ELSEVIER

European Journal of Pharmacology 315 (1996) 213–219

ejp

Involvement of $G_{\alpha q/11}$ in the contractile signal transduction pathway of muscarinic M_3 receptors in caecal smooth muscle

Pierre Cuq [a,*], Robert Zumbihl [b], Thierry Fischer [b], Bruno Rouot [b], Jean-Pierre Bali [a], Richard Magous [a]

[a] *Laboratoire de Biochimie des Membranes, INSERM CJF 92-07, Université de Montpellier I, Faculté de Pharmacie, 15 avenue Charles Flahault, F-34060 Montpellier Cédex, France*

[b] *INSERM U431, Université Montpellier II, place Eugène Bataillon, CC100, F-34095 Montpellier Cédex, France*

Received 25 April 1996; revised 25 June 1996; accepted 26 July 1996

Abstract

The nature of the pertussis toxin-insensitive G-protein involved in muscarinic-mediated phosphoinositides breakdown and contraction of isolated smooth muscle cells from the circular layer of the rabbit caecum was investigated. Immunoblotting of membrane proteins using affinity purified antibodies directed against different G-protein α-subunits revealed the expression of $G_{\alpha q/11}$, $G_{\alpha i1}$ and $G_{\alpha i2}$ in these cells. The carbachol-mediated [^{3}H]inositol phosphates accumulation in saponin-permeabilized cells was abolished by anti-$G_{\alpha q/11}$-antibodies whereas anti-$G_{\alpha i1,2}$-antibodies were ineffective. Moreover, the carbachol-induced contraction of permeabilized cells, as determined by videomicrocopic measurements, was reversed by anti-$G_{\alpha q/11}$-antibodies but not affected by anti-$G_{\alpha i1,2}$-antibodies. From these data, we conclude that carbachol stimulates phosphoinositides hydrolysis and cell contraction through activation of specific muscarinic M_3 receptors coupled to the pertussis toxin-insensitive $G_{\alpha q/11}$-protein. This is the first demonstration of $G_{\alpha q/11}$ implication in the contractile signal transduction pathway of muscarinic M_3 receptors in smooth muscle cells.

Keywords: Smooth muscle; Contraction; Phospholipase C; Muscarinic; G-protein; Antibody

1. Introduction

The muscarinic acetylcholine receptors are members of the superfamily of G-protein-coupled cell surface receptors, predicted to have seven transmembrane domains (Nathanson, 1987). Five genes for the muscarinic receptors have been cloned (m1–m5) (Bonner, 1989), but only three (M_1, M_2 and M_3) (Hulme et al., 1990) and perhaps four receptor subtypes (M_4) (Dörje et al., 1990) have been pharmacologicaly identified. On the basis of binding profiles, tissue distribution and functional properties, the M_1, M_2, M_3 and M_4 receptor subtypes are considered to be equivalent to the products of the m1, m2, m3 and m4 genes, respectively (Maeda et al., 1988; Buckley et al., 1989). Multiple effectors are regulated by the muscarinic receptors: phospholipases A_2, C and D, adenylate cyclase, guanylate cyclase, tyrosine kinase and ionic channels (Felder, 1995). The muscarinic m1, m3 and m5 receptors expressed in various cell types predominantly activate phosphoinositides breakdown and can stimulate adenylate cyclase (Peralta et al., 1988; Felder et al., 1989) whereas the m2 and m4 subtypes efficiently inhibit adenylate cyclase but poorly activate phosphoinositides hydrolysis (Peralta et al., 1988). While the G-proteins mediating muscarinic-induced adenylate cyclase inhibition are all sensitive to pertussis toxin (Birnbaumer et al., 1990), the G-proteins involved in the coupling of muscarinic receptors to phospholipase C appear to be heterogeneous, distinguished by their pertussis toxin sensitivity (Ashkenazi et al., 1989; Lazareno et al., 1993).

To date, the pertussis toxin-sensitive G-proteins involved in the stimulation of phospholipase C by muscarinic receptors are not clearly defined. Two forms of G_o (G_{oA} and G_{oB}) specifically increase the muscarinic-mediated stimulation of phospholipase C when injected into *Xenopus* oocytes (Padrell et al., 1991). Subsequent studies linked the muscarinic m2 receptor with phospholipase C through $G_{\alpha i2}$ and $G_{\alpha i3}$ (Dell'Acqua et al., 1993) but in

Abbreviations: $Ins(1,4,5)P_3$, Inositol 1,4,5-trisphosphate

* Corresponding author. Tel.: (33) 6754-3344; Fax: (33) 6754-8039.

0014-2999/96/$15.00 Copyright © 1996 Elsevier Science B.V. All rights reserved.
PII S0014-2999(96)00605-X

vitro reconstitution with the purified elements (receptor, phospholipase C and α-subunits) has not been reported. It has also been shown that G-protein $\beta\gamma$ dimers activate certain phospholipase C isoenzymes, and that this activity may account for pertussis toxin-sensitive inositol lipid signalling (Boyer et al., 1994). On the other hand, numerous data indicate that the muscarinic-mediated pertussis toxin-insensitive stimulation of phospholipase C is due to members of the G_q family (Sternweis and Smrcka, 1992). Reconstitution experiments have shown that muscarinic m1 receptor can activate phospholipase C-β1 via G_q or G_{11} α-subunits (Berstein et al., 1992). Additional studies performed in rat parotid gland membranes have demonstrated, by using polyclonal antibodies directed against $G_{q/11}$ α-subunits, the involvement of $G_{q/11}$ in the muscarinic m3 receptor-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) (Sawaki et al., 1993).

Muscarinic acetylcholine M_2 and M_3 receptors are co-expressed in smooth muscles from gastrointestinal tract, urinary bladder, vascular and airway tissues (Eglen et al., 1994). In the gastrointestinal tract, mixed populations of muscarinic M_2 and M_3 receptors have also been detected in ileal (Maeda et al., 1988; Candell et al., 1990), caecal (Cuq et al., 1994a) and colonic (Zhang et al., 1991; Gomez et al., 1992) smooth muscles. The co-existence of two receptor subtypes in gastrointestinal smooth muscles suggested that both subtypes would play a role in the contraction of these tissues. Little is known about the function of muscarinic M_2 receptors. As reported by Ehlert and collaborators, their contribution to contraction may be indirect, by inhibiting the relaxation elicited by other receptors (Candell et al., 1990; Griffin and Ehlert, 1992; Thomas et al., 1993; Thomas and Ehlert, 1994). On the contrary, it has been shown that muscarinic M_3 receptors activation induced contraction of different gastrointestinal smooth muscles (Candell et al., 1990; Barocelli et al., 1993; Cuq et al., 1994a).

It has been previously shown that the activation of muscarinic M_3 receptors leads to phosphoinositide breakdown via the activation of a pertussis toxin-insensitive G-protein in ileal (Candell et al., 1990; Thomas and Ehlert, 1994) and colonic (Zhang and Buxton, 1991) smooth muscle tissues. We also observed a pertussis toxin-insensitive muscarinic regulation of phosphoinositides hydrolysis in circular smooth muscle cells from the rabbit caecum (Cuq et al., 1994b); however, the molecular nature of the pertussis toxin-insensitive G-protein remained to be determined. Using antibodies raised against the C-terminal part of the G_q α-subunit, Zhou et al. (1994) recently showed that $G_{\alpha q}$ was expressed in bovine iris sphincter and mediated the muscarinic-induced PIP_2 hydrolysis. However, this study performed on reconstituted smooth muscle membranes did not allow to prove the involvement of $G_{\alpha q}$ in M_3-stimulated smooth muscle cells contraction.

The aim of this work was to identify the G-protein involved in the contractile signal transduction pathway of muscarinic M_3 receptors in smooth muscle cells from the circular layer of the rabbit caecum. The expression of G-proteins in smooth muscle cell membranes was studied by immunodetection using affinity purified antibodies directed against the C-terminal part of the $G_{i1,2}$, $G_{q/11}$ α-subunits (Zumbihl et al., 1994). The effects of these antibodies on carbachol-mediated phospholipase C stimulation and smooth muscle cells contraction were also investigated.

2. Materials and methods

2.1. Materials

Collagenase from *Clostridium histolyticum*, creatine phosphate, creatine phosphokinase and pronase were from Boehringer-Mannheim (Germany). Soybean trypsin inhibitor (STI), ATP, carbachol, saponin and pertussis toxin were from Sigma (St. Louis, MO, USA). Bovine serum albumin was from Fluka (Buchs, Switzerland). AG-1X-4 (anion exchange resin) was from Bio-Rad (Ivry-sur-Seine, France). *myo*-[^{3}H]inositol (20 Ci/mmol) was from ICN (Irvine, CA, USA). [^{125}I]protein A ($2-3 \times 10^5$ cpm/ml) and polyvinyl-difluoride sheets (Polyscreen®) were from DuPont NEN (Les Ulis, France). Affinity purified antibodies directed against the C-terminal part of the $G_{i1,2}$ (750 μg/ml) and $G_{q/11}$ (450 μg/ml) α-subunits were prepared as already described (Zumbihl et al., 1994).

Medium A: 132 mM NaCl, 5.4 mM KCl, 5 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 25 mM Hepes, 0.2% glucose, 0.2% bovine serum albumin, 0.02% Phenol red, pH 7.4.

Medium B: Earle's balanced salt solution containing 10 mM Hepes, 0.1% bovine serum albumin, pH 7.4.

Medium C: 20 mM NaCl, 100 mM KCl, 5 mM $MgSO_4$, 1 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 1 mM EGTA, 10 mM Hepes, 2% bovine serum albumin, 1.5 mM ATP, 5 mM creatine phosphate, 10 U/ml creatine phosphokinase, pH 7.2.

2.2. Isolation of smooth muscle cells from the caecum

Smooth muscle cells were isolated from the circular muscle layer of the rabbit caecum as described previously (Cuq et al., 1994a). Briefly, 3-month-old New-Zealand male rabbits were killed by stunning and bleeding. The mucosal and submucosal layers were peeled off the muscular and serosal layers. The circular muscle, separated from longitudinal muscle and serosa, was incubated in medium A containing 0.27% collagenase, 0.03% pronase, 0.01% STI and gassed with 100% O_2. After 10 min at 30°C, the incubation medium was diluted in medium A and filtered through a nylon mesh. The remaining tissue was re-incubated in fresh medium A for 10 min at 30°C and fragments were dispersed into single cells by passages in

and out the inverted wide end of a 5-ml pipette. The resulting cell suspension was filtered through a nylon mesh. The filtrate which contained isolated cells was diluted in fresh medium A and centrifuged at $150 \times g$ for 2 min. The cell pellet was then diluted in medium B (for *myo*-[^{3}H]inositol incorporation) or in medium C (for cell permeabilization). Viability (estimated by Trypan blue exclusion) was always > 90%. This method yielded about 10^7 isolated circular smooth muscle cells/caecum. It is emphasized that only those cells that are dissociated in enzyme-free medium were used for subsequent studies.

2.3. Smooth muscle cell membrane preparation

Plasma membranes were prepared following the method of Bursten et al. (1991), with minor modifications. Dispersed smooth muscle cells, pelleted by centrifugation at $150 \times g$ for 2 min as described above, were suspended in a ice-cold 10 mM imidazole, pH 7.4, 1 mM phenylmethylsulphonyl fluoride containing medium and disrupted with a Dounce homogenizer. The broken cell suspension was centrifuged at $600 \times g$ for 10 min at 4°C and the supernatant recentrifuged at $15\,200 \times g$ for 25 min at 4°C. The resultant crude microsomal fraction, resuspended in 20 mM Hepes buffer, pH 7.4, was layered on 35% (w/w) sucrose cushion in 20 mM Hepes buffer, pH 7.4 and centrifuged at $70\,000 \times g$ for 30 min at 4°C, to obtain a plasma membrane-enriched fraction at the interface. The plasma membrane-enriched fraction was then washed by centrifugation at $70\,000 \times g$ for 15 min at 4°C in 20 mM Hepes buffer, pH 7.4. After washing, the plasma membrane-enriched fraction was resuspended in the Hepes buffer at a protein concentration of 5–12 mg/ml and stored at −25°C until use.

2.4. Sodium dodecylsulphate-polyacrylamide gel electrophoresis and immunoblotting

Plasma membrane proteins of caecal smooth muscle cells and rat cortex particulate fraction were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as already described (Toutant et al., 1987). Proteins were then electrotransferred onto polyvinyl-difluoride sheets and stained with Coomassie blue for visualization. After destaining, quenching and washing, strips were incubated for 2 h at 25°C with the affinity purified antipeptide antibodies. Labeling of the immune complexes was performed for 1 h incubation at 30°C with [^{125}I]protein A followed by autoradiography without intensifying screen.

2.5. Measurements of [^{3}H]inositol phosphates accumulation in permeabilized cells

[^{3}H]inositol phosphates accumulation measurements in saponin-permeabilized smooth muscle cells were performed as previously described (Cuq et al., 1994b), with minor modifications: smooth muscle cells (4×10^6/ml) were incubated for 4 h at 37°C under continuous gassing (95% O_2–5% CO_2) in medium B containing *myo*-[^{3}H]inositol (45 μCi/ml). Next, *myo*-[^{3}H]inositol-pre-labeled smooth muscle cells were washed with medium C. Then, smooth muscle cells (0.5×10^6/ml) were equilibrated for 20 min at 37°C in medium C containing 10 mM LiCl. Permeabilization was performed by incubation with saponin (35 μg/ml in medium C) for 10 min at 37°C. Cells were then washed and resuspended in medium C. Finally, smooth muscle cells were incubated for 10 min at 37°C with the agents to be tested (final volume 0.5 ml). When used, antibodies were added 20 min before incubation with agents. The reaction was stopped by the addition of 100 μl ice-cold 60% $HClO_4$ and centrifugation at $8000 \times g$, for 1 min in a microfuge at 4°C. The pH of the supernatants were adjusted to pH 7 with a 3 M KOH/0.15 M Hepes buffer. After 10 min at 4°C and centrifugation for 2 min at $2000 \times g$, the [^{3}H]inositol phosphates present in the resulting supernatant were separated by anion-exchange chromatography on Dowex AG-1-X4 (formate form).

2.6. Measurement of saponin-permeabilized cell contraction

Smooth muscle cells were permeabilized by saponin as previously described (Bitar et al., 1986), with minor modifications. Briefly, smooth muscle cells incubated for 20 min at 30°C in the presence of 35 μg/ml saponin, were washed and resuspended in medium C. The cell suspension (0.25×10^6 cells/ml) was pre-incubated for 20 min at 30°C with the antibodies and then incubated for 30 s at 30°C in the presence of 0.1 μM carbachol. The reaction was stopped by adding 50 μl of glutaraldehyde solution to a final glutaraldehyde concentration of 2% (v/v). In con-

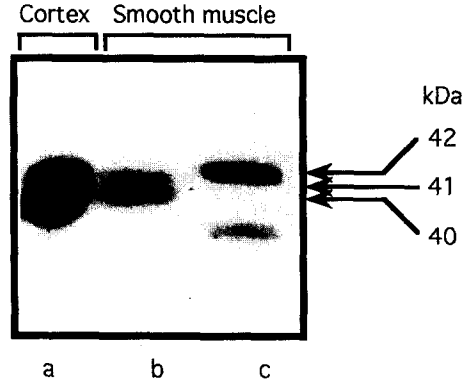


Fig. 1. Expression of $G_{q/11}$, G_{i1} and G_{i2} α-subunits in membranes of smooth muscle cells from the circular layer of the rabbit caecum. Membrane proteins from rat cortex and rabbit caecal smooth muscle cells were separated by SDS-PAGE and electroblotted onto polyvinyl-difluoride sheets. The G-protein α-subunits immunoreactivity was assessed using antibodies raised against $G_{\alpha i1,2}$ (lanes a and b) and $G_{\alpha q/11}$ (lane c) as described in Section 2.

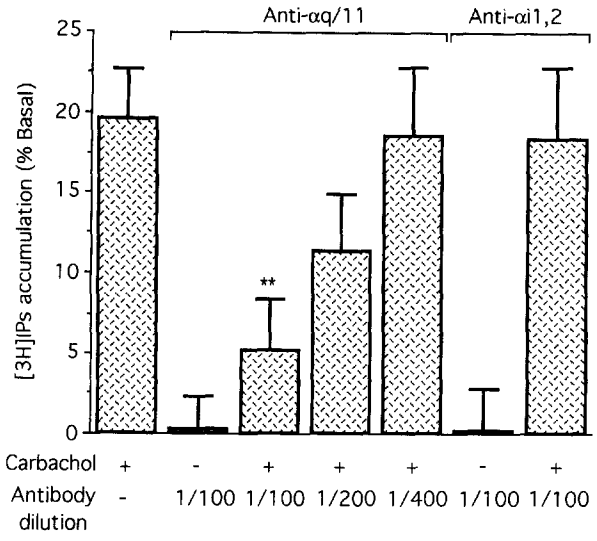


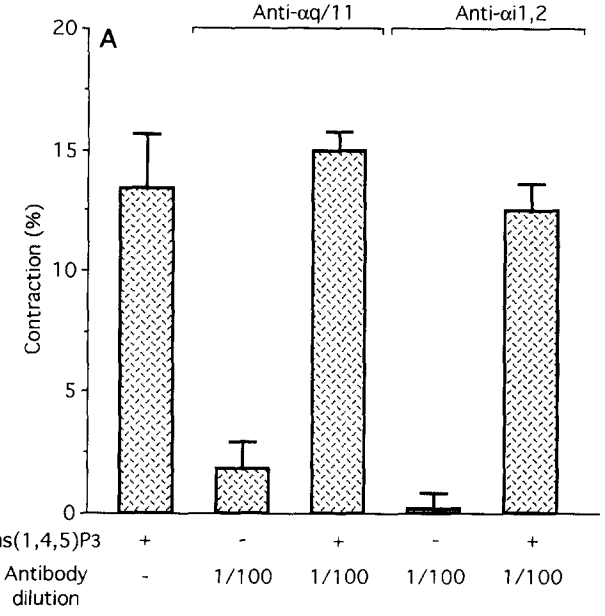
Fig. 2. Effects of antibodies directed against the G-protein $G_{q/11}$ and $G_{i1,2}$ α-subunits on carbachol-induced [^{3}H]inositol phosphates accumulation in saponin-permeabilized smooth muscle cells. Permeabilized cells, pre-incubated for 20 min at 37°C in the absence (control cells) or the presence of antibodies directed against $G_{\alpha q/11}$ (1:100, 1:200 and 1:400 final dilution) and $G_{\alpha i1,2}$ (1:100 final dilution), were incubated for 10 min at 37°C with 0.1 mM carbachol. Data, expressed as percentage of increase in the basal [^{3}H]inositol phosphates radioactivity (3675 ± 630 dpm), are means ± S.E.M. of 5 separate experiments. Significantly different from the effect of carbachol alone at ** $P < 0.01$ (Student's t-test).

trol experiments, 100 μl of medium C was used instead of the agent solution. The mean cell length was statistically determined by video-microscopic measurements of 100 cells. The contractile response corresponds to the percentage of decrease in mean cell length compared to the mean length of control cells.

3. Results

3.1. Expression profile of $G_{i1,2}$ and $G_{q/11}$ α-subunits

In order to identify the G-proteins expressed in circular smooth muscle from the rabbit caecum, plasma membranes were fractionated from dispersed smooth muscle cells, the proteins present in this fraction were separated by SDS-PAGE, electrotransferred onto polyvinyl-difluoride sheets and probed with affinity purified antibodies raised against the C-terminal part of the $G_{i1,2}$ and $G_{q/11}$ α-subunits. The specificity of these antibodies has been described elsewhere (Zumbihl et al., 1994). The distribution of these G-proteins in the rabbit caecum is shown in Fig. 1. Probing smooth muscle cells and rat cortex membrane proteins with anti-$G_{\alpha i1,2}$ antibodies revealed 2 protein bands in the 40- and 41-kDa regions corresponding to G_{i2} and G_{i1} α-subunits, respectively. Interestingly, the 41-kDa protein band was weakly immunoreactive in rabbit smooth muscle cell membranes. Polyclonal anti-$G_{\alpha q/11}$ antibodies recognized, in comparison with purified rat cortex $G_{\alpha i1,2}$-proteins, a 42-kDa polypeptide in smooth muscle cell membrane fraction, indicating that $G_{\alpha q/11}$ is expressed in circular smooth muscle cells.



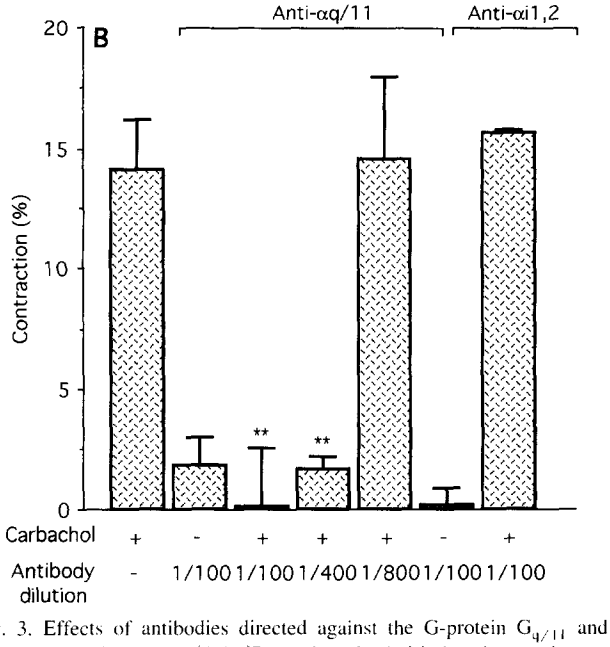


Fig. 3. Effects of antibodies directed against the G-protein $G_{q/11}$ and $G_{i1,2}$ α-subunits on $Ins(1,4,5)P_3$ and carbachol-induced saponin-permeabilized smooth muscle cell contraction. Permeabilized cells were pre-incubated for 20 min at 30°C in the absence (control cells) or the presence of antibodies directed against $G_{\alpha q/11}$ (1:100, 1:400 and 1:800 final dilution) and $G_{\alpha i1,2}$ (1:100 final dilution). Then, smooth muscle cells were incubated for 30 s at 30°C in the presence of $Ins(1,4,5)P_3$ (1 μM) (A) or carbachol (0.1 μM) (B). Data, expressed as percentage of decrease in mean cell length compared to the mean length of control cells, are means ± S.E.M. of 3 separate experiments. Significantly different from the effect of carbachol alone at ** $P < 0.01$ (Student's t-test).

3.2. Identification of the G-protein mediating muscarinic-induced [^{3}H]inositol phosphates accumulation

We next investigated the possible involvement of $G_{\alpha q/11}$ in the muscarinic receptor-mediated phospholipase C stimulation in saponin permeabilized smooth muscle cells, by using antibodies directed against $G_{i1,2}$ and $G_{q/11}$ α-subunits. We previously showed that saponin-treated smooth muscle cells retained their ability to respond to muscarinic receptor-linked agonists by an increase in [^{3}H]inositol phosphates production (Cuq et al., 1994b). As shown in Fig. 2, the 0.1 mM carbachol-induced [^{3}H]inositol phosphates accumulation in saponin-permeabilized cells (19.53 $\pm$ 3.09% over basal level) was abolished in a concentration-dependent fashion by anti-$G_{\alpha q/11}$ antibodies; when used alone, these antibodies did not affect basal [^{3}H]inositol phosphates level. In contrast, the anti-$G_{\alpha i1,2}$ antibodies did not modify the 0.1 mM carbachol-induced [^{3}H]inositol phosphates accumulation (Fig. 2).

3.3. Identification of the G-protein mediating muscarinic-induced contraction

Finally, we attempted to identify which G-protein mediated the contractile effect of carbachol in saponin-permeabilized smooth muscle cells, by using anti-$G_{\alpha q/11}$ and anti-$G_{\alpha i1,2}$ antibodies. As indicated in Fig. 3, exogeneous Ins(1,4,5)P_3 and carbachol significantly contracted the saponin-permeabilized smooth muscle cells (13.50 $\pm$ 2.30 and 14.04 $\pm$ 2.06%, respectively, decrease in mean cell length). The anti-$G_{\alpha q/11}$ and anti-$G_{\alpha i1,2}$ antibodies did not modify the contraction elicited by Ins(1,4,5)P_3 (Fig. 3A). On the other hand, the 0.1 μM carbachol-induced contraction of saponin-treated smooth muscle cells was abolished by anti-$G_{\alpha q/11}$ antibodies (Fig. 3B). However, the antibodies directed against $G_{i1,2}$ α-subunits did not affect the carbachol-induced cell contraction (Fig. 3B).

4. Discussion

We previously reported the existence of a mixed population of muscarinic receptors in smooth muscle cells from the circular layer of the rabbit caecum: muscarinic M_2 receptors inhibiting adenylate cyclase via a pertussis toxin-sensitive G-protein and muscarinic M_3 receptors mediating, via a pertussis toxin-insensitive G-protein, Ins(1,4,5)P_3 accumulation and smooth muscle cell contraction (Cuq et al., 1994a,b). In this report, we identified the G-protein-coupling muscarinic receptors to phospholipase C and contraction in these cells, by using affinity purified antibodies raised against the C-terminal part of different G-protein α-subunits. Such antibodies directed against C-terminal sequences have been demonstrated to interfere with receptor-G-protein interactions and hence with agonist control of effector functions (Milligan, 1994), so they can be used as molecular probes to identify G-proteins in functional experiments.

We first studied the expression of G-proteins in circular smooth muscle cells. The probing, by anti-$G_{\alpha i1,2}$ antibodies, of two protein bands in the 40–41-kDa region indicated the expression of both G_{i1} and G_{i2} α-subunits in these cells. Moreover, the weak immunoreactivity of the 41-kDa protein suggested that G_{i1} α-subunits were poorly expressed in this tissue. A similar pattern of G_{i1} and G_{i2} α-subunits expression was previously reported in human myometrial smooth muscle (Zumbihl et al., 1994). As already shown using purified muscarinic receptors in reconstituted systems (Parker et al., 1991), $G_{\alpha i1}$ and/or $G_{\alpha i2}$ could be involved, in the circular smooth muscle from the rabbit caecum, in the muscarinic M_2 receptor-induced pertussis toxin-sensitive adenylate cyclase inhibition.

Using polyclonal anti-$G_{\alpha q/11}$ antibodies, we also detected a 42-kDa polypeptide, indicating that $G_{q/11}$ α-subunits were expressed in the rabbit caecal smooth muscle cells. As previously described (Wilkie et al., 1992), the amino-acid sequences of α_q and α_{11} are about 80% homologous and the differences are restricted to the N-terminus of these proteins. Thus, anti-$G_{\alpha q/11}$ antibodies could not discriminate between α_q and α_{11}. Recently, Ibarrondo et al. (1995) showed a close association of α_q and α_{11} with actin filaments in WRK_1 cells and in human myometrium, suggesting that actin-associated $G_{\alpha q/11}$ proteins play a role in G-protein-mediated phospholipase C activation. In our study, we were not able to detect $G_{\alpha q/11}$ in the Triton-insoluble cytoskeletal fraction of smooth muscle cells from the rabbit caecum (data not shown), suggesting that $G_{\alpha q/11}$ is preferentially associated to the plasma membrane in this tissue.

We next investigated the role of $G_{\alpha q/11}$ in the muscarinic-mediated phospholipase C stimulation. This study was based on the property of antibodies directed against C-terminal sequences of G-protein α-subunits to interfere with agonist regulation of effector functions (Milligan, 1994). However, as antibodies had to enter the cells to interact with the G-proteins, smooth muscle cells were permeabilized with saponin prior to muscarinic stimulation. We previously had shown (Cuq et al., 1994b) that muscarinic agonists are able to increase [^{3}H]inositol phosphates accumulation in saponin-permeabilized smooth muscle cells. The carbachol-induced [^{3}H]inositol phosphates accumulation in saponin-treated cells was abolished in a concentration-dependent fashion by anti-$G_{\alpha q/11}$ antibodies. The specificity of this inhibition was demonstrated by using the anti-$G_{\alpha i1,2}$ antibodies which were previously shown to inhibit substance P- and mastoparan-stimulated GTPase activity of purified G-proteins from calf brain (Mousli et al., 1990): these anti-$G_{\alpha i1,2}$ antibodies did not affect the carbachol-induced [^{3}H]inositol phosphates accumulation in saponin-permeabilized smooth muscle cells. This lack of effect was not due to an inability of these antibodies to recognize G-proteins because they detected

$G_{i1,2}$ α-subunits in immunoblotting experiments. Taken together, these data indicate that muscarinic M_3 receptors stimulate [^{3}H]inositol phosphates accumulation in rabbit caecal smooth muscle cells via activation of the pertussis toxin-insensitive $G_{q/11}$-protein. These results were in good agreement with a previous report where G_q was shown to mediate muscarinic-stimulated PIP_2 hydrolysis in reconstituted smooth muscle membranes (Zhou et al., 1994).

It has been reported that the contraction of smooth muscle cells derived from the circular layer of the intestine was dependent on the release of intracellular Ca^{2+} by Ins(1,4,5)P_3 (Murthy et al., 1991). We also showed that muscarinic M_3 receptors activation led to Ins(1,4,5)P_3 production in circular smooth muscle cells from the rabbit caecum (Cuq et al., 1994a). As exogeneous Ins(1,4,5)P_3 and carbachol significantly contracted the saponin-permeabilized smooth muscle cells, it seems likely that an Ins(1,4,5)P_3-induced Ca^{2+} release from internal stores was involved in the muscarinic-stimulated contraction of circular smooth muscle cells from the rabbit caecum. Moreover, this result indicates that saponin-permeabilized cells from gastrointestinal smooth muscles retain their contractile potentiality, as previously mentioned (Makhlouf, 1987).

Finally, we identified the G-protein involved in the transduction of the contractile signal of muscarinic receptors in rabbit caecal permeabilized smooth muscle cells. The carbachol-stimulated contraction of saponin-permeabilized smooth muscle cells was abolished by anti-$G_{\alpha q/11}$ antibodies whereas the antibodies raised against $G_{i1,2}$ α-subunits were ineffective. Moreover, both anti-$G_{\alpha q/11}$ and anti-$G_{\alpha i1,2}$ antibodies did not modify the Ins(1,4,5)P_3-induced smooth muscle cell contraction. Taken together, these data strongly suggest that the inhibition of the muscarinic-stimulated contraction is due to a specific interaction of anti-$G_{\alpha q/11}$ antibodies with the corresponding G-protein. This is, to our knowledge, the first demonstration of the implication of $G_{q/11}$ α-subunits in the transduction of the contractile signal of muscarinic receptors in smooth muscle cells.

In conclusion, the pertussis toxin-insensitive $G_{\alpha q/11}$-protein is involved in M_3 subtype muscarinic receptor-mediated phospholipase C activation in dispersed smooth muscle cells from the circular layer of the rabbit caecum. Moreover, this G-protein is involved in the transduction of the muscarinic contractile signal in these cells.

Acknowledgements

We thank Dr. Serge Roche for helpful comments and revision of the manuscript. This work was supported by grants from Centre National de la Recherche Scientifique (CNRS), from Institut National de la Santé et de la Recherche Médicale (INSERM) and from Université de Montpellier I.

References

Ashkenazi, A., E.G. Peralta, J.W. Winslow, J. Ramachandran and D.J. Capon, 1989, Functionally distinct G proteins selectively couple different receptors to PI hydrolysis in the same cell, Cell 56, 487.

Barocelli, M., M. Chiavarini, V. Ballabeni, F. Bordi and M. Impicciatore, 1993, Interaction of selective compounds with muscarinic receptors at dispersed intestinal smooth muscle cells, Br. J. Pharmacol. 108, 393.

Berstein, G., J.L. Blank, A.V. Smrcka, A.T. Higashijima, P.C. Sternweis, J.H. Exton and E.M. Ross, 1992, Reconstitution of agonist-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis using purified muscarinic receptor, $G_{q/11}$, and phospholipase C-β1, J. Biol. Chem. 267, 8081.

Birnbaumer, L., J. Abramowitz and A.M. Brown, 1990, Receptor-effector coupling by G proteins, Biochim. Biophys. Acta 1031, 163.

Bitar, K.N., P.G. Bradford, J.W. Putney and G.M. Makhlouf, 1986, Stoichiometry of contraction and Ca^{2+} mobilization in isolated guinea-pig and human gastric smooth muscle cells, J. Biol. Chem. 261, 16591.

Bonner, T.I., 1989, The molecular basis of muscarinic receptor diversity, Trends Neurosci. 12, 148.

Boyer, J.L., A. Paterson and T.K. Harden, 1994, G-protein-mediated regulation of phospholipase C: involvement of $\beta\gamma$ subunits, Trends Cardiovasc. Med. 4, 88.

Buckley, N.J., T.I. Bonner and M.R. Brann, 1989, Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells, Mol. Pharmacol. 35, 469.

Bursten, S.L., W.E. Harris, K. Bomszty and D. Lovett, 1991, Interleukin-1 rapidly stimulates lysophosphatidate acyltransferase and phosphatidate phosphohydrolase activities in human mesanglial cells, J. Biol. Chem. 266, 20732.

Candell, L.M., S.H. Yun, L.L.P. Tran and F.J. Ehlert, 1990, Differential coupling of subtypes of the muscarinic receptors to adenylate cyclase and phosphoinositide hydrolysis in the longitudinal muscle of the rat ileum, Mol. Pharmacol. 38, 689.

Cuq, P., R. Magous and J.P. Bali, 1994a, Pharmacological coupling and functional role for the muscarinic receptor subtypes in isolated cells from the circular smooth muscle of the rabbit cecum, J. Pharmacol. Exp. Ther. 271, 149.

Cuq, P., R. Magous and J.P. Bali, 1994b, Signal transduction pathways of muscarinic receptors in circular smooth muscle from the rabbit caecum, Mol. Cell. Biochem. 140, 65.

Dell'Acqua, M.L., R.C. Carroll and E.G. Peralta, 1993, Transfected m2 muscarinic acetylcholine receptors couple to $G_{\alpha i2}$ and $G_{\alpha i3}$ in chinese hamster ovary cells. Activation and desensitization of the phospholipase C signaling pathways, J. Biol. Chem. 268, 5676.

Dörje, F., T. Friebe, R. Tacke, E. Mutschler and G. Lambrecht, 1990, Novel pharmacological profile of muscarinic receptors mediating contraction of the guinea-pig uterus, Naunyn-Schmiedeberg's Arch. Pharmacol. 342, 284-289.

Eglen, R.M., H. Reddy, N. Watson and R.A.J. Challiss, 1994, Muscarinic acetylcholine receptor subtypes in smooth muscle, Trends Pharmacol. Sci. 15, 114.

Felder, C.C., 1995, Muscarinic acetylcholine receptors: signal transduction through multiple effectors, FASEB J. 9, 619.

Felder, C.C., A.L. Ma and B.R. Conklin, 1989, Carbachol-induced reverse transformation of Chinese hamster ovary cells transfected with and expressing the m5 muscarinic acetylcholine receptor, FEBS Lett. 245, 75.

Gomez, A., F. Martos, I. Bellido, E. Marquez, A.J. Garcia, J. Pavia and F. Sanchez De La Cuesta, 1992, Muscarinic receptor subtypes in human and rat colon smooth muscle, Biochem. Pharmacol. 43, 2413.

Griffin, M.T. and F.J. Ehlert, 1992, Specific inhibition of isoproterenol-stimulated cyclic AMP accumulation by M_2 muscarinic receptors in rat intestinal smooth muscle, J. Pharmacol. Exp. Ther. 263, 221.

Hulme, E.C., N.J.M. Birdsall and N.J. Buckley, 1990, Muscarinic receptor subtypes, Annu. Rev. Pharmacol. Toxicol. 30, 633.

Ibarrondo, J., D. Joubert, M.N. Dufour, A. Cohen-Solal, V. Homburger, S. Jard and G. Guillon, 1995, Close association of the α subunits of G_q and G_{11} G proteins with actin filaments in WRK1 cells: relation to G protein-mediated phospholipase C activation, Proc. Natl. Acad. Sci. USA 92, 8413.

Lazareno, S., T. Farries and N.J.M. Birdsall, 1993, Pharmacological characterization of guanine nucleotide exchange reactions in membranes from CHO cells stably transfected with human muscarinic receptor M1-M4, Life Sci. 52, 449.

Maeda, A., T. Kubo, M. Mishima and S. Numa, 1988, Tissue distribution of mRNAs encoding muscarinic acetylcholine receptor subtypes, FEBS Lett. 239, 339.

Makhlouf, G.M, 1987, Isolated smooth muscle cells of the gut, in: Physiology of the Gastrointestinal Tract, 2nd Ed., ed. L.R. Johnson (Raven, New York) p. 555.

Milligan, G, 1994, Specificity and functional applications of antipeptide antisera which identify G-protein α subunits, in: Methods in Enzymology, 237, ed. R. Iyengar (Academic Press) p. 268.

Mousli, M., C. Bronner, J. Bockaert, B. Rouot and Y. Landry, 1990, Interaction of substance P, compound 48/80 and mastoparan with the α-subunit C-terminus of G-protein, Immunol. Lett. 25, 355.

Murthy, K.S., J.R. Grider and G.M. Makhlouf, 1991, InsP3-dependent Ca^{2+} mobilization in circular but not longitudinal muscle cells of intestine, Am. J. Physiol. 261, G937.

Nathanson, N.M., 1987, Molecular properties of the muscarinic acetylcholine receptor, Annu. Rev. Neurosci. 10, 195.

Padrell, E., D.J. Carty, T.M. Moriarty, J.D. Hildebrandt, E.M. Landau and R. Iyengar, 1991, Two forms of the bovine brain Go that stimulate the inositol trisphosphate-mediated Cl- currents in *Xenopus* oocytes. Distinct guanine nucleotides binding properties, J. Biol. Chem. 266, 9771.

Parker, E.M., K. Kameyama, T. Higashijima and E.M. Ross, 1991, Reconstitutively active G protein-coupled receptors purified from baculovirus-infected insect cell, J. Biol. Chem. 266, 519.

Peralta, E.G., A. Ashkenazi, J.W. Winslow, J. Ramachandran and D.J. Capon, 1988, Differential regulation of PI hydrolysis and adenylyl cyclase by muscarinic receptor subtypes, Nature (London) 334, 434.

Sawaki, K., Y. Hiramatsu, B.J. Baum and S.I. Ambudkar, 1993, Involvement of $G_{\alpha q/11}$ in m3-muscarinic receptor stimulation of phosphatidylinositol 4,5 bisphosphate-specific phospholipase C in rat parotid gland membranes, Arch. Biochem. Biophys. 305, 546.

Sternweis, P.C. and A.V. Smrcka, 1992, Regulation of phospholipase C by G proteins, Trends Biochem. Sci. 17, 502.

Thomas, E.A., S.A. Baker and F.J. Ehlert, 1993, Functional role for the M_2 muscarinic receptor in smooth muscle of guinea-pig ileum, Mol. Pharmacol. 44, 102.

Thomas, E.A. and F.J. Ehlert, 1994, Pertussis toxin blocks M_2 muscarinic receptor-mediated effects on contraction and cyclic AMP in the guinea-pig ileum, but not M_3-mediated contractions and phosphoinositides hydrolysis, J. Pharmacol. Exp. Ther. 271, 1042.

Toutant, M.D., D. Aunis, J. Bockaert, V. Homburger and B. Rouot, 1987, Presence of three pertussis toxin substrates ang $G_o\alpha$ immunoreactivity in both plasma and granule membranes of chromaffin cells, FEBS Lett. 215, 339.

Wilkie, T.M., D.J. Gilbert, A.S. Olsen, X.N. Chen, T.T. Amatruda, J.R. Korenberg, B.J. Trask, P. De Jong, R.R. Reed, M.I. Simon, N.A. Jenkins and N.G. Copeland, 1992, Evolution of the mammalian G protein α subunit multigene family, Nat. Genet. 1, 85.

Zhang, L. and I.L.O. Buxton, 1991, Muscarinic receptors in canine colonic circular smooth muscle. II. Signal transduction pathways, Mol. Pharmacol. 40, 352.

Zhang, L., B. Horowitz and I.L.O. Buxton, 1991, Muscarinic receptors in canine colonic circular smooth muscle. I. Coexistence of M_2 and M_3 subtypes, Mol. Pharmacol. 40, 343.

Zhou, C.J., R.A. Akhtar and A.A Abdel-Latif, 1994, Identification of phosphoinositide-specific phospholipase C-β1 and GTP-binding protein, $G_q\alpha$, in bovine iris sphincter membranes: characteristics of the phospholipase and its coupling to cholinergic muscarinic receptors, Exp. Eye Res. 59, 377.

Zumbihl, R., M. Breuiller-Fouché, J. Carrette, M.N. Dufour, F. Ferré, J. Bockaert and B. Rouot, 1994, Up-regulation in late pregnancy of both $G_{o1}\alpha$ and $G_{o2}\alpha$ isoforms in human myometrium, Eur. J. Pharmacol. Mol. Pharmacol. Sect. 288, 9.