The C-terminal Tail of the M₃-muscarinic Receptor Possesses Anti-apoptotic Properties*

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This study investigates the mechanisms by which the muscarinic receptor gene family can protect against apoptosis. Chinese hamster ovary cells transfected with human muscarinic receptor subtypes underwent apoptotic cell death following treatment with the DNA-damaging agent etoposide. Apoptosis was significantly reduced following muscarinic receptor stimulation of cells that were transfected with receptor subtypes that couple to the Gq/11/phospholipase C pathway, namely M₁, M₃, and M₅. No protection was detected in cells transfected with the Gt-coupled M₈ and M₉ receptors. Further analysis of the Gq/11-coupled M₃ receptor revealed that truncation of the carboxyl-tail (Δ565-M₃ mutant) removed the ability of the receptor to protect against etoposide-induced cell death. This mutation did not affect the ability of the receptor to signal through the phospholipase C pathway. Furthermore, activation of the Δ565-M₃ receptor resulted in robust activation of the extracellular-regulated kinase (ERK) and c-Jun kinase (JNK). The Δ565-M₃ receptor mutant also underwent agonist-driven phosphorylation in a similar manner to the wild-type receptor indicating that the anti-apoptotic effect of the M₃ receptor is independent of receptor phosphorylation. Consistent with this was the fact that two M₃-muscarinic receptor mutants deficient in agonist-induced receptor phosphorylation were capable of producing a full anti-apoptotic response. We conclude that the anti-apoptotic response of the muscarinic receptor family was confined to the Gq/11-coupled members of this family. The direct involvement of Gq/11/phospholipase C signaling and the ERK-1/2 and JNK pathways together with receptor phosphorylation in the anti-apoptotic response were eliminated. Mutation of a poly-basic region within the short C-terminal tail of the M₃-muscarinic receptor inhibited the ability of the receptor to induce an anti-apoptotic response. We conclude that the conserved poly-basic region in the C-terminal tail of the M₁, M₃, and M₅ receptors contributes to the ability of these receptors to mediate protection against apoptotic cell death.

Apoptosis or programmed cell death is the process by which cells initiate a series of biochemical reactions that ultimately result in the breakdown of the cell cytoskeleton, destruction of the integrity of the nucleus, and cleavage of cellular DNA, leading to cell death. Apoptosis is manifested during a number of physiological processes, including embryogenesis and development where specific populations of cells are targeted for elimination. However, it is also now widely appreciated that the process of programmed cell death can play a central role in the onset and development of various disease states, particularly in the mammalian CNS, and is characteristic of such neuronal disorders as Alzheimer’s disease and ischemia.

The process of cellular degradation during apoptosis involves the activation of intracellular cysteine proteases (caspases) that cleave substrates after specific aspartate residues. Caspases exist in healthy cells aszymogens with low degradative activity but become fully activated via autocatalytic processing in response to an apoptotic signal. Apoptosis can be induced in mammalian cells by receptor-specific mechanisms (e.g. Fas ligand, tumor necrosis factor-related apoptosis-inducing ligand or TRAIL) or by the addition of cytotoxic agents (4). However, in the case of UV irradiation or the DNA-damaging agent etoposide, apoptosis in cells occurs via biochemical pathways that appear to involve mitochondrial depolarization, an increase in the mitochondrial permeability transition pore, and release of apoptogenic factors such as cytochrome c from the inner mitochondrial membrane to the cytosol. Cytosolic cytochrome c is able to interact with Apaf-1 and pro-caspase 9 to form the apoptosome (5, 6). The formation of this large multimeric complex is the signal for the autocatalytic processing and activation of pro-caspase 9, which in turn lead to the sequential activation of downstream executioner caspases and degradation of cellular proteins, which ultimately leads to the destruction of the cell cytoskeleton, nuclear structure, and DNA.

It is now clear that a number of G-protein-coupled receptors (GPCRs) have the ability to control apoptosis, either initiating a pro- or anti-apoptotic signal depending on the receptor subtype and cell type in which the receptor is expressed (7, 8). Among those receptors initiating an anti-apoptotic signal is the muscarinic receptor family, which is composed of five distinct members, three of which (M₁, M₃, and M₅) are coupled to the Gq/11-phospholipase C pathway and two (M₂ and M₄) that in-
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Habit adenylate cyclase via coupling to G<sub>i0</sub> (9). Muscarinic receptors (most likely the M<sub>2</sub>-subtype) expressed endogenously in cerebellar granule cells have been shown to protect against apoptotic-cell death induced by culturing in non-depolarizing conditions (10). Similarly, the G<sub>i1</sub>-coupled M<sub>1</sub>-muscarinic receptor expressed as a recombinant protein in PC-12 cells protected against apoptosis following serum deprivation (11). The mechanism by which muscarinic receptors are able to attenuate apoptosis is not clear with some studies demonstrating a role, the cell survival pathway mediated by phosphatidyli-
nositol 3-kinase/Akt (8), and others reporting that this pathway is in addition to the mitogen-activated protein kinase (MAPK) pathway and that downstream G-protein-mediated second messenger pathways (e.g., calcium, cAMP, and protein kinase C) are not important (11, 12).

In the current study we use apoptosis induced by the DNA-damaging agent, etoposide, in Chinese hamster ovary (CHO) cells as our experimental model to investigate the properties of the anti-apoptotic response elicited by the muscarinic receptor family. We show that only the muscarinic receptor subtypes coupled to G<sub>i1</sub>-proteins (M<sub>1</sub>, M<sub>3</sub>, and M<sub>5</sub>) elicit an anti-apo-
ptotic response. Further analysis of the M<sub>3</sub>-muscarinic receptor revealed that the anti-apoptotic signal does not involve direct activation of the G<sub>i1</sub>/phospholipase C-signaling pathway, the MAPK pathway, or receptor phosphorylation but that a conserved polybasic region within the C-terminal tail of the receptor contributes to the anti-apoptotic response.

**MATERIALS AND METHODS**

Etoposide and phosphor-12,13-dibutyrate were from Calbiochem (Not-
tingham, UK). DEVD-pNA was purchased from Bachem (Merseyside, UK). G418 sulfate and all tissue culture reagents were purchased from Invitrogen (Glasgow, UK). Rabbit anti-ERK antisera were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Carbachol chloride, atropine sulfate, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazo-
lium bromide (MTT) were from Sigma (Poole, UK). All other chemicals were of research grade and purchased from Fisher (Loughborough, UK). All radiochemicals were purchased from Amersham Biosciences (UK).

The specific activities of radionucleotides were 3000 Ci/mmol, 81 Ci/
ml of [3H]Ile, and 550 nm. All radiochemicals were purchased from Amersham Biosciences (UK).

**Generation of Receptor Mutants**—The T→A tail mutant was generated by mutating threonines 551, 553, and 554 to alanines by site-
directed mutagenesis (QuikChange, Stratagene) and PCR using the 5′-
primer, GACCGACTGTCTCTGCTGGTACTGCTGCGCGGCCGCAGCAGCAGCATCTT.

Mutant 6 was produced by the sequential mutation of 16 serines in the third intracellular loop. All serines were mutated to alanine except for serine 450, which was mutated to a glycine. Serine-alanine muta-
tions were at positions 286, 287, 289, 291, 292, 332, 333, 334, 336, 419, 423, 425, 433, 445, and 446. Serine-glycine mutations were carried out at positions 450. The K<sub>19566</sub> generation of receptor mutants—

**Receptor Phosphorylation**—Stably transfected CHO cells were grown in six-well plates. Cells were incubated at 37°C for 1 h, and then floating and adhered cells were collected in 200 μl of PBS. Cells were lysed with 200 μl of lysis buffer (6 μg guanidine-HCl, 10 mM urea, 10 mM Tris-HCL, 2% Triton x-100 (v/v), pH 4.4). Samples were then processed as described in the manufacturer’s instructions.

**Caspase 3 Assay**—Sub-confluent cells plated in 10-cm² plates were treated with the appropriate concentration of etoposide for the desired time. Floating and attached cells were harvested in PBS/0.5 mM EDTA, and cells were centrifuged at 1500 rpm in a bench-top centrifuge. The pellets were washed once with cold PBS and resuspended in 50 mM HEPES, 4 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 10% glycerol in plastic 96-well plates. 11.10⁻⁴ mM DEVD-pNA was added to the each well of the 96-well plate, and the plate was kept at 37°C for 2–4 h. Cleavage of the DEVD-pNA substrate was monitored colorimetrically at 405 nm in a microplate reader.

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ous-free Krebs/HEPES buffer (KHB: 118 mM NaCl, 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM diethiothreitol, 0.1 mM EDTA), and the cells were kept on ice for 10 min. The cell lysates were pre-cleared by centrifugation at 10,000 × g for 1 g. A Bradford assay was performed on the lysate, and 200–400 μg of μg of cell lysate was diluted 1:2 in reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) in plastic 96-well plates. 10⁻⁴ mM DEVD-pNA was added to the each well of the 96-well plate, and the plate was kept at 37°C for 2–4 h. Cleavage of the DEVD-pNA substrate was monitored colorimetrically at 405 nm in a microplate reader.

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EDTA, 3 mM EGTA, 1 mM phenylmethylsulfon fluoride, 1 mM Na3VO4, 1 mM dithiothreitol, 5 µg/ml benzamidine), and cells were placed on ice for 5 min. Cells were then lysed by scraping, and lysates were pre-cleared by centrifugation. For ERK assays, 0.2 µg of anti-ERK antisera (Santa Cruz Biotechnology) was added and immunoprecipitation was performed for 1 h at 4°C. For JNK assays, 5 µg of GST-c-Jun was added and samples placed on a roller for 1 h at 4°C. For the ERK assay immunocomplexes were isolated on protein A-Sepharose beads, and for the JNK assay JNK-c-Jun complexes were isolated on glutathione-Sepharose beads. Samples were washed twice with lysis buffer and twice with assay buffer (20 mM HEPES, pH 7.2, 20 mM β-glycerophosphate, pH 7.2, 10 mM MgCl2, 1 mM dithiothreitol, 50 µM Na3VO4). ERK activity was measured by resuspending the immunocomplexes in assay buffer containing 2 µCi of [32P]ATP, 20 µM ATP, and 200 µM epidermal growth factor receptor peptide (encompassing the region 661–681 of the epidermal growth factor receptor). For JNK assays, JNK-c-Jun complexes were resuspended in assay buffer containing 2 µCi/ml [32P]ATP, 20 µM ATP. For ERK assays, the reactions were terminated by the addition of 25% trichloroacetic acid, and samples were spotted on P81 phosphocellulose squares. P81 squares were washed four times with 0.05% orthophosphoric acid, and the incorporation of radioactivity was measured by scintillation counting. For JNK experiments, the assays were terminated by the addition of sample buffer. Samples were resolved on 12% SDS-PAGE gels, and phospho-c-Jun was visualized by autoradiography. Quantification of radioactivity incorporated into c-Jun was performed by excising the protein from the Coomassie Blue-stained gel and measuring by scintillation counting.

Measurement of [Ca2+]i—CHO cells grown on 22-mm coverslips were loaded with 5 µM fura-2-acetoxymethyl ester for 30 min at 37°C. Measurement of [Ca2+]i, was performed as previously described (13).

RESULTS

Anti-apoptotic Response of Gq/11-coupled Muscarinic Receptor Subtypes—Apoptosis is mediated by the activation of caspases that was measured colorimetrically in this study by the cleavage of the peptide substrate DEVD-pNA (see “Materials and Methods”). Treatment of CHO cells stably expressing recombinant human M1–M5 muscarinic receptors showed an increase in caspase activity following an overnight treatment with the DNA-damaging agent, etoposide (Fig. 1). This increase in caspase activity was significantly attenuated by muscarinic receptor stimulation in cells expressing the M1, M3, or M5 muscarinic receptor subtypes (Fig. 1: 59.0 ± 1.0%, n = 4; 51.6 ± 1.2%, n = 3; and 54.4 ± 0.2%, n = 4 for M1, M3, and M5, respectively). In contrast, muscarinic receptor stimulation of cells expressing the M2- or M4-muscarinic receptor subtypes had no significant affect on etoposide-mediated caspase activation (Fig. 1: 95.0 ± 11.0%, n = 3; 93.1 ± 1.0%, n = 4 for the M2- and M4-muscarinic receptor, respectively).

Characterization of the Anti-apoptotic Response of the M3-muscarinic Receptor—Detailed characterization of the anti-apoptotic response shown by the Gq11-coupled muscarinic receptors was investigated in CHO cells expressing the recombinant human M3-muscarinic receptor (CHO-M3 cells). The appearance of phosphatidylserine on the outer leaflet of the plasma membrane is a marker of apoptosis and can be detected by utilizing the extremely high affinity that annexin V exhibits for this phospholipid (14). Phase-contrast images of CHO-M3 cells showed that an overnight treatment with etoposide resulted in a marked reduction in cell number with a large percentage of cells displaying phenotypic features characteristic of apoptosis such as cell rounding, membrane blebbing, and annexin V-FITC binding (Fig. 2). Significant inhibition of these markers of apoptosis was observed in CHO-M3 cells treated with the muscarinic agonist carbachol (Fig. 2). In particular, the increase in annexin V-FITC staining observed following etoposide treatment was markedly reduced by muscarinic receptor stimulation (Fig. 2). As a control for necrotic cell death, CHO-M3 cells treated with etoposide and carbachol, or vehicle were stained with propidium iodide as a marker of necrosis. No significant difference in propidium iodide staining was observed with the different cell treatments, consistent with apoptotic rather than necrotic cell death (data not shown).

During the later stages of apoptosis caspase-dependent DNase activation results in the degradation of genomic DNA that is characterized by a 200-bp DNA ladder (15). Following an overnight treatment of CHO-M3 cells with etoposide, the extracted genomic DNA shows a distinctive apoptotic DNA ladder that was attenuated by muscarinic receptor stimulation (Fig. 3). These data, combined with the measurement of caspase activity (Fig. 1) and the determination of annexin V staining (Fig. 2), are consistent with etoposide mediates apoptosis in CHO-M3 cells, and stimulation of the M3-muscarinic receptor results in attenuation of this apoptotic response.

The reduction of MTT to an insoluble formazen product is catalyzed by mitochondrial succinate dehydrogenase and is used as measure of cellular viability (16). An overnight treatment of CHO-M3 cells with etoposide resulted in an inhibition in the level of MTT reduction compared with CHO-M3 cells.

![Fig. 1. Muscarinic receptor subtype-specific inhibition of etoposide-mediated cell death.](image1)

![Fig. 2. M3-muscarinic receptor stimulation attenuates etoposide-induced apoptosis as measured by Annexin V-FITC staining.](image2)
treated with vehicle only, confirming that the apoptotic agent reduces the viability of the cell population (Fig. 4). Stimulation of the M3-muscarinic receptor prevented this reduction in cellular viability induced by etoposide treatment (Fig. 4).

Characterization of the M3-muscarinic Receptor Anti-apoptotic Response—We explored the kinetics of the muscarinic-induced protection of CHO-M3 cells by treating cells with carbachol for various time periods. M3-muscarinic receptor signaling was terminated by the addition of atropine (0.5 µM) followed by three washes with α-MEM. Apoptosis was then induced by an overnight treatment of cells with etoposide. Surprisingly, muscarinic receptor-mediated protection was induced extremely rapidly, because a brief 0.5-min exposure of CHO-M3 cells to carbachol resulted in a maximal anti-apoptotic response to a subsequent overnight exposure to etoposide (Fig. 5A). It should be noted that the continued presence of muscarinic receptor stimulation was not necessary to provide protection from etoposide-induced cell death. The suppression of cell death by the M3-muscarinic receptor was also dose-dependent with an EC50 of 4.9 µM (Fig. 5, B and C).

We have utilized chemical inhibitors of ERK, p38, and phosphatidylinositol 3-kinase (PI3K) to determine whether these signaling components play any role in mediating the anti-apoptotic response of the M3-muscarinic receptor. All the chemical inhibitors were tested for their expected activity in cell signaling experiments in CHO-M3 cells (data not shown). Inhibition of ERK and p38 with PD98059 (50 µM) and SB202190 (10 µM), respectively, had little affect on the M3-muscarinic receptor protective response (data not shown). Importantly, these inhibitors did not mediate an apoptotic response in their own right. Likewise, inhibition of PI3K with wortmannin (100 nM) alone did not induce cell death. However, incubation of CHO-M3 cells with wortmannin (100 nM) had no significant affect on the ability of the M3-muscarinic receptor to induce protection against etoposide-mediated cell death (data not shown).

Functional Role of the C-terminal Tail of the M3-muscarinic Receptor in the Anti-apoptotic Mechanism—Sequence align-

To test the importance of the poly-basic region of the C-terminal tail in mediating the anti-apoptotic response of the M3-muscarinic receptor, we produced a receptor mutant where the basic residues in the region 565KKKRRK570 were mutated to alanine (K → A) (Fig. 6B). These receptors were stably expressed in CHO cells at the plasma membrane as determined by ligand binding using the hydrophilic muscarinic receptor antagonist [3H]N-methylscopolamine (see Materials and Methods). Overnight treatment of CHO cells stably expressing the Δ565-M3 receptor with etoposide lead to a significant increase in caspase activity. However, in contrast to the wild-type receptor, activation of the Δ565-M3 muscarinic receptor was unable to prevent etoposide-mediated caspase activation (Fig. 7A). These data, together with the sequence alignment of the C-terminal tails of muscarinic receptor, suggest that the C-terminal tail region of the M3-muscarinic receptor and particularly the poly-basic region in the membrane proximal region (Fig. 6A) may be central in allowing the receptor to inhibit etoposide-mediated cell death in CHO cells.

Fig. 3. M3-muscarinic receptor stimulation attenuates etoposide-induced apoptosis as measured by DNA laddering. CHO-M3 cells seeded on 10-cm² plates were treated with carbachol (CCH, 1 mM) and/or etoposide (Etop, 250 µM) overnight. Genomic DNA was isolated and electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining and UV transillumination. Data shown are representative of three separate experiments.

Fig. 4. M3-muscarinic receptor stimulation inhibits the etoposide-mediated decrease in cell viability. CHO-M3 cells seeded on 10-cm² plates were treated with carbachol (CCH, 1 mM) and/or etoposide (Etop, 250 µM) overnight. 0.5 mg/ml MTT was added to the plates, and the cells were incubated at 37 °C for 1 h. The insoluble, colored formazan product was solubilized and measured spectrophotometrically at 550 nm as described under “Materials and Methods.” Data shown are the mean ± S.E. (n = 3).
ined the role of receptor phosphorylation in the M₃-muscarinic receptor anti-apoptotic response by generating two receptor mutants that were deficient in their ability to undergo agonist-mediated receptor phosphorylation. The T³₃A tail mutant was produced by site-directed mutagenesis of three threonine residues (threonines 551, 553, and 554) to alanine in the membrane proximal region of the C-terminal tail of the M₃-muscarinic receptor (Fig. 6B). This mutant receptor showed a 54.3% decrease in agonist-mediated receptor phosphorylation compared with wild-type receptor (Fig. 8, A and D). A second mutant, designated mutant-6, was generated by site-directed mutagenesis of the serine phospho-acceptor sites in the third intracellular loop of the M₃-muscarinic receptor (Fig. 6C). Mutant-6 also showed a significant reduction in agonist-mediated receptor phosphorylation (Fig. 8, B and D, 62.9% reduction compared with wild-type receptor). It should be noted that both mutant-6 and the T³₃A tail mutants were expressed at the cell surface and were able to couple to downstream signaling pathways such as calcium/phospholipase C and the MAPK pathway (data not shown).

Despite the fact that the mutant-6 and the T→A tail mutant receptors showed reduced levels of agonist-mediated phospho-

FIG. 6. Amino acid sequences of the C-terminal tails of the M₁- through M₅-muscarinic receptor subtypes and M₃-muscarinic receptor mutants. A, alignment of the C-terminal tails of the human muscarinic receptor family. The sequences are divided into the proximal region, which is the region N-terminal to the putative cysteine palmitoylation site (marked by a star), and the distal region, which is C-terminal to the palmitoylation site. The box indicates the conserved poly-basic region identified in the Gₐ1-coupled muscarinic receptor subtypes. B, C-terminal tail mutations of the human M₃-muscarinic receptor showing the position of the threonine to alanine (highlighted in boldface and underlined) substitutions in the T→A mutant and the truncation mutant 565-M₃ are shown. The substitution of basic residues in the K→A mutant are also shown. C, graphical representation of the 15 serine to alanine mutations and 1 serine to glycine mutation made in the third intracellular loop of the M₃-muscarinic receptor to create mutant-6.
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Fig. 7. The Δ565-M3 and K → A receptor mutants are unable to protect CHO cells from etoposide-mediated cell death. A. CHO cells stably expressing the wild-type M3-muscarinic receptor (Wt-M3) or the Δ565-M3 were treated with carbachol (1 mM) and/or etoposide (250 μM) overnight, and cell lysates were processed for caspase activity. Note the cell surface expression levels of the wild type and mutant receptor were 0.40 and 0.35 pmol/mg of protein, respectively. Data shown are the mean ± S.E. (n = 6). B. CHO cells stably expressing the wild-type M3-muscarinic receptor (Wt-M3) or the K → A receptor were treated with carbachol (1 mM) and/or etoposide (250 μM) overnight, and cell lysates were processed for caspase activity. Note the cell surface expression levels of the wild type and mutant receptor were 0.40 and 0.30 pmol/mg of protein, respectively. Data shown are the mean ± S.E. (n = 3).

Signaling Properties of the Δ565-M3 Mutant—GPCR activation of the MAPK signaling pathways has been shown to be central in the regulation of cell death in a variety of cell lines (19–22). To test this in the current study, the ability of the truncation mutant Δ565-M3 to stimulate the ERK and JNK pathways was investigated. Stimulation of the Δ565-M3 receptor induced a robust increase in ERK activity in a manner similar to the wild-type M3-muscarinic receptor (Fig. 10A). Similarly, stimulation of the Δ565-M3 receptor also resulted in activation of the JNK pathway that was similar to that seen following wild-type M3-muscarinic receptor activation (Fig. 10, B and C; 134.2 ± 26.0 and 118.7 ± 33.1 fmol of phosphate incorporated/mg/min following a 60-min stimulation with 1 mM carbachol for the wild-type M3-muscarinic receptor and Δ565-M3, respectively; n = 3). These data, coupled with the inhibitor studies above, strongly imply that the M3-muscarinic receptor anti-apoptotic affects are not mediated via stimulation of the MAPK family.

It has been widely recognized that cytosolic increases in intracellular calcium [Ca^{2+}] can either protect or induce apoptosis under certain experimental conditions (23). In this study we have demonstrated that the G_{q/11}/phospholipase C-coupled muscarinic receptors are able to protect CHO cells against etoposide-mediated cell death, whereas the G_{q/11}-coupled muscarinic receptor family members are not (Fig. 1). To determine whether activation of the phospholipase C pathway is essential in mediating the anti-apoptotic effects of the G_{q/11}-coupled members of the muscarinic receptor family, we examined whether the Δ565-M3 receptor, which is unable to protect against etoposide-mediated cell death, was able to produce a [Ca^{2+}] transient following receptor activation. Fig. 11A shows that activation of the Δ565-M3 receptor with a maximal dose of carbachol initiates a [Ca^{2+}] transient that was similar to that observed following activation of wild-type M3-muscarinic receptors. Similarly, submaximal doses of carbachol gave a comparable response in the mutant and wild-type expressing cell lines (Fig. 11B). As an internal control, endogenous purinergic receptors expressed in CHO cells were stimulated with 100 μM ATP, which resulted in a robust [Ca^{2+}] transient (Fig. 11A). The conclusion from these data is that the anti-apoptotic properties of the G_{q/11}-coupled muscarinic receptors is not due to their ability to activate the phospholipase C pathway but is due to a conserved poly-basic region found within the membrane distal portion of the C-terminal tail of these receptors that is not shared by the G_{q/11}-coupled members of the receptor family.

DISCUSSION

We report here that the G_{q/11}-coupled members of the muscarinic receptor family expressed in CHO cells protect against apoptotic cell death. Detailed analysis of the M3-muscarinic receptor demonstrated that the distal portion of the C-terminal tail provided a motif that was essential in this anti-apoptotic response.

One of most intriguing characteristics of the M3-muscarinic receptor cell survival response was the rapid time course. A short 0.5-min pulse of muscarinic receptor agonist was sufficient to protect against apoptosis induced by an overnight treatment with etoposide. This suggested that the mechanism of the M3-muscarinic receptor response was rapid in its onset and was then maintained for an extended period after agonist withdrawal. A strong candidate for mediating such a mechanism was rapid changes in intracellular calcium, which in the case of a number of GPCRs has been shown to encode for longer adenosine 5’-monophosphate response elements (24, 25). However, analysis of a truncated mutant of the M3-muscarinic receptor (Δ565-M3), which was unable to mediate an anti-apoptotic response, revealed that this receptor was still coupled to the calcium/PLC pathway in a manner similar to the wild-type receptor. These data would appear to eliminate a role.
for calcium/PLC signaling in the M₃-muscarinic receptor cell survival response. In this regard our work is consistent with that of Lindenboim and colleagues (11) who demonstrated that the M₁-muscarinic receptor in PC-12 cells protected against FIG. 8.

Comparison of the agonist-mediated phosphorylation of wild-type M₃-muscarinic receptors, T → A tail receptors, mutant-6 receptors, and Δ565-M₃ receptors stably expressed in CHO cells. CHO cells incubated with [³²P]orthophosphate for 1 h (see "Materials and Methods") were stimulated with 0.1 mM CCH for 5 min. Muscarinic receptors were solubilized and immunoprecipitated, and receptors were resolved on 8% SDS-PAGE gels. Muscarinic receptor expression was determined by ligand binding, and equal numbers of receptor were loaded in each lane. Data shown are representative of at least three separate experiments. A, comparison of the phosphorylation profile of Wt-M₃ and T → A tail receptors. B, comparison of the phosphorylation profile of wild-type M₃-muscarinic receptors (Wt-M₃) and mutant-6 receptors (Mut 6) C, comparison of the phosphorylation profile of Wt-M₃ and Δ565-M₃ receptors, D, densitometric analysis of receptor phosphorylation profiles of Wt-M₃, T → A tail, Mut 6, and Δ565-M₃ receptors. Phosphorylations of mutant M₃-muscarinic receptors were normalized to the phosphorylation of Wt-M₃ receptors, which is given as 100%.

Fig. 9. Phosphorylation-deficient mutants, mut-6 and T → A tail receptor, are capable of inhibiting etoposide-mediated cell death. CHO cells stably expressing either the wild-type M₃-muscarinic receptor (Wt-M₃), mutant-6 receptor (Mut 6), or the T → A tail receptor were treated with carbachol (1 mM) and/or etoposide (250 μM) overnight, and cell lysates were processed for caspase activity. Data shown are the mean ± S.E. (n = 3–6).

FIG. 10. ERK and JNK are activated following Δ565-M₃ receptor stimulation. A, ERK activation following stimulation of cells expressing wild-type M₃-muscarinic receptors (Wt-M₃) and Δ565-M₃ receptors with 1 mM CCH. Data shown are mean ± S.E. (n = 3). B, JNK activation following stimulation of CHO cells expressing Wt-M₃ and Δ565-M₃ receptors with 1 mM CCH. The gel shown is representative of three separate experiments. C, JNK activation following stimulation of Wt-M₃ and Δ565-M₃ receptors with 1 mM CCH. Data shown are mean ± S.E. (n = 3).

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Previous studies in COS-7 cells have demonstrated that M₁- and M₂-muscarinic receptors can protect against cell death, in part, through the pro-survival PKB/Akt pathway (8). Activation of PKB/Akt is downstream of the phosphoinositide lipid products of the PI3Ks (27). By using wortmannin, an inhibitor of PI3K, previous studies have demonstrated that M₂-muscarinic receptors can activate PKB/Akt via PI3K (28). However, the involvement of PKB/Akt in the M₃-muscarinic receptor anti-apoptotic response in the current study appears unlikely, because treatment with wortmannin at a concentration reported to inhibit PI3K had no significant effect on the anti-apoptotic response.

There appear to be discrepancies in the muscarinic anti-apoptotic responses reported here in CHO cells compared with those previously reported in COS-7 cells (8). The anti-apoptotic response in CHO cells is confined to the Gq/11-coupled subtypes, whereas in COS-7 cells both Gq/11- and Gi/o-coupled receptor subtypes provide protection. These discrepancies may be due to cell and receptor-specific differences, but it is also possible that the mechanisms adopted by GPCRs to modulate cell death might be influenced by the method of inducing cell death (e.g., UV irradiation in COS-7 cells and etoposide in the current study). We are currently testing in CHO cells whether muscarinic receptors can protect against cell death induced by apoptotic stimuli other than etoposide.

We addressed the requirement of M₃-muscarinic receptor phosphorylation in the induction of the anti-apoptotic response. This is important given the growing body of evidence that suggests the formation of phosphorylated GPCR-arrestin complexes appears to be essential in allowing for GPCR modulation of the cell death pathway (20, 29). Indeed, the formation of rhodopsin-arrestin complexes is essential in inducing retinal degeneration in Drosophila (30). Light-induced photoreceptor apoptosis in Drosophila appears to involve the formation of membrane complexes of phosphorylated and activated rhodopsin and arrestin and, subsequently, clathrin-dependent endocytosis of these complexes into a cytoplasmic compartment (31). It has been proposed that similar phosphorylated GPCR-arrestin complexes may be required to allow GPCR regulation of apoptosis in mammalian cells (29). Indeed prevention of stable neurokinin-1 receptor-β-arrestin complexes by mutating the C-terminal tail of the receptor is sufficient to prevent substance P-mediated anti-apoptosis (20). Intriguingly, in the current study phosphorylation of the M₃-muscarinic receptor does not appear to be central in allowing for the anti-apoptotic effects of the receptor, because the Δ565-M₃ receptor, which lacks the anti-apoptotic properties of the wild-type receptor, is phosphorylated normally in response to agonist addition. Furthermore, we have also shown that two M₃-muscarinic receptor mutants that exhibit significantly reduced agonist-induced phosphorylation, are able to protect CHO cells against etoposide-induced cell death in an identical manner to wild-type M₃-muscarinic receptors. Therefore, it appears that the ability of Gq/11-coupled muscarinic receptors to inhibit apoptotic cell death proceeds via a mechanism that does not involve receptor phosphorylation.

The inability of the C-terminal tail truncation mutant, Δ565-M₃, to mediate an anti-apoptotic response indicates the existence of structural determinants within the distal portion of the C-terminal tail that are essential for mediating the pro-survival response of the receptor. Unlike many other type I GPCRs (e.g., the adrenergic receptor family) the muscarinic receptor family have relatively short C-terminal tails (23–39 amino acids). There is a large degree of conservation between the five muscarinic receptor subtypes in the membrane proximal region...
of the C-terminal tail up to the putative cysteine palmitoylation site (see Fig. 5A). This conservation across the family is, however, lost downstream of the cysteine palmitoylation site. In the case of the G_{q/11}-coupled receptors (M₁, M₃, and M₅) there is a poly-basic motif that is not present in the G_{i/o}-coupled members (M₂ and M₄). We assessed whether this poly-basic region was important in mediating the anti-apoptotic effects of the G_{q/11}-coupled muscarinic receptors by mutating the basic residues in this region of the M₃-muscarinic receptor to alanine (K → A). Indeed activation of the K → A mutant significantly reduced the ability of etosopside to induce activation of caspase 3 in CHO cells. Because it is only the G_{q/11}-coupled members of the muscarinic receptor family that are able to protect against cell death, these data strongly imply that this poly-basic region is the common structural element that is essential for the anti-apoptotic response of the M₁, M₃, and M₅-muscarinic receptors.

The conclusions from the current study are that the M₃-muscarinic receptor (and most probably the M₁ and M₅ receptors) are able to protect against etosopside-mediated cell death in CHO cells by a mechanism that is rapid in its onset, is independent of calcium/PLC signaling, receptor phosphorylation, and the MAPK and PI3K pathways, and is dependent on a conserved poly-basic region within the distal region of the C-terminal tail. The physiological importance of these findings has yet to be clearly defined. However, an anti-apoptotic response mediated by M₃-muscarinic receptors in native tissues has been demonstrated. For example, muscarinic activation in cerebellar granule cells has been shown to protect from apoptosis induced by culturing in non-depolarizing conditions suggesting that this process may be involved in regulating neuronal apoptosis in the developing central nervous system (10). Furthermore, the recent findings that acetylcholine can be released from cells of hematopoietic lineage (32) and that both T- and B-lymphocytes express functional M₃-muscarinic receptors (33), lead to the intriguing possibility that the immune function of circulating T- and B-lymphocytes may be controlled in an autocrine/paracrine manner by circulating acetylcholine via M₃-muscarinic receptors. Because the function, growth, and differentiation of lymphocytes are highly dependent on apoptosis, this may provide a novel, physiological setting whereby M₃-muscarinic receptor modulation of programmed cell death may be important in mediating immune responses.

Acknowledgment—We thank the Wellcome Trust for their financial support.

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The C-terminal Tail of the M<sub>3</sub>-muscarinic Receptor Possesses Anti-apoptotic Properties

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J. Biol. Chem. 2003, 278:19565-19573.
doi: 10.1074/jbc.M211670200 originally published online March 20, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211670200

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