British Journal of Pharmacology (2003) 139, 1219–1227
www.nature.com/bjp

Adrenomedullin upregulates M_2-muscarinic receptors in cardiomyocytes from P19 cell line

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1 The effects of AM on expression of muscarinic (M) receptors from P19-derived cardiomyocytes were examined.

2 RT–PCR experiments revealed expression of M_1–M_4 receptor genes. Immuno-histochemistry indicated that M_2 expression is restricted to contractile cells. Carbachol inhibition of isoprenaline-induced increase in beating rate was prevented by atropine and methoctramine (pA_2: 8.1). Inhibition of [^{3}H]-NMS binding by atropine (pK_i: -8.4 ± 0.2) and methoctramine (pK_i: -8.3 ± 0.2) suggests that M_2 is the functional expressed isoform.

3 [^{3}H]-NMS binding and semiquantitative RT–PCR studies showed a dome shaped time course of M_2 expression with a maximum at 7 days of differentiation followed by a progressive decline.

4 AM concentration-dependently upregulated M_2 receptor mRNA during late differentiation stages in P19 cells but also in rat atrial cardiomyocytes. This effect was potentiated by factor H. AM (100 nM) plus factor H (50 nM) treatment of P19 cells for 24 h significantly increased [^{3}H]-NMS-specific binding (B_{max}: 81 ± 7 vs 31 ± 6 fmol mg^{-1} prot). The effect of AM on mRNA levels was prevented by AM receptor antagonist AM_{22-52} (1 μM) but not by CGRP antagonist, $CGRP_{8-37}$ (1 μM).

5 The mRNA levels encoding CRLR receptor declined with culture duration, whereas those encoding L1/G10D receptor remained stable.

6 Our findings demonstrate that AM regulates M_2 receptors expression in cardiomyocytes probably through a mechanism involving L1/G10D receptors. The '*in vivo*' significance of this phenomenon remains to be demonstrated.

British Journal of Pharmacology (2003) **139,** 1219–1227. doi:10.1038/sj.bjp.0705350

Keywords: P19 cells; muscarinic receptors; adrenomedullin; L1/G10D receptor

Abbreviations: AM, adrenomedullin; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; GPCR, G protein-coupled receptor; [^{3}H]-NMS, [^{3}H]-*N*-methylscopolamine; IUPHAR, International Union of Pharmacology; RAMP, receptor-associated modifying protein

Introduction

Reduced heart rate variability has been shown to be associated with an increased risk of cardiac death in patients suffering from various heart diseases (La Rovere *et al*., 1998; Galinier *et al*., 2000; Sevre *et al*., 2001) and also in the elderly (Dekker *et al*., 1997; Kikuya *et al*., 2000). Because of its prognostic significance, understanding the pathophysiological mechanisms involved in the decrease in heart rate variability in these conditions and the search for drugs able to normalize or to enhance heart rate variability is relevant.

Various experiments have shown that vagal nerve stimulation (Hedman *et al*., 1995) or presynaptic muscarinic receptor pharmacological blockade increase heart rate variability (La Rovere & De Ferrari, 1995). However, these approaches lack simple applicability in humans and also assume preserved functionality of cardiac muscarinic receptors and overall of the M_2 subtype (Caufield, 1993; Stengel *et al*., 2000). Concerning this aspect of the question, a decrease in heart M_2 receptors has been reported in left ventricular hypertrophy (Mansier *et al*., 1993), in the elderly (Brodde *et al*., 1998) and in obesity (Pelat *et al*., 1999). Thus, a more promising appproach would be, starting from the mechamisms accounting for M_2 muscarinic receptor downregulation, which are still far from understood (Haddad & Roussel, 1998), to search for factors able to correct or to prevent heart M_2 muscarinic receptor decline.

One candidate for such an effect is AM. This 52-amino-acid peptide, originally isolated from human pheochromocytoma (Kitamura *et al*., 1993), belongs to the calcitonin family of peptides and exhibits potent vasorelaxing and natriuretic actions. Elevated plasma levels of AM are found in arterial hypertension, myocardial infarction and heart failure (Hinson *et al*., 2000; Jougasaki *et al*., 2001). In the heart , AM inhibits cardiac fibrosis (Zhang *et al*., 2000) and was also shown to prevent cardiomyocyte hypertrophy induced by angiotensin II (Tsuruda *et al*., 1998; Luodonpää *et al*., 2001), phenylephrine or endothelin (Autelitano *et al*., 2001). These data together with those from mice lacking (Shindo *et al*., 2001) or overexpressing AM (Shindo *et al*., 2000) currently suggest that AM is involved in mechanisms that compensate cardio-

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Advance online publication: 23 June 2003

vascular changes. Most of the biological actions of AM are mediated by GPCRs formed by heterodimerization of CRLRs with one RAMP resulting in various protein complexes named $CGRP_1$ ($CRLR/RAMP_1$), AM_1 ($CRLR/RAMP_2$) and AM_2 ($CRLR/RAMP_3$), following the recent recommendations of IUPHAR (Poyner *et al*., 2002). Furthermore, two additional proteins, canine orphan receptor (RDC1) and L1/G10D, have been reported to be involved in the mediation of some AM effects (Kapas *et al*., 1995; Kapas & Clark, 1995) despite available data are being quite controversial, making these receptors still considered as orphans.

Despite numerous investigations of the role of AM in the heart, its effect on gene expression remains poorly understood. Because of the prominent role of muscarinic M_2 receptors in heart rate control, we decided to investigate the putative role of AM on the regulation of muscarinic receptor expression in cardiomyocyte-like cells derived from the totipotent murine P19 cell line. In this cell line, which has been extensively used to study mechanisms of cardiogenesis (Grepin *et al*., 1997), the presence of muscarinic receptors was never reported. Therefore, the first part of the study focuses on the pharmacological and functional characterization of M_2 muscarinic receptors in differentiated P19 cells. The second part shows that AM is able to upregulate M_2 expression by a mechanism more probably involving L1/G10D receptor but not CRLR/RAMPs receptor complexes.

Methods

Cell culture

Totipotent murine P19 cells were cultured as previously reported (Skerjanc, 1999). Briefly, the cells were first cultured in minimal essential medium (MEM) supplemented with 2.5% fetal bovine (FBS) and 7.5% newborn calf serum (NBCS) at 37°C under 5% CO_2. Embryoid bodies were obtained by cultivating 10^5 cells ml^{-1} in MEM supplemented with 10% FBS and 0.8% dimethyl sulfoxide (DMSO) on Petri dishes for 4 days. Embryoid bodies were then replated into tissue culture dishes with MEM supplemented with 10% FBS and 1% penicillin–streptomycin mix (1 mg ml^{-1}). The differentiation days were counted after this seeding. As preliminary experiments indicated that the percentage of cells that developed the spontaneously beating phenotype was dependent on the quality of serum (data not shown), all experiments were carried out using ATCC serum. Under these conditions, spontaneous beating was observed from the seventh day of culture. As previously described, the beating rate and percentage of beating cells reached a maximun at day 9 and remained stable until at least 15 days of culture.

Isolated cardiac myocytes were prepared from the atria of Wistar rats weighing 250 g as previously described (Schwarzfeld & Jacobson, 1981).

Immunohistochemistry

Cells were fixed in methanol for 1.5 min and incubated with rabbit anti-M_2 and mouse anti-desmin antibodies. Then, anti-M_2 antibodies were detected with anti-rabbit antibody coupled to rhodamine and anti-desmin antibodies were detected with anti-mouse antibody coupled to FITC. Each antibody was incubated for 1 h at 37°C and washed with phosphate-buffered saline solution.

mRNA extraction and relative quantitative RT–PCR

Total RNA was extracted from P19 cells and rat cardiomyocytes using Trizol reagent (Invitrogen) following the instructions of the manufacturer and then treated with RNAse-free DNAse to eliminate genomic DNA. RNA was quantified using a fluorometer (Fuoroscan, Lab System, Inc.) after addition of 100 μl ribogreen (0.5%) in TE buffer (10 mM Tris hydroxymethyl aminoethane and 5 mM EDTA).

For reverse transcription (RT), 2.5 μg total RNA were retrotranscribed at 42°C for 60 min in a Biometra ThermalCycler in a reaction mixture containing 1 μl (200 U) SuperScript II reverse transcriptase, 2 μl random primers (50 μM), 4 μl 5 × First-Strand Buffer, 2 μl DTT (0.1 M), 2 μl dNTP (10 mM).

A total of 25 PCR cycles were chosen for PCR product quantification since it was found to be in the PCR linear range for all the genes tested (data not shown). For the M_1, M_2, M_3, M_4 and M_5 muscarinic receptors, $RAMP_{1,2}$, CRLR and L1 receptors, $GATA_4$, α-cardiac actin, MLC-2a, MLC-2b and Nkx 2.5, PCR was performed in 50 μl of a reaction mixture containing 5 μl PCR buffer 10 ×, 1.5 μl $MgCl_2$ (50 mM), 1 μl dNTP (10 mM), 2 μl of each primer (100 nM), 0.5 μl *Taq* polymerase, 34 μl water and 4 μl cDNA. For M_2, after heating to 94°C for 4 min, denaturation, annealing and elongation were carried out at 94°C for 45 s, 54°C for 60 s and 72°C for 90 s, respectively. The reaction was repeated for 25 cycles followed by a final elongation step of 7 min at 72°C. Annealing was performed at 58°C for M_3 and $RAMP_3$, whereas for α-MHC and β-MHC the annealing reaction was carried out at 60°C. All other cDNA were amplified in cycles set with a 54°C annealing temperature. Primer sequences are displayed in Table 1.

The expression level of mRNA encoding the ribosome 18S subunit was monitored as an internal control and used to normalize the variation of mRNA levels in semiquantitative RT–PCR experiments. PCR was performed with competimers (Ambion) and 5 μCi [γ-^{33}P]dATP. Incorporated radioactivity in the PCR product was revealed with a Si445 phosphorimager (Molecular Dynamics) and quantified with ImagequaNT software (Molecular Dynamics).

Real-time PCR mRNA quantification

A measures of 2 μg of DNAse I-treated (DNAfree, Ambion) total RNA was retrotranscribed in the presence of random primers and Superscript II enzyme (Invitrogen, France) according to the manufacturer's protocol. Gene-specific primers were designed using Primer Express software (Applied Biosystems) based on sequencing data from the National Center for Biotechnology Information databases, U.S.A. Primer sequences were as follows: mouse AM: forward CCTGGACGAGCAGAACACAA and reverse TGGCGGTAGCGTTTGACA; mouse M_2: forward ATGGCGGCCTGGAACAC and reverse CCTCCCCTTGAACACAGGTTT; mouse 18S: forward TCGTATTGCGCCGCTAGAG and reverse TGAAAACATTCTTGGCAAATGC. Real-time PCR reactions were carried out with Cybergreen PCR Master Mix (Applied Biosystems) in a GenAmp 5700 apparatus. The standard curve method was used for the relative quantification

Table 1 Sequences of primers used during RT-PCR experiments

Target		*Sequence*
M_1 mouse	S	CTGGTTTCCTTCGTTCTCTG
	AS	GCTGCCTTCTTCTCCTTGAC
M_2 mouse	S	GGCAAGCAAGAGTAGAATAAA
	AS	GCCAACAGGATAGCCAAGATT
M_3 mouse	S	GTGGTGTGATGATTGGTCTG
	AS	TCTGCCGAGGAGTTGGTGTC
M_4 mouse	S	AGTGCTTCATCCAGTTCTTGTCCA
	AS	CACATTCATTGCCTGTCTGCTTTG
M_5 mouse	S	CTCATCATTGGCATCTTCTCCA
	AS	GGTCCTTGGTTCGCTTCTCTGT
RDC-1	S	CCGCTATCTCTCCATCACCTACTTC
	AS	GTCCTTCTCCTCTTCATACCACTCA
L1	S	AGCGCCACCAGCACCGAATACG
	AS	AGAGGATGGGGTTGGCGACACAGT
CRLR	S	TGCTCTGTGAAGGCATTTAC
	AS	CAGAATTGCTTGAACCTCTC
$RAMP_1$	S	GACTGGGGAAAGACCATACAGA
	AS	CTCCTCCAGACCACCAGTGCA
$RAMP_2$	S	GGACGGTGAAGAACTATGAG
	AS	ATCATGGCCAGGAGTACATC
$RAMP_3$	S	TGGAAGTGGTGCAACCTGTC
	AS	CACGGTGCAGTTGGAGAAGA
GATA 4	S	CTGTCATCTCACTATGGGCA
	AS	CCAAGTCCGAGCAGGAATTT

S-sense; AS = antisemse.

of the PCR products and gene expression was normalized to 18S RNA quantification.

Binding studies

All binding experiments were performed with crude membranes obtained as previously described (Pelat *et al*., 1999) using [^{3}H]-NMS, a nonspecific muscarinic antagonist. All incubations were performed at 25°C for 30 min. For saturation experiments, a fixed concentration of membranes were incubated with increasing [^{3}H]-NMS concentrations (from 0.1 to 3 nM) and nonspecific binding was defined using methoctramine (20 μM). The reaction was stopped by the addition of incubation buffer followed by rapid filtration through Whatmann GF/C filters. The filters were then washed twice with ice-cold incubation buffer. The radioactivity retained on the filters was measured with a Packard-scintillation counter. Data were analysed with a Scatchard plot allowing B_{max} and K_d calculations.

For inhibition binding studies, a fixed concentration of [^{3}H]-NMS (2 nM) was incubated in the presence of increasing concentrations (ranging from 10 pM to 10 μM) of methoctramine, atropine or pirenzepine. Buffers used and incubation procedures, rapid filtration and measurement of radioactivity retained on filters were as described above. Inhibition curves were analysed using Prism software. pK_i and K_i values were calculated from IC_{50} values according to Cheng & Prusoff (1973) for competitive interactions taking into account the concentration and K_d of tritiated ligand. Slope factor (Hill coefficient, n_H) was calculated from a one-site receptor interaction model. The decision to fit data according to a one- or a two-site model was moreover taken after comparison of the residual sum of squares from both analysis.

Functional assessment of muscarinic receptors on P19 cells

These experiments were carried out on fully 9-day differentiated cells, that is, at the time when the maximum number of beating cells was reached. For each dish, the mean resting beating rate was counted on at least five different groups of cells and only those with a stable beating rate were retained for functional studies. The cells were first stimulated by addition of 10 μM isoprenaline, a β-adrenoreceptor agonist, which induced a rapid (15 s to reach maximal effect) and lasting (15 min) two-fold increase in beating frequency. At 2 min after isoprenaline stimulation, six cumulative concentrations of carbachol (from 1 nM to 10 μM) alone or of carbachol plus a fixed concentration (10 μM) of muscarinic antagonist (atropine or pirenzepine) were added at 2 min intervals and mean beating rate calculated during the second minute of drug exposure. Similar experiments were performed using three different concentrations of methoctramine (0.1, 1 and 10 μM) in order to calculate pA_2 for this antagonist. Analysis of dose–response curves was carried out from at least six experiments for each experimental condition performed in four independent cultures of P19 cells using Prism software (GraphPad Software Incorporated).

Drugs and reagents

Alpha minimum essential medium (αMEM), Dulbecco's modified Eagle's medium (DMEM), NBCS, penicillin and streptomycin, oligo(dT), 5 × First-Strand Buffer, dithiothreitol (DTT), deoxyribonucleoside triphosphate (dNTP), Superscript II, PCR buffer 5 ×, *Taq* DNA polymerase, random primers, Hanks' medium and laminin were purchased from Invitrogene, Inc. Competimers and deoxyribonuclease (Rnase-free DNAse) were from Ambion. P19 cells, FBS and DMSO were from ATCC, Inc., ATCC: Virginia, U.S.A. Mouse adrenomedullin, $CGRP_{8-37}$, ADM_{22-52} were purchased from Cal Biochem, Inc., Cal Biochem: Meudon, France. deoxyadenosine 5′-[γ^{33P}] triphosphate (dATP), [^{3}H]-NMS were from NEN, Inc., NEN: Perkin Elmer, Life Science, Cartaboeuf, France. Ribogreen was from Molecular probes and random primers were purchased from Amersham, Inc. Anti-M_2, anti-rabbit and anti-mouse antibodies from Alomon, Inc; primers from Genset, Inc., Genset: Prolico, Paris, France. TriZOL reagent, cytosine β-D-arabino-furanoside, collagenase, protease, carbachol, pirenzepine, methoctramine, atropine, isoprenaline, anti-desmine antibodies and from Sigma, Sigma: St Quentin Fallavier, France.

Satistical analysis

Student's test was used for comparison between two variables. Multiple comparisons were made with one-way ANOVA. All data are expressed as mean ± s.e.m., and P-values of <0.05 were considered significant.

Results

Muscarinic receptor characterization of P19-derived cardiomyocytes

The presence of mRNA encoding M_1, M_2, M_3 and M_4 receptors in P19 cells at full differentiation stage (9 and 15 days) was demonstrated by RT–PCR experiments whereas no mRNA encoding the M_5 subtype was found at the cycle number sufficient to detect expression of other muscarinic receptor subtypes (Figure 1a).

In order to assess if the encountered mRNA were able to encode functional muscarinic proteins, we measured both spontaneous and isoprenaline-stimulated beating rate in cardiomyocyte-like cells derived from P19 cells after 9 days of differentiation. The spontaneous beating rate of resting P19 cells was stable over at least 30 min under our experimental conditions (52 ± 10 bpm). Addition of isoprenaline (10 μM) to the medium was followed by a rapid and long-lasting significant increase in the beating rate (120 ± 13 bpm, $P < 0.001$). The effects of isoprenaline were reversed, in a concentration-dependent manner by carbachol, a nonspecific muscarinic agonist. When cells were pretreated with a fixed concentration of atropine (10 μM) prior to carbachol addition, the effects of carbachol were no longer observed, whereas no significant change in response to carbachol occurred with pretreatment by 10 μM pirenzepine (Figure 1b). In this same experimental setting, methoctramine (0.1, 1 and 10 μM) shifted the carbachol curve to the right in a concentration-dependent way and the pA_2 value calculated for methoctramine was 8.1 (plot not shown). Results of inhibition experiments of [^{3}H]-NMS binding by atropine, methoctramine and pirenzepine are depicted in Figure 1c. Curves obtained with atropine (n_H: 0.98 ± 0.01) and methoctramine (n_H: 0.99 ± 0.01) were clearly monophasic and pK_i values (atropine: -8.4 ± 0.2 and methoctramine: -8.3 ± 0.2) compatible with binding to M_2 as well as to M_4 receptors. Unexpectedly, pirenzepine hardly inhibited [^{3}H]-NMS binding even at the highest concentration used making the estimated curve characteristics difficult to interpret. According to n_H values (0.486 ± 0.121) and F-test, inhibition curves obtained with pirenzepine were analyzed using a two-site model, which allowed to determine the presence of 15.3% receptors with high affinity for pirenzepine (K_{ihigh}: 0.6 ± 0.2 nM) while the K_{ilow} for the remaining population was > 10 μM. Saturation experiments of [^{3}H]-NMS using methoctramine (20 μM) to assess nonspecific binding in membranes ($n = 10$) from 9 days differentiated P19 cells indicated the presence of 57 ± 5 fmol mg^{-1} protein and a K_d value of 1.0 ± 0.2 nM.

As only 30–40% of cells were beating after 9 days of differentiation, we undertook immunohistochemical experiments to define the cellular expression of M_2 receptors. The percentage of cells stained after incubation with an anti-M_2 antibody was similar to the percentage of beating cells (not

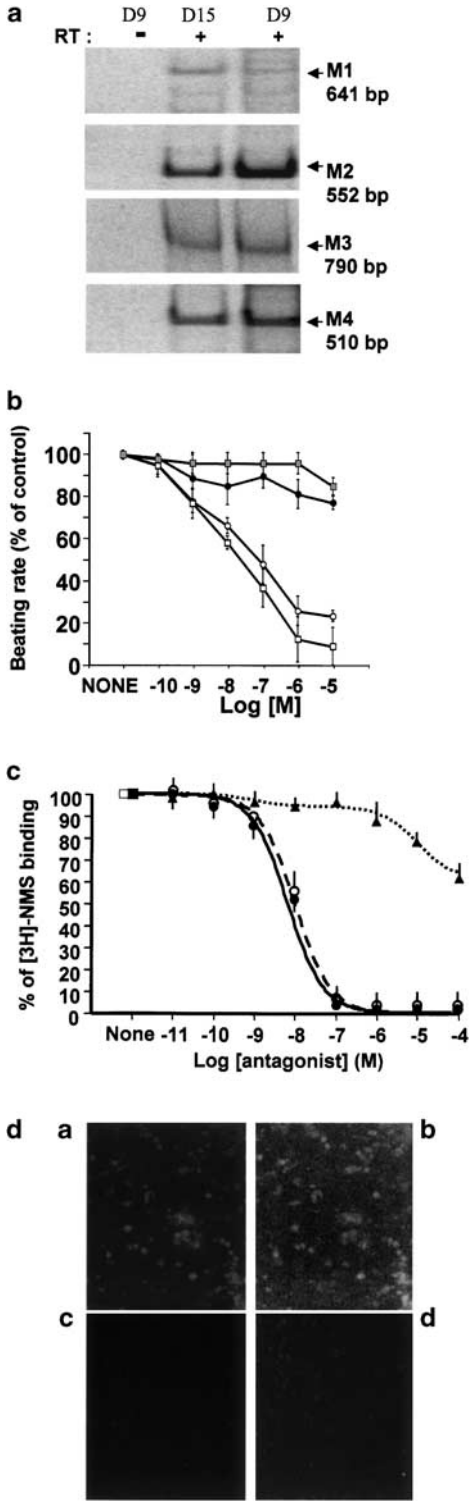

Figure 1 Characterization of muscarinic receptors on P19-derived cardiomyocytes. (A) RT–PCR analysis of mRNA expression for muscarinic receptors M_1–M_4 in P19 differentiated cardiomyocytes. D9 and D15 indicate 9 and 15 days of differentiation respectively. (−) is a control PCR without reverse transcription of the mRNA. Band sizes are indicated. (B) Inhibition of the isoprenaline-induced increase in beating rate by increasing concentrations of carbachol alone ($n = 8$, open squares) or in the presence of a fixed concentration (10 μM) of atropine ($n = 8$, filled squares), methoctramine ($n = 12$, filled circles) or pirenzepine ($n = 8$, open circles). (C) Inhibition of [^{3}H]-NMS binding by atropine ($n = 6$, filled line), methoctramine ($n = 6$, hatched line) and pirenzepine ($n = 6$, dotted line). (D) Top: Colocalization of M_2 muscarinic receptor and desmin by immunofluorescent staining of P19 differentiated cardiomyocytes (representative example of four experiments). M_2 is revealed by a rhodamine-coupled antibody (a) and desmin is revealed by a FITC-coupled antibody (b). Bottom: negative controls: c and d.

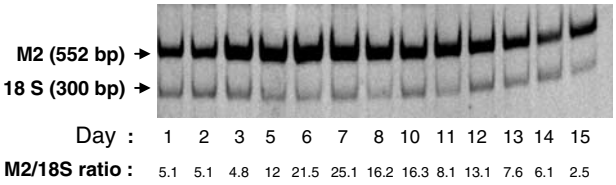

Figure 2 Time course expression of M_2 muscarinic receptor mRNA in P19 cells during differentiation. Semiquantitative RT–PCR were performed from day 1 (D1) to day 15 (D15). 18S mRNA are used as internal standard. Values of M2/18S ratio are depicted on bottom line of the figure.

shown). Moreover, M_2 immunostaining was strictly superposable on immunostaining for desmin (Figure 1d).

Evolution of M_2 expression according to cell differentiation

In RT–PCR experiments, the expression level of mRNA encoding the M_2 receptor was dome shaped. Expression was detected from the first day of differentiation, which increased until day 7 and then progressively declined (Figure 2). This pattern of evolution was also seen when measuring protein expression during [^{3}H]-NMS saturation experiments ($n=4$). The B_{max} measured on day 1 (24 ± 5 fmol mg^{-1} protein) reached 57 ± 5 fmol mg^{-1} protein at day 7 and returned to values comparable to day 1 after 15 days of culture (26 ± 6 fmol mg^{-1} protein).

The way in which M_2 expression varied over time is probably not due to cell dedifferentiation since the levels of expression of RNA encoding MHC, MLC or cardiac transcription factors Nkx2.5 (data not shown) and GATA4 (Figure 6) remained stable until at least day 15.

Effect of AM on M2 muscarinic receptor expression

This was assessed at two different times (9 and 15 days of differentiation) by measuring the levels of M_2 receptor mRNA using real-time PCR with ribosome 18S as control. No change in M_2 mRNA levels was seen at day 9 (data not shown). By contrast, a 24-h incubation of 15 days differentiated P19 cells with AM led to a concentration-dependent increase in M_2 receptor mRNA levels reaching 50% at 1 μM AM (Figure 3a). As factor H has been shown to bind AM and to potentiate its cellular effects (Pio *et al.*, 2001), we investigated the consequence of adding factor H to the cell culture media on M_2 mRNA levels. When combined with AM (100 nM), factor H (50 and 200 nM) concentration-dependently potentiated the effect of AM leading to a larger increase in the M_2 mRNA levels than observed with AM alone (Figure 3b). Factor H alone failed to modify M_2 mRNA levels (Figure 3b). Saturation experiments carried out on crude membranes from 15 days differentiated P19 cardiomyocytes using [^{3}H]-NMS confirmed that exposure of cells to AM plus factor H induced a significant increase in B_{max}, thus reflecting M_2 protein upregulation (81 ± 7 vs 31 ± 6 fmol mg^{-1} protein in controls, $P<0.05$) without significant modification of K_d values (0.9 ± 0.3 vs 0.8 ± 0.2 nM).

Upregulation of mRNA encoding M_2 receptors by AM (100 nM) and potentiation by factor H (50 nM) was also found

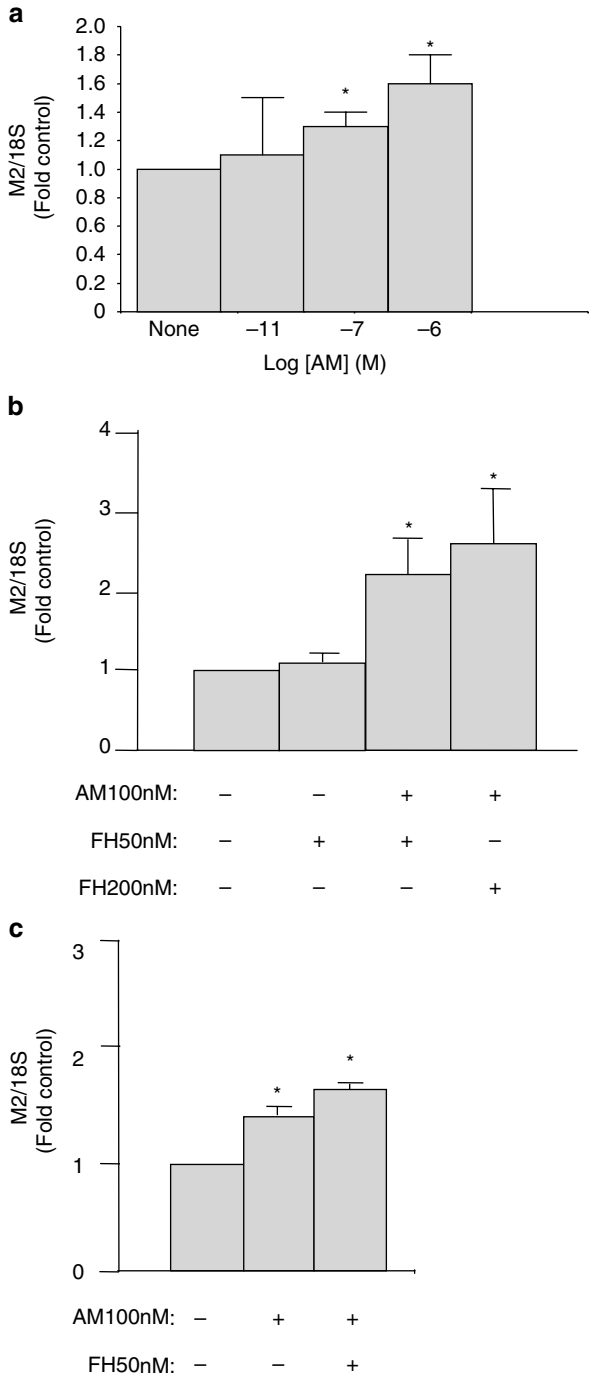

Figure 3 Effects of AM with or without factor H on M_2 muscarinic receptor mRNA expression in P19 cardiomyocytes and rat atrial cardiomyocytes. Mean$\pm$s.e.m. of six separate experiments using real-time quantitative PCR with 18S mRNA used as standard for normalization. *$P<0.05$ vs control values. (a) Effect of increasing AM concentrations on 15 days differentiated P19 cardiomyocytes. Statistical analysis was performed using ANOVA. (b) Effect of two concentrations of factor H added to a fixed concentration of AM. (c) Effect of AM alone or associated with factor H on isolated rat atrial cardiomyocytes.

in isolated myocardial cells from rat atrium confirming that the effect is not limited to the P19 cell line (Figure 3C). Potentiation of AM effects by factor H was less pronounced in rat atrial cells than in P19-derived cardiomyocytes.

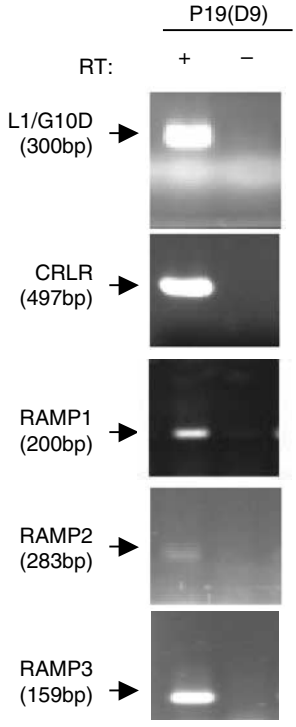

Figure 4 RT–PCR analysis of mRNA expression from L1/G10D and CRLR receptors and $RAMP_{1-3}$ proteins. Sizes of the bands are indicated between parentheses.

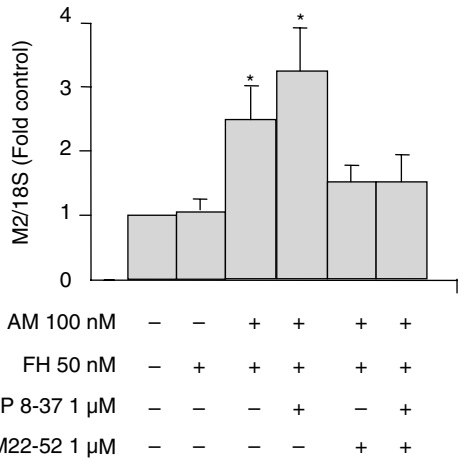

Figure 5 Effect of antagonists of L1/G10D (AM_{22-52}) and CRLR ($CGRP_{8-37}$) receptors on the induction of M_2 mRNA expression by AM plus factor H. Experiments were monitored by real-time PCR in P19 cardiomyocytes (D15 of differentiation) using 18S RNA as standard for normalization. Mean ± s.e.m. of three independent experiments. $*P<0.05$.

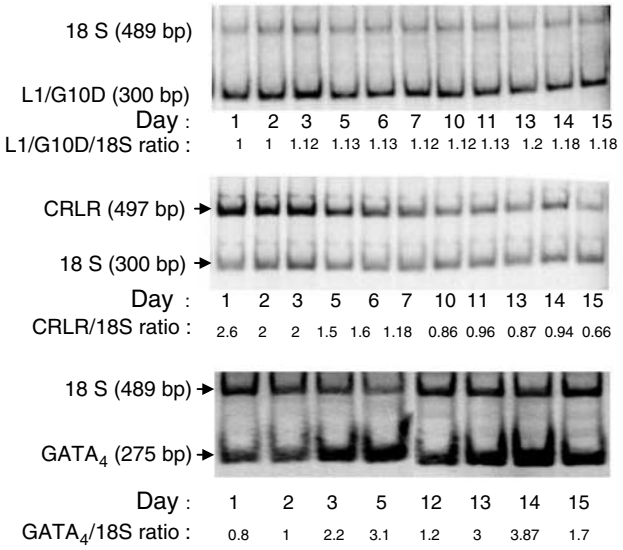

Figure 6 Temporal evolution of L1/G10D and CRLR receptors and of GATA4 mRNAs in P19 cells during culture from day 1 (D1) to day 15 (D15). Results of semiquantitative RT–PCR analysis using 18S mRNA as internal standard. For L1/G10D and CRLR the value of ratio of expression over 18S expression is provided at each time.

Analysis of AM receptors involved in increased M_2 expression

We first looked for potential receptors able to bind AM. RT–PCR experiments identified the presence of both CRLR and L1/G10D receptors (Figure 4) but not of RDC1 (data not shown). Moreover, significant amounts of $RAMP_1$, $RAMP_2$ and $RAMP_3$ mRNA (Figure 4) were also detected, leading to a complex situation in which AM effects on M_2 receptors could be mediated either by the various CRLR-RAMP receptor complexes ($CGRP_1$, AM_1 and AM_2) or by L1/G10D receptor as well. Cell pretreatement with a CGRP antagonist, $CGRP_{8-37}$ (1 μM) failed to antagonize the AM-induced increase in M_2 mRNA, whereas pretreatment with AM_{22-52} (1 μM) or with a mixture of both antagonists prevented it (Figure 5). Moreover, CRLR expression progressively declined starting from day 5 and its expression was dramatically reduced after 15 days of differentiation (Figure 6). In contrast, normalization of levels of L1/G10D receptor mRNA by 18S mRNA levels indicated that expression of L1/G10D receptor remained unchanged with culture time (Figure 6). Thus, it can be proposed that AM-induced increase in M_2 expression is probably not mediated by the classical AM receptors but could result from interaction of AM with orphan L1/G10D receptor.

Discussion

The main results of the present study first clearly indicate the presence of a fully characterized M_2 receptor on P19-derived cardiomyocytes. They also demonstrate that AM is able to upregulate muscarinic M_2 receptor expression during late differentiation stages probably through a pathway involving L1/G10D receptor.

Totipotent P19 cells have been shown to differentiate into spontaneously beating cardiomyocyte-like cells under suitable cell culture conditions (Skerjanc, 1999). Extensive data are available on the phenotypic characteristics of these cells, which can be considered as a suitable model of nodal cells (Wobus *et al*., 1994). We found a higher percentage of beating cells (30–40%) than previously reported (Grepin *et al*., 1997), and this can be explained by careful choice of appropriate serum. P19 cells differentiate into spontaneously beating myocytes that express cardiac-specific transcription factors GATA-4 and Nkx2.5 as well as the various isoforms of heavy and light

chains of myosin and ANP (Grepin *et al*., 1997). However, the question of their ability to express muscarinic receptors has not been resolved although this is an important point. Only one work investigated the presence of M_2 receptors using classical pharmacological methods and concluded their absence (Wobus *et al*., 1994). In the present work, we definitively show that cardiomyocytes derived from P19 cells express mRNA for four of the five known muscarinic receptors. Despite this wide expression pattern, only the M_2 subtype seems to be functional as assessed by suppression of the carbachol-induced reversion of chronotropic effects of isoprenaline by methoctramine and atropine pretreatment. This result is in accordance with those obtained in M_2 or M_4 receptor knockout mice showing that in isolated atria, carbachol-induced bradycardia is abolished in M_2 receptor knockout mice, whereas it is only slightly decreased in M_4 knockout ones (Stengel *et al*., 2000). A striking result from functional experiments was the inability of pirenzepine to prevent carbachol-induced reversion of isoprenaline effects on beating rates. One possible explanation could be that, as reported in pig atria by Daeffler *et al*. (1999), pirenzepine behaves as an inverse agonist at M_2 muscarinic receptors. The lack of selective antagonists does not allow to exclude definitively the expression of a functional M_4 receptor in P19-derived cardiomyocytes. In fact, binding experiments indicated that pK_i values for atropine and methoctramine are compatible with binding at both M_2 and M_4 receptors. Whatever the case, concerning M_2 receptor, which is considered as the main effector of parasympathetic nervous system in the heart (Caufield, 1993), its expression was confirmed by immunohistochemistry and was shown to be limited to beating cells that also exhibit positive staining for desmin antibodies whereas nonbeating cells did not. Interestingly, radioligand binding studies and RT–PCR indicated that M_2 expression varied with the number of days of differentiation. The expression level, which is low on poorly differentiated cells, progressively increases and reaches maximal levels shortly before the cells arrive at their maximal differentiation state and beating rate. The mechanisms accounting for this temporal pattern of M_2 expression were not the topic of the present work. However, it seems unlikely that this pattern is due to a loss of cardiomyocyte-like differentiation, since the percentage of beating cells and their beating rate as well as the expression of specific cardiac transcription factors $GATA_4$ and Nkx2.5 in our experimental conditions, remained unchanged even in late days of culture. A decline of cardiac M_2 expression during aging has been reported in humans, even in the absence of left ventricular hypertrophy (Brodde *et al*., 1998). This observation needs further experiments to be elucidated. It also explains why we tested the effects of AM not only in fully mature but also in late differentiated P19 cells.

The rationale for starting the present study was based on the report that AM is able to prevent the myocardial hypertrophy induced by angiotensin II, phenylephrine or endothelin (Tsuruda *et al*., 1998; Autelitano *et al*., 2001) and to inhibit the angiotensin II-induced increase in ANP and BNP gene expression in rat neonatal ventricular cardiomyocytes (Luodonpää *et al*., 2001). We show that AM *per se* increases, in a dose-dependent manner, mRNA encoding M_2 receptors in P19-derived cardiomyocytes. This effect on mRNA is dependent on the status of the cells, since it is only observed in late differentiation stages and was confirmed at the protein level in binding experiments with [^{3}H]-NMS, which indicate a large increase in M_2 receptor density. The effect of AM is not limited to P19 cells since it also was observed in isolated cardiomyocytes from adult rat atrium cells suggesting that it is a relevant effect.

As previously reported, factor H potentiated the cellular effect of AM (Pio *et al*., 2001) thus explaining why most experiments were carried out in the presence of factor H. The role of factor H desserves some comments. This 150-kDa glycoprotein present in human plasma (Whaley & Ruddy, 1976) is an important regulator in the activation of the complement system (Zipfel, 2001). It was also shown as the major AM binding protein in human plasma able to bind almost half of the total plasma AM (Pio *et al*., 2001) but the details of the interactions between AM and factor H are not yet entirely known. This point may be important for the discussion of the physiological relevance of our findings. As reviewed by Hinson *et al*. (2000) the plasma concentrations of AM in healthy humans (1–10 pM) or even in pathophysiological conditions such as cardiac failure (around 100 pM, Pousset *et al*., 2000) remain far lower from those able to induce upregulation of M_2 receptor in our '*in vitro*' study. However, the significance of plasma AM levels may be questioned since most of published data have measured free AM and not factor H-bound fraction, which seems the most active one. The mechanisms by which factor H potentiates AM biological action are not known but one possibility is that through its binding to cell surface, factor H will modulate AM bioavaibility leading to high concentrations of AM at the proximity of its receptors. Whatever the case, using the same concentrations of factor H as we did, it was shown that the concentration of AM able to induce proliferation of breast cancer cell line T-47D (Pio *et al*., 2001) or to increase cAMP production on Rat2 cells was in the same range as those able to upregulate M_2 receptors in our study.

The mechanisms by which AM achieves its effect on cardiomyocytes are not fully understood. Recent data suggest that AM signalling depends on combination of CRLR and $RAMP_2$ (Autelitano & Ridings, 2001) resulting in AM_1 receptor (Poyner *et al*., 2002) and that antihypertrophic action is mediated by cAMP in cultured neonatal rat ventricular myocytes (Luodonpää *et al*., 2001). However, the presence of other putative receptors able to bind AM including RDC1 (Autelitano, 1998) and L1/G10D (Cormier-Regard *et al*., 1998) in rat cardiac myocytes has also been reported even if their characterization has not been achieved, making them still considered as orphans (Poyner *et al*., 2002). Our results indicate that in P19-derived cardiomyocytes, RDC1 is not expressed, thus limiting the mediation of AM-induced upregulation of M_2 receptors to CRLR/RAMP complexes (AM_1 and AM_2) or to L1/G10D receptors.

The signalling pathways that participate in the AM-induced M_2 muscarinic receptor regulation probably do not involve CRLR/RAMP complexes. In fact, as previously reported in other models, P19 cells express CRLR as well as the three isoforms of RAMPs (Autelitano, 1998; Husmann *et al*., 2000) but two points from the present study make the involvment of this pathway unlikely. First, AM-induced upregulation of M_2 receptor expression was limited to long-term-cultured P19-derived cardiomyocytes and the follow-up of CRLR expression indicated that CRLR expression declines during culture and is very low in late differentiation stages. Second, AM-

induced upregulation of M_2 expression was not reversed by $CGRP_{8-37}$, a reasonable antagonist of AM responses mediated *via* CRLR receptors (Han *et al*., 1997).

In the present study, we found that P19-derived cardiomyocytes express L1/G10D receptor. Concerning cardiac cells, it has been shown that L1/G10D is expressed in rat ventricular myocytes with higher expression level in embryonic and neonatal than in adult hearts (Cormier-Regard *et al*., 1998) and in human hearts (Hanze *et al*., 1997). However, the exact physiological significance of L1/G10D receptor remains a matter of controversy. In fact, despite initial reports considering L1/G10D as an AM receptor (Kapas *et al*., 1995), later attempts to further characterize these receptors were unsuccessful (Kennedy *et al*., 1998). We propose that L1/G10D receptor participate in the AM-induced upregulation of M_2 receptors in view of various arguments. The first is that, as discussed above, $CGRP_{8-37}$, which has only weak antagonistic properties against L1/G10D-mediated AM responses (Kapas *et al*., 1995), failed to prevent AM-induced upregulation of M_2, whereas AM_{22-52} totally blocked it. The second is that its expression level does not depend on the time of the culture by contrast to that we observed for CRLR. However, we cannot exclude that a not yet described AM receptor different from L1/G10D and CRLR complexes with RAMPs will mediate the AM effects in our experimental model. Further experiments are needed to definitively prove that L1/G10D is able to mediate AM responses.

To conclude, the present work completes the pharmacological characterization of P19 cells and describes for the first time the presence of a functional muscarinic M_2 receptor in these cardiomyocyte-like cells. It also proves that, apart from inhibition of myocyte hypertrophy, AM upregulates expression of the M_2 muscarinic receptor. The relevance of this '*in vitro*'AM effect, in both P19 and adult rat atrial isolated cells remains, however, to be demonstrated '*in vivo*' in pathophysiological conditions associated with M_2 receptor downregulation and decreased heart rate variability such as, for instance, heart failure or obesity.

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(Received October 25, 2002
Revised March 25, 2003
Accepted April 30, 2003)