Activation of second messenger-dependent protein kinases induces muscarinic acetylcholine receptor desensitization in rat thyroid epithelial cells

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Abstract
Internalization and phosphorylation of G protein-coupled receptors (GPCR) are considered two important regulatory events of receptor signal transduction. In Fischer rat thyroid (FRT) epithelial cells, we have shown that muscarinic acetylcholine receptor (mAChR) stimulation induces intracellular Ca2+ mobilization via Ca2+ store release, capacitative Ca2+ entry and voltage-dependent Ca2+ channels activation. In the present study, the role of mAChR internalization and phosphorylation on receptor signalling pathway was examined by means of intracellular Ca2+ measurement in these cells. Exposure of FRT cells to carbachol (Cch), a mAChR agonist, resulted in a desensitization of receptor-mediated intracellular Ca2+ mobilization and induced the internalization of constitutively expressed mAChR in this cell type. Treatment of FRT cells with hypertonic sucrose, which markedly reduced agonist–receptor complex internalization, or phenylarsine oxide (PAO) diminished the Cch-induced intracellular Ca2+ response. Moreover, pretreatment of cells with phorbol-12-myristate-13-acetate (PMA), an activator of protein kinase C (PKC), completely abolished Cch-evoked Ca2+ mobilization, whereas it was significantly increased by the preincubation of cells with GF109203X, a selective inhibitor of PKC. We also found a marked decrease on Cch-stimulated Ca2+ mobilization in pretreated FRT cells with forskolin, an activator of protein kinase A (PKA), but the preincubation of cells with genistein, an inhibitor of protein tyrosine kinases, had no effect on Ca2+ mobilization induced by Cch. These findings seem to indicate that mAChR in FRT cells exhibit a desensitization, which may be mediated, at least in part, through activation of second messenger-dependent protein kinases and that receptor internalization could be necessary for signalling.
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Keywords: Muscarinic acetylcholine receptor desensitization; Receptor signalling pathway; Agonist-receptor complex

1. Introduction
Muscarinic acetylcholine receptors (mAChR) are members of the seven transmembrane domain receptor family, which couple to heterotrimeric G-proteins. The five different mAChR subtypes identified, termed m1, m2, m3, m4 and m5, can be divided into two functional groups on the basis of their cellular distribution and signal transduction pathway coupling. The mAChR subtypes m3 and m5 are normally linked to Gq and adenylate cyclase inhibition, whereas the m1, m2 and m4 mAChR subtypes are typically coupled to activation of the Gsα11-phosphatidylinositol pathway (Ashkenazi et al., 1989; Pasquali et al., 1992).

In primary cultures of dog thyroid cells and in non-differentiated Fischer rat thyroid (FRT) epithelial cells, mAChR stimulation induces a transient increase in the intracellular free Ca2+ mobilization via phospholipase C (PLC) activation, and Ca2+ store depletion, capacitative Ca2+ entry and voltage-dependent Ca2+ channel activation (Rani et al., 1989; Montiel et al., 2001). In addition, in dog thyroid cells, activation of mAChR reduces the adenylate cyclase activity by a process that does not involve Ca2+ mobilization or activation of phosphodiesterase (Pasquali et al., 1992).

It is well-documented that adenylate cyclase/AMP and Ca2+-phosphatidylinositol are the main intracellular signalling pathways involved in the regulation of thyroid function by activation of mAChR. In dog thyroid cells, the membrane iodine transport to the follicular lumen, the H2O2 generation and the protein iodination are regulated...
by both signalling transduction pathways through activation of mAChR (Raspe et al., 1991; Raspe and Dumont, 1994, 1995). Moreover, in rat thyroid epithelial cells we have reported that mAChR stimulation induces a phosphorylation of mitogen-activated protein kinase (MAPK) that was dependent on intracellular Ca\(^{2+}\) mobilization (Jiménez et al., 2002).

Many GPCR have been shown to desensitize during the course of exposure to agonist and several mechanisms have been proposed to regulate the length and strength of GPCR signals. Since desensitization of a particular GPCR is regulated by a number of factors that include receptor structure and cellular environment, the mechanism of mAChR desensitization was studied in FRT epithelial cells. Our results indicate that the desensitization of constitutively expressed mAChR in FRT epithelial cells is mediated through activation of second messenger-dependent protein kinases and that mAChR internalization could be necessary for signalling.

2. Material and methods

2.1. Materials

Calf serum was from Gibco (Paisley, UK). Bisindolylmaleimide I (GF109203X), phorbol-12-myristate-13-acetate (PMA), genistein, okadaic acid, thapsigargin and fura maleimide I (GF109203X), phorbol-12-myristate-13-acetate (PMA), genistein, okadaic acid, thapsigargin and fura 2-deoxy-m-glucose, antibiotics and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell culture

FRT epithelial cells were routinely grown in Coon’s modified Ham’s F12 medium, supplemented with 5% calf serum, containing amphotericin B (0.25 \(\mu\)g/ml) and gentamicin (50 \(\mu\)g/ml). The cells were maintained in a water-saturated atmosphere of 5% CO\(_2\) and 95% air at 37 °C. Before the experiments, cells were harvested with 0.05% trypsin-EDTA solution and plated onto plastic 150-mm culture. The cells were grown for 7–8 days, with two or three changes of the cultured medium. Fresh medium was always added 24 h before an experiment.

2.3. Measurement of mAChR internalization

Confluent FRT cells were serum-starved for 1 h in Krebs–Henseleit buffer and then stimulated with 100 \(\mu\)M Cch for appropriated times at 37 °C. Reactions were terminated by aspiration and the cells were washed three times with ice-cold Krebs–Henseleit buffer, and incubated with 0.2 \(\mu\)Ci of [\(^{3}H\)]NMS overnight at 4 °C. Cells were washed twice in ice-cold Krebs–Henseleit buffer, solubilized by the addition of 1 ml ice-cold, 10 mM Tris, 500 mM NaCl, 10 mM EDTA, 1% Nonidet P-40, 0.1% SDS and 0.5% sodium deoxycholate, and receptor number was determined by liquid scintillation counting. Non-specific binding was determined in the presence of 10 \(\mu\)M atropine.

2.4. Determination of [Ca\(^{2+}\)]

The medium was aspirated and the cells were then harvested with a 0.05% trypsin-EDTA solution. After washing the cells three times by pelleting (50 g for 3 min), the cells were incubated with 5 \(\mu\)M fura 2-acetoxyethyl ester for 45 min at 37 °C with continuous shaking (100 cycles/min).

Following the loading period, the cells were washed twice with a modified Krebs–Ringer buffer, in which the bicarbonate was replaced by 20 mM HEPES, pH 7.4, incubated again for at least 10 min at room temperature to facilitate hydrolysis of the esterified probe, and washed once again. The cells were resuspended in the same buffer containing 0.1% BSA and 2 ml of the cell suspension was added to a fluorescence cuvette kept at 37 °C, and stirred throughout the experiment. The fluorescence intensity was measured with a Hitachi F2000 fluorimeter. The excitation wavelengths were 340 and 380 nm, and emission was measured at 510 nm. The maximal fluorescence was determined at the end of the assay by adding 20 \(\mu\)l 10% SDS and the minimal fluorescence by adding 15 \(\mu\)l 0.5 M EGTA solution, pH 9.0. The cytoplasmic Ca\(^{2+}\) concentration at the time \(t\) was calculated using the software of the spectrofluorometer and assuming a dissociation constant for the fura 2:Ca\(^{2+}\) complex of 224 nM, according to the Grynkiewicz equation (Grynkiewicz et al., 1985):

\[
[Ca^{2+}] = \frac{224(F_0 - F_{\text{min}})F_{\text{min}}}{(F_{\text{max}} - F_{\text{min}})F_0 - F_{\text{min}}}
\]

where \(F\) denotes the time course of the fluorescence at 510 nm after dual excitation at 340/380 nm and \(F_{\text{380}}\) was the fluorescence at 510 nm after excitation at 380 nm.

3. Results

3.1. Endocytosis of mAChR in FRT cells

Initial experiments set out to investigate mAChR internalization from the surface of FRT epithelial cells as function of time. As shown in Fig. 1, treatment of cells with 100 \(\mu\)M Cch for 1 h reduced numbers of cell surface mAChR by approximately 15% and reached an apparent plateau by 120 min (data not shown). Since internaliza-
Fig. 1. Internalization of mAChR in FRT epithelial cells. Cells were pretreated with or without 0.45 M sucrose and then incubated with 100 μM Cch for different periods. mAChR remaining on the cell surface were determined as described in Section 2.

Fig. 2. Desensitization of mAChR in FRT epithelial cells. Representative trace showing change in free-intracellular Ca^{2+} mobilization in response to 100 μM Cch and desensitization towards subsequent Cch stimulation. A calcium response was triggered following 100 μM ATP.

Fig. 3. Effect of PAO on Cch-induced free-intracellular Ca^{2+} mobilization in FRT epithelial cells. Fura 2-loaded cells were preincubated in the absence (trace 1) or presence of 5 μM (trace 2) or 20 μM (trace 3) PAO for 5 min, and then 100 μM Cch was added. Results shown are representative of at least three similar experiments.

3.2. Effect of Cch on mAChR desensitization

It is well-recognized that exposure of cells to agonists often leads to a rapid desensitization of cell surface GPCR. As shown in Fig. 2, the stimulation of FRT epithelial cells with 100 μM Cch provoked a transient increase in intracellular calcium concentration ([Ca^{2+}]_i), suggesting the existence of other routes for agonist–receptor complex internalization.

3.3. Effect of mAChR internalization on Cch-evoked Ca^{2+} mobilization

The event of GPCR deactivation has been associated with various processes, including receptor internalization. To study the function of mAChR internalization on Cch-evoked Ca^{2+} mobilization we used two independent methods: treatment of cells with PAO, a trivalent arsenic reagent that inhibits internalization of several cell surface G-protein-coupled receptors (Sorensen et al., 1998) and cell incubation with hypertonic sucrose. As shown in Fig. 3, when FRT epithelial cells were pretreated with two different concentrations of PAO (5 or 20 μM) for 5 min, the sustained phase of Cch-induced Ca^{2+} mobilization was quickly achieved, while the first phase of Ca^{2+} mobilization markedly diminished with the higher concentration of PAO. On the other hand, preincubation of FRT epithelial cells with 0.45 M sucrose for 1 min, almost completely blocked Cch-induced Ca^{2+} mobilization, but did not affect basal [Ca^{2+}]_i or mobilization of Ca^{2+} induced by ATP (Fig. 4). Moreover, exposure of the cells to hyperosmolar sucrose clearly reduced thapsigargin-induced Ca^{2+} capacitative entry (Fig. 5).

3.4. Effect of PKC, PKA and protein tyrosine kinases on Cch-evoked Ca^{2+} mobilization

Several families of protein kinases, as the second messenger-dependent protein kinases, exhibit capacity to phosphorylate GPCR and agonist-induced phosphorylation
Fig. 4. Effect of hyperosmolar sucrose on ATP- and Cch-induced free-intracellular Ca\(^{2+}\) mobilization in FRT epithelial cells. Fura 2-loaded cells were exposed in the presence of 0.45 M sucrose for 1 min, and then 100\(\mu\)M ATP (trace 1) or 100\(\mu\)M Cch (trace 2) was added. Results shown are representative of at least three similar experiments.

has been shown to facilitate desensitization of GPCR. In order to explore whether PKC, a second messenger-dependent protein kinase, was involved in the mAChR desensitization, FRT epithelial cells were pretreated with PMA or GF109203X, an activator and selective inhibitor of PKC respectively. Preincubation of FRT cells with 1 \(\mu\)M PMA for 5 min completely abolished Cch-evoked Ca\(^{2+}\) mobilization, whereas it was significantly increased in pretreated cells with 5 \(\mu\)M GF109203X for 30 min. However, PMA did not significantly change the ATP-evoked Ca\(^{2+}\) response (Fig. 6).

We have previously described that in pretreated FRT cells with 1 mM IBMX, a phosphodiesterase inhibitor, the addition of 5 \(\mu\)M forskolin, an activator of PKA, rapidly produced an increment in the intracellular cAMP concentration (Montiel et al., 2001). Under these conditions, Cch-evoked Ca\(^{2+}\) mobilization in these cells was also markedly diminished (Fig. 7).

Since tyrosine residues of GPCR have been described to be responsible for agonist binding and receptor function (Wess et al., 1991; Bluml et al., 1994) and tyrosine represents an essential structural element for receptor phosphorylation

Fig. 5. Effect of hyperosmolar sucrose on thapsigargin-induced free-intracellular Ca\(^{2+}\) mobilization in FRT epithelial cells. Fura 2-loaded cells were exposed in the absence (trace 1) or presence of 0.45 M sucrose for 1 min (trace 2), and then 1 \(\mu\)M thapsigargin (TG) was added. Results shown are representative of at least three similar experiments.

Fig. 6. Effect of PKC on Cch-induced free-intracellular Ca\(^{2+}\) mobilization in FRT epithelial cells. Fura 2-loaded cells were preincubated in the absence (trace 1) or presence of 5 \(\mu\)M GF109203X for 30 min (trace 2) and then stimulated with 100\(\mu\)M Cch, or treated with 1 \(\mu\)M PMA for 5 min before addition of 100 \(\mu\)M Cch (trace 3) or 100 \(\mu\)M ATP (trace 4). Results shown are representative of at least three similar experiments.

Fig. 7. Effect of PKA on Cch-induced free-intracellular Ca\(^{2+}\) mobilization in FRT epithelial cells. Fura 2-loaded cells were preincubated in the absence (trace 1) or presence of 1 mM IBMX for 20 min (trace 2), and then stimulated with 100\(\mu\)M Cch or 5 \(\mu\)M forskolin + 100 \(\mu\)M Cch (trace 3). Results shown are representative of at least three similar experiments.

Fig. 8. Effect of PTK on Cch-induced free-intracellular Ca\(^{2+}\) mobilization in FRT epithelial cells. Fura 2-loaded cells were preincubated in the absence (trace 1) or presence of 50 \(\mu\)M genistein for 30 min (trace 2) and then 100 \(\mu\)M Cch was added. Results shown are representative of at least three similar experiments.
response of Cch-evoked Ca\textsuperscript{2+} receptor deactivation, since both agents diminished the initial of agonist-mAChR complex could be important for the re-
ishment receptor internalization through clathrin-coated vesicles.
and hypertonic sucrose, two well known agents, which abol-
ishment mechanisms.

To investigate the role of PKC, experiments were designed to test whether activation or inhibition of PKC was capable to modulate mAChR signalling in FRT epithelial cells. We observed that preincubation of cells with PMA, an activator of PKC, was able to completely abolish Cch-evoked Ca\textsuperscript{2+} mobilization, whereas it was increased in cells pretreated with GF109203X, a selective PKC inhibitor. These find-
ings are consistent with the hypothesis that agonist-induced mAChR homologous desensitization is PKC dependent in FRT epithelial cells.

In FRT epithelial cells, we have previously demonstrated that stimulation of mAChR did not couple to G-proteins re-

4. Discussion
In cultures of different cell types, which constitutively express mAChR, such as NG108 neuroblastoma cell line, ventricular myocytes or smooth muscle cells (Holroyd et al., 1999; Sakai et al., 1999; Mia et al., 1997) and in cell lines stably expressing a particular mAChR receptor subtype (Schlador and Nathanson, 1997; Mou and Jackson, 2001) agonist stimulation causes a rapid desensitization of this member of GPCR family. Nevertheless, the molecular mechanisms that lead to agonist-dependent desensitization are not completely defined. Following agonist activation, mAChR subtypes can be phosphorylated by various protein kinases or internalized through multiple pathways, in a highly regulated manner depending on receptor subtype and cell type (van Koppen and Kaiser, 2003).

In the present study, we demonstrated that following exposure to agonist a desensitization process occurs of constitu-
tively expressed m\textsubscript{3} mAChR subtype in FRT epithelial cells. After initial exposure to Cch, which induced a rapid and transient increase in intracellular Ca\textsuperscript{2+} mobilization (Montiel et al., 2001), a second dose of agonist was ineffective to evoke a further increase of this intracellular messenger.

It is well established that many GPCR undergo internal-
ization or endocytosis after the binding of agonists, but the consequences of GPCR internalization process on receptor signalling is uncertain. For some GPCR, receptor endocyto-
sis has been described not to be necessary for generation of second messengers (Yu and Hinkle, 1998), while for others the internalization was required for the production of intra-
cellular signals (Hunyady et al., 1991; Kapas et al., 1994).
To determine whether mAChR-mediated endocytosis was related to the process of receptor deactivation, we inhibited mAChR internalization by preincubation of cells with PAO and hypertonic sucrose, two well known agents, which abol-
ishment receptor internalization through clathrin-coated vesicles. The results of the present study suggest that internalization of agonist-mAChR complex could be important for the re-
ceptor deactivation, since both agents diminished the initial response of Cch-evoked Ca\textsuperscript{2+} mobilization. Moreover, hy-
perosmolar sucrose, which has been found to be ineffective to block both IP\textsubscript{3} formation and Ca\textsuperscript{2+} response induced by TRH (Yu and Hinkle, 1998) and ATP-induced Ca\textsuperscript{2+} mobilization, markedly reduced the capacitative Ca\textsuperscript{2+} entry evoked by thapsigargin in FRT epithelial cells (Montiel et al., 2001). Since multiple mechanisms for capacitative Ca\textsuperscript{2+} entry has been described in several cell types, we can only move in the field of hypothesis. One such mechanism is conformational coupling, in which IP\textsubscript{3} receptors may sense the fall in Ca\textsuperscript{2+} levels through Ca\textsuperscript{2+}-binding sites on their luminal domains, and convey this conformational information directly by physically interacting with Ca\textsuperscript{2+} channels in the plasma membrane (Putney et al., 2001). According to this mechanism, hypertonic sucrose could interfere with the coupling between IP\textsubscript{3} receptors and plasma membrane Ca\textsuperscript{2+} channels, although this possibility requires further studies to be confirmed.

Previous data have demonstrated that phosphorylation of GPCR also induces receptor desensitization by reducing the ability to interact with biochemical effectors and/or reduc-
ting the affinity for binding of agonists. This functional mod-
ulation has been shown to be associated with phosphory-
lation of serine/threonine residues of the receptor proteins. In particular, cloning of mAChR has shown the presence of serine/threonine-rich regions susceptible for phosphory-
lation in the third intracellular loop of the protein that may participate in the regulation of receptor desensitization in response to agonist (Lee and Fraser, 1993).

At least two families of protein kinases, the GPCR ki-
nases (GRK) and the second messenger-dependent protein kinases, PKA and PKC, phosphorylate serine/threonine residues within the intracellular domains GPCR, and they appear to play an important role in initiating the desensiti-
ization for most receptors (Ferguson et al., 1996; Bunemann and Hosey, 1999; Dale et al., 2002). Although stimulation of m\textsubscript{3} mAChR subtype induces PKC activation, the role of this serine/threonine kinase in receptor desensitization is unclear. In developing oligodendrocytes, PKC activation desensitizes mAChR, although Cch-stimulated desensitiza-
tion has been found to be independent of PKC activation (Molina-Holgado et al., 2003) and in human SH-SY5Y neuroblastoma cells, the PKC inhibitor Ro-31-8220 was ineffective in preventing the agonist-mediated desensitization (Bundey and Nahorski, 2001). Nevertheless, PKC activa-
tion was found to alter the functionality of mAChR and to contribute to the initial period of desensitization, reducing Cch-stimulated inositol phosphates generation in rat my-
ometrium (Lajat et al., 1998). The differences between these observations may be the results of species differences in the nature of target cell specific signal transduction and/or desensitization mechanisms.

In FRT epithelial cells, we have previously demonstrated that stimulation of mAChR did not couple to G-proteins re-

(Paxton et al., 1994), we examined whether agonist-induced protein-tyrosine kinases (PTK) activation was related with mAChR desensitization. As shown in Fig. 8, pretreatment of cells with 50\mu M genistein, a non-specific PTK inhibitor, for 30 min did not significantly alter the Cch-evoked Ca\textsuperscript{2+} mobilization, indicating that PTK activation was not responsi-
ble for mAChR desensitization in FRT cells.
lated with the regulation of intracellular cAMP levels, but forskolin, a reversible activator of adenylate cyclase, caused a pronounced increase in the cytosolic cAMP concentration (Montiel et al., 2000). As various studies have shown that GPCR may be desensitized by activation of other receptors (heterologous desensitization), being this particularly true for several members of the mAChR family (Shimada et al., 1991; Lee and Fraser, 1993), we then investigated whether cAMP-dependent kinase activation could be implicated in the mAChR desensitization. Upon activation with forskolin, Cch-evoked Ca\(^{2+}\) response in the cells was noticeably diminished. These results agree with those showing heterolo-
gous desensitization of the m1 mAChR in CHO cells upon activation of the adenylylate cyclase system (Lee and Fraser, 1993) and suggest that in FRT epithelial cells mAChR de-
sensitization may also be due to receptor phosphorylation by cAMP-dependent kinase PKA.

In the angiotensin II A\(_1\) receptor, which also couples to the G\(_{q/11}\) family of G-proteins, a highly conserved tyrosine residue located in the fifth transmembrane domain of nu-
merous GPCR has been found to be important in receptor activation and signal transduction (Hunyady et al., 1995).

Structure–function relationship studies of the m3 mAChR have also identified a tyrosine residue, which is critically involved in acetylcholine binding and in receptor-induced G\(_q\) activation (Wess et al., 1991; Wess et al., 1995). Since various GPCR have been described to be direct substrates for PTK and tyrosine residue phosphorylation may represent an essential structural element for receptor function (Paxton et al., 1994; Pak et al., 1999; Martin et al., 2002), we tested the hypothesis that tyrosine residues phosphorylation were necessary for mAChR desensitization in FRT epithelial cells. The results of our studies using genistein, a non-specific PTK inhibitor, seem to indicate that tyrosine residue phos-
phorylation may not be involved in mAChR desensitization.

In summary, our findings suggest that mAChR exhibit a desensitization in FRT epithelial cells, which could be me-
diated, at least in part, through receptor phosphorylation via activation of second messenger-dependent protein kinases, PKA and PKC, and receptor internalization.

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