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Role of κ opioid receptors in modulating cholinergic twitches in the circular muscle of guinea-pig colon

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1 Single pulse electrical field stimulation (EFS, 0.5 ms pulse width, 60 V at a frequency of 0.05 Hz) induced twitch contractions of mucosa-free circular muscle strips from the guinea-pig proximal colon which were abolished by atropine (0.3 μM), tetrodotoxin (0.3 μM) or ω-conotoxin GVIA (0.1 μM).

2 Various opioid receptor agonist concentration-dependently inhibited twitches with the following rank order of potency (EC_{50} values in brackets): U 50488 (0.31 nM) > dermorphin (4.3 nM) = dynorphin A (1-13) (6.2 nM) > [D-Ala^2, N-MePhe^4, Gly^5-ol]-enkephalin (DAMGO, 33.5 nM) = [D-Ala^2, D-Leu^5]-enkephalin (DADLE, 60 nM) > [D-Pen^2, D-Pen^5]-enkepahlin (DPDPE, 1144 nM).

3 Peptidase inhibitors (captopril, thiorphan and bestatin, 1 μM each) did not modify the amplitude of twitches. In the presence of peptidase inhibitors the concentration-response curve to dynorphin A (1-13) was displaced to the left to yield an EC_{50} of 0.35 nM, comparable to that of the selective κ receptor agonist, U50488. The curves to the other opioid receptor agonist were unaffected by peptidase inhibitors.

4 DPDPE, DADLE, dermorphin and DAMGO consistently induced a concentration-unrelated transient increase in basal tone and a small and transient facilitation of twitches before development of their inhibitory effect. These transient excitatory effects were not observed upon application of dynorphin A (1-13) or U 50488. The contraction produced by DPDPE (30 nM) was largely inhibited (>80%) by 1 μM atropine.

5 Twitches suppression induced by dynorphin A (1-13) (30 nM) was partly reversed (46 $\pm$ 8%, $n=6$) by naloxone (0.3 μM). The potent and selective κ opioid receptor antagonist nor-binaltorphimine (Nor-BNI, 3-100 nM)) did not affect the amplitude of twitches and potently antagonized (pK_B 9.83 $\pm$ 0.09, $n=10$) the inhibitory effect of dynorphin.

6 Naloxone (1-300 nM) concentration-dependently depressed the cholinergic twitches: this depressant effect was largely counteracted in the presence of apamin (0.1 μM) and N^G-nitro-L-arginine (30 μM) which potentiated cholinergic twitches on their own.

7 Dynorphin A (1-13) (10 nM, $n=6$) did not affect the contractile response to exogenous acetylcholine (1 μM), indicating that depression of evoked twitches occurs prejunctionally.

8 We conclude that multiple opioid receptors modulate cholinergic twitches in the circular muscle of guinea-pig proximal colon. While μ and δ opioid receptor agonists produced mixed excitatory and inhibitory effects, κ opioid receptors, activated by sub-nanomolar concentrations of dynorphin A (1-13), mediate a powerful and pure prejunctional inhibition of acetylcholine release.

Keywords: Guinea-pig proximal colon; circular muscle; opioids; cholinergic contractions; κ opioid receptor

Introduction

Various neuronal populations in the myenteric plexus of guinea-pig intestine express opioid-like immunoreactivity (Schultzberg *et al.*, 1980; Furness & Costa, 1982; Furness *et al.*, 1983; Kobayashi *et al.*, 1985) and binding sites for both μ, δ and κ opioid receptors have been localized on various target cells of the gut (see Leslie, 1987 for review), providing a background for a role of endogenous opioids in regulating intestinal motility.

Opioids, by acting via multiple receptors, exert a potent modulatory effect on intestinal motility which involves changes in the release of both excitatory and inhibitory mediators. Evidence has been presented that opioid receptor agonists can inhibit the release of acetylcholine and tachykinins from enteric excitatory nerves (Kosterlitz & Waterfield, 1972; Gintzler & Scalisi, 1982; Barthò *et al.*, 1982; Cherubini *et al.*, 1985; Johnson *et al.*, 1987; Fox & Morton, 1991; Kojima *et al.*, 1994) as well as the release of inhibitory transmitters from nonadrenergic non-cholinergic (NANC) nerves (Shimo & Ishii, 1978; Tonini *et al.*, 1985; Grider & Makhlouf, 1986; Glass *et al.*, 1986). Thus complex and possibly multiphasic changes in spontaneous and evoked contractility of the intestine are produced by stimulating opioid receptors (Kromer, 1988 for review).

An extensive literature deals with the effect of opioids on motility of the guinea-pig small intestine: at this level, a depression of evoked acetylcholine release (Paton, 1957) is the most documented effect, involving both μ and κ receptors (Waterfield & Kosterlitz, 1975; Johnson *et al.*, 1987; Fox & Morton, 1991). Administration of naloxone enhances the evoked release of acetylcholine, implying a tonic inhibitory role of endogenous opioids on acetylcholine release (e.g. Waterfield & Kosterlitz, 1975), and release of substance P appears to be similarly regulated (Holzer, 1984). Moreover, opioid receptor antagonists improve the efficiency of the peristaltic reflex of the guinea-pig ileum (Kromer, 1990; Waterman *et al.*, 1992). Therefore, a tonic inhibition on the release of excitatory mediators represents the prevalent effect of opioids in the guinea-pig small intestine. The effects of opioids in the guinea-pig large intestine have been less extensively investigated, yet some data suggest the existence of regional differences and, especially, a more complex role involving inhibitory effects on both excitatory and inhibitory transmitters (Shimo & Ishii, 1978; Tonini *et al.*, 1985; Marino *et al.*, 1993).

The aim of this study was to analyse the effect of opioid receptor agonists on cholinergic twitches produced by electrical field stimulation (EFS) in the circular muscle of the guinea-pig proximal colon: this preparation has been extensively used in the past years in our laboratory to char-

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acterize excitatory and inhibitory neuromuscular transmission (Maggi & Giuliani, 1993; Zagorodnyuk *et al.*, 1993; 1995; Maggi *et al.*, 1994a, b; Zagorodnyuk & Maggi, 1994; Giuliani & Maggi, 1995). With this aim we studied the effects of a number of selective agonists for opioid μ, δ and κ opioid receptors. Evidence has been obtained that κ opioid receptors mediate a powerful suppressant effect of EFS-evoked acetylcholine release, while μ and δ receptor agonists produce complex effects, probably involving a modulation of the release of both excitatory and inhibitory mediators.

Methods

Male albino guinea-pigs (300–350 g) were stunned and bled. A ring of proximal colon taken at 2–3 cm from the caecum was excised and placed in oxygenated (96% CO_2 and 4% CO_2, pH 7.4 at 37°C) Krebs solution of the following composition (mM): NaCl 119, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.5, KCl 4.7, $CaCl_2$ 2.5 and glucose 11. Circularly-oriented, mucosa-free muscle strips, were excised and prepared for isometric recording (load 10 mN) of mechanical activity in 5 ml organ bath, as described previously (Maggi & Giuliani, 1993; Giuliani & Maggi, 1995). The strips were allowed to equilibrate for 90 min. Electrical field stimulation (EFS) was made by means of two platinum wire electrodes placed at the top and the bottom of the organ bath. Single pulses (60 V, 0.5 ms duration) were automatically delivered through a GRASS S88 at a frequency of 0.05 Hz.

Most experiments were performed in the presence of bestatin, captopril and thiorphan (1 μM each) in order to reduce peptide degradation.

Cumulative concentration-response curves to opioid receptor agonists were constructed, the next concentration being added to the bath when the effects of the preceding one had reached a steady state. To validate this protocol and rule out tachyphylaxis, preliminary experiments were performed in which single submaximally effective concentration of U 50488 (1 nM, $n=4$), dynorphin A (1-13) (1 nM, $n=5$), dermorphin (10 nM, $n=4$) DAMGO (30 nM, $n=4$), DADLE (100 nM, $n=4$) or DPDPE (3 μM, $n=4$) were administered and their effect compared to the effect produced in the same strips by the same concentration of the agonist during a cumulative concentration-response curve. The results of these experiments (data not shown) failed to indicate any significant tachiphylaxis for twitch inhibition during the cumulative protocol of agonist administration.

In some experiments with unstimulated preparations, acetylcholine 1 μM was administered every 15 min with the aim of inducing a contractile response of similar amplitude to those induced by EFS. The effect of dynorphin A (1-13) on these contractions was checked by administering the opioid agonist 10 min before the next challenge with acetylcholine and after having obtained two reproducible responses to acetylcholine.

Statistical analysis

All data in the text are mean ± standard error (s.e.) of the mean. Statistical analysis was performed by means of Student's *t* test for paired or unpaired data or by analysis of variance, when applicable. Regression analysis was performed by the least squares method. EC_{50} and 95% confidence limits were calculated accordingly. Schild plot analysis was performed for the competitive antagonist Nor-BNI and the affinity was expressed in terms of pK_B calculated using the constrained plot method (slope constrained to −1) according to Tallarida & Murray (1981).

Drugs

Dermorphin, dynorphin A (1-13), [D-Ala^2, N-MePhe^4, Gly^5-ol]-enkephalin (DAMGO), ω-conotoxin GVIA were from Peninsula (St Helens, UK), atropine hydrochloride (Serva, Heidelberg, Germany), [D-Pen^2, D-Pen^5]-enkephalin (DPDPE), [D-Ala^2, D-Leu^5]-enkephalin (DADLE), naloxone hydrochloride, apamin and N^G-nitro-L-arginine (L-NOARG) were from Sigma (St. Louis, MO, U.S.A.), tetrodotoxin (Sankyo, Tokyo, Japan), nor-binaltorphimine dihydrochloride (Nor-BNI) and U 50488(−(±)-3,4,Dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methanesulphonate) (R.B.I., Natick, U.S.A.), acetylcholine chloride (Merck, Darmstadt, Germany) and GR 82,334 ([[(S,S)Pro-Leu(spiro-γ-lactam)]^{9,10},Trp^{11}] Physalaemin (1–11)) was purchased from Neosystem (Strasbourg, France). GR 94800 (PhCO-Ala-Ala-DTrp-Phe-DPro-Pro-NleNH$_2$) was synthesized by conventional solid phase methods in the Chemistry Dept. of Menarini Pharmaceuticals.

Results

General

Single pulse EFS (0.5 ms pulse width, 60 V), automatically delivered at a frequency of 0.05 Hz produced a series of regular twitch contractions of mucosa-free circular muscle strips from the guinea-pig proximal colon. The force developed was 24.8 ± 3.3 mN ($n=11$) in normal Krebs and 25.4 ± 1.6 mN ($n=24$, NS) in the presence of peptidase inhibitors (captopril, thiorphan and bestatin, 1 μM each).

Twitches were abolished by atropine (1 μM, $n=5$), tetrodotoxin (0.3 μM, $n=4$) or by the N-type calcium channel blocker ω-conotoxin (0.1 μM, $n=5$). The selective tachykinin NK_1 receptor antagonist, GR 82,334 (10 μM) and the selective tachykinin NK_2 receptor antagonist, GR 94,800 (1 μM) slightly inhibited twitches by 18 ± 3% and 33 ± 4% ($n=5$ and 8), respectively.

Effects of opioids on electrically-induced contractions

All the opioid receptor agonists tested produced a concentration-dependent depression of the EFS-evoked twitch contractions (Figure 1 and 2; Table 1 for EC_{50} values). In the absence of peptidase inhibitors, the selective κ opioid receptor agonist, U 50488 was most potent and effective inhibitor tested: the EC_{50} was 0.31 nM and full suppression of twitches was produced at 3 nM (Figure 2a). The natural κ receptor preferring agonist, dynorphin A (1-13) likewise produced an almost complete suppression of twitches (96 ± 4% inhibition, $n=5$) but, in the absence of peptidase inhibitors, it was about 20 fold less potent that U 50488 (Table 1). The μ opioid receptor preferring agonist, dermorphin, potently inhibited but did not suppress twitches (E_{max} 84 ± 4% inhibition, $n=4$) and was about 14 fold less potent than U50488 (Figure 2a, Table 1). Another μ receptor selective agonist, DAMGO, inhibited twitches by no more than 60% and was about 108 fold less potent than U50488 (Figure 2a, Table 1). The selective μ

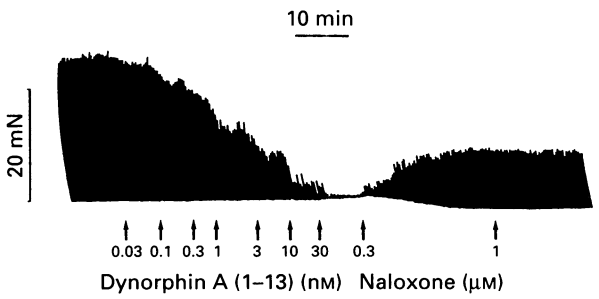

Figure 1 Typical tracing showing the concentration-related inhibitory effect of dynorphin A (1-13) on the twitch contractions (0.05 Hz, 0.5 ms, 60 V) of circular muscle strips of guinea-pig isolated proximal colon. This inhibition was partially antagonized by 0.3 μM naloxone. At steady state, increasing the naxolone concentration to 1 μM did not induce additional antagonism.

Table 1 EC_{50} values of various opioid receptor agonists in inhibiting EFS-evoked twitches of circular muscle strips from the guinea-pig proximal colon in the absence and presence of peptidase inhibitors (captopril, bestatin and thiorphan, 1 μM each)

Agonist	*EC_{50}* (nM) (controls)	*Potency Ratio*	*EC_{50}* (nM) (peptidase inhibitors)	*Potency Ratio*
U 50488	0.31 (0.2–0.4)	1	0.32 (0.1–0.5)	1
Dynorphin A(1-13)	6.2 (3.6–23.4)	20	0.35 (0.3–0.5)	1.1
Dermorphin	4.3 (3.0–8.6)	14	3.6 (3.0–4.7)	11
DAMGO	33.5 (12.5–314)	108	28.5 (19.9–45)	86
DADLE	60 (25–267)	192	29 (17–63)	91
DPDPE	1144 (260–14789)	3700	1643 (138–15400)	5134

Each value is the mean ± s.e.mean of 4–6 experiments.

opioid receptor agonists, DADLE and DPDPE inhibited twitches by about 90 and 50% at the highest concentrations tested (1 and 10 μM) respectively and were about 192 and 3700 fold less potent than U50488, respectively (Figure 2a, Table 1).

When tested in the presence of peptidase inhibitors (thiorphan, captopril and bestatin, 1 μM each), the order of potency of the opioid receptor agonists did not change (Figure 2b; Table 1), except for dynorphin A (1-13): the concentration-response curve to this neuropeptide was significantly shifted to the left in the presence of peptidase inhibitors and the corresponding EC_{50} value (0.35 nM) was not significantly different from that of U50488 (Figure 2b, Table 1).

Effect of opioid receptor agonist on resting tone

Upon cumulative bath application, dermorphin, DADLE, DPDPE and DAMGO consistently produced a small (< 15% of twitches amplitude) and transient tonic-type contraction of the strips, at threshold concentrations ranging between 1–10 nM for different agonists. Concomitant with this contractile response, DADLE and DPDPE also produced a small and transient enhancement of twitches amplitude which was then replaced by the sustained inhibitory effect described above. For example, 30 nM DADLE applied during a cumulative concentration-response curve produced a transient tonic contraction of 1.9 ± 1 mN, concomitant with a transient increase of twitch amplitude (28 ± 18% increase) and followed by a sustained depression of twitches (38 ± 12% inhibition, $n = 4$). The tonic type contraction and transient twitch facilitation produced during the cumulative-concentration-response curves to dermorphin, DADLE, DPDPE and DAMGO were not concentration-dependent and were not analysed further.

In 4 experiments, the contractile response produced by 30 nM DPDPE (2.0 ± 0.5 mN) was markedly inhibited (81 ± 7%) by 1 μM atropine.

In sharp contrast, neither U50488 nor dynorphin A (1-13) had consistent effects on tone and a pure inhibitory effect on twitch amplitude was observed in all strips tested.

Effect of opioid antagonists on twitch amplitude and on dynorphin inhibition of twitches

Naloxone (0.1–300 μM, $n = 8$) alone produced a concentration-dependent inhibition of twitches: the maximal effect of naloxone averaged 87 ± 3% inhibition at 0.3 μM with an EC_{50} of 3.5 nM (2.7–5.2 nM are 95% c.l.). The inhibitory effect of naloxone was reversed by washout and could be reproduced upon a second application of the drug. This inhibitory effect precluded the analysis of the effect of naloxone on the concentration-response curve to dynorphin A (1-13). To overcome this problem, we administered naloxone (0.3–1 μM) after the development of the suppressant effect of dynorphin A (1-13): as shown in Figure 1, naloxone (0.3 μM) promptly reversed the suppressant effect of dynorphin A (1-13): the amplitude of twitches recovered to 46 ± 8% of pre-drug amplitude ($n = 5$) in about 5–10 min. At 1 μM naloxone did not further reverse the inhibitory effect of dynorphin.

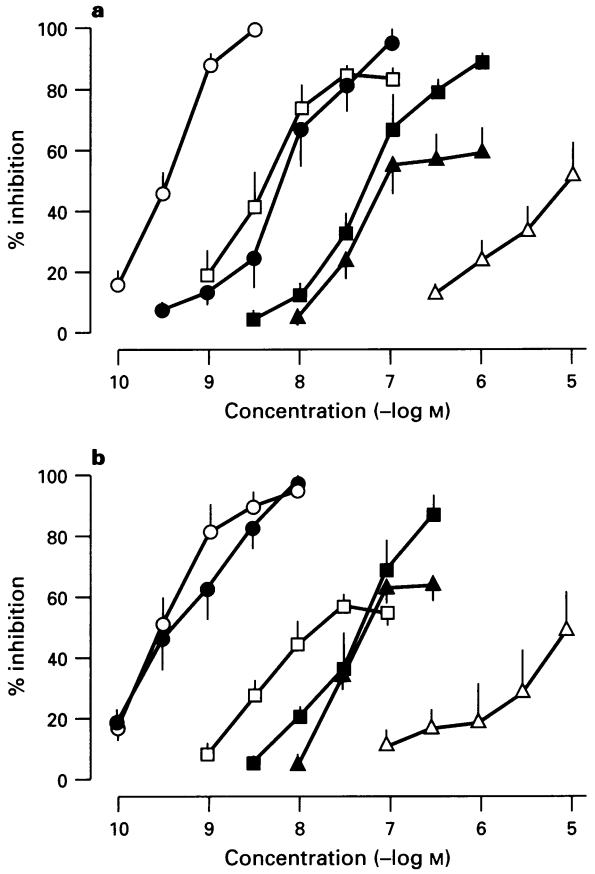

Figure 2 (a) Concentration-dependent inhibition of guinea-pig isolated proximal colon contractions, induced by EFS (single pulses at 0.05 Hz, 0.5 ms, 60 V), by the selective opioid receptors agonists (○) U 50488, (●) dynorphin A (1-13), (□) dermorphin, (■) DADLE, (▲) DAMGO and (△) DPDPE. (b) The same as in (a) but in the presence of peptidase inhibitors (thiorphan, captopril and bestatin, 1 μM each) in the bath. Note that the maximal inhibition induced by both dermorphin and DAMGO was approximately 60%. Each value is mean ± s.e.mean of 4–6 experiments.

The hypothesis was advanced that the inhibitory effect of naloxone may originate from blockade of an opioid tone inhibiting NANC inhibitory mechanisms which regulate cholinergic twitches (see discussion). To test this hypothesis the effect of naloxone was investigated in the presence of apamin (0.1 μM) and L-NOARG (30 μM). Apamin and L-NOARG (30 min contact time) enhanced twitches amplitude from 20.5 ± 1.6 to 41.4 ± 3.4 mN (102 ± 10% increase, $n = 10$). In the presence of apamin and L-NOARG the inhibitory effect of naloxone was markedly reduced as compared to control: the EC_{50} was increased to 796 nM (465 ± 1510 nM are 95% c.l.) and the maximal inhibition of twitches averaged 40 ± 8% at 300 nM ($P < 0.05$ vs control) (Figure 3).

The selective κ opioid receptor antagonist Nor-BNI did not affect the amplitude of twitches up to 100 nM (Figure 4). Nor-BNI (3–100 nM, 15 min before) induced a parallel rightward shift of the control concentration-response curve to dynorphin A (1-13) without depression of the maximal response to the agonist: the slope of Schild plot (−0.88) was not significantly different from unity and an apparent pK_B value of 9.83 ± 0.09 ($n = 10$) was calculated by the constrained Schild plot method.

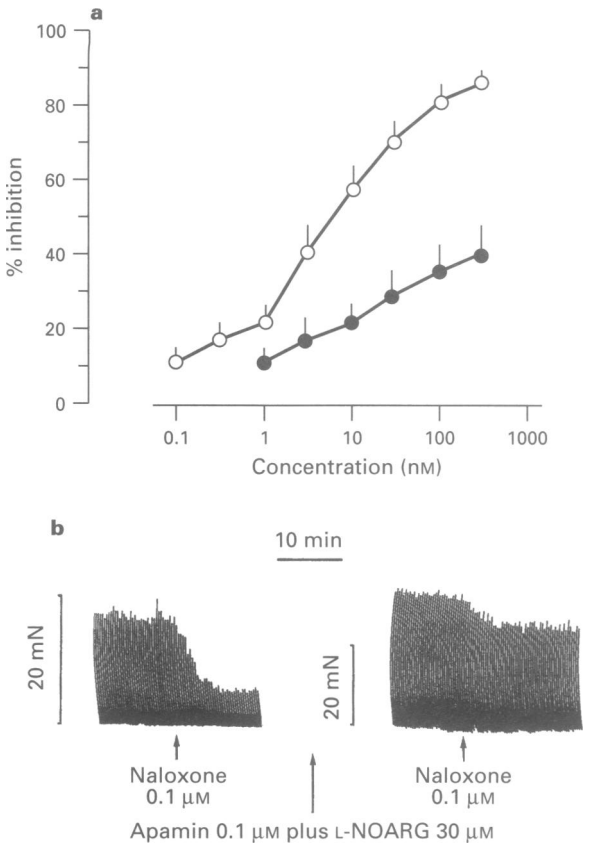

Figure 3 (a) Concentration-related inhibitory effect of naloxone on twitches in the circular muscle strips of guinea-pig proximal colon in control (○) and in the presence (●) of apamin (0.1 μM) plus L-NOARG (30 μM). Each value is mean ± s.e.mean of 10 experiments. (b) Typical tracings illustrating the inhibition of the twitches produced by a selected concentration (0.1 μM) of naloxone in the absence (left tracing) and in the presence of apamin plus L-NOARG (0.1 and 30 μM respectively, 30 min contact time) (right tracing). The pretreatment produced a consistent enhancement of the amplitude of twitch contractions.

Effect of dynorphin A (1-13) on the response to exogenous acetylcholine

Acetylcholine (1 μM) induced a contraction of the circular muscle of the guinea-pig proximal colon, averaging 8.8 ± 1.1 mN ($n = 6$), which was not significantly affected (8.4 ± 0.9 mN, $n = 6$) by a previous application of dynorphin A (1 = 13) (10 nM, 10 min before).

Discussion

Mucosa-free circular muscle strips from the guinea-pig proximal colon respond to single pulse EFS with cholinergic twitch contractions: owing to the importance of circular muscle excitatory cholinergic innervation for peristalsis, this preparation is a convenient model for studying the effect of drugs on evoked acetylcholine release. The cholinergic nature of the EFS-evoked twitches is demonstrated by the suppressant effect of atropine: the limited inhibitory effects on twitches amplitudes produced by the selective tachykinin receptor antagonists, GR 82334 and GR 94800, is in keeping with previous data indicating that even a single pulse of EFS can produce a slow atropine-resistant response in this preparation (Maggi *et al.*, 1994a). It appears likely that, in response to single pulse EFS, minute amounts of tachykinins may facilitate the contractile action of acetylcholine.

Our findings indicate that κ opioid receptors mediate a powerful suppressant effect of prejunctional origin on the EFS-evoked cholinergic twitches. This is indicated by the potent action of U 50488 and of the natural κ-receptor preferring agonist, dynorphin A (1-13). The potency of the latter neuropeptide was distinctly increased by the addition of peptidase inhibitors, to suggest its degradation by membrane bound peptidases. Moreover, the suppressant action of dynorphin A (1-13) was potently antagonized (pK_B 9.83) by the selective κ opioid receptor antagonist, Nor-BNI (Portoghese *et al.*, 1987).

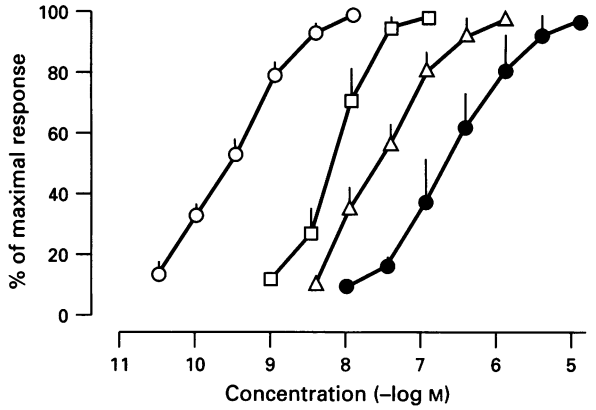

Figure 4 Concentration-response curves to dynorphin A (1-13) in inhibiting twitches of circular muscle strips of guinea-pig isolated proximal colon in the absence (○) and in the presence of 3 nM (□), 10 nM (△) and 100 nM (●) concentration of the selective κ opioid receptor antagonists nor-binaltorphimine. Each value is mean ± s.e.mean of 3–4 experiments.

The motor effects produced by the μ and δ opioid receptor preferring agonists, dermorphin, DADLE, DAMGO and DPDPE were more complex: all these ligands effectively depressed the cholinergic twitches, as U 50488 and dynorphin A (1-13) did, but with a lower potency and, in some cases, with a lower E_{max} than κ receptor agonists. More importantly, the response to dermorphin, DADLE, DPDPE and DAMGO was qualitatively different from that produced by U 50488 and dynorphin A (1-13), since a small and transient contraction was observed as the initial effect, concomitant with a slight twitch potentiation and followed by a more sustained inhibition. Contrary to the concentration-dependent inhibition of twitches, the excitatory effects of dermorphin, DAMGO, DADLE and DPDPE were not concentration-dependent. Tonini *et al.* (1985) reported previously a naloxone-reversible contractile effect of morphine in guinea-pig taenia coli. As illustrated by experiments with DPDPE, the contractile response obtained in guinea-pig colon is atropine-sensitive and may involve either stimulation or disinhibition of cholinergic neurones.

The second alternative appears more likely when considering the results of experiments with naloxone, apamin and L-NOARG. Previously, we obtained evidence that NANC inhibitory mechanisms are tonically active in this preparation since apamin and L-NOARG produce a sustained contraction (Maggi & Giuliani, 1993); the marked enhancement in the amplitude of the EFS-evoked cholinergic twitches after apamin and L-NOARG observed here suggests a tonic influence on the excitability of cholinergic neurones, thus reducing the evoked aetylcholine release. Moreover a fast and a slow NANC inhibitory junction potential (i.j.p.) are evoked by single pulse EFS in the presence of atropine, which are blocked by apamin and L-NOARG, respectively; and DPDPE inhibits the apamin-sensitive NANC i.j.p. (Zagorodnyuk & Maggi, 1994).

From the above, we speculate that, during single pulse EFS, NANC inhibitory mechanisms are co-activated along with cholinergic neurones and the amplitude of cholinergic twitches is reduced by the concomitant release of NANC inhibitory transmitters. Mirroring the atropine-sensitive contraction produced by DPDPE is the concentration-dependent inhibition of cholinergic twitches produced by low concentrations of naloxone, suggestive of a specific effect on opioid receptors. This inhibitory effect was greatly reduced when tested in the presence of

apamin and L-NOARG, suggesting again the involvement of NANC inhibitory transmission. Marino *et al.* (1993) have shown a facilitatory effect of naloxone on acetylcholine and noradrenaline release in the guinea-pig distal colon suggesting the existence of a tonic control exerted by opioids on the NANC inhibitory pathways. The effect of naloxone may thus involve the blockade of a tonic inhibitory influence by endogenous opioids on NANC inhibitory neurones controlling acetylcholine release. On the other hand κ opioid receptors do not appear to be tonically involved in controlling acetylcholine release, since Nor-BNI did not affect twitch amplitude.

In conclusion, the present findings indicate that κ opioid receptors exert a powerful modulatory influence on acetylcholine release in the circular muscle of the guinea-pig proximal colon. On the other hand μ and δ opioid receptors are likely to be involved in a more complex modulation of acetylcholine release involving mixed effects, both excitatory and inhibitory. In particular, naloxone-sensitive opioid receptors could be involved in controlling acetylcholine release indirectly, by modulating the release of NANC inhibitory transmitters.

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