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EFFECTS OF OPIOID COMPOUNDS ON BASAL AND MUSCARINIC INDUCED ACCUMULATION OF INOSITOL PHOSPHATES IN CULTURED BOVINE CHROMAFFIN CELLS

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Abstract—The mammalian adrenal medulla expresses a variety of both opioid peptides and opioid receptors. The function of this adrenal opioid system is, however, largely unknown. We have examined the ability of a number of opioid compounds to influence basal and muscarinic stimulated accumulation of inositol phosphates in cultured bovine chromaffin cells. Muscarine produced a dose-dependent 1.5-fold increase in total inositol phosphates. This response was sensitive to atropine inhibition. The ten opioid compounds examined were chosen because between them they possess selectivity for all of the identified opioid receptor subtypes. However, none of these opioids in the concentration range 10 nM–10 μM had any significant effect on either basal or muscarinic induced total inositol phosphate accumulation. We conclude that it is unlikely that opioid peptides released from either the chromaffin cells themselves or the splanchnic nerve can modulate the inositol phosphate second messenger system within the adrenal chromaffin cells.

The mammalian adrenal medulla contains a large number of neuroactive substances, including high concentrations of the opioid peptides [1]. Immunohistochemical studies indicate that these adrenal opioid peptides are present both within the chromaffin cells and within the nerves innervating the adrenal medulla [2, 3]. Opioid peptides are released from the adrenal gland *in vivo* by splanchnic nerve stimulation [4, 5]. The mechanism of release has been studied, *in vitro*, using isolated chromaffin cells stimulated with nicotinic agonists [6, 7].

In addition to opioid peptides the adrenal chromaffin cells possess high affinity, stereospecific opioid binding sites [8–14]. It is possible, therefore, that opioid peptides released from the splanchnic nerve, or the chromaffin cells themselves, act through these opioid receptors to modify chromaffin cell function.

Opioids have been reported to inhibit nicotinic induced catecholamine secretion from cultured bovine chromaffin cells [9–12, 15–19]. These actions are, however, unlikely to be mediated via the opioid receptor sites identified with binding studies because they are weak, lack stereospecificity and are poorly antagonized by opioid antagonists [15, 17, 18].

In addition to nicotinic acetylcholine receptors the bovine adrenal medulla possesses muscarinic receptors [20–24]. In some species, these muscarinic receptors mediate catecholamine secretion (see Ref. 25). Their role in the bovine adrenal is, however, less well established. Both muscarinic mediated inhibition and enhancement of the nicotinic induced catecholamine secretion have been reported [23, 26–28]. Activation of these muscarinic receptors leads to an increased cGMP formation [19, 20, 21, 23, 29], a stimulation of inositol phospholipid turnover [22, 27, 30, 31] and an increase in intracellular calcium concentration [25, 32].

In this study we have measured the accumulation of inositol phosphates as an index of muscarinic receptor activation to examine the influence of a number of different opioid compounds on muscarinic receptor function.

MATERIALS AND METHODS

Preparation of chromaffin cells. Cells were prepared by collagenase digestion of bovine adrenal glands, purified by Percoll (TM) density gradient centrifugation and cultured in 6 well collagen-coated, plastic culture dishes at a density of 1.5 to 2×10^6 cells per well [33]. Cells were cultured for 2 days prior to loading with [^{3}H]inositol. The culture medium was replaced with 1 ml of the same medium (Dulbecco's modified Eagles medium and Hams F12 (1:1), with fetal calf serum reduced from 10% to 2%, 25 μg/ml fluorodeoxyuridine, 2.5 μg/ml cytosine arabinoside, 2.5 μ/ml uridine, 5 μg/ml nystatin, and 100 μg/ml each of penicillin and streptomycin) containing 5 μCi/ml myo-[2-^{3}H]inositol and cultured for a further 24 hr.

Stimulation of inositol phosphate accumulation. The loading medium was removed and each well received two consecutive 5-min washes at 37° in incubation buffer (149 mM NaCl, 2.6 mM KCl,

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† Abbreviations used: PI, phosphatidylinositol; TCA, trichloroacetic acid; IP, inositol phosphates (including mono, bis and tris phosphates) IP_1, inositol 1-phosphate; IP_2, inositol 1,4-biphosphate; IP_3, inositol trisphosphate; EC_{50}, the concentration of drug producing half maximal response; GTP, guanosine 5′-triphosphate; DAGO, H-Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; EKC, ethylketocyclazocine.

2.15 mM K_2HPO_4, 0.85 mM KH_2PO_4, 1.18 mM $MgSO_4$, 5 mM LiCl and 10 mM glucose). The cells were then stimulated over a further 37° incubation period with the same incubation buffer containing appropriate drugs. When assessing the effect of an opioid compound on muscarinic-induced accumulation of inositol phosphates, the compound was included in both the second wash and stimulation period. The incubation was terminated by replacing the buffer with 1 ml ice-cold 15% TCA. The cells were extracted for 1 hr, the TCA collected in glass tubes and the cells rinsed with a further 0.5 ml of 15% TCA.

Analysis of inositol phosphates. Inositol phosphates were extracted and analysed using a modified procedure of Berridge *et al.* [34]. TCA was removed from the cell extracts with 3 ether washes, the aqueous phase neutralized and applied to an anion exchange column (1 ml of a 1:1 mixture of water and Bio-Rad AG1-X8 resin 100–200 mesh formate form, to give a column of approximately 2 cm). [^{3}H]Inositol and [^{3}H]glycerophosphoinositol were removed from the columns by eluting with 10 ml of water and 10 ml of 60 mM sodium formate, respectively. Total [^{3}H]inositol phosphates were eluted with 2 × 5 ml of 1.0 M ammonium formate in 0.1 M formic acid. In some experiments [^{3}H]inositol phosphates were fractionated into [^{3}H]IP, [^{3}H]IP_2 and [^{3}H]IP_3 by eluting with 2 × 5 ml of increasing concentrations (0.2, 0.4, 1.0 M) of ammonium formate in 0.1 M formic acid. Each fraction was collected and the radioactive content determined by gel-phase liquid scintillation counting (18–25% efficiency with Amersham ACS).

Materials. Etorphine and diprenorphine HCl were gifts from Mr I. Mawhinney (C-Vet, U.K.), EKC a gift from Dr Michne (Sterling Winthrop, Columbia, U.S.A.), Bremazocine a gift from Dr Maurer (Sandoz Basle, Switzerland), and Thiorphan a gift from Dr J. C. Schwartz (Paris). Dynorphin(1-13) and metorphamide were synthetized within the department. All other peptides were obtained from Peninsula Lab. (San Carlos, CA), or Cambridge Research Biochemicals (Harston, U.K.). Muscarine was obtained from Sigma Chem. Co. (St Louis, MO). Myo-[2-^{3}H]inositol (1 mCi/ml, 15–20 Ci/mmol), and ACS scintillation fluid were obtained from Amersham Int. (U.K.).

RESULTS

Muscarine-stimulated accumulation of inositol phosphates

During a 30-min stimulation period muscarine produced a dose-dependent increase in the production of [^{3}H]inositol phosphates from cultured bovine adrenal chromaffin cells. The threshold for the response was in the region of 1 μM. The EC_{50} was approximately 25 μM, and maximal increases were observed between 100 μM and 1 mM muscarine (Fig. 1). This muscarine-induced accumulation of inositol phosphates was sensitive to inhibition by atropine. At 0.1 μM, atropine produced a partial and variable suppression of the response to a 30-min stimulation with 25 μM muscarine. At 1 μM atropine fully inhibited this response and in some experiments

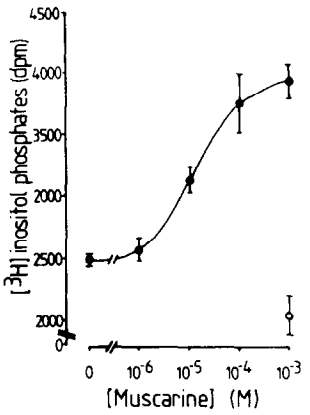

Fig. 1. Dose response curve for muscarine stimulated inositol phosphate accumulation in cultured bovine chromaffin cells. Cells were incubated for 30 min with increasing concentrations of muscarine and the total inositol phosphates extracted as described in Materials and Methods. Values expressed as dpm of total [^{3}H]inositol phosphate extracted per culture well ± SEM (N = 6 data pooled from 3 separate cell preparations). The maximal response was fully inhibited by 1 μM atropine (open circle).

suppressed the accumulation of inositol phosphates to below basal levels (as in Fig. 1). At higher concentrations this atropine-induced suppression of the basal response became very marked.

The time course of the response to 50 μM muscarine was examined in more detail. Each muscarinic stimulation was compared to the response produced by incubating cells with buffer alone for the same time period, under identical conditions. There was a rapid increase in IP_3 following muscarinic stimulation. IP_3 was 136 ± 14% ($P < 0.025$) of basal after 1 min incubation, the increase slowed after 5 min at 172 ± 5% ($P < 0.01$) to reach a maximal 189 ± 23% ($P < 0.005$) after 15 min (Fig. 2c). IP_2 levels increased more slowly than IP_3, rising from slightly greater than basal levels after 1 min of incubation, to a maximal of 185 ± 14% ($P < 0.005$) after 15 min (Fig. 2b). IP levels increased linearly throughout the time course, from basal levels after 1 min to an accumulated maximum of 194 ± 16% ($P < 0.005$) of basal after 30 min (Fig. 2a).

Effect of etorphine on basal and muscarine-stimulated accumulation of inositol phosphates

The universal opioid agonist etorphine (100 nM to 1 mM) had no significant effect on basal IP_3, IP_2 or IP levels over a 15-min incubation period (Fig. 3). Similarly, etorphine over the same concentration range had no significant effect on the IP_3, IP_2 or IP levels produced during a 15-min incubation with maximal (100 μM) or EC_{50} (25 μM) muscarine stimulations (Fig. 3).

Effect of opioid receptor-subtype selective agonists on basal and muscarinic-stimulated accumulation of inositol phosphates

The effect of a number of opioid receptor-subtype selective ligands on accumulation of inositol phosphates was assessed by measuring total inositol phos-

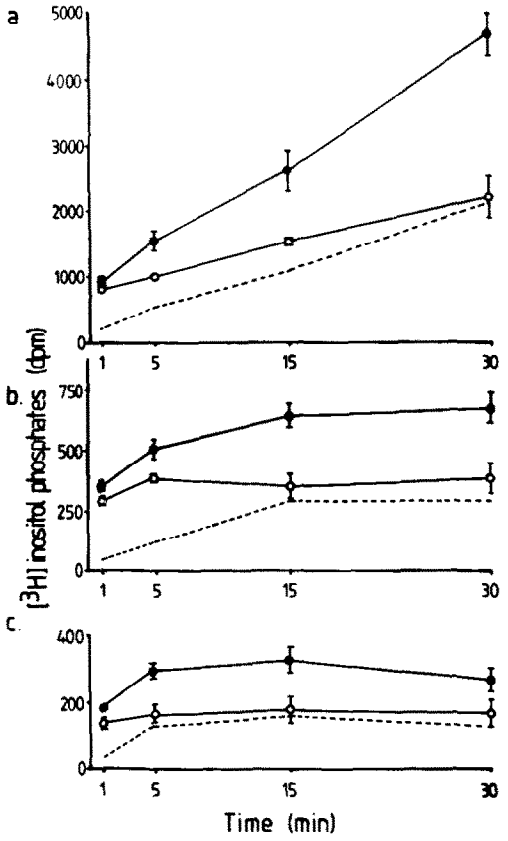

Fig. 2. Time course of muscarine stimulation of accumulation of inositol phosphates metabolism. Values expressed as dpm [^{3}H]inositol phosphate extracted per culture well ± SEM (N = 6 data pooled from 3 separate cell preparations). (a) [^{3}H]IP accumulation, (b) [^{3}H]IP_2 accumulation, (c) [^{3}H]IP_3 accumulation. Cells were incubated and inositol phosphates extracted as described in Materials and Methods. Open circles represent the unstimulated levels of the various inositol phosphates, filled circles represent the muscarine stimulated (50 μM) levels and the dotted line represents the muscarine response with the basal levels subtracted.

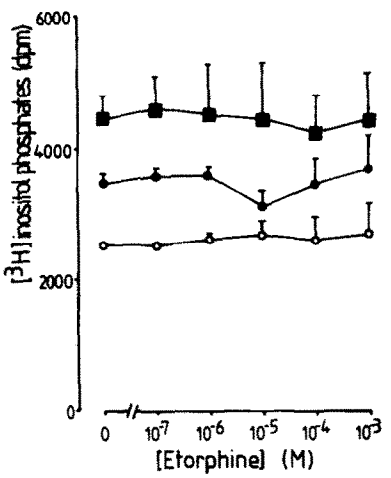

Fig. 3. Effect of etorphine on basal (open circles), 25 μM muscarine (filled circles) or 100 μM muscarine (square symbols) stimulated accumulation of inositol phosphates metabolism. Cells were incubated for 30 min and the total inositol phosphates extracted as described in Materials and Methods. Values expressed as dpm [^{3}H]inositol extracted per culture well ± SEM (N = 6 data pooled from 3 separate cell preparations).

phate accumulation following a 15 min incubation with the opioid or the opioid in the presence of 50 μM muscarine. None of the selective opioid ligands at the two concentrations examined (10 nM and 10 μM) had any significant effect on either basal (Table 1) or muscarine stimulated IP accumulation (Table 2). The inclusion of a peptidase inhibitor-cocktail (15 μM bestatin, 0.1 μM thiorphan and 37 U/ml bacitracin, see Ref. 17) for the opioid peptides tested suppressed muscarinic stimulation but did not reveal any opioid influence (data not shown). The opioid antagonist diprenorphine (at 10 nM and 10 μM) was also without effect on either the basal or muscarine stimulated accumulation of inositol phosphates.

Table 1. Effect of opioid compounds on the accumulation of inositol phosphates

	10 nM			10 μM		
Opioid	Activity	SEM	N	Activity	SEM	N
Ethylketocyclazocine	92	10.0	6	102	3.0	6
Bremazocine	103	9.0	6	107	5.0	6
DAGO	107	4.0	6	98	9.0	5
DSLET	105	2.0	6	102	8.0	6
Dynorphin(1-13)	105	9.0	6	93	4.0	6
Met-enk-Arg-Gly-Leu	105	5.0	5	102	8.0	5
Met-enk-Arg-Phe	107	9.0	4	96	1.0	4
Metorphamide	107	4.0	4	103	7.0	4
Diprenorphine	103	3.0	6	102	7.0	6

Cells incubated in the presence or absence of 10 nM or 10 μM opioid for 30 min. Activity expressed as a percentage of inositol phosphate accumulation following incubation with buffer alone. Data pooled from 2–3 cell separate cell preparations. No significant difference from basal levels under any condition $P > 0.05$ (two-tailed Student t-test).

Table 2. Effect of opioid compounds on muscarine stimulated accumulation of inositol phosphates

Opioid	10 nM			10 μM		
	Activity	SEM	N	Activity	SEM	N
Ethylketocyclazocine	96	2.0	4	100	3.0	6
Bremazocine	102	6.0	4	103	1.0	4
DAGO	99	3.0	6	93	13.0	5
DSLET	109	1.0	5	98	7.0	5
Dynorphin(1-13)	106	12.0	4	100	1.0	5
Met-enk-Arg-Gly-Leu	98	7.0	4	89	3.0	5
Met-enk-Arg-Phe	89	2.0	4	88	5.0	5
Metorphamide	103	1.0	4	104	4.0	4
Diprenorphine	101	1.0	6	103	3.0	6

Cells incubated with 50 μM muscarine in the presence or absence of 10 nM or 10 μm opioid for 30 min. Activity expressed as a percentage of total inositol phosphates accumulated in cultures incubated with muscarine alone. Data pooled from 2–3 cell separate cell preparations. No significant difference from control muscarine stimulated levels under any condition $P > 0.05$ (two-tailed Student *t*-test).

DISCUSSION

It is well established that muscarinic stimulation of adrenal chromaffin cells activates inositol phospholipid metabolism [22, 24, 30, 31]. It was only recently, however, that direct measurements were made of IP_3 accumulation in these cells following muscarinic stimulation with the non-selective cholinergic agonist carbachol [27]. In these current studies we have reexamined this phenomenon using the selective muscarinic agonist muscarine. Our observations reported here, of an early IP_3 increase, with delayed IP_2 and IP accumulations are in full agreement with this latter report. The earliest time point that we could reliably examine in our system was 1 min. Therefore, although there was a significant increase in IP_3 after this time, we cannot confirm the evidence for a more rapid rise in this inositol phosphate [27]. The muscarinic nature of the stimulated accumulation of inositol phosphates reported here is clearly illustrated by its sensitivity to atropine inhibition. The concentration–response relationship for muscarine-induced accumulation of inositol phosphates in cultured chromaffin cells is very similar to that reported for muscarine-induced accumulation of inositol phosphates in the sympathetic ganglia [35]. This micromolar EC_{50} would be consistent with the recent suggestion of Yamanaka *et al.* [24] that muscarinic induced accumulation of inositol phosphates in the bovine chromaffin cells is mediated via low (μM) affinity receptors, while cGMP formation may perhaps be stimulated by high (nM) affinity muscarinic receptors.

A great deal of evidence has accumulated to support the existence of opioid receptors on the bovine adrenal chromaffin cells. This work culminated in a detailed examination by Castanas *et al.* [13, 14] of a membrane preparation of the bovine adrenal medulla, which reported the existence of multiple subtypes of kappa opioid receptors with lower levels of the mu and delta receptor subtypes. The function of these adrenal opioid receptors is, however, largely unknown.

In other systems, delta opioid receptors appear to be negatively coupled through a GTP-binding protein, to the adenylate cyclase system [36]. A recent report indicates that although adrenal medullary opioid receptors are also linked to a GTP-binding protein, it is unlikely that these are coupled to adenylate cyclase [37]. This raises the possibility that the adrenal opioid receptors may be coupled to an alternative second messenger system, such as poly-phosphoinositol metabolism. Interestingly, Leach *et al.* [38] have recently reported that the opioid peptide Leu-enkephalin can stimulate inositol phosphate accumulation in isolated hepatocytes.

The opioid agonist etorphine was chosen for this study because it is a potent agonist at mu, delta and kappa opioid receptors. A number of opioid receptor subtype-selective ligands were also examined because it is possible that etorphine may not be a good agonist at the novel opioid receptor subtypes in the bovine adrenal medulla [14], or that an effect at one receptor subtype could be masked by an opposing action at another subtype. This possibility seems unlikely, however, because none of the subtype selective opioid compounds tested had any significant influence on the accumulation of inositol phosphates.

DAGO is a highly selective mu agonist while DADLE interacts with both mu and delta sites. EKC and bremazocine are kappa drugs while dynorphin $A_{(1-13)}$ is a possible endogenous ligand for the kappa opioid receptor, and the kappa 1 subtype of the bovine adrenal medulla (see Refs 13, 14, 39 and 40 for ligand selectivities). Metorphamide is a novel endogenous adrenal opioid peptide [41], and Met-enk-Arg-Gly-Leu and Met-enk-Arg-Phe show relative selectivity for the proposed kappa 2 and kappa 3 adrenal opioid binding sites respectively [14].

It is noteworthy that although in previous studies we have found dynorphin$_{(1-13)}$ and metorphamide to be the most potent opioids in inhibiting catecholamine secretion from these cells [17, 18], these opioid peptides, however, exhibited no influence on the accumulation of inositol phosphates. Incubation with the opioid antagonist diprenorphine was also without influence, indicating that receptor occupancy by endogenous opioids was not a significant factor.

The findings reported here indicate that it is

unlikely that any of the adrenal opioid receptors are coupled to the poly-phosphoinositol second messenger system because neither etorphine nor any of the opioid receptor-subtype selective ligands tested had any significant effect on accumulation of inositol phosphates. The action of peptidases in degrading the opioid peptide ligands did not appear to be a significant factor in preventing an opioid action, because the addition of peptidase inhibitors did not enhance opioid potency. This failure of opioid ligands to influence accumulation of inositol phosphates in cultured adrenal chromaffin cells is in agreement with a less extensive study which examined the influence of DADLE and dynorphin [42]. In contrast to these observations on chromaffin cells and other neuronal systems [43, 44, 28] Leu-enkephalin has been reported to stimulate inositol phosphate accumulation in isolated hepatocytes [38]. This is probably due to tissue specific differences in opioid receptor mechanisms because similar high concentrations of the metabolically stable Leu-enkephalin analogue DADLE failed to induce inositol phosphate accumulation in this present study.

Furthermore, none of the opioids tested had any significant influence on muscarine-induced accumulation of inositol phosphates in the chromaffin cells. This observation suggests that the adrenal opioid receptors, and by inference the adrenal opioid peptides, are unlikely to be involved in modulating the muscarinic stimulation of the chromaffin cells produced by acetylcholine released from the splanchnic nerve.

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