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The properties of the clonidine withdrawal response of guinea-pig isolated ileum

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1 The dependence-inducing effects of clonidine were investigated on the guinea-pig isolated ileum. Clonidine produced relaxation of the ileum with a threshold concentration between 0.01 and 0.1 μmol l^{-1}. Washout of clonidine did not induce a withdrawal contraction.

2 Following 2 min contact of the ileum with clonidine, 1 μmol l^{-1}, addition of phentolamine, 5 μmol l^{-1}, induced a contracture. The phentolamine-precipitated withdrawal contracture did not increase in height with a longer period of contact (32 min) of the ileum with clonidine.

3 The phentolamine-precipitated withdrawal contracture following 2 min contact of ileum with clonidine was abolished by atropine, 5 μmol l^{-1}, and substance P (SP) antagonists, (D-Pro^2,D-Phe^7,D-Trp^9)-SP and spantide, 10 μmol l^{-1}.

4 [Met^5]enkephalin, 1 μmol l^{-1}, abolished the withdrawal response to clonidine and clonidine, 1 μmol l^{-1}, abolished the withdrawal response to [Met^5]enkephalin.

5 Following 2 min contact of the ileum with noradrenaline, 5 μmol l^{-1}, washout or addition of phentolamine or yohimbine, 5 μmol l^{-1}, also induced a withdrawal response. The noradrenaline washout withdrawal response was abolished by atropine, 5 μmol l^{-1}, and spantide, 10 μmol l^{-1}.

6 Since clonidine dependence may be induced as rapidly as opiate dependence in the ileum and the pharmacology of the withdrawal responses is similar, it is suggested that they both induce the same post-receptor neuronal feedback disturbance in which substance P neurones play a major role.

Introduction

It is now well known that sudden withdrawal of the antihypertensive drug clonidine can give rise to withdrawal phenomena characterized by hyperirritability and rebound hypertension in man (Hansson *et al.*, 1973) and experimental animals (Thoolen *et al.*, 1981; 1983). Clonidine dependence of the guinea-pig isolated ileum following incubation for 24 h with clonidine, has also been demonstrated by the presence of a withdrawal contracture on addition of α-adrenoceptor blocking drugs (Collier *et al.*, 1981).

A recent study has shown that 0.5 to 2 min exposure of guinea-pig ileum to [methionine^5]enkephalin (ME) resulted in dependence as revealed by a withdrawal contracture on washout of ME or addition of naloxone (Chahl, 1983). Furthermore the response was inhibited by substance P (SP) antagonists and was presumably mediated by SP. These findings demonstrated that dependence on opiates occurred more rapidly than previously suspected and also provided a simpler system for study of the mechanisms involved in dependence and tolerance. The present study was undertaken to determine whether dependence of guinea-pig ileum on clonidine occurred following short-term contact and if so, to determine the pharmacological mediators of the withdrawal response.

Methods

Adult guinea-pigs were stunned by a blow to the head and the spinal cord severed. Segments, 1.5–2 cm long, of distal ileum were suspended under 1 g tension in 2 ml organ baths containing Tyrode solution at 37°C and gassed with O_2. Tension changes were recorded by means of Grass force transducers (FT03C) and Grass 7D polygraph. To determine the maximum response to acetylcholine (ACh), the response to a high concentration of ACh, 5 μmol l^{-1}, was obtained at the start of each experiment and all responses were expressed as percentages of the ACh maximum taken as 100%. Submaximal responses to ACh and SP were obtained so that the effectiveness of antagonist drugs could be assessed. In preliminary experiments the effects of clonidine in concentrations ranging from 0.05 to 5 μmol l^{-1} were investigated. For subsequent experiments a concentration of 1 μmol l^{-1} was chosen. A

similar concentration of ME was also tested in several experiments.

Responses to overflow washout and addition of the α-adrenoceptor antagonist drug, phentolamine (1 and 5 μmol l^{-1}), following periods of contact of ileum with clonidine of 2 and 32 min were investigated. With drawal contractures were measured as the height of the response above the baseline prior to addition of clonidine. In experiments following 32 min contact with clonidine, the preparations were washed every 5 min and the clonidine replaced immediately. The effects of yohimbine (1 and 5 μmol l^{-1}) and naloxone (1 μmol l^{-1}) were also tested following 2 min contact of the ileum with clonidine. Responses to overflow washout and addition of phentolamine (5 μmol l^{-1}) or yohimbine (5 μmol l^{-1}) were also tested following 2 min contact of the ileum with noradrenaline (1 and 5 μmol l^{-1}).

The pharmacology of the contractures produced on addition of phentolamine (5 μmol l^{-1}) to preparations exposed to clonidine, 1 μmol l^{-1}, for 2 min was investigated. Since these clonidine withdrawal contractures were not reproducible, responses on untreated preparations were compared with those on preparations pretreated with either phentolamine (5 μmol l^{-1}), naloxone (1 μmol l^{-1}), atropine (5 μmol l^{-1}) or the SP antagonists (D-Pro^2,D-Phe^7,D-Trp^9)-SP or (D-Arg^1,D-Trp^{7,9},Leu^{11})-SP (spantide, 10 μmol l^{-1}). Phentolamine, naloxone and atropine were added to the bath fluid 5 min before addition of clonidine. The SP antagonists were added 30 s before addition of phentolamine i.e. following 1.5 min contact with clonidine. The effects of all antagonists were tested on responses to ACh (0.1 μmol l^{-1}) and SP (2.5 nmol l^{-1}) on several preparations. On another 5 preparations the responses to ACh and SP were tested in the presence of clonidine before and after 2 and 32 min contact with clonidine. The effects of atropine and spantide were also tested on the noradrenaline washout withdrawal response.

The interactions between clonidine and ME were examined by adding clonidine and ME (both at a concentration of 1 μmol l^{-1}) together to the organ bath. Following 2 min contact the preparations were treated with phentolamine (5 μmol l^{-1}) to precipitate clonidine withdrawal in the presence of ME, or either washed out or treated with naloxone (1 μmol l^{-1}) to precipitate ME withdrawal in the presence of clonidine. In other preparations combined withdrawal was precipitated by simultaneous addition of phentolamine and naloxone.

Statistics

Responses obtained on the same preparation before and after antagonist drugs, were compared by paired *t* tests. Responses obtained on different preparations were compared by Student's *t* tests.

Drugs

The following drugs were used: acetylcholine chloride (Sigma); atropine sulphate (Macfarlane Smith); clonidine hydrochloride (Boehringer Ingelheim); [Met^5]enkephalin (Protein Research Foundation, Osaka, Japan); naloxone hydrochloride (Endo Laboratories); (−)-noradrenaline bitartrate (Sigma); phentolamine mesylate ('Regitine', Ciba); substance P (Protein Research Foundation, Osaka, Japan, or Sigma); (D-Pro^2,D-Phe^7,D-Trp^9)-substance P (Peninsula Laboratories, San Carlos, California); (D-Arg^1,D-Trp^{7,9},Leu^{11})-substance P (Bachem); yohimbine hydrochloride (Sigma).

Stock solutions of ME, SP and SP antagonists were made in acetic acid, 20 mmol l^{-1}. The composition of the Tyrode solution was (mmol l^{-1}): 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

Properties of the clonidine withdrawal response

On contact with the guinea-pig ileum, clonidine produced relaxation with a threshold concentration between 0.01 and 0.1 μmol l^{-1}. No contracture of the ileum occurred on washout following 2 min or 32 min contact with clonidine in concentrations ranging from 0.01 to 5 μmol l^{-1}. This was in contrast to the response of the same preparations to washout of ME, 1 μmol l^{-1} (Figure 1). However addition of phentolamine (1 to 5 μmol l^{-1}) produced a withdrawal contracture following 2 min contact with clonidine 0.1 to 1 μmol l^{-1} (Figure 1). Within these concentration ranges the height of the clonidine withdrawal contracture increased with increasing concentration of both clonidine and phentolamine. On some preparations there were marked, regular spontaneous movements during the withdrawal contracture, which might reflect an action on the circular muscle of the ileum. The heights of contracture on these preparations were measured by drawing a smooth curve by eye through the midpoints of the excursions of the spontaneous contractions. The heights of all withdrawal contractures were measured from the baseline prior to the addition of clonidine. The mean height of the withdrawal contracture precipitated by phentolamine (5 μmol l^{-1}) following 2 min contact of ileum with clonidine (1 μmol l^{-1}) was found to be 20 ± 2% (mean ± standard error (s.e.) from 20 individual observations expressed as a percentage of the ACh maximum). This was identical to the mean height of the responses on

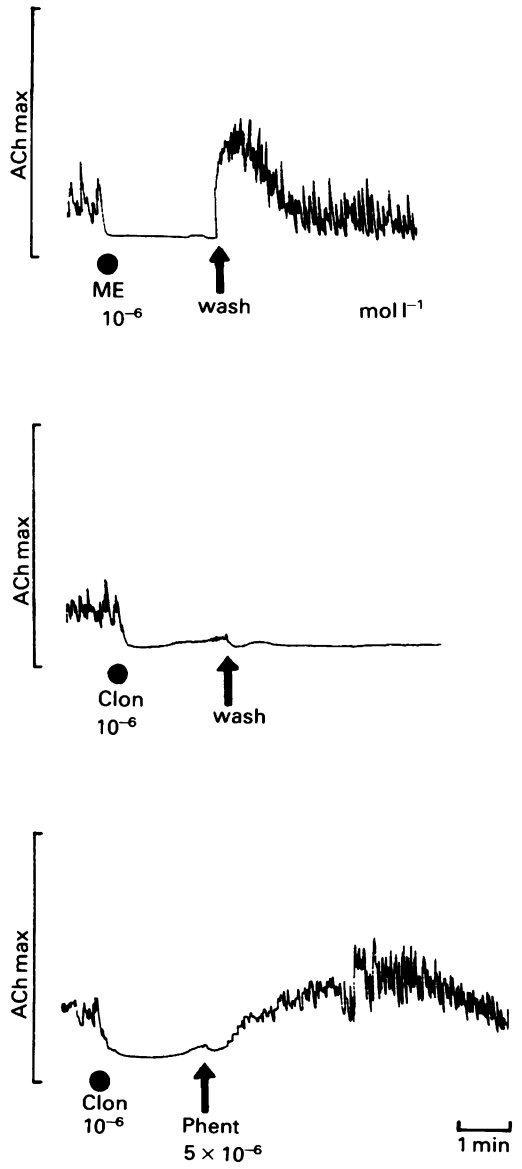

Figure 1 Withdrawal responses to [Met^5]enkephalin (ME) and clonidine (Clon) at a concentration of 1 μmol l^{-1}. Note that following 2 min contact of ME with the ileum a withdrawal response was precipitated by washout, whereas following contact with clonidine, washout was ineffective in producing a withdrawal response. Addition of phentolamine, 5 μmol l^{-1} (Phent) induced a response. All three responses were obtained on the same preparation.

washout following 2 min contact with ME (1 μmol l^{-1}) (20 ± 2%) on the same preparations. Addition of naloxone, 1 μmol l^{-1}, following contact of the ileum with clonidine did not precipitate a withdrawal contracture. In agreement with the findings of Collier *et al.* (1981), phentolamine alone did not produce contracture of ileum from freshly killed guinea-pigs which had not previously been exposed to clonidine. Although the mean heights of the clonidine and ME withdrawal contractures were similar the time courses were different, the clonidine withdrawal response being slower in onset and recovery (Figure 1).

The ME withdrawal response precipitated by washout was previously found to be reproducible and increased with a longer period of contact with ME (Chahl, 1983). However the clonidine withdrawal response precipitated by phentolamine was not reliably reproducible, possibly because phentolamine was not completely removed by washing. For this reason, only one clonidine withdrawal response was obtained on each preparation. The clonidine withdrawal contracture following 32 min incubation of ileum with clonidine was not significantly different from that obtained on preparations exposed for 2 min (mean response following 32 min contact = 18 ± 5% of ACh maximum, $n = 13$).

Pharmacology of the clonidine withdrawal response

Since the most reliable results were obtained by addition of phentolamine (5 μmol l^{-1}) to preparations in contact with clonidine (1 μmol l^{-1}) for 2 min these conditions were chosen for study of the pharmacology of the clonidine withdrawal response. It was found that atropine (5 μmol l^{-1}) added 5 min before addition of clonidine (6 experiments) and the SP antagonists, (D-Pro^2,D-Phe^7,D-Trp^9)-SP (5 experiments) and spantide (5 experiments) 10 μmol l^{-1}, added 1.5 min after addition of clonidine i.e. 30 s before addition of phentolamine, abolished the clonidine withdrawal response. In the presence of naloxone, 1 μmol l^{-1}, added 5 min before addition of clonidine, the withdrawal response could still be evoked (14 ± 2%, $n = 5$) but addition of phentolamine, 5 μmol l^{-1}, 5 min before addition of clonidine, abolished the response in all 5 experiments. Atropine, 5 μmol l^{-1}, abolished the response to ACh, 0.1 μmol l^{-1}, but did not affect the response to SP, 2.5 nmol l^{-1}. The SP antagonists at a concentration of 10 μmol l^{-1} did not affect the response to ACh but reduced the response to SP, 2.5 nmol l^{-1}, (D-Pro^2,D-Phe^7,D-Trp^9)-SP by 30 ± 10%, $n = 5$ (Chahl, 1983) and spantide by 82 ± 6%, $n = 13$. Neither phentolamine nor clonidine in contact with the ileum for 2 min (or clonidine for 32 min) in the concentrations used affected responses to SP or ACh.

Interaction between clonidine and [Met^5]enkephalin

It was found that ME, 1 μmol l^{-1}, added at the same time as clonidine abolished the clonidine withdrawal response and clonidine, 1 μmol l^{-1}, abolished the ME withdrawal response precipitated by either washout (6 experiments) or naloxone, 1 μmol l^{-1} (8 experiments). When the combined ME and clonidine withdrawal response was precipitated by simultaneous addition of phentolamine plus naloxone, the mean response, (30 $\pm$ 6% of ACh maximum, $n = 10$), was not significantly different from either the ME or clonidine withdrawal response alone.

Effect of noradrenaline

Noradrenaline produced relaxation of the ileum at concentrations above 0.1 μmol l^{-1}. Noradrenaline was less potent than clonidine in producing a withdrawal response, contact with a concentration of 5 μmol l^{-1} usually being required to induce a response. In contrast to experiments with clonidine, washout of ileum following 2 min contact with noradrenaline, 5 μmol l^{-1}, produced a contracture, the mean height of which (22 $\pm$ 4% of ACh maximum, $n = 8$) was similar to that of the clonidine withdrawal response. However, the time course of the noradrenaline withdrawal response resembled that of the ME withdrawal response more closely than that of the clonidine withdrawal response (Figures 1 and 2). Noradrenaline withdrawal contractures were also precipitated by addition of phentolamine, 5 μmol l^{-1} (mean response, 11 $\pm$ 4%, $n = 6$), or yohimbine (5 μmol l^{-1}) (mean response, 13 $\pm$ 3%, $n = 10$) (Figure 2). Yohimbine was significantly less effective than phentolamine in precipitating a clonidine withdrawal response (mean response 5 $\pm$ 3%, $n = 5$; $0.01 > P > 0.001$) (Figure 2). The noradrenaline withdrawal response precipitated by washout was abolished by atropine, 5 μmol l^{-1}, and by spantide, 10 μmol l^{-1} (5 experiments).

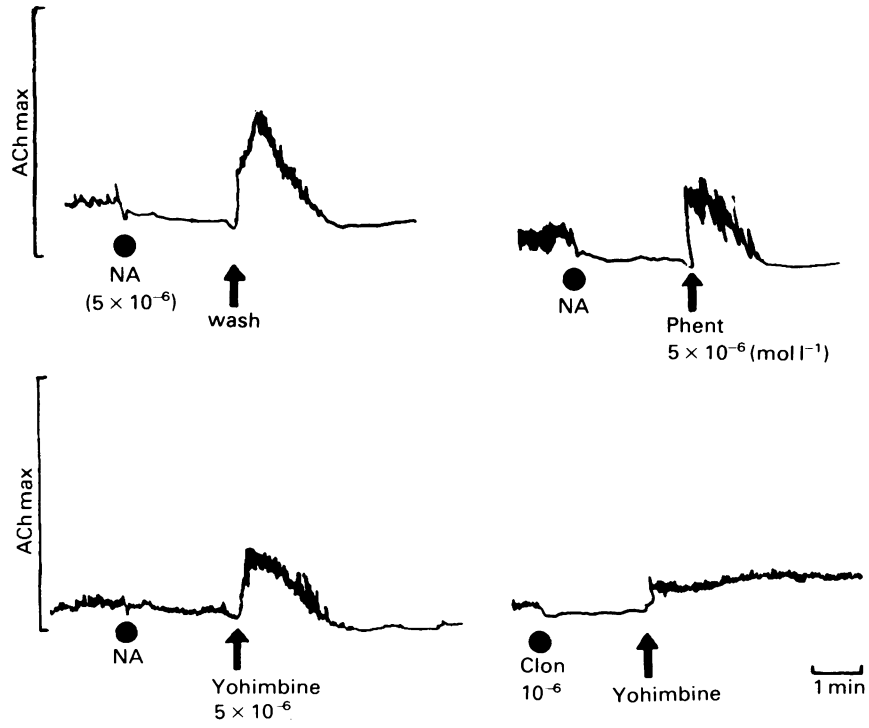

Figure 2 Withdrawal responses to noradrenaline (NA, 5 μmol l^{-1}) and clonidine (Clon, 1 μmol l^{-1}). Note that following 2 min contact of NA with the ileum either washout of NA or addition of α-adrenoceptor blocking drugs, phentolamine (Phent) or yohimbine (both at 5 μmol l^{-1}) induced withdrawal responses. Yohimbine was less effective in precipitating withdrawal from clonidine than from NA. Responses were obtained on different preparations.

Discussion

The present study has confirmed the findings of Collier *et al.* (1981) that the guinea-pig ileum may be made 'dependent' on clonidine as shown by a withdrawal contracture precipitated by addition of α-adrenoceptor blocking drugs. Furthermore, it has shown that dependence of ileum on clonidine occurs just as rapidly as dependence on ME. The pharmacology of the clonidine withdrawal response following 2 min contact was similar to that reported for the ME withdrawal response precipitated by washout (Chahl, 1983), being abolished by atropine and SP antagonists and therefore apparently mediated by ACh and SP. From these findings it might be concluded that the endogenous SP released during withdrawal acts only on cholinergic neurones to release ACh and has no detectable direct effect on the smooth muscle of the ileum. Following either more prolonged exposure or precipitation of the response by naloxone, the ME withdrawal response has been shown to be atropine-resistant indicating that some SP acted on the smooth muscle directly under these conditions (Chahl, 1983).

The clonidine withdrawal response differed in some respects from ME withdrawal. Firstly, it could not be precipitated by simply washing clonidine out of the organ bath, presumably because clonidine was not removed as rapidly from the α-adrenoceptors as ME was from the opiate receptors. Slower removal from receptors was also reflected in the slower time course of the phentolamine precipitated clonidine withdrawal contracture, compared with the ME and noradrenaline withdrawal contractures. Secondly, the height of the clonidine withdrawal response was not increased by increasing the period of contact of the ileum with clonidine from 2 min to 32 min. In this respect it was similar to the naloxone-precipitated ME withdrawal response but differed from the ME withdrawal response precipitated by washout which was increased by a longer period of contact (Chahl, 1983). In contrast to the present experiments Collier *et al.* (1981) found that ileum incubated for 24 h with clonidine produced a greater response to phentolamine than ileum incubated for 0.5 h. Experiments using several periods of incubation with clonidine might provide some explanation for these different results. It should be noted that Collier *et al.* (1981) found a small contracture to phentolamine in preparations that were incubated for 24 h without clonidine, indicating that some functional change occurred in preparations incubated for longer periods of time. On the other hand the results might simply reflect the fact that the withdrawal response following 2 min contact with clonidine, 1 μmol l^{-1}, precipitated by phentolamine, 5 μmol l^{-1} was approaching maximum. Whatever the mechanism responsible for lack of increase in the clonidine withdrawal response after 32 min contact it could not be attributed to an effect of clonidine on responses to ACh or SP as these were not affected by either 2 min or 32 min prior contact with clonidine.

The finding that clonidine and opiate withdrawal phenomena, although involving different receptor mechanisms, could both be precipitated following the same short period of contact and were both mediated by similar putative neurotransmitters, would suggest that a similar neuronal pathway was activated by these drugs. It has been proposed that the ME withdrawal response could be explained by the existence of a negative feedback mechanism which controls the excitability of SP and ACh neurones (Chahl, 1983). Any drug which inhibited release of ACh from cholinergic neurones and of SP from SP neurones, would cause disinhibition of SP neurones. Both clonidine and opiates inhibit enteric neuronal activity (Hirst & Silinsky, 1975; Morita & North, 1981; North, 1982; North & Egan, 1983) and inhibit release of ACh and SP in guinea-pig ileum (clonidine–Deck *et al.*, 1971; Drew, 1978; Tanaka & Starke, 1979; Bartho *et al.*, 1983; opiates–Paton, 1957; Bartho *et al.*, 1982; Gintzler & Scalisi, 1982). It would seem reasonable to assume that similar post-receptor actions of these drugs are the basis of their ability to activate the same neuronal events which lead to withdrawal contractures.

Clonidine has been reported to suppress opiate withdrawal in man (Gold *et al.*, 1978), rats (Tseng *et al.*, 1975) and guinea-pig isolated ileum (Collier *et al.*, 1981). The present results have confirmed the findings of Collier *et al.* (1981) since clonidine abolished ME withdrawal and ME abolished clonidine withdrawal. This observation is entirely consistent with the proposal outlined above that both clonidine and opiate withdrawal are mediated by the same post-receptor neuronal mechanism which cannot be triggered in the continued presence of the other drug. If, however, both drugs were added to the preparation and removed from their receptors simultaneously by addition of both naloxone and phentolamine, the withdrawal response was precipitated.

Since clonidine is an α-receptor agonist, it was expected that noradrenaline would mimic the response of the ileum to clonidine. This was indeed found to be so in the present study. Presumably because of its more rapid removal from the receptors, a withdrawal response following 2 min contact with noradrenaline could be precipitated either by washout or addition of α-receptor antagonists. This finding might have important implications in the functioning of certain regions of the central nervous system, possibly those involved in cardiovascular control, following states of stress.

The present experiments did not include a detailed study of the type of α-adrenoceptors in the guinea-pig ileum activated by clonidine to induce dependence.

However it has been shown that the α-receptors in the guinea-pig ileum are of the α_2-receptor type and are located on cholinergic neurones (Drew, 1978; Wikberg, 1978a, b; Tanaka & Starke, 1979). The present findings were consistent with previous observations since clonidine was more potent than noradrenaline in inducing a withdrawal response (Wikberg, 1978a).

In conclusion, this study lends support to the previous proposal (Chahl, 1983) that withdrawal responses are essentially a reflection of disturbance in feedback control of neuronal networks in which SP neurones play a major role.

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