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OPIOID INHIBITION OF NICOTINE-INDUCED $^{45}Ca^{2+}$-UPTAKE INTO CULTURED BOVINE ADRENAL MEDULLARY CELLS

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Abstract—The ability of a number of opioid agonists and antagonists to affect nicotine-induced $^{45}Ca^{2+}$-uptake into cultured bovine adrenal medullary cells has been investigated. High (10 μM) concentrations of the opioid agonist bremazocine produced a significant inhibition of nicotine-induced $^{45}Ca^{2+}$-uptake throughout the 15 min time course examined. The opioid subtype-selectivity of this inhibition was investigated; mu and delta selective agonists produced only minor effects whereas the kappa selective agonist U50-488H and the endogenous opioid peptides dynorphin$_{(1-13)}$ and metorphamide almost abolished nicotine-induced $^{45}Ca^{2+}$-uptake. The U50-488H inhibition was significant at 10 nM concentrations with an IC_{50} of approximately 1 μM. U50-488H inhibition could not be reversed or reduced by the opioid antagonists naxolone, diprenophine or Mr2266. Furthermore, Mr2266 and its optical isomer Mr2267 also produced marked inhibition of $^{45}Ca^{2+}$-uptake. The inhibition was specific to nicotine-induced $^{45}Ca^{2+}$-uptake in that a similar level of uptake evoked by potassium depolarization was unaffected by high concentrations of U50-488H. These data indicate that opioid inhibition of nicotine-induced $^{45}Ca^{2+}$-uptake does not involve classical, stereospecific opioid receptors and suggests the involvement of a pharmacologically distinct opioid recognition site. It is speculated that this may be associated with the nicotine receptor–ionophore complex.

In common with many biologically active substances opioid drugs and opioid peptides mediate their effects by interacting with highly specific receptors located at the cell surface. Extensive radioligand binding studies in many different tissues have demonstrated the presence of multiple subtypes of high affinity stereospecific opioid binding sites. These binding sites are generally believed to correspond to multiple subtypes of the opioid receptor termed mu (at which morphine is the prototype agonist), delta (enkephalin), kappa (ketazocine) and sigma (*N*-allylnormetazocine).

A number of investigations have demonstrated the presence of these opioid binding sites in membranes or homogenates prepared from the bovine adrenal medulla [1–8]. Castanas *et al.* [9, 10] used subtype-selective radioligands to demonstrate the presence of moderate levels of mu and delta and higher levels of kappa opioid binding sites in membranes prepared from the bovine adrenal medulla.

Opioid receptors located within the adrenal medulla are likely to be stimulated by endogenous opioid peptides. Immunohistochemical investigations have demonstrated that opioid peptides are found both in the adrenal chromaffin cells and in the terminals of the splanchnic nerve innervating the tissue [11]. Stimulation of the intact gland [12–15] or isolated chromaffin cells [16–22] results in release of a variety of opioid peptides which would in theory then be available to interact with adrenal medullary opioid receptors.

In contrast to this substantial evidence supporting the existence of multiple opioid binding sites within the bovine adrenal medulla, the function of these presumed opioid receptors has remained controversial. Opiate drugs and opioid peptides have been reported to inhibit nicotinic-induced catecholamine secretion from adrenal medullary chromaffin cells *in vitro* [3, 5, 6, 23–26]. It seems unlikely however, that this inhibition is mediated through the opioid binding sites identified from radioligand studies because in contrast to these binding sites opioid mediated inhibition of nicotinic-induced catecholamine secretion required relatively high concentrations of the opioids and in some [6, 23–26] but not all [3, 5] studies was found to lack stereospecificity and to be insensitive to opioid antagonists.

It is well established that the exocytotic release process from adrenal medullary chromaffin cells, and indeed many other tissues, is dependent on the influx of extracellular calcium ions. Interestingly, there is considerable evidence to suggest that opioid receptors, particularly those of the kappa subtype, mediate their effects by inhibiting calcium-influx [27, 28]. In the light of this evidence, and the presence of a multiplicity of opioid binding sites within the adrenal medulla, we have conducted the first examination of the effect of subtype-selective opioids on nicotinic-induced $^{45}Ca^{2+}$-uptake in cultured bovine adrenal chromaffin cells as an indicator of the possible primary site of opioid action in these cells.

MATERIALS AND METHODS

Cultured bovine adrenal medullary cells. Isolated

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bovine adrenal medullary cells were prepared by collagenase digestion followed by purification by Percoll™ density gradient centrifugation. This preparation has been described in detail elsewhere [29]. The cells were plated on rat tail collagen-coated 24-well plastic culture plates at a density of 0.5×10^6 cells per well (chromaffin cell number determined by neutral red staining, Ref. 30) in a medium comprising DMEM supplemented with 15 mM HEPES, 10% foetal calf serum, 2.5 μg/mL each of fluorodeoxyuridine and cytosine arabinoside, 5 μg/NL nystatin, 50 μg/mL gentamysin and 100 μg/mL each of penicillin and streptomycin. Cultures were equilibrated with 95% O_2/5% CO_2 in a 37° incubator. In a recent study we identified 79–92% of the isolated cells after 3 days in culture as chromaffin cells on the basis of dopamine-β-hydroxylase immunostaining [31]. Three days after plating, and every second day from then on, the cells were fed by removing half the medium and replacing with an equal volume of fresh culture medium.

$^{45}Ca^{2+}$-*uptake*. After 5–9 days in culture the cells were removed from the incubator and given two 5 min washes with 1 mL of a buffer of the following composition (mM): NaCl 154, KCl 2.6, K_2HPO_4 2.15, KH_2PO_4 0.85, $MgSO_4$ 1.18, $CaCl_2$ 2.2 and glucose 10, pH 7.4 at 37°. The cells were then stimulated by a further incubation with 1 mL of the same buffer containing an appropriate concentration of nicotine (routinely an EC_{50} concentration of 30 μM) in the presence of 1 μCi/mL $^{45}Ca^{2+}$. The stimulation period was terminated by removal of the buffer followed by three rapid (approximately 1 min) washes with 1 mL ice-cold Ca^{2+}-free buffer (composition as above with $CaCl_2$ replaced with increased $MgSO_4$ and the addition of 1 mM EGTA). $^{45}Ca^{2+}$-uptake was determined by extracting the cells for 1 hr with 500 μL of 10% TCA followed by liquid scintillation counting with 4 mL ACSII (Amersham). The effect of opioids on nicotine-induced $^{45}Ca^{2+}$-uptake was determined by including an appropriate concentration of the opioid in both the second wash and stimulation period. When the effects of opioid antagonists on opioid action were tested, the antagonist was included in both the first and second wash periods as well as the stimulation period. Where appropriate statistical analysis was performed using a Student's *t*-test.

Materials. Metorphamide and dynorphin$_{(1-13)}$ were gifts from Dr P. Marley (University of Melbourne, Australia). ^{45}Calcium chloride (10–40 mCi/mg) was purchased from Amersham International (Amersham, U.K.), DAGO (Tyr-D-Ala-Gly-NMe-Phe-Gly-ol)* and DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr) from Cambridge Research Biochemicals (Cambridge, U.K.), diprenophine–HCl from C-Vet Limited (U.K.), and nicotine and naloxone–HCl from the Sigma Chemical Co. (St Louis, MO, U.S.A.). U50-488H, bremazocine, Mr2266 and Mr2267 were generous gifts from Dr P. F. VonVoigtlander (The Upjohn Company, Kalamazoo, MI, U.S.A.), Dr Maurer (Sandoz, Switzerland) and Dr Merz (Boehringer, Ingelheim, F.R.G.), respectively. The two opioid peptides used in this study were dissolved in 100 mM sodium phosphate buffer (pH 7.0) and the concentration of these stock solutions determined from their absorption at 280 nm assuming a molar extinction coefficient for tyrosine of 1280.

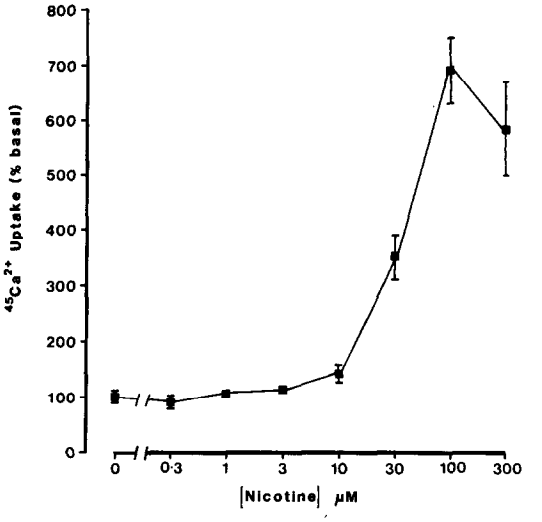

Fig. 1. Concentration–response relationship for nicotine-induced $^{45}Ca^{2+}$-uptake into cultured bovine adrenal medullary cells. $^{45}Ca^{2+}$-uptake was determined over a 10 min incubation period as described in Materials and Methods, and is presented as a percentage of the experimental mean uptake occurring in the presence of buffer alone (539 ± 54 dpm per well). Data represents the mean ± SE (N = 6) from two independent cell preparations.

RESULTS

Nicotine evoked a concentration dependent increase in $^{45}Ca^{2+}$-uptake (Fig. 1). Following a 10 min stimulation period $^{45}Ca^{2+}$-uptake was threshold at 10 μM and maximal at 100 μM (690 ± 63% of basal uptake) with an EC_{50} of approximately 30 μM. The response was slightly (although not significantly) reduced at high (300 μM) concentrations of nicotine which may suggest a degree of densensitization.

The time course of this response was examined in more detail (Fig. 2). Incubation in buffer alone (basal uptake) produced an initial rapid increase in $^{45}Ca^{2+}$-uptake followed by a slower and sustained increase over a 15 min incubation period (Fig. 2 upper panel). A half maximal concentration of nicotine (30 μM) stimulated a rapid and sustained increase in $^{45}Ca^{2+}$-uptake. This nicotine-evoked uptake was significantly ($P < 0.05$) above basal uptake after only 10 sec of incubation and the rate was greatest during this initial period of the stimulation. Equilibrium was reached after approximately 5 min at about 380% basal uptake, when no further increase above basal was observed.

* Abbreviations used: DAGO, H-Tyr-D-Ala-Gly-*N*-Me-Phe-Gly-ol; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; U50-488H, *trans*-3,4,dichloro-*N*-methyl-*N*-[2(1-pyrrolidinyl) cyclohexol] benzeneacetamide; Mr 2266 (−)-α-5,9-methyl-2-(3-furylmethyl)-2′-hydroxy-6,7-benzomorphan.

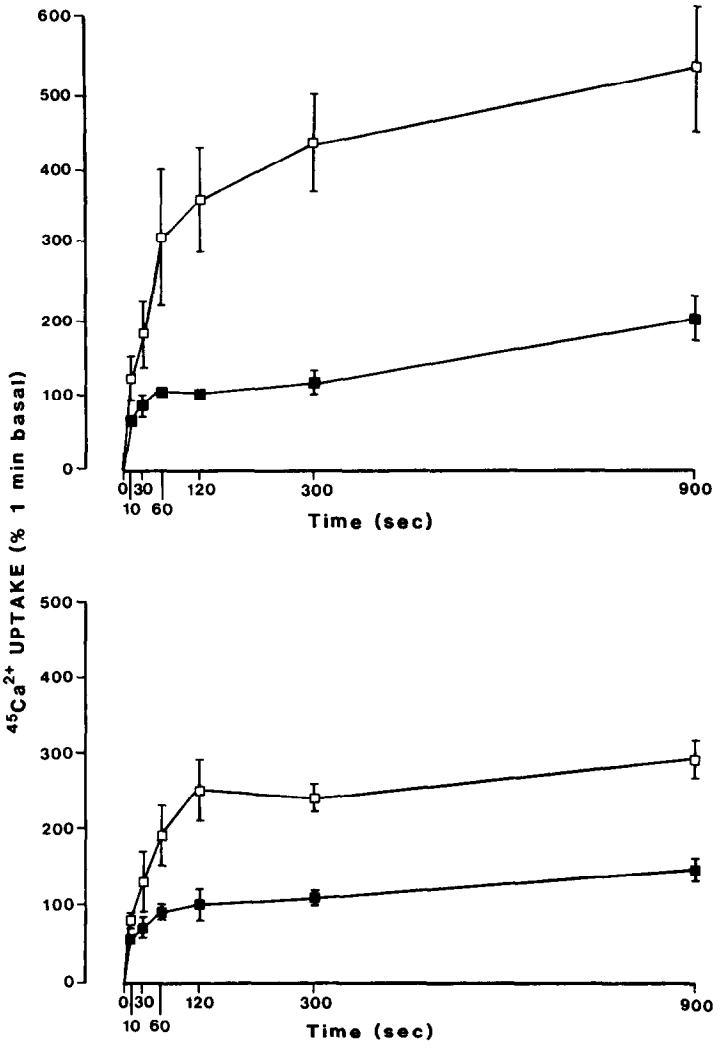

Fig. 2. Upper panel: the time course of nicotine-induced $^{45}Ca^{2+}$-uptake. Lower panel: the time course of nicotine-induced $^{45}Ca^{2+}$-uptake in the presence of the opioid agonist bremazocine. $^{45}Ca^{2+}$-uptake was determined by incubating the cells with (□) or without (■) 30 μM nicotine in the presence or absence of 10 μM bremazocine and is expressed as a percentage of the experimental mean of the uptake occurring over 1 min in the presence of buffer alone. This 1 min basal uptake varied between 360 and 2639 dpm per culture well. Data represents the mean ± SE (N = 8) from three separate cell preparations. Bremazocine inhibition of $^{45}Ca^{2+}$-uptake is significantly different from that occurring in the presence of nicotine alone at all time points examined ($P < 0.05$ Student's t-test).

The opioid agonist bremazocine (at 10 μM a concentration sufficient to interact with mu, delta and kappa sites) caused a marked inhibition of the nicotine-induced $^{45}Ca^{2+}$-uptake (Fig. 2 lower panel). This inhibition was apparent at all times points examined and led to a reduced equilibrium accumulation of $^{45}Ca^{2+}$ after 5 min of approximately 250% basal uptake. Bremazocine was without effect on the basal $^{45}Ca^{2+}$-uptake with incubation times of up to 5 min. At 15 min incubation there was however a small but significant reduction in basal uptake (Fig. 2 lower panel). The concentration dependence of bremazocine inhibition of nicotine-induced $^{45}Ca^{2+}$-uptake was investigated further (Fig. 3). Bremazocine inhibition of $^{45}Ca^{2+}$-uptake evoked by a 5 min stimulation with 30 μM nicotine was limited to high concentrations of the opioid (greater than 1 μM). Ten micromolar bremazocine (the highest concentration tested) reduced $^{45}Ca^{2+}$-uptake to 42 ± 6% of that occurring in the presence of nicotine alone.

The opioid subtype-selectivity of the inhibition of nicotine-evoked $^{45}Ca^{2+}$-uptake (over 5 min with 30 μM nicotine) was investigated using mu (DAGO), delta (DSLET) and kappa (U50-488H) selective agonists (Fig. 4A). The delta selective agonist DSLET was without significant effect over the concentration range tested (1 nM–10 μM). The mu agonist DAGO was largely without effect up to

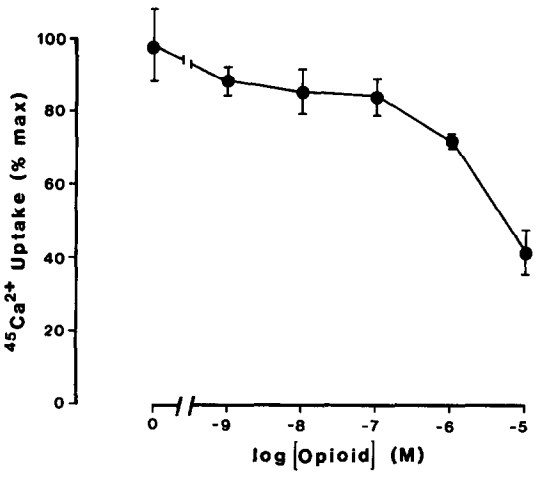

Fig. 3. The concentration–response relationship for bremazocine inhibition of nicotine-induced $^{45}Ca^{2+}$-uptake. Cultures were incubated for 5 min with 30 μM nicotine in the presence of increasing concentrations of bremazocine. Data is expressed as a percentage of the experimental mean uptake stimulated by nicotine in the absence of bremazocine (corrected for basal uptake). Data represents the mean ± SE (N = 4) from two separate cell preparations.

10 μM, except for a small but significant inhibition (77 ± 8% of the $^{45}Ca^{2+}$-uptake occurring in the presence of nicotine alone) at 100 nM. In contrast to the mu and delta opioid agonists the kappa selective agonist U50-488H produced a pronounced inhibition of nicotine-induced $^{45}Ca^{2+}$-uptake (Fig. 4A). U50-488H inhibition was significant at 10 nM (90.8 ± 1.5% of the $^{45}Ca^{2+}$-uptake occurring in the presence of nicotine alone. $P < 0.02$), and became more complete with higher concentrations of the drug (23 ± 6% of uninhibited uptake at 10 μM) with an IC_{50} of approximately 1.8 μM.

The opioid mediated inhibition of nicotine-induced $^{45}Ca^{2+}$-uptake was not limited to opioid alkaloids. The kappa-selective opioid peptide dynorphin$_{(1-13)}$ and the C-terminal amidated octapeptide metorphamide also produced almost complete inhibition of the response with IC_{50} values in the micromolar range (Fig. 4B).

The effect of opioid antagonists on nicotine-induced $^{45}Ca^{2+}$-uptake in bovine adrenal medullary cells were investigated. Naloxone (1 nM–10 μM) was without effect on either basal or nicotine-evoked $^{45}Ca^{2+}$-uptake (Fig. 4C). Low concentrations of diprenorphine (1 nM–100 nM) were similarly without effect on $^{45}Ca^{2+}$-uptake, although higher concentrations (1 μM) of the antagonist produced a small but significant inhibition of nicotine-induced $^{45}Ca^{2+}$-uptake (85 ± 4% of that occurring in the presence of 30 μM nicotine alone, significant at $P < 0.02$, Fig. 4C). This inhibition was maintained at 10 μM diprenorphine but greater experimental variation in the data prevented this from being statistically significant ($P > 0.05$). In contrast to the weak inhibition produced by these two drugs, the opioid antagonist Mr2266 produced a considerable inhibition of the nicotine-induced $^{45}Ca^{2+}$-uptake (Fig. 4D). The highest concentration of Mr2266 examined (10 μM) inhibited nicotine-induced $^{45}Ca^{2+}$-uptake to approximately 35 ± 3% of that occurring in the presence of 30 μM nicotine alone. Interestingly, the normally biologically inactive optical isomer of this antagonist, Mr2267, produced an essentially identical inhibition of the nicotine-induced $^{45}Ca^{2+}$-uptake (Fig. 4D).

The ability of opioid antagonists to effect the opioid-mediated inhibition in nicotine-induced $^{45}Ca^{2+}$-uptake was also examined. The inhibition in $^{45}Ca^{2+}$-uptake produced by 10 μM U50-488H was not significantly reduced by the addition of an equal concentration of the opioid antagonists naloxone, diprenorphine or Mr2266 responses equivalent to 106 ± 13%, 98 ± 5% and 87 ± 5% of those occurring in the presence of U50-488H (N = 6 from three separate cell preparations). This apparent lack of antagonistic action of these classical opioid antagonists was investigated further. The potency of U50-488H to inhibit nicotine-induced $^{45}Ca^{2+}$-uptake was examined in the presence of increasing concentrations of diprenorphine. As illustrated in Fig. 5 the addition of the antagonist produced a concentration-dependent increase in the potency of the U50-488H inhibition of nicotine-induced $^{45}Ca^{2+}$-uptake.

The U50-488H mediated inhibition of $^{45}Ca^{2+}$-uptake demonstrated some degree of selectivity towards nicotine-induced responses. While the degree of $^{45}Ca^{2+}$-uptake induced by a 3 min exposure to a depolarizing concentration of K^+ (56 mM) was similar to that produced by 30 μM nicotine (257 and 300% basal $^{45}Ca^{2+}$-uptake, respectively), the K^+ response was unaffected by a concentration of U50-488H sufficient to markedly inhibit the nicotine response (Fig. 6).

The basal $^{45}Ca^{2+}$-uptake, that is $^{45}Ca^{2+}$ accumulation occurring in the absence of nicotine, was routinely determined in all experiments. This basal response was unaffected over the time course of these experiments by all concentrations of opioid agonists and antagonists examined.

DISCUSSION

Nicotine stimulated $^{45}Ca^{2+}$-uptake into bovine adrenal medullary cells with an EC_{50} of approximately 30 μM. Interestingly, nicotine is rather more potent in evoking catecholamine release from these cells (EC_{50} of approximately 5 μM, [32], and routinely found in this laboratory). A similar difference in the sensitivities of these two responses has previously been reported between carbachol-induced catecholamine secretion and $^{45}Ca^{2+}$-uptake [33]. The explanation for these differences probably lies in the fact that $^{45}Ca^{2+}$-uptake as determined in these experiments measures the net uptake over the incubation period, that is the balance between $^{45}Ca^{2+}$-influx and $^{45}Ca^{2+}$-efflux from the cells.

Bremazocine is a potent opioid agonist, and while often referred to as a kappa opioid it will interact with mu and delta sites as well [34]. We and others have previously demonstrated that the bovine adrenal medulla contains a substantial number of

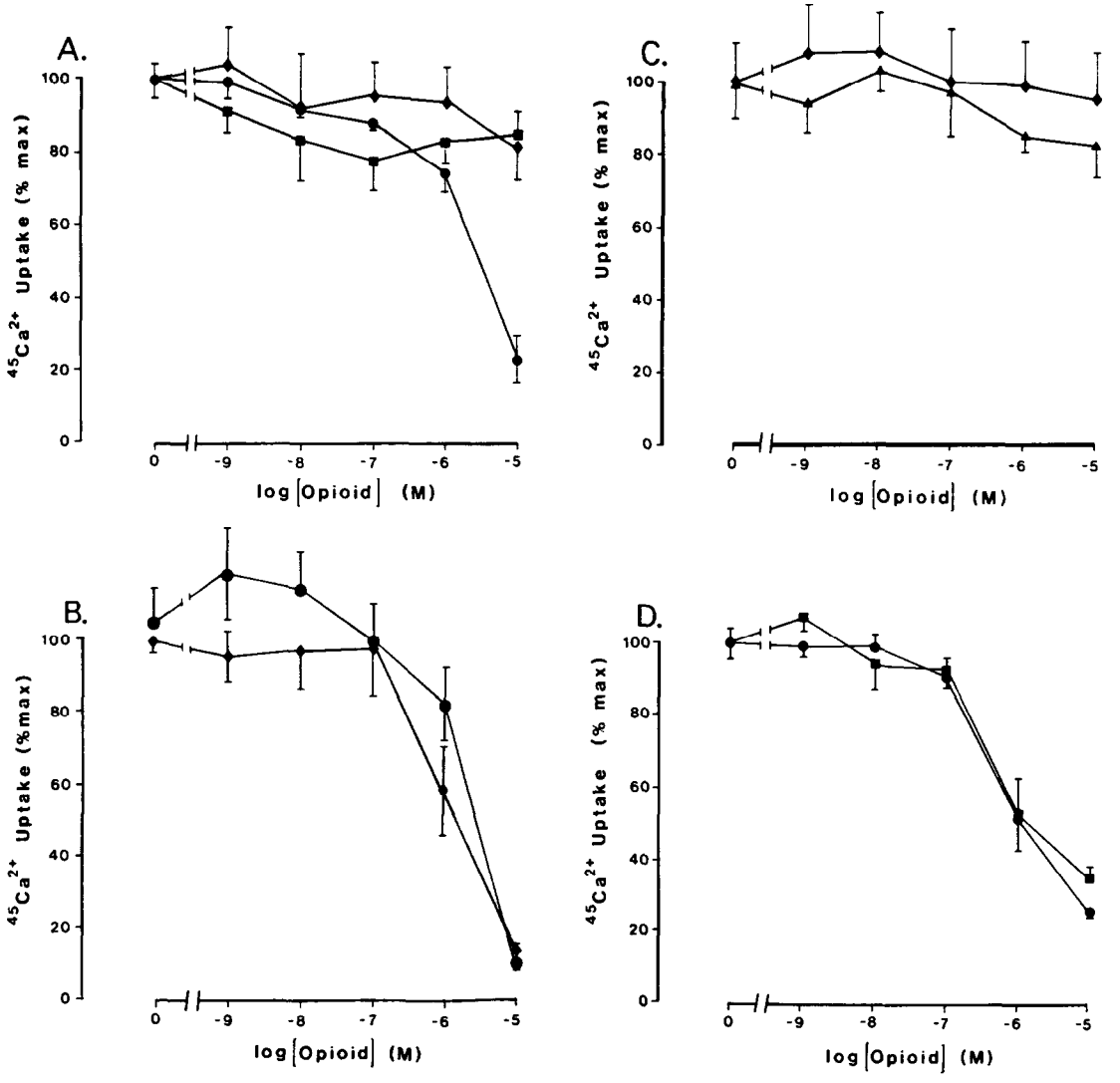

Fig. 4. Opioid receptor subtype and antagonist inhibition of nicotine-induced $^{45}Ca^{2+}$-uptake. Cells were incubated for 5 min with 30 μM nicotine in the presence of increasing concentrations of: (A) the mu-selective agonist DAGO (■), the delta-selective agonist DSLET (◆) or the kappa-selective agonist U50-488H (●); (B) the opioid peptides dynorphin$_{(1-13)}$ (●) or metorphamide (◆); (C) the opioid antagonists naloxone (◆) or diprenophine (▲); and (D) the opioid antagonists Mr2266 (●) or Mr2267 (■). Data is presented as a percentage of the experimental mean uptake occurring in the presence of nicotine alone (corrected for basal uptake) and represents the mean ± SE from two (N = 4 for DSLET, dynorphin$_{(1-13)}$, metorphamide, diprenorphine, Mr2266 and Mr2267), three (N = 6 for naxolone and DAGO) or five (N = 10 for U50-488H) separate cell preparations.

pharmacologically heterogeneous [^{3}H]bremazocine binding sites [10, 35]. In this study we have demonstrated that bremazocine produced a dose dependent inhibition of nicotine-evoked $^{45}Ca^{2+}$-uptake, but it should be noted that this inhibition required some 1000-fold higher concentrations of bremazocine than the reported K_d values of the binding sites [10, 35]. While lacking in potency this opioid inhibition of nicotine-induced $^{45}Ca^{2+}$-uptake displayed a high degree of selectivity towards kappa selective opioids. DAGO and DSLET are modified opioid peptides with marked selectivities towards mu and delta sites, respectively. The adrenal medulla contains a significant number of delta opioid binding sites and a smaller but still significant number of mu sites [9, 35]. However, despite the presence of mu and delta opioid binding sites DAGO and DSLET at concentrations of up to 10 μM were almost without effect on the nicotine-induced $^{45}Ca^{2+}$-uptake. The small inhibition seen with 100 nM DAGO is difficult

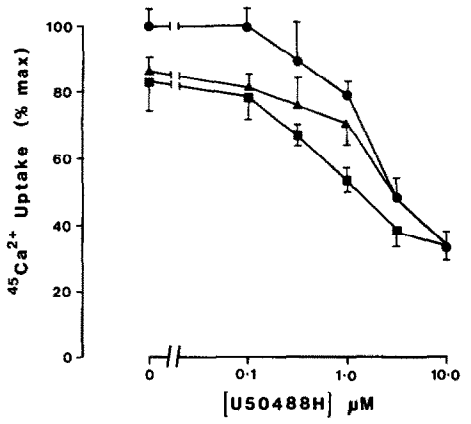

Fig. 5. The effect of diprenorphine on the concentration–response relationship for U50-488H inhibition of nicotine-induced $^{45}Ca^{2+}$-uptake. Cells were incubated for 5 min with 30 μM nicotine in the presence of increasing concentrations of U50-488H (●) together with 1 μM (▲) or 10 μM (■) diprenorphine. Data is presented as a percentage of the experimental mean uptake occurring in the presence of nicotine alone (corrected for basal uptake) and represents the mean ± SE (N = 6) from three separate cell preparations.

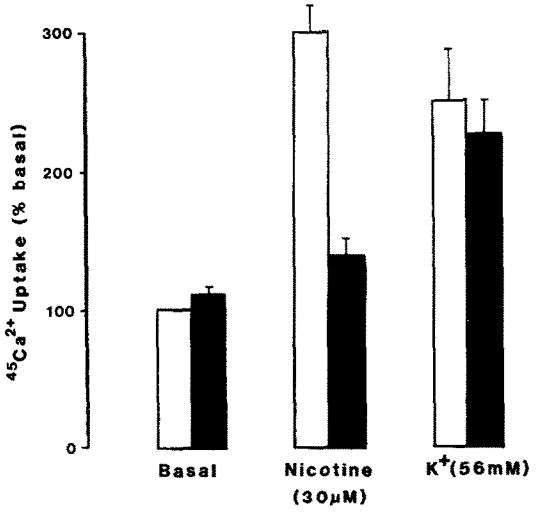

Fig. 6. A comparison of the effect of U50-488H on nicotine- and K^+ depolarization-induced $^{45}Ca^{2+}$-uptake. Cells were incubated for 5 min with 30 μM nicotine or 56 mM K^+ with (■) or without (□) 10 μM U50-488H. Data is expressed as a percentage of the mean experimental uptake occurring in the presence of buffer alone (% basal) and represents the mean ± SE (N = 6) from two separate cell preparations.

to explain because it was not sustained at higher concentrations of the peptide. These observations are consistent with the suggestion that mu and delta opioid receptors do not mediate their effects by directly modifying Ca^{2+}-uptake [27].

In contrast to the mu and delta agonists, U50-488H, a highly selective kappa agonist [36], almost completely abolished the nicotine-induced $^{45}Ca^{2+}$-uptake. Although relatively high concentrations of the drug were required to produce this degree of inhibition, a significant reduction in $^{45}Ca^{2+}$-uptake was obtained in some experiments (Fig. 4A) with concentrations as low as 10 nM. The ability to inhibit nicotine-induced $^{45}Ca^{2+}$-uptake was not limited to U50-488H, the kappa selective peptide dynorphin$_{(1-13)}$, and the amidated met-enkephalin analogue metorphamide also produced almost complete inhibition of the response with IC_{50} values in the micromolar range. These data are supported by previous observations that while kappa opioids and metorphamide inhibit nicotine-induced catecholamine amine release from bovine chromaffin cells, mu and delta opioids are ineffective in this action [3, 23, 26, 37].

Given the close association between calcium ion influx and the exocytotic process this data provides new evidence that strongly suggests that opioid-mediated inhibition of catecholamine secretion from adrenal medullary chromaffin cells is the result of an inhibition of Ca^{2+}-influx. As described above U50-488H-sensitive opioid binding sites are present on membranes obtained from the bovine adrenal medulla [37] and could conceivably mediate the inhibition of nicotine-induced $^{45}Ca^{2+}$-uptake reported here. Dynorphin-sensitive sites have been described in this tissue [10, 35], but in our autoradiographic examination of the distribution of these sites we found that they were restricted to the nerve terminals within the gland and did not appear to occur within the chromaffin tissue of the medulla. For example, 96% of the specific [^{3}H]bremazocine binding (in the presence of excess unlabelled mu and delta ligands) was unaffected by the addition of 1 μM dynorphin$_{(1-13)}$, a concentration sufficient to cause significant inhibition of the nicotine-induced $^{45}Ca^{2+}$-uptake. This suggests that the dynorphin inhibition of $^{45}Ca^{2+}$-uptake is unlikely to be mediated by the kappa-like bremazocine binding sites identified in these autoradiographic binding studies. The binding characteristics of metorphamide in the adrenal medulla have not been investigated, however metorphamide inhibition of nicotine-induced catecholamine secretion also suggests the involvement of a pharmacologically distinct opioid recognition site [25, 38].

A further complication in attributing the inhibition of nicotine-induced $^{45}Ca^{2+}$-uptake to the action of the opioid binding sites identified in radioligand studies occurs when examining the action of opioid antagonists. While naloxone and diprenorphine had little effect on their own against nicotine-induced $^{45}Ca^{2+}$-uptake, Mr2266 produced a marked inhibition with a potency similar to that observed for opioid agonists. The inhibition produced by this opioid antagonist displayed no stereoselectivity, in that the generally inactive stereoisomer of this compound, Mr2267, produced an essentially identical inhibition of the response. These data contrast with the radioligand binding data where Mr2266 and Mr2267 displayed marked differences in their ability to inhibit [^{3}H]bremazocine binding in the bovine adrenal medulla [35]. None of the opioid antagonists examined were able to reverse or reduce the

inhibition of $^{45}Ca^{2+}$-uptake produced by U50-488H. Indeed, as demonstrated in Fig. 5, the degree of inhibition was greater in the presence of the antagonist than with the agonist alone. Interestingly, a similar lack of opioid antagonism has been reported for U50-488H-mediated inhibition of depolarization-induced $^{45}Ca^{2+}$-uptake into rat brain synaptosomes [39]. This data clearly indicates that opioid inhibition of nicotine-induced $^{45}Ca^{2+}$-uptake is not mediated through the classical stereospecific opioid binding sites identified in radioligand binding studies [1–8].

In contrast to synaptosomes [39] $^{45}Ca^{2+}$-uptake evoked by potassium depolarization was not inhibited by concentrations of U50-488H sufficient to almost completely abolish the nicotine-induced uptake. A similar contrast in the effect of opioids on nicotinic and potassium depolarization-induced responses in these cells has been reported for catecholamine secretion [5, 6]. Furthermore, we have previously demonstrated that secretion evoked by other agents such as angiotensin II, histamine and the prostaglandins (PGE_1, and PGE_2) and the stimulation of inositol phospholipid metabolism by these agents and muscarine are also insensitive to opioid modulation [40–43]. It is possible therefore that the opioid recognition site mediating the inhibition of $^{45}Ca^{2+}$-uptake and catecholamine secretion is associated with the nicotinic-receptor ionophore complex. A similar suggestion has been made with regard to the inhibitory action of aliphatic alcohols, anaesthetics, substance P and the α_2-adrenoceptor agonist clonidine, on nicotinic-induced catecholamine secretion from sympathetic neurons or adrenal chromaffin cells [13, 24, 44].

This study indicates that the opioid recognition site responsible for mediating inhibition exhibits a non-classical pharmacology, lacking both stereospecificity and antagonist sensitivity. Further studies are required to more fully characterize this site and to establish its relationship to the mu, delta, kappa, sigma and PCP binding sites identified in radioligand binding studies [7, 9, 10, 35]. It is noteworthy however that the most potent opioid alkaloids in mediating this inhibition (bremazocine, Mr2266 and Mr2267) are all benzomorphanes, and that U50-488H may interact with a benzomorphan binding site in some tissues [45]. It is therefore of interest that a benzomorphan binding site has been reported to be associated with the nicotinic-receptor ionophore complex prepared from the *Torpedo* electric organ [46]. The apparent lack of stereospecificity and antagonist-agonist selectivity of the opioid recognition site identified in these current studies may arise from an inappropriate choice of opioid ligands. The adrenal medulla is known to contain a large number of different opioid peptides and some of these may be specific high affinity agonists or indeed antagonists at these non-classical opioid sites. An alternate interesting, although purely speculative suggestion at this stage, is that the opioid recognition site involved in mediating this inhibition may not be coupled to an effector system. The inhibition could thus arise as a direct consequence of ligand binding to or near the nicotinic receptor–ionophore complex perhaps allowing traditional antagonists to mimic the effect of agonists.

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