Effects of putative neurotransmitters and related drugs on withdrawal contractions of guinea-pig isolated ileum following brief contact with [Met⁵]enkephalin

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1 Brief exposure for 2 min of guinea-pig isolated ileum to [Met⁵]enkephalin (MENk) and noradrenaline has been shown previously to produce withdrawal contractions on washout of the agonist or addition of naloxone (MENk) or phentolamine (noradrenaline).

2 The present study was undertaken to investigate firstly, whether other putative neurotransmitters and/or related drugs which inhibit transmitter release also produced withdrawal responses following 2 min contact with the ileum and secondly, whether they affected the opioid withdrawal response.

3 Adenosine (1–5 μ M), but not U-50,488H (1–5 μ M), somatostatin (0.01–5 μ M), oreotide (1–5 μ M), baclofen (1–25 μ M) or dopamine (5, 50 μ M), produced a contraction on washout following 2 min contact with the ileum. The adenosine (5 μ M) washout contraction, in common with MENk and noradrenaline washout contractions, was inhibited by the substance P antagonist, spantide (10 μ M).

4 Added 30 s before washout at a concentration of 5 μ M, noradrenaline, U-50,488H, adenosine, somatostatin and oreotide significantly inhibited the washout withdrawal response following 2 min contact of the ileum with MENk, 1 μ M. A higher concentration of baclofen, 250 μ M, also inhibited this response.

5 The naloxone (1 μ M)-precipitated withdrawal response following contact of the ileum with MENk, 1 μ M, for 2 min, was inhibited only by noradrenaline (5 μ M) and U-50,488H (5 μ M).

6 It is concluded that during naloxone-precipitated opioid withdrawal an additional population of enteric motor neurones is recruited which is not involved in the washout withdrawal response and these neurones have less diversity of presynaptic receptors mediating inhibition of transmitter release than cholinergic motor neurones.

Introduction

Brief exposure for 2 min to [Met⁵]enkephalin (MENk), several enkephalin analogues and noradrenaline produced dependence in guinea-pig ileum as measured by withdrawal contractions on washout (Chahl, 1983; 1985; 1986). Withdrawal responses were also precipitated by naloxone following 2 min contact with several opioids including morphine, β -endorphin and dynorphin A- (1–13), and by phentolamine following contact with clonidine (Chahl, 1985). Furthermore it has been shown that the α_2 -adrenoceptor agonist clonidine (Chahl, 1985), and both μ and κ -opioid receptor agonists inhibited the opioid withdrawal response of the guinea-pig ileum (Chahl, 1986).

Opioids and α -adrenoceptor agonists have in common an action to inhibit release of acetylcholine (ACh) and substance P (SP) from enteric neurones (see Barthó & Holzer, 1985). Several other putative enteric neurotransmitters, including adenosine (see Fredholm & Dunwiddie, 1988), somatostatin (Guillemin, 1976; Yau *et al.*, 1986) and γ -aminobutyric acid (GABA) (Cherubini & North, 1984), have also been reported to inhibit transmitter release from enteric neurones. In order to obtain further information on possible mechanisms involved in withdrawal responses, the present study was undertaken to investigate firstly, whether these putative neurotransmitters and/or related drugs produced dependence as manifest by a withdrawal response following brief contact with the ileum, and secondly whether they affected the opioid withdrawal response.

Methods

Adult guinea-pigs of either sex were killed by a blow to the head and a 2 cm segment of ileum was removed and suspended under 1 g tension in a 2 ml organ bath containing oxygenated Tyrode solution at 37°C. Responses were recorded with a force transducer and Grass polygraph. At the start of each experiment a maximum response to ACh (5 μ M) was

obtained so that all responses could be expressed as percentages of the ACh maximum. Standard responses were then obtained to ACh (0.1 μ M), SP (2.5 nM), and to washout following 2 min contact with MENk, 1 μ M.

Effects of agonists and antagonists

Neurotransmitters and related drugs, were each tested for their effects on the ileum on contact, and on washout following 2 min contact. The effect of the SP antagonist, spantide, 10 μ M, added 30 s before washout, was tested on the washout response to adenosine.

The effect of the somatostatin antagonist, cyclo(7-aminoheptanoyl-Phe-D-Trp-Lys-Thr[Bzl]) (Fries *et al.*, 1982) added 2 min after addition of somatostatin to the organ bath was tested. The effect of naloxone, 1 μ M, was tested following 2 min contact of the ileum with the κ -opioid agonist, U-50,488H. Likewise the effect of the GABA_B antagonist, phaclofen (Kerr *et al.*, 1987), was tested following 2 min contact of the ileum with baclofen.

Effects of agonists and related drugs on the opioid withdrawal response

The effects of the neurotransmitters and related drugs were tested on the washout withdrawal response to MENk. In these experiments MENk, 1 μ M was added to the bath for 2 min before washout and the substance to be tested was added 0.5 min before washout (1.5 min after addition of MENk). In some experiments oreotide was added 5 min before addition of MENk. Control responses to MENk washout were obtained on the same preparations.

In some experiments the effects of the substances were tested as above on the naloxone-precipitated withdrawal response to MENk. In these experiments MENk, 1 μ M, was added to the bath and naloxone, 1 μ M, was added 2 min later. Since only one response to naloxone was obtained on each preparation, control responses were obtained on other preparations from the same animals.

Statistics

Responses were compared by Student's *t* tests for unpaired observations, or paired *t* tests for paired observations. The *n* values represent preparations from different animals.

Drugs

Drugs used were: acetylcholine chloride (Sigma); adenosine (Merck); baclofen (Ciba-Geigy); dopamine hydrochloride (Sigma); γ -aminobutyric acid (Sigma); [Met⁵]enkephalin (Peptide Institute, Osaka, Japan); naloxone hydrochloride (Sigma); neurotensin (Peptide Institute); (–)-noradrenaline bitartrate (Sigma); ocreotide (Sandostat, Sandoz); phaclofen (Professor David Kerr and Dr Jennifer Ong); 8-phenyltheophylline (Research Biochemicals Inc.); somatostatin (Sigma); cyclo(7-aminoheptanoyl-Phe-D-Trp-Lys-Thr[Bzl]) (somatostatin antagonist) (Sigma); substance P (Peptide Institute); [D-Arg¹,D-Trp^{7,9},Leu¹¹] substance P Spantide, Bachem); U-50,488H (trans-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methane sulphonate Upjohn) (Stephen Johnson); vasoactive intestinal polypeptide (Peptide Institute).

Results

Effects of agonists on guinea-pig ileum

The effects of putative neurotransmitters and related drugs on the tension of the guinea-pig ileum are shown in Table 1. On preparations where tone was slightly elevated, some fall in tone was observed on addition of U-50,488H, adenosine, somatostatin and its analogue ocreotide, baclofen, and dopamine, in the concentrations shown in Figures 1, 2 and 4 respectively. None of these drugs produced contraction of the ileum. However, GABA, 5 μ M, neurotensin, 0.05 μ M, and VIP, 0.1 μ M, produced marked contractions on contact with the ileum. At a concentration of 0.1 μ M, GABA produced no effect on the ileum whereas at 1 μ M it produced relaxation only. The

Table 1 Effects of 2 min contact of guinea-pig ileum with putative neurotransmitters and related drugs and the effect of washout

Drug	Effect on ileum	Response on washout
[Met ⁵]enkephalin (Chahl, 1985)	relaxation	contraction (1 μ M) 20 $\pm$ 2† (20)
Noradrenaline (Chahl, 1985)	relaxation	contraction (5 μ M) 22 $\pm$ 4 (8)
U-50,488H 1–5 μ M	relaxation	nil (9) (5 μ M)
Adenosine 1–5 μ M	relaxation	contraction (5 μ M) 32 $\pm$ 4 (15)
Somatostatin 0.01–5 μ M	relaxation	nil (6) (5 μ M)
Ocreotide 1–5 μ M	relaxation	nil (5) (5 μ M)
GABA 0.1 μ M	nil	nil (3)
1 μ M	relaxation	nil (3)
5 μ M	'spike' contraction 60;50	nil (2)
Baclofen 1–25 μ M	relaxation	nil (7) (5 μ M)
Dopamine 5 μ M	slight relaxation	nil (5)
50 μ M	slight relaxation	nil (11)
VIP 0.1 μ M	maximal contraction (tachyphylaxis) (5)	return to baseline
Neurotensin 0.05 μ M	maximal contraction (5)	return to baseline

† Percentage of ACh maximum $\pm$ s.e.

Numbers in parentheses are the numbers of preparations from different animals.

contractions to VIP, and to a lesser extent neurotensin, exhibited tachyphylaxis on subsequent additions.

Effects of washout

On washout following 2 min contact, adenosine, 1–5 μ M, was the only drug tested which produced a contracture (Table 1, Figure 1). The washout contracture to adenosine, 5 μ M, was abolished in seven preparations (from seven animals) by addition of the SP antagonist, spantide, 10 μ M, 30 s before washout of the bath (Figure 1). Thus adenosine produced similar actions on the ileum to those previously described for MENk and noradrenaline (Chahl, 1983; 1985). The lack of response to washout of somatostatin, 5 μ M, is shown in Figure 2.

Effects of addition of antagonists

Addition of the somatostatin antagonist, cyclo(7-aminoheptanoyl-Phe-D-Trp-Lys-Thr[Bzl]), at a concentration of 5 μ M, following 2 min contact of the ileum with concentrations of somatostatin of 1 μ M (*n* = 5) and 0.1 μ M (*n* = 3) did not produce contraction of the ileum. In one experiment the somatostatin antagonist at a concentration of 50 μ M did not produce contraction following 2 min contact of the ileum with somatostatin, 5 μ M. Naloxone, 1 μ M, added 2 min after addition of U-50,488H, 1 or 5 μ M, did not result in contracture of the ileum (*n* = 9). Higher concentrations of naloxone were not tested as previous experiments indicated that naloxone was not specific at these concentrations (Chahl, 1983). The GABA_B antagonist, phaclofen, 500 μ M, produced a small contraction (23 $\pm$ 6% of the ACh maximum, *n* = 7) following 2 min contact of ileum with the GABA_B agonist, baclofen, 250 μ M. However, this response was not significantly greater than the contraction produced by phaclofen alone on the ileum (16 $\pm$ 3%, *n* = 8). The effect of the adenosine A₁ antagonist, 8-phenyltheophylline (5 μ M), was tested on two preparations following 2 min contact with adenosine 5 μ M and found to produce contractions (14% and 25%).

Effects of agonists on the opioid withdrawal response

The effects of the drugs tested on the withdrawal response following 2 min contact with MENk, 1 μ M, precipitated by washout or addition of naloxone, 1 μ M, are shown in Table 2.

Figure 1 Inhibition by the substance P antagonist, spantide, 10 μ M, of the washout withdrawal response of guinea-pig ileum to adenosine (Aden), 5 μ M. Adenosine was washed out after 2 min contact with the tissue. Spantide was added 30 s before washout and replaced immediately after washout.

Figure 2 Comparison of the effects of washout of [Met⁵]enkephalin (MENk) 1 μ M, and of somatostatin (Som) 5 μ M, on guinea-pig ileum. Each drug was present in the bath for 2 min before washout.

Table 2 Effect of putative neurotransmitters and related drugs on the withdrawal responses precipitated either by washout or naloxone ($1 \mu\text{M}$) following 2 min contact with $[\text{Met}^5]\text{enkephalin}$ ($1 \mu\text{M}$)

	MENk washout withdrawal	MENk naloxone-precipitated withdrawal
Control	44 ± 10 (7)	62 ± 9 (12)
Noradrenaline ($5 \mu\text{M}$)	$*19 \pm 6$ (7)	$***3 \pm 2$ (9)
Control	27 ± 4 (5)	56 ± 7 (8)
U-50,488H ($5 \mu\text{M}$)	$**0 \pm 0$ (5)	$***0 \pm 0$ (8)
Control	50 ± 9 (7)	62 ± 9 (12)
Adenosine ($5 \mu\text{M}$)	$***6 \pm 4$ (7)	43 ± 17 (6)
Control	47 ± 7 (8)	52 ± 9 (8)
Somatostatin ($5 \mu\text{M}$)	$***11 \pm 6$ (8)	47 ± 9 (7)
Control	31 ± 4 (5)	
Ocreotide ($5 \mu\text{M}$)	$**10 \pm 6$ (5)	
Control	50 ± 7 (9)	52 ± 9 (8)
Ocreotide ($5 \mu\text{M}$, added 5 min before MENk)	$***7 \pm 4$ (9)	51 ± 12 (6)
Control	40 ± 11 (5)	56 ± 7 (8)
Baclofen ($5 \mu\text{M}$)	36 ± 9 (5)	60 ± 19 (5)
Control	48 ± 7 (7)	62 ± 9 (12)
Baclofen ($250 \mu\text{M}$)	$***11 \pm 2$ (7)	76 ± 13 (8)

Except where indicated, drugs were added 30 s before washout or naloxone.

Responses are expressed as mean percentages of the ACh maximum $\pm$ s.e.mean. The numbers of preparations from different animals are shown in parentheses. Asterisks indicate statistically significant differences from control values, obtained in paired *t* tests (washout withdrawal) or Student's *t* tests (naloxone-precipitated withdrawal). $*0.05 > P > 0.01$; $**0.01 > P > 0.001$; $***P < 0.001$.

At a concentration of $5 \mu\text{M}$, noradrenaline, U-50,488H, adenosine, somatostatin (Figure 3) and ocreotide, added 30 s before washout, significantly inhibited the MENk washout withdrawal response. None of these agonists inhibited standard responses to SP or ACh. Ocreotide was more effective added 5 min before addition of MENk, indicating that it took time to reach equilibrium with the receptors (Figure 4). A higher concentration of baclofen, $250 \mu\text{M}$, inhibited the MENk washout withdrawal response. However, the naloxone-precipitated MENk withdrawal response was inhibited only by noradrenaline and U-50,488H (Table 2). Lack of effect of ocreotide on naloxone-precipitated MENk withdrawal is shown in Figure 4. In three preparations somatostatin at a concentration of $50 \mu\text{M}$, did not inhibit the naloxone-precipitated MENk withdrawal response, but in another three preparations, baclofen,

$500 \mu\text{M}$, abolished the naloxone-precipitated MENk response. This concentration of baclofen produced little effect on the SP standard response (10% reduction).

Discussion

The present study has demonstrated that following brief (2 min) contact with guinea-pig ileum, adenosine, in common with MENk and noradrenaline, produced a washout withdrawal contracture which was inhibited by an SP antagonist. The basis of the common actions of these three agonists is most probably due to their ability to inhibit transmitter release from enteric cholinergic neurones following activation of their own specific receptors (see Introduction). Although U-50,488H, somatostatin, ocreotide, baclofen and dopamine all produced relaxation of the ileum presumably due to inhibition of ACh release, none of these agonists produced a washout withdrawal contracture. A likely explanation for the lack of withdrawal responses to these agonists is that they were less readily removed from their receptors on washout than MENk, noradrenaline and adenosine. This possibility is supported by the observation that those agonists which did not exhibit a washout withdrawal response tended to produce a prolonged inhibition of tone and spontaneous movements after washout. However, it is also possible that the two groups of agonists did not have similar actions on neurotransmitter release (see below).

In order to produce withdrawal responses to substances that are not rapidly removed from receptors by washout it would be necessary to use a specific receptor antagonist to precipitate withdrawal. For example, naloxone has been shown to precipitate withdrawal following 2 min contact of ileum with morphine (Chahl, 1986), and phentolamine precipitated withdrawal following 2 min contact with clonidine (Chahl, 1985). In the present experiments concentrations of naloxone ($1 \mu\text{M}$) which precipitated withdrawal from μ -receptor agonists (Chahl, 1986), did not produce a withdrawal contracture from the κ -agonist, U-50,488H. Higher concentrations of naloxone were not tested as previous experiments indicated that naloxone was not specific at higher concentrations (Chahl, 1983). The finding that the adenosine A_1 antagonist, 8-phenyltheophylline, produced contraction of the ileum following 2 min contact with adenosine confirms previous findings in which withdrawal of ileum following 16–21 h incubation with adenosine was demonstrated by 8-phenyltheophylline challenge (Collier & Tucker, 1983). The lack of effect of the somatostatin antagonist and the GABA_B receptor antagonist, phaclofen, in producing withdrawal responses from somatostatin and baclofen respectively, probably reflected the low potency of these antagonists.

It has been proposed previously (Chahl, 1983) that a feedback circuit of neurones might be responsible for mediating the opioid withdrawal response and that the contracture response might involve release of transmitters (SP and ACh) from more than one type of neurone. If such a circuit is indeed

Figure 3 Reversible inhibition of the washout withdrawal response to $[\text{Met}^5]\text{enkephalin}$ (MENk) $1 \mu\text{M}$, by somatostatin (Som) $5 \mu\text{M}$, added 30 s before washout. Washout shown as triangles.

Figure 4 Effect of ocreotide on [Met⁵]enkephalin (MENk) withdrawal responses on three ileum preparations from the same animal. On each preparation a control withdrawal response was obtained by washout following 2 min contact with MENk, 1 μ M. On preparation (a) a control naloxone (1 μ M)-precipitated withdrawal response to MENk (1 μ M) was obtained. Naloxone was added 2 min after MENk. On preparation (b) a MENk naloxone-precipitated withdrawal response was obtained 5 min after addition of the somatostatin analogue, ocreotide, 5 μ M. On preparation (c), a MENk washout withdrawal response was obtained 5 min after addition of ocreotide, 5 μ M. No marked difference between the heights of responses to MENk washout and naloxone-precipitated withdrawal (as in these preparations) was consistently observed over a number of preparations (see Table 2 for mean values), although the naloxone-precipitated response was often slightly larger and usually more prolonged.

involved, it is not known whether the other agonists tested here acted in the same circuit and on the same neurones in the circuit as the opioids. The agonists used in this study reduce transmitter release from neurones by different mechanisms. Thus μ -opioid and α_2 -adrenoceptor agonists, and possibly adenosine and somatostatin, increase potassium conductance (see North, 1986; Fredholm & Dunwiddie, 1988), whereas κ -opioid and GABA_B receptor agonists decrease an inward calcium conductance (see North, 1986; Cherubini & North, 1984). The relative proportions of these receptors on different neuronal types in the guinea-pig ileum and their relative effectiveness in inhibiting transmitter release are unknown. Nevertheless, if the receptors for all agonists tested were present in similar numbers on the same population of enteric neurones and acted through the same intracellular mechanism to inhibit transmitter release to a similar extent, it would be expected that each agonist could substitute for any one of the others and thus prevent its withdrawal response. This was tested in the present experiments by examining whether each agonist would inhibit the MENk washout and naloxone-precipitated withdrawal responses. The results obtained for the MENk washout withdrawal response were as expected, that is, each agonist tested inhibited this response. Thus it would seem reasonable to conclude that the population(s) of neurones involved in the MENk washout withdrawal response had not only μ -opioid receptors, but also κ -opioid receptors, α -adrenoceptors, somatostatin, adenosine and GABA_B receptors, stimulation of which resulted in the same functional response.

Results for the experiments on MENk naloxone-precipitated withdrawal, however, differed from those for the MENk washout withdrawal. Only noradrenaline and U-50,488H inhibited the MENk naloxone-precipitated withdrawal at concentrations that inhibited the washout withdrawal response. A two fold higher concentration of baclofen inhibited the MENk naloxone precipitated withdrawal response, a finding which

agreed with previous findings on the inhibition by baclofen (1 μ M) of the naloxone contracture in morphine-tolerant guinea-pig ileum (Luzzi *et al.*, 1985). However, even a ten fold increase in the concentration of somatostatin was ineffective in reducing the response. Two possible explanations for these findings should be considered. Firstly, since the MENk naloxone-precipitated withdrawal response was much more resistant to inhibition by some agonists than others, at concentrations which markedly inhibited the MENk washout withdrawal response, it might be concluded that an additional population(s) of neurones is involved in the MENk naloxone-precipitated response, and that this population(s) is equipped with μ - and κ -opioid receptors, α -adrenoceptors and perhaps GABA_B receptors, but with few or no adenosine or somatostatin receptors. Alternatively, it could be postulated that the number/effectiveness of receptors for adenosine and somatostatin is less than that of α_2 - and κ -receptors and thus adenosine and somatostatin were unable to inhibit the naloxone-precipitated response, which was often slightly larger and more prolonged than the washout response. This latter proposal seems less likely in view of the fact that no reduction or delay in response was noted with a ten times higher concentration of somatostatin than that which inhibited the washout response.

A difference in the pharmacology of the MENk washout and naloxone-precipitated withdrawal responses was reported previously (Chahl, 1983). Although both responses were inhibited by antagonism of SP, the MENk washout response was more atropine-sensitive than the naloxone-precipitated response. It is also known that SP (and ACh) are present in more than one type of neurone in the myenteric plexus (Costa *et al.*, 1981). It is therefore possible that a population of non-cholinergic, SP-containing neurones might play a greater role in MENk naloxone-precipitated withdrawal than in washout withdrawal, and that these neurones do not have as extensive a range of receptor types as cholinergic neurones. In support of

the concept of differing receptor types on myenteric neurones, Yau *et al.* (1986) found that MENK but not somatostatin inhibited the efflux of ACh evoked by SP in the myenteric plexus. Nevertheless, it is difficult to explain why withdrawal induced by naloxone should recruit additional neurones to those recruited by washout, unless naloxone more effectively reaches certain compartments of the preparation than washout.

In conclusion, the present study has elucidated a pharmacological difference between washout and naloxone-precipitated withdrawal, in their different sensitivities to agonists which inhibit ACh release from enteric cholinergic neurones. Although these agonists have in common the ability to inhibit release from cholinergic neurones, it is by no means

clear that their action is limited to these neurones, or that these neurones are the only type involved in withdrawal responses. It is now proposed that in naloxone-precipitated withdrawal, not only cholinergic motor neurones are involved but an additional, distinct population of motor neurones containing SP (see Barthó & Holzer, 1985) is recruited which has less diversity of presynaptic receptors.

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