Characterisation of the muscarinic receptor subtype M_3 in the bovine zona fasciculata-reticularis cells by receptor binding, mRNA and functional studies

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Abstract

In the present work we have investigated which muscarinic (M) receptor subtype is responsible for the steroidogenic effect of muscarinic agonists in bovine zona fasciculata-reticularis (ZFR) cells in culture. Radioligand binding studies using the muscarinic antagonist [³H]quinuclidinyl benzilate ([³H]QNB) demonstrated binding sites of high affinity ($K_d=0.45$ nM) and low capacity (~ 8000 sites/cell). Pharmacological characterisation of muscarinic receptors was assessed by evaluating the effects of the $M_3>M_1>>M_2$ antagonist 4-DAMP (4-diphenylacetyl-N-methylpiperidine) and the $M_1=M_4>M_3>>M_2$ antagonist pirenzepine on the binding of [³H]QNB and carbachol-induced cortisol production. For both parameters, the potency of 4-DAMP was about two orders of magnitude higher than that of pirenzepine.

Reverse transcriptase (RT)-PCR analysis of bovine ZFR mRNAs using specific primers for M_2 , M_3 and M_4 receptors revealed the expression of only M_3 mRNA. Moreover, carbachol significantly stimulated inositol phosphate accumulation, but had no inhibitory effect on basal or ACTH-induced cAMP production. Indeed, carbachol potentiated ACTH-induced cAMP production and this effect was, in part, mediated through protein kinase C. Lastly, neomycin, an inhibitor of phosphoinositide turnover, significantly attenuated carbachol-evoked cortisol production. Thus, pharmacological, biochemical and mRNA studies indicate that the M_3 receptor subtype is responsible for the biological effects of muscarinic agonists in bovine ZFR cells.

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Introduction

It has been widely accepted that the secretion of glucocorticoids from the adrenal cortex is determined principally by the action of adrenocorticotrophin (ACTH). However, in addition to ACTH, other factors, including neuropeptides, neuroamines and growth factors, may influence steroidogenic responses (Vizi *et al.* 1992, 1993, Ehrhart-Bornstein *et al.* 1998, Szalay *et al.* 1998).

Cholinergic regulation of adrenal cortex has been suggested by the presence of cholinergic nerve fibres in the adrenal cortex of several species (Stern *et al.* 1989, Charlton *et al.* 1991, Parker *et al.* 1993, Janossy *et al.* 1998) and by the observation that acetylcholine was able to stimulate steroid production by bovine zona fasciculata-reticularis (ZFR) cells (Hadjian *et al.* 1981, 1982, 1984, Walker *et al.* 1990, 1991) and by bovine and rat zona glomerulosa cells (Kojima *et al.* 1986, Janossy *et al.* 1998). Moreover, binding studies have demonstrated the presence of muscarinic (M) receptors in membranes prepared from rat zona glomerulosa (Janossy *et al.* 1998) and bovine ZFR (Hadjian *et al.*

1981). Pharmacological studies have suggested that the steroidogenic action of acetylcholine is probably mediated through the M_3 receptor subtype (Walker *et al.* 1990, 1991, Janossy *et al.* 1998). However, the pharmacological approach is probably not sufficient to discriminate clearly between multiple subtypes, because molecular cloning has demonstrated the existence of five muscarinic receptors (M_1 – M_5) (Bonner *et al.* 1987, 1988) rather than three as defined by the pharmacological approach.

In the present work using radioligand binding experiments, pharmacological studies and analysis of muscarinic receptor mRNAs, we demonstrate that the M_3 subtype is the main, if not the sole receptor involved in the steroidogenic effects of muscarinic agonists in bovine ZFR cells.

Materials and Methods

Synthetic angiotensin II (ANGII) was obtained from Bachem (Bubenford, Switzerland); corticotrophin

Figure 1 Dose-response curve of carbachol-stimulated cortisol secretion by bovine ZFR cells. Cells were incubated with the indicated concentrations of carbachol or with ACTH (10^{-9} M) or ANGII (10^{-7} M) for 2 h. Results, expressed as a percentage of control, are the means $\pm$ S.E.M. of four experiments each performed in quadruplicate. The mean basal cortisol level was 3.2 ± 1.1 ng/ 10^6 cells.

(ACTH) (Synacthen) from Ciba (Rueil-Malmaison, France); carbachol, pirenzepine hydrochloride, insulin, transferrin, bacitracin and HEPES from Sigma Chemicals Co. (St Louis, MO, USA); 4-DAMP (4-diphenylacetyl-N-methylpiperidine) from RBI (Natick, MA, USA). Ham's F-12 medium and Dulbecco's modified Eagle's medium (DMEM) in powder form, nystatin, penicillin/streptomycin, trypsin/EDTA, fetal calf serum were obtained from Gibco (Paris, France); Dowex 1×8 resin (100–200 mesh chloride form) from BioRad (Paris, France). D-myo- ^3H inositol (specific activity 16 Ci/mmol) and ^3H quinuclidinyl-benzylate (^3H QNB; specific activity 60 Ci/mmol) were purchased from Amersham International (Amersham, Bucks, UK). Reverse transcriptase MMLV was obtained from Gibco-BRL (Cergy-Pontoise, France); Taq DNA polymerase from Appligene-Oncor (Illkirch, France) and the primers indicated below were from Genosys (Cambridge, Cambs, UK).

Isolation and culture of bovine ZFR cells

Cells were isolated and cultured as previously described (Langlois *et al.* 1990). Briefly, bovine adrenal glands were freed of fat, dissected, and sliced with a Stadie-Riggs microtome delivering slices of 0.2 cm. The first slice containing the zona glomerulosa was discarded, whereas the second and third slices were saved. Dispersed cells

were obtained by sequential treatment with trypsin (0.19%) in F-12/DMEM (1:1) medium containing gentamycin (20 $\mu\text{g}/\text{ml}$), nystatin (10 U/ml), penicillin (50 U/ml), streptomycin (50 $\mu\text{g}/\text{ml}$), NaHCO_3 (14 mM), and HEPES (15 mM, pH 7.4). The cells were harvested, washed, and placed in growth medium composed of F-12/DMEM (1:1), containing insulin (10 $\mu\text{g}/\text{ml}$), transferrin (10 $\mu\text{g}/\text{ml}$), vitamin C (10^{-4} M), and fetal calf serum (1%). The next day the medium was changed and replaced by the same medium without serum. Most experiments were performed on days 3 and 4 of culture.

Treatments and measurements of cortisol and cAMP production

Treatments were conducted in the medium described above for two hours, except in the case of the antagonists, in which the cells were pre-treated for 15 min with the antagonists prior to stimulation. The following drugs were assayed: ACTH(1–24), ANGII, carbachol (carbamylcholine chloride), pirenzepine hydrochloride and 4-DAMP. Cortisol and cAMP levels in culture medium were determined by direct RIAs, as described previously (Langlois *et al.* 1992).

Receptor binding studies

Muscarinic cholinergic receptor binding studies were performed with the non-selective receptor antagonist ^3H QNB as radioligand. Incubation of bovine ZFR cells was carried out in the same medium as used for culture for 30 min at 37 °C. At each concentration of radioligand total binding was determined in triplicate, and non-specific binding, defined as radioligand binding in the presence of 1 μM atropine, was determined in duplicate. In competition studies, binding of ^3H QNB (0.7 nM) was measured in the presence of different concentrations of drugs. At the end of incubation, the cells were rapidly washed 3 times with ice-cold 0.9% NaCl, dissolved in 0.5 M NaOH 0.4% sodium deoxycholate and bound radioactivity was estimated in a liquid scintillation spectrometer.

Measurement of the accumulation of ^3H inositol phosphates

Inositol phosphate (IP) accumulation was determined as described previously (Bégeot *et al.* 1988). Briefly, adrenal cells were incubated with myo- ^3H inositol (5 $\mu\text{Ci}/\text{ml}$) for 48 h at 37 °C. At the end of the incubation period, the medium was removed and the cells were washed and incubated for 10 min in medium containing 10 mM inositol. The medium was again removed and the cells incubated for 30 min in medium containing 10 mM lithium chloride. The medium was once more removed and replaced by fresh medium containing 10 mM lithium chloride, with or without the indicated effectors. After

30 min, the reaction was terminated by adding ice-cold trichloroacetic acid at a final concentration of 10%. After centrifugation the supernatant was neutralised with NaOH (1 M). The [³H]inositol phosphates were separated on 0.6 ml Dowex 1 × 8 resin packed in small columns as described (Berridge *et al.* 1983). Briefly, [³H]inositol was eluted with 5 × 5 ml of H₂O. Absorbed products (inositol-monophosphate, -bisphosphate and -triphosphate) were eluted by 10 ml 0.1 M formic acid/1 M ammonium formate.

Analysis of muscarinic receptors mRNAs by reverse transcriptase (RT)-PCR

Total RNA was isolated from bovine ZFR cells by the method described in Chomczynski and Sacchi (1987).

For RT-PCR total RNA (2 µg in 20 µl) was reverse transcribed using oligo dT primers. PCR amplification was performed with primers derived from the sequences of cDNAs cloned from bovine adrenal gland cDNA libraries (Gene-Bank accession numbers M₂-L27102, M₃-U08286, M₄-L27104). The primers used were: M₂ sense 5'-GCCGAATAACAACAACAT-3', antisense 5'-GGC TGCTTGCTCATTTTCAC-3'; M₃ sense 5'-ACGTA CCAAAGAAGT-TGCTC-3', antisense 5'-GAAGCTT TTCTGAAAGCTGC-3'; M₄ sense 5'-GAAGCAGA GC-GTCAAGAAAC-3', antisense 5'-CTGTCTGCTT CGTTACAATC-3'.

The reaction mixture (100 µl) contained 1 µl RT products or 100 ng genomic DNA (control), MgCl₂ (1.5 mM), deoxynucleotide triphosphate (0.25 mM), primers (0.5 µM) and the reaction was initiated by addition of 1 unit Taq polymerase after denaturation at 94 °C for 5 min. The following reaction cycles were used: 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min for 35 cycles. The amplification procedure ended with a 10-min extension at 72 °C. PCR products were electrophoresed on 1% agarose gels and visualised by ethidium bromide staining.

Results

Pharmacological characterisation of muscarinic receptors in the ZFR cells

We have investigated the effect of carbachol on cortisol production using cultured ZFR cells. In order to define the time needed to measure accurately carbachol-stimulated cortisol production we analysed the time-course of both carbachol and ACTH. The cortisol output (means ± s.e.m. ng/10⁶ cells) at 0, 15, 30, 60, 120 min for carbachol were 1.5 ± 0.01, 2.29 ± 0.28, 4.07 ± 0.17, 20.10 ± 2.97 and 35.1 ± 0.72, and for ACTH 1.5 ± 0.09, 3.59 ± 0.30, 17.49 ± 0.92, 80.1 ± 8.57 and 194.6 ± 14.5. Therefore a treatment of 2 h was chosen for all subsequent experiments. Carbachol dose-dependently stimulated cor-

Figure 2 Dose-dependent inhibition of carbachol (10^{-4} M)-induced cortisol secretion by 4-DAMP and pirenzepine. 4-DAMP and pirenzepine (Pir) were added to the incubation medium 15 min prior to carbachol. Results, expressed as a percentage of the response induced by carbachol alone, are the means ± s.e.m. of two to four experiments each performed in quadruplicate. Half maximal inhibitions (ID_{50}) were 3.2×10^{-10} and 3.3×10^{-7} M for 4-DAMP and pirenzepine respectively. The mean basal cortisol level was 2.0 ± 0.9 ng/10⁶ cells.

tisol secretion with a threshold response at 10^{-6} M and a maximum response at 10^{-4} M. The ED_{50} value was 1.2×10^{-5} M. The maximal effect of carbachol was three-fold lower than that induced by ACTH (10^{-9} M) or ANGII (10^{-7} M) (Fig. 1). Inhibition of the cortisol response to carbachol was investigated by using the M₃>M₁ selective antagonist 4-DAMP and the M₁>M₃ selective antagonist pirenzepine. Both 4-DAMP and pirenzepine dose-dependently inhibited cortisol secretion stimulated by carbachol (10^{-4} M) with an ID_{50} of 2.9×10^{-10} and 3.0×10^{-7} M respectively (Fig. 2). The muscarinic receptor responsible for cortisol stimulation was also characterised by receptor binding assays. [³H]QNB (non subtype selective muscarinic antagonist) bound with high affinity (K_d : 0.45 nM) to muscarinic receptors in low abundance in bovine ZFR cells (~8000 sites/cell)

Figure 3 Saturation curve of specific binding of [³H]QNB on ZFR cells. Each point represents total binding, determined in triplicate, minus non-specific binding, determined in duplicate. Non-specific binding, measured in the presence of atropine (10^{-6} M), was less than 30% of total binding. The experiment shown is representative of three independent experiments. K_d , 0.47 nM and B_{max} , 6500 sites/cell. Inset: Scatchard transformation of the specific binding data.

(Fig. 3). In competition studies, 4-DAMP was again more potent than pirenzepine. The respective K_i were 2.1×10^{-10} and 2×10^{-7} M (Fig. 4).

Coupling of muscarinic receptors in ZFR cells

To investigate whether Ca^{2+} was required for the steroidogenic effects of carbachol, cells were pre-treated for 15 min before hormonal stimulation with EGTA (6 mM), the Ca^{2+} channel antagonist nifedipine (100 μ M), or the phospholipase C inhibitor neomycin (3 mM) (Fig. 5). EGTA completely abolished the steroidogenic action of both carbachol and ANGII, but reduced the effect of ACTH by only 60%. Nifedipine also totally blocked the effect of carbachol, but only partially that of ANGII. Lastly, neomycin reduced by 70% and 50% the steroidogenic effects of carbachol and ANGII respectively. Thus it appears that the steroidogenic action of carbachol, as well as that of ANGII, required activation of phospholipase C and Ca^{2+} . The results in Fig. 6 show that carbachol significantly stimulates inositol accumulation, although the effect was less marked than that induced by ANGII.

To test the possible involvement of G-proteins in the coupling of muscarinic receptors, the cAMP production by ZFR cells under various conditions was studied (Table 1). Neither carbachol (10^{-4} M), nor ANGII (10^{-7} M) alone had a significant effect on cAMP production. However, both potentiated ACTH-induced cAMP production. Pertussis toxin (PT) pre-treatment at concentrations which

completely ADP-ribosylates all PT-sensitive G-proteins (Bégeot *et al.* 1988), decreased cAMP production only slightly. In contrast, neomycin reduced the potentiating effects of ANGII and carbachol on ACTH-induced cAMP production by 40% and 60% respectively. Similar inhibitory effects were observed in the presence of staurosporine, a protein kinase C (PKC) inhibitor. Taken together these results indicate that neither the muscarinic nor the ANGII receptors were negatively coupled to adenylate cyclase in ZFR cells and that the potentiating effects of both factors on ACTH-induced cAMP production were mediated through the phosphoinositide pathway.

Identification of the muscarinic receptor mRNA expressed in bovine ZFR cells by RT-PCR

Nothern blot analysis of 40 μ g total RNA or 4 μ g poly A⁺ RNA with the five human labelled cDNAs gave a very weak signal for M_3 only (data not shown). To confirm these results, RT-PCR amplification was performed for M_2 , M_3 and M_4 , the only receptors for which bovine cDNA sequences are available. Control experiments were conducted using genomic DNA isolated from bovine adrenal cortex tissue. The length of the DNA fragments delimited by the M_2 , M_3 , M_4 primers were 359, 492 and 304 bp respectively. The amplification products migrated as single bands with the expected length for each muscarinic receptor subtype suggesting that they were

Figure 4 Displacement of [³H]QNB by increasing concentrations of 4-DAMP and pirenzepine (Pir). The [³H]QNB concentration used was 0.7 nM. Curves are representative of three experiments carried out in triplicate. In the experiment shown, the *K_i* were 1.9×10^{-10} M and 1.6×10^{-7} M for 4-DAMP and pirenzepine respectively.

specific sequences of the corresponding genes (Fig. 7a). In addition, the amplification products were digested by specific restriction enzymes. NsiI, SacI and AvaI digested M₂, M₃ and M₄ DNA sequences into the expected 243–146, 100–392 and 33–120–157 bp fragments confirming the specificity of these sequences (Fig. 7b).

First strand cDNAs from bovine ZFR cells were similarly amplified to provide evidence for expression of the M₂, M₃ or M₄ receptors. Amplification product displaying both the expected size and pattern of digestion by SacI, was only detected for M₃ mRNA. Signals were not detected when first strand cDNAs were amplified with the primers specific for the M₂ and M₄ sequences, although simultaneous amplification of genomic DNA with the same primers was successful (Fig. 7a,b).

Figure 5 Effects of neomycin (3 mM), nifedipine (100 μM) and EGTA (6 mM) on carbachol (10^{-4} M)-, ANGII (10^{-7} M)- and ACTH (10^{-9} M)-induced cortisol response. Cortisol production of cells treated only by the specific agonists is taken as 100% (control). The values are the means $\pm$ S.E.M. of four experiments each performed in quadruplicate. All lettered bars differ significantly from controls and bars of the same type with different letters differ significantly from each other ($P < 0.05$).

Figure 6 Effect of carbachol (10^{-4} M) and ANGII (10^{-7} M) on inositol phosphate formation in ZFR cells pre-labelled with myo-[³H]inositol. Each point is the mean $\pm$ S.E.M. of four wells from a single cell preparation. Lettered bars a and b differ significantly ($P < 0.02$) from controls and differ significantly from each other.

Discussion

The present results confirm and extend previous studies showing that cholinergic stimulation of ZFR cells (Hadjian *et al.* 1981, 1982, 1984; Walker *et al.* 1990, 1991) and zona glomerulosa cells (Kojima *et al.* 1986) increases steroid production in a dose-dependent manner, and that these steroidogenic effects are mediated mainly through phosphoinositide (PI) hydrolysis and Ca²⁺ mobilisation.

Although muscarinic receptors were initially classified pharmacologically as M₁ or M₂ based on their sensitivity to pirenzepine, five subtypes have now been identified by

Table 1 cAMP production by ZFR under various conditions. The results are the means ± S.E.M. of 3–5 independent experiments, each done in triplicate

	cAMP production (pmol/10 ⁶ cells per/h)				
	– ACTH	ACTH (10 ^{–9} M)			
		Control	+PT	+Neomycin	+Staurosporine
Basal	0.28 ± 0.12 ^a	35 ± 3 ^b	37 ± 5 ^b	34 ± 3 ^b	30 ± 4 ^c
ANGII (10 ^{–7} M)	0.42 ± 0.02 ^a	88 ± 4 ^d	81 ± 4 ^d	68 ± 6 ^e	62 ± 6 ^e
Carbachol (10 ^{–4} M)	0.37 ± 0.02 ^a	58 ± 5 ^f	52 ± 4 ^f	44 ± 4 ^g	41 ± 3 ^g

On the third day of culture, ZFR cells were preincubated with or without neomycin (3 mM) or staurosporine (1 µM) for 15 min, or with pertussis toxin (PT) (0.5 µg/ml) for 24 h and stimulated with ANGII or carbachol in the absence or presence of ACTH. cAMP was measured in the medium after 1 h. Different superscripts represent a significant difference (*P* < 0.05) among groups.

molecular cloning (M₁ to M₅) (Bonner *et al.* 1987, 1988). Analysis of the functional coupling of each of these receptors ectopically expressed in mammalian cells have shown two main couplings, either stimulation of phosphoinositide hydrolysis (M₁, M₃ and M₅) or inhibition of adenylate cyclase (M₂ and M₄) (Fukuda *et al.* 1988, Felder 1995). Moreover, it has been reported that the affinity profile of the antagonist 4-DAMP was M₃ > M₁ > M₂, whereas that of pirenzepine was M₁ = M₄ > M₃ > M₂ (Bonner *et al.* 1987). Scant information con-

cerns the M₅ subtype which is expressed at a low level in the brain but not in the peripheral tissues (Hulme *et al.* 1990, Hosey 1992).

Our results show that in ZFR cells muscarinic receptors are not coupled to inhibition of adenylate cyclase. Indeed, they show that carbachol which by itself has no effect on cAMP production, potentiates the effect of ACTH and that this effect, as previously shown for ANGII (Langlois *et al.* 1992), is in part mediated by PKC. On the other hand, carbachol stimulates inositol phosphates accumulation, which is consistent with previous results (Hadjian *et al.* 1982, Walker *et al.* 1990, Clyne *et al.* 1992, 1994). Moreover, neomycin, a drug known to block PI turnover by direct binding to PIP₂ and PIP (Carney *et al.* 1985), inhibited both carbachol- and ANGII-induced cortisol production but had a weak effect on ACTH action. Taken together these results indicate that the receptor involved in the action of carbachol on ZFR cells could be M₁, M₃ or M₅.

The dose-dependent inhibition of carbachol-induced cortisol secretion by 4-DAMP and pirenzepine, as well as the radioligand binding studies with these compounds, clearly demonstrated that 4-DAMP is two orders of magnitude more potent than pirenzepine, suggesting that M₃ is the muscarinic receptor involved in the action of carbachol on ZFR cells.

This hypothesis was confirmed by the identification of the muscarinic receptor subtype expressed in bovine ZFR cells. RT-PCR amplification of RNA using primers specific for the M₂, M₃ and M₄ receptors clearly indicated the expression of the M₃ receptor in ZFR cells. We failed to detect the M₂ or M₄ mRNAs, although the M₂ and M₄ sequences were consistently amplified from genomic DNA. It was not possible to investigate M₁ and M₅ receptor expression by RT-PCR because the bovine sequences have not been published. Nonetheless, in the light of the pharmacological data and given that neither M₁ nor M₅ mRNAs could be detected by Northern blot, it is very unlikely that either of these receptors is expressed in bovine ZFR cells.

Figure 7 (a) RT-PCR amplification products corresponding to bovine M₂, M₃, M₄ cDNA or genomic DNA sequences. RNA was extracted, reverse transcribed and the resultant cDNA or genomic DNA was amplified as described in Materials and Methods. Lanes 1 and 10, 6-kb ladder DNA marker; lane 2, negative control of PCR; lane 3, negative control, enzyme was omitted from reverse transcription (RT); lane 4, M₂ cDNA; lane 5, M₃ cDNA; lane 6, M₄ cDNA; lane 7, M₂ DNA; lane 8, M₃ DNA; lane 9, M₄ DNA. (b) PCR amplification products digested by specific restriction enzymes. M₂ (lane 2), M₃ (lane 4) and M₄ (lane 8) DNA or M₃ (lane 6) cDNA sequences were digested by NsiI, SacI and Aval respectively into the expected 243–146, 392–100 and 157–120-33 bp fragments confirming the specificity of these sequences. Lanes 3, 5, and 9 show the non digested products of M₂, M₃, M₄ DNA respectively and lane 7 the non digested product of M₃ cDNA; in these cases enzymes were omitted from the reaction mixtures. Lanes 1 and 10: 1-kb ladder DNA marker.

It has been reported that, in ZFR cells, ANGII and carbachol act through a common mechanism and a common pool of phosphoinositides to promote steroidogenesis (Clyne *et al.* 1992). However, the effects of carbachol on all the parameters measured (IP accumulation, potentiation of ACTH on cAMP production, cortisol secretion) were significantly lower than those induced by ANGII. Similarly, the inhibitory effects of neomycin in hormone-induced cortisol production were more pronounced following carbachol than ANGII stimulation. These differences between ANGII and carbachol are consistent with recent data obtained with bovine adrenal glomerulosa cells showing that, whereas ANGII induced a sustained activation of phospholipase D and therefore a sustained diacylglycerol accumulation, the effect of carbachol was less pronounced and more transient (Jung *et al.* 1998). The reasons for these quantitative differences between the biological effects of ANGII and carbachol are not clear, but might be related, at least in part, to the fact that in ZFR cells ANGII type 1 receptors (Ouali *et al.* 1997) are about ten times more numerous than muscarinic receptors.

In conclusion, the present results indicate that muscarinic receptor subtype M₃ is the receptor involved in the steroidogenic action of muscarinic agonists in bovine ZFR cells. The presence in the adrenal cortex in several species of both cholinergic fibres and specific acetylcholine receptors suggests that this neurotransmitter might take a part in the regulation of adrenal cortex functions, the extent and the physiological significance of which are still unknown.

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