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Muscarinic acetylcholine receptor trafficking in streptolysin O-permeabilized MDCK cells

Abstract We investigated the validity of streptolysin O (SLO)-permeabilized Madin-Darbin canine kidney (MDCK) cells which express muscarinic acetylcholine receptors (mAChRs) coupled to pertussis toxin-sensitive guanine nucleotide-binding proteins (G proteins) for the study of the molecular machinery that regulates mAChR internalization and recycling. Exposure of SLO-permeabilized cells to carbachol-reduced cell surface receptor number by up to 40% without changing total receptor number. The kinetics and maximal extent of receptor internalization as well as the potency of carbachol to induce receptor internalization were almost identical in SLO-permeabilized and non-permeabilized cells. Using this semi-intact cell system, we studied the effect of various agents affecting components potentially involved in receptor trafficking. Internalization was prevented by treatment of the SLO-permeabilized MDCK cells with (i) the stable ATP analogues, adenosine 5′-O-(3-thiotriphosphate) and adenylylimidodiphosphate, to block ATP-dependent processes, and (ii) heparin to block G protein-coupled receptor kinases. Inclusion of the stable GTP analogue, guanosine 5′-O-(3-thiotriphosphate), increased the rate but not the extent of receptor internalization. None of the membrane-impermeant agents affected receptor internalization in intact MDCK cells. This model system also allowed recycling of internalized receptors back to the plasma membrane. After removal of the agonist, cell surface receptor number in SLO-permeabilized cells returned to control values within 90 min with the same kinetics as seen in intact cells. Inclusion of guanosine 5′-O-(3-thiotriphosphate) shortened the recovery time. These data suggest that both ATP-dependent kinases including G protein-coupled receptor kinases and G proteins participate in receptor internalization and recycling. In summary, the SLO-permeabilized MDCK cell is a feasible model system for the study of mAChR internalization and recycling and allows manipulation of the intracellular milieu with membrane-impermeable macromolecules.

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Key words Muscarinic acetylcholine receptor · Receptor internalization · G proteins · Streptolysin O

Introduction

Similar to many other receptors coupled to guanine nucleotide-binding proteins (G proteins), muscarinic acetylcholine receptors (mAChRs) not only transmit signals across the plasma membrane to intracellular effector systems but also adapt to chronic stimulation. One mechanism of desensitization involves the phosphorylation of the agonist-occupied, active receptor by receptor-specific kinases, and the binding of the inhibitory protein, β-arrestin, to the phosphorylated receptor, thereby preventing the coupling and activation of the G protein (Haga et al. 1994). Within minutes, a second mechanism can come into play in which mAChRs internalize from the plasma membrane into an intracellular storage depot which probably represents endosomes, from which the receptors can recycle back to the plasma membrane. This trafficking represents an important desensitization mechanism as it markedly prolongs the desensitization state of the receptors (Bogatkewitsch et al. 1996). A third but slower adaptive process is receptor down-regulation in which total number of cellular mAChRs decreases. This loss of receptors results predominantly from increased degradation of receptor protein (Lenz et al. 1994).

The molecular machinery that regulates internalization and recycling of G protein-coupled receptors, including mAChRs, has remained largely unknown. The lack of information probably relates to the fact that receptor traffick-

ing cannot as yet be either reconstituted with purified components alone or studied in cell homogenates, but requires an intact intracellular architecture as is found in intact cells. However, in intact cells, the plasma membrane clearly hinders access to the intracellular milieu to explore the regulation of intracellular mAChR trafficking. We therefore investigated the validity of a permeabilized cell system of Madin-Darbin canine kidney (MDCK) cells which endogenously express m3 mAChRs coupled to pertussis toxin-sensitive G proteins (Mohuczy-Dominiak and Garg 1992). The MDCK cells were chosen as model system as they have been extensively used for the study of membrane-traffic regulation by G proteins (Zerial and Stenmark 1993). The MDCK cells were permeabilized with the bacterial toxin streptolysin O (SLO). SLO binds to cholesterol within the plasma membrane and introduces pores sufficiently large to allow free passage of macromolecules into the cytoplasm (Bhakdi et al. 1993). Our results suggest that this model system may facilitate the identification of the factors which regulate mAChR internalization and recycling.

Materials and methods

Materials. [^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB) (43 Ci/mmol) and [*N*-methyl-^{3}H]scopolamine methylchloride ([^{3}H]NMS) (80 Ci/mmol) were obtained from DuPont-New England Nuclear. Pertussis toxin (PTX) was purchased from List Biological Laboratories. Recombinant SLO was produced in *E. coli* as maltose-binding-protein-streptolysin-O fusion protein which has equivalent permeabilizing activity as native SLO (Weller et al. 1996). The activity of SLO was titrated by lysis of human erythrocytes (1 hemolytic activity unit was defined as causing 50% lysis of a 2% human erythrocyte suspension (100 µl) in 40 min at 37°C). All nucleotides were from Boehringer Mannheim. Propidium iodide and heparin (low molecular weight of approx. 3000) were from Sigma. All other reagents were of analytical grade-quality.

Cell culture. MDCK cells obtained from the European Collection of Animal Cell Cultures (Salisbury, UK) were grown in 24-well plates in DME-medium containing 10% fetal calf serum, penicillin G (100 U/ml) and streptomycin (0.1 mg/ml) in 10% CO_2. For PTX treatment, MDCK cells were incubated with 100 ng/ml PTX for 24 h.

MDCK cell permeabilization with SLO. The lyophilized SLO preparation was dissolved at a concentration of 1 mg/ml (1 µg corresponds to 130 hemolytic units) in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (126 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) with 0.1% bovine serum albumin, and activated by reduction with 10 mM dithiothreitol for 10 min at 37°C. The SLO solution was diluted in intracellular transport (ICT) buffer (50 mM HEPES-KOH, pH 7.2, 78 mM KCl, 5 mM D-glucose, 4 mM $MgCl_2$, 8.37 mM $CaCl_2$, 10 mM EGTA, 1 mM dithiothreitol, 3 mM benzamidine, 2 µg/ml trypsin inhibitor and 1 mM leupeptin) (Ullrich et al. 1995). Confluent MDCK cells grown on 24-well plastic dishes were placed on ice and washed once with 500 µl ICT buffer. Permeabilization was accomplished by a two-step procedure (Ahnert-Hilger et al. 1989a). In the first step, cells were incubated with 250 µl ICT buffer containing SLO (0.04, 0.4 or 4.0 µg/ml) at 4°C to allow toxin binding to the cell surface but not pore formation. After 15 min, excess of SLO was aspirated and the cells were washed once with 500 µl ice-cold ICT buffer. Then, 250 µl prewarmed (37°C) ICT buffer was added and cells were permeabilized by incubation for 15 min at 37°C. This procedure ensures that the plasma membrane is selectively permeabilized and intracellular organelles are left intact (Ahnert-Hilger et al. 1989a). Agonist-induced mAChR internalization was then initiated by addition of carbachol at a final concentration of 1 mM or as indicated (final volume 278 µl/well).

Muscarinic receptor-binding assays. After incubation for the indicated periods of time at 37°C, permeabilized cells were washed three times with 500 µl ice-cold ICT buffer and incubated with the membrane-impermeable mAChR antagonist [^{3}H]NMS (300 pM). Atropine (3 µM) was included in the appropriate wells for the determination of nonspecific binding. After incubation for 4 h at 4°C, cells were washed three times with 500 µl ice-cold ICT buffer. Following solubilization in 500 µl Triton X-100 (1%), cells were scraped into scintillation vials, and 3.5 ml of scintillation fluid was added for radioactivity counting. Total binding of [^{3}H]NMS was measured in quadruplicate, nonspecific binding in duplicate. Typically, nonspecific binding of [^{3}H]NMS was 17±7% and 22±14% of total binding in intact cells and cells permeabilized with SLO (0.4 µg/ml), respectively. Specific [^{3}H]NMS binding varied between 290 and 700 cpm/well. Binding of [^{3}H]NMS was less than 10% of total radioactivity added. For equilibrium saturation binding experiments, the concentration of [^{3}H]NMS varied in the range of 9 to 1750 pM. The incubation of intact MDCK cells with carbachol and subsequent binding of [^{3}H]NMS to intact cells was performed in HEPES-buffered saline, pH 7.4 (25 mM HEPES, 116 mM NaCl, 2.7 mM KCl, 0.8 mM $MgSO_4$ 1.8 mM $CaCl_2$, 1.0 mM NaH_2PO_4 and 5.5 mM D-glucose). Total receptor number was determined by binding of the membrane-permeable mAChR radioligand [^{3}H]QNB (500 pM) to cell homogenates. Cells on 60-mm plates were washed three times with phosphate-buffered saline and scraped in 1 ml of 25 mM HEPES-buffered saline, pH 7.4, containing 113 mM NaCl, 6 mM glucose, 3 mM $CaCl_2$, 3 mM KCl, 5 mM $MgCl_2$, 1 mM NaH_2PO_4 and supplemented with 1 mM dithiothreitol, 3 mM benzamidine, 2 µg/ml trypsin inhibitor and 1 mM leupeptin (HBS). The cells were pelleted by centrifugation at 13000 rpm in an Eppendorf-centrifuge for 10 min (4°C) and resuspended in 3 ml HBS by Potter homogenization (5 strokes). Polypropylene tubes received 100 µl [^{3}H]QNB in HBS, 100 µl HBS with or without atropine (final concentration 3 µM) and 800 µl membrane homogenate in HBS. Incubations were carried out for 60 min at 37°C, followed by filtration through Whatman GF/C filters and washing three times with 4 ml ice-cold phosphate-buffered saline. Radioactivity on filters was determined after transferring the filters to scintillation vials to which 3.5 ml of scintillant was added. Specific binding of [^{3}H]QNB was determined in quadruplicate, nonspecific binding in duplicate. Membrane protein concentrations were determined by the Lowry method.

Preparation of rat brain cytosol extract. Brains from 5 male Wistar rats were homogenized in two volumes of ice-cold ICT buffer containing 0.2 M sucrose using a Wheaton glass homogenizer with 10–12 passes of the pestle. The homogenate was centrifuged at 10000 rpm for 15 min in a Sorvall SS-34 rotor and the supernatant was subjected to a second centrifugation at 45000 rpm for 60 min in a Beckman 50Ti rotor. The high-speed cytosol fraction (12 mg protein/ml) was supplemented with an ATP-regenerating system (5 mM creatine phosphate and 5 U/ml creatine phosphokinase), prewarmed to 37°C and added to the cells after toxin binding at 4°C.

Fluorescence microscopy. Permeabilization of MDCK cells was monitored with the plasma membrane-impermeable dye propidium iodide (molecular weight of 668) which renders the nuclei of the cells brightly fluorescent (Bhakdi et al. 1993). SLO (0.04, 0.4 or 4.0 µg/ml) was added to the cells grown on glass coverslips, and incubated for 15 min at 37°C in the presence of propidium iodide (10 µg/ml). The resulting influx of propidium iodide was detected using a Zeiss Axiophot fluorescence microscope.

Lactate dehydrogenase assay. The release of cytosolic lactate dehydrogenase (LDH, MW of 134000) from SLO-permeabilized MDCK cells was determined according to the method of Suttorp et al. (1985). MDCK cells on 35-mm plates were permeabilized with 0.4 µg/ml SLO (total volume 1.0 ml) and incubated for 60 min at 37°C. Then, 900 µl of the medium was transferred to a quartz cuvette containing 100 µl ICT buffer with 2 mM NADH and 15 mM pyruvate, and ex-

tinction of light was measured at 340 nm. Total LDH activity was measured in the supernatant fraction of homogenized (non-treated) MDCK cells following Potter homogenization in ICT buffer and centrifugation at 13000 rpm as described above. The release of cellular LDH activity was defined as the amount of activity measured in the medium of permeabilized cells minus the amount of activity determined in the medium of non-permeabilized cells (i.e., 2.2±0.7% of total LDH activity).

Results

Permeabilization of MDCK cells with SLO

We first tested the efficiency of permeabilization at varying concentrations of SLO as different cell types exhibit widely varying sensitivities to SLO (Ahnert-Hilger et al. 1989a; Bhakdi et al. 1993). Permeabilization was assessed by studying the cellular uptake of the membrane-impermeable nuclear dye, propidium iodide (Fig. 1). At all SLO concentrations used, i.e. 0.04, 0.4 or 4.0 μg/ml, virtually all cells were found to be permeabilized. However, the intensity of staining became stronger with increasing SLO concentrations suggesting that number of pores per cell increases with SLO concentration.

mAChR internalization in SLO-permeabilized MDCK cells

Specific binding of [^{3}H]NMS to non-permeabilized cells and cells permeabilized with 0.4 μg/ml SLO was saturable and of similar magnitude (B_{max} values of 87±7 and 85±10 fmol/mg protein, respectively) (n=5 experiments). When intact MDCK cells were exposed to 1 mM carbachol, cell surface receptor number was reduced, which amounted to 40% after 1 h of agonist treatment (Fig. 2, left panel). In cells permeabilized with SLO (0.4 μg/ml) prior to agonist exposure, addition of carbachol also induced mAChR internalization. In contrast to the effect of carbachol on cell surface receptor number, there was no change in total receptor number as measured by binding of the membrane-permeable radioligand [^{3}H]QNB to cell homogenates of non-permeabilized and SLO-permeabilized cells (Fig. 2, right panel). The kinetics and the maximal extent of receptor internalization were identical in intact and SLO-permeabilized cells (Fig. 3). In contrast to permeabilization with 0.4 μg/ml SLO, the permeabilization of the cells with 4.0 μg/ml SLO markedly reduced mAChR internalization (Fig. 3, insert). This reduction is probably caused by a larger efflux of cytosolic factors from the SLO-permeabilized cells as internalization was fully reconstituted by inclusion of donor cytosol from rat brain. Thus, MDCK cells permeabilized with 0.4 μg/ml SLO but not with 4.0 μg/ml SLO, support mAChR internalization. We next tested other mAChR characteristics in cells permeabilized with 0.4 μg/ml SLO. The equilibrium dissociation constants (K_d) for SLO-permeabilized cells and non-permeabilized cells were also similar (K_d values of 48±16 pM and 51±19 pM, respectively, n=2 independent experiments). Furthermore, there was no difference in the potency of carbachol (half-maximal value of about

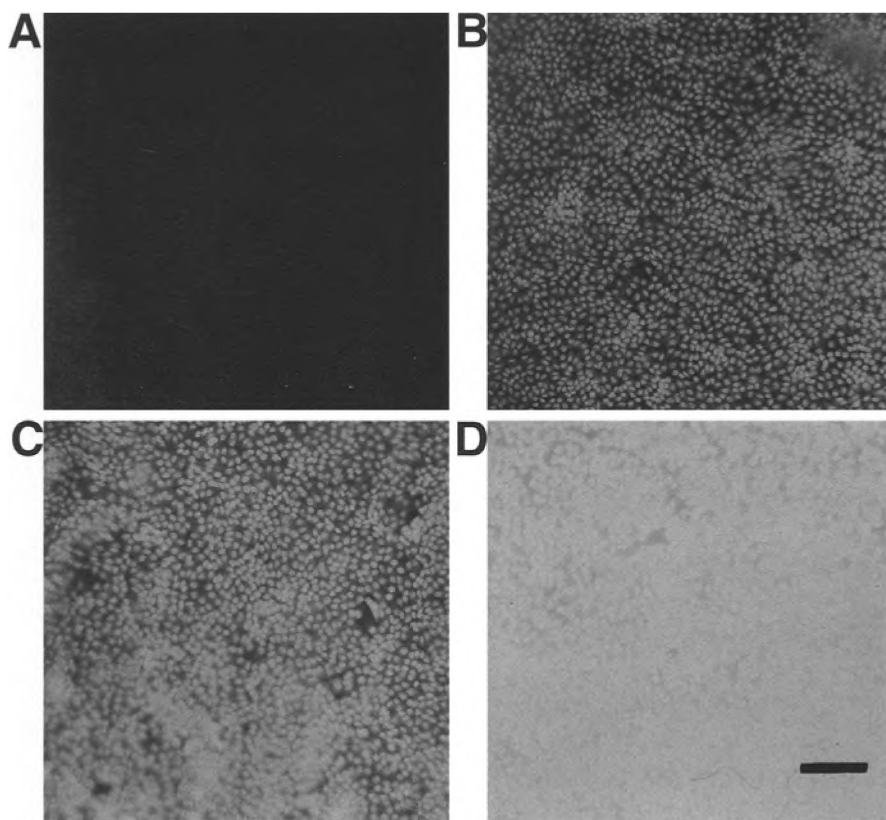

Fig. 1A–D Fluorescence microscopy of SLO-permeabilized MDCK cells. MDCK cells grown on glass cover slips were incubated in HEPES-buffered saline (**A**), or permeabilized in ICT buffer with 0.04 μg/ml SLO (**B**), 0.4 μg/ml SLO (**C**) or 4.0 μg/ml SLO (**D**) for 15 min at 37°C. The incubation media contained the membrane-impermeable nuclear dye propidium iodide (10 μg/ml). Staining of the nuclei was viewed by fluorescence microscopy. The *bar* represents 150 μm

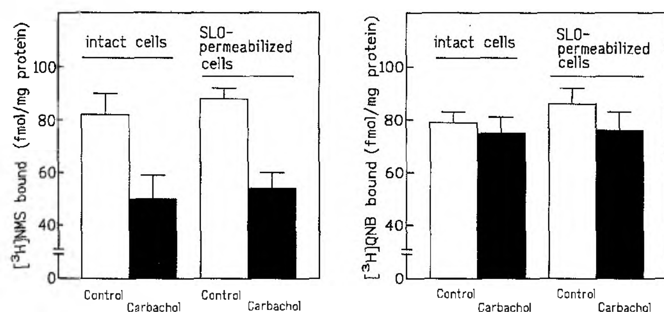

Fig. 2 [^{3}H]NMS and [^{3}H]QNB binding to non-permeabilized and SLO-permeabilized MDCK cells. Effect of carbachol. MDCK cells were incubated in HEPES-buffered saline or permeabilized in ICT buffer with 0.4 µg/ml SLO. Then, cells were exposed to 1 mM carbachol for 60 min, and cell surface receptor number was determined by specific [^{3}H]NMS binding to intact or SLO-permeabilized cells. The total receptor number was measured by specific [^{3}H]QNB binding to homogenates from cells incubated in parallel. Membrane protein concentration was determined by the Lowry method. The data (mean±SEM) are from three separate experiments

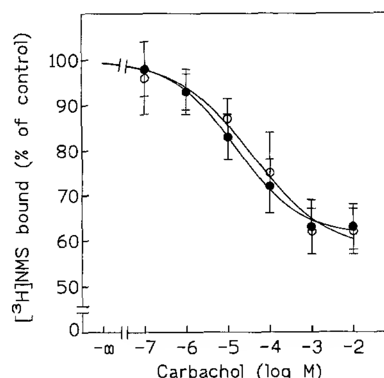

Fig. 4 Dependence of mAChR internalization on carbachol concentration in non-permeabilized and SLO-permeabilized MDCK cells. Intact MDCK cells (●) or MDCK cells permeabilized with 0.4 µg/ml SLO (○) were exposed to the indicated concentrations of carbachol for 1 h, and cell surface receptor number was determined by specific [^{3}H]NMS binding as described in the Methods section. The data represent the means±SEM of 4 experiments

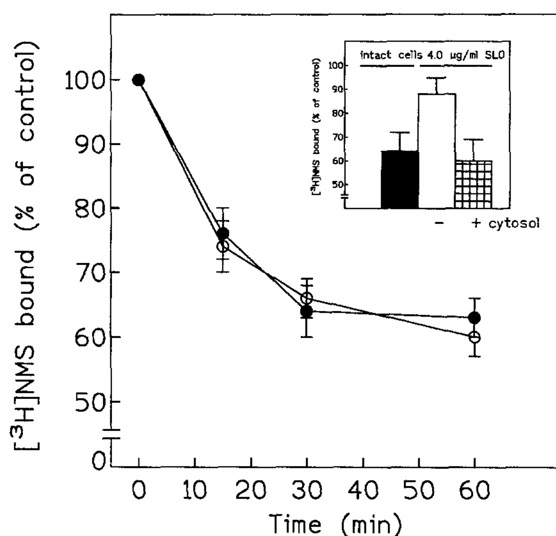

Fig. 3 Kinetics of carbachol-induced mAChR internalization in non-permeabilized and SLO-permeabilized MDCK cells. MDCK cells were incubated in HEPES-buffered saline (○) or permeabilized in ICT buffer with 0.4 µg/ml SLO (●). Then, cells were exposed to 1 mM carbachol for the indicated periods of time, and cell surface receptor numbers were determined by specific [^{3}H]NMS binding as described in the Methods section. The data represent the mean±SEM of 13 experiments. *Insert:* MDCK cells were incubated in HEPES-buffered saline or permeabilized with 4.0 µg/ml SLO in the absence and presence of a high speed supernatant cytosol from rat brain. Inclusion of rat brain cytosol did not affect permeabilization of the cells. Cell surface receptor number was determined after 1 h of incubation with 1 mM carbachol. The data represent the means±SD from 2 sets of independent experiments

25 µM) to induce receptor internalization in SLO-permeabilized cells and non-permeabilized cells (Fig. 4). We therefore choose to use a concentration of 0.4 µg/ml SLO to permeabilize MDCK cells in all subsequent experiments.

Modulation of mAChR internalization in SLO-permeabilized MDCK cells

In the SLO-permeabilized MDCK cells, we tested a variety of exogenously applied, membrane-impermeable compounds which affect intracellular components potentially involved in mAChR internalization. As receptor internalization in intact cells has been shown to require intracellular ATP (Von Zastrow and Kobilka 1994), we investigated whether inhibition of ATP hydrolysis-dependent processes by the stable ATP analogues, adenylylimidodiphosphate (App(NH)p) and adenosine 5′-O-(3-thiotriphosphate) (ATPγS), affects mAChR internalization. Figure 5 (A and B) clearly shows that App(NH)p and ATPγS almost completely abolished mAChR internalization. Second, we tested whether inhibition of G protein-coupled receptor kinases by heparin (Haga et al. 1994; Tobin et al. 1996) blocks mAChR internalization in SLO-permeabilized cells. This compound was tested because it was previously shown that overexpression of a dominant-negative G protein-coupled receptor kinase GRK-2 significantly reduces m2 mAChR internalization in COS-7 cells (Tsuga et al. 1994). As depicted in Fig. 5C, heparin (1 µM) as nonselective inhibitor of mAChR phosphorylation reduced the extent of receptor internalization by about 50%. Third, we examined whether persistent activation of G proteins by the stable GTP analogue, guanosine 5′-O-(3-thiotriphosphate) (GTPγS), influences mAChR internalization, since a number of G proteins have been implicated in regulating different stages of vesicular trafficking (Nuoffer and Balch 1994). As shown in Fig. 6A, inclusion of GTPγS (100 µM) during MDCK cell permeabilization significantly increased the rate but not the extent of mAChR internalization. Unexpectedly, pretreatment of the permeabilized cells with the stable GDP analogue, guanosine 5′-O-(2-thiodiphosphate) (GDPβS) (2 mM), which is frequently used as a competitive inhibitor of GTP, also seemed to increase the rate but not the extent of mAChR internaliza-

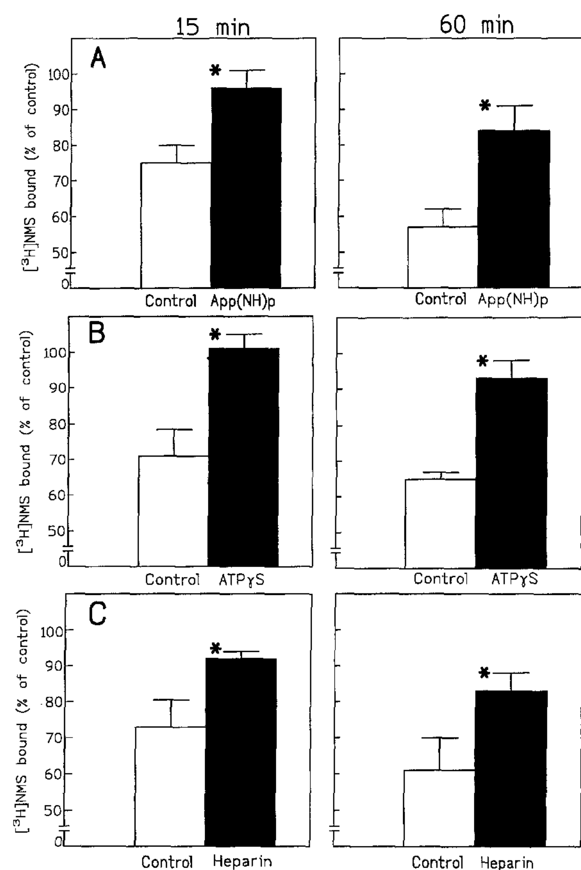

Fig. 5 Effects of App(NH)p, ATPγS and heparin on mAChR internalization in SLO-permeabilized MDCK cells. MDCK cells were permeabilized with 0.4 μg/ml SLO in the presence and absence of 1 mM App(NH)p (**A**), 100 μM ATPγS (**B**) or 1 μM heparin (**C**), and then incubated with or without 1 mM carbachol for either 15 or 60 min. Cell surface receptor number was determined by specific [^{3}H]NMS binding. Inclusion of App(NH)p and ATPγS but not heparin reduced cell surface receptor number in control SLO-permeabilized MDCK cells by about 15%. The data shown are means±SEM of 3 experiments. *, Internalization is significantly different from untreated cells ($P<0.05$, two-tailed paired *t*-test)

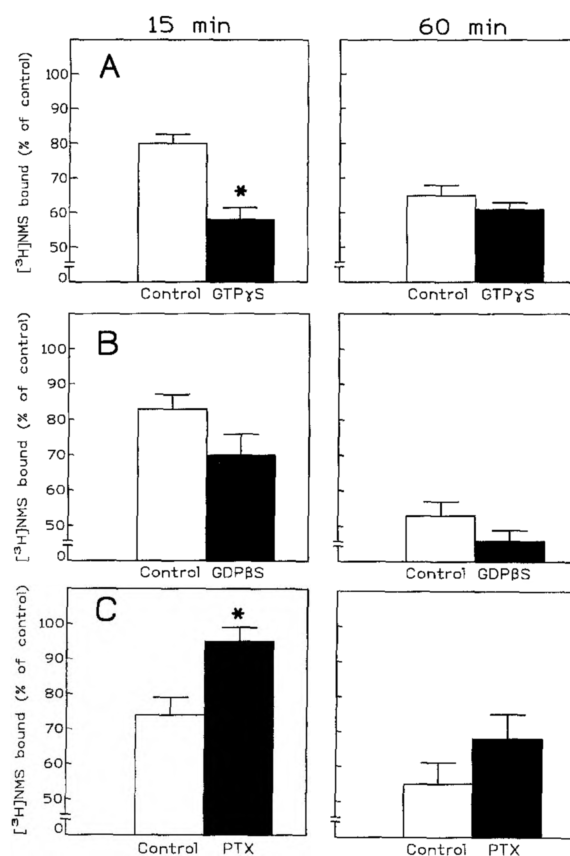

Fig. 6 Effects of GTPγS, GDPβS and PTX treatment on mAChR internalization. (**A** and **B**) MDCK cells were permeabilized with 0.4 μg/ml SLO in the presence and absence of 100 μM GTPγS (**A**) or 2 mM GDPβS (**B**), and then incubated with or without 1 mM carbachol for either 15 or 60 min. Cell surface receptor number was determined by specific [^{3}H]NMS binding. There was no effect of GTPγS or GDPβS on cell surface receptor number in control SLO-permeabilized cells. The data shown are means±SEM of 3 independent experiments. **C** Following pretreatment with 100 ng/ml PTX or vehicle for 24 h, intact MDCK cells were incubated in the presence or absence of 1 mM carbachol for either 15 or 60 min. Cell surface receptor number was not altered by the PTX treatment. The data shown are means±SEM of 5 experiments. *, Internalization is significantly different from untreated cells ($P<0.05$, two-tailed paired *t*-test)

tion (Fig. 6B). None of the compounds described above did change cell surface receptor number or mAChR internalization in intact MDCK cells. Furthermore, at the concentrations indicated above, neither compound altered the extent of permeabilization. The release of cellular LDH from SLO-permeabilized cells (6.2±0.8% of total LDH activity, n=2 experiments) was similar in control cells and cells that were incubated with the compounds during the complete permeabilization and internalization procedure. The G proteins that possibly mediate the GTPγS-induced stimulation of mAChR internalization may be the receptor-coupled heterotrimeric G proteins. PTX treatment of intact cells significantly reduced the rate of mAChR internalization in MDCK cells without changing the maximal extent of receptor internalization (Fig. 6C).

Recycling of internalized mAChRs in SLO-permeabilized MDCK cells

In a previous study, plasma membrane permeabilization by SLO has been used to investigate the final events of exocytosis in PC12 cells (Ahnert-Hilger et al. 1989b). We therefore studied whether SLO-permeabilized MDCK cells also retain their capacity to recycle internalized receptors back to the plasma membrane. For this, SLO-permeabilized cells were first treated with 1 mM carbachol, followed by agonist wash out and further incubation in the absence of agonist. As illustrated in Fig. 7, recovery of internalized mAChRs proceeded rather slowly but was essentially complete after 90 min of incubation in non-permeabilized and SLO-permeabilized MDCK cells. Using GTPγS to activate G proteins, we studied whether G proteins are also involved in mAChR recycling in MDCK

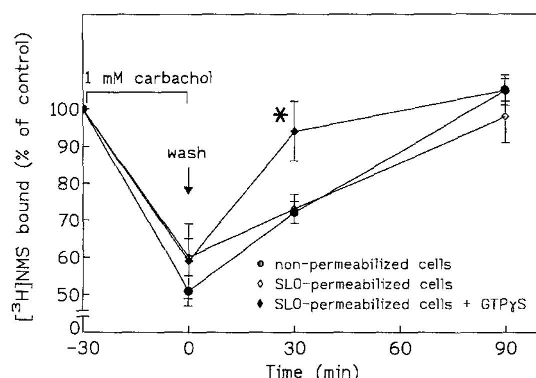

Fig. 7 Kinetics of mAChR recycling in non-permeabilized and SLO-permeabilized MDCK cells. Effect of GTPγS. Intact MDCK cells or MDCK cells permeabilized with 0.4 μg/ml SLO were incubated for 30 min with 1 mM carbachol. Then, cells were washed free of agonist (0 time point) and incubated for 30 or 90 min in the absence or presence of 100 μM GTPγS. GTPγS did not change cell surface receptor number in control SLO-permeabilized cells. The data represent means±SEM of 3–7 experiments. *, [^{3}H]NMS binding is significantly different from that to SLO-permeabilized cells not treated with GTPγS (P<0.05, two-tailed paired t-test)

cells. GTPγS (100 μM) was added to the cells following receptor internalization and removal of the agonist. As shown in Fig. 7, inclusion of GTPγS significantly shortened the recovery time, indicating the participation of G proteins in mAChR recycling.

Discussion

Receptor endocytosis is a process that is mediated by transport vesicles which bud from the plasma membrane into an intracellular storage depot which probably represents endosomes. From here, receptors can recycle back to the plasma membrane. In order to dissect the factors that regulate mAChR trafficking, we have successfully developed a semiintact cell system of SLO-permeabilized MDCK cells that support this trafficking. MDCK cells which were permeabilized with 0.4 μg/ml SLO demonstrated mAChR internalization and recycling characteristics as similar as observed in intact cells. Internalized mAChRs thus remained inaccessible for the positively charged [^{3}H]NMS despite disruption of the plasma membrane. This phenomenon reflects the selective interaction of SLO with cholesterol molecules in the plasma membrane since many intracellular membranes in contrast to the plasma membrane appear to be largely devoid of cholesterol (Montesano et al. 1979). Two other permeabilization methods have been described that involve digitonin to produce pores in the plasma membrane. However, these studies reported that permeabilization with digitonin either reduced the magnitude of agonist-induced loss of cell surface receptors by 40% in SH-SY-5Y cells (Slowiejko et al. 1994) or even precluded a differentiation between internalized and cell surface receptors by binding studies with a hydrophilic radioligand to A431 cells (Lohse et al. 1990). Thus, SLO as used in the two-step permeabilization procedure in the present study appears to be superior over digitonin to permeabilize the plasma membrane without affecting mAChR internalization.

The SLO-permeabilized cells allowed manipulation of the intracellular milieu by a variety of exogenously applied hydrophilic agents. None of these compounds had any effect on mAChR internalization in intact non-permeabilized cells. Inclusion of the poorly hydrolyzable ATP analogues, App(NH)p and ATPγS, to inhibit ATP hydrolysis-dependent intracellular processes almost completely blocked the mAChR internalization. These results are in agreement with previous findings on β-adrenoceptor internalization in intact HEK 293 cells. Preincubation of these cells with sodium azide in the absence of glucose to reduce intracellular ATP levels caused a complete loss of agonist-induced β-adrenoceptor internalization (Von Zastrow and Kobilka 1994). Probably, several intracellular processes that require ATP hydrolysis, including membrane fusion (Whiteheart and Kubalek 1995), have been disrupted by the stable ATP analogues. A phosphorylation event that likely has been inhibited is receptor phosphorylation. Inclusion of heparin, a blocker of mAChR kinases (Haga et al. 1994; Tobin et al. 1996), which in certain cases may block receptor kinases also indirectly by inhibiting receptor-G protein coupling (Gerstin et al. 1992), inhibited mAChR internalization in SLO-permeabilized cells almost as effectively as App(NH)p and ATPγS. Thus, our results lend support to the hypothesis that mAChR internalization involves G protein-coupled receptor kinase activity (Tsuga et al. 1994), a hypothesis which has been challenged very recently (Pals-Rylaarsdam et al. 1995).

Members of the family of G proteins have been implicated as key regulators of many intracellular membrane-trafficking steps (Nuoffer and Balch 1994; Zerial and Stenmark 1993). It is the GTP-bound form that possesses active effector function. Hydrolysis of GTP returns the protein into its basal inactive conformation. We therefore tested the effect of the poorly hydrolyzable GTP analogue GTPγS on mAChR internalization. The rate of mAChR internalization was significantly increased by inclusion of 100 μM GTPγS, indicating that activation of G proteins but not GTP hydrolysis is important for the loss of cell surface receptors. However, inclusion of 2 mM GDPβS to inhibit activation of GTP-binding proteins seemed also to increase mAChR internalization although the effect did not reach the level of statistical significance. GDPβS at high concentrations has been found to activate G proteins in some model systems, mimicking the effects of GTPγS after a lag period of 15–20 min (Otero 1990; Paris and Pouysségur 1990). As GTPβS is able to activate G proteins (Paris and Eckstein 1992), it was postulated by the authors that GDPβS is phosphorylated to GTPβS by cellular enzymes, including the ubiquitous and membrane-associated nucleoside diphosphate kinase. Such transphosphorylation reaction may have occurred in SLO-permeabilized MDCK cells before agonist exposure during the 15 min permeabilization period in which GDPβS was present at a high concentration (2 mM).

The precise mechanism of stimulatory action of GTPγS on mAChR internalization is unclear at present time. The stimulation may have resulted from the GTPγS-induced activation of heterotrimeric G proteins in the plasma membrane. As the data presented here suggest that phosphorylation of mAChRs by receptor kinases in MDCK cells facilitates receptor internalization (Tsuga et al. 1994), the increased presence of free $\beta\gamma$ subunits in the plasma membrane as anchor sites for receptor kinases may augment m3 receptor internalization in MDCK cells. This mechanism of GTPγS action is supported by the observation that PTX pretreatment to inhibit mAChR-G protein coupling significantly attenuated mAChR internalization. A similar PTX effect on mAChR internalization has been observed in CHO cells expressing m4 mAChRs (Van Koppen et al. 1994). While GTPγS-induced release of free $\beta\gamma$ subunits is an attractive model of stimulatory action, GTPγS may have activated other G proteins as well, including dynamin and Rab5 proteins. These monomeric G proteins have been implicated as key regulators of receptor endocytosis (Nuoffer and Balch 1994; Zerial and Stenmark 1993). In light of the assumption that GTPγS will attenuate the endocytosis process at later stages which require GTP hydrolysis, one would expect that inclusion of GTPγS reduces mAChR internalization. However, several experimental findings suggest that this prediction is too simple. Recent studies have shown that inhibition of Rab5 GTPase activity by site-directed mutagenesis of the corresponding cDNA, in fact, stimulates membrane fusion in endocytosis both in vitro and in vivo, indicating that GTP hydrolysis is not required for membrane docking and fusion of clathrin-coated vesicles to the endosomes (Stenmark et al. 1994). In addition, both stimulatory and inhibitory effects of GTPγS on vesicle trafficking have been demonstrated in cell-free assays. These effects appeared to be dependent on cytosol concentration. At low concentration of cytosol, GTPγS stimulates membrane fusion, while this guanine nucleotide inhibits membrane fusion at optimal cytosol concentration (Mayorga et al. 1991; Colombo et al. 1992; Carter et al. 1993). The finding that GTPγS increases the rate of mAChR internalization in SLO-permeabilized cells is therefore not in contradiction with data from the literature.

In addition to supporting receptor internalization, SLO-permeabilized MDCK cells also allow recycling of internalized receptors to the plasma membrane. The recycling kinetics of internalized mAChRs in SLO-permeabilized cells was similar to that found in intact cells. Most interestingly, inclusion of GTPγS significantly increased the rate of mAChR recycling. While after 30 min in control SLO-permeabilized cells about 30% of the internalized receptors had returned to the plasma membrane, in GTPγS-treated, SLO-permeabilized cells almost all (80%) had reappeared on the cell surface. These results suggest that G proteins are also involved in the recycling of mAChRs to the plasma membrane. Our data are in agreement with previous studies reporting stimulatory effects of GTPγS on transport of various types of vesicles to the plasma membrane in permeabilized cells. For example, GTPγS has been shown to stimulate insulin secretion from pancreatic β-cells independent of calcium (Vallar et al. 1987), to promote transferrin receptor recycling in CHO cells under suboptimal cytosol conditions (Martys et al. 1995), to stimulate ACTH secretion from AtT-20 cells (McFerran and Guild 1995) and to augment exocytosis of GLUT4 glucose transporter in rat adipocytes (Shibata et al. 1995). Both monomeric and heterotrimeric G proteins are present on exocytic vesicles and are thought to play an important role in exocytosis (Nuoffer and Balch 1994; Konrad et al. 1995). Further work will be required to understand which G proteins are activated during the mAChR recycling process.

In conclusion, we have developed and characterized a semi-intact cell system of SLO-permeabilized MDCK cells that support mAChR internalization and recycling. This model system allows manipulation of the intracellular milieu through SLO pores and should therefore be helpful in dissecting the regulatory factors involved in mAChR trafficking.

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References

Ahnert-Hilger G, Mach W, Föhr KJ, Gratzl M (1989a) Poration by α-toxin and streptolysin O: an approach to analyze intracellular processes. Methods Cell Biol 31:63–90

Ahnert-Hilger G, Bader M-F, Bhakdi S, Gratzl M (1989b) Introduction of macromolecules into bovine adrenal medullary chromaffin cells and rat pheochromocytoma cells (PC12) by permeabilization with streptolysin O: inhibitory effect of tetanus toxin on catecholamine secretion. J Neurochem 52:1751–1758

Bhakdi S, Weller U, Walev I, Martin E, Jonas D, Palmer M (1993) A guide to the use of pore-forming toxins for controlled permeabilization of cell membranes. Med Microbiol Immunol 182:167–175

Bogatkewitsch GS, Lenz W, Jakobs KH, Van Koppen CJ (1996) Receptor internalization delays muscarinic acetylcholine receptor resensitization at the plasma membrane. Mol Pharmacol (in press)

Carter LL, Redelmeier TE, Woollenweber LA, Schmid SL (1993) Multiple GTP-binding proteins participate in clathrin-coated vesicle-mediated endocytosis. J Cell Biol 120:37–45

Colombo MI, Mayorga LS, Casey PJ, Stahl PD (1992) Evidence of a role for heterotrimeric GTP-binding proteins in endosome fusion. Science 255:1695–1697

Debburman SK, Kunapuli P, Benovic JL, Hosey MM (1995) Agonist-dependent phosphorylation of human muscarinic receptors in *Spodoptera frugiperda* insect cell membranes by G protein-coupled receptor kinases. Mol Pharmacol 47:224–233

Gerstin EH, Luong T, Ehlert FJ (1992) Heparin, dextran and trypan blue allosterically modulate m2 muscarinic receptor binding properties and interfere with receptor-mediated inhibition of adenylate cyclase. J Pharmacol Exp Ther 263:910–917

Haga T, Haga K, Kameyama K (1994) G protein-coupled receptor kinases. J Neurochem 63:400–412

Konrad RJ, Young RA, Record RD, Smith RM, Butkerait P, Manning D, Jarett L, Wolf BA (1995) The heterotrimeric G-protein G_i is localized to the insulin secretory granules of β-cells and is involved in insulin exocytosis. J Biol Chem 270:12869–12876

Lenz W, Petrusch C, Jakobs KH, Van Koppen CJ (1994) Agonist-induced down-regulation of the m4 muscarinic acetylcholine receptor occurs without changes in receptor mRNA steady-state levels. Naunyn-Schmiedeberg's Arch Pharmacol 350:507–513

Lohse MJ, Benovic JL, Caron MG, Lefkowitz RJ (1990) Multiple pathways of rapid β_2-adrenergic receptor desensitization. Delineation with specific inhibitors. J Biol Chem 265:3202–3209

Martys JL, Shevell T, McGraw TE (1995) Studies on transferrin recycling reconstituted in streptolysin O permeabilized Chinese hamster ovary cells. J Biol Chem 270:25976–25984

Mayorga LS, Bertini F, Stahl PD (1991) Fusion of newly formed phagosomes with endosomes in intact cell and in a cell-free system. J Biol Chem 266:6511–6517

McFerran BW, Guild SB (1995) Effects of mastoparan upon the late stages of the ACTH secretory pathway of AtT-20 cells. Br J Pharmacol 115:696–702

Mohuczy-Dominiak D, Garg LC (1992) Muscarinic receptors in MDCK cells are coupled to multiple messenger systems. Am J Physiol 263:C1289–C1294

Montesano R, Perrelet A, Vassalli P, Orci L (1979) Absence of filipin-sterol complexes from large coated pits on the surface of culture cells. Proc Natl Acad Sci USA 76:6391–6395

Nuoffer C, Balch WE (1994) GTPases: multifunctional molecular switches regulating vesicular traffic. Annu Rev Biochem 63:949–990

Otero AS (1990) Transphosphorylation and G protein activation. Biochem Pharmacol 39:1399–1404

Pals-Rylaarsdam R, Xu Y, Witt-Enderby P, Benovic JL, Hosey MM (1995) Desensitization and internalization of the m2 muscarinic acetylcholine receptor are directed by independent mechanisms. J Biol Chem 270:29004–29011

Paris S, Eckstein F (1992) Activation of G proteins by (R_p) and (S_p) diastereomers of guanosine 5′-[β-thio]triphosphate in hamster fibroblasts. Differential stereospecificity of G_i, G_s and G_p. Biochem J 284:327–332

Paris S, Pouysségur J (1990) Guanosine 5′-O-(3-thiotriphosphate) and guanosine 5′-O-(2-thiodiphosphate) activate G proteins and potentiate fibroblast growth factor-induced DNA synthesis in hamster fibroblasts. J Biol Chem 265:11567–11575

Shibata H, Suzuki Y, Omata W, Tanaka S, Kojima I (1995) Dissection of GLUT4 recycling pathway into exocytosis and endocytosis in rat adipocytes. Evidence that GTP-binding proteins are involved in both processes. J Biol Chem 270:11489–11495

Slowiejko DM, Levey AI, Fisher SK (1994) Sequestration of muscarinic cholinergic receptors in permeabilized neuroblastoma cells. J Neurochem 62:1795–1803

Stenmark H, Parton RG, Steele-Mortimer O, Lütcke A, Gruenberg J, Zerial M (1994) Inhibition of Rab5 GTPase activity stimulates membrane fusion in endocytosis. EMBO J 13:1287–1296

Suttorp N, Seeger W, Dewein E, Bhakdi S, Roka L (1985) Staphylococcal α-toxin-induced PGI_2 production in endothelial cells: role of calcium. Am J Physiol 248:C127–C134

Tobin AR, Keys B, Nahorski SR (1996) Identification of a novel receptor kinase that phosphorylates a phospholipase C-linked muscarinic receptor. J Biol Chem 271:3907–3916

Tsuga H, Kameyama K, Haga T, Kurose H, Nagao T (1994) Sequestration of muscarinic acetylcholine receptor m2 subtypes. Facilitation by G protein-coupled receptor kinase (GRK2) and attenuation by a dominant-negative mutant of GRK2. J Biol Chem 269:32522–32527

Ullrich O, Horiuchi H, Alexandrov K, Zerial M (1995) Use of Rab-GDP dissociation inhibitor for solubilization and delivery of Rab proteins to biological membranes in streptolysin O-permeabilized cells. Methods Enzymol 257:243–253

Vallar L, Biden TJ, Wollheim CB (1987) Guanine nucleotides induce Ca^{2+}-independent insulin secretion from permeabilized RINm5F cells. J Biol Chem 262:5049–5056

Van Koppen CJ, Sell A, Lenz W, Jakobs KH (1994) Deletion analysis of the m4 muscarinic acetylcholine receptor. Molecular determinants for activation of but not coupling to the G_i guanine-nucleotide-binding regulatory protein regulate receptor internalization. Eur J Biochem 222:525–531

Van Koppen CJ, Lenz W, Nunes JPL, Zhang C, Schmidt M, Jakobs KH (1995) The role of membrane proximal threonine residues conserved among guanine-nucleotide-binding-protein-coupled receptors in internalization of the m4 muscarinic acetylcholine receptor. Eur J Biochem 234:536–541

Von Zastrow M, Kobilka BK (1994) Antagonist-dependent and -independent steps in the mechanism of adrenergic receptor internalization. J Biol Chem 269:18448–18452

Weller U, Müller L, Messner M, Palmer M, Valeva A, Tranum-Jensen J, Agrawal P, Biermann C, Döbereiner A, Kehoe M, Bhakdi S (1996) Expression of active streptolysin O in *Escherichia coli* as a maltose-binding-protein-streptolysin-O fusion protein. The N-terminal 70 amino acids are not required for hemolytic activity. Eur J Biochem 236:34–39

Whiteheart SW, Kubalek EW (1995) SNAPs and NSF: general members of the fusion apparatus. Trends Cell Biol 5:64–68

Zerial M, Stenmark H (1993) Rab GTPases in vesicular transport. Curr Opinion Cell Biol 5:613–620