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Differential agonist-induced regulation of human M_2 and M_3 muscarinic receptors

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Abstract

We have compared the regulation of M_2 and M_3 muscarinic receptors heterologously expressed in HEK-293 cells upon long-term exposure towards the agonist carbachol. Carbachol time- and concentration-dependently reduced M_2 receptor density with a maximum reduction of about 60%. Treatment with 1 mM carbachol for 24 hr was accompanied by desensitisation of carbachol-induced Ca^{2+} elevations (maximum response reduced by 70%) but not by alterations in the expression of various G-protein α-subunits. Consistently, heterologous desensitisation of Ca^{2+} elevations by the purinergic receptor agonist ATP or by sphingosine-1-phosphate was not detected. In contrast, carbachol time- and concentration-dependently up-regulated M_3 receptors with maximum increases to about 350% of control values. The up-regulation was fully blocked by cycloheximide indicating that it was dependent on protein synthesis. Concomitant with the up-regulation of the M_3 receptor was a reduction in the expression of the α-subunit of $G_{q/11}$. The net effect of these two opposite regulatory mechanisms was a lack of alteration of carbachol-stimulated Ca^{2+} elevation. However, the reduction of $G_{q/11}$ was accompanied by a heterologous desensitisation of Ca^{2+} elevations by ATP and sphingosine-1-phosphate. Levels of M_2 and M_3 receptor mRNA as assessed by real-time PCR were not significantly altered by carbachol exposure for either receptor, suggesting that alterations of mRNA stability did not contribute to the observed changes in receptor number. We conclude that M_2 and M_3 receptor expression within the same cell undergoes differential agonist-induced regulation being accompanied by distinct regulation of G-protein expression leading to differential effects on signal transduction by other receptor systems.

Keywords: Muscarinic receptor; G-protein; Calcium; Regulation; ATP; Sphingosine-1-phosphate

1. Introduction

Muscarinic acetylcholine receptors mediate a wide variety of functions in mammals [1,2]. As with many other G-protein-coupled receptors, the expression and function of muscarinic receptors is not a static entity but rather dynamically regulated by a variety of factors, most importantly the ambient level of agonist exposure [3–5]. Numerous studies have assessed the acute desensitisation of M_2 and M_3 receptors upon short-term (minutes) agonist treatment which involves receptor phosphorylation by protein kinase C and/or G-protein-coupled receptor kinases. Based upon the differential presence of phosphorylation sites for these kinases, it is not surprising that the two subtypes exhibit somewhat different patterns of acute agonist-induced desensitisation [5]. Since the relevance of such acute alterations in receptor function for chronic pathophysiological states is unclear, several studies focused on the effect of chronic agonist exposure on muscarinic receptors. Such studies were performed using exogenous agonist, antagonist or *in vivo* denervation procedures [6–8]. Due to a lack of highly subtype-selective ligands, however, only very few studies have reported on the regulation of defined populations of M_2 or M_3 receptors at the protein level by extended agonist treatment and only one of them directly compared the regulation of the two subtypes [8,9]. Such comparisons are further hampered by the fact that within a

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK, human embryonic kidney.

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given tissue it is not always possible to determine whether observed differences are intrinsically due to the receptor subtypes under investigation and/or the cell types expressing them.

Therefore, we have used human embryonic kidney (HEK-293) cells stably transfected with the human M_2 or M_3 receptors to directly compare the effects of extended carbachol treatment on their expression and function in order to determine whether intrinsic differences exist between M_2 and M_3 receptors in their susceptibility for agonist-induced regulation.

2. Methods

2.1. Cell culture

HEK-293 cells stably transfected with the human M_2 and M_3 receptors [10] were cultured in Dulbecco's modified Eagle's/F-12 medium containing 10% foetal calf serum. Unless otherwise stated, cells were treated with 1 mM carbachol or its vehicle for the last 24 hr and subsequently washed intensively prior to harvesting.

2.2. Chemicals

Darifenacin hydrobromide was a gift from Pfizer Central Research and Fura-2 from Molecular Probes; they were dissolved at 10 mM in DMSO. Atropine hemisulfate, carbachol hydrochloride, cycloheximide and methoctramine tetrahydrochloride were from Sigma and dissolved at 10 mM in water. [^{3}H]-L-Quinuclidinylbenzylate (specific activity 48 Ci/mmol) was obtained from Amersham. The G-protein-specific antisera $G_{q/11}$ (QL), $G_{i1/2}$ (AS/7), G_{i3} (EC/2) and G_s (RM/1) were obtained from NEN Life Science Products.

2.3. Radioligand binding

Muscarinic receptors were identified by [^{3}H]-L-quinuclidinylbenzylate as previously described [11]. Briefly, cells were homogenised with an Ultra-Turrax in ice-cold preparation buffer (50 mM Tris, 10 mM $MgCl_2$, 0.5 mM EDTA, pH 7.5) for 10 s at full speed and thereafter twice for 20 s each at 2/3 speed. The homogenates were centrifuged for 20 min at 50,000 *g* at 4°. The pellets were re-suspended in buffer, re-homogenised shortly (10 s at full speed) and washed by an additional centrifugation step. The final pellets were re-suspended and re-homogenised in binding buffer (see below).

Equilibrium saturation and competition binding experiments were performed in binding buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4 at pH 7.4) in a total volume of 1000 μL containing 50–100 μg protein per assay. Incubations were performed for 60 min at 37° and terminated by rapid vacuum filtration over Whatman GF/C filters. Each filter was washed four times with 5 mL each of ice-cold binding buffer. Nonspecific binding was defined by 3 μM atropine. Six concentrations of radioligand were tested in the saturation binding experiments. In competition binding experiments a single concentration of radioligand (approximately 100–150 pM) and 21 narrowly spaced concentrations of competitors were used.

2.4. G-protein immunoblots

G-protein α-subunits of the $G_{q/11}$, $G_{i1/2}$, G_{i3}, $G_{s\ short}$ or $G_{s\ long}$ type were quantified by immunoblotting as previously described for several tissues [12] with minor modifications. Briefly, membrane preparations (100–150 μg protein/sample) were separated on SDS–PAGE, and the separated proteins were transferred to nitrocellulose membranes (Hybond ECL). For detection of the α-subunits of $G_{q/11}$, $G_{1/2}$, G_3 and G_s the QL, AS/7, EC/2 and RM/1 antisera were used, respectively, in a 1:500 dilution. Antirabbit horseradish peroxidase-conjugated IgG was used as the secondary antibody. The resulting autoradiographs were analysed by quantitative two-dimensional densitometry using commercially available software (Herolab). Samples from carbachol- and vehicle-treated cells were assayed in parallel, and the band intensity of the carbachol-treated cells was expressed as % of that of the vehicle-treated cells analysed within the same blot.

2.5. Ca^{2+} measurements

Alterations of intracellular free Ca^{2+} concentrations were determined using the fluorescent indicator dye Fura-2 as previously described [10]. Briefly, experiments were performed in buffer containing 137 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mg/mL BSA and 1 mg/mL D-glucose at pH 7.2 at room temperature using approximately $(0.5-1) \times 10^6$ cells/mL. Excitation was alternating at 340 and 380 nm and emission being read at 510 nm.

2.6. RT-PCR

Total mRNA was prepared from 1 μg of total RNA (RNeasy Mini Kit; Qiagen) carried out with the Oligotex mRNA Kit (Qiagen). Reverse transcription was performed with an oligo(dT_{15}) primer using SuperScript II reverse transcriptase (Invitrogen). To obtain full-length cDNA's PCR (*Taq* PCR Master Mix Kit; Qiagen) was carried out with 1 μL cDNA and specific primers encompassing the open reading frames (ORF) of the M_2 mAChR mRNA and M_3 mAChR mRNA, respectively (M_2 mAChR ORF forward: 5′-aagcggccgcatgaataactcaacaaactcctct-3′, M_2 mAChR ORF reverse: 5′-ttaccttgtagcgcctatgttc-3′, M_3 mAChR ORF forward: 5′-aagcggccgcatgaccttgcacaataacagtac-3′, M_3 mAChR ORF reverse: 5′-ctacaaggcctgctcggtg-3′). PCR lacking the reverse transcription served as

negative controls, PCR with genomic DNA (QIAamp DNA Mini Kit; Qiagen) served as positive controls.

2.7. Real-time RT-PCR

Quantification of M_2 and M_3 receptor mRNA from cells treated for 24 hr with 1 mM carbachol or its vehicle was performed by real-time RT-PCR, monitoring the increase in fluorescence of the SYBR Green dye (QuantiTect SYBR Green PCR Kit) on an ABI Prism 7700 Sequence Detection System in real time (Applied Bioscience). mRNA preparations and reverse transcriptase reactions were done as described above. PCR was carried out with primer pairs resulting in specific 151 bp cDNA fragments of M_2 receptor, M_3 receptor, and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, respectively. Quantification of the housekeeping gene mRNA level was used for normalisation. The sequences of these primers were: M_2 receptor forward: 5′-cccgcaagattgtgaagatgact-3′, M_2 receptor reverse: 5′-ggtgttaatgagcaccatgacatt-3′, M_3 receptor forward: 5′-ggacattggctccgagacga-3′, M_3 receptor reverse: 5′-ctttcctctccaagtccacca-3′, GAPDH forward: 5′-ggcgatgctggcgctgagt-3′, and GAPDH reverse: 5′-catggttcacacccatgacga-3′. Each real-time quantitative PCR was performed twice using duplicate samples and the sequence of each RT-PCR fragment was verified by sequencing. Analysis of the data were done by the $2^{-\Delta\Delta C_T}$ method [13] resulting in the relative transcription level of the target genes in induced cells compared to untreated cells.

2.8. Data analysis

Data are means ± SEM of N experiments. When two groups were compared, statistical significance of differences was assessed by the Student's paired *t*-test. When multiple groups were compared, a repeated measures ANOVA followed by Bonferroni-corrected *t*-tests was performed. All statistical tests were performed with the Prism programme (Graphpad Software), and a $P < 0.05$ was considered significant.

3. Results

Under control conditions HEK-293 cells expressed 1043 ± 233 fmol/mg protein M_2 receptors (N = 8) or 2396 ± 545 fmol/mg protein M_3 receptors (N = 12). Their affinities for the radioligand [^{3}H]-L-quinuclidinylbenzylate (K_d values) were 56 ± 9 pM and 64 ± 22 pM, respectively. None of the subsequent treatments consistently affected the K_d at either muscarinic receptor subtype (data not shown).

The identity of the stably transfected receptors was confirmed in competition binding studies with the M_2-selective methoctramine and the M_3-selective darifenacin. Methoctramine had 24-fold higher affinity than darifenacin at cloned human M_2 receptors (pK_i 8.84 ± 0.04 vs. 7.46 ± 0.10, N = 4), whereas darifenacin had 26-fold higher affinity than methoctramine at cloned human M_3 receptors (pK_i 8.46 ± 0.01 vs. 7.05 ± 0.05, N = 4). Moreover, the identity of the two cell lines was confirmed by RT-PCR, resulting in the generation of specific full-length cDNAs encoding for the complete open reading frame of the M_2 and M_3 receptor, respectively; such RT-PCR products were not detected in HEK-293 wild-type cells (data not shown).

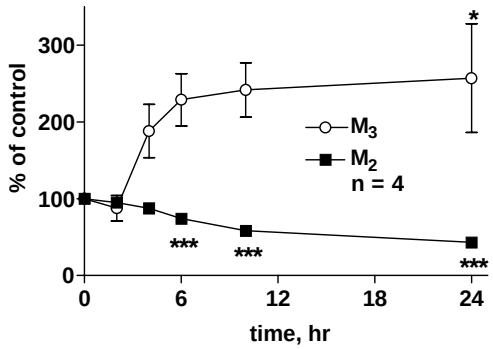

Fig. 1. Time course of M_2 and M_3 receptor protein expression upon treatment with 1 mM carbachol. Data are derived from saturation radioligand binding experiments and expressed as % of control, i.e. receptor expression at time point 0 (control, see Section 3). $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. control (0 hr time point) in a one-way ANOVA followed by Bonferroni-corrected *t*-tests.

Treatment with 1 mM carbachol for 2–24 hr reduced the density of M_2 receptors by approximately half (Fig. 1). At the 24 hr time point, this down-regulation was concentration-dependent, and the threshold for statistically significant reductions was 10 μM carbachol (Fig. 2). In contrast, treatment with carbachol increased M_3 receptor number more than 2-fold, and at the 24 hr time point the threshold concentrations for statistically significant elevations was 0.1 μM carbachol (Figs. 1 and 2). To characterise this up-regulation of M_3 receptors upon carbachol treatment in more detail, experiments in the absence and presence of the

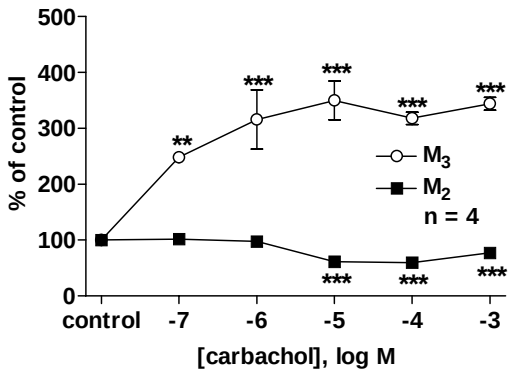

Fig. 2. Concentration–response curve for carbachol-induced regulation of M_2 and M_3 receptor protein expression during a 24 hr incubation. Data are derived from saturation radioligand binding experiments and expressed as % of control, i.e. receptor expression in the absence of carbachol (control, see Section 3). $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control (no carbachol added) in a one-way ANOVA followed by Bonferroni-corrected *t*-tests.

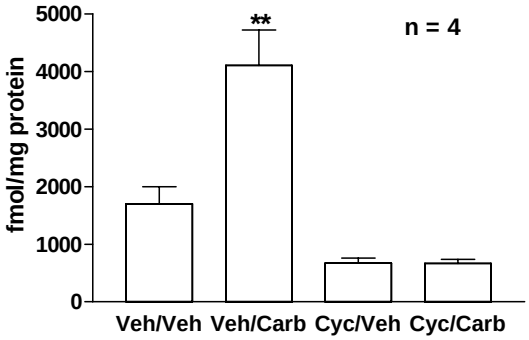

Fig. 3. Effects of cycloheximide (Cyc, 350 μM) on 1 mM carbachol (Carb)-induced regulation of M_3 receptors. $^{**}P < 0.01$ vs. the group receiving both vehicles (Veh/Veh) in a repeated measures ANOVA followed by Bonferroni-corrected *t*-tests.

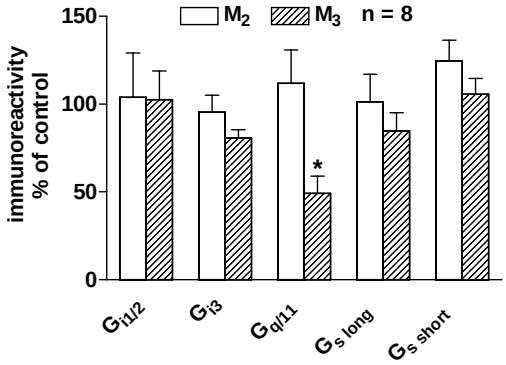

Fig. 4. Effects of treatment with 1 mM carbachol for 24 hr on the immunodetectable expression of various G-protein α-subunits. Data are expressed as percent of values in paired vehicle-treated cells (control). $^{*}P < 0.05$ in a one-sample *t*-test.

protein synthesis inhibitor cycloheximide (350 μM) were performed. As in the previous series of experiments, treatment with 1 mM carbachol significantly increased receptor number, but this increase was completely abolished in cycloheximide-treated cells (Fig. 3). Neither the down-regulation of M_2 nor the up-regulation of M_3 receptors at the protein level was accompanied by changes in the abundance of the corresponding mRNA as assessed by real-time PCR (Table 1).

Table 1
Effect of treatment with 1 mM carbachol for 24 hr on the mRNA level of M_2 and M_3 receptors in HEK-293 cells as assessed by real-time quantitative RT-PCR

	C_T M_2 receptor	C_T GAPDH
M_2 receptor-expressing cells		
Untreated	16.40	16.05
	16.55	16.06
	16.88	16.24
	16.53	16.15
Average	16.59	16.13
Carbachol	16.05	15.78
	16.11	15.81
	16.14	15.61
	16.46	15.69
Average	16.19	15.72
	C_T M_3 receptor	C_T GAPDH
M_3 receptor-expressing cells		
Untreated	17.15	16.80
	17.37	16.73
	17.27	17.17
	17.23	16.71
Average	17.26	16.85
Carbachol	17.73	16.84
	17.00	16.56
	17.01	16.77
	16.79	17.00
Average	17.13	16.79

ΔC_T, $\Delta\Delta C_T$ and $2^{-\Delta\Delta C_T}$ values for untreated and carbachol-treated M_2 receptor-expressing cells were 0.46, 0.0 and 1.00 and 0.47, 0.01 and 0.99, respectively. In M_3 receptor-expressing cells these values were 0.41, 0.0 and 1.00 and 0.34, −0.07 and 1.04, respectively.

α-Subunits of the $G_{q/11}$, $G_{i1/2}$, G_{i3}, $G_{s\ short}$ or $G_{s\ long}$ type which were detected as bands with apparent molecular weights of approximately 42, 41, 41, 42 and 45 kDa, respectively, as previously reported [14]. Treatment of M_3 receptor-expressing cells with 1 mM carbachol for 24 hr significantly reduced immunodetectable expression of $G_{q/11}$ by about 50%, whereas that of the other G-protein α-subunits was not significantly altered (Fig. 4). Carbachol treatment of M_2 receptor-expressing cells did not significantly affect the expression of any of the G-protein α-subunits (Fig. 4).

Basal intracellular Ca^{2+} concentrations were 403 ± 4 nM and 380 ± 3 nM in M_2 and M_3 receptor-expressing cells, respectively (N = 120 measurements each). Treatment with 1 mM carbachol for 24 hr had no major effect on basal Ca^{2+} concentrations (413 ± 10 nM and 396 ± 6 nM). Carbachol treatment of M_2 receptor-expressing cells markedly reduced maximum carbachol-stimulated Ca^{2+} elevations, whereas it did not significantly affect it in M_3 receptor-expressing cells (Table 2). The potency of the carbachol response was not significantly affected in either cell line (Fig. 5). In contrast, Ca^{2+} elevations by 100 μM ATP or by 10 μM sphingosine-1-phosphate were significantly reduced in M_3 but not in M_2 receptor-expressing cells (Table 2).

Table 2
Effect of treatment with 1 mM carbachol for 24 hr on Ca^{2+} elevations (delta nM) induced by carbachol (maximum of concentration–response curve, see Fig. 5), ATP (100 μM) and sphingosine-1-phosphate (S1P, 10 μM)

	M_2 receptor-expressing cells		M_3 receptor-expressing cells	
	Treatment		Treatment	
	Vehicle	Carbachol	Vehicle	Carbachol
Carbachol	526 ± 36	158 ± 30*	438 ± 31	398 ± 49
ATP	522 ± 55	483 ± 40	370 ± 71	156 ± 25*
S1P	380 ± 42	331 ± 41	389 ± 63	188 ± 38*

Data are means ± SEM of six experiments.
* $P < 0.05$ vs. vehicle-treated cells in a paired *t*-test.

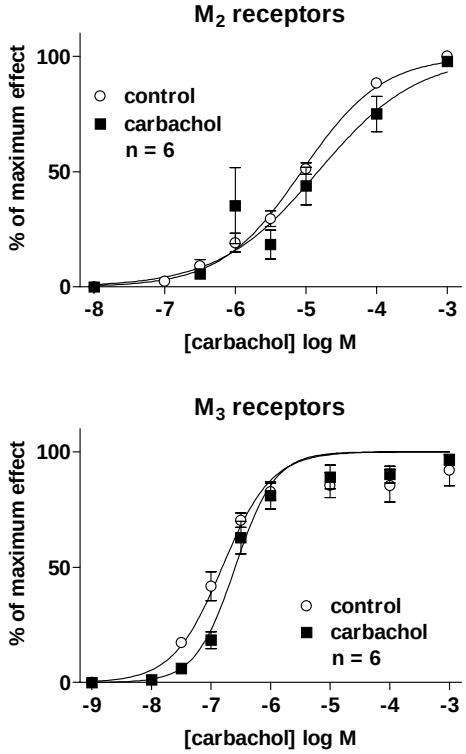

Fig. 5. Effects of treatment with 1 mM carbachol for 24 hr on Ca^{2+} elevations induced by fresh carbachol addition to washed cells. Data are normalised to maximum responses given in each experiment as shown in Table 2. Note that curves from carbachol- and vehicle-treated cells were not significantly different.

4. Discussion

Several tissues such as airways or urinary bladder co-express the M_2 and M_3 subtype of muscarinic receptors, and denervation experiments suggest that these may undergo a differential agonist-mediated regulation *in vivo* [7,15]. Therefore, the primary aim of the present study was to directly compare the regulation of human M_2 and M_3 muscarinic receptors upon long-lasting agonist exposure. The susceptibility of a receptor to agonist-induced regulation depends both on intrinsic properties of the receptor and on properties of the cell expressing it. In order to study receptor-intrinsic differences between M_2 and M_3 receptor and to avoid cell type-specific differences in their regulation, we have used stably transfected HEK-293 cells. Such approach may allow detection of receptor-intrinsic differences in their regulation, but it does not exclude distinct receptor-intrinsic regulation in other cell types. The identity of the cell lines was confirmed by competition experiments with the M_2-selective methoctramine and the M_3-selective darifenacin, which exhibited affinities in good agreement with previously reported values [2,16], and by RT-PCR upon detection of specific full-length cDNAs encoding for the complete open reading frame of the M_2 and M_3 receptors, respectively [16].

As shown before in cultured rat cerebellar granule cells [8] and in human embryonic lung HEL-299 cells [9], we have detected a time- and concentration-dependent down-regulation of human M_2 receptors. The time interval to achieve maximal down-regulation of the human M_2 receptor differed only slightly between the distinct cell types. Approximately, 8 hr were required to down-regulate the M_2 receptor in the cerebellar granule cells, 12 hr in HEL-299 cells and 24 hr in our stably transfected HEK-293 cells. The maximum extent of down-regulation was similar in all three cell types. While concentration–response relationships for agonist-induced down-regulation of M_2 receptors have not previously been reported, we found that a concentration of at least 10 μM carbachol was required for statistically significant reductions of receptor number. Previous studies on the agonist-induced regulation of M_2 receptor mRNA have reported increases at early time points [17] and reductions [8,18] or unchanged levels at later time points [9]. Our experiments also failed to detect altered steady-state mRNA levels, but it should be noted that the stably transfected M_2 receptor in our study was under the control of a heterologous promoter, and hence our assays could only detect alterations of steady-state mRNA levels due to altered stability. Two previous studies, which found M_2 receptor mRNA reductions at late time points, did not detect alterations in mRNA half-life and concluded that the steady-state reductions are due to an altered transcription [9,18]. Therefore, the overall data demonstrate that M_2 receptors undergo agonist-induced down-regulation which, at least at late time points, involves reduced mRNA levels which might be due to decreased transcription rates of the gene.

Immunoprecipitation studies with subtype-selective antibodies revealed that extended agonist treatment-induced down-regulation of M_3 receptors in rat cerebellar granule cells [8]. In contrast, in the present study we did not detect such down-regulation but surprisingly a time- and concentration-dependent up-regulation of M_3 receptors upon carbachol treatment. This up-regulation of M_3 receptors occurred somewhat faster than the down-regulation of M_2 receptors (maximal at 10 hr as compared to 24 hr) and already reached statistical significance with 0.1 μM carbachol (as compared to 10 μM for M_2 receptors). The observation that the carbachol-induced up-regulation of M_3 receptors was completely abolished upon cycloheximide treatment indicates that it depended on *de novo* protein synthesis. Similar to our findings for M_2 receptors, agonist-induced regulation of M_3 receptor protein was not accompanied by alterations in corresponding mRNA levels. In previous studies with rat cortico-striatal neurons, cerebellar granule cells and SH-SY5Y cells agonist treatment caused M_3 receptor mRNA up-regulation at early time points and down-regulation at late time points [8,17,19]. The down-regulation at late time points was due to a reduced transcriptional activity, which explains why it had not been detected in our system where

the receptor is under the control of a heterologous promoter. Taken together these data indicate that the up-regulation of M_3 receptors upon agonist treatment may occur at the translational level. While it remains to be determined why M_3 receptor down-regulation was seen at the protein level in rat cerebellar granule cells [8] but not with the cloned human receptor stably expressed in HEK-293 cells used in the present study, the data demonstrate clearly that M_2 and M_3 receptors can undergo qualitative different regulation within the same cell type. Similar differences have previously reported for subtypes of α_1-adrenoceptors [20].

We have assessed the functional consequences of M_2 receptor down-regulation and M_3 receptor up-regulation using acute carbachol-induced Ca^{2+} elevations, a cell response which has been characterised in these cells in detail before [10]. The down-regulation of M_2 receptors was accompanied by a marked attenuation of maximum Ca^{2+} elevations. Similarly, 24 hr carbachol treatment of M_2 receptor-expressing HEL-299 cells was accompanied by an almost complete loss of inhibition of forskolin-stimulated cAMP accumulation [9]. Unchanged basal Ca^{2+} levels and Ca^{2+} elevations in response to ATP and sphingosine-1-phosphate in the present study indicate lack of heterologous desensitisation of Ca^{2+} signalling. Indeed carbachol treatment of M_2 receptor-expressing cells did not cause major alterations in G-protein expression. Despite the up-regulation of M_3 receptors, we did not observe any sensitisation of the Ca^{2+} response in agonist-treated cells. Since the M_3 receptor-mediated Ca^{2+} elevation is largely pertussis toxin-insensitive and hence likely to be $G_{q/11}$-mediated [10], this lack of sensitisation may be explained by the concomitant reduction in $G_{q/11}$ expression. This may also explain why carbachol treatment of M_3 receptor-expressing HEK-293 cells caused a heterologous desensitisation of Ca^{2+} responses to ATP or sphingosine-1-phosphate. Alternatively, it is possible that prolonged M_3 receptor stimulation causes heterologous desensitisation *via* phosphorylation of the receptors mediating the effects of ATP and sphingosine-1-phosphate [21].

Taken together the present study demonstrates distinct mechanisms of agonist-induced regulation of human M_2 and M_3 receptors upon stable expression in HEK-293 cells. M_2 receptors are down-regulated which leads to a desensitised Ca^{2+} response. The lack of concomitant alterations in G-protein expression probably accounts for the unaltered functional responses by other receptors. In contrast, M_3 receptors are up-regulated in a process requiring *de novo* protein synthesis. This up-regulation is not accompanied by enhanced functional responsiveness, since it is counterbalanced by reduced expression of $G_{q/11}$ G-proteins and hence by heterologous desensitisation of Ca^{2+} signalling. This differential pattern of M_2 and M_3 receptor regulation could explain why denervation of the urinary bladder leads to an up-regulation of M_2 but not M_3 receptors [7,15].

Acknowledgments

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