J. Membrane Biol. 133, 183–190 (1993)

The Journal of
Membrane
Biology

A G_q-type G Protein Couples Muscarinic Receptors to Inositol Phosphate and Calcium Signaling in Exocrine Cells from the Avian Salt Gland

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Summary. Muscarinic acetylcholine receptor (mAChR) activation in isolated cells from the nasal salt gland of the domestic duck (*Anas platyrhynchos*) results in a rapid increase in the rate of phosphatidylinositol hydrolysis and pronounced intracellular calcium signals. Both responses can be elicited by treating these cells with fluoroaluminate (AlF_4^-) indicating the involvement of a heterotrimeric G protein in the transmembrane signaling process. To characterize this G protein, electrophoretically separated membrane proteins were blotted onto nitrocellulose filters and probed with peptide-antibodies raised against portions of different α-subunits of mammalian G proteins. We could demonstrate the presence of at least four different G proteins in salt gland cell membranes. Two of these proteins (40 and 41 kD) were ADP-ribosylated by pertussis toxin and were recognized by an antiserum against a common sequence in all G protein α-subunits. One protein (46 kD) was a cholera toxin-substrate and was recognized by a G_s-specific antiserum; the other (42 kD) was recognized by G_q-specific antisera and was resistant to ADP-ribosylation. Since the initial inositol phosphate production upon receptor activation with carbachol and the resulting calcium signals were not affected by pertussis toxin-pretreatment of salt gland cells, we conclude that muscarinic receptors are coupled to phospholipase C by a G_q-type G protein.

Key Words signal transduction · G_q-type G protein · intracellular calcium signals · inositol phosphates · avian salt gland

Introduction

Many types of cell surface receptors transduce signals to the inside of the cell by activating heterotrimeric GTP-binding proteins (G proteins) located on the inside of the cell membrane. Upon binding of an agonist to the receptor, the α-subunit of the G protein exchanges bound guanosine diphosphate (GDP) against guanosine triphosphate (GTP), dissociates from the β- and γ-complex and activates a particular effector system (Birnbaumer, Abramowitz & Brown, 1990). The specificity of a G protein with respect to an effector system is generally determined by the type of its α-subunit (Simon, Strathmann & Gautam, 1991). Different classes of G protein α-subunits have been defined, among them the G_s class (stimulation of adenylate cyclase) and the G_i class (inhibition of adenylate cyclase). Members of these classes are characterized by their susceptability to ADP-ribosylation by cholera toxin (CTX) or pertussis toxin (PTX), respectively. CTX-ribosylation activates G_s and results in the accumulation of cyclic AMP in cells, while PTX-ribosylation disrupts the G protein-receptor coupling (*cf.* Gill & Woolkalis, 1991; Kopf & Woolkalis, 1991).

Muscarinic acetylcholine receptors (mAChR) are important members of the group of G protein coupled receptors (Sokolovsky, 1989). In many tissues, activation of mAChRs results in the production of inositol phosphates and diacylglycerol and the generation of intracellular calcium signals. While in most tissues these responses are insensitive to pertussis toxin-treatment of cells, there are reports of attenuation of inositol phosphate production and calcium signals due to pretreatment of cells with PTX (Inoue & Kenimer, 1988; Ransom et al., 1991). This may reflect the presence of different subtypes of the muscarinic receptor in these tissues (*cf.* Bonner, 1989). It has been shown that muscarinic activation of cells stably transfected with DNA for different mAChR-subtypes resulted in PTX-sensitive or PTX-insensitive inositol phosphate accumulation depending upon the mAChR-subtype expressed (Ashkenazi et al., 1989). Recently, a new G protein class, G_q, has been described (Strathmann & Simon, 1990) whose members specifically activate phospholipase Cβ and are resistant to toxin treatment—an effect apparently due to a lack of the relevant reactive amino acid residues (Strathmann & Simon, 1990). Although the coupling of m1 muscarinic receptors to phospholipase Cβ by G_q has recently been shown in a reconstitution system (Berstein et al., 1992), structural and functional evidence of such a coupling in natural tissues is rare.

Activation of muscarinic receptors (mAChR) in isolated cells from the avian nasal salt gland results in a pronounced increase in the rate of phosphatidylinositol hydrolysis (Santiago-Calvo et al., 1964; Shuttleworth & Thompson, 1989; Hildebrandt & Shuttleworth, 1991) resulting in the production of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and a subsequent increase in the intracellular calcium concentration (Shuttleworth & Thompson, 1989). The calcium signal comprises two different components, a transient elevation in the free intracellular calcium concentration ($[Ca^{2+}]_i$) caused by the release of calcium from intracellular stores by the action of $Ins(1,4,5)P_3$, and a sustained signal caused by calcium entering the cytosol from the extracellular space (Shuttleworth & Thompson, 1989; Shuttleworth, 1990*a*). It is this sustained elevation in $[Ca^{2+}]_i$ that is required for the secretory response of the salt gland. Shuttleworth (1990*b*) has shown that activation of phosphatidylinositol hydrolysis and intracellular calcium signaling in salt gland cells can be achieved by treating isolated cells with fluoroaluminate indicating the involvement of a heterotrimeric G protein in the transmembrane signal transduction process.

This study was undertaken to characterize the type of G protein involved in the signal transduction process linking the muscarinic receptor in salt gland cells with inositol phosphate and calcium signaling. We performed ^{32}P-ADP-ribosylation assays and Western blot analyses using specific antisera against G protein α-subunits to identify the subtypes of α-subunits present in plasma membranes prepared from salt gland cells. Furthermore, we tested the PTX-sensitivity of inositol phosphate production and calcium signal generation upon muscarinic activation of intact cells.

Materials and Methods

Animals

One-day-old ducklings (*A. platyrhynchos*) were purchased from a commercial hatchery and fed on chick starter crumbs with drinking water *ad libitum*. The experimental animals were between 4 and 10 days old. Suspensions of isolated cells from the salt gland were obtained as described (Shuttleworth & Thompson, 1989; Hildebrandt & Shuttleworth, 1991).

Western Blot Experiments

Membranes from isolated salt gland cells were prepared as described by Brass, Woolkalis and Manning (1988). Briefly, cells were lyzed in hypotonic buffer solution (10 mmol/liter triethanolamine, 5 mmol/liter EDTA, 2 μg/ml aprotinin, pH 6.8), rapidly frozen and thawed and centrifuged at 14,000 $\times$ *g* for 10 min at 4°C. The pellet was washed twice in hypotonic buffer solution and resuspended in 10 mmol/liter triethanolamine containing 2 μg/ml aprotinin, pH 6.8. The final protein concentration was 4 mg/ml. Aliquots of 10 μl were stored at −85°C.

Membranes (40 μg protein) were mixed with 2 μl of 2.5 mmol/liter dithiothreitol and 5 μl sodium dodecyl sulfate (10%) and heated (90°C for 3 min). The cooled samples were mixed with 1 μl of a N-ethylmaleimide solution (100 mmol/liter) and incubated for 15 min at room temperature. After adding 45 μl sample buffer (50 mmol/liter Tris · HCl, pH 6.8, 1% SDS, 0.2% bromophenolblue, 4% β-mercaptoethanol, 40% glycerol) and 4.5 μl 1 mol/liter dithiothreitol, the samples were heated to 90°C for 5 min. Membrane proteins as well as molecular weight standards (normal and prestained, Biorad) were then separated by SDS-PAGE in a Biorad Mini Protean II cell at 160 V on 13.5% gels using a Tris/glycine running buffer (Sambrook, Fritsch & Maniatis, 1989).

Proteins were blotted from the gel onto nitrocellulose filters in a Biorad semi-dry blotting apparatus at a constant current of 350 mA for 30 min using a Tris/glycine transfer buffer (48 mmol/liter Tris, 39 mmol/liter glycine, 20% (v/v) methanol, pH 9.2). The prestained molecular weight markers were quantitatively transferred under these conditions. Proteins on the blots were stained with Ponceau S to locate the positions of the molecular weight markers. Blots were dried and stored at 4°C.

In preparation for the antibody experiments, blots were incubated in Tris-buffer solution (100 mmol/liter Tris, 0.9% NaCl and 0.1% Tween 20, pH 7.5) containing 1.35% (v/v) goat blocking serum (Pierce ImmunoPure ABC Peroxidase Rabbit IgG staining kit) for 24 hr at 4°C. After adding the primary antibody, blots were incubated overnight at 4°C, washed three times in fresh buffer solution without antibody and incubated with the secondary antibody (biotinylated affinity-purified goat anti-rabbit IgG, Pierce). After another washing cycle, blots were incubated for 30 min at room temperature in buffer solution containing staining reagent (Pierce biotinylated horseradish-peroxidase previously coupled to avidin). Blots were developed using ICI Enzygraphic™ Web. The enzymatic reaction between peroxidase and the reagent transferred from the web onto the blots resulted in a dark blue stain.

Antisera

A polyclonal antiserum against a peptide sequence common to different G protein α-subunit classes (8645: Carlson, Brass & Manning, 1989) was a gift from Dr. D.R. Manning and was used at a dilution of 1 : 200. A $G\alpha_s$-specific polyclonal antiserum (RM/1: Simonds et al., 1989) was obtained from NEN Dupont and used at a dilution of 1 : 1,000. An antiserum against a peptide sequence of $G\alpha_{q/11}$ (QL: Shenker et al., 1991) was also obtained from NEN Dupont and used at a dilution of 1 : 1,000. Another $G\alpha_{q/11}$-specific antiserum (Z808), which was raised against a peptide containing a cysteine and the last 12 amino acids of $G\alpha_q$ and $G\alpha_{11}$—CILQLNLKEYNLV—was a gift from Dr. P.C. Sternweis and used at a dilution of 1 : 400. Also a gift from Dr. Sternweis was an antiserum specifically designed for the detection of a peptide sequence in $G\alpha_q$ (WO82: Pang & Sternweis, 1990). It was used at a dilution of 1 : 800. Control immunoblot experiments were performed using preimmune sera from rabbits used to raise the antisera Z808 and WO82. These sera were also provided by Dr. Sternweis and were used at the same dilutions as the respective immune sera.

PTX- and CTX-incubation of Membranes

Cell membranes were prepared as described above and used in aliquots of 10 μl (40 μg membrane protein). The A promoter subunit of pertussis toxin (Calbiochem) was reconstituted in sodium phosphate (10 mmol/liter), sodium chloride (50 mmol/liter) buffer at pH 7.0. Ribosylation was performed for 30 min at 30°C in a total volume of 25 μl containing membranes, 1 mmol/liter EDTA, 5 mmol/liter dithiothreitol, 10 mmol/liter thymidine, 0.2 mg/ml BSA, 0.025% SDS, 10 μCi ^{32}P-NAD (Sp. Act. = 30 Ci/mmol), 10 μg/ml PTX (A Promoter) or vehicle (controls) and 10 mmol/liter HEPES, pH 8.0.

The A subunit of cholera toxin (Calbiochem) was reconstituted in (mmol/liter) 50 Tris, 200 NaCl, 1 EDTA, 3 NaN, pH 7.5. Aliquots of this suspension were warmed to 30°C for 10 min in 0.5% SDS, mmol/liter) 5 dithiothreitol, 130 NaCl, 10 HEPES, pH 7.3 before use. Membranes (40 μg protein) were mixed with 4.5 μl diluted cytosol (supernatant of the first centrifugation step during membrane preparation, protein concentration adjusted to 4 mg/ml) and 4 μl ribosylation buffer, and incubated at 37°C for 10 min. Ribosylation was performed for 30 min at 30°C after adding ^{32}P-NAD and toxin or vehicle (controls) and 1.5 μl ribosylation buffer. The final concentrations in the reaction mixture (total volume 25 μl) were 1.6 mg/ml membrane protein, 110 mmol/liter NaCl, 10 mmol/liter thymidine, 10 μmol/liter GTP, 0.025% SDS, 0.25 mmol/liter dithiothreitol, 0.5 mmol/liter HEPES, 1.5 μg/ml CTX-A and 10 μCi ^{32}P-NAD (Sp. Act. = 30 Ci/mmol).

Samples were mixed with 5 μl 2.5 mmol/liter dithiothreitol and 10 μl 10% SDS-solution and incubated for 5 min at 30°C. N-ethylmaleimide (5 μl of a 100 mmol/liter solution) was added and samples incubated for 10 min at room temperature. Samples were then prepared for SDS-PAGE as described above.

After electrophoresis, proteins were blotted from the gel onto nitrocellulose filters as described above. Blots were stained in Ponceau S, dried and exposed to Kodak X-OMAT AR film between two intensifying screens at −85°C for various time periods between 1–12 hr. The radiolabeled protein bands were cut out of the blots and counted in a scintillation counter to obtain quantitative ^{32}P-ADP-ribosylation data.

Blotting the proteins to nitrocellulose had some advantages over drying and exposing the gel to X-ray film. The ribosylation-reaction mixtures could directly be used for electrophoresis without washing the membranes which could result in loss of material. In addition, free ^{32}P-ADP-ribose, which is produced by endogeneous NADase-activity in the membrane preparation, binds to polyacrylamide and can cause a smear of radioactivity concealing specific signals on autoradiograms of gels (Gill & Woolkalis, 1991). Blotting leaves this smear on the gels, but effectively transfers the ribosylated proteins onto nitrocellulose. Consequently, autoradiography of these blots results in clear signals without excess background. Furthermore, the determination of the molecular weight of ribosylated proteins is more accurate on blots than on dried gels, and blots can still be used for detection of ribosylated proteins with antibodies.

Calcium Measurements in Intact Cells

After isolation from the salt gland, cells were incubated in minimum essential medium (GIBCO 320-2570) for 4 hr at 38°C and gassed with 5% carbon dioxide in air, either in the presence or absence of 500 ng/ml of whole pertussis toxin (Calbiochem). Cells were washed in toxin-free medium and resuspended in HEPES-buffered saline (Shuttleworth & Thompson, 1989) before being loaded with the calcium-sensitive dye indo-1 in the ester form (Grynkiewicz, Poenie & Tsien, 1985). Loading of the cells with dye, recording of the intracellular free calcium concentration ($[Ca^{2+}]_i$) and calibration of the signals were performed as described previously (Shuttleworth & Thompson, 1989).

Inositol 1,4,5-Trisphosphate Generation

Cells were isolated from the salt gland and treated with pertussis toxin as described above. Cells were washed and resuspended in HEPES-buffered saline (Shuttleworth & Thompson, 1989) and kept at 38°C. Both the control and the PTX-treated cells were activated with 0.5 mmol/liter carbachol for a period of 15 sec. We showed previously (Hildebrandt & Shuttleworth, 1991) that the turnover in the phosphatidylinositol pathway and the accumulation of intracellular Ins(1,4,5)P_3 reach maximum levels within this time period. Reactions were terminated by adding an equal volume of ice-cold trichloroacetic acid (1 mol/liter). The samples were processed and assayed for the content of inositol 1,4,5-trisphosphate using a receptor binding assay as described previously (Hildebrandt & Shuttleworth, 1991).

Results

G Protein α-Subunits in Salt Gland Cells

Membrane proteins from duck nasal salt gland cells separated by denaturating gel electrophoresis and transferred to nitrocellulose filters were probed with polyclonal antisera against different types of G protein α-subunits. All antisera tested gave positive results with membrane proteins prepared from salt gland cells as well as from rat brain. This cross-reactivity indicates a high degree of conservation of G protein α-subunit structure between birds and mammals. The signals seen in the immunoblots were due to the presence of proteins containing the G protein-specific amino acid sequences used for immunizing the rabbits, since no bands could be detected at the respective positions on immunoblots incubated with preimmune sera (*data not shown*).

Antiserum 8645, which was raised against a peptide sequence common to $G\alpha_i$ and $G\alpha_s$, recognized a series of at least four proteins in salt gland cell membranes in the molecular weight range of 46 to 40 kD (Fig. 1). A 46 kD protein was labeled, which was also recognized by the $G\alpha_s$-specific RM/1-antiserum and was a substrate for ADP-ribosylation by cholera toxin. In addition, there were signals associated with proteins of relative molecular masses of 44, 41 and 40 kD. Whereas the nature of the 44 kD protein remains unknown, it seems likely that the 41 and the 40 kD proteins belong to the $G\alpha_i$ and/or $G\alpha_o$ family (or an as yet unknown group) since both were substrates for ADP-ribosylation by PTX (Fig. 1), a reaction which was specifically dependent upon

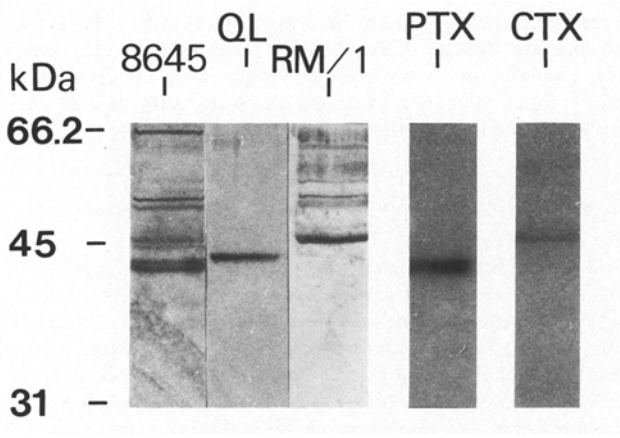

Fig. 1. Immunoreactivity of G proteins in salt gland cell membranes and detection of pertussis- and cholera-toxin substrates.

Membrane proteins (20 μg per lane) from salt gland cells were separated by SDS-PAGE (13.5% gel) and transferred to nitrocellulose filters. Blots were incubated with one of the primary antisera (*8645* is a $G\alpha_{common}$-antiserum, *QL* is specific for $G\alpha_{q/11}$, and *RM/1* is specific for $G\alpha_s$). As described in Materials and Methods, detection was accomplished using a secondary antibody coupled to an avidin/biotin-enhancer system and horseradish peroxidase. The positions of Biorad molecular weight markers are indicated. Membranes prepared from salt gland cells were incubated with *PTX* (pertussis toxin, A promoter) or *CTX* (cholera toxin, A subunit), respectively, in the presence of ^{32}P-NAD. Proteins in the reaction mixture were separated by SDS-PAGE and transferred onto nitrocellulose filters. Radiolabeled proteins were detected by autoradiography.

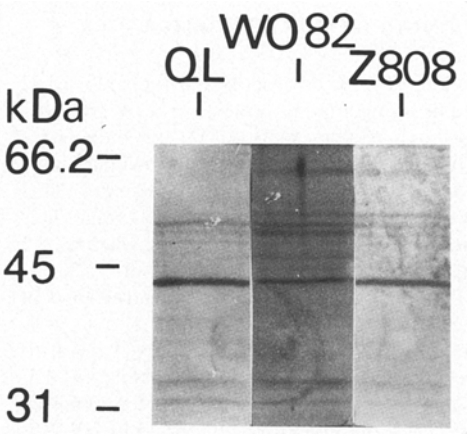

Fig. 2. Immunoreactivity of a 42 kD G protein α-subunit in salt gland cells membranes. Membrane proteins (20 μg per lane) from salt gland cells were separated by SDS-PAGE on a 13.5% gel and transferred onto nitrocellulose filters. Blots were incubated with one of the primary antisera (*QL* and *Z808* antisera are specific for $G\alpha_{q/11}$; *WO82* antiserum is specific for $G\alpha_q$) and processed as described in the legend to Fig. 1. The positions of Biorad molecular weight markers are indicated.

the presence of PTX in the assay (Fig. 3). There was no incorporation of radiolabeled ADP-ribose into the protein band (42 kD) recognized by the QL-antiserum indicating that this G protein α-subunit is not a substrate for ribosylation by either pertussis or cholera toxin, a finding that is consistent with members of the $G\alpha_{q/11}$ group lacking specific ribosylation sites in their amino acid sequences (Strathmann & Simon, 1990; Simon et al., 1991). To investigate the nature of this 42 kD protein in greater detail, another $G\alpha_{q/11}$-specific antiserum (Z808) and a $G\alpha_q$-specific antiserum (WO82) were used in Western blot analyses. Both antisera recognized the 42 kD protein (Fig. 2) identifying it as a member of the $G\alpha_q$-family.

Ins(1,4,5)P_3-Generation and Calcium Signaling

Activation of phospholipase C by G protein-coupled receptors is refractory to PTX-treatment in some cell systems but PTX-sensitive in others (*cf.* Fain, Wallace & Wojcikiewicz, 1988; Sternweis, Smrcka & Gutowski, 1992). Therefore, we tested whether or not inositol phosphate generation and calcium signaling in salt gland cells was affected by pretreatment of cells with pertussis toxin.

Preliminary experiments were performed to determine the extent of PTX-ribosylation of α-subunits under the incubation conditions used. Isolated salt gland cells were preincubated with 500 ng/ml PTX (holotoxin) or vehicle (controls) for 4 hr at 38°C. Membranes were prepared from these cells and subjected to ^{32}P-ADP-ribosylation as described. PTX-preincubation of intact cells resulted in a reduction of specific radioactivity in the blotted material in the molecular weight range of 39 to 42 kD from 1230 ± 150 to 235 ± 56 dpm (means ± SEM, n = 8 preparations) (*cf.* Fig. 4) indicating that more than 80% of the 40/41 kD proteins were ribosylated in intact cells during preincubation with PTX-holotoxin for 4 hr.

Ins(1,4,5)P_3 accumulation in cells pretreated with PTX and stimulated with the muscarinic agonist carbachol (0.5 mmol/liter) for 15 sec was not different from that in stimulated cells pretreated with vehicle alone (Fig. 5). Both values were 8–10 times higher than the Ins(1,4,5)P_3 content in unstimulated cells but similar to values reported for carbachol-activated cells in a previous study (Hildebrandt & Shuttleworth, 1991). This indicates that the G protein α-subunit involved in coupling of the muscarinic receptor to phospholipase C is functionally not affected by the treatment of cells with PTX.

To determine whether the calcium signals in salt gland cells upon muscarinic receptor activation were affected by PTX pretreatment, isolated cells were treated with PTX as described above, loaded with

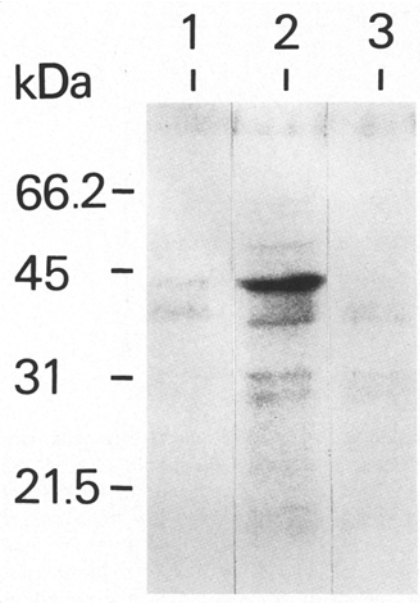

Fig. 3. Specificity of ^{32}P-ADP-ribosylation by PTX. ^{32}P-ADP-ribosylation experiments were performed to show that the transfer of radiolabeled ADP-ribose onto the 40 and 41 kD proteins in salt gland cell membranes was specifically dependent upon PTX as a catalyst. Lane *2* (control): Cell membranes were incubated with ^{32}P-NAD and PTX (A promoter). Lane *1*: Cell membranes were incubated with ^{32}P-NAD, A promoter of PTX and 1 mmol/liter unlabeled NAD. The transfer of ^{32}P-ADP-ribose from ^{32}P-NAD onto the 40 and 41 kD proteins was competitively blocked by increasing concentrations of unlabeled NAD in the incubation mixture. Lane *3*: Cell membranes were incubated with ^{32}P-NAD in the absence of PTX. PTX was required to achieve a detectable incorporation of radiolabeled ADP-ribose into proteins within the incubation period.

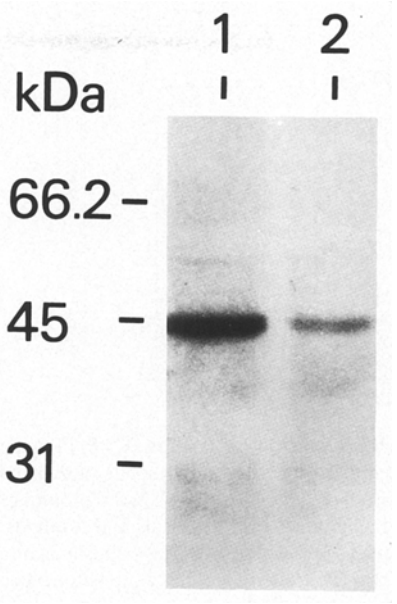

Fig. 4. Extent of ADP-ribosylation of 40 and 41 kD proteins in intact cells during PTX incubation. Salt gland cells were isolated and incubated without (lane *1*) or with 500 ng/ml pertussis toxin (holotoxin) (lane *2*) for 4 hr. Membranes were prepared from both batches of cells as described in Materials and Methods. Cell membranes were then incubated with ^{32}P-NAD and PTX (A promoter). Membrane proteins were separated by SDS-PAGE and transferred onto nitrocellulose filters. Detection of ^{32}P-ADP-ribosylation was achieved by autoradiography. The radioactive portions of the filter were then cut out and radioactivity quantified by scintillation counting. Preincubation of intact cells with PTX for 4 hr reduced the incorporation of radiolabeled ADP-ribose into 40 and 41 kD membrane proteins by more than 80% (*see text*).

the calcium-sensitive dye indo-1, resuspended in saline with a reduced calcium concentration (40 μmol/liter) in the cuvette of the spectrofluorimeter and stimulated with 0.5 mmol/liter carbachol (Fig. 6). The initial peak of the free intracellular calcium concentration ($[Ca^{2+}]_i$) upon addition of carbachol, which is known to be caused by the Ins(1,4,5)P_3-mediated release of calcium from intracellular stores (Shuttleworth & Thompson, 1989), was not different in cells pretreated with PTX or vehicle. The sustained portion of the calcium signal, which is dependent upon calcium entry from the extracellular space, showed a very low plateau due to the low calcium concentration in the medium. Upon readdition of extracellular calcium (1.3 mmol/liter), the already activated entry mechanism allowed calcium ions to enter the cells and raise $[Ca^{2+}]_i$ to a sustained plateau value of 200–300 nmol/liter) (Fig. 6). Neither the rate of calcium entry nor the sustained values of $[Ca^{2+}]_i$ during the subsequent plateau phase were significantly affected by the pretreatment of cells with PTX.

Discussion

Signals initiating salt secretion in the exocrine avian nasal gland are generated by activation of muscarinic acetylcholine receptors (mAChR) in the secretory cells (Shuttleworth & Thompson, 1987). Muscarinic activation of these cells increases the rate of phosphoinositide hydrolysis and generates intracellular calcium signals (Shuttleworth & Thompson, 1989; Hildebrandt & Shuttleworth, 1991). Both of these intracellular signaling systems can be activated by incubation of salt gland cells with fluoroaluminate (Shuttleworth, 1990*b*) indicating the involvement of a heterotrimeric G protein in the transmembrane signal transduction process.

There are different subtypes of G proteins whose effector specificity is largely determined by the nature of their α-subunits (Simon et al., 1991). G protein α-subunits of the G_s class are substrates for ADP-ribosylation by *Vibrio cholerae* toxin (CTX). Members of the G_i and G_o classes are ADP-ribosylated by pertussis toxin (PTX). The activated

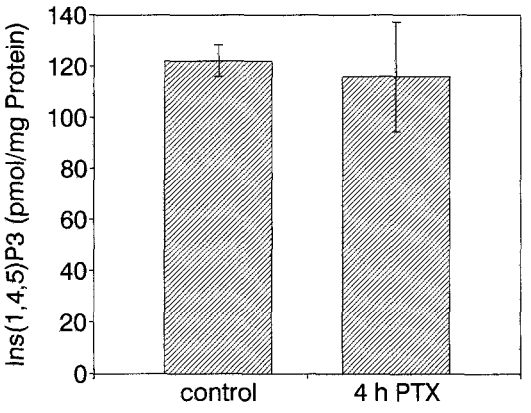

Fig. 5. Receptor-mediated accumulation of $Ins(1,4,5)P_3$ in intact salt gland cells pretreated with pertussis toxin. Isolated salt gland cells were pretreated for 4 hr with 500 ng/ml PTX (holotoxin) or with a vehicle (control) as described in Materials and Methods. Cells were then resuspended in HEPES-buffered saline and stimulated with 0.5 mmol/liter carbachol for 15 sec. Inositol phosphates were extracted from the cytosolic fraction and the content of $Ins(1,4,5)P_3$ analyzed using a receptor binding kit (Amersham). The content of $Ins(1,4,5)P_3$ in activated cells pretreated with PTX was not significantly different from the controls (Student's t-test, $P > 0.1$) (means ± SEM, $n = 5$).

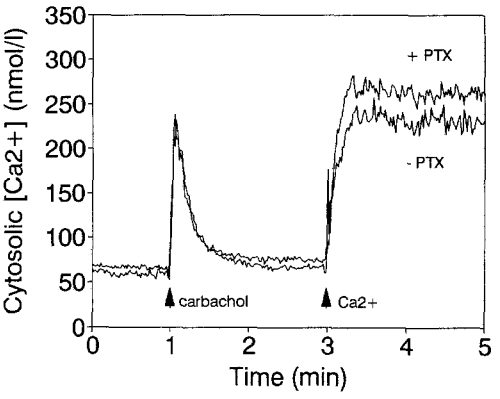

Fig. 6. Intracellular calcium signals in PTX-pretreated cells. Isolated cells were pretreated for 4 hr with 500 ng/ml PTX (+PTX) or vehicle (−PTX). Cells were then loaded with the fluorescent calcium probe indo-1, resuspended in HEPES-buffered saline with a reduced free calcium concentration of 40 μmol/liter and transferred into the cuvette of an LKB LS-5B spectrofluorimeter. At 1 min, cells were stimulated by addition of 0.5 mmol/liter carbachol. At 3 min, the normal extracellular calcium concentration of 1.3 mmol/liter was re-established. No differences were seen in intracellular calcium signals of cells pretreated with PTX (+PTX) or vehicle (−PTX). Representative example curves out of six experiments with similar results are shown.

forms of these toxins can be used to characterize G protein α-subunits in cell membranes (Gill & Woolkalis, 1991; Kopf & Woolkalis, 1991). However, comparison of the amino acid sequences of different α-subunit classes revealed that members of the newly described G_q class lack the amino acid residues for ADP-ribosylation (Strathmann & Simon, 1990). Consequently, signal transduction pathways involving members of this G protein class are not affected by PTX- or CTX-treatment of cells. In this study, we have used polyclonal antisera against mammalian G protein α-subunits and ^{32}P-ADP-ribosylation tests to identify the type of G protein coupling muscarinic receptors to phospholipase C in salt gland cells.

Western blot analyses of electrophoretically separated membrane proteins using an antiserum directed against a common sequence in G protein α-subunits demonstrated the presence of up to six types of G protein α-subunits in avian salt gland cells (Fig. 1), ranging in molecular weight from 40 to 52 kD. Two forms (40 and 41 kD) are substrates for ADP-ribosylation by PTX indicating that they may be related to the G_i or G_o class. A 46 kD protein was ribosylated by CTX and recognized by antiserum RM/1, identifying this protein as a member of the G_s class (Fig. 1). Two antisera (QL and Z808) directed against a peptide sequence in $G_{q/11}$ recognized a protein of 42 kD which was not a substrate for ADP-ribosylation (Figs. 1 and 2). The G_q-specific antiserum WO82 recognized the same protein (Fig. 2), indicating that it is a member of the G_q class of G proteins.

Incubation of isolated salt gland cells in 500 ng/ml pertussis toxin for 4 hr resulted in ADP-ribosylation of more than 80% of the ribosylatable proteins in the molecular weight range of 40 to 42 kD (Fig. 4). Yet, cells treated in such a way and stimulated with carbachol showed no difference in the accumulation rate of $Ins(1,4,5)P_3$ compared with cells pretreated with vehicle alone (Fig. 5). This indicates that the G protein coupling the muscarinic receptor to phosphoinositide hydrolysis in these cells is not sensitive to PTX. A similar insensitivity to PTX has been described for the muscarinic activation of inositol phosphate production in many other cell types (Dunlop & Larkins, 1986; Merritt et al., 1986; Marc, Leiber & Harbon, 1988; Ambudkar et al., 1990) and is consistent with the notion that the G protein mediating mAChR-induced phosphoinositide hydrolysis in salt gland cells is identical with the protein recognized by the G_q-specific antiserum (Fig. 2).

Since the generation of $Ins(1,4,5)P_3$ is refractory to PTX-pretreatment of salt gland cells, we expected to see no difference in the initial $Ins(1,4,5)P_3$-dependent transient calcium signal upon carbachol stimulation of PTX- or vehicle-pretreated cells. This was confirmed by the observation that addition of carbachol increased $[Ca^{2+}]_i$ transiently without any differ-

ence in size or duration of the peak due to PTX-pretreatment of cells (Fig. 6). There are, however, several reports in the literature suggesting that the sustained phase of the calcium signal, which is dependent upon calcium influx, may be affected by PTX-pretreatment in certain cell types (Inoue & Kenimer, 1988; Sjölander et al., 1990; Ransom et al., 1991). Such findings suggest that, in contrast with (or, at least, in addition to) current models in which calcium influx is secondary to either depletion of intracellular calcium stores (Putney, 1990) or production of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (Petersen, 1989; Irvine, 1990), the receptor-activated calcium influx mechanism may be directly controlled by a PTX-sensitive G protein in the plasma membrane. In this context, it is interesting to note the results previously obtained in fluoroaluminate-activated salt gland cells (Shuttleworth, 1990*b*). In these cells, fluoroaluminate ions activated the same calcium entry pathway as muscarinic activation, but the evidence suggested that calcium influx was activated significantly before any detectable release of calcium from intracellular stores. This observation suggested the presence of a more direct link between receptor activation and the initiation of calcium influx, possibly via a G protein which may or may not be PTX-sensitive. In view of this, we compared the rates of increase and sustained plateau levels of $[Ca^{2+}]_i$ in PTX- and vehicle-pretreated cells upon reestablishing the normal extracellular calcium concentration (1.3 mmol/liter) following carbachol stimulation in a low-Ca^{2+} medium. This protocol results in an influx of calcium via the carbachol-activated entry mechanism and a rapid rise in $[Ca^{2+}]_i$. We found that, neither the rate of $[Ca^{2+}]_i$ increase nor the sustained $[Ca^{2+}]_i$ during the plateau phase were significantly affected by PTX-pretreatment of cells (Fig. 6). There are two possible explanations for this observation, and both need further investigation. Either other mechanisms (depending upon store depletion or inositol phosphates) are overriding the G protein effects during the sustained portion of the calcium signal (*cf.* Clementi et al., 1992), or there is a PTX-insensitive G protein controlling the function of the calcium influx pathway.

In summary, muscarinic activation of phosphoinositide hydrolysis and calcium signaling in avian nasal salt gland cells is mediated by a PTX-insensitive G protein, most likely a member of the G_q family. To date, this is one of the few direct demonstrations of probable G_q action in inositol phosphate and calcium signaling in intact cells. Furthermore, this observation implies that the muscarinic receptor in salt gland cells is probably related to the mammalian m1- or m3-mAChR (Bonner, 1989) since only these activated phospholipase C in a PTX-insensitive manner when transfected into CHO cells (Ashkenazi et al., 1989). The existence of multiple mAChR subtypes coupled to different types of G proteins may provide an explanation for some results reported in the literature indicating a PTX-sensitivity of muscarinic receptor-induced inositol phosphate generation and calcium signaling in other cell systems (Inoue & Kenimer, 1988; Takashima & Kenimer, 1989; Lechleiter et al., 1990).

This study was supported by National Institutes of Health Grant GM-40457 (to T.J.S.) and, in part, by a fellowship (Hi 448/1) from the Deutsche Forschungsgemeinschaft (to J.-P.H.). We are grateful to Drs. D.R. Manning and P.C. Sternweis for their kind gifts of antisera.

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Received 20 August 1992; revised 3 December 1992