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EFFECTS OF OPIOID COMPOUNDS ON DESENSITIZATION OF THE NICOTINIC RESPONSE OF ISOLATED BOVINE ADRENAL CHROMAFFIN CELLS

PHILIP D. MARLEY* and BRUCE G. LIVETT
Department of Biochemistry, University of Melbourne, Parkville, 3052, Victoria, Australia

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Abstract—Opioid compounds have been assessed for their ability to modify desensitization of nicotine-induced catecholamine secretion from cultured, bovine, adrenal chromaffin cells. Dynorphin-1-13 and metorphamide produced protection against desensitization of the nicotinic response at concentrations between 1 and 20 μM while etorphine and morphine only produced this effect at 100 μM. The opioid antagonists, naloxone and diprenorphine, at 100 μM mimicked the weak ability of the opioid agonists to protect against nicotinic desensitization. All opioid compounds tested were considerably more potent at inhibiting nicotine-induced catecholamine secretion from the cells than at protecting against desensitization of this response. It is concluded that adrenal opioid peptides probably do not act on adrenal opioid binding sites characterised from ligand binding studies to prevent the nicotinic response from desensitizing. They are unlikely, therefore, to be involved in such a mechanism to maintain catecholamine secretion during stress.

A number of neuroactive peptides have been identified in the mammalian adrenal medulla (see ref. 1). However, their functional significance is not yet known. Two groups of these peptides, the tachykinins (including substance P) and the opioid peptides, have been assessed in some detail for their ability to modify the nicotinic secretory response of bovine adrenal medullary cells. Both groups of peptides inhibited this response but did not affect potassium-induced secretion [2–7]. In the case of substance P, the inhibition of nicotine- or acetylcholine-induced catecholamine (CA)† secretion from cultured chromaffin cells was seen with peptide concentrations in the low micromolar range. The opioid peptides, on the other hand, were very weak at inhibiting the nicotinic secretory response, some being active only at 100–1000 μM. In addition, the effects of opioid peptides and of opiate alkaloid agonists were mimicked and not reversed by opioid antagonists, such as naxolone or diprenorphine, and opiate alkaloid steroisomers did not show any stereoselectivity [4–7]. These data suggest that adrenal medullary opioid peptides do not act on the opioid binding sites identified in the adrenal medulla from ligand binding studies [8, 9], to modify the nicotinic secretory response of the chromaffin cells. Recent studies also indicate that these putative opioid receptors do not modify the secretory responses of chromaffin cells to histamine [10], angiotensin II or prostaglandins [11, 12].

Another feature of adrenal medullary chromaffin cells that is affected by substance P and other tachykinins is the desensitization of the nicotinic secretory response [13]. Exposure of these cells to high concentrations, to prolonged periods or to repeated application of nicotinic agonists renders them progressively less sensitive to further nicotinic stimulation of CA secretion [14–16]. Substance P can fully protect chromaffin cells against such "desensitization" of the nicotinic response [13]. An important factor of this action of substance P is that it occurs at *lower* concentrations than are required to inhibit the nicotinic secretory response directly. Thus at low concentrations, substance P may maintain chromaffin cell CA secretion by preventing the nicotinic response from desensitizing. This mechanism may be of importance *in vivo*, to maintain adrenal CA secretion during stress by preventing the cholinergic stimulation of the chromaffin cells from inactivating. That such a system does operate *in vivo* during stress has recently been demonstrated in rats subjected to hypoglycaemia, hypothermia or histamine-stress [17, 18; Khalil, Livett and Marley, submitted].

In the present study, we have investigated whether adrenal opioid peptides act like the tachykinins to prevent desensitization of the nicotinic secretory response of adrenal medullary chromaffin cells. Preliminary abstracts of this work have been published [11, 12].

MATERIALS AND METHODS

Cultured bovine adrenal chromaffin cells. Dispersed bovine adrenal chromaffin cells were prepared from bovine adrenal medullae by collagenase digestion followed by Percoll™ gradient purification. The dispersed cells were plated at a density of 2.5–3.5 × 10^5/well on rat-tail collagen-coated 24-well plastic culture dishes and cultured for 3 days before use to allow attachment of the cells. Full details of the preparation and culturing of the cells have been published [19].

* Author for correspondence and reprint requests.

† Abbreviations used: CA, catecholamines; NA, noradrenaline; A, adrenaline.

Desensitization of CA release. Desensitization of the nicotinic secretory response of cultured bovine adrenal chromaffin cells was studied essentially as described previously [13, 15]. Cultured cells were removed from the incubator for 5 min to equilibrate to room temperature. All wells then had their culture medium replaced with 400 μl incubation buffer of the 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; D-glucose, 10; bovine serum albumin (fraction V) 0.5% w/v; pH 7.4. Each well then received six sequential 400 μl incubations at room temperature (22–25°). These were two 10-sec washes, a 5½ min desensitizing incubation (incubation I), a further two 10-sec washes and a 5½ min test incubation (incubation II). When studying desensitization of the nicotinic response, incubations I and II both contained 5 μM nicotine: 5 μM nicotine in incubation I to desensitize the cells, and 5 μM nicotine in incubation II to test the degree of desensitization produced. The degree of desensitization seen in incubation II was assessed by comparison with control wells that were incubated without nicotine in incubation I. Desensitization of potassium-depolarization-induced CA secretion from the cultured cells was studied in a similar manner by including 50 mM excess KCl (in exchange for NaCl) in incubations I and II. The effects of opioid compounds on desensitization of the nicotinic or potassium secretory responses were tested by including them in the first (desensitizing) incubation, to observe their effect on the subsequent degree of desensitization measured in incubation II. The effect of nicotine concentration in incubation I on the subsequent degree of desensitization in incubation II was assessed by using 1–1000 μM nicotine in incubation I. Nicotine solutions of 300 μM and above required adjustment of pH back to 7.4 with HCl.

Measurement of CA secretion. The buffer from incubations I and II was collected separately and acidified with 100 μl of 2 M perchloric acid (PCA) to precipitate protein. The samples were then stored at −20° until assayed for CA content. Following clarification by centrifugation, endogenous noradrenaline (NA) and adrenaline (A) were measured by separation of the amines by HPLC and their electrochemical detection (for details, see refs 6 and 7). The cellular content of CA remaining at the end of the six incubations was measured for each well by extraction into PCA and assay as above. The release of CA during incubations I and II was expressed as a fraction (%) of the cellular CA levels, present in the well at the beginning of that incubation period. Data presented represent mean ± SEM for the stated number of observations. Where error bars are not shown, the SEM was smaller than the symbol used. In Figs 3–8, the top panel shows the release of CA induced by 5 μM nicotine in the presence of opioid compounds in incubation I, plotted as a % of the release produced by 5 μM nicotine alone. The bottom panels show the release of CA induced by 5 μM nicotine in incubation II, plotted as a % of the response obtained from un-desensitized cells (i.e. cells not exposed to nicotine in incubation I). Figure 10 is presented similarly for 50 mM potassium-induced desensitization. In the upper panels of Figs 1 and 9 and in all panels of Fig. 2 the release of CA has been plotted directly.

Drugs. Etorphine HCl and diprenorphine HCl were generous gifts from Mr I. Mawhinney (C-Vet Ltd, U.K.); metorphamide was a generous gift from Dr E. Weber (Stanford, CA); naloxone HCl was a generous gift from Dr V. Nicholson (DuPont, U.S.A.); morphine HCl was from Prosana Labs (Sydney, Australia); and dynorphin-1-13 was from Peninsula Labs (CA).

RESULTS

Nicotine-induced desensitization of CA secretion

In a previous study [13, 15], desensitization of nicotine-induced CA secretion was followed by preloading the cells with ^{3}H-noradrenaline and monitoring its subsequent release. In the present experiments, the release of endogenous NA and A were followed using HPLC with electrochemical detection to quantify the CA released. This allows the desensitization of nicotine-induced CA secretion to be assessed with the *endogenous* CA pools being studied, rather than those that may be selectively

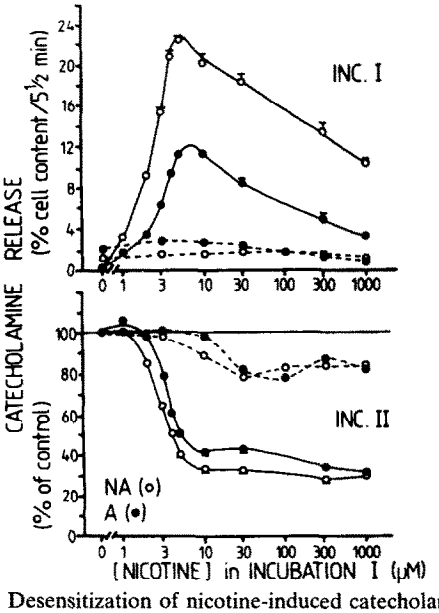

Fig. 1. Desensitization of nicotine-induced catecholamine secretion from cultured, bovine, adrenal chromaffin cells. Top panel: secretion of CA induced by 0–1000 μM nicotine in incubation I in the presence (complete lines) and absence (dotted lines) of 2.2 mM Ca^{2+}. For experiments in the absence of Ca^{2+}, the 2.2 mM $CaCl_2$ in the medium was replaced with an additional 2.2 mM $MgSO_4$. Bottom panel: subsequent secretion of CA induced by 5 μM nicotine in incubation II following exposure to nicotine under the indicated conditions in incubation I. Results are plotted as a % of the control response to 5 μM nicotine seen in incubation II from cells not treated with nicotine in incubation I (i.e. undesensitized cells). Data pooled from three cell preparations, N = 6–12. NA (open circles), noradrenaline; A (closed circles), adrenaline. Control responses in incubation II: for complete lines—NA 19.7 (±0.5) % of cell content released/5½ min, N = 28; A 10.8 (±0.3) % released/5½ min, N = 28: for dotted lines—NA 17.4 (±3.5) % released/5½ min, N = 12; A 16.3 (±0.34) % released/5½ min, N = 11.

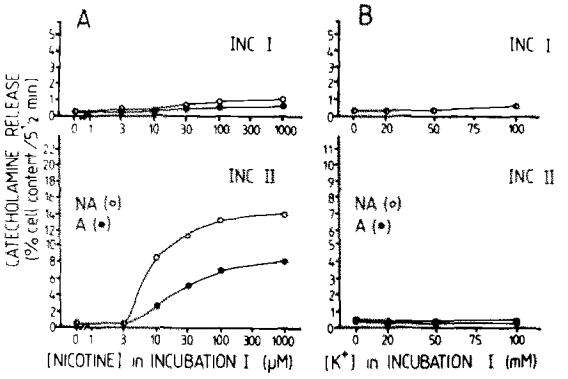

Fig. 2. (A) Effect of preincubating cultured, bovine, adrenal chromaffin cells with 0–1000 μM nicotine in calcium-free buffer (top panel) on their subsequent secretion of CA in control buffer (bottom panel). Cells were exposed to nicotine in the absence of calcium (2.2 mM $CaCl_2$ replaced with $MgSO_4$) in incubation I, and to control buffer containing 2.2 mM $CaCl_2$ (but no nicotine) in incubation II. N = 4. (B) Effect of preincubating cultured bovine adrenal chromaffin cells with 0–100 mM extra KCl (replacement of NaCl) in calcium-free buffer (top panel) on their subsequent secretion of CA in control buffer (bottom panel). Details similar to Fig. 2A. N = 6. NA (open circles), noradrenaline; A (closed circles), adrenaline.

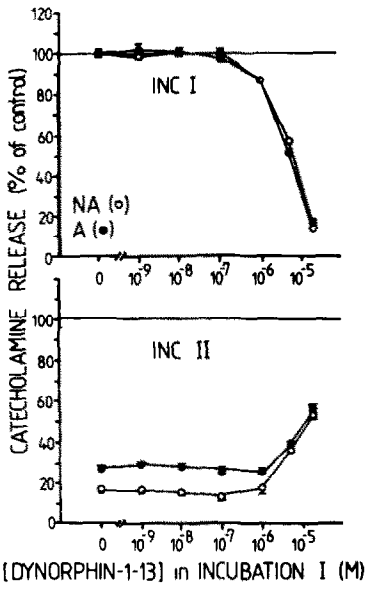

Fig. 3. Effect of dynorphin-1-13 (10^{-9} M–2×10^{-5} M) on desensitization of nicotine-induced catecholamine secretion from cultured, bovine, chromaffin cells. Top panel: effect of including dynorphin-1-13 in incubation I at the indicated concentrations on the secretion of CA induced by 5 μM nicotine. Bottom panel: subsequent secretion of CA induced by 5 μM nicotine in incubation II, following their exposure to 5 μM nicotine and the indicated concentrations of dynorphin-1-13 in incubation I. Data pooled from two cell preparations, N = 6–9. Control responses to 5 μM nicotine: incubation I—NA 24.7 (±0.5)% cell content released/5½ min, N = 20; A 11.5 (±0.3)% released/5½ min, N = 20: incubation II—NA 23.5 (±0.3)% released/5½ min, N = 19; A 11.0 (±0.2)% released/5½ min, N = 19.

labelled by the chromaffin cell CA uptake system. It also enables any differences between NA and A cells to be seen.

As reported previously [13, 15], the secretion of CA from cultured chromaffin cells by nicotine was reduced ("desensitized") by prior exposure of the cells to nicotine (Fig. 1). When cells were exposed to a range of nicotine concentrations in the first incubation, they secreted both NA and A in a concentration-dependent manner (Fig. 1, top panel, solid lines). Subsequent restimulation of the cells with a standard test concentration of nicotine (5 μM) showed that both NA and A cells were desensitized by their prior exposure to nicotine (Fig. 1, bottom panel, solid lines). The degree of desensitization increased as the nicotine concentration in the first incubation was increased from 1 to 7 μM. However, when the nicotine concentration in the first incubation was increased further to 1 mM, little further desensitization of the secretory response was seen. These findings are in agreement with those of Boksa and Livett [15] using ^{3}H-NA loading. It was noteworthy that nicotine consistently induced almost twice as much NA release (as a % of cellular NA levels) as A release in incubation I, but only induced slightly more desensitization of NA secretion than A secretion (see Figs 1, 3–8).

The calcium-dependence of desensitization of the nicotinic response is also shown in Fig. 1 (dotted lines). If the cultured cells were exposed to increasing concentrations of nicotine in incubation I in the absence of Ca^{2+}, the secretion of CA during incubation I was virtually abolished. Subsequent testing of the sensitivity of the cells to 5 μM nicotine (in the presence of Ca^{2+}) in incubation II showed that desensitization of the nicotinic response of both NA and A cells appeared to have calcium-dependent and independent components (Fig. 1, bottom panel, dotted lines). The desensitization appeared to be completely dependent on calcium in the first incubation up to nicotine concentrations of approximately 10 μM. Thereafter there was a small component of calcium-independent desensitization. This pattern of dependence on calcium was similar for both cell types and is identical to that reported by Boksa and Livett [15] using release of preloaded ^{3}H-NA.

This, however, is not a true reflection of the calcium dependence of nicotinic desensitization. If cells were exposed to increasing concentrations of nicotine in incubation I in the absence of calcium, the secretion of CA in incubation I was virtually abolished, as described above. If now the cells are washed and then exposed in incubation II to control buffer (i.e. buffer containing calcium but not containing nicotine), substantial CA secretion is seen, in spite of the absence of nicotine (Fig. 2A, bottom panel). Such secretion is not seen with cells exposed to calcium-free buffer without nicotine in incubation I. It therefore represents a sensitization of the cells to the reintroduction of Ca^{2+} by exposure of the cells to nicotine in calcium-free buffer.

This finding suggests that most of the CA secretion seen in the dotted lines of the lower panel of Fig. 1 is due simply to the reintroduction of Ca^{2+}, and is not

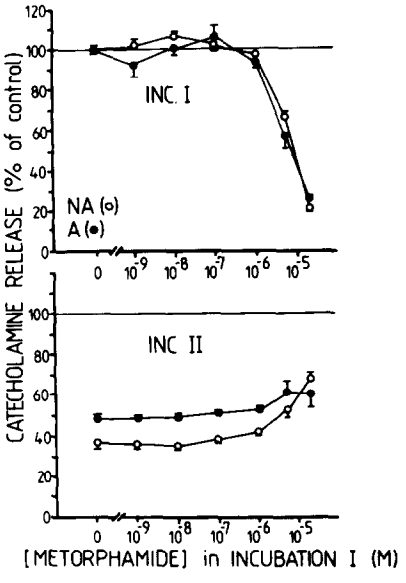

Fig. 4. Effect of metorphamide (10^{-9} M–2×10^{-5} M) on desensitization of nicotine-induced CA secretion. Details as for Figs 1 and 3. Data are pooled from two cell preparations, N = 6–9. Control responses to 5 μM nicotine: incubation I—NA 19.1 (±0.5)% released/5½ min, N = 21; A 7.48 (±0.5)% released/5½ min, N = 21; incubation II—NA 19.3 (±0.4)% released/5½ min, N = 21; A 8.40 (±0.4)% released/5½ min, N = 21.

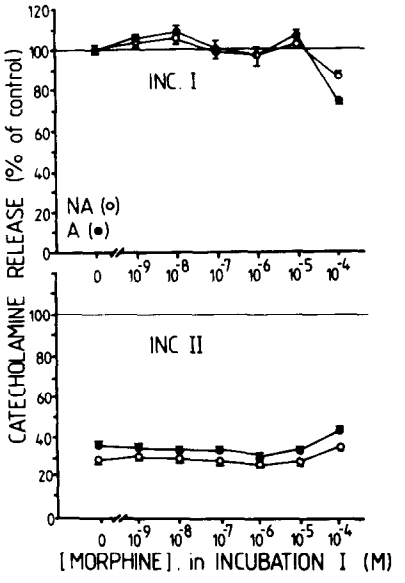

Fig. 5. Effect of morphine (10^{-9} M–10^{-4} M) on desensitization of nicotine-induced CA secretion. Details as for Figs 1 and 3. Data are pooled from three cell preparations, N = 9 or 10. Control responses to 5 μM nicotine: incubation I—NA 21.5 (±0.3)% released/5½ min, N = 25; A 8.64 (±0.2)% released/5½ min, N = 25; incubation II—NA 20.7 (±0.2)% released/5½ min, N = 25; A 7.95 (±0.2)% released/5½ min, N = 25.

due to the 5 μM nicotine present in this incubation II. Consequently, if this nicotine-independent secretion (Fig. 2A, lower panel)) is subtracted from the data in Fig. 1 (lower panel, dotted lines), it is apparent that the cells are almost equivalently desensitized by exposure to 30 μM or more nicotine in incubation I, whether or not calcium is present in this incubation. This is clearly different from the situation seen in Fig. 1, where the cells were apparently desensitized by about 60% in the presence of calcium and by only 20% in the absence of calcium.

Due to the stimulatory effect of reintroducing Ca^{2+} to cells previously exposed to nicotine in calcium-free buffer, it was not possible to assess separately the effects of opioid compounds on calcium-dependent and calcium-independent components of desensitization. Instead, we have studied the effects of opioid compounds on nicotine-induced desensitization only in the presence of calcium, since this is the situation that would occur *in vivo*. A desensitizing concentration of 5 μM nicotine for incubation I was chosen for all further experiments for two main reasons. Firstly, this concentration of nicotine is not supramaximal either for NA and A secretion or for desensitization of secretion for either CA. It is thus in the normal operating range of the nicotinic dose–response curve for both parameters. Secondly, there is no "carry-over" of CA secretion from incubation I to incubation II when 5 μM nicotine is used for the former (desensitizing) incubation. When high nicotine concentrations are used in incubation I, very significant CA secretion is recorded in the second test incubation, even in the absence of nicotine in this latter incubation (see [13, 15]).

Effects of opioid compounds on desensitization of the nicotinic secretory response

The adrenal medulla contains more than 30 different opioid peptides, many of them in substantial quantities. Of a total of 19 tested for their ability to inhibit nicotine-induced secretion from cultured bovine chromaffin cells, dynorphin-1-13 and metorphamide were the two most potent [6, 7]. Consequently, these two peptides were tested for their ability to modify desensitization of the nicotinic secretory response of these cells. The peptides were included only in incubation I to assess their effects on desensitization.

Figures 3 and 4 show the effects of dynorphin-1-13 and metorphamide (1 nM–20 μM) on the desensitization of the nicotinic response. As reported previously, both peptides produced a concentration-dependent inhibition of NA and A secretion during incubation I. They both also protected against desensitization of the nicotinic response, as seen by the increase in responsiveness of the cells to a second stimulation with 5 μM nicotine in incubation II (Figs 3 and 4, bottom panels). Concentrations of dynorphin-1-13 or metorphamide (20 μM) that inhibited the nicotinic secretory response by 80%, only protected against desensitization by 30–50%.

The adrenal medulla possess several distinct types of opioid receptor, defined by ligand binding studies [8, 9]. However, the two peptides tested are selective for only certain of these types. Consequently, the two opioid peptides tested may not be able to act on the appropriate opioid receptor subtype to produce an appreciable effect on desensitization of the nicotinic response. Instead of testing a wide range of

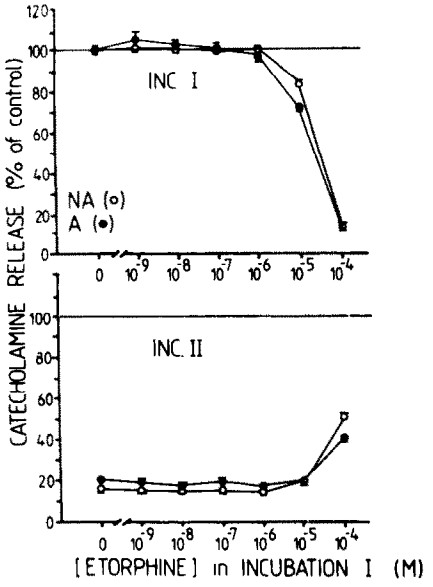

Fig. 6. Effect of etorphine (10^{-9}–10^{-4} M) on desensitization of nicotine-induced CA secretion. Details as for Figs 1 and 3. Data are pooled from two cell preparations, N = 6–9. Control responses to 5 μM nicotine: incubation I—NA 17.5 (±0.6)% released/5½ min, N = 21; A 9.50 (±0.4)% released/5½ min, N = 21; incubation II—NA 18.1 (±0.3)% released/5½ min, N = 20: A 10.2 (±0.3)% released/5½ min, N = 20.

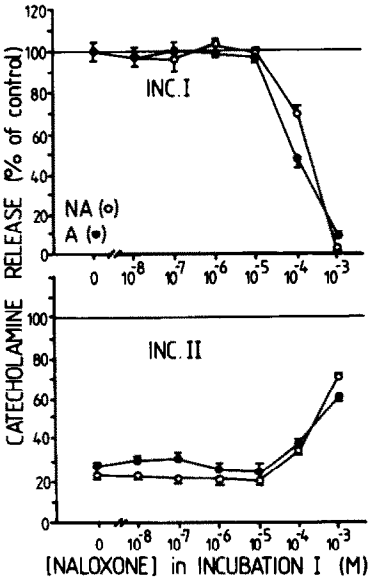

Fig. 7. Effect of naxolone (10^{-8}–10^{-3} M) on desensitization of nicotine-induced CA secretion. Details as for Figs 1 and 3. Data are pooled from three cell preparations, N = 6–12. Control responses to 5 μM nicotine: incubation I—NA 21.3 (±0.5)% released/5½ min, N = 21; A 10.6 (±0.6)% released/5½ min, N = 21: incubation II—NA 20.7 (±0.4)% released/5½ min, N = 21; A 10.9 (±0.6)% released/5½ min, N = 21.

opioid peptides for such effects, we chose to test two opiate alkaloid agonists, morphine and etorphine, which between them are potent agonists on all known opioid binding sites in the bovine adrenal medulla [8, 9]. Morphine only weakly inhibited the nicotinic response in incubation I (Fig. 5, top panel; see [5]), and produced only minor protection against desensitization even at 0.1 mM (Fig. 5, bottom panel). Etorphine, on the other hand, was more potent at inhibiting the secretory response in incubation I (Fig. 6, top panel: see also [20]); nevertheless, concentrations that inhibited this secretory response by almost 90% only protected the nicotinic response from desensitization by 25–45%. The apparent weaker ability of morphine and etorphine at inhibiting 5 μM nicotine-induced CA release in incubation I seen here, in comparison with previous studies [5, 20] was due to the absence of a pre-incubation period with the alkaloid alone, before the cells were stimulated with nicotinic agonists. Such prior incubation shifts the inhibition curves to the left by approximately twofold.

The low potency of morphine or etorphine at preventing desensitization of nicotine-induced CA secretion may be due to the secretion of substantial quantities of endogenous opioid peptides during the nicotinic stimulation in incubation I. These endogenous peptides may already saturate the opioid receptors, so preventing any effect of exogenous opioid agonists on desensitization from being seen. To test for this possibility, we used two opioid receptor antagonists, naloxone and diprenorphine, which between them are able to bind to all the known opioid binding sites in the bovine adrenal medulla [8, 9]. Both antagonists mimicked the ability of the agonists to inhibit the nicotinic secretory response in incubation I (Figs 7 and 8, top panels: see [6]), and they both weakly protected against desensitization of the nicotinic response (Figs 7 and 8, bottom panels). Neither antagonist enhanced desensitization induced by nicotine, as would have been expected if endogenous opioid peptides were being released and already protecting the nicotinic response from desensitization.

Effects of opioid compounds on desensitization of potassium-induced CA secretion

The exceptionally high concentrations of opioid agonists (and antagonists) required to protect the nicotinic response against desensitization may be acting on the nicotinic-ionophore complex or at some later element in the exocytotic mechanism. When inhibiting the nicotine- (or acetylcholine-) induced CA secretion, the opioid compounds appear to act on the nicotinic receptor–ionophore complex, since they do not inhibit potassium-induced CA secretion at concentrations that do inhibit nicotinic secretion. However, the interaction of nicotine with chromaffin cells appears to activate two distinct events, one leading to CA secretion, the other leading to inactivation ("desensitization") of this secretion (see Discussion). It is possible therefore that the opioid compounds may be producing their weak protection against desensitization of nicotinic CA secretion at a site distinct from the nicotinic receptor–ionophore complex. To test for this possibility, two opioid compounds (etorphine and dynorphin-1-13) were assessed for their ability to affect desensitization of pot-

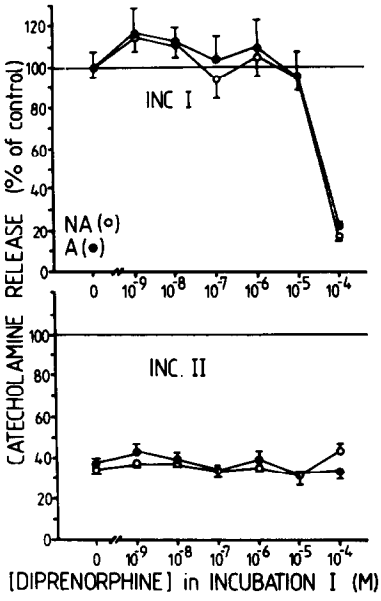

Fig. 8. Effect of diprenorphine (10^{-9} M–10^{-4} M) on desensitization of nicotine-induced CA secretion. Details as for Figs 1 and 3. Data are pooled from two cell preparations, N = 6–9. Control responses to 5 μM nicotine: incubation I—NA 15.0 (±1.0)% released/5½ min, N = 17; A 4.55 (±0.7)% released/5½ min, N = 17: incubation II—NA 15.5 (±0.8)% released/5½ min, N = 18; A 4.88 (±0.7)% released/5½ min, N = 18.

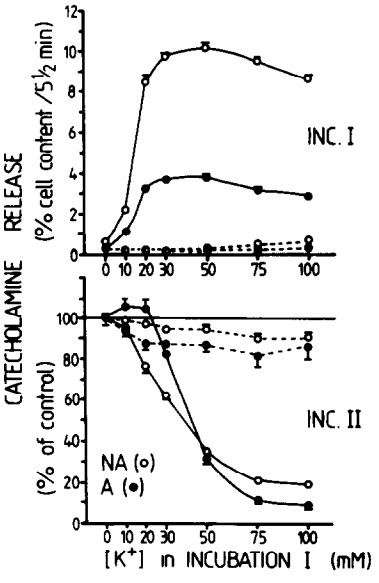

Fig. 9. Desensitization of potassium-induced catecholamine secretion from culture, bovine, chromaffin cells. Top panel: secretion of CA induced by 0–100 mM extra KCl (replacement of NaCl) in incubation I in the presence (complete lines) and absence (dotted lines) of 2.2 mM Ca^{2+} (see Fig. 1 for details). Bottom panel: subsequent secretion of CA induced by 50 mM extra KCl (replacement of NaCl) in incubation II following exposure to extra KCl at the indicated concentrations in incubation I. Results in incubation II are plotted as a % of the control response to 50 mM extra KCl in incubation II of un-desensitized cells. Data are pooled from 3 cell preparations, N = 6–12. NA (open circles)—noradrenaline; A (closed circles)—adrenaline. Control responses in incubation II: for complete lines—NA 12.9 (±0.9)% released/5½ min, N = 18; A 3.98 (±0.1)% released/5½ min, N = 18: for dotted lines NA—12.4 (±0.3)% released/5½ min, N = 12; A 4.36 (±0.2)% released/5½ min, N = 12.

assium-induced CA secretion from the cultured cells.

For this, extra KCl was substituted for NaCl in incubation I to induce desensitization, and 50 mM extra KCl was substituted for NaCl in incubation II as a standard test concentration to assess the degree of desensitization. Increasing the K^+ concentration in incubation I by 10–30 mM produced a progressive increase in CA secretion, and a progressive desensitization to subsequent stimulation with 50 mM excess K^+ (Fig. 9). Increasing the K^+ concentration in incubation I further produced a slight decline in the amount of CA secreted, but produced still more desensitization to subsequent K^+ stimulation. This was true for both NA and A cells. As with nicotine, K^+ induced considerably more secretion of NA than A in incubation I, but little difference in the desensitization of the two cell types to subsequent K^+ stimulation. Potassium-induced CA secretion in incubation I was completely dependent on calcium (Fig. 9, dotted lines) and only slight (10%) desensitization of K^+-induced CA secretion in incubation II was seen if Ca^{2+} was omitted from incubation I. Unlike nicotine (cf. Fig. 2A), K^+ stimulation in incubation I in the absence of Ca^{2+} did not sensitize the cells to the reintroduction Ca^{2+}. Cells exposed to K^+ in calcium-free buffer in incubation I secreted CA at the basal rate in incubation II when incubated in control buffer (i.e. containing Ca^{2+}, but no additional K^+—Fig. 2B). Consequently the apparent calcium-dependence of desensitization of K^+ induced CA secretion seen in Fig. 9 probably reflects the true calcium-dependence of this event. These properties of K^+-induced CA secretion and its desensitization are similar to those reported previously [15] using the ^{3}H-NA as a marker for CA secretion.

Etorphine (10^{-5} and 10^{-4} M) and dynorphin-1-13 (5×10^{-6} and 2×10^{-5} M) included in incubation I with 50 mM extra K^+ failed to protect the cells against desensitization to subsequent K^+ stimulation (Fig. 10, bottom panels) even though they did protect significantly against nicotinic desensitization (Figs 6 and 3, bottom panels). However, etorphine at 10^{-4} M did inhibit the K^+-induced CA release in incubation I, suggesting at such extremely high concentrations, etorphine may act non-specifically to reduce chromaffin cell excitability. A similar inhibition of K^+-induced CA secretion was seen with 10^{-3} M but not with 10^{-4} M naloxone (not shown). At the lower concentration, etorphine, and dynorphin-1-13 at both concentrations, did not affect K^+-induced CA secretion in incubation I.

DISCUSSION

Early studies on the secretion of CA from the adrenal medulla found that prolonged stimulation of the gland with acetylcholine led to a rapid decline in the amount of CA released [21, 22]. Such desen-

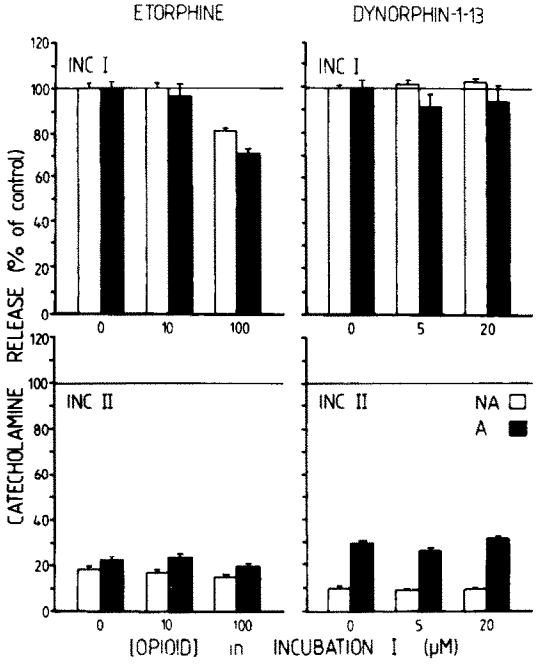

Fig. 10. Effects of etorphine (10^{-5} M and 10^{-4} M—left panels) or dynorphin-1-13 (5×10^{-6} and 2×10^{-5} M—right panels) on desensitization of potassium-induced CA secretion. Top panels: effects of including etorphine or dynorphin 1-13 in incubation I on the secretion of CA induced by 50 mM extra KCl. Bottom panels: subsequent secretion of CA induced by 50 mM extra KCl in incubation II, following the exposure to 50 mM extra KCl and the indicated concentrations of etorphine or dynorphin-1-13 in incubation I. N = 6. Control responses to 50 mM extra KCl for etorphine and dynorphin-1-13 experiments, respectively: incubation I—NA 13.3 (±0.3) and 12.0 (±0.1)% released/5½ min, N = 6 and 6; A 4.70 (±0.1) and 8.81 (±0.3)% released/5½ min, N = 6 and 6: incubation II—NA 13.3 (±0.8) and 12.5 (±0.2)% released/5½ min, N = 6 and 6; A 4.82 (±0.2) and 8.13 (±0.2)% released/5½ min, N = 6 and 6.

sitization of the chromaffin cells secretory response has been observed during prolonged exposure to nicotinic agonists, during exposure to high concentrations of nicotinic agonists, and following repetitive exposure to such agonists [14–16, 21–24]. Desensitization has also been observed in a variety of different species, including the adrenal medulla of cat [14, 21, 22], cow [15, 16], pig [16], and rat [25]. During times of stress, acetylcholine will be secreted from adrenal medullary terminals of the splanchnic nerve at an appreciable rate, and the medullary chromaffin cells may be exposed to high concentrations of acetylcholine for prolonged periods. As a consequence, the nicotinic secretory response of the chromaffin cells is likely to become desensitized and the secretion of adrenal CA will be severely impaired. Clearly there is a need for a mechanism to act *in vivo* to prevent such an inactivation of adrenal medullary CA secretion during stress. This would ensure that adrenal CA could continued to contribute to the body's adaptation to prolonged or severe stress..

Previous studies [13] have shown that substance P, a neuropeptide found endogenously in the adrenal medulla [26], can act to prevent the nicotinic secretory response of medullary chromaffin cells from desensitizing. Subsequent studies *in vivo* in the rat indicate that this action of substance P may be physiologically important in enabling the adrenal medulla to maintain CA secretion during times of stress [17, 18]. Substance P protected against desensitization of the nicotinic secretory response of cultured bovine chromaffin cells at one fifth of the concentration required to inhibit nicotine-induced CA release directly [13]. This suggests that the mechanisms involved in nicotine-induced secretion and in desensitization of this nicotinic response are different. Four further pieces of evidence support this view. Firstly, the degree of desensitization is not correlated to the quantity of CA secreted. For example, 3 μM and 300 μM nicotine both induced the secretion of similar amounts of NA and A from the cultured cells (Fig. 1), but 3 μM nicotine desensitized the cells by 30% and 300 μM nicotine desensitized them by 70% (see also [15]). Consequently, a component of desensitization is independent of CA secretion. Secondly, although nicotine-induced CA secretion is completely dependent on the presence of calcium (Fig. 1, top panel), desensitization of the nicotinic secretory response can occur almost unchanged in calcium-free buffer (Figs 1 and 2A, see Results). Desensitization hence still occurs under conditions when secretion and depletion of cellular CA has been prevented. Thirdly, substance P (as noted above), a substance P antagonist D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]-substance P, and the α_2 adrenergic agonist clonidine are all capable of inhibiting nicotine-induced CA release from cultured bovine chromaffin cells, however substance P protects against desensitization of this nicotinic response [13], the substance P antagonist enhances the degree of desensitization (Khalil, Marley and Livett, submitted), and clonidine has no effect on the degree of desensitization (Wan, Powis and Livett, submitted). Finally, the secretion of adrenaline from cultured chromaffin cells by 50 mM K^+ stimulation is enhanced almost three-fold by prior stimulation of the cells with nicotine, even though such prior treatment depletes up to 20% of the cellular adrenaline levels [27].

Taken together, these observations clearly indicate that agonist-evoked secretion and agonist-induced desensitization are quite separate phenomena that occur in parallel and which can be regulated independently. Although the above data suggest that desensitization can occur independently of CA secretion, it is quite possible that depletion of cellular CA stores contributes to the degree of desensitization seen in experimental models. As described in the Results section, it is not possible to study the depletion-independent component of desensitization by desensitizing cells with nicotine in the absence of Ca^{2+}, since reintroduction of Ca^{2+} after such treatment itself induces substantial CA secretion (Fig. 2A). These data suggest the previous study on the effect of substance P on calcium-dependent and independent components of desensitization need to be reassessed [13].

The various mechanisms capable of producing desensitization of the nicotinic response, and their relative contributions to the degree of desensitization are of great interest but are outside the scope of the present study. The fact that CA secretion and desensitization of CA secretion can be independently regulated (see above) is sufficient grounds to study the effects of drugs on both processes, irrespective of their mechanisms.

Previous studies have shown that opioid peptides, which are present in the adrenal medulla in high concentrations, do not act on the high affinity, stereospecific opioid binding sites identified in the adrenal medulla from ligand binding studies, to modify the secretion of CA from adrenal chromaffin cells induced by nicotinic agonists [4–7, 20]. In the present study the ability of opioid compounds to modify the desensitization of this nicotinic response has been assessed.

Although opioid agonists, (such as etorphine, dynorphin-1-13 and metorphamide), did produce some protection against desensitization of the nicotinic response, this action was mimicked by opioid antagonists (such as naloxone), was very weak and only occurred at concentrations of the agonists that already produced greater inhibition of the nicotinic secretory response directly. The results suggest that adrenal opioid peptides do not act on the putative opioid receptors identified in the adrenal medulla to affect desensitization. At no concentrations were the opioid agonists able to protect against desensitization to a greater extent than they inhibited the nicotinic response. Consequently, the adrenal opioid peptides are unlikely to play a role *in vivo* in maintaining adrenal medullary CA secretion by preventing the nicotinic response of the chromaffin cells from desensitizing. The functions of the adrenal opioid receptors and of the adrenal opioid peptides remain to be determined.

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