120 min with 50 μM ATP were 8.4 and 4.1 mol/mol of receptor in the presence and absence of $\beta\gamma$ subunits, respectively.

4. DISCUSSION

It is known that both cerebral [7–9,21,24] and atrial [10,25] muscarinic receptors reconstituted with one of the three G proteins (Gi, Go, Gn) show high affinity for agonists in the absence of a guanine nucleotide but low affinity in its presence, and that the proportion of guanine nucleotide-sensitive high affinity agonist binding increases with an increase in the G protein concentration. The concentrations of G proteins required for the maximum effect differ depending on the experimental conditions, being reported to be a only 3-fold excess of cardiac Gi over atrial receptors by Tota et al. [10], and an 800-fold excess of Go over cerebral receptors by Florio and Sternweis [24]. Under the present experimental conditions, the guanine nucleotide-sensitive high affinity agonist binding increased from a 2- to 20-fold excess Go over atrial receptors, in accordance with the previous results [9,21,25]. The inhibition of agonist-dependent phosphorylation by Go in the absence of GTPγS occurred in the same Go concentration range (Fig. 2). In addition, the high affinity agonist binding and the inhibition of agonist-dependent phosphorylation, both of which are induced on the addition of excess G proteins, are canceled on the addition of either GTP or GDP, as well as GTPγS [9,16]. The concentrations of GTP and GDP giving half-maximum effects as to restoration of the inhibition of agonist-dependent phosphorylation were 0.3 and 0.8 μM, respectively (data not shown). These levels are a little higher than those of GTP and GDP giving half-maximum effects on the high affinity agonist binding (0.07 and 0.18 μM [9], respectively). Furthermore, the finding that both α and $\beta\gamma$ subunits are required for the inhibition of agonist-dependent phosphorylation is consistent with the observation that the interaction of muscarinic receptors with both α and $\beta\gamma$ subunits is required for the guanine nucleotide sensitive high affinity agonist binding [7,24]. These results, taken together, indicate that the inhibition by G proteins of the agonist-dependent phosphorylation of muscarinic receptors occurs in parallel with the high affinity agonist binding of muscarinic receptors reconstituted with G proteins. It would be reasonable to assume that the guanine nucleotide-sensitive high affinity agonist binding represents the formation of a ternary complex of an agonist, a receptor and a G protein, which dissociates on the addition of GTP or GDP, and that the agonist-dependent phosphorylation of muscarinic receptors is inhibited on the formation of the ternary complex, probably through masking by G proteins of the phosphorylation sites on receptors. Consistent with the present results, Kelleher and Johnson [26] reported that the light-dependent phosphorylation of rhodopsin by rhodopsin kinase was inhibited by a retinal G protein, transducin, and that the inhibition was relieved in the presence of GTPγS.

The stimulation of phosphorylation by $\beta\gamma$ subunits, on the other hand, is the phenomenon which was found for the first time in the present system, and such a stimulation was not observed in the rhodopsin-transducin system [26] and it is not known if the phosphorylation of β-adrenergic receptors by β-adrenergic receptor kinase is regulated by G proteins or not. The target of the $\beta\gamma$ subunit has not been determined and may be the receptor or the receptor kinase. Whichever the target of $\beta\gamma$ subunits is, it is tempting to speculate that the $\beta\gamma$ subunit stimulates the

phosphorylation of receptors thereby facilitating their desensitization, whereas the α subunits affect the effectors.

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