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Muscarinic receptor modulation of release of [Met^5]enkephalin immunoreactive material and catecholamines from the bovine adrenal gland

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1 Muscarinic receptor modulation of secretion from the adrenal medulla was studied using retrogradely perfused bovine tissue.

2 Atropine, at a dose not affecting 1,1 dimethyl-4-phenylpiperizinium (DMPP)-stimulated release, inhibitied the acetylcholine (ACh)-stimulated release of noradrenaline and [Met^5]enkephalin-immunoreactive material (ME-IRM).

3 DMPP-stimulated release of catecholamines and ME-IRM was potentiated by the addition of methacholine.

4 Pilocarpine significantly potentiated the release of noradrenaline only.

5 Methacholine, pilocarpine or muscarine, when infused alone, had no stimulatory affect on the basal release of ME-IRM.

6 These findings suggest that the muscarinic receptors in the bovine adrenal medulla function by potentiating the release of ME-IRM and catecholamines induced by nicotinic receptor stimulation.

Introduction

Control of catecholamine release from the bovine adrenal gland has been thought to be exclusively via the nicotinic cholinoceptor (Wilson & Kirshner, 1977). However, muscarinic receptors have been detected in the bovine adrenal medulla by different techniques. [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding to muscarinic receptors was demonstrated by Kayaalp & Neff (1979) and Barron & Hexum (1986). Indirect evidence for the existence of muscarinic receptors was shown by examining changes in phosphatidylinositol labelling (Mohd. Adnan & Hawthorne, 1981) and alterations in cytosolic Ca^{2+} concentrations (Kao & Schneider, 1985) after stimulation by muscarinic agents. An inhibition of nicotine-stimulated catecholamine release by muscarinic agonists was shown by Swilem *et al.* (1983).

Kao & Schneider (1985) demonstrated that muscarinic stimulation led to an increase in cytosolic Ca^{2+} concentration regardless of external Ca^{2+} concentration. This increase in internal Ca^{2+} was subthreshold for induction of exocytosis but may be the basis for muscarinic receptor modulation of the exocytosis mediated by nicotinic receptor stimulation. The present study was carried out to provide further information on the role of muscarinic receptors in the modulation of catecholamine release from the adrenal medulla and to expand on this by monitoring the concomitant release of [Met^5]enkephalin-immunoreactive material (ME-IRM).

Methods

Bovine adrenal glands were obtained from a local slaughterhouse and transported on ice to the laboratory. The glands were dissected free of fat and connective tissue and cannulated via the adrenal lumbar vein within one hour of death of the animal. Retrograde perfusion utilized oxygenated-Locke solution at a flow rate of 2 ml min^{-1} at ambient temperature. The adrenal capsule was punctured with a needle at multiple sites to permit the perfusate to leave the gland. Glands were equilibrated for approx-

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imately 60 min to allow a steady state release of catecholamines and ME-IRM to occur before collecting baseline (non-stimulated) fractions. All fractions were collected for 3 min periods into tubes on ice containing 0.1 ml of 0.15 M HCl and 0.1% 2-mercaptoethanol. The fractions were heated at 100°C for 10 min to destroy proteases. The HCl was then neutralized with 0.1 ml of 0.15 M NaOH and an aliquot taken for catecholamine determination by high performance liquid chromatography with electrochemical detection (h.p.l.c.-e.c). Fractions for catecholamine analysis were preserved with 0.2 mM $NaHSO_3$ and 0.78 M $HClO_4$ immediately after neutralization with NaOH, and then frozen. The remaining perfusate was centrifuged at 12,350 *g* for 10 min and a 0.3 ml aliquot taken for radioimmunochemical analysis of ME-IRM (Barron & Hexum, 1984).

Physostigmine was added to the perfusing medium (1×10^{-5} M, final concentration) when ACh was used as the stimulating drug. Drugs were dissolved in Locke solution and infused into the cannulating lines at 0.02 ml min^{-1}. Acetylcholine perchlorate (ACh), 1,1 dimethyl-4-phenylpiperizinium iodide (DMPP), acetyl-β-methylcholine bromide (methacholine), muscarine chloride, pilocarpine HCl and atropine sulphate were obtained from Sigma Chemical Co., St. Louis, Mo.

Catecholamines were isolated from perfusates by alumina extraction (Davis & Kissinger, 1981; Goldstein & Fuerstein, 1981); 3, 4 dihydroxybenzylamine (DHBA) was added as the internal standard. Processed catecholamine samples were applied to a 5 micron C-18 μBondapak h.p.l.c. column through a Rheodyne injector. The mobile phase was 0.1 M potassium phosphate, pH 3.0, containing 0.1 mM EDTA, 0.6 mM Na octyl sulphate, and 10% methanol, pumped at 1 ml min^{-1}. Noradrenaline and adrenaline (1–10 pmol) were detected by a BAS electrochemical detector with a glassy carbon electrode set at 0.65 V with a sensitivity of 0.1 to 1 nA.

Results

The dose of ACh or DMPP necessary to stimulate the release of ME-IRM and catecholamines from the perfused adrenal gland was determined by infusing increasing concentrations of each drug. ACh or DMPP was infused for a 9 min period with a re-equilibration period of 30 min followed by stimulation with another concentration of drug. Glands were stimulated up to four times. The increased outflow of ME-IRM stimulated by ACh and DMPP was concomitant with the release of catecholamines (Figures 1 and 2). Stimulated release of catecholamines and ME-IRM are expressed as the % change from baseline

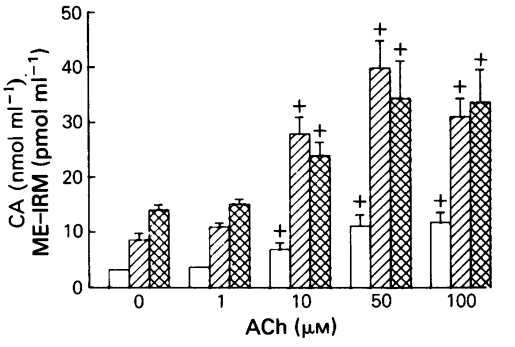

Figure 1 Dose-response pattern of release [Met^5] enkephalin-immunoreactive material (ME-IRM) and catecholamines from bovine adrenal glands during stimulation by increasing concentrations of acetylcholine (ACh). ACh was infused in the presence of 1×10^{-5} M physostigmine. A baseline sample of perfusate was collected followed by the lowest concentration of ACh. Each stimulation of the gland was for a 9 min period followed by a 30 min re-equilibration period. The lowest concentration of ACh was infused first, two stimulated samples were collected, followed by a rest period then the next higher dose of ACh. Perfusates were treated as described in Methods. ME-IRM was determined radioimmunochemically. Catecholamines were assayed by h.p.l.c.-e.c. after alumina extraction. Open columns = ME-IRM, hatched columns = noradrenaline, cross-hatched columns = adrenaline. Each column represents the mean and vertical lines show s.e.mean ($n = 6$). All values of ME-IRM and catecholamines (CA) released were compared to the baseline values (no drug) by Student's paired *t* test. †$P < 0.05$.

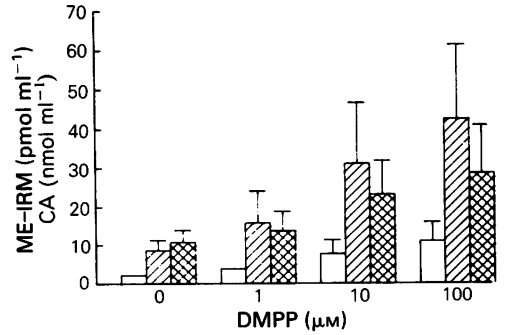

Figure 2 Release of [Met^5] enkephalin-immunoreactive material (ME-IRM) and catecholamines (CA) from bovine adrenal glands stimulated by increasing concentrations of 1, 1 dimethyl-4-phenylpipenizinium (DMPP). A baseline sample of perfusate was collected followed by the lowest concentration of DMPP. See Figure 1 for procedures and methods. Open columns = ME-IRM, hatched columns = noradrenaline, cross-hatched columns = adrenaline. Each column represents the mean and vertical lines show s.e.mean ($n = 3$).

release to minimize differences between individual glands. Noradrenaline constitutes approximately 25–30% of the total amount of catecholamines stored in the bovine adrenal gland (Hillarp & Hokfelt, 1953). However, secretion of noradrenaline is nearly equal to or greater than that of adrenaline at all doses of ACh or DMPP. This suggests a difference in the responsiveness of noradrenaline and adrenaline containing chromaffin cells.

Cholinoceptor involvement in adrenomedullary secretion was investigated by examining the influence of atropine or hexamethonium (C-6) on ACh or DMPP stimulation of secretion. Cholinoceptor antagonists were infused for 3 min before the introduction of antagonist. C-6 (5×10^{-4} M) significantly inhibited the ACh (5×10^{-5} M)-induced release of ME-IRM, adrenaline and noradrenaline. However, atropine (5×10^{-7} M) inhibited the release of noradrenaline ($P < 0.05$) but not adrenaline or ME-IRM (Figure 3). As expected, hexamethonium (5×10^{-4} M) but not atropine (5×10^{-7} M), significantly inhibited the DMPP-stimulated release of ME-IRM and catecholamines (Figure 4). The secretion of noradrenaline being more sensitive, as indicated by the dose-response curves, is reduced by muscarinic blockade whereas the secretion of adrenaline or ME-IRM is not.

The inhibition of noradrenaline but not adrenaline or ME-IRM release by atropine suggests that the optimal conditions for blockade of the muscarinic receptor had not been selected. The concentration of ACh in the perfusing medium is 100 fold higher than that of atropine and atropine is only infused for 3 min before the administration of ACh. We anticipated that by either increasing the concentration of atropine or the time of infusion we might be able to demonstrate an effect on the secretion of ME-IRM. We chose to increase the infusion time for atropine rather than its concentration since atropine at higher concentrations (1×10^{-5} M) can also block nicotinic receptors (Barron & Hexum, unpublished).

We examined this possibility by perfusing the gland with atropine (5×10^{-7} M) for 1 h before the infusion of increasing concentrations of ACh. The ACh-stimulated release of ME-IRM in the presence of atropine was compared to that in the absence of atropine by use of Student's 2 mean t test. Atropine significantly inhibited the stimulated release of ME-IRM at ACh concentrations of 5×10^{-5} and 10^{-4} M (Figure 5). Catecholamines were not determined. The shape of the resultant does-response curve (with and without atropine) may indicate a non-competitive action of atropine in inhibiting the ACh-stimulated release.

Muscarinic agonists are capable of eliciting an increase in the release of catecholamines from the adrenal glands of various species (Douglas *et al.*, 1967;

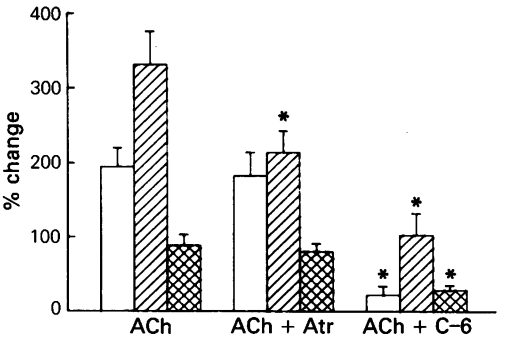

Figure 3 Acetylcholine (ACh)-stimulated release from bovine adrenal glands with or without either atropine or hexamethonium. Increased release of noradrenaline, adrenaline, and [Met^5] enkephalin-immunoreactive material (ME-IRM) is expressed as % change from baseline release and vertical lines show s.e.mean. Perfusates were treated as described in Methods. ME-IRM was determined radioimmunochemically. Catecholamines were assayed by h.p.l.c.-e.c. after alumina extraction. Drugs used were ACh (5×10^{-5} M), atropine (Atr, 5×10^{-7} M), or hexamethonium (C-6, 5×10^{-4} M). The antagonists were infused 3–6 min before the infusion of ACh. Open columns = ME-IRM, hatched columns = noradrenaline, cross-hatched columns = adrenaline. ACh ($n = 34$), ACh plus atropine ($n = 14$), ACh plus C-6 ($n = 10$). *$P < 0.05$.

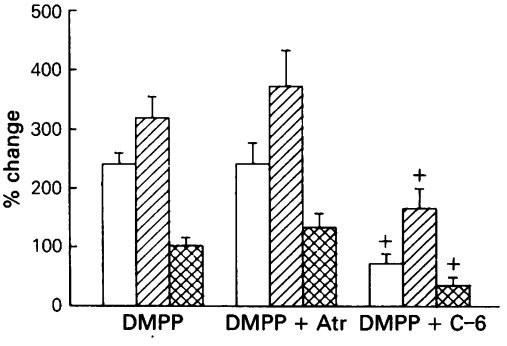

Figure 4 1, 1 Dimethyl-4-phenylpiperizinium (DMPP)-stimulated release from bovine adrenal glands with or without either atropine or hexamethonium. Increased release of noradrenaline, adrenaline, and [Met^5] enkephalin-immunoreactive material (ME-IRM) is expressed as % change from baseline release and vertical lines show s.e.mean. Perfusates were treated as described in Methods. ME-IRM was determined radioimmunochemically. Catecholamines were assayed by h.p.l.c.-e.c. after alumina extraction. Drugs used were DMPP (5×10^{-5} M), atropine (Atr, 5×10^{-7} M) or hexamethonium (C-6, 5×10^{-4} M). The antagonists were infused 3–6 min before the infusion of DMPP. Open columns = ME-IRM, hatched columns = noradrenaline, cross-hatched columns = adrenaline, DMPP ($n = 42$). DMPP plus atropine ($n = 24$), DMPP plus C-6 ($n = 15$). †$P < 0.05$).

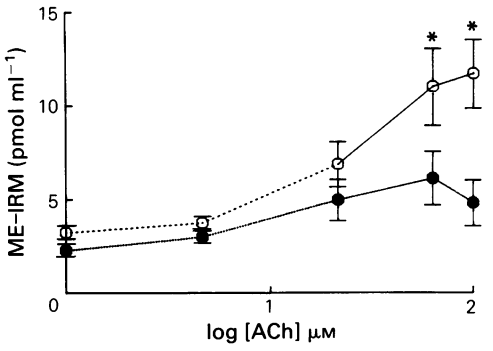

Figure 5 Bovine adrenal gland stimulation by increasing concentrations of acetylcholine (ACh) with or without continuous infusion of atropine (5×10^{-7} M). Glands were equilibrated with atropine for one hour. Infusion of ACh was for a 9 min period separated by a 30 min rest period between each stimulation. Each gland was stimulated four times with a higher concentration of ACh each time. Perfusates were assayed for [Met^5] enkephalin-immunoreactive material (ME-IRM) radioimmunochemically. Each point represents the mean with vertical lines showing s.e.mean ($n = 6$). ACh-stimulated ME-IRM release was compared to ACh-stimulated release in the presence of atropine (*$P < 0.05$) by Student's 2 mean t test. Values obtained at 10, 50 and 100 μM ACh were significantly different from the values obtained in the absence of ACh (analysed by Student's paired t test, $P < 0.05$). (●) ACh-stimulated in the presence of atropine, (○) ACh only.

Tsujimoto & Nishikawa, 1975; Corder *et al.*, 1982). The bovine adrenal gland, however, has previously been shown to be insensitive to the action of muscarinic agonists (Wilson & Kirshner, 1977). In agreement with these findings we determined that the introduction of either muscarine or pilocarpine (5×10^{-5} M) in the absence of a nicotinic agent had no affect on the release of catecholamines or ME-IRM from the bovine adrenal medulla (data not shown). The partial inhibition of ACh-stimulated release from this same tissue by atropine suggests the possibility that muscarinic receptor stimulation could modulate the release of catecholamines and ME-IRM brought about by nicotinic receptor stimulation. Our approach was to examine the effect of various muscarinic agents in the presence of DMPP, a pure nicotinic receptor agonist. The dose of DMPP previously used (5×10^{-5} M) was near the top of the dose-response curve (Figure 2). Thus a positive modulatory role for the muscarinic agonist may not be apparent under these conditions where the gland is already maximally stimulated. The dose of DMPP was therefore lowered to 1×10^{-5} M before adding the muscarinic agonists. The addition of methacholine (4×10^{-5} M) to the perfusion medium during stimulation by DMPP resulted in a significant increase in the release of both catecholamines and ME-IRM (Table 1). Pilocarpine (4×10^{-5} M) addition to the DMPP-stimulated glands resulted in a significant increase of only noradrenaline release. The stimulated release of catecholamines and ME-IRM was analysed by Student's paired t test (DMPP alone compared to DMPP plus methacholine or plus pilocarpine).

Discussion

The role of muscarinic receptors in stimulus-secretion coupling in the adrenal gland is undisputed in the rat, gerbil, cat and dog (Douglas *et al.*, 1967; Tsujimoto & Nishikawa, 1975; Role & Perlman, 1983; Wakade &

Table 1 Effect of methacholine and pilocarpine on 1, 1 dimethyl-4-phenylpiperizinium (DMPP) stimulation of release from the bovine adrenal gland

	ME-IRM (pmol ml^{-1})	*Noradrenaline* (nmol ml^{-1})	*Adrenaline* (nmol ml^{-1})
Baseline	2.08 ± 0.26	7.96 ± 0.85	11.61 ± 1.49
DMPP	5.38 ± 1.67**	26.35 ± 4.6**	18.69 ± 2.03**
DMPP + methacholine	6.13 ± 1.7*,**	32.36 ± 5.28*,**	20.49 ± 2.16*,**
Baseline	2.94 ± 0.45	8.32 ± 0.5	10.30 ± 1.26
DMPP	5.50 ± 1.08**	19.50 ± 4.7**	16.97 ± 3.23**
DMPP + pilocarpine	6.36 ± 1.47**	24.40 ± 5.97*,**	18.08 ± 3.48**

Bovine adrenal glands retrogradely perfused as described in Methods were stimulated with DMPP (1×10^{-5} M). Methacholine ($n = 6$) or pilocarpine ($n = 6$) (each at 4×10^{-5} M) were infused 5 min after the start of the DMPP infusion. Stimulated samples were collected for 3 min periods starting 3 min after the beginning of the DMPP infusion. Samples were assayed for [Met^5] enkephalin-immunoreactive material (ME-IRM) and catecholamines as described in Methods. Values given are the mean ± s.e.mean Statistical analysis was by Student's paired t test. DMPP plus methacholine or pilocarpine were compared to DMPP alone (*$P < 0.05$). DMPP, DMPP plus methacholine or pilocarpine were compared to baseline (**$P < 0.05$).

Wakade, 1983). In these species, muscarinic agonists when infused alone stimulate secretion of catecholamines from the adrenal gland. The bovine adrenal gland does not respond to the infusion of muscarinic agonists by an increase in secretion of catecholamines (Wilson & Kirshner, 1977; Mizobe & Livett, 1983; this paper). Nevertheless, this tissue has been shown to be responsive to this type of stimulation. Muscarinic agonists increase the incorporation of ^{32}P into phosphatidylinositol (Mohd. Adnan & Hawthorne, 1981) and increase guanosine 3', 5'-monophosphate levels in bovine adrenal medullary cells (Yanagihara *et al.*, 1979). Using the fluorescent probe, 2-{[2-bis-(carboxymethyl)-amino-5-methylphenoxy]-methyl}-6-methoxy-8-bis-(carboxymethyl)-amino-quinoline (Quin-2) for intracellular Ca^{2+}, Kao & Schneider (1985) revealed that muscarinic agonists could increase cytosolic Ca^{2+} independent of the extracellular Ca^{2+} concentration. This increase was subthreshold for exocytosis of catecholamines. In conjunction with these findings, muscarinic receptors have been characterized in the bovine adrenal gland (Kayaalp & Neff, 1979; Barron & Hexum, 1986). The action of muscarinic agonists in the bovine adrenal gland may therefore be a modulatory one rather than a direct effect.

Recently, Swilem *et al.*, (1983) showed that muscarinic agonists modulate the nicotinic activation of catecholamine secretion in an inhibitory fashion. This inhibition of catecholamine release from the bovine adrenal gland may be attributable to the high concentration of nicotine used (3×10^{-4} M). The muscarinic agonist potentiation of nicotine-stimulated release of catecholamines may not be demonstrable at this dose of nicotine. The addition of a muscarinic agent may have added to the depolarization blockade that is expected at 3×10^{-4} M nicotine. In studies of this type it may be more advantageous to choose a concentration of the agent in question that is at an intermediate point on the dose-response curve to permit the demonstration of either inhibition or potentiation.

We re-examined this issue by investigating the dose-response relationship of adrenal gland stimulation to ACh or DMPP in the release of catecholamine and ME-IRM. The bovine adrenal gland responded to either ACh or DMPP with an increased release of noradrenaline, adrenaline and ME-IRM. The secretion of noradrenaline was nearly equal to or greater than that of adrenaline. Since the bovine adrenal medulla contains four times more adrenaline than noradrenaline (Hillarp & Hokfelt, 1953) it is of some interest that the secretion of noradrenaline is equal to or greater than that of adrenaline. This may be attributable to the technique of retrograde perfusion and the anatomy of the adrenal gland wherein the noradrenaline-containing chromaffin cells are adjacent to the adrenal lumbar vein and the adrenaline-containing chromaffin cells are adjacent to the adrenal cortex. Thus, the likelihood of interaction between the agonist and the noradrenaline-containing chromaffin cells is greater than with the adrenaline-containing cells.

The blockade of the nicotinic receptor by hexamethonium (C-6) resulted in a greatly diminished response of the gland to ACh or DMPP in release of both catecholamines and ME-IRM. These findings confirm data published earlier (Kilpatrick, *et al.*, 1980; Barron & Hexum, 1984). In these experiments, C-6 did not completely abolish the response of the gland to stimulation, despite the fact that the concentration of C-6 was greater than the concentration of stimulating drug. This further supports the role of receptors other than the nicotinic cholinoceptor as being involved in stimulus-secretion coupling in the bovine adrenal medulla.

The role of the muscarinic receptor was examined by studying the ability of atropine to alter the action of cholinoceptor agonists. The retrograde infusion of atropine (5×10^{-7} M) into bovine adrenal glands inhibited the release of ME-IRM and noradrenaline induced by ACh. Atropine did not block the DMPP-stimulated release of ME-IRM and catecholamines from the bovine adrenal gland. Garrett *et al.* (1965) demonstrated the inhibition of neostigmine-stimulated release of catecholamines with a similar dose of atropine (1×10^{-7} M). Swilem *et al.* (1983) used pilocarpine or methacholine in a high dose (1×10^{-4} M), each combined with a high dose of nicotine (3×10^{-4} M), to demonstrate the inhibition of catecholamine release by the muscarinic agonists. This finding does not agree with that of Garrett *et al.* 1965, or the data shown here. When methacholine or pilocarpine (4×10^{-5} M) was added to a submaximal dose of DMPP (1×10^{-5} M) there was a significant potentiation in the release of ME-IRM and catecholamines (Table 1). The doses of methacholine and pilocarpine infused alone were the same as the dose of ACh, yet had no effect on the basal release of catecholamines and Me-IRM. We have shown that pilocarpine is slightly more potent than ACh in displacing [^{3}H]-QNB from muscarinic binding sites in the bovine adrenal medulla (Barron & Hexum, 1986).

The evidence presented here, i.e. the inhibition by a low dose of atropine (which was ineffective in blocking nicotinic-stimulated catecholamine and ME-IRM secretion) and the potentiation of release by methacholine and pilocarpine, along with the previous finding of muscarinic receptors in the bovine adrenal gland through [^{3}H]-QNB binding studies (Kayaalp & Neff, 1979; Barron & Hexum, 1986), indicates that there is a muscarinic receptor in the bovine adrenal gland that is involved in stimulus-secretion coupling. The stimulation of the muscarinic receptor in the bovine adrenal gland by itself is not sufficient to elicit

release of ME-IRM and catecholamines but does appear to modulate secretion stimulated by nicotinic receptor activation.

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