Pergamon

Biochemical Pharmacology, Vol. 47, No. 7, pp. 1145–1150, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(93)E0100-L

THE M_3 MUSCARINIC RECEPTOR MEDIATES ACETYLCHOLINE-INDUCED CORTISOL SECRETION FROM BOVINE ADRENOCORTICAL ZONA FASCICULATA/RETICULARIS CELLS

COLIN D. CLYNE,* SIMON W. WALKER,† MOIRA R. NICOL† and BRENT C. WILLIAMS‡

Departments of Pharmacology, 1 George Square, †Clinical Biochemistry, The Royal Infirmary, and ‡Medicine, Western General Hospital, Edinburgh University, Edinburgh, U.K.

(*Received* 20 *August* 1993; *accepted* 30 *November* 1993)

Abstract—In order to characterize the receptor subtype mediating acetylcholine (ACh)-induced cortisol secretion from purified bovine adrenocortical zona fasciculata/reticularis cells in primary culture, the potencies of a range of selective muscarinic antagonists of ACh-induced steroidogenesis were assessed by Schild analysis. Basal secretion of cortisol was 10.2 ± 1.4 pmol/well/30 min. ACh stimulated a dose-dependent increase in cortisol secretion and was maximally effective at 10^{-5} M, at which concentration cortisol secretion was 143.4 ± 12.9 pmol/well/30 min. Hexahydro-sila-difenidol and *para*-fluoro-hexahydro-sila-difenidol were potent competitive antagonists of ACh-stimulated cortisol secretion, with pA_2 values of 8.68 ± 0.28 and 7.96 ± 0.29, respectively. Pirenzepine (pA_2 = 6.95 ± 0.28) and methoctramine (pA_2 = 6.06 ± 0.27) were relatively weak competitive antagonists. The pA_2 values determined in this study are characteristic of the M_3 muscarinic receptor, and we conclude that this receptor subtype mediates ACh-induced cortisol secretion from bovine zona fasciculata/reticularis cells.

Key words: cetylcholine; adrenal contex; M_3 receptor; bovine

Stimulation of adrenocortical steroid secretion in response to ACh§ was first demonstrated in the isolated perfused bovine adrenal gland [1]. ACh has since been reported to stimulate steroidogenesis from the perfused rat and amphibian adrenal [2, 3] and from cells isolated from both the bovine zg and zfr [4–6]. It is significant that direct cholinergic innervation of the adrenal cortex has been observed in several species including sheep [7] and man [8]. Furthermore, a possible role for ACh in regulating adrenocortical steroidogenesis in man has been proposed [9].

While no effect of ACh on cAMP levels has been demonstrated in adrenocortical inner zone cells [5, 6], ACh clearly stimulates PtdIns turnover. Dose-dependent increases in $^{32}P_i$ labelling of PtdIns occur in response to ACh [10], and in purified bovine zfr cells, ACh stimulates the formation of inositol tris- and bis-phosphates, the time course of appearance of which is consistent with the activation of a polyphosphoinositide specific phosphoinositidase C [6].

Where studied, stimulation of steroidogenesis and second messenger formation by ACh have been sensitive to muscarinic, but not nicotinic antagonists [3, 5, 6, 10, 11]. However, the muscarinic receptor subtype involved in the steroidogenic response to ACh has not been established.

Of the five muscarinic receptor subtypes which have been cloned [12–15], the m1, m2 and m3 receptors appear to correlate with the pharmacologically defined M_1, M_2 and M_3 subtypes [16]. Since none of the muscarinic antagonists currently available is more than 10-fold selective for a given receptor subtype, it is necessary to obtain affinity profiles for a range of antagonists in order to classify muscarinic receptor subtypes definitively. Such antagonists include pirenzepine ($M_1 > M_2 = M_3$), methoctramine ($M_2 > M_1 > M_3$), HHSD ($M_1 = M_3 > M_2$) and p-FHHSD ($M_3 > M_2 = M_1$) [17–20]. Recently a fourth receptor subtype has been pharmacologically identified (M_4) exhibiting high affinity for the M_1- and M_2-selective antagonists pirenzepine and AF-DX-116 [21, 22]. This putative M_4 receptor subtype can be distinguished from the M_1 receptor by its high affinity for himbacine and methoctramine [21, 23], and appears to correspond to the muscarinic m4 gene product expressed in chick heart and brain [24].

Preliminary observations indicated that stimulation of steroidogenesis from primary cultures of bovine zfr cells by ACh may occur through the M_3 muscarinic receptor [6]. The present study was undertaken to definitively characterise the receptor subtype involved in this response, by determining the affinities of a range of selective antagonists of ACh-stimulated cortisol secretion by Schild analysis [25, 26].

MATERIALS AND METHODS

Materials. The source of all cell culture and

* Corresponding author, present address: University College Dublin, Department of Pharmacology, Foster Avenue, Blackrock, Co. Dublin, Ireland. FAX 353-12692749.

§ Abbreviations: ACh, acetylcholine; zg, zona glomerulosa; zfr, zona fasciculata/reticularis; EBS, Earle's Balanced Salts solution; HHSD, hexahydro-sila-difenidol; p-FHHSD, *para*-fluoro-hexahydro-sila-difenidol; PtdIns, phosphatidylinositol.

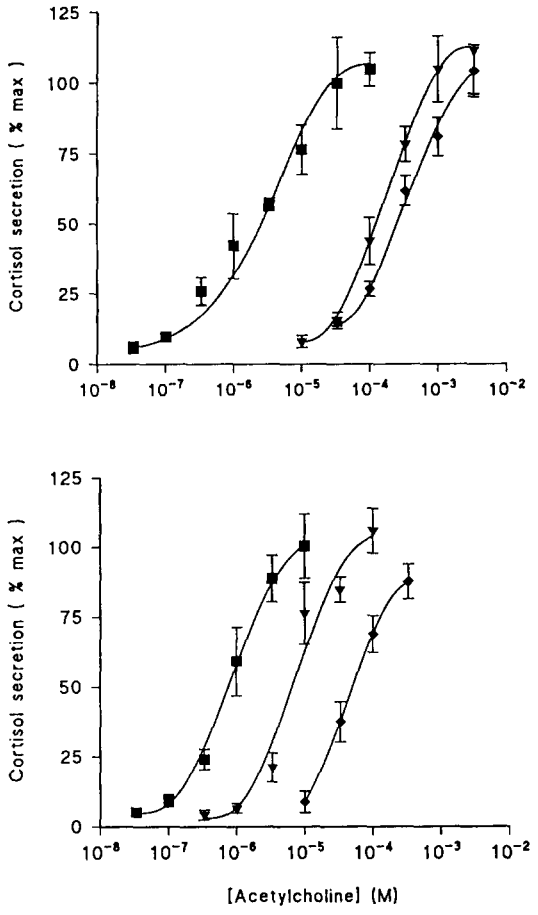

Fig. 1. Dose–response curves for the secretion of cortisol by cultured bovine zfr cells produced on stimulation with ACh alone (■) and in the presence of (upper panel) pirenzepine (▼) 3.3×10^{-6}M, (◆) 10^{-5}M; (lower panel) methoctramine (▼) 10^{-5}M, (◆) 10^{-4}M. Data points are the means ± SD of triplicate determinations from one representative experiment for each antagonist.

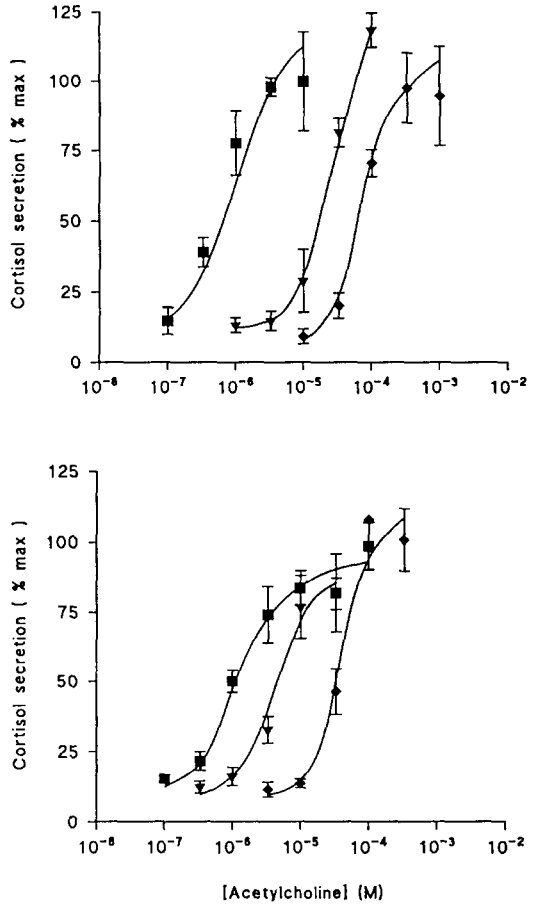

Fig. 2. Dose–response curves for the secretion of cortisol by cultured bovine zfr cells produced on stimulation with ACh alone (■) and in the presence of (upper panel) HHSD (▼) 3.3×10^{-8}M, (◆) 10^{-7}M; (lower panel) p-FHHSD (▼) 3.3×10^{-8}M, (◆) 3.3×10^{-7}M. Data points are the means ± SD of triplicate determinations from one representative experiment for each antagonist.

radioimmunoassay materials is described in Ref. 27. Controlled Process Serum Replacement No. 5 (CPSR-5), acetylcholine and pirenzepine were from the Sigma Chemical Co. (Poole, U.K.). HHSD hydrochloride and p-FHHSD hydrochloride were kind gifts from Drs G. Lambrecht, E. Mutschler and R. Tacke of the Johann-Wolfgang-Gothe University (Frankfurt am Main, Germany). *N,N'*-Bis[6-(2-methoxybenzly)amino]hexyl - 1,8 - octane - diamine tetrahydrochloride (methoctramine) was a generous gift from Prof. Dr C. Melchiorre, Universita' Degli Studi di Bologna (Italy).

Cell culture and stimulation. Bovine adrenal glands were obtained from freshly slaughtered 1–2 year old steers (Gorgie Abattoir, Edinburgh, U.K.). Purified zfr cell suspensions were prepared by the collagenase digestion and column filtration procedure described previously [28], producing a zfr cell preparation essentially free from glomerulosa and medullary cell contamination [28]. Cells were cultured at 37° under 5% CO_2 in 12-well culture dishes (25 mm diameter wells) at 333,000 cells/mL, in 1 mL of Ham's F10 medium containing 10% (v/v) CPSR-5, penicillin (50 IU/mL), streptomycin (50 μg/mL) and amphotericin B (2.5 μg/mL) (F10 growth medium). After 24 hr the overlying medium was replaced with 0.5 mL of identical fresh medium.

Experiments were performed 48 hr after initial plating, at which time cells showed peak responsiveness to ACh [29]. F10 growth medium was removed and cells washed twice with 1 mL of EBS solution with added BSA and glucose [0.2 and 0.1% (w/v) respectively] (EBSBG). Cells were pre-incubated for 5 min under 0.4 mL EBSBG after which ACh and/or antagonists, dissolved in EBSBG, were added to the cells to a final volume of 0.5 mL. Stimulation was allowed to proceed for 30 min at 37° after which the overlying medium was removed and stored at −20° prior to cortisol radioimmunoassay as described previously [28].

Data analysis. In order to obtain estimates of

dose–response curve parameters, data sets were fitted by iterative non-linear least squares regression analysis using the software package Fig P (Biosoft) on an IBM computer, allowing the maximum and half-maximum responses to be calculated. Dose–response curves were tested for parallelism according to Kenakin [30], and antagonist pA_2 values estimated by the method of Arunlakshana and Schild [26]:

$$\log_{10}(r - 1) = n\log_{10}[B] + pK_B$$

in which r is the dose ratio produced by an antagonist of concentration [B], n is the Schild plot slope and pK_B is the antagonist affinity constant. Where n was found to be not significantly different from unity, pK_B was estimated with n constrained to unity [31].

RESULTS

A series of concentration–response curves to ACh were set up in the presence of increasing doses of the muscarinic antagonists pirenzepine, methoctramine, HHSD and p-FHHSD. Representative experiments are shown in Figs 1 and 2. Six such experiments were carried out for each antagonist in order to determine pA_2 values. Basal cortisol secretion was 10.2 ± 1.4 pmol/well/30 min (mean ± SEM, N = 24 experiments). The dose–response curve for ACh fell in the concentration range 10^{-7}–10^{-5}M, and the ED_{50} for ACh-stimulated cortisol secretion was 1.1×10^{-6}M (range 0.4–7.2 × 10^{-6}M). Maximal stimulation of cortisol secretion occurred at 1×10^{-5}M ACh, at which concentration cortisol secretion was 143.4 ± 12.9 pmol/well/30 min (mean ± SEM, N = 24).

The effect of each of the antagonists studied was to shift the log dose–response curve to the right. None of the antagonists had any effect on the maximal response attainable by ACh or the slope of the dose–response curve, consistent with competitive antagonism (Figs 1 and 2). None of the antagonists had any intrinsic effect on cortisol secretion at any of the concentrations employed (up to 1×10^{-5}M HHSD, 1×10^{-6}M p-FHHSD, 1×10^{-4}M pirenzepine and methoctramine) (data not shown).

For estimation of antagonist affinities, the dose ratio (r) for each concentration of antagonist was obtained and Schild plots [26] constructed (Fig. 3). The estimated antagonist pA_2 values and slope of the Schild regressions are shown in Table 1.

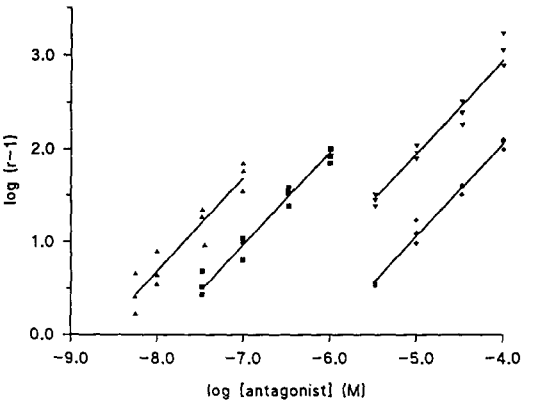

Fig. 3. Schild analysis ($\log_{10}$ (dose-ratio (r)-1) vs $\log_{10}$[antagonist]) of the antagonism by (▲) HHSD, (■) p-FHHSD, (▼) pirenzepine and (◆) methoctramine of ACh-stimulated cortisol secretion from bovine zfr cells. Cumulative data from six separate cell preparations for each antagonist (two concentrations of antagonist per experiment).

DISCUSSION

Bovine adrenocortical ACh receptors have previously been characterized as muscarinic both in the outer zg and in the inner zfr [4, 5]. Walker *et al.* [6] reported the effects of a number of cholinergic agonists and antagonists on cortisol secretion from zfr cells, and obtained preliminary evidence for the presence of an M_3 receptor. However, definitive investigation of the muscarinic receptor subtypes present in the adrenal cortex has not been carried out, either by radioligand binding or by functional analysis, in any species. The present study establishes that ACh-stimulated cortisol secretion from bovine adrenocortical inner zone cells occurs through activation of the M_3 muscarinic receptor, as discussed below.

The use of Schild analysis for calculation of antagonist pA_2 values is valid only for competitive antagonists. The antagonists employed in this study produced parallel shifts of the log dose–response curve to ACh without depressing the maximum response to ACh, and produced linear Schild regressions with slopes not significantly different from unity, consistent with competitive antagonism. The rank potency order (HHSD > p-FHHSD > pirenzepine > methoctramine) is characteristic of that of the M_3 muscarinic receptor: the relatively low potencies of pirenzepine ($pA_2 = 6.95$) and methoctramine ($pA_2 = 6.06$) indicate that the steroidogenic response to ACh is not mediated by M_1 or M_2 receptors respectively (see Ref. 32 for review), while the high pA_2 values for HHSD ($pA_2 = 8.68$) and p-FHHSD ($pA_2 = 7.96$) are characteristic of M_3 receptor function [19, 20].

Recently a fourth muscarinic receptor subtype has been pharmacologically characterized which appears to correspond to the m4 gene product [21]. While the bovine adrenal medulla has been shown to express mRNA transcripts exclusively for the m4 receptor, a weak signal for the m4 receptor mRNA was also detected in the cortex [33]. Although the putative M_4 receptor subtype exhibits high affinity for the M_3-selective compounds HHSD and p-FHHSD [21], it also has moderate to high affinity for methoctramine similar to that of pirenzepine in both ligand binding and functional assays [21, 23]. The low pA_2 for methoctramine observed in bovine zfr cells argues against any significant involvement of the M_4 receptor in ACh-stimulated cortisol secretion.

While the observed pA_2 values for pirenzepine, methoctramine and p-FHHSD correlate well with the known values at M_3 receptors in other systems

Table 1. Comparison of experimental pA_2 values for muscarinic antagonists in bovine zfr cells with previously published values

Antagonist	$pA_2 \pm$ SEM	slope ± SEM	Published pA_2				Reference
			M_1	M_2	M_3	M_4	
Pizenzepine	6.95 ± 0.28	1.06 ± 0.06	8.0–8.3	6.5–6.8	6.7–7.0	7.2–7.7	[21, 23, 38–43]
Methoctramine	6.06 ± 0.27	1.01 ± 0.04	6.8–7.6	7.6–8.3*	5.9–6.9	7.6–7.8	[21, 23, 38, 39, 42, 43]
HHSD	8.68 ± 0.28	1.02 ± 0.10	7.9–8.7	6.5–6.9	7.6–8.5	7.6–7.9*	[21, 38, 40, 44]
p-FHHSD	7.96 ± 0.29	0.95 ± 0.05	6.7–7.2	6.0–6.1	7.6–8.0	7.8	[16, 38–40, 42, 43]

* $-\log_{10}K_i$.

(Table 1), that for HHSD was slightly higher in zfr cells than would be expected at any muscarinic receptor. While this value is not inconsistent with an M_3 receptor classification, since HHSD exhibits highest affinity at M_1/M_3 receptors, it does require comment. A similarly high value (8.49) has been reported previously in ovine detrusser smooth muscle [34]. Although carbachol was used as the agonist, this would not be expected to affect the apparent antagonist affinity, since measurements of pA_2 are independent of the agonist used to elicit the functional response [30]. It seems unlikely that this discrepancy is due to inadequate antagonist equilibration time, since the existence of a non-equilibrium steady-state would be expected to produce a Schild slope greater than unity [30]: the Schild slopes for each antagonist in the present study were not significantly different from unity. It is possible that the unusually high pA_2 for HHSD reported in the present study reflects a species-dependent heterogeneity of M_3 receptors arising from differences in receptor or G protein structure. The precise reasons for this anomaly are unclear at present, and require further investigation.

The presence of the M_3 muscarinic receptor is consistent with the previously reported effects of ACh in bovine zfr cells: (i) stimulation of phosphoinositidase C activity [6, 10], the second messenger system associated with the cloned m1, m3 and m5 gene products, and with native M_1 and M_3 cholinoceptors; (ii) the lack of effect of ACh on cAMP levels in adrenocortical cells [5, 6]; (iii) the inability of the M_1-selective agonist McN-A-343 to stimulate cortisol secretion from bovine zfr cells [6].

Although the significance of ACh in controlling adrenocortical steroidogenesis remains to be established, a number of observations clearly support an *in vivo* function. ACh stimulates cortisol secretion from both the isolated perfused bovine adrenal gland and from freshly isolated bovine zfr cells [1, 6]. In addition, acetylcholinesterase-positive innervation of the adrenal cortex has been detected in many species including sheep [7] and man [8]. While administration of muscarinic agonists to humans had no intrinsic effect on plasma steroid concentration, the plasma aldosterone response to angiotensin II infusion was inhibited by atropine [9]. Observations such as these suggest that cholinergic mechanisms may exert a modulatory effect on the actions of other adrenocortical agonists. Such modulatory effects could occur either through interactions between ACh and other agonists at the level of the steroidogenic cells, as have been reported to occur in the amphibian adrenal [35, 36], or through effects of ACh on adrenocortical blood flow: in conscious calves, infusion of ACh resulted in a decrease in adrenocortical vascular resistance and an enhancement of the steroidogenic effect of adrenocorticotropin [37]. Thus, while data presented here and elsewhere clearly indicate a direct steroidogenic action of ACh on purified adrenocortical cells, it seems likely that ACh may also enhance agonist-stimulated steroidogenesis *in vivo* by increasing adrenal blood flow, thereby increasing the delivery of such agonists to the steroidogenic tissue.

In conclusion, the antagonist affinity profiles observed in the present study most closely resemble those of the M_3 muscarinic receptor, and we propose that this receptor subtype mediates ACh-stimulated cortisol secretion from bovine zfr cells. The functional significance of this muscarinic cholinergic element in the regulation of adrenocortical steroidogenesis *in vivo* warrants further investigation.

Acknowledgements—The award of a grant from the Wellcome Trust to B.C.W. and S.W.W. in support of this work is gratefully acknowledged. C.D.C. holds a Medical Research Council studentship.

REFERENCES

1. Rosenfield G, Stimulative effect of acetylcholine on the adrenocortical function of isolated perfused calf adrenals. *Am J Physiol* **183**: 272–278, 1955.
2. Porter ID, Whitehouse BJ, Taylor AH and Nussey SS, Effect of arginine-vasopressin and oxytocin on acetylcholine stimulation of corticosteroid and catecholamine secretion from rat adrenal gland perfused *in situ*. *Neuropeptides* **12**: 265–271, 1988.
3. Benyamina M, Leboulenger F, Lirhmann I, Delarue C, Feuilloley M and Vaudry H, Acetylcholine stimulates steroidogenesis in isolated frog adrenal gland through muscarinic receptors: evidence for a desensitization mechanism. *J Endocrinol* **113**: 339–348, 1987.
4. Kojima I, Kojima K, Shibata H and Ogata E, Mechanism of cholinergic stimulation of aldosterone secretion in bovine adrenal glomerulosa cells. *Endocrinology* **119**: 284–291, 1986.
5. Hadjian AJ, Guidicelli C and Chambaz E, Cholinergic muscarinic stimulation of steroidogenesis in bovine adrenal cortex fasciculata cell suspensions. *Biochim Biophys Acta* **714**: 157–163, 1982.

6. Walker SW, Strachan MJW, Lightly ERT, Williams BC and Bird IM, Acetylcholine stimulates cortisol secretion through the M_3 muscarinic receptor linked to phosphoinositidase C in bovine adrenal fasciculata/reticularis cells. *Mol Cell Endocrinol* **72**: 227–238, 1990.
7. Robinson PM, Perry RA, Hardy KG, Coghlan JP and Scoggins BA, The innervation of the adrenal cortex in the sheep *Ovis ovis*. *J Anat* **124**: 117–129, 1977.
8. Charlton BG, Nkomavana OF, McGadey J and Neal DE, A preliminary study of acetylcholinesterase-positive innervation in the human adrenal cortex. *J Anat* **176**: 99–104, 1991.
9. Stern N, Pullen W, Plasko R, Eggena P and Tuck ML, Evidence for a cholinergic muscarinic modulation of aldosterone secretion in man. *J Clin Endocrinol Metab* **69**: 294–298, 1989.
10. Hadjian AJ, Culty M and Chambaz EM, Stimulation of phosphatidylinositol turnover by acetylcholine, angiotensin II and ACTH in bovine adrenal fasciculata cells. *Biochim Biophys Acta* **804**: 427–433, 1984.
11. Kawamura M, Yonezawa Y, Tanaka J, Imagawa N, Tomita C and Matsuba M, Corticoidogenic effect of acetylcholine in bovine adrenocortical cells. *Endocrinol Jpn* **32**: 17–19, 1985.
12. Bonner T, Buckley NJ, Young AC and Brann MR, Identification of a family of muscarinic receptor genes. *Science* **237**: 527–533, 1987.
13. Peralta EG, Ashkenazi A, Winslow JW, Smith DH, Ramachandran J and Capon DJ, Distinct primary structures, ligand binding properties and tissue specific expression of four human muscarinic acetylcholine receptors. *EMBO J* **6**: 3923–3929, 1987.
14. Bonner TI, Young AC, Brann, MR and Buckley NJ, Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. *Neuron* **1**: 403–410, 1988.
15. Liao C-F, Themmen APN, Joho R, Barberis C, Birnbaumer M and Birnbaumer L, Molecular cloning and expression of a fifth muscarinic acetylcholine receptor. *J Biol Chem* **264**: 7328–7337, 1989.
16. Buckley NJ, Bonner TI, Buckley CM and Brann MR, Antagonist binding profiles at the five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol Pharmacol* **35**: 469–476, 1989.
17. Hammer R, Berrie CP, Birdsall NMJ, Burgen ASV and Hulme EC, Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature* **283**: 90–92, 1980.
18. Melchiorre CA, Cassinelli A and Quaglia W, Differential blockade of muscarinic receptor subtypes by polymethylene tetraamines. Novel class of selective antagonists of cardiac M-2 receptors. *J Med Chem* **30**: 201–204, 1987.
19. Fuder H, Kilbinger H and Muller H, Organ selectivity of hexahydrosiladifenidol in blocking pre- and post-junctional muscarinic receptors studied in guinea-pig ileum and rat heart. *Eur J Pharmacol* **113**: 125–127, 1985.
20. Lambrecht G, Feifel R, Forth B, Strohmann C, Tacke R and Mutschler E, *p*-Fluoro-hexahydro-sila-difenidol: the first $M_{2\beta}$-selective muscarinic antagonist. *Eur J Pharmacol* **152**: 193–194, 1988.
21. Lazareno S, Buckley NJ and Roberts FF, Characterisation of muscarinic M_4 binding sites in rabbit lung, chicken heart and NG108-15 cells. *Mol Pharmacol* **38**: 805–815, 1990.
22. Garcia-Villalon AL, Krause DN, Ehlert FJ and Duckles S, Heterogeneity of muscarinic receptor subtypes in cerebral blood vessels. *J Pharm Exp Ther* **258**: 304–310, 1991.
23. Caulfield MP and Brown DA, Pharmacology of the putative M_4 muscarinic receptor mediating Ca-current inhibition in neuroblastoma × glioma hybrid (NG 108-15) cells. *Br J Pharmacol* **104**: 39–44, 1991.
24. Tietje KM, Goldman PS and Nathanson NM, Cloning and functional analysis of a gene encoding a novel muscarinic acetylcholine receptor expressed in chick heart and brain. *J Biol Chem* **265**: 2828–2834, 1991.
25. Schild HO, pA, A new scale for the measurement of drug antagonism. *Br J Pharmacol Chemother* **2**: 189–206, 1947.
26. Arunlakshana O and Schild HO, Some quantitative uses of drug antagonists. *Br J Pharmacol* **14**: 48–58, 1959.
27. Clyne CD, Williams BC, Walker SW and Bird IM, Studies of hormone-sensitive and -insensitive pools of phosphoinositides in cultured bovine zona fasciculata/reticularis cells. *Biochem Pharmacol* **44**: 441–446, 1992.
28. Williams BC, Lightly ERT, Ross AR, Bird IM and Walker SW, Characterisation of the steroidogenic responsiveness and ultrastructure of purified zona fasciculata/reticularis cells from bovine adrenal cortex before and after primary culture. *J Endocrinol* **121**: 317–324, 1989.
29. Bird IM, Clyne CD, Lightly ERT, Williams BC and Walker SW, Further characterisation of the steroidogenic responsiveness of purified zona fasciculata/reticularis cells from bovine adrenal cortex before and after primary culture: changing responsiveness to phosphoinositidase C agonists. *J Endocrinol* **133**: 21–28, 1992.
30. Kenakin TP, Drug antagonism. In: *Pharmacologic Analysis of Drug-Receptor Interaction* pp. 205–244. Raven Press, New York, 1987.
31. Mackay D, How should values of pA_2 and affinity constants for pharmacological competitive antagonists be estimated? *J Pharm Pharmacol* **30**: 312–313, 1978.
32. Mei L, Roeske WR and Yamamura HI, Molecular pharmacology of muscarinic receptor heterogeneity. *Life Sci* **45**: 1831–1851, 1989.
33. Fernando JCR, Abdallah E-SAM, Evinger M, Forray C and El-Fakahany EE, The presence of an M_4 subtype muscarinic receptor in the bovine adrenal medulla revealed by mRNA and receptor binding analyses. *Eur J Pharmacol* **207**: 297–303, 1991.
34. Rivera L, Prieto D, Hernandez M, Benedito S and Garcia-Sacristan A, Distribution and function of cholinergic receptors in the sheep detrussor muscle. *J Auton Nerv Syst* **34**: 95–102, 1991.
35. Larcher A, Delarue C, Idres S and Vaudry H, Interactions between vasotocin and other corticotropic factors on the frog adrenal gland. *J Ster Chem Mol Biol* **41**: 795–798, 1992.
36. Leboulenger F, Benyamina M, Delarue C, Netchitailo P, Saint-Pierre S and Vaudry H, Neuronal and paracrine regulation of adrenal steroidogenesis: interactions between acetylcholine, serotonin and vasoactive intestinal peptide (VIP) on corticosteroid production by frog interrenal tissue. *Brain Res* **453**: 103–109, 1988.
37. Jones CT, Edwards AV and Bloom SR, Endocrine responses to intra-aortic infusions of acetylcholine in concious calves. *J Physiol* **439**: 481–499, 1991.
38. Lambrecht G, Feifel R, Wagner-Röder M, Strohmann C, Zilch H, Tacke R, Waelbroeck M, Christophe J, Boddeke H and Mutschler E, Affinity profiles of hexahydro-sila-difenidol analogues at muscarinic receptor subtypes. *Eur J Pharmacol* **168**: 71–80, 1989.
39. Eglen RM, Adham N and Whiting RL, Acute desensitisation of muscarinic receptors in the isolated guinea-pig ileal longitudinal muscle. *J Auton Pharmacol* **12**: 137–148, 1992.
40. Bungardt E, Vockert E, Feifel R, Moser U, Tacke R, Mutchler E, Lambrecht G and Surprenant A, Characterisation of muscarinic receptors mediating vasodilation in guinea-pig ileum submucosal arterioles

by the use of computer-assisted videomicroscopy. *Eur J Pharmacol* **213**: 53–61, 1992.

41. Russo C, Marchi M, Andrioli GC, Cavazzani P and Raiter M, Enhancement of glycine release from human brain cortex synaptosomes by acetylcholine acting at M_4 muscarinic receptors. *J Pharmacol Exp Ther* **266**: 142–146, 1993.
42. Racke K, Hey C and Wessler I, Endogenous noradrenaline release from guinea-pig isolated trachea is inhibited by activation of M_2 receptors. *Br J Pharmacol* **107**: 3–4, 1992.
43. Boddeke HW and Buttini M, Pharmacological properties of cloned muscarinic receptors expressed in A9 L cells; comparison with *in vitro* models. *Eur J Pharmacol* **202**: 151–157, 1991.
44. Stephan CC and Sastry BV, Characterization of the subtype of muscarinic receptor coupled to the stimulation of phosphoinositide hydrolysis in 132-1N1 human astrocytoma cells. *Cell Mol Biol* **38**: 701–712, 1992.