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Adenosine receptor-mediated control of in vitro release of pain-related neuropeptides from the rat spinal cord

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Abstract

Although it is well established that adenosine exerts antinociceptive effects at the spinal level in various species including human, the mechanisms responsible for such effects are still a matter of debate. We presently investigated whether adenosine-induced antinociception might possibly be related to an inhibitory influence of this neuromodulator on the spinal release of neuropeptides implicated in the transfer and/or control of nociceptive signals. For this purpose, the K^+-evoked overflow of substance P-, calcitonin gene-related peptide (CGRP)- and cholecystokinin-like materials was measured from slices of the dorsal half of the rat lumbar enlargement superfused with an artificial cerebrospinal fluid supplemented with increasing concentrations of various adenosine receptor ligands. The data showed that stimulation of adenosine A_1 and (possibly) A_3 receptors, but not A_{2A} receptors, exerted an inhibitory influence on the spinal release of CGRP-like material. In contrast, none of the adenosine A_1, A_{2A} and A_3 receptor agonists tested within relevant ranges of concentrations significantly affected the release of substance P- and cholecystokinin-like materials. These results support the idea that adenosine-induced antinociception at the spinal level might possibly be caused, at least partly, by the stimulation of inhibitory adenosine A_1 receptors located presynaptically on primary afferent fibres containing CGRP but not substance P.

Keywords: Adenosine receptor; K^+-evoked release; Substance P; (CGRP) calcitonin gene-related peptide; Cholecystokinin; Spinal cord; (Rat)

1. Introduction

Numerous data are in favor of a role for adenosine in nociceptive processes, at the level of peripheral nerve terminals of sensory fibres as well as at central sites, particularly at the first central relay of pain transmission, i.e. the dorsal horn of the spinal cord (see Sawynok, 1998; Sawynok and Poon, 1999). Indeed, adenosine-containing terminals are precisely concentrated within the superficial layers of the dorsal horn, as shown by the distribution of adenosine deaminase activity (Geiger and Nagy, 1986) and adenosine-like immunoreactivity (Braas et al., 1986). Intrathecal administration of adenosine produces antinociceptive effects in rats (Holmgren et al., 1986; Sjölund et al., 1997, 1998) and mice (Post, 1984; Karlsten et al., 1991). Conversely, intrathecal administration of adenosine receptor antagonists elicits nociceptive behavior in mice (Nagaoka et al., 1993), and reverses morphine antinociception in rats (Sawynok et al., 1991). In human, systemic infusion (Sollevi et al., 1995) or intrathecal administration (Belfrage et al., 1999) of adenosine has been reported to alleviate chronic neuropathic pain.

Four types of adenosine receptors, named A_1, A_{2A}, A_{2B} and A_3, have been identified in the central nervous system (CNS) (Fredholm et al., 1994; Ralevic and Burnstock, 1998; Klotz, 2000). That adenosine A_1 receptors mediate the antinociceptive effect of adenosine at the spinal level has been well established (Sawynok and Poon, 1999), but some roles for the other adenosine A_2 and A_3 receptors in the control of nociception have also been reported in the relevant literature (DeLander and Hopkins, 1987; Ledent et al., 1997; Sawynok et al., 1997). Interestingly, adenosine A_1 and A_2 receptors, at least, are expressed in the spinal cord, predominantly in the superficial layers of the dorsal horn (Goodman and Snyder, 1982; Geiger et al., 1984; Choca et al., 1987, 1988), where the terminals of primary afferent fibres which convey noxious inputs are located.

Because adenosine has been repeatedly shown to exert an inhibitory influence, through local presynaptic receptors, on the release of various neurotransmitters from nerve terminals (Fredholm and Dunwiddie, 1988), one possible mechanism

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accounting for its antinociceptive effect would be a direct inhibition of the release of excitatory amino acids and/or neuropeptides from the terminals of primary afferent fibres projecting within the dorsal horn. Relevant studies have already been conducted with particular attention to two of these peptides, i.e. substance P and calcitonin gene-related peptide (CGRP) (Hökfelt et al., 1977; Lee et al., 1985; Skofitsch and Jakobowitz, 1985; Pohl et al., 1990), but the results reported so far are controversial. For some authors, adenosine even at high concentration, as well as adenosine A_1 receptor agonists [10–50 μM N^6-cyclohexyladenosine, 10 μM N^6-(2-phenylisopropyl) adenosine)] had no effect on the K^+-evoked, Ca^{2+}-dependent, release of substance P from rat spinal cord slices (Vasko and Ono, 1990), whereas for others, adenosine (0.3–300 μM) inhibited the spinal release of both this peptide and CGRP evoked by electrical field stimulation (Santicioli et al., 1992).

The present study was undertaken in an attempt to solve this controversial issue and to determine the receptor type(s) which mediate(s) the effects, if any, of adenosine on the spinal release of substance P and/or CGRP. The release of cholecystokinin-(1–8) (CCK), another peptide also contained within the superficial layers of the spinal cord but not in primary afferent fibres (Pohl et al., 1990), was also studied, and served as a control. Experiments consisted of exposing slices of the dorsal part of the rat spinal cord to ligands of the various types of adenosine receptors, and quantifying their effects on basal outflow and K^+-evoked overflow of the three peptides from these tissues.

2. Materials and methods

2.1. Animals

Experiments were performed on adult male Sprague–Dawley rats (3-month-old, 250–300 g body weight, Centre d'Élevage R. Janvier, Le Genest-St. Isle, France). They were kept under controlled environmental conditions (12 h light–dark cycle, 22 ± 1 °C, 60% relative humidity, food and water ad libitum) for at least 7 days before the experiments.

All the procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (Council directive #87.848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions #0299 to M.H., #0313 to F.C. and #6228 to S.B.).

2.2. Chemicals

[^{125}I][Iodo-tyrosyl^{10}]human CGRP ([^{125}I]CGRP, 70 TBq/mmol) was purchased from Amersham-Pharmacia Biotech (Buckinghamshire, UK) and [^{125}I]Tyr^8-substance P ([^{125}I] substance P, 74 TBq/mmol) from New England Nuclear (Boston, MA, USA). [^{125}I]Human gastrin (74 TBq/mmol) was from CIS-Bio International (Gif-sur-Yvette, France).

Other compounds were: N^6-2-(4-aminophenyl)ethyladenosine (APNEA), N^6-cyclopentyl-adenosine (CPA), 2-[4-[2-carboxyethyl]-phenylethylamino]-5′-*N*-ethylcarboxamido-adenosine (CGS 21680), 5′-*N*-ethyl-carboxamido-adenosine (NECA) and 8-(4-[{([{2-aminoethyl}amino]carbonyl)methyl}oxy]phenyl)-1,3-dipropylxanthine (xanthine amine congener, XAC). All these drugs were purchased from Research Biochem. Int. (Natick, MA, USA).

2.3. Methods

2.3.1. In vitro release experiments

Animals were killed by decapitation, and the dorsal half of the lumbar enlargement of the spinal cord was dissected at 0 °C as described (Cesselin et al., 1984). For each experiment, tissues from 12 rats were suspended in an artificial cerebrospinal fluid (ACSF, in mM: NaCl, 136; $NaHCO_3$, 16.2; KCl, 5.4; NaH_2PO_4, 1.2; $CaCl_2$, 2.2; $MgCl_2$, 1.2; Na_2SO_4, 0.5; glucose, 5.0, adjusted to pH 7.3 by bubbling with an O_2/CO_2 mixture, 95:5, v/v), then transferred to a filter paper and sliced (thickness: 0.3 mm) using a McIlwain tissue chopper. The pooled slices were resuspended in 6.5 ml of ACSF and equally dispersed into 12 thermostated (37 °C) chambers. Tissues in the chambers were then continuously superfused at a flow rate of 0.25 ml/min with the same medium. Following a 20 min washing period, superfusate fractions (1 ml) were collected in polystyrene tubes maintained at 0 °C and immediately divided into aliquots to be kept at − 30 °C until the measurement of their contents in CCK-, CGRP-, and substance P-like materials.

Fifteen fractions (corresponding to 60 min of superfusion) were collected for each experiment. During collection of fractions 3 and 4 (K_1) and 12 and 13 (K_2), the concentration of KCl was increased from 5.4 to 30 mM (while that of NaCl was reduced to 111.4 mM) in order to depolarize the tissues (and trigger the Ca^{2+}-dependent release of the peptides; Mauborgne et al., 1987; Pohl et al., 1989; Benoliel et al., 1992). Compounds to be tested on the release of the peptides were added to the superfusing ACSF at the beginning of fraction 8 up to the end of the experiment. Since, for each peptide, the ratio of K^+-induced overflow during the second depolarization (K_2) over that during the first one (K_1) was constant in the absence of drugs, any change in this ratio in the presence of a given substance could be ascribed to the effect of this particular substance on the Ca^{2+}-dependent release of the peptide (see Mauborgne et al., 1987; Pohl et al., 1989; Benoliel et al., 1992).

2.3.2. Radioimmunoassays of CGRP, substance P- and CCK-like materials

The sensitive radioimmunoassay (RIA) procedure described in detail elsewhere (Pohl et al., 1992) was used to quantify CGRP-like material in 200 μl aliquots of each collected superfusate fraction. The detection limit of the

assay was 1 pg/tube, and 50% displacement of [^{125}I]CGRP bound to the antibodies was obtained with 25 pg of the peptide. Besides rat α-CGRP-(23–37) (cross-reactivity 225%), none of the peptides tested, including CCK-(1–8) S, porcine and human calcitonins, enkephalins and related peptides, dynorphins and related peptides, and substance P, showed a cross-reactivity >0.001%, compared to 100% with synthetic CGRP-(1–37) (Pohl et al., 1992). Substance P-like material was radioimmunoassayed using monoradioiodinated [^{125}I]Tyr^{8}-substance P as the tracer (Arvieu et al., 1996). Briefly, an aliquot (50 μl) of each collected superfusate fraction was mixed with 250 μl of buffer (0.05 M Tris–HCl containing 0.1% bovine serum albumin and 0.1% Triton X 100, pH 7.5) and 100 μl of an anti-substance P antiserum dilution (1:500,000). After 48 h at 4 °C, 100 μl of a [^{125}I]substance P solution (corresponding to 2000–2500 cpm) was added and the incubation proceeded for 24 h. The assay was stopped by adsorbing unbound [^{125}I]substance P onto active dextran T70-coated charcoal (2.5 and 12.5 g/l, respectively, in 10% horse serum). The detection limit of the substance P assay was 0.05 pg/tube, and 50% displacement of [^{125}I]substance P bound to antibodies was obtained with 1 pg of the peptide. The cross-reactivities of substance P-(2–11) and substance P-(3–11) were 2% and 0.4%, compared to 100% for substance P, respectively, whereas shorter C-terminal fragments and N-terminal fragments did not show any cross-reactivity (i.e. less than 0.1%). Similarly, neurokinins A and B were devoid of immunoreactivity (<0.1%) (Arvieu et al., 1996).

Quantification of CCK-like material in 200 μl aliquots of superfusate fractions was made according to Benoliel et al. (1992) with [^{125}I]human gastrin as radiotracer. The detection limit of this RIA was 0.5 pg of CCK-(1–8)S per tube, and half displacement of [^{125}I]gastrin bound to antibodies was obtained with 5 pg of the peptide. Compared to 100% for the latter peptide, cross-reactivity was 233% with nonsulphated CCK-(1–8), 204% with gastrin, 31% with sulphated CCK-7 [CCK-(2–8)S], 26% with nonsulphated CCK-7 [CCK-(2–8)], 25% with CCK-5 [CCK-(4–8)], 11% with CCK-33, 1% with CCK-4 [CCK-(5–8)], and was undetectable with CCK-3 [CCK-(6–8)], rat α-CGRP, substance P, met- and leu-enkephalins (up to 2.5 pg/tube) (Benoliel et al., 1992, 1994).

2.4. Expression of results and calculations

In all experiments, peptide overflow due to the first and second depolarizations corresponded to the total peptide-like material in fractions 3, 4 and 5 (K_1) and 12, 13 and 14 (K_2), respectively. The K_2/K_1 ratio was calculated for each superfusion chamber. Results are expressed as percentage of this ratio in the absence of drug.

Statistical analyses were performed using ANOVA followed by unpaired Student's *t*-test. When the *P* value was higher than 0.05, a difference was considered as being nonsignificant.

3. Results

3.1. Spontaneous outflow and K^+-evoked-overflow of substance P-, CGRP- and CCK-like materials from spinal cord slices

Under the conditions used, i.e. after a 20 min washing period, the spontaneous outflow of the three peptide-like materials from spinal cord slices superfused with normal ACSF remained constant for at least 1 h. Absolute basal values were 1.32 ± 0.12 pg of substance P-like material, 9.61 ± 0.71 pg of CGRP-like material, and 1.84 ± 0.02 pg of CCK-like material per minute (means ± S.E.M., $n = 36$ for each value). These values were not significantly changed by the addition to normal ACSF ($[K^+] = 5.4$ mM) of any of the drugs tested in the present study (data not shown). K^+-induced depolarization triggered a marked overflow of each peptide-like material, which was larger for the first K_1 pulse (substance P-like material: +247 ± 21% over baseline; CGRP-like material: +298 ± 22%; CCK-like material: +346 ± 29%; means ± S.E.M., $n = 54$) than for the second one, K_2, but the ratio of these overflows, K_2/K_1, was remarkably constant in the absence of drugs (Table 1; see also Mauborgne et al., 1987; Pohl et al., 1989; Benoliel et al., 1992, 1994).

3.2. Effects of CPA on the K^+-evoked release of substance P-, CGRP- and CCK-like materials from spinal cord slices

Up to 10 nM, only the overflow of CGRP-like material was significantly affected by the addition of the preferential adenosine A_1 receptor agonist CPA (Jacobson and Van Rhee, 1997; Klotz, 2000) to the ACSF superfusing spinal cord slices. As illustrated in Fig. 1, the K_2/K_1 ratio for this peptide-like material was reduced by −16% ($p < 0.05$) in

Table 1
Concentration-dependent effects of XAC on the K^+-evoked release of CGRP-, substance P-and CCK-like materials from rat spinal cord slices

[XAC] (nM)	K_2/K_1		
	CGRP-like material	Substance P-like material	CCK-like material
None	0.296 ± 0.016	0.467 ± 0.016	0.468 ± 0.037
0.1	0.287 ± 0.017	0.403 ± 0.026	0.466 ± 0.026
1	0.288 ± 0.013	0.412 ± 0.014	0.458 ± 0.018
10	0.280 ± 0.018	0.381 ± 0.013[a]	0.442 ± 0.016
100	0.273 ± 0.008	0.371 ± 0.016[a]	0.503 ± 0.023

Slices of the dorsal half of the lumbar enlargement were depolarized twice (K_1, K_2) by 30 mM K^+ in the course of superfusion with artificial cerebrospinal fluid, and XAC (0.1–100 nM) was added to the superfusing medium only for the second half of the experiment (see Materials and methods). CGRP-, substance P- and CCK-like materials were measured in superfusate fractions, and the ratio K_2/K_1 was calculated for each peptide-like material. Each value is the mean ± S.E.M. of at least nine independent determinations.

[a] $P < 0.05$ when compared to the respective K_2/K_1 value in the absence of XAC (none).

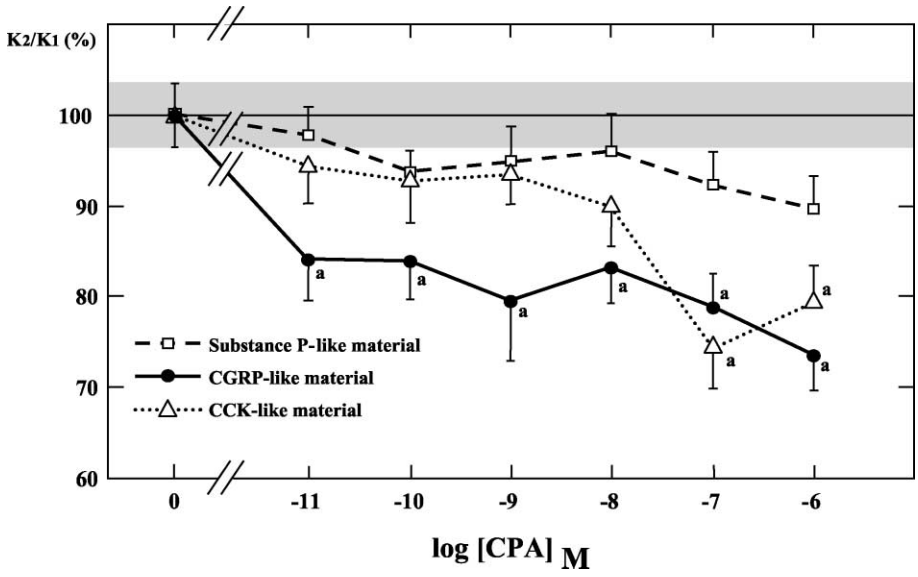

Fig. 1. Concentration-dependent effects of CPA on the K^+-evoked release of substance P-, CGRP- and CCK-like materials from rat spinal cord slices. Slices of the dorsal half of the lumbar enlargement of the spinal cord were depolarized twice (K_1, K_2) by 30 mM K^+ in the course of superfusion with artificial cerebrospinal fluid, and CPA (10 pM–1 μM, abscissa) was added to the superfusing medium only for the second half of the experiment (see Materials and methods). Substance P (□)- CGRP (●)- and CCK (△)- like materials were measured in superfusate fractions, and the ratio K_2/K_1 was calculated for each peptide-like material and expressed as percentage (ordinate) of that determined under control conditions (in the absence of CPA, 0 on abscissa). Each point is the mean ± S.E.M. of at least nine independent determinations. The grey area indicates the range (mean ± S.E.M.) of K_2/K_1 values for each peptide-like material under control conditions (absolute K_2/K_1 values corresponding to 100% were 0.436 ± 0.015 for substance P-like material, 0.286 ± 0.010 for CGRP-like material and 0.494 ± 0.016 for CCK-like material). $^aP < 0.05$ when compared to the K_2/K_1 value in the absence of CPA (0 on abscissa).

the presence of only 10 pM of CPA, and this effect reached up to −27% with 1 μM of the agonist. In contrast, the K^+-evoked overflow of substance P-like material remained unchanged by CPA at concentrations ranging between 10 pM and 1 μM, and that of CCK-like material was affected (−20 to −25%) only by the two highest concentrations tested: 0.1 and 1.0 μM (Fig. 1).

3.3. Effects of NECA and CGS 21680 on the K^+-evoked release of substance P-, CGRP- and CCK-like materials from spinal cord slices

Tissue superfusion with various concentrations of the mixed adenosine A_1, A_{2A}, A_3 receptor agonist NECA (Klotz, 2000) produced no changes in the K_2/K_1 ratio for each peptide-like material up to 10 nM. However, at higher concentrations, NECA exerted an inhibitory influence on the release of the three peptides; for CGRP-like material, this effect reached the critical level of significance ($p < 0.05$) already at 0.1 μM, and increased up to −25% of the control K_2/K_1 value in the presence of 10 μM of the agonist (Fig. 2). The K^+-evoked overflow of CCK-like material and even more that of substance P-like material were much less sensitive to the inhibitory effect of NECA since 1 and 10 μM of the agonist were required to significantly reduce the K^+-evoked release of these peptide-like materials, respectively (Fig. 2).

In contrast to NECA, addition to the superfusing ACSF of the preferential adenosine A_{2A} receptor agonist CGS 21680 (Jarvis et al., 1989; Klotz, 2000), at concentrations ranging between 1 nM and 1 μM, exerted no significant effect on the K^+-evoked overflow of the three peptide-like materials studied (data not shown).

3.4. Effects of APNEA on the K^+-evoked release of substance P-, CGRP- and CCK-like materials from spinal cord slices

As illustrated in Fig. 3, the preferential adenosine A_3 receptor agonist APNEA (Fredholm et al., 1994; Okada et al., 1999) did not affect the K_2/K_1 ratio for both substance P- and CCK-like materials when added at concentrations ranging between 10 pM and 0.1 μM into the superfusing ACSF. In contrast, a significant decrease in CGRP-like material was noted at the highest concentrations tested (−15% at 10 nM and 0.1 μM).

3.5. Prevention by the adenosine receptor antagonist XAC of the inhibitory effect of CPA on the K^+-evoked release of CGRP-like material from spinal cord slices

On its own, the mixed preferential adenosine A_1, A_{2A}, receptor antagonist XAC (Klotz, 2000) did not affect the K^+-evoked overflow of CGRP- and CCK-like materials when added in the range of 0.1–100 nM into the ACSF superfusing spinal cord slices (Table 1). Similarly, the K^+-evoked release of substance P-like material did not significantly change with 0.1 or 1.0 nM of this antagonist. In

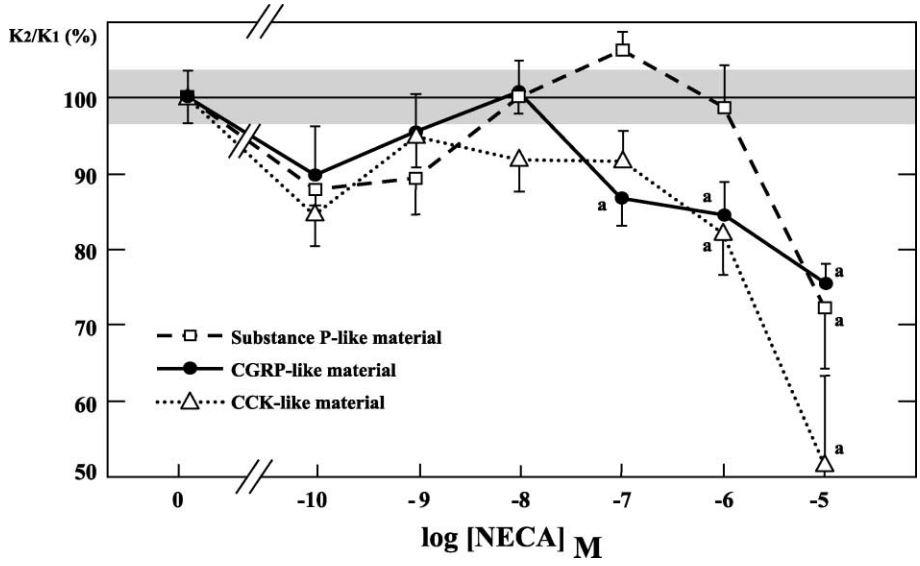

Fig. 2. Concentration-dependent effects of NECA on the K^+-evoked release of substance P-, CGRP- and CCK-like materials from rat spinal cord slices. The same protocol as that described in the legend to Fig. 1 was used, except that NECA (0.1 nM–10 μM) instead of CPA was added into the superfusing fluid for the second half of the experiment. Each point is the mean ± S.E.M. of at least nine independent determinations of K_2/K_1 values expressed as percentages of respective values under control conditions (no NECA, 0 on abscissa). The grey area indicates the range (mean ± S.E.M.) of K_2/K_1 value for each peptide-like material under control conditions (absolute K_2/K_1 values corresponding to 100% were 0.451 ± 0.019 for substance P-like material, 0.297 ± 0.011 for CGRP-like material and 0.457 ± 0.019 for CCK-like material). $^aP<0.05$ when compared to the K_2/K_1 value in the absence of NECA (0 on abscissa).

contrast, at higher concentrations (10 and 100 nM) of this drug, a significant decrease (~ −20%) in the ratio K_2/K_1 for substance P-like material was noted (Table 1).

Further experiments that consisted of superfusing spinal cord slices with or without 10 nM XAC in the presence of CPA at a concentration (0.1 nM) high enough to significantly reduce the overflow of CGRP-like material, showed that the antagonist completely prevented this effect (Fig. 4). On the other hand, like that observed with 0.1 nM CPA alone (Fig. 1), the combination of this agonist plus 10 nM

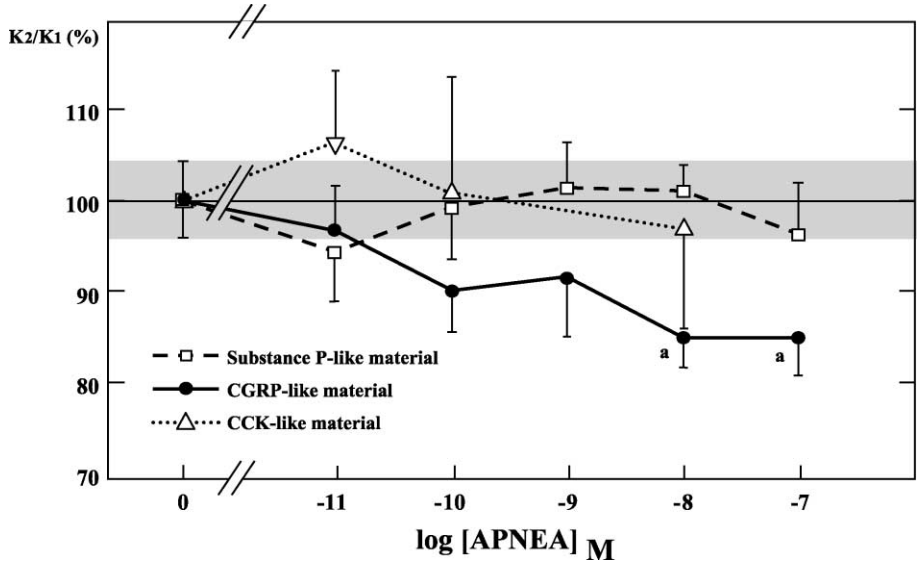

Fig. 3. Concentration-dependent effects of APNEA on the K^+-evoked release of substance P-, CGRP- and CCK-like materials from rat spinal cord slices. The same protocol as that described in the legend to Fig. 1 was used, except that APNEA (10 pM–0.1 μM) instead of CPA was added into the superfusing fluid for the second half of the experiment. Each point is the mean ± S.E.M. of at least eight independent determinations of K_2/K_1 values expressed as percentages of respective values under control conditions (no APNEA, 0 on abscissa). The grey area indicates the range (mean ± S.E.M.) of K_2/K_1 value for each peptide-like material under control conditions (absolute K_2/K_1 values corresponding to 100% were 0.392 ± 0.018 for substance P-like material, 0.279 ± 0.013 for CGRP-like material and 0.442 ± 0.018 for CCK-like material). $^aP<0.05$ when compared to the K_2/K_1 value in the absence of APNEA.

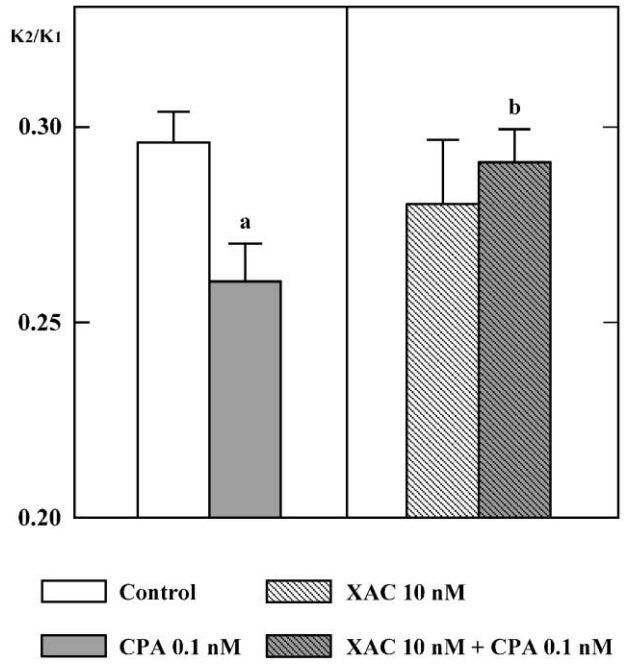

Fig. 4. Prevention by XAC (10 nM) of the inhibitory effect of CPA (0.1 nM) on the K^+-evoked release of CGRP-like material from rat spinal cord slices. The same protocol as that described in the legend to Fig. 1 was used, except that tissues were exposed to either 0.1 nM CPA or 10 nM XAC alone, or to the combination of both drugs. CGRP-like material was measured in superfusate fractions and the K_2/K_1 ratio was calculated for each pharmacological conditions. Each bar is the mean ± S.E.M. of at least 11 independent determinations of respective K_2/K_1 values. [a]$P<0.05$ when compared to control (no drug); [b]$P<0.05$ when compared to CPA alone.

XAC exerted no significant influence on the overflow of both substance P- and CCK-like materials (data not shown).

4. Discussion

Previous studies using the same superfusion conditions as those used herein have shown that the three peptide-like materials measured in superfusates from slices of the dorsal half of the rat spinal cord in fact corresponded almost exclusively to the authentic peptides, i.e. CGRP-like material to CGRP (Pohl et al., 1989), substance P-like material to substance P (Mauborgne et al., 1987), and CCK-like material to CCK (Benoliel et al., 1992, 1994). In addition, thanks to the high sensitivity of respective RIAs, these peptide-like materials could be measured in aliquots of the *same* superfusate fractions, thereby allowing the analysis of possible *simultaneous* changes in their release after application of a given drug onto spinal tissues. These conditions were used here for investigating the effects of various adenosine receptor ligands on the K^+-evoked, Ca^{2+}-dependent, spinal release of CGRP-, substance P- and CCK-like materials, and identifying the adenosine receptors involved. The data obtained mainly indicate that adenosine A_1 receptors play a key role in an adenosine-mediated inhibitory control that affects solely the release of CGRP-like material.

The present study thus confirmed the previous report by Vasko and Ono (1990) claiming that adenosine receptor agonists do not affect the K^+-evoked release of substance P-like material from spinal cord slices. Indeed, neither CPA, a preferential adenosine A_1 receptor agonist, CGS 21680, a preferential adenosine A_{2A} receptor agonist, nor APNEA, a preferential adenosine A_3 receptor agonist (Fredholm et al., 1994; Ralevic and Burnstock, 1998), in a wide range of concentrations, significantly affected the overflow of substance P-like material from spinal cord slices. Among agonists, only NECA, which does not discriminate between adenosine receptor subtypes (Fredholm et al., 1994; Klotz, 2000), exerted an inhibitory influence on the K^+-evoked overflow of this peptide-like material, but only at a high concentration (10 μM). In addition, at the highest concentrations tested, 10 and 100 nM, the mixed adenosine A_1, A_{2A} receptor antagonist XAC was also found to decrease the K^+-evoked release of substance P-like material. The fact that both an agonist and an antagonist produced similar effects would favor the idea that these effects, observed at high concentrations only, were in fact not specific, i.e. not mediated by adenosine receptors. However, further investigations with more selective adenosine receptor ligands are needed to solve this question.

In the ranges of concentrations ensuring preferential stimulation of adenosine A_1, A_{2A} or A_3 receptors, neither CPA (up to 0.1 μM), CGS 21680 (at 1 nM–1 μM), nor APNEA (10 pM–10 nM), respectively, significantly affected the spinal overflow of CCK-like material. Indeed, only high concentrations (1–10 μM) of NECA were found to reduce the spinal release of this peptide, leading to the question of the actual mediation of this effect by adenosine receptors. However, it has to be emphasized that micromolar concentrations of NECA are needed for the stimulation of adenosine A_{2B} receptors (Klotz, 2000), and the latter receptors might therefore possibly contribute to the inhibitory effects of this agonist on the release of both substance P- and CCK-like materials from spinal cord slices. Whether a selective antagonist at the adenosine A_{2B} receptor type can prevent the latter effects is a key question to be addressed as soon as such a drug will be available.

In contrast to that of the former two peptide-like materials, the release of CGRP-like material was significantly affected by relatively low concentrations of some of the adenosine receptor agonists tested. Thus, CPA, NECA and APNEA, but not CGS 21680, were able to decrease the K^+-evoked overflow of this material from spinal cord slices. The respective potencies of agonists at the various receptor types allow us to attribute their effects on the release of CGRP-like material to the stimulation of adenosine A_1, and possibly A_3, receptors. Indeed, CPA, which was shown to reduce the spinal release of the latter peptide-like material at a concentration as low as 10 pM, is 20 and 400 times more potent at adenosine receptors of the A_1 type than at those of the A_3 and A_{2A} types, respec-

tively, and is inactive at the A_{2B} type (Fredholm et al., 1994; Klotz, 2000). Clearly, its agonistic action at adenosine A_{2A} receptors did not probably contribute to its effect on the release of CGRP-like material because the selective stimulation of these receptors by CGS 21680 (Fredholm et al., 1994; Klotz, 2000) exerted no influence on the peptide overflow from spinal tissues. On the other hand, like CPA, but at higher concentrations, both the preferential adenosine A_3 receptor agonist APNEA, and the mixed adenosine $A_1/A_2/A_3$ receptor agonist NECA (Fredholm et al., 1994; Klotz, 2000), also decreased the K^+-evoked overflow of CGRP-like material, thereby suggesting that adenosine A_3 receptors might also participate in an adenosine-mediated inhibitory control of spinal CGRP release. However, the latter receptors are much less abundant than adenosine A_1 receptors in the CNS (Fredholm et al., 1994; Ralevic and Burnstock, 1998), and we presently found that the inhibitory effect of CPA on the spinal release of CGRP-like material could be completely prevented by the adenosine A_1, A_{2A} receptor antagonist XAC, making the hypothesis of the participation of adenosine A_3 receptors in the latter effect rather unlikely. Altogether, these data are congruent with the report of Santicioli et al. (1993) claiming that adenosine A_1 receptor stimulation exerts an inhibitory influence on the release of CGRP from primary afferent fibres.

Because both CGRP and substance P are partly colocalised in the same primary afferent fibres (Lee et al., 1985; Skofitsch and Jakobowitz, 1985; Franco-Cereceda et al., 1987), it is rather surprising that the K^+-evoked release of CGRP-like material but not that of substance P-like material was negatively controlled by adenosine A_1 receptor stimulation. In fact, if virtually all substance P-ergic primary afferent fibres contain CGRP, the reverse is not true (Lee et al., 1985; Franco-Cereceda et al., 1987), and such differences probably account for previous data which already showed that the K^+-evoked overflow of substance P- and CGRP-like materials from spinal cord slices can be differentially affected by drugs (Bourgoin et al., 1993; Collin et al., 1994). In the present case, it can be hypothesized that the CGRP-ergic primary afferent fibres under the negative control of adenosine A_1 receptors are those that do not also contain substance P. Such an action of adenosine receptor agonists on only a restricted contingent of CGRP-containing primary afferent fibres might explain why their inhibitory effect on the release of CGRP-like material remained moderate, even at high, saturating, concentrations.

Because initial studies showed that dorsal rhizotomy did not decrease the specific binding of selective radioligands at adenosine A_1 receptors in the rat spinal cord (Geiger et al., 1984; Choca et al., 1988), it was inferred that these receptors are not located on the terminals of primary afferent fibres within the dorsal horn. Accordingly, as CGRP is contained exclusively in primary afferent fibres within the dorsal part of the spinal cord (Pohl et al., 1990), the inhibitory effect of adenosine receptor agonists on the release of CGRP-like material would have to be mediated indirectly through the stimulation of adenosine A_1 receptors on interneurones exerting a presynaptic inhibitory influence on CGRP-ergic primary afferent fibres. However, more recent in situ hybridization (Reppert et al., 1991) and immunocytochemical (Carruthers et al., 2001) studies did demonstrate the expression of adenosine A_1 receptors by neurons in trigeminal ganglia, which correspond at the cephalic level to dorsal root ganglia for the rest of the body. Furthermore, electrophysiological investigations on rat dorsal root ganglion neurones in culture demonstrated that agonists at adenosine A_1 receptors *directly* inhibit calcium-dependent currents in these cells (Dolphin et al., 1986). Altogether, these results support the idea that the latter receptors do exist on primary afferent fibres, and that their stimulation probably accounted for the negative influence of their agonists on the release of CGRP-like material, like that recently found on rat trigeminal neurones in culture (Carruthers et al., 2001). However, as emphasized above, only a relatively small proportion of primary afferent fibres probably express adenosine A_1 receptors, i.e. those containing CGRP but not substance P. Compared to the bulk of adenosine A_1 receptors which are mainly located on intrinsic neurones (Geiger et al., 1984; Choca et al., 1988), those on CGRP-ergic primary afferent fibres probably represent only a very small fraction, which might explain why the expected loss of specific adenosine A_1 receptor binding sites within the dorsal horn after dorsal rhizotomy could not be detected using quantitative autoradiography (Choca et al., 1988).

A general agreement exists in the literature about the analgesic effects of intrathecally administered adenosine and adenosine receptor agonists in relevant animal models (Sawynok, 1998; Millan, 1999; Sawynok and Poon, 1999). This holds notably for acute (thermal and chemical) as well as chronic (inflammatory and neuropathic) pain. Those effects are generally attributed to the stimulation of spinal adenosine A_1 receptors (Sawynok and Poon, 1999). Indeed, intrathecal administration of selective agonists at these receptors such as CPA and UP 202-56 has been shown to reduce both the responses of dorsal horn neurones to primary afferent C fibre stimulation or subcutaneous injection of formalin (Reeve and Dickenson, 1995) and noxious stimuli-evoked c-Fos expression in these cells (Honoré et al., 1998). Our data indicate that these antinociceptive effects cannot be mediated through some inhibition by adenosine receptor stimulation of the release of CCK, whose pro-nociceptive anti-opioid effects have been extensively characterized at the spinal level (Cesselin, 1995). In addition, some inhibitory influence of adenosine receptor stimulation on nociceptive primary afferent substance P-ergic fibres can also be excluded on the basis of the present and previous (Vasko and Ono, 1990) data. In contrast, because CGRP is a well-established neurotransmitter of nociceptive fibres entering the dorsal horn of the spinal cord (Pohl et al., 1990; Collin et al., 1993; Millan, 1999), the inhibitory effect of adenosine A_1 receptor stimulation on the release of CGRP-like material from such fibres might well contribute to the antinociceptive

effects of intrathecally administered adenosine and related agonists. However, the present findings do not exclude the participation of other mechanisms in the latter effects. In particular, an inhibitory action of adenosine on cytokine expression by activated glial cells can be suspected as several nociceptive cytokines (interleukin-1β, interleukin-6, interleukin-10, tumor necrosis factor) appear to be upregulated in spinal tissues of neuropathic pain suffering rats (Winkelstein et al., 2001), and intrathecal administration of adenosine is especially efficient to reduce neuropathic pain (Belfrage et al., 1999). Assessment of the respective contributions of (i) the presynaptic effect presently observed, (ii) postsynaptic inhibitory action of adenosine receptor stimulation on intrinsic spinal neurones (Geiger et al., 1984; Choca et al., 1988; DeLander and Wahl, 1988) and (iii) possible modulation of glial expression of cytokines to adenosine-mediated antinociception at the spinal level is a key question to be addressed in further investigations.

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