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Research Paper

Acute withdrawal induced by adenosine A_1-receptor activation in isolated guinea-pig ileum: role of opioid receptors and effect of cholecystokinin

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Abstract

Objectives In isolated guinea-pig ileum, the μ-opioid acute withdrawal response is under control of several neuronal systems, including the κ-opioid and the A_1-adenosine systems, which are involved in the μ-withdrawal response inhibitory control. After μ-opioid system stimulation, indirect activation of both κ-opioid and A_1-adenosine systems is prevented by the peptide cholecystokinin-8 (CCk-8). Guinea-pig ileum exposed to A_1-adenosine agonist (CPA), shows a withdrawal contracture precipitated by the A_1-adenosine antagonist (CPT). We investigated this response.

Methods We investigated the involvement of the opioid system in the A_1-adenosine acute withdrawal response in guinea-pig ileum, the potential induced cross-dependence between the A_1 and the opioid system and also the interaction between the CCk-8 and A_1 systems.

Key findings We found that in the guinea-pig ileum preparation exposed to CPA, μ- and κ-opioid antagonists increased the withdrawal response to CPT. Tissues exposed to CPA showed a contractile response to the opioid receptor antagonist naloxone only after complete removal of the A_1-agonist. In the presence of CPA, the response to CCk-8 was inhibited while a significant increase in CPT response intensity was observed.

Conclusions In guinea-pig ileum, stimulation of the A_1 system indirectly activates both μ- and κ-opioid systems; this indirect activation is significantly, albeit not completely, antagonised by CCk-8. Cross dependence between A_1 and opioid systems was also observed.

Keywords acute withdrawal; adenosine A_1-receptor; cholecystokinin; guinea-pig ileum; indirect activation; opioid receptor

Introduction

The enteric nervous system can control gastrointestinal function independently of direct connections with the central nervous system, using multiple mechanisms of excitatory and inhibitory neurotransmission in enteric ganglia. There are two broad types of neurons in the enteric nervous system: S and AH neurons. In guinea-pig ileum, μ- and κ-opioid receptors are primarily localised in S-type myenteric neurons,[1] which are motor neurons and interneurons.[2–4] Adenosine A_1-receptors are not present in a significant proportion in S neurons, since their primary sites of action are in the after-hyperpolarizing AH neurons, which are intrinsic primary afferent sensory neurons. In the enteric nervous system, adenosine acts as a presynaptic neuromodulator that influences the release of inhibitory neurotrasmitters, such as noradrenaline (norepinephrine) and somatostatin,[5,6] and excitatory neurotransmitters, such as acetylcholine and tachykinins,[7–9] hyperpolarising the AH neurons.[10,11]

Guinea-pig isolated preparations are widely used to study the mechanisms underlying the opioid withdrawal syndrome.[12,13]

The opioid withdrawal response usually occurs upon the abrupt discontinuation/separation or a decrease in dosage of opioid drugs. It is generally accepted that the opioid antagonist-induced withdrawal response results from the displacement of the agonist from its receptor binding sites. Its symptoms and signs persist for a long time after removal of

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the agonist.[14–16] Some of the μ-opioid withdrawal symptoms are similar to those characterising adenosine A_1-withdrawal responses; this suggests the existence of a functional relationship between μ-opioid and A_1-adenosine systems.[17–19] Specifically, bi-directional cross-withdrawal responses have been observed in rats treated systemically with an adenosine A_1- or μ-opioid agonist. In particular, in rats pre-treated with an adenosine A_1-agonist, some signs of the μ-opioid withdrawal syndrome are precipitated by the μ-opioid antagonist administration, whereas in rats pre-treated with a μ-opioid agonist, some signs of A_1-adenosine withdrawal symptoms are precipitated by adenosine A_1-antagonist administration.[20,21] A possible explanation for cross-withdrawal between the μ-opioid and the A_1-adenosine systems is that the exposure to μ-opioid agonists induces an up-regulation of the adenosine A_1-receptors and that the adenosine A_1-antagonists may block, acting at the up-regulated adenosine A_1-receptors, the inhibitory tone exerted by the endogenous adenosine A_1-agonist.[18] Similarly, exposure to adenosine A_1-agonists might up-regulate the μ-opioid receptors with a consequent inhibitory tone induced by the endogenous μ-opioid agonists. In this case, the μ-opioid antagonists may induce withdrawal signs by blocking this inhibitory tone.[20] Alternatively, cross-withdrawal may be the result of receptor modification (caused by the indirect interaction between the above-mentioned systems), that in turn induces an active state of the receptors, at which the antagonist may act as an inverse agonist.[22] Both hypotheses are based on indirect activation of the adenosine A_1-system by μ-opioid agonists and *vice versa*. In addiction, several in-vivo observations show that, in μ-opioid dependent rats and mice, adenosine A_1-receptor agonists decrease some withdrawal signs precipitated by naloxone.[23–26]

The interactions observed *in vivo* between the μ-opioid and A_1-adenosine systems can also be observed in guinea-pig ileum isolated preparations, where a clear sign of a withdrawal response is represented by a tissue contraction, that occurs even after the acute exposure to a μ-opioid agonist.[27,28] Guinea-pig ileum contains many neurotransmitters, modulators and their receptors, including μ- and κ-opioid receptors,[29] and adenosine A_1- and A_2-receptors.[30] In guinea-pig ileum, withdrawal signs of A_1-adenosine dependence were first observed by Collier and Tucker,[31] but the authors did not observe any interaction with the opioid system. Chahl and co-workers observed that the withdrawal contraction in guinea-pig ileum preparations, incubated for a brief period with adenosine, is substance P-mediated, but no further experiments aimed at elucidating the mechanisms underlying this process were carried out.[12] Our previous in-vitro studies have shown that in guinea-pig isolated ileum preparation, acute exposure to a κ-opioid agonist indirectly activates the μ-opioid system, which inhibits the κ-antagonist-induced withdrawal response.[13] We have also shown that acute μ-opioid agonist stimulation of guinea-pig ileum preparation indirectly activates both adenosine A_1- and κ-opioid systems, which in turn inhibit the μ-antagonist-induced withdrawal response.[28] In our present study we have first investigated whether the A_1-adenosine acute withdrawal response in guinea-pig ileum is controlled by μ- or κ-opioid systems (or both), and whether a cross-dependence occurs between the adenosine A_1 and opioid systems.

For a better understanding of our present in-vitro studies, it is important to keep in mind that the withdrawal responses in guinea-pig ileum preparations, after repeated tests with μ- or κ-agonist/antagonist, show a declining responsiveness of the antagonist-precipitated withdrawal contracture.[12,13,32,33] We attribute this effect to a development of tolerance. It has been reported in the literature that the peptide cholecystokinin-8 (CCk-8) is able both to reduce or prevent the withdrawal signs precipitated by naloxone and counteract the tolerance development in morphine-dependent mice.[34–39] In our previous in-vitro studies, we have also shown that: (i) in guinea-pig ileum preparations, CCk-8 induces a contracture, that in turn can be inhibited by opioid agonists;[27] (ii) tissues treated only with CCk-8 show withdrawal contracture precipitated by μ- or κ-opioid antagonists;[35] and finally (iii) CCk-8 is able to prevent development of tolerance after repeated tests with agonist/antagonist opioid receptor.[27,28] Based on the observations mentioned above, the final aims of the present study were to further investigate whether CCk-8 is also able to activate the A_1-adenosine system and whether it is involved in the A_1-antagonist precipitated withdrawal contracture.

Materials and Methods

Drugs

Acetylcholine chloride (2-(acetyloxy)-*N*,*N*,*N*-trimethylethanaminium chloride), atropine (endo-(±)-α-(hydroxymethyl) benzeneacetic acid 8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester), cholecystokinin octapeptide sulfate, naloxone hydrochloride ((5a)-4,5-epoxy-3,14-dihydro-17-(2-propenyl)morphinan-6-one hydrochloride) and tetrodotoxin were purchased from Sigma Chemical Co. (St Louis, USA); *nor*-binaltorphimine dihydrochloride (17,17′-(dicyclopropylmethyl)-6,6′,7,7′-6,6′-imino-7,7′-binorphinan-3,4′,14,14′-tetrol dihydrochloride), cyprodime hydrochloride (17-(cyclopropylmethyl)-4,14-dimethoxymorphinan-6-one hydrochloride), 8-cyclopentyl-1,3-dimethylxanthine and N^6-cyclopentyladenosine were purchased from Research Biochemical International (Natick, USA).

Stock solutions containing 1 mg/ml in de-ionised water and ethanol (1 : 1) were prepared, from which further dilutions were prepared freshly before use; 8-cyclopentyl-1,3-dimethylxanthine was dissolved in 0.05 N NaOH to yield a 1 mg/ml concentration and used as such.

Guinea-pig ileum isolated preparation

The experimental procedure was essentially performed as previously described.[27,28] Male guinea-pigs, 300–400 g (Morini, Italy), were used. They were housed in a room with controlled temperature (21 ± 1°C) and light (12 h per day) for at least four days before being used. Four to six segments of ileum, 2–3 cm long, were excised from the same guinea-pig, discarding the 10 cm nearest the caecum. The segments were cleaned with Tyrode's solution and set up, under 1 g tension, over the end of a J-shaped stainless steel 10-ml organ bath containing Tyrode's solution, maintained at

37°C and gassed with 95% O_2 and 5% CO_2. Changes in tension were recorded by an isotonic force transducer connected to a pen recorder (Ugo Basile, Italy) and calibrated before each experiment. The preparations were allowed to equilibrate for 30–40 min and then stimulated two or three times with acetylcholine (10^{-7} M) to ascertain their responsiveness. The preparations were generally used for several consecutive tests and were washed out once immediately after each test; thereafter they were allowed to rest for 25–30 min with three washouts. During the experiments, after three consecutive tests, the tissues were exposed again to acetylcholine (10^{-7} M) to ascertain their responsiveness. Each experimental test was performed in tissue preparations coming from at least four guinea-pigs. Animals were handled according to guidelines published in the European Communities Council directives (86/609/EEC), the Italian National regulations (D.L. 116/92) and the Declaration of Helsinki. The experimental procedure has been approved by the local University Ethic Committee 'La Sapienza', concerning the cure and use of Mammals in experimental practice.

The effect of μ- and κ-opioid receptor blockade on adenosine A_1 withdrawal responses

To eliminate the possible interference of spontaneous responses to the antagonists, before starting the experiments, tissue preparations were preliminarily exposed to μ- and κ-opioid and A_1-adenosine antagonists, naloxone (5×10^{-7} M), *nor*-binaltorphimine (BNI, 3.4×10^{-8} M) and 8-cyclopentyl-1,3-dimethylxanthine (CPT, 1.2×10^{-6} M), respectively (2 min exposure for each antagonist). We had previously observed that some naïve tissue preparations gave a contractile response to the addition of the antagonists but these responses normally disappeared after two antagonist contacts. After this preliminary procedure and three washouts, the tissues were exposed for 5 min to the selective adenosine A_1-receptor agonist, N^6-cyclopentyladenosine (CPA, 2.9×10^{-9} or 2.9×10^{-8} M) and then challenged with the selective A_1-antagonist CPT (1.2×10^{-6} M). The μ- and κ-opioid receptor antagonists, cyprodime (1.4×10^{-6} M) and BNI (3.4×10^{-8} M) were injected 1 min before the adenosine antagonist (or 2 min and 1 min, respectively, when administered in combination). The concentrations of CPA and CPT used were in the range used to previously characterise the effect of the adenosine A_1 system on opioid withdrawal.[28] The opioid antagonists were the same as those used in previous works investigating μ/κ and CCk-8/opioid system interactions.[13,27]

Naloxone and CPT challenges in tissues previously tested with CPA/CPT, after washing out CPA; effect of naloxone on the responses to CPT

After a first CPA/CPT test (CPA: 2.9×10^{-8} M; CPT: 1.2×10^{-6} M), isolated preparations were exposed for 5 min to CPA (2.9×10^{-8} M) and then challenged with naloxone (5.4×10^{-7} M) and, 5 min later, with CPT (1.2×10^{-6} M). After the usual three washouts, preparations were exposed for 5 min to CPA (2.9×10^{-8} M) and then challenged with naloxone (5.4×10^{-7} M) and CPT (1.2×10^{-6} M), after removing CPA with one or three washouts. Naloxone was added 5 min after the last washout; CPT was added 5 min after naloxone.

Responses to CPT after tissue stimulation with cholecystokinin; effects of μ- and κ-opioid antagonists on the inhibition of the cholecystokinin contractile response elicited by CPA

To investigate whether CCk-8 activates the adenosine A_1 system, tissue preparations were exposed to the excitatory peptide (0.1×10^{-9} M to 10×10^{-9} M). After each exposure, CPT (1.2×10^{-6} M) was added to the declining tonic response to CCk-8. To investigate the effect of an adenosine A_1-agonist on CCk-8 contraction and the withdrawal response to CPT in presence of the peptide, tissues were exposed to CPA (2.9×10^{-9} M to 2.9×10^{-8} M); CCk-8 (0.8×10^{-9} M) was added 5 min after the adenosine agonist, CPT (1.2×10^{-6} M) was added 5 min after CCk-8. In some preparations, after the test CPA/CCk-8/CPT and three washouts, the control CCk-8/CPT test was repeated to study the possible persistence of the adenosine agonist effect. To investigate the effect of opioid antagonists on the effect on the CPA-induced response to CCk-8 and CPT, opioid antagonists, BNI (3.4×10^{-8} M), cyprodime (1.4×10^{-6} M) or naloxone (5.4×10^{-7} M), were added 5 min before CPA (1.6×10^{-8} M). The concentrations of CCk-8 were the same as those used in previous works investigating CCk-8/opioid system interactions.[13,27]

Statistical analysis

The contractile responses were expressed as percentage of the maximal response to acetylcholine (ACh max). The response intensity was calculated as the mean (± SE) of all tissues; those yielding a response ≤ 10% of ACh max were assigned a 0 value. To express the responses also quantitatively, we considered as responding tissue preparations those with response intensity higher than 10% of ACh max. For each experimental protocol, two initial control tests were carried out; the intensity of the control test response was calculated from the first control test response, when there was no statistically significant difference between the two control test responses. Statistical significance of differences of response intensities was evaluated by one-way analysis of variance or one-way analysis of variance for repeated measure, as appropriate, followed by Holm–Sidak multiple comparison tests. The chi-square test was used to evaluate the significance of differences of response frequencies.

Results

The effect of μ- and κ-opioid receptor blockade on adenosine A_1 withdrawal responses

The opioid antagonists BNI (3.4×10^{-8} M) and naloxone (5.4×10^{-7} M), and the adenosine A_1-antagonist CTP (1.2×10^{-6} M), administered at a 2-min interval, elicited contractions (> 10% ACh max) in naïve ileum preparations from eight guinea-pigs out of a total of 28 used. These contractile responses disappeared following a second contact to the antagonists in almost all preparations. The few

preparations that still showed a contraction (> 10% ACh max) were disregarded.

We think that these contractures induced by antagonists administered alone could be due either to the presence, in naïve guinea-pig ileum preparations, of receptors constitutively activated, and to the presence of a constitutive opioid and/or adenosine endogenous tone. It is unlikely that these contractures may be ascribed to tissue stretch during dissection, because we observed that all tissue preparations from the same animal behaved in the same way (i.e. either responded or did not respond to the addition of opioid and adenosine A_1-antagonists). Moreover, guinea-pig ileum preparations were repeatedly washed out before adding the opioid or the adenosine A_1-antagonists, and this procedure should probably eliminate both the possible differences in the endogenous level and the constitutive activity of the receptors.[11–15,40–44]

A typical trace showing the responses obtained in these tests is shown in Figure 1. In guinea-pig ileum preparations exposed to CPA (2.9×10^{-9} M) for 5 min, the addition of CPT (1.2×10^{-6} M) elicited a withdrawal contraction (i.e. a contractile response with intensity of at least 10% ACh max) in 7 out of 14 tested preparations (Figure 1). In a second CPA/CPT test, the number of responding tissues and the mean response amplitude did not change significantly, although after three tests the CPT withdrawal contracture decreased significantly (data not shown). Both the selective μ-opioid antagonist cyprodime (1.4×10^{-6} M) and the selective κ-opioid antagonist BNI (3.4×10^{-8} M) significantly increased the amplitude of withdrawal response to CPT; however, the increase elicited by BNI was significantly higher than that elicited by cyprodime (Figures 1 and 2). The two opioid antagonists added together increased the CPT-precipitated withdrawal contraction more than both BNI and cyprodime alone, and the difference reached statistical significance. The opioid antagonists did not cause a statistical increase in the frequency of responding preparations (Figure 2), and elicited the same effects on the withdrawal response to CPT, when added before the adenosine agonist (data not shown). Increasing the concentration of CPA to 2.9×10^{-8} M did not significantly change either the number of preparations showing CPT-precipitated contractions in CPA/CPT control tests (10 out of 21), or the contraction intensity (20.2 ± 10.5). The administration of BNI and cyprodime before CPT increased both the number of responding preparations (17/21 vs 10/21) and the amplitude of the precipitated contractions. This increase was significantly lower than that observed with lower CPA concentration (Table 1). Naloxone (5.4×10^{-7} M), added 5 min after CPA, increased the number of responding tissues and the contraction intensity to CPT similarly to the co-administration of both selective opioid antagonists (not shown). None of the tissues showed a contractile response after the addition of an opioid antagonist at the concentrations of CPA used. In a separate series of experiments, CPT failed to elicit a withdrawal contraction in the presence of tetrodotoxin (1.6×10^{-7} M), while when atropine (1×10^{-6} M)

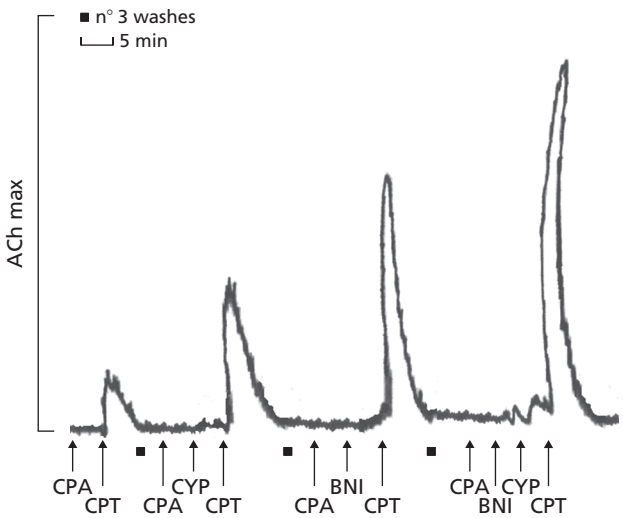

Figure 1 Typical trace showing the effect of cyprodime (CYP, 1.4×10^{-6} M), *nor*-binaltorphimine (BNI, 3.4×10^{-8} M), and their combination, on the adenosine A_1-withdrawal response in a guinea-pig isolated ileum preparation. The withdrawal contractions were precipitated by the selective A_1-adenosine antagonist 8-cyclopentyl-1,3-dimethylxanthine (CPT, 1.2×10^{-6} M), after 5 min exposure to adenosine A_1-receptor agonist, N^6-cyclopentyladenosine (CPA, 2.9×10^{-9} M). In tissues exposed to CPA/CPT after two tests, the withdrawal contracture showed an intensity approx. 20% of ACh max (maximal response to acetylcholine). The figure shows an experiment in which the contractile response to CPT is significantly increased (approx 70% ACh max) in the presence both of μ-opioid (CYP) and κ-opioid (BNI) antagonists. To ascertain the tissue responsiveness, acetylcholine control test was performed after three agonist/antagonist consecutive tests (data not shown). Each pharmacological test was performed after three wash outs, performed at 10-min intervals

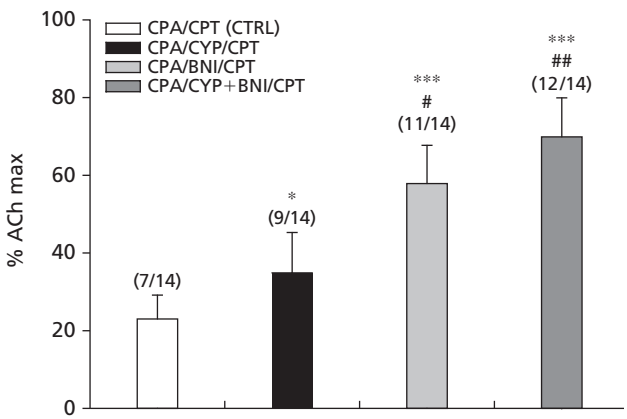

Figure 2 Effect of the opioid antagonists cyprodime (CYP, 1.4×10^{-6} M), *nor*-binaltorphimine (BNI, 3.4×10^{-8} M) and CYP + BNI on the intensity of withdrawal contractions of guinea-pig isolated ileum to the adenosine A_1-receptor antagonist, 8-cyclopentyl-1,3-dimethylxanthine (CPT, 1.2×10^{-6} M), after 5 min exposure to the adenosine A_1-receptor agonist N^6-cyclopentyladenosine (CPA, 2.9×10^{-9} M). Response intensity is expressed as percentage of maximal response to acetylcholine (ACh max, mean ± SE). The number of tested and responding preparations (> 10% of ACh max) is shown in brackets. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs CPA/CPT (CTRL); $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs CPA/CYP/CPT (one-way analysis of variance for repeated measure followed by Holm–Sidak multiple comparison test)

Table 1 Effect of opioid system blockade with cyprodime and *nor*-binaltorphimine on the withdrawal response to 8-cyclopentyl-1,3-dimethylxanthine after exposure to two N^6-cyclopentyladenosine concentrations

CPA concentration (M)	CPA/CPT	CPA/cyprodime + BNI/CPT
2.9×10^{-9}	21.0 ± 9.9 (7/14)	72.0 ± 20.1 (12/14)$^{***,\#}$
2.9×10^{-8}	20.2 ± 10.5 (10/21)	48.0 ± 17.3 (17/21)***

CPA, N^6-cyclopentyladenosine; CPT, 8-cyclopentyl-1,3-dimethylxanthine; BNI, *nor*-binaltorphimine. Response intensity is expressed as percentage of ACh max (maximal response to acetylcholine; mean ± SE). The number of tested and responding preparations (> 10% ACh max) are shown in brackets. $^{***}P < 0.001$ vs the response in CPA/CPT control test, with the corresponding CPA concentration; $^{\#}P < 0.05$ vs the response to CPT in presence of cyprodime + BNI, with CPA 2.9×10^{-9} M.

was added before the adenosine antagonist, the CPT-induced withdrawal contracture was inhibited by 40–60% of ACh max (data not shown).

Naloxone and CPT challenges in tissues previously tested with CPA/CPT, after washing out CPA; effect of naloxone on the responses to CPT

With the aim of investigating whether a cross-dependence occurred between adenosine A_1 and opioid systems, isolated preparations were first tested with CPA/CPT and CPA/naloxone/CPT (CPA: 2.9×10^{-8} M; CPT: 1.2×10^{-6} M; naloxone: 5.4×10^{-7} M). As described above, naloxone increased both the number of responding tissues and the contraction intensity to CPT, but no contractile response to naloxone was observed. CPA was then removed by performing one or three washouts. Thereafter, challenges with naloxone and CPT, at 5-min intervals, in the absence of CPA, were repeated. After one washout, naloxone evoked a response in 5 out of 16 preparations and markedly increased the amplitude of the response to the subsequent CPT challenge. The amplitude of these responses was similar to that obtained in the presence of CCk-8 (Figure 5). After three washouts (performed at 5-min intervals), all tissue preparations responded to naloxone and the response intensity markedly increased, with respect to the response to naloxone after one washout (Figure 3). The relationship between the frequency of response to naloxone and washouts was highly significant ($P < 0.001$). The response to the subsequent CPT challenge was markedly higher than that of the previous CPA/CPT and CPA/naloxone/CPT tests, but did not differ from that observed after one washout (Figure 3).

Responses to CPT after tissue stimulation with cholecystokinin; effects of μ- and κ-opioid antagonists on the inhibition of CPA-induced cholecystokinin contractile response

The injection into the bath of increasing doses of CCk-8 (0.1×10^{-9} M to 10×10^{-9} M) evoked a dose-dependent contraction of the ileum, consisting of the following two components: (i) the phasic response, characterised by a sharp

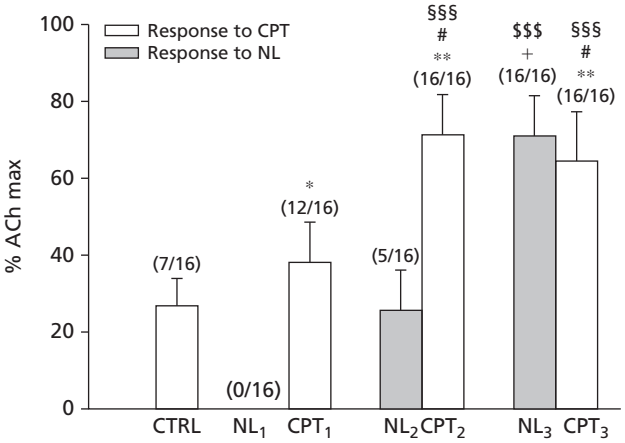

Figure 3 Responses of guinea-pig isolated ileum to naloxone (NL, 5.4×10^{-7} M) and 8-cyclopentyl-1,3-dimethylxanthine (CPT, 1.2×10^{-6} M) challenges after removal of the A_1-agonist N^6-cyclopentyladenosine (CPA, 2.9×10^{-8} M) with one or three washouts. Guinea-pig ileum preparations were first tested with CPA/CPT (control test, CTRL) and then, after washout, with CPA/NL/CPT (NL_1 and CPT_1). After washouts, preparations were exposed for 5 min to CPA and after washing out the preparations once (one washout), challenges with NL and CPT were repeated (CPT was added to the bath 5 min after NL), in the absence of CPA (NL_2 and CPT_2). Preparations were then re-exposed to CPA and the agonist was removed by performing three washouts, at 5-min intervals; thereafter, challenges with NL and CPT were repeated (NL_3 and CPT_3). Response intensity is expressed as percentage of maximal response to acetylcholine (ACh max, means ± SE). The number of tested and responding preparations to NL and CPT is shown in brackets. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs response to CPT in control test CPA/CPT (CTRL); $^{\#}P < 0.05$ vs response to CPT in CPA/CPT/NL test (CPT_1); $^{+}P < 0.05$ vs response to NL in CPT/NL test challenge after one wash out (NL_2); $^{§§§}P < 0.001$ vs number of responding tissue to CPT (CTRL); $^{\$\$\$}P < 0.001$ vs number of responding tissues to NL (NL_1). Statistical significance was evaluated by one-way analysis of variance for repeated measure followed by Holm-Sidak multiple comparison test. Statistical significance of frequency responding tissues was evaluated by chi-square test

spike, lasting a few seconds and (ii) a tonic response, which declined slowly. The maximal amplitude reached was at 0.4×10^{-9} M to 0.8×10^{-9} M (Table 2, Figure 4). The addition of CPT (1.2×10^{-6} M) during the declining tonic phase induced a subsequent contraction, with intensity increasing upto about 30% of ACh max with increasing CCk-8 concentrations in the range 0.1×10^{-9} M to 0.8×10^{-9} M. At higher CCk-8 concentrations the amplitude of CPT-induced contraction decreased, thus showing a bell shaped trend (Table 2). As shown in Figure 5, CPA (2.9×10^{-9} M to 2.9×10^{-8} M), added to the bath 5 min before CCk-8, inhibited, in a dose-related way, the contractile response to CCk-8 (0.8×10^{-9} M). It also caused a marked increase of the contractile response to CPT (1.2×10^{-6} M). This increase did not change with increasing concentrations of CPA (Figures 4 and 5). However, it should be noted that at the lower dose of CPA (from 2.9×10^{-9} M to 8×10^{-9} M), the variability of the response to CPT (as indicated by the SE values shown in Figure 5) was very low; this variability was markedly increased at the higher concentrations of CPA (from 1.6×10^{-8} M to 2.9×10^{-8} M). This difference in the

Table 2 Contraction of guinea-pig ileum in response to cholecystokinin-8 and to 8-cyclopentyl-1,3-dimethylxanthine added at the declining response to cholecystokinin-8

CCk-8 concentration (nM)	Tonic contraction	Response to CPT
0.1 (10)	47.2 ± 15.5	$9.3 \pm 5.6^{\#\#\#,+++}$
0.2 (10)	51.4 ± 13.8	$16.3 \pm 8.2^{\#\#\#,++}$
0.4 (10)	$66.1 \pm 12.3^{***,\S\S}$	28.7 ± 12.6
0.8 (10)	$71.7 \pm 9.2^{***,\S\S\S}$	30.2 ± 10.1
2.0 (15)	$72.9 \pm 7.0^{***,\S\S\S}$	$15.5 \pm 2.8^{\#\#\#,+++}$
10.0 (14)	$70.8 \pm 6.0^{***,\S\S\S}$	17.2 ± 6.7

CCk-8, cholecystokinin-8; CPT, 8-cyclopentyl-1,3-dimethylxanthine. Response intensity is expressed as percentage of maximal response to acetylcholine (ACh max, mean ± SE). The number of tested preparations is shown in brackets. $^{***}P < 0.001$ vs 0.1 nM; $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$ vs 0.2 nM; $^{\#\#\#}P < 0.001$ vs 0.8 nM; $^{++}P < 0.01$, $^{+++}P < 0.001$ vs 0.4 nM.

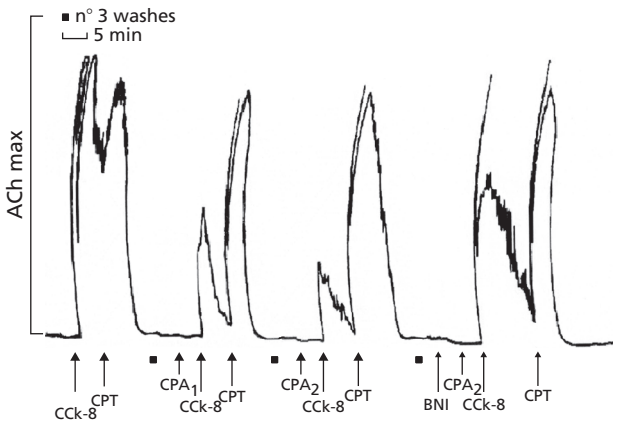

Figure 4 Typical tracing showing both the enhancing effect of cholecystokinin (CCk-8, 0.8×10^{-9} M) on the withdrawal response to 8-cyclopentyl-1,3-dimethylxanthine (CPT, 1.2×10^{-6} M) and the concentration-dependent inhibition of N^6-cyclopentyladenosine (CPA_1, 2.9×10^{-9} M; CPA_2, 2.9×10^{-8} M) on the response to CCk-8 in guinea-pig isolated ileum. The CPA inhibitory effect on cholecystokinin contraction, partially reverted by the κ-opioid antagonist *nor*-binaltorphimine (BNI, 3.4×10^{-8} M) has been shown. The responses to CPT precipitated in the presence of CPA and CCk-8 reached the maximal intensity that remained unchanged at increasing doses of CPA (CPA_2). The contractile response to CCk-8 was inhibited in a dose-related way by different doses of CPA (CPA_1 and CPA_2). Finally, in the presence of the κ-opioid antagonist, BNI, the A_1-adenosine agonist-induced CCk-8 response inhibition was significantly reverted. Each pharmacological test was performed after three wash outs at 10-min intervals. To ascertain the tissue responsiveness, acetylcholine (ACh) control test was performed after three agonist/antagonist consecutive tests (data not shown)

response to CPT is due to the fact that with the higher concentrations of CPA, 30% of preparations showed a response intensity of about 100% of ACh max, while 20% of preparations showed an intensity markedly lower (i.e. ≤ 40% of ACh max) than that observed with the lower CPA concentrations. The CPA inhibitory effect on CCk-8 contraction and the intensity of withdrawal contraction to CPT were reproducible in several subsequent CPA/CCk-8/CPT tests

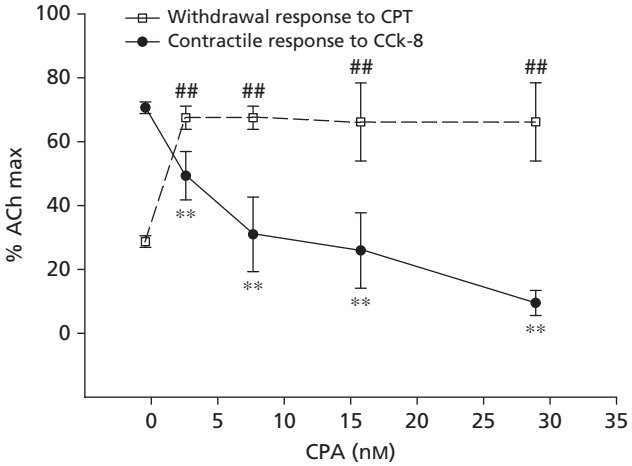

Figure 5 Effect of N^6-cyclopentyladenosine (CPA, from 2.9×10^{-9} M to 2.9×10^{-8} M) on the response to cholecystokinin (CCk-8, 0.8×10^{-9} M) and 8-cyclopentyl-1,3-dimethylxanthine (CPT, 1.2×10^{-6} M). Response intensity is expressed as percentage of maximal response to acetylcholine (ACh max, means ± SE, $n = 17$ for all concentrations tested). $^{**}P < 0.01$ vs the response to CPT in control CCk-8/CPT test; $^{\#\#}P < 0.01$ vs the response to CCk-8 in control CCk-8/CPT test (one-way analysis of variance for repeated measure followed by Holm–Sidak multiple comparison test)

(data not shown). After the tests with CPA/CCk-8/CPT and three washouts, the contractile responses to both CCk-8 and the adenosine antagonist showed the same intensity as in the initial control tests, thus indicating that three wash outs were enough to completely remove CPA from the bath (data not shown). The μ-opioid selective receptor antagonist, cyprodime (1.4×10^{-6} M), when added 5 min before CPA (2.9×10^{-8} M), only partially antagonised the inhibitory effect of CPA on the response to CCk-8, while both the selective κ-opioid receptors antagonist BNI (3.4×10^{-8} M) and the non-selective opioid antagonist naloxone (5.4×10^{-7} M) significantly antagonised the CPA inhibitory effect on the CCk-8-induced contractile response (Figure 6). In this case, the withdrawal contractions to CPT were unaffected by the opioid antagonists (data not shown), and the responses to CPT were again completely abolished by tissue pre-treatment both tetrodotoxin (1.6×10^{-7} M) and partially (40–60%) with atropine (1×10^{-6} M), added before the adenosine antagonist. In a similar way, both tetrodotoxin and atropine also abolished CCk-8-mediated guinea-pig ileum contractile response (data not shown).

Discussion

Isolated guinea-pig ileum preparations, briefly exposed to opioid agonists, show a typical withdrawal contracture precipitated by opioid receptor antagonists. This contracture is controlled by the integration of a complex network of both inhibitory and excitatory systems. The withdrawal contracture precipitated by an opioid antagonist could be considered as a physiological adaptive response of the guinea-pig ileum to a disruption of this balance induced by the opioid system stimulation.[27,28]

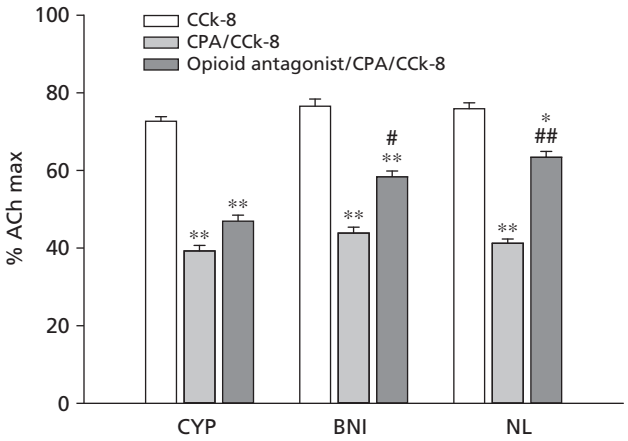

Figure 6 Effect of the opioid antagonists cyprodime (CYP, 1.0×10^{-6} M), *nor*-binaltorphimine (BNI, 3.4×10^{-8} M) and naloxone (NL, 5.4×10^{-7} M) on the adenosine A_1-agonist N^6-cyclopentyladenosine (CPA; CPA_1, 2.9×10^{-8} M) induced cholecystokinin (CCk-8, 0.8×10^{-9} M) contractile response inhibition in guinea-pig isolated ileum. Response intensity is expressed as percentage of maximal response to acetylcholine (ACh max, means ± SE, $n = 16$ for the three series of tests). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs control response to CCk-8; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs the response to CCk-8 in the presence of CPA (CPA/CCK-8 test) (one-way analysis of variance for repeated measure followed by Holm–Sidak multiple comparison test)

In naïve guinea-pig ileum tissues, a brief exposure to an A_1-agonist (CPA) induces a dependence status represented by a precipitated withdrawal contracture that is elicited in presence of an A_1-antagonist (CPT). The withdrawal contractures to CPT are completely abolished in the presence of tetrodotoxin (data not shown), demonstrating the neuronal nature of these responses.[45,46] On the other hand, in the presence of atropine, CPT withdrawal contractures are partially blocked, in accordance with the finding that, in guinea-pig ileum, the A_1 withdrawal contracture is not only characterised by cholinergic release, but also other neurotransmitters, such as substance P, can be involved.[47–49]

The incubation time (5 min) used in our experimental conditions was much shorter than that reported in previous works (16–22 h), suggesting that the processes responsible for adenosine A_1 dependence begin very early, after a single exposure.[12,31]

In this study, we have demonstrated for the first time that, in guinea-pig ileum, A_1 withdrawal contracture is controlled by the opioid system, since both μ- and κ-opioid antagonists increased the response to the A_1-antagonist CPT, following a 5-min exposure to the A_1 agonist, CPA (Figure 1). The κ-opioid system seems to play a major role in inhibiting the withdrawal response to CPT (Table 1, Figure 2), and this is supported by the observation that the increase of this response induced by the selective κ-opioid antagonist BNI was higher than that elicited by the μ-selective antagonist cyprodime (Figure 2). Our data show that the opioid system is activated by acute exposure to the A_1-agonist and that the activated opioid system modulates the intensity of the adenosine A_1 withdrawal contracture.[16,18,19]

Interestingly, we observed that, at the highest CPA concentration tested, the increase in withdrawal response to CPT elicited by the opioid antagonists was lower than that observed with the lowest CPA concentration (Table 1). In contrast, previous in-vivo findings have shown that the adenosine antagonist-precipitated withdrawal signs increased at increasing doses of CPA.[21] These apparent contradictory results suggest that, in guinea-pig ileum, other neuronal inhibitory systems, activated at the highest CPA concentration, could be involved in the control of the A_1-withdrawal response. One of them might be the endogenous cannabinoid system, which, as we have previously shown, modulates the μ-opioid withdrawal response in isolated guinea-pig ileum preparations.[50] In fact, exposure to a μ-opioid agonist (such as dermorphine) leads to an indirect activation of the cannabinoid CB_1 receptors, which in turn inhibit the opioid acute withdrawal response. The effect exerted by the CB_1 receptors can only be observed in presence of κ-opioid and adenosine A_1-antagonists, suggesting that the endocannabinoid system plays a marginal inhibitory role in the regulation of the μ-opioid withdrawal response.[50]

We have also demonstrated that, following acute exposure to CPA, a cross-dependence occurs, because tissue preparations exposed to CPA yielded contractile responses when challenged with naloxone. These responses could only be observed after washing out CPA, which would have otherwise inhibited the withdrawal response to naloxone. This view is strengthened by the finding that the frequency and intensity of the responses to naloxone increased with the number of washouts performed (Figure 3). This explains the apparent contrast of our results with those reported by other authors,[31] who found that in guinea-pig ileum preparations incubated with an adenosine A_1-agonist, naloxone did not precipitate withdrawal contractions. In their work, the A_1-agonist was not washed out before challenging the tissue with naloxone.

In our previous work, we have already shown that in guinea-pig ileum a cross-dependence between opioid and adenosine systems occurs, since after removal of the μ-opioid agonist (dermorphine), guinea-pig ileum preparation yielded a contracture response to CPT.[28] Therefore, our studies demonstrate that bi-directional cross-withdrawal responses can be observed in isolated guinea-pig ileum, after a very short-term exposure to either an adenosine A_1 or a μ-opioid agonist.

In vivo, bi-directional cross-withdrawal responses have been demonstrated after acute[20,51] and sub-acute administration.[21]

It is worth noting that also after removal of the A_1-agonist, the activated opioid system still inhibits the withdrawal expression, since naloxone is able to increase the CPT-induced contractile response (Figure 3). The fact that a withdrawal response to CPT could be obtained after removal of CPA indicates that this response does not result from the agonist displacement from its receptor binding site. This conclusion is in agreement with the results both of in-vivo in-vitro studies on opioid withdrawal, demonstrating that withdrawal symptoms and signs persist for a long time after removal of the agonists.[14–16]

Some authors have proposed that opioid agonists produce dependence by inducing a spontaneously active state of opioid receptors at which the opioid antagonist naloxone acts

as an inverse agonist. Our data suggest that this hypothesis might also apply to adenosine A_1 withdrawal response.[22,52–55] The mechanisms involved in the development of acute adenosine dependence and its expression are not well known yet. We know that activation of adenosine A_1-receptors has neurochemical characteristics similar to those of opioid receptor activation, such as inhibition of adenylyl cyclase activity,[56–58] closing of voltage-gated Ca^{2+} channels,[59] activation of inwardly rectifying K^+ channels,[60] modulation of inositol triphosphate turnover[61,62] and inhibition of neurotransmitter release.[63]

An important adaptive response to chronic opioid exposure is a sensitisation in the cAMP signal transduction system, which is represented by a rebound of cAMP production above the basal level after administration of opioid antagonists or abrupt cessation of treatment. Modulation of adenylyl cyclase, also observed in guinea-pig longitudinal muscle myenteric plexus preparations,[64] has long been considered the cellular adaptation to drug exposure.[65]

Also *in vivo*, it seems that adaptive increase in cAMP, which is consequent to a cAMP decrease induced by an adenosine A_1-agonist, could be modulated by endogenous opioid inhibitory tone.[21] It is unlikely that this mechanism occurred under our experimental conditions, both because we used a short exposure time and because opioid antagonists did not inhibit, but increased, the CPT-induced withdrawal response.

With the aim of explaining the possible mechanisms underlying tolerance and withdrawal, many hypotheses have been proposed. Both the cAMP–protein kinase A (through $G_{i/o}$) and MAPK (at least through $G\beta\gamma$ subunits) cascade are of interest because they lie more or less directly downstream from opioid or adenosine receptor activation.[66,67] Other kinase cascades may also be directly involved in tolerance and withdrawal, including protein kinase C[68] as well as other signalling systems.[69,70] Moreover, a possible inhibition of adenosine uptake induced by morphine has been proposed to explain the cross-withdrawal between opioid and adenosine systems.[71,72] Finally, a physical interaction (heterodimerisation) cannot be excluded.[73,74]

Finally, the withdrawal response in guinea-pig ileum preparations, after repeated tests with μ- or κ-agonist/antagonist, shows a declining responsiveness of the antagonist precipitated withdrawal contracture.[12,13,32,33] We attribute this effect to a development of tolerance. The peptide CCk-8 is able to reduce or prevent the withdrawal signs precipitated by naloxone, and counteract the tolerance development in morphine-dependent mice.[34,35,38,39,75,76] For this reason in our in-vitro experiments CCk-8 was used.

CCk-8, when administered alone, is able to induce a contracture in guinea-pig ileum tissues as well as to activate the A_1-adenosine system. This is supported by our observation that after CCk-8 administration, an A_1-antagonist (CPT)-induced withdrawal contracture has been observed (Table 2, Figure 4).

In addition, as shown in Figure 4, in the presence both of CPA and CCk-8, the CPT precipitated withdrawal response reached the maximal intensity that remains unchanged at increasing doses of CPA (CPA_2). On the other hand, the withdrawal responses to CPT in the presence of CCk-8 were unaltered by opioid antagonist, which suggests that CCk-8 counteracts indirect activation of the opioid system, and this may explain why CCk-8 increased the frequency and intensity of adenosine A_1-withdrawal responses (Figure 5).[77,78]

We also found that the CPA inhibitory effect on CCk-8-induced contraction (Figure 4) was partially antagonised by opioid antagonists, showing that this effect is also mediated by activation of opioid receptors (Figures 4, 6). However, we previously found that CCk-8 antagonised the inhibitory control induced by indirectly activated systems, since in the presence of the peptide the μ-opioid withdrawal responses were maximal and not increased by κ- and A_1-antagonists.[27,28] Also in the presence of an A_1-adenosine agonist, CCk-8 inhibits the opioid system indirect activation induced by the A_1-adenosine receptor stimulation. Therefore, CCk-8 appears to interfere with the mechanisms controlling the expression of both μ-opioid and A_1-adenosine acute dependence. A further confirmation of the interaction between CCk-8 and the opioid system is given by our observation that CPA-induced inhibition contracture to CCk-8 was partially reverted when an opioid antagonist was administered before the A_1-receptor agonist. In this process, the κ-opioid system seems to be mainly involved, since in the presence of the κ-opioid selective antagonist (BNI), the CCk-8 induced contracture was increased (Figures 4, 6). The exact mechanism underlying CCk-8-induced effects in guinea-pig ileum are not well understood yet. A possible mobilisation of Ca^{2+} from intracellular stores, has been reported in the literature.[79]

In this study we found that on increasing the CPA concentration in about 20% of guinea-pig ileum preparations, the enhancing effect of CCk-8 on the A_1 withdrawal response was lower than the enhancing effect observed with lower concentrations of the A_1-agonist. Further, we observed that opioid antagonists did not increase the A_1-withdrawal contraction in the presence of CCk-8 and this finding confirms that exposure to CPA also activates systems other than the μ- and κ-opioid ones (i.e. the endocannabinoid system). These systems, which are activated at concentrations higher than those necessary to activate indirectly the opioid system, also inhibit the A_1-withdrawal response, although their inhibitory effect might not be counteracted by CCk-8.[50]

The adenosine A_1-agonist inhibition of the contractile response to CCk-8 was reproducible in many consecutive tests; the same was previously found for opioid-induced inhibition of the CCk-8 response.[27,35] These findings provide evidence against the development of tolerance in the myenteric plexus, as observed with opioids also using different experimental procedures.[15,80]

Conclusions

In conclusion, taken together our results suggest that, in guinea-pig ileum, the withdrawal contracture induced by A_1-receptor stimulation is under the control of the opioid system. Moreover, the adenosine system induces cross-withdrawal with the opioid system, since withdrawal contracture to the opioid antagonist (naloxone) is precipitated after A_1-receptor

stimulation, although only after complete removal of the A_1-agonist. This indirect interaction between the A_1-adenosine and the opioid system is counteracted by CCk-8, indicating a functional role of this peptide in the induction of the A_1-adenosine withdrawal syndrome. Further investigations need to be performed to explain the mechanisms underlying the indirect activation between the A_1-adenosine and the opioid system and to elucidate the physiological implications of CCk-8 in this complex bi-directional intersystemic relationship.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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