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Contracture of guinea-pig ileum on withdrawal of methionine^5-enkephalin is mediated by substance P

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1 The effects of methionine^5-enkephalin (Met-enkephalin, ME) 5×10^{-8}–5×10^{-6} moll^{-1}) were investigated on the resting guinea-pig ileum.

2 While in contact with the ileum, ME reduced the natural tone and movements, but following washout a contracture occurred which increased with increasing duration of the contact period from 0.5 to 32 min and with increasing concentration of the ME present during the contact period.

3 The washout contractures after 2 min contact with ME, 10^{-6} moll^{-1}, were abolished by naloxone, 10^{-6}moll^{-1}, added prior to the addition of ME, atropine, 5×10^{-6}moll^{-1} and the substance P (SP) antagonist, (D-Pro^2, D-Phe^7, D-Trp^9)-SP, 10^{-5}moll^{-1} and were reduced by 5-hydroxytryptamine (5-HT)-autodesensitization. Washout contractures following 32 min contact with ME, 10^{-6}moll^{-1}, were significantly inhibited by the SP antagonist and naloxone and were abolished by a combination of atropine and the SP antagonist, but were not significantly reduced by atropine alone or by 5-HT-desensitization.

4 Contractures of ileum occurred on addition of naloxone to ileal segments exposed to ME for 2 or 32 min. These contractures were also inhibited by the SP antagonist but a combination of atropine and SP-desensitization was required to abolish them.

5 It was concluded that gut dependence occurs following very brief exposure to an opioid and that SP plays a central role in the withdrawal response precipitated either by washout or addition of naloxone.

Introduction

Methionine^5-enkephalin (Met-enkephalin, ME) is an opioid peptide found in neurones of the gastrointestinal tract and in the central nervous system (Hökfelt, Elde, Johansson, Terenius & Stein, 1977; Simantov, Kuhar, Uhl & Snyder, 1977; Larsson, Childers & Snyder, 1979; Furness, Costa, Franco & Llewellyn-Smith, 1980; Jessen, Saffrey, Van Noorden, Bloom, Polak & Burnstock, 1980; Schultzberg, Hökfelt, Nilsson, Terenius, Rehfeld, Brown, Elde, Goldstein & Said, 1980). The guinea-pig ileum has been used extensively as a model system for the study of opiates and opioid peptides, since their actions in the enteric nervous system are thought to mimic those in the central nervous system. In the guinea-pig ileum, ME inhibits electrically-induced contractions of the longitudinal muscle (Hughes, Smith, Kosterlitz, Fothergill, Morgan & Morris, 1975), and also inhibits firing in myenteric neurones (North, Katayama & Williams, 1980). However, the effect of ME on resting, unstimulated guinea-pig ileum has been less well investigated, presumably because ME has little apparent effect on a preparation with such low tone. The present study was prompted by the observation that a contracture occurred on washout of ME. The properties of the withdrawal contracture, precipitated either by washout or addition of naloxone, following contact of resting guinea-pig ileum with ME were therefore investigated.

Methods

Isolated segments of guinea-pig distal ileum, 1.5–2 cm long, were suspended under 1 g tension in oxygenated Tyrode solution at 37°C in a 2 ml organ bath. Tension changes were recorded by means of a Grass force transducer (FTO3C) and Grass 7D polygraph. To determine the maximum response to acetylcholine (ACh), the response to a high concentration of ACh, 5×10^{-6}moll^{-1}, was obtained at the start of each experiment and all responses were expressed as % of the ACh maximum taken as 100%. Submaximal responses to ACh, substance P (SP) and 5-hydroxytryptamine (5-HT) were obtained so that

the effectiveness of antagonist drugs could be assessed. In preliminary experiments the effects of ME in concentrations ranging from 5×10^{-8} to 5×10^{-6} mol l^{-1} were investigated. In all subsequent experiments a concentration of ME of 10^{-6} mol l^{-1} was used.

The contracture on washout of Met-enkephalin

The responses to overflow washout following periods of contact of ileum with ME of 0.5, 1, 2, 5, 10 and 15 min were investigated. Experiments with 17 and 32 min contact periods were also performed in which ME was washed out every 5 min and replaced immediately. The contracture on the final washout was measured following a 2 min contact period with ME.

The pharmacological properties of the washout contracture to ME were investigated on responses following 2 and 32 min contact of preparations with ME, 10^{-6} mol l^{-1}. Responses following 2 min contact with ME were obtained before and after addition of atropine 5×10^{-6} mol l^{-1}, naloxone 10^{-6} mol l^{-1}, or the SP antagonist, (D-Pro^2, D-Phe^7, D-Trp^9)-SP 10^{-5} mol l^{-1} (Folkers, Horig, Rosell, & Bjorkroth, 1981). Atropine or naloxone were placed in the bath 5 min before addition of ME and replaced immediately following washout of ME. The SP antagonist was added either 30 s before addition of ME or 30 s before washout of ME. Responses were also obtained before and after preparations were desensitized to 5-HT by addition of 5×10^{-7} mol l^{-1}. ME was added in the presence of 5-HT when the preparations had regained resting tone. The desensitizing concentration of 5-HT was replaced immediately after washout of ME. Under these conditions there was selective loss of the response to low concentrations of 5-HT but responses to ACh and SP were unaffected (see Results). On 5-HT-desensitized preparations, readdition of the desensitizing concentration of 5-HT immediately following washout did not usually produce a contraction. This was in contrast to SP-desensitized preparations where readdition of SP after washout usually produced a contraction of the preparation. Thus it was possible to use 5-HT-desensitized preparations in experiments where ME withdrawal was precipitated by washout but it was not possible to use SP-desensitized preparations for these experiments since the contraction induced by readdition of SP might have confused interpretation of the height of contracture due to withdrawal of ME. The effects of the antagonists used were also tested on responses to ACh (10^{-7} mol l^{-1}), SP (2.5×10^{-9} mol l^{-1}) and 5-HT (10^{-7} mol l^{-1}). Similar experiments were performed on the washout response following 32 min contact with ME but since preparations produced unreliable responses following a second 32 min period of exposure to ME, control responses were obtained on different preparations from the responses in the presence of antagonists. In these experiments, atropine and 5-HT for desensitization were added 5 min before the final addition of ME and the SP antagonist was added 30 s before washout of ME.

The naloxone-precipitated contracture

The pharmacology of the contractures produced by addition of naloxone, 10^{-6} mol l^{-1}, without washout to preparations exposed to ME 10^{-6} mol l^{-1}, for 2 and 32 min was also investigated using similar concentrations of antagonists and experimental procedures to those described above. The above concentration of naloxone was chosen to precipitate withdrawal since in preliminary experiments it abolished the inhibitory response of the ileum to ME 10^{-6} mol l^{-1}, but unlike higher concentrations, did not inhibit the response to excitatory agonists such as ACh. Naloxone added before exposure of preparations to ME did not produce a contracture.

In addition, the effects of SP desensitization in the presence and absence of atropine 5×10^{-6} mol l^{-1}, on the naloxone-precipitated withdrawal response were investigated, since the procedure did not necessitate the washout and readdition of the desensitizing concentration of SP. SP-desensitization was carried out by addition of SP 5×10^{-8} mol l^{-1}. When tension had returned to baseline, responses were tested in the presence of the desensitizing concentration of SP, since following washout of desensitizing concentrations of SP there was reduced sensitivity to ACh which was not apparent in the presence of SP (Chahl, unpublished).

Statistics

Responses obtained on the same preparation before and after antagonist drugs, were compared by paired *t* tests. Responses in the presence of antagonist drugs which were obtained on different preparations from control responses, were compared with their control responses by Student's *t* tests.

Drugs

The following drugs were used: acetylcholine chloride (Sigma); atropine sulphate (Macfarlane Smith); 5-hydroxytryptamine creatinine sulphate (Sigma); (Met^5)-enkephalin (Protein Research Foundation, Osaka, Japan); naloxone hydrochloride (Endo Laboratories); substance P (Protein Research Foundation, Osaka, Japan, or Sigma); (D-Pro^2, D-Phe^7, DTrp^9)-substance P (Peninsula Laboratories, San Carlos, California). Stock solutions of ME, SP and SP antagonist were made in acetic acid, 2×10^{-2} mol l^{-1}.

The composition of the Tyrode solution was (mM):

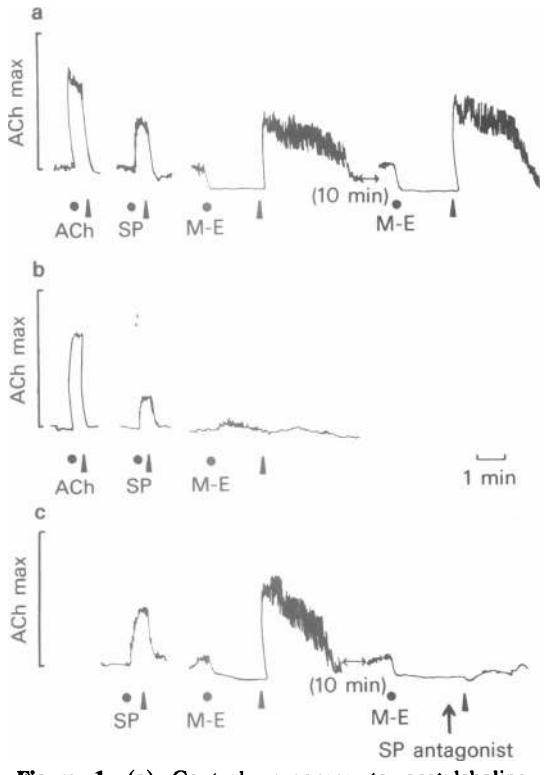

Figure 1 (a) Control responses to acetylcholine (ACh 10^{-7}mol l^{-1}), substance P (SP, 2.5×10^{-9}mol l^{-1}) and two responses to Met-enkephalin (ME 10^{-6}mol l^{-1}) obtained 10 min apart. Drug additions are shown by closed circles and washouts by closed triangles. Note relaxation to ME during the 2 min contact period and contracture on washout. (b) Responses to ACh, SP and ME in presence of SP antagonist, (D-Pro^2, D-Phe^7, D-Trp^9)-SP, 10^{-5}mol l^{-1}. Note reduction in response to SP and abolition of response to ME. The SP antagonist appears to have had a greater effect on the response to ME than on response to SP. (c) Control responses to SP and ME following recovery after washout of SP antagonist, and response to ME when SP antagonist was given following 2 min contact of ME with the ileum. Using this procedure the preparation was relaxed but the washout contracture was abolished.

NaCl 136.9, KCl 2.7, $MgCl_2$ 1.05, $CaCl_2$ 1.8, NaH_2PO_4 0.42 $NaHCO_3$ 11.9 and glucose, 5.55.

Results

The contracture on washout of Met-enkephalin

ME produced inhibition of movement and relaxation in those ileum preparations which had natural tone and/or spontaneous movements (Figure 1). This inhibitory response was concentration-related, the threshold concentration usually being 0.5 to 1×10^{-7}mol l^{-1}. Following a period of contact of 0.5 to 2 min, removal of ME by overflow washout induced a contracture which was reproducible if the concentration of ME and the period of contact were constant (Figure 1). However, the height of the contracture increased with increasing period of contact from 0.5–2 min (Figure 2), and with increasing concentrations of ME from 5×10^{-8} to 5×10^{-6}mol l^{-1}. Further increases in the period of contact beyond 2 min of a single addition of ME 10^{-6}mol l^{-1}, did not increase the washout contracture (Figure 2). However, ME is rapidly destroyed by guinea-pig ileum (Geary, Wiley, Scott & Cohen, 1982) and further experiments with 17 and 32 min periods of contact were performed, the ME being washed out and replaced every 5 min. Using this procedure it was noted that the duration of the inhibitory action of ME progressively decreased with each addition and the tendency of the preparation to contract during the brief washout and replacement period, increased. The contracture following the final washout was significantly greater following 17 min ($0.01 > P > 0.001$) and 32 min ($P = 0.001$) contact with ME (Figure 3), compared with the response following 2 min contact. This enhancement of the washout contracture was reversible since recovery of the response following 2 min contact to control height occurred by 15 min after removal of ME (Figure 3). Thus increased duration of contact of the ileum with ME induced a reversible enhancement of the washout withdrawal contracture.

The results obtained from investigation of the pharmacological properties of the washout contrac-

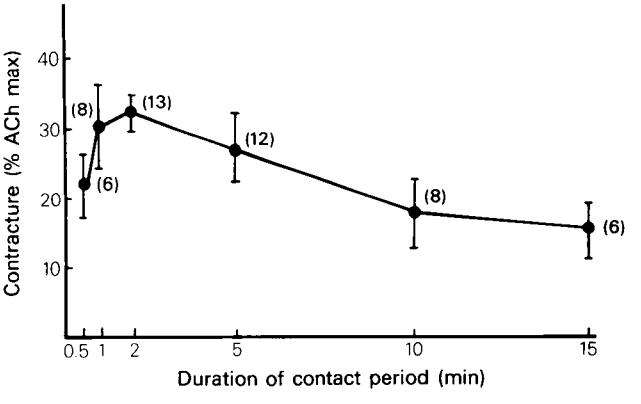

Figure 2 Contracture responses on washout of Met-enkephalin (ME, 10^{-6}mol l^{-1}) with increasing duration of the contact period of a single addition of ME. Responses, expressed as percentages of the ACh maximum, are the means from the numbers of experiments shown in parentheses and the bars represent s.e.means. Note that an increase in response occurred only with increase in duration of contact from 0.5 to 2 min.

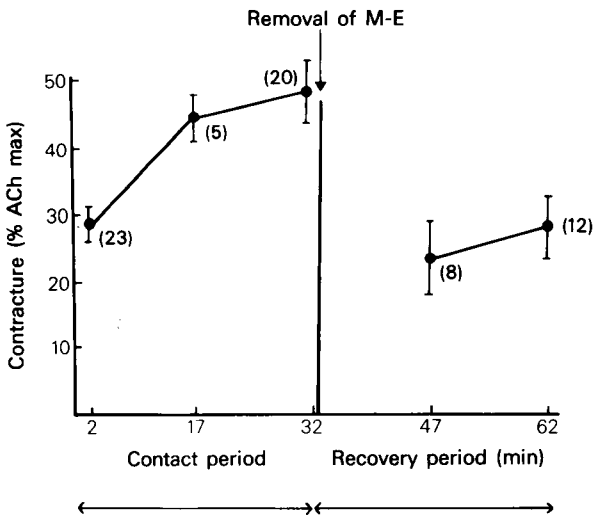

Figure 3 Contracture responses on washout of Met-enkephalin (ME, 10^{-6}moll^{-1}) following 2, 17 and 32 min periods of contact. In experiments with 17 and 32 min contact periods, ME was washed out and replaced every 5 min, the contact period following the final addition being 2 min. Note the increase in response with increasing duration of the contact period. Note also that the response following 2 min contact returned to control levels by 15 min following removal of ME after the 32 min contact period (47 min after start of experiment). Responses, expressed as percentages of the acetylcholine (ACh) maximum, are the means from the numbers of experiments shown in parentheses and the bars represent s.e.means.

ture following 2 min contact with ME 10^{-6}moll^{-1}, are shown in Figures 4 and 5. The response following 2 min contact was found to be abolished by atropine 5×10^{-6}moll^{-1}, and by naloxone 10^{-6}moll^{-1}, provided that naloxone was added to the bath fluid before addition of ME and was replaced immediately after the ME was washed out. If naloxone was added only on washout, a larger contracture occurred because naloxone then precipitated the withdrawal response (see below). This dose of naloxone significantly enhanced responses to ACh, SP and 5-HT (Figure 4). The washout contracture was also found to be completely inhibited by the SP antagonist (D-Pro^2, D-Phe^7, D-Trp^9)-SP, 10^{-5}moll^{-1}, given either 30 s before addition of ME or 30 s before washout (Figure 1). This concentration of the SP antagonist produced a small but significant reduction of the response to SP and as previously reported, a more marked reduction of the response to 5-HT (Chahl, 1983), but did not affect the response to ACh (Figure 5). The effect of the SP antagonist was reversible within 10 min of its removal. In Figure 4 it may also be seen that 5-HT-desensitization reduced but did

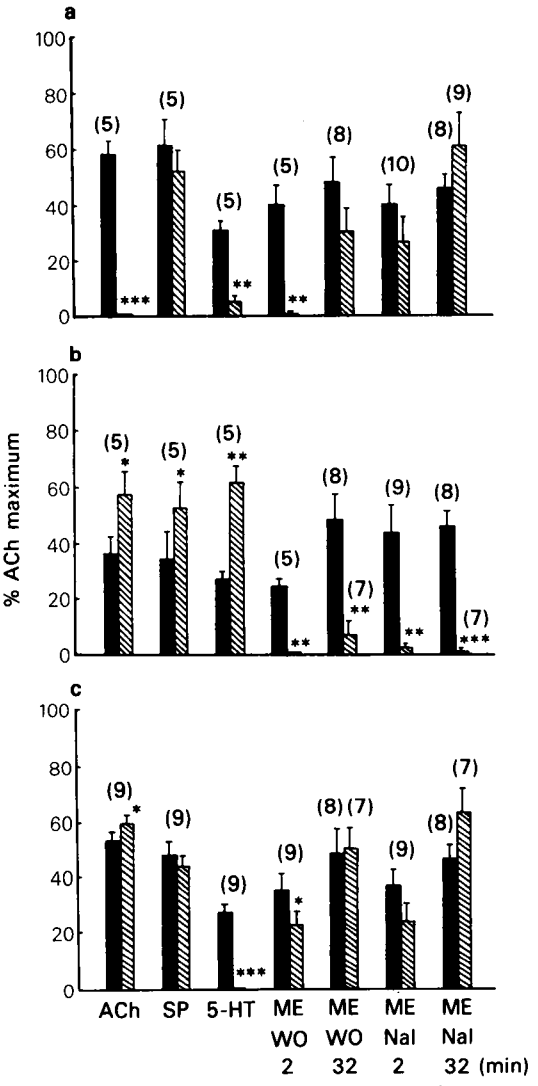

Figure 4 Effects of (a) atropine (5×10^{-6}moll^{-1}), (b) pretreatment with naloxone (10^{-6}moll^{-1}) and (c) 5-hydroxytryptamine (5-HT)-desensitization (5×10^{-7}moll^{-1}) on contractures of guinea-pig ileum precipitated by washout (WO) and naloxone, 10^{-6}moll^{-1}, (Nal) following 2 and 32 min contact with Met-enkephalin (ME, 10^{-6}moll^{-1}). Columns represent mean responses, expressed as % of the acetylcholine (ACh) maximum, obtained from the numbers of experiments shown in parentheses and the bars represent s.e.means. Solid columns represent control responses and hatched columns represent responses in the presence of antagonists. Mean responses to ACh (10^{-7}moll^{-1}), substance P (SP, 2.5×10^{-9}moll^{-1}) and 5-HT (10^{-7}moll^{-1}) are also shown. Asterisks indicate significant differences from controls obtained from paired or unpaired t tests as appropriate (see text). *$0.05 > P > 0.01$; **$0.01 > P > 0.001$; ***$P < 0.001$.

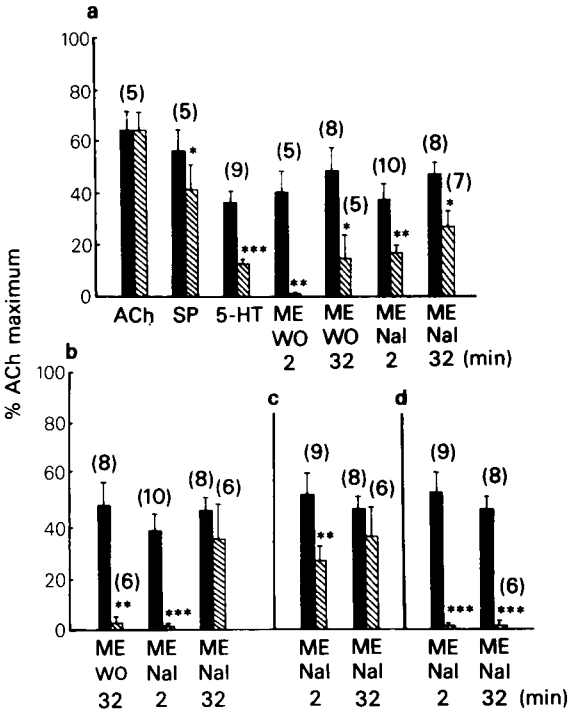

Figure 5 Effects of the substance P (SP) antagonist, (D-Pro^2, D-Phe^7, D-Trp^9)-SP, (10^{-5}mol l^{-1}) alone (a) and with atropine (b) and of SP-desensitization (5×10^{-8}mol l^{-1}) alone (c) and with atropine (5×10^{-6}mol l^{-1}) (d) on contractures of guinea-pig ileum precipitated by washout (WO) or naloxone 10^{-6}mol l^{-1} (Nal), following 2 or 32 min contact with Met-enkephalin (ME, 10^{-6}mol l^{-1}) as indicated. The effects of the SP antagonist on responses to acetylcholine (ACh, 10^{-7}mol l^{-1}), SP (2.5×10^{-9}mol l^{-1}) and 5-hydroxytryptamine (5-HT 10^{-7}mol l^{-1}) are also shown. For further explanation see legend to Figure 4.

not abolish the washout contracture following 2 min contact with ME.

The effects of antagonists on the washout withdrawal response following 32 min contact with ME are also shown in Figures 4 and 5. The response was significantly reduced by the SP antagonist and was abolished by a combination of atropine plus the SP antagonist and greatly reduced by naloxone added before addition of ME. However, the response was not significantly reduced by atropine alone or by 5-HT-desensititization.

The naloxone-precipitated contracture

The mean contractures induced by addition of naloxone following exposure of preparations to ME for 2 and 32 min were not significantly different in height from those induced by washout following similar periods of exposure to ME. However, unlike the washout contractures, the response following 32 min contact with ME was not significantly greater than that following 2 min contact. The naloxone-precipitated contractures following both 2 and 32 min exposures to ME were significantly reduced by the SP antagonist (Figure 5) and were abolished by naloxone pretreatment of preparations (Figure 4). They were not significantly affected by 5-HT-desensitization or atropine (Figure 4). The response following atropine, however, was often delayed by 1–2 min after addition of naloxone compared with controls. Although the response precipitated by naloxone following 2 min exposure to ME was abolished by a combination of atropine plus the SP antagonist, the response following 32 min was abolished only on SP-desensitized preparations treated with atropine (Figure 5).

Discussion

The present study has shown that following brief exposure of guinea-pig ileum to ME, withdrawal by either washout of ME or addition of naloxone resulted in a contracture which was mediated predominantly by SP and which was an opiate-specific response since it was absent on preparations pretreated with naloxone. In previous studies SP has been implicated in gut dependence on opiates (Gintzler, 1980; Tsou, Louie & Way, 1982) as have ACh (Schulz & Herz, 1976) and 5-HT (Gintzler, 1979), but conflicting results have been obtained regarding the relative importance of these mediators. The results from the present study provide an explanation for previous observations since it was found that SP played a central role in the withdrawal responses whereas the contribution of ACh and 5-HT varied with the duration of contact of the preparation with ME and with the manner of precipitation of the withdrawal.

In experiments in which preparations were exposed to ME for 2 min and withdrawal was precipitated by washout, both atropine and the SP antagonist completely inhibited the response, thus suggesting that ACh and SP were sequentially, rather than independently, released. These results would be explained if SP released ACh or if ACh released SP in the ileum. Evidence in favour of the former possibility has been obtained in experiments on the nature of the response of ileum to capsaicin (Chahl, 1982). In these experiments a cholinergic response to capsaicin and to high concentrations of SP was demonstrated on SP-desensitized preparations and it was concluded that the response of ileum to capsaicin was due to released SP producing an action on cholinergic

neurones to produce ACh release (Chahl, 1982) as well as a direct effect on the smooth muscle. Several other workers have also found evidence of a cholinergic component in the action of SP in guinea-pig ileum (Hedqvist & von Euler, 1975; Holzer & Lembeck, 1980) although at low concentrations of exogenously applied SP the contribution of ACh to the response appeared to be negligible (Bury & Mashford, 1977; Chahl, 1982). Nevertheless, it is possible that even small amounts of endogenously released SP might preferentially activate SP receptors on cholinergic neurones since the atropine-sensitive response to low concentrations of 5-HT was also found to be mediated by SP (Chahl, 1983). Thus the weight of current evidence would suggest that SP releases ACh in guinea-pig ileum. However, the possibility that ACh releases SP cannot be dismissed, particularly since ganglion stimulating agents, DMPP (Franco, Costa & Furness, 1979) and nicotine (Chahl, 1983), release SP indicating that nicotinic receptors for ACh exist on SP neurones in the ileum.

Since there is no specific receptor antagonist for the neuronal action of 5-HT in guinea-pig ileum, a technique of selective 5-HT autodesensitization was used to determine the extent of involvement of 5-HT in the washout contracture to ME. Although the molecular mechanisms underlying receptor desensitization are not certain and conclusions based on these techniques must remain tentative, the results indicated that 5-HT did play some role in the washout response following 2 min contact with ME. Since low concentrations of 5-HT have been shown to act via release of SP and subsequent release of ACh (Chahl, 1983) the contribution of 5-HT to the washout contracture would be abolished by atropine or the SP antagonist.

The washout contractures following 17 and 32 min contact with ME were greater than that following 2 min contact. Thus increased duration of contact of guinea-pig ileum with ME induced a reversible enhancement of the washout withdrawal response and this was accompanied by a decrease in the duration of the inhibitory response produced by successive additions of ME. These observations would suggest that the ileum developed tolerance to the inhibitory action of ME because of progressive activation of a superimposed excitatory mechanism. The pharmacology of the washout response following 32 min contact with ME exhibited some differences from that of the response following 2 min contact. Firstly, the response was not significantly inhibited by atropine or 5-HT-desensitization, implying that ACh and 5-HT did not contribute to the response. However, the SP antagonist alone significantly reduced the response and in combination with atropine virtually abolished it, showing that ACh played some part in the response although its effect alone did not appear to be significant. Nevertheless, the role of ACh and 5-HT was apparently less important in the washout withdrawal response following more prolonged exposure to ME.

The situation appeared even more complex when the naloxone-precipitated withdrawal responses were investigated. Following 2 min exposure to ME, naloxone induced a contracture of similar height to that produced by washout. The pharmacology of this response resembled the washout withdrawal response following 32 min contact with ME, in that atropine and 5-HT-desensitization did not significantly affect the response although it was abolished by a combination of atropine and the SP antagonist. The naloxone-precipitated withdrawal following 32 min contact with ME was not greater than that following 2 min contact. It is possible that the concentration of naloxone used was not sufficient to displace ME completely from its receptors after 32 min contact or that prolonged contact with ME involved naloxone-resistant opiate receptors. In contrast to the other withdrawal responses, the naloxone-precipitated withdrawal response following 32 min contact with ME was not significantly reduced in height by a combination of atropine and the SP antagonist, although it was reduced by the SP antagonist alone. Atropine was not without effect on the response, however, since it delayed the onset of contracture. The naloxone-precipitated withdrawal following 32 min contact with ME was abolished by a combination of atropine and SP-desensitization, indicating that the response was mediated by SP. It has been previously suggested that the SP antagonist might inhibit the SP receptors on cholinergic neurones more effectively than those on smooth muscle (Chahl, unpublished observations). If this were so, then it would appear that the naloxone-precipitated withdrawal following 32 min contact with ME involved mainly SP acting on smooth muscle SP receptors with little contribution from a cholinergic neurone action. Thus not only the magnitude of the withdrawal response but also its nature altered with longer periods of contact with ME.

There is now considerable evidence that SP plays an important role in the gastrointestinal tract. Apart from its well known contractile action on the smooth muscle of the gut, there is evidence that SP is present in both intrinsic and extrinsic neurones (Costa, Furness, Llewellyn-Smith & Cuello, 1981), that it is released from intestinal nerves (Franco *et al.*, 1979) and has an excitatory action on neurones of the myenteric plexus (Morita, North & Katayama, 1980). The neuronal source of the SP released during opiate withdrawal is not certain but it is more likely to be the intrinsic nerves since preliminary experiments have shown that capsaicin, which acts on extrinsic (possibly sensory) nerves (Barthó & Szolcsányi,

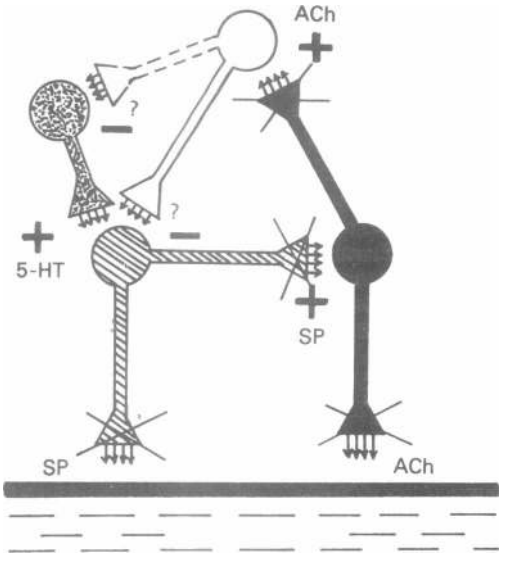

Figure 6 Proposed mechanism of the Met-enkephalin (ME) withdrawal response. A negative feedback mechanism is suggested to control the excitability of substance P (SP) and acetylcholine (ACh) neurones via a tonically-active inhibitory neurone which is activated by ACh to release an unknown inhibitory transmitter onto SP neurones and perhaps also onto 5-hydroxytryptamine (5-HT) neurones. If ME inhibited release of ACh from cholinergic neurones and of SP from SP neurones (shown by crosses), disinhibition of the SP neurone would occur which would result in enhanced release of SP only after removal of ME (see text).

1978) to release SP (Chahl, 1982; Barthó, Holzer, Lembeck & Szolcaányi, 1982) did not inhibit the withdrawal response on washout of ME following 2 min contact, provided the preparations had recovered sensitivity to SP following treatment with capsaicin (Chahl, unpublished observations).

Paton (1957) showed that opiates inhibit release of ACh from cholinergic neurones in the guinea-pig ileum. If ME also inhibits release of ACh, a possible mechanism whereby increased excitability of neurones in the ileum could appear on withdrawal of ME following even very brief exposure might be as shown in Figure 6. In this proposal a negative feedback mechanism is suggested to control the excitability of SP and ACh neurones via a tonically-active inhibitory neurone which is activated by ACh to release an unknown inhibitory transmitter onto SP neurones and perhaps also onto 5-HT neurones. Inhibition of release of ACh would result not only in relaxation of the ileum but also in disinhibition of the SP neurone. On washout of ME the inhibition of cholinergic neurones would be removed and the action of SP on cholinergic neurones unmasked. This proposal depends on the assumption that after washout of ME, the excitatory action outlasts the inhibitory action. Although an opiate-specific excitatory action of ME on enteric neurones has not yet been observed (North *et al.*, 1980) excitation by disinhibition induced by opiates has been found in certain regions of the central nervous system (see North, 1979). If this mechanism were to operate it might be expected that contraction of the ileum would occur even in the presence of ME because SP acts directly on smooth muscle as well as on cholinergic neurones. However, a response did not occur on most preparations until ME was washed out, indicating that ME might also inhibit the release of SP in the ileum. Indeed, it has previously been proposed that opioids might inhibit release of SP from enteric neurones (Gintzler & Scalisi, 1982; Barthó, Sebok & Szolcsányi, 1982; Barthó *et al.*, 1982) as they have been found to do at the central terminals of primary afferent neurones (Jessell & Iversen, 1977).

Although the above proposal provides an adequate explanation for the involvement of SP in the withdrawal response it does not explain why the proportion of its action on smooth muscle receptors (atropine-resistant response) to cholinergic neurone receptors should have been greater with longer exposure to ME and with naloxone-precipitated withdrawal compared with washout withdrawal. It could be speculated that the kinetics of offset of ME from opiate receptors located at SP and ACh neuronal terminals induced by washout and naloxone might differ. Alternatively, following 32 min exposure there might have been some change in the function of the neurones associated with the development of tolerance which resulted in the different proportions of atropine-sensitive and atropine-resistant SP action. Further experiments are necessary to determine the explanation for this finding.

Several methods using guinea-pig ileum have recently been employed for the study of dependence and tolerance to opiates (Schulz & Herz, 1976; Gintzler, 1979; 1980; Lujan & Rodriguez, 1981; Collier, Cuthbert & Francis, 1981). The presence of an atropine-resistant component in the naloxone-precipitated withdrawal response of morphine-dependent guinea-pig ileum has led to the suggestion that there is activation of at least two neuronal pathways in the withdrawal response, one of which involves ACh and the other 5-HT and SP (Schultz & Herz, 1976; Gintzler, 1979; 1980; Gintzler & Scalisi, 1982; Tsou *et al.*, 1982). In light of the dual action of SP on cholinergic neurones and on smooth muscle (Hedqvist & von Euler, 1975; Holzer & Lembeck, 1980; Chahl, 1982) and the SP-releasing action of

5-HT (Chahl, 1983), it is more likely that only one major neuronal pathway is involved in the expression of gut dependence on opioids and it is tempting to suggest that this pathway functions physiologically as a negative feedback control of the complex cholinergic neuronal activity involved in gut movements. The present findings of a rapidly-induced withdrawal response to ME offers a simpler system for the study of tolerance and dependence and extends our knowledge of these phenomena since it has clearly shown that the withdrawal response is mediated by SP. Furthermore, the response may be inhibited in the ileum either before or after contact with ME by antagonism of SP, a finding, which, if it applies to the central nervous system, offers a therapeutic goal for the development of more powerful SP antagonists.

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