Carbachol-induced desensitization of PLC-β pathway in rat myometrium: downregulation of G_qα/G_11α

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Carbachol-induced desensitization of PLC-β pathway in rat myometrium: downregulation of G_qα/G_11α. Am. J. Physiol. 275 (Cell Physiol. 44): C636–C645, 1998.—In the estrogen-treated rat myometrium, carbachol increased the generation of inositol phosphates by stimulating the muscarinic receptor-G_a/G_11α-phospholipase C-β3 (PLC-β3) cascade. Exposure to carbachol resulted in a rapid and specific (homologous) attenuation of the subsequent muscarinic responses in terms of inositol phosphate production, PLC-β3 translocation to membrane, and contraction. Refractoriness was accompanied by a reduction of membrane muscarinic binding sites and an uncoupled state of residual receptors. Protein kinase C (PKC) altered the function of muscarinic receptors and contributed to the initial period of desensitization. A delayed phase of the muscarinic desensitization was PKC independent and was associated with a downregulation of G_qα/G_11α. Atropine failed to induce desensitization as well as G_qα/G_11α downregulation, indicating that both events involve active occupancy of the receptor. Prolonged exposure to AlF_4 reduced subsequent A IF_4 as well as carbachol-mediated inositol phosphate responses and similarly induced downregulation of G_qα/G_11α. Data suggest that a decrease in the level of G_qα/G_11α is subsequent to its activation and may account for heterologous desensitization.

IN MANY INSTANCES, prolonged exposure of various tissues to agonists at a G protein-linked receptor trigger a counterregulatory process that attenuates receptor signaling, a phenomenon commonly denoted as desensitization (2, 18). Desensitization may be either agonist specific (homologous) or associated with decreased responsiveness to other activators (heterologous). The direct uncoupling of the receptor from its respective G protein underlies the rapid phase of desensitization and is mediated, at least in part, by receptor phosphorylation. Two classes of serine/threonine kinases, phosphorylating G protein-coupled receptors: the G protein-coupled receptor kinases (GRKs) and the second-messenger-dependent protein kinases, PKA and PKC (2, 18, 25). Agonist-induced refractoriness may be further associated with receptor sequestration and ultimately with receptor recycling or degradation "down-regulation." While regulation at the receptor level appears to be the predominant site of desensitization of G protein-coupled receptors, there has been recently increasing evidence that such regulation may also exist at the level of the G proteins (21). It has been reported that chronic exposure to a G protein-linked receptor can result in a reduction in the level of the G protein that interacts specifically with this receptor. Agonist-induced downregulation of cellular G_α proteins has been observed for members of the G_1 (1, 21) and the G_2 (10, 21) families regulating the stimulatory and inhibitory pathways of adenyl cyclase and more recently for G_9 proteins coupled to the phospholipase C (PLC) stimulatory cascade (12, 22).

It is well recognized that the generation of ω-myo-inositol 1,4,5-trisphosphate with the accompanying rise in cytosolic Ca_2+ is an important determinant in uterine contractility. Many reports, including ours (7, 9, 20), have demonstrated that the phosphoinositide-PLC transducing system can be activated by agonists that induce contraction in different myometrial preparations (15, 24). For most contractile agonists, the receptor PLC coupling was insensitive to pertussis toxin (7, 9, 20). We have recently shown that in rat myometrium the responsiveness of the PLC pathway, associated with receptor- and/or direct G protein-mediated activation, increased during pregnancy. The enhancement of PLC activity could not be ascribed to changes in the amount of PLC-β3, the predominant PLC-β isoform expressed in rat myometrium, but clearly coincided with the increase in the amount of G_9α (16).

In the present study it is demonstrated that the PLC pathway can also be negatively regulated. Exposure of the nonpregnant rat myometrium to carbachol resulted in a dramatic decline in the ability of the muscarinic agonist to increase the generation of inositol phosphates and the tension in the desensitized tissue. Carbachol-induced refractoriness was associated with 1) both quantitative and functional alterations of muscarinic receptors and 2) downregulation of G_qα/G_11α. The decrease in G_9 levels appears to be subsequent to its sustained activation, whether direct or receptor mediated, and may contribute to the development of heterologous desensitization. Our observations provide additional support for a pivotal role of G_9 levels in the control of agonist-mediated activation of the PLC-β pathway.

MATERIALS AND METHODS
Materials. Lithium chloride, carbamylcholine chloride (carbachol), β-estradiol 3-benzoate, oxytocin, phosphatidylinositol, leupetin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), and phorbol 12,13-di-butyrate (PDBu) were obtained from Sigma Chemical (St. Louis, MO). Endothelin-1 (ET-1) was from Neosystem (Strasbourg, France). Myo-[2-3H]inositol (10–20 Ci/mmol) was obtained from Amersham International (Les Ulis, France). Ro-31-8220 was generously provided by Dr. D. Bradshaw (Roche, Hertfordshire, UK). Anti-G_qα/G_11α (QL) and anti-G_9δ (RM1) polyclonal antibodies and [N-methyl-3H]scopolamine methylcholylate ([^3H]NMS) at 80 Ci/mmol were obtained from New England Nuclear Product.
Division (DuPont de Nemours, Les Ulis, France). The antibody directed against β-subunits of heterotrimeric G proteins was generously provided by Dr. B. Rouot [Centre National de la Recherche Scientifique (CNRS), Montpellier, France]. PLC-β3 antibody was from Santa Cruz Biotechnology. Silica gel plates were from Merck (Darmstadt, Germany), and AG1-X8 was from Bio-Rad (Ivry, France). Other chemicals were of the highest grade commercially available.

Animals and tissue processing. Immature female rats (Wistar, 5 wk old) were treated with 30 µg estradiol for 2 days and used the following day. Rats were killed by decapitation. Uteri were removed and the myometrium was prepared free of endometrium (9, 16).

Tissue incubation and [3H]inositol preloading experiments. Myometrial strips (–25 mg) were allowed to equilibrate for 25 min in 5 ml Krebs bicarbonate buffer (pH 7.4) containing (in mM) 117 NaCl, 4.7 KCl, 1.1 MgSO4, 1.2 KH2PO4, 24 NaHCO3, 0.8 CaCl2, and 1 glucose (gas phase 95% O2–5% CO2) under constant agitation. Tissues were incubated with 7 µCi of myo-[3H]inositol (0.4 µM) in 1 ml of fresh buffer for 4 h as described (9, 16). Incubations were continued for the time indicated without (control) or with carbachol (pretreated). All tissues were then washed three times with 10 ml of agonist-free Krebs buffer and transferred into 1 ml of fresh buffer, during 5 min before the addition of 10 mM LiCl. After 10 min, agonists to be tested were added at the indicated concentration, and incubation was further continued for the time indicated for the specific experiment. Reactions were stopped by immersing the myometrical strips into 1.5 ml of cold 7% (wt/vol) TCA, followed by homogenization and centrifugation at 10,000 g for 15 min at 4°C.

Measurement of [3H]phosphoinositides. The pellets obtained after centrifugation of the TCA homogenates were washed with 0.5 ml of TCA to remove any residual [3H]inositol obtained after centrifugation. The upper phase was discarded, HCl (1,700 µl) were then added, and two phases were separated by centrifugation. The lower phase was neutralized with Tris base, and applied to a column (40:80:1, vol/vol/vol; 2.8 ml) was then added and the phospholipids were extracted for 30 min at room temperature. Chloroform (930 µl) and 0.1 N HCl (1,700 µl) were then added, and two phases were obtained by centrifugation. The upper phase was discarded, and the lower phase was dried under a stream of nitrogen and used to determine the radioactivity incorporated into [3H]inositol phospholipids. In some experiments phosphatidylinositol (PtdIns), phosphatidylinositol monophosphate (PtdInsP), and phosphatidylinositol bisphosphate (PtdInsP2) were separated by TLC, and the associated radioactivity was determined as previously described (7, 16).

Measurement of [3H]inositol phosphates. The TCA-soluble supernatants were extracted four times with 6 ml diethyl ether, neutralized with Tris base, and applied to a column (0.7 x 2 cm) of the anion-exchange resin (AG1-X8; formate form, 200–400 mesh). Free inositol was eluted with 10 ml water, and the individual inositol phosphates [inositol trisphosphate (Ins3P), inositol bisphosphate (InsP2), and inositol monophosphate (InsP)] were separated as described (9, 20). Alternatively, total inositol phosphates (InsP3 + InsP2 + InsP) were eluted together in a single step with 12 ml of 1 M ammonium formate/0.1 M formic acid. The [H] content of the various fractions was determined by scintillation counting in QuickSafe A. Production of [3H]inositol phosphates was calculated as a percentage of radioactivity incorporated into phosphoinositides obtained from the corresponding sample (16).

Method for recording uterine contractile response. The contractile activity of isolated myometrial strips was measured with an isometric transducing device. The segments were loaded at a basal tension of 0.2–0.3 g and bathed at 37°C in 10 ml Krebs buffer under 95% O2–5% CO2. The contractile activity was integrated during a 1-min exposure to the indicated agonist.

Membrane preparation. Myometrial strips (100 mg) were homogenized with an Ultra-Turrax homogenizer in 0.6 ml buffer A containing 10 mM Tris-HCl, 0.5 mM EDTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM PMSF and were centrifuged for 5 min at 700 g. The 700-g supernatant (total homogenate) was then centrifuged for 20 min at 100,000 g (Beckman TLC 100–4, fixed rotor). The resulting supernatant represented the cytosolic fraction, and the corresponding pellet was suspended in buffer A at 4–6 mg protein/ml and constituted the membrane or particulate fraction. Proteins were estimated using the Lowry reagent (19).

Detergent-extracted proteins. Myometrial strips (100 mg) were homogenized in 0.6 ml of cold buffer A supplemented with 1% Triton X-100 and 2 mM EGTA (16). After 30 min at 4°C, the lysates were desalted by centrifugation at 10,000 g for 20 min, and the resulting supernatant was used as detergent-extracted proteins.

Receptor binding assay. Tissues were incubated in Krebs buffer with or without the addition of the indicated agonist for various times depending on the experiment. Myometrial strips were washed with hormone-free Krebs buffer, and membrane preparations were obtained as described above. Membrane proteins (150–300 µg) were incubated with the indicated concentration of [3H]NMS for 1.5 h at 30°C in 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2 in 1 ml final volume. Triplicate 300-µl aliquots were filtered through Whatman GF/C glass fiber filters, and the bound radioactivity was determined by scintillation counting (16). Nonspecific binding was defined as the amount of radioactivity bound to the filter when incubations were performed in the presence of 1 µM atropine and was subtracted from total binding to obtain specific binding. Under these conditions, nonspecific binding represented <5% of total binding at [3H]NMS concentrations near its dissociation constant (Kd) values. Binding data were analyzed using a nonlinear least-squares curve-fitting program (Multifit program from Day Computing, Cambridge, MA) to obtain IC50 values. Inhibition constant (Ki) values were calculated from IC50 values by applying the equation of Cheng and Prusoff (4).

Immunologic analysis of Gqα/ G11α. Protein samples were resolved by SDS-PAGE (10% wt/vol acrylamide). Proteins were transferred to nitrocellulose and blocked as described (16, 26). Primary antiserum QL (1:500 dilution) in 0.5% nonfat dried milk in Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, 500 mM NaCl) was then added and left overnight at 4°C. Secondary antiserum (swine anti-rabbit IgG coupled to horseradish peroxidase) was used at 1:2,000 dilution in 0.5% nonfat dried milk/TBS and left for 3 h at room temperature. The immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham). Quantification of the developed blots was performed using a Molecular Dynamics Densitometer.

Immunologic analysis of PLC-β3. Membrane proteins were resolved by SDS-PAGE (7.5% wt/vol acrylamide). The separated proteins were transferred to a nitrocellulose sheet for immunoblotting. The nitrocellulose sheet was blocked for 90 min at 37°C with 5% nonfat dried milk/TBS. Primary antiserum, anti-PLC-β3 (dilution 1:100) in 5% nonfat dried milk/TBS, was then added and left for 1 h at room temperature. The immunoreactive bands were visualized by the ECL detection system, after incubation with swine anti-rabbit IgG coupled to horseradish peroxidase (dilution 1:2,000) for 60 min at room temperature.
Methods

Production of [3H]inositol phosphates was expressed as a percentage of maximal carbachol response (69.3 ± 6.0% of label in phosphoinositides). Values are means ± SE of three different experiments, each done in duplicate.

Results

Desensitization of the inositol phosphate response to carbachol. When [3H]inositol-prelabeled myometrial strips that had been treated with 100 µM carbachol during different times were washed to remove the agonist and challenged with the muscarinic agonist for 15 min in the presence of LiCl, there was a diminished response in terms of inositol phosphate accumulation, compared with similarly treated tissues in carbachol-free medium (Fig. 1). Agonist-induced desensitization was rapid, being significantly detectable (50%) at 15 min and maximal (80% desensitization) at 60 min. Carbachol-induced desensitization was also observed for individual inositol phosphates (InsP2, InsP3, and InsP6), indicating that desensitization occurred at the level of the receptor-Gq-PLC cascade that hydrolyses PtdInsP2.

Figure 1 further illustrates that the oxytocin-stimulated inositol phosphate response was also reduced after carbachol treatment, albeit with a differential time course: the onset of this heterologous desensitization was slower, not apparent before 15 min, and a maximal decline was obtained after 60 min of exposure to carbachol. When myometrial strips were treated with 100 µM carbachol during 60 min, there was also a marked decrease in the ability of AlF4 to stimulate the generation of inositol phosphates (63.4 ± 7.0 and 38.7 ± 5.2% of label in phosphoinositides in control and carbachol-treated tissues, respectively, n = 3). The attenuated inositol phosphate responses were not brought about by a limiting supply of the PLC substrate. Indeed, after 120 min of treatment with carbachol, there was no significant alteration in the [3H]inositol incorporated into PtdInsP2, PtdInsP3, and PtdIns (85.3 ± 7.0, 51.1 ± 0.5, and 8.5 ± 0.8% of total [3H]phosphoinositides, respectively) compared with the control (86.1 ± 4.9, 49.0 ± 0.4, and 8.7 ± 0.8% of [3H]phosphoinositides, respectively).

As indicated in Fig. 2, the magnitude of the inositol phosphate refractory response was progressively larger with increasing concentrations of carbachol during the initial 2-h incubation period. It is interesting to note that the concentration-dependent curve of carbachol-induced increases in inositol phosphates was strikingly similar to the dose dependency of carbachol-mediated inositol phosphate refractoriness, with virtually the same half-maximal concentration (EC50: 15.1 ± 1.4 and 10.2 ± 1.2 µM, respectively). In the presence of atropine, carbachol failed to attenuate the subsequent inositol phosphate response. These observations indicated that carbachol-induced desensitization was triggered by receptor activation.

Absence of carbachol-mediated membrane association of PLC-β3 in desensitized tissues. It has been reported (15, 16) that PLC-β3 but not PLC-β1 or PLC-β2 was immunodetected in detergent-extracted proteins derived from estrogen-treated rat myometrium. It was further noted (experiments not shown) that, under basal conditions, PLC-β3 was immunodetected mainly in cytosol, with a minor amount (25–30%) consistently detected in membranes. Experi-
Fig. 3. Effect of carbachol on level of membrane-associated phospholipase C-β3 (PLC-β3) in control and desensitized tissues. A: myometrial strips were incubated for 2 h in absence or presence of 100 µM carbachol. Tissues were then washed and further incubated for 3 and 5 min without or with 100 µM carbachol. When used, atropine (0.1 µM) was added 5 min before carbachol. Equal amounts (20 µg) of membrane proteins were resolved by SDS-PAGE (7.5% wt/vol acrylamide) and were immunoblotted in presence of PLC-β3 antibody (1:100 dilution). B: myometrial strips were incubated 2 h in absence (● and lane 1; ▲ and lane 2) or presence of 100 µM carbachol (○ and lane 3) and stimulated for 5 min by carbachol (▲ and lane 2; ○ and lane 3). Membrane proteins (10, 20, and 30 µg) from each preparation were resolved and immunoblotted with PLC-β3 antibody. Top (A and B) is a representative immunoblot. Immunoreactive bands from 3 separate experiments were quantified by densitometric scanning. Data are means ± SE.

Fig. 4. Influence of carbachol-induced desensitization on muscarinic-mediated myometrial contraction. Cumulative dose-response curves obtained with indicated concentrations of carbachol before (control) and after 2 h of carbachol pretreatment (desensitized). Isometric contractions were recorded during 1-min exposure of loaded myometrial strips to indicated concentrations of carbachol. Values were expressed as percentage of maximal contractile response due to carbachol in control tissue. Values are means ± SE of 3–4 independent experiments.
Carbachol pretreatment induced both decrease and uncoupling of muscarinic receptors 

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<th>[3H]NMS</th>
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<td>Kᵦ(i) nM</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.1</td>
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<td>Bmax fmol/mg protein</td>
<td>31 ± 5</td>
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Values are means ± SE of 3–4 separate experiments. Myometrial strips were incubated 2 h in absence of presence of 100 µM carbachol (CB) before membrane preparation. [N-methyl-3H]scopolamine methylchloride ([3H]NMS) binding experiments were performed as described in MATERIALS AND METHODS. Dissociation constant (Kᵦ(i)) for NMS and maximal binding capacity (Bmax) were determined by Scatchard analysis of data. Data from displacement curves of [3H]NMS (1.2 nM) binding by different concentrations of carbachol in absence or presence of 100 µM guanosine 5'-O-(3-thiotriphosphate) (GTPγS) were used to calculate IC₅₀ values. Inhibition constant (Kᵦ(i)) values were calculated from equation of Cheng and Prusoff (4).

Carbachol (Kᵦ(i) = 0.5 ± 0.1 and 2.5 ± 0.2 µM, in the absence and presence of 100 µM GTPγS, respectively). By contrast, in desensitized membranes, the ability of the residual membrane receptors to form a high-affinity state seems to be impaired. Thus, in the absence of GTPγS, the Kᵦ(i) for carbachol in the desensitized membranes was shifted to values fourfold greater than that observed in control membranes, and GTPγS did not affect the apparent affinity of the receptor for carbachol (2.0 ± 0.2 and 2.2 ± 0.3 µM in the absence and presence of GTPγS, respectively). The data demonstrate that carbachol-induced desensitization was associated with both a reduction in the number of muscarinic binding sites and an uncoupled state of residual muscarinic receptors.

Role of PKC in carbachol-mediated stimulation and desensitization. Figure 6 shows that, in the presence of 2 µM PDBu, an exogenous activator of PKC, there was a marked and consistent reduction (60%) in the generation of inositol phosphates triggered by carbachol. The PDBu inhibitory effect was completely prevented by 10 µM Ro-31-8220, a selective PKC inhibitor (6), suggesting the involvement of a PKC-mediated process. Under similar conditions, PDBu failed to alter the production of inositol phosphates induced by ET-1 and by AlF₄⁻. The findings demonstrate that PKC did not exert any modulatory effect either at the ET-1 receptor level or at the level of the G protein and PLC activation. They rather indicate that PKC may elicit, presumably through a phosphorylation reaction, a specific inhibition at the level of the muscarinic receptor function.

Because the activation of the PLC pathway by carbachol leads to PKC activation, we examined a possible contribution of PKC in the desensitization process triggered by carbachol. Figure 7 illustrates that the
presence of Ro-31-8220 caused a delay in the development of the process of carbachol-induced desensitization. Thus a 15-min pretreatment with carbachol plus Ro-31-8220 resulted in a barely detectable (10%) attenuation of the subsequent muscarinic inositol phosphate response, compared with a 50% attenuation after a pretreatment with carbachol alone. However, with increasing time of pretreatment (60 min), the extent of the attenuated carbachol response was identical whether refractoriness was induced by carbachol alone or combined with Ro-31-8220. These results demonstrate the involvement of another, PKC-independent process, in the long-term desensitization triggered by carbachol.

Carbachol-mediated downregulation of \( G_{q/11\alpha} \). Our previous work (16) demonstrated the presence of \( G_{q/11\alpha} \) in rat myometrial membranes and its involvement in the PLC pathway. We next examined the possible modulation of the \( G_{q/11\alpha} \) level in myometrial membranes derived from postdesensitized tissues. Figure 8A displays an immunoblot of rat myometrial membranes obtained from untreated and carbachol-pretreated tissues, resolved by SDS-PAGE. The blot identified an apparent single band of 42 kDa, corresponding to \( G_{q/11\alpha} \) in all preparations. Figure 8 further illustrates that pretreatment of myometrial strips with 100 µM carbachol led to a marked decrease at the level of \( G_{q/11\alpha} \) associated with the membranes. Densitometric scanning of the immunoblot indicated that, compared with control, the amount of \( G_{q/11\alpha} \) was decreased by 60–65% after 2 h of carbachol pretreatment, as assayed with 10- and 20-µg loaded proteins.

**Fig. 7.** Differential time course of carbachol-induced refractoriness in absence and presence of Ro-31-8220. Prelabeled [³²H]inositol myometrial strips were incubated with 100 µM carbachol in absence or presence of 10 µM Ro-31-8220. At indicated times, tissues were washed and incubated for 10 min in presence of 10 mM LiCl before being rechallenged for 15 min with 100 µM carbachol. Production of total [³²H]inositol phosphates was expressed as percentage of carbachol response obtained in untreated tissues. Values are means ± SE of 3 independent experiments, each done in duplicate.

**Fig. 8.** Sustained exposure to carbachol-induced downregulation of membrane-associated \( G_{q/11\alpha} \). A: myometrial strips were incubated for 2 h without or with 100 µM carbachol. When used, atropine (0.1 µM) was added 5 min before carbachol. Equal amounts of membrane proteins were resolved by SDS-PAGE (10% wt/vol acrylamide) and then immunoblotted with \( G_{q/11\alpha} \) antisemur (1:500 dilution), anti-\( G_{q/11\alpha} \) (1:1,000 dilution), and anti-\( G_{\beta/\gamma} \) subunits (1:500 dilution). B: tissues were incubated for 2 h in absence or presence of 100 µM carbachol. Total homogenates (H), cytosolic fraction (C), membrane proteins (M), and detergent-extracted myometrial proteins (DE) were prepared as described in MATERIALS AND METHODS. Equal amounts of proteins (10, 25, 30, and 100 µg for M, DE, H, and C, respectively) for each specific fraction derived from treated and untreated tissues were subjected to SDS-PAGE. The resolved proteins were probed by immunoblotting with \( G_{q/11\alpha} \) antisemur (1:500 dilution). Data represent 1 of 3 separate experiments.

Figure 9 shows the time course of the muscarinic-induced decrease in immunoreactive \( G_{q/11\alpha} \) in myometrial membranes. Compared with untreated tissue, a 15-, 30-, 60-, and 120-min treatment with 100 µM carbachol resulted in a 25 ± 3, 42 ± 5, 56 ± 6, and 66 ± 7% decrease in the level of \( G_{q/11\alpha} \), respectively, with a maximal reduction (73 ± 8%) being achieved by 4 h. When myometrial strips were exposed to carbachol for 4 h, the half-maximal decrease in membrane-associated \( G_{q/11\alpha} \) was observed at 30 µM and the maximal effect at 100 µM carbachol (not shown). In the presence of atropine, carbachol failed to induce the loss of membrane-associated \( G_{q/11\alpha} \) (Fig. 8A), indicating that the process was triggered by muscarinic receptor activation. Results in Fig. 8A also demonstrate that, in membranes derived from carbachol-desensitized tissues, the amount of \( G_{q/11\alpha} \) isoforms (45 and 52 kDa) that are not coupled to muscarinic receptor activation in rat myometrium remained unchanged. Similarly, the amount of immunodetected \( \beta \)-subunits was not affected in the desensitized preparations.
Data in Fig. 8B show that the carbachol-induced decrease in membrane-associated Gqα/G11α is not due to the transfer of Gα proteins to cytoplasm. Indeed, no significant signals were obtained by immunoblotting the corresponding cytosolic protein fractions from either carbachol-treated or untreated tissues. Furthermore, the decrease in membrane-associated Gqα/G11α levels due to carbachol treatment was similarly observed in both total myometrial homogenates (700-g supernatants) and in detergent-extracted myometrial proteins. Taken together, these results indicated that the decline of Gqα/G11α levels observed in membranes derived from carbachol-desensitized tissues most probably reflects the downregulation of these proteins.

Experiments were then designed to evaluate the potential role of PKC in the quantitative regulation of Gqα/G11α. Myometrial strips were incubated for 20 min in the presence of 2 µM PDBu, conditions under which this agent was demonstrated to stimulate PKC activities (23) and to attenuate the carbachol-mediated inositol phosphate response (Fig. 6). Myometrial strips were similarly incubated with 2 µM PDBu for 2 h (a time period comparable with carbachol-induced desensitization). As shown in Table 2, short as well as prolonged PDBu treatments were unable to affect the level of membrane-associated Gqα/G11α, indicating that agonist-induced downregulation of Gqα/G11α did not occur subsequent to activation of phorbol ester-sensitive PKC isoforms. The inability of Ro-31-8220 to prevent downregulation of Gqα/G11α triggered by carbachol supports the contention that PKC did not participate in this process.

AlF4− induced both desensitization of the PLC pathway and downregulation of Gqα/G11α. To investigate whether Gqα/G11α downregulation is dependent or not on downregulation of muscarinic receptors, we used AlF4− that activates G proteins by circumventing the need for receptor engagement. Results in Fig. 10A illustrate the increased production of inositol phosphates due to a 10-min stimulation by AlF4−. When myometrial tissues were pretreated for 1 h with AlF4−, there was a marked attenuation of the inositol phosphate response (50 ± 6%) to a subsequent challenge with AlF4−. A 1-h exposure to AlF4− similarly led to a subsequent refractory state of the myometrium to carbachol in terms of inositol phosphate generation. The extent of such a heterologous desensitization averaged 52 ± 6%. Heterologous desensitization induced by AlF4− indicated an alteration in the PLC pathway at a step distal to the specific agonist receptor. Results in Fig. 10B further show that membranes derived from AlF4−-pretreated tissues displayed an important decrease (72 ± 7%) in Gqα/G11α levels. The data suggested that Gqα/G11α downregulation occurred in a manner consecutive to its activation and independent from any receptor participation.

**DISCUSSION**

Our previous observations showed that, in rat myometrium, carbachol stimulates the generation of inositol phosphates by activating muscarinic receptors that are coupled to PLC-β3 via Gq/G11 proteins (16). Evidence is now presented that the myometrium is endowed with regulatory mechanisms that control the extent of its responsiveness to the muscarinic agonist. Prolonged exposure of myometrial strips to carbachol resulted in a progressive desensitization of muscarinic receptor-mediated inositol phosphate response. Refractoriness could be observed at the level of individual inositol phosphates (InsP1, InsP2, and InsP3), indicating that desensitization occurred at the level of the receptor-G protein-PLC cascade that hydrolyzes PtdIns(4,5)P2. An interesting indication of the muscarinic refractoriness was further revealed at the level of PLC-β3, which is the predominant PLC-β isoform expressed in rat myometrium (15, 16). We found that, in rat myometrial preparations, PLC-β3 was predominantly present in the cytosol, as reported for other systems (8). Notewor-
intracellular Ca²⁺. Such an agonist-induced membrane translocation of PLC-β3 that correlates with the activated by ET-1 and AlF₄⁻. Tissues were then washed and rechallenged for 15 min with carbachol or AlF₄⁻. Production of total [³H]inositol phosphates was expressed as percentage of the responses to carbachol or AlF₄⁻. Membrane proteins were prepared and Gq/11α-immunoprecipitation and Gq/11α-immunoprecipitation were performed as described in Fig. 8. Immunoreactive bands were quantified by densitometric scanning. Results were expressed as relative levels of Gq/11α compared with membranes from untreated control. Results are presented as means ± SE from 3 independent experiments.

Fig. 10. AlF₄⁻ induced both desensitization of [³H]inositol phosphate responses and downregulation of Gq/11α. A: [³H]inositol-phosphate-labeled myometrial strips were incubated for 1h in the presence or absence of 100 µM carbachol or AlF₄⁻ (20 mM NaF + 10 µM AlCl₃). Tissues were then washed and rechallenged for 15 min with carbachol or AlF₄⁻. Production of total [³H]inositol phosphates was expressed as percentage of the responses to carbachol or AlF₄⁻. B: myometrial strips were incubated for 1 h in absence or presence of AlF₄⁻ or carbachol. Membrane proteins were prepared and Gq/11α-immunoprecipitation procedure was performed as described in Fig. 8. Immunoreactive bands were quantified by densitometric scanning. Results were expressed as relative levels of Gq/11α compared with membranes from untreated control. Results are presented as means ± SE from 3 independent experiments.

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Carbachol-evoked desensitization was found to be a time- and dose-related phenomenon. A correlation was observed between muscarinic agonist concentrations that stimulate inositol phosphate generation and those concentrations that trigger refractoriness, indicating that carbachol-induced desensitization required active occupancy of the receptor. The interpretation was strengthened by the observation that atropine, a muscarinic antagonist, prevented carbachol-mediated desensitization. Kinetic analysis of desensitization triggered by carbachol suggested the existence of two processes: a rapid, receptor-specific, homologous refractoriness that appeared as early as 10–30 min after exposure of the tissue to carbachol, followed by a heterologous desensitization process that could be detected only after prolonged (30 min) exposure to carbachol and that leads to an attenuated response to oxytocin as well as to AlF₄⁻.

Many studies provide evidence that the initial phase of agonist-mediated desensitization is subsequent to functional and/or quantitative alteration of the receptor itself (2, 18, 25, 31). In this view, our binding studies with the hydrophilic ligand, namely [³H]NMS, demonstrated a decrease in muscarinic receptor density present at the membrane surface as well as an uncoupling state of the residual receptors in carbachol-postdesensitized myometrial preparations. Our data could not provide any information about receptors that could be internalized and/or downregulated, a phenomenon that has been described for desensitized muscarinic receptors (18). A relationship was noted between the time course of muscarinic receptor alteration and the impairment of inositol phosphate response triggered by the muscarinic agonist in carbachol-treated tissues.

Agonist-dependent phosphorylation of many G protein-coupled receptors (2, 18, 25), including different muscarinic receptor subtypes (11, 31), by GRK and/or second-messenger-activated kinases, is proposed to be a key event in receptor desensitization. The data described here for the myometrium imply that PKC, the kinase activated by signaling pathways downstream of the receptor, exerts an inhibitory feedback regulation at the muscarinic receptor function. Indeed, we demonstrate that a short incubation with PDBu markedly reduced the generation of inositol phosphates stimulated by carbachol. The effect is mediated by PKC, since the selective inhibitor Ro-31-8220 was able to completely prevent the action of PDBu. The failure of the phorbol ester to affect the PLC pathway when activated by ET-1 and AlF₄⁻ clearly indicated that the target for the PKC inhibitory effect was the muscarinic receptor itself and not an element downstream of the receptor. This is in accord with earlier observations that purified muscarinic receptors may act as in vitro substrates for PKC (11) and that muscarinic receptor-mediated production of inositol phosphates in numerous cell lines is significantly affected by PKC activation (14, 28). The potential contribution of PKC in the muscarinic desensitization was further provided by the failure of carbachol, in the presence of Ro-31-8220, to induce the early phase of inositol phosphate refractoriness. It is now well established that desensitization or inactivation of G protein-coupled receptor signaling at the receptor level is mediated in part by members...
of the GRKs (25). Muscarinic receptors have also been shown to be agonist-dependent substrates for purified GRKs in vitro (11, 31). Furthermore, specific GRK subtypes have been demonstrated to contribute to the desensitization in intact cells of at least two (11) subclasses of muscarinic receptors. In view of these observations, the contribution of GRKs in the negative regulation of the muscarinic receptor function in the myometrium has to be retained. Although this possibility was not investigated in the present study, the potential activation of GRK 2 by PKC is worth mentioning (5, 29). Thus the combined effects of PKC, i.e., alterations at the muscarinic receptor function and enhancement of GRK activities, may contribute to the PKC-dependent process of the early phase of the muscarinic desensitization.

Recently, it has been shown that G proteins represent an additional target of agonist-induced desensitization (21). Chronic exposure to a G protein-linked receptor agonist can frequently result in a decrease in membrane levels of the G protein that interacts with this receptor. Such effects have been observed for members of the Gq and Gi (10, 21) families and more recently for Gq/G11 proteins coupled to PLC (12, 22). In the present study, we demonstrate that sustained activation of myometrial strips with carbachol induced a marked and selective decrease in the amount of membrane-associated Gq/G11 α-subunits without affecting the levels of Gq/α and G protein β-subunits. Evidence is further provided that the reduction of Gqα/G11α was subsequent to muscarinic receptor activation, since atropine by itself was unable to cause any quantitative change in the amount of Gqα/G11α but markedly attenuated the agonist-mediated effect. Our data argued that the decline observed at the level of membrane-associated Gqα/G11α corresponded most probably to a downregulation of these proteins, consequent to their proteolytic degradation. Within the limits of the immunologic assay, we were unable to detect any increase in cytosolic levels of Gqα/G11α in carbachol-exposed tissues. This is in line with current observations reporting that G protein α-subunits activated by a receptor are degraded considerably more rapidly than those in the inactive state (30). Additionally, our observations support the contention that, in rat myometrium, activation of PKC subsequent to muscarinic stimulation did not contribute to downregulation of Gqα/G11α. First, direct activation of PKC by PDBu failed to mimic carbachol-induced downregulation of Gqα/G11α, and, second, the PKC inhibitor, Ro-31-8220, did not attenuate carbachol regulation of Gqα/G11α levels. Agonist-mediated downregulation of Gqα/G11α, independent of PKC activation, has similarly been reported for angiotensin in vascular smooth muscle cells (12) and for gonadotrophin-releasing hormone in α3T–1 pituitary cells (3). It is reasonable to conclude that, in myometrium, downregulation of Gqα/G11α may account for the PKC-independent process that developed during long-term desensitization triggered by carbachol.

In rat myometrium, exposure to carbachol appeared to induce a negative regulation at the level of both Gqα/G11α and muscarinic receptors. A similarly concurrent regulation of the receptor and the related G protein was observed in the prostacyclin receptor-Gα protein system (21) and more recently in the muscarinic M1 receptor and the AT1 receptor-Gq/G11 system (12, 22). Although these observations may tend to suggest that a functional interaction between a receptor and its cognate G protein is required to trigger downregulation of each, recent reports provide evidence that receptor downregulation was not a prerequisite process for Gqα/G11α downregulation (27). This is in line with our present data. Indeed AlF4−, which directly activates G proteins without any receptor engagement, was able to cause heterologous refractoriness in terms of inositol phosphate production, with a concomitant downregulation of Gqα/G11α. Hence it is conceivable to postulate that downregulation of Gqα/G11α is subsequent to its persistent activation whether direct or receptor mediated.

In conclusion, the present study illustrates that prolonged exposure of myometrium to carbachol results in diverse negative regulatory mechanisms operating at the level of both muscarinic receptors and Gq/G11 proteins. Our data further suggest that the agonist-induced Gq/G11 downregulation may be involved in part in mechanisms of long-term desensitization of the Gq/G11-mediated signaling system. Such a heterologous refractory state of the PLC/InsP3 pathway would ultimately display a negative control on myometrial activities triggered by diverse receptor-mediated contractile agonists. These adaptive responses might be considered as an example of autoregulation to protect the tissue from excessive fluctuations in local concentrations of vasoactive agents that do occur in the vicinity of the myometrium in certain physiological conditions.

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