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Authors

Cahill, CM
White, TD
Sawynok, J

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INFLUENCE OF CALCIUM ON THE RELEASE OF ENDOGENOUS ADENOSINE FROM SPINAL CORD SYNAPTOSOMES

Catherine M. Cahill, Thomas D. White and Jana Sawynok

Department of Pharmacology, Dalhousie University
Halifax, Nova Scotia, Canada B3H 4H7

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Summary

Intrathecal administration of Ca^{2+} has been shown to produce antinociception which is thought to be partially mediated by the release of adenosine. In the present study we have examined directly the effects of varying Ca^{2+} concentrations on the release of endogenous adenosine, measured by HPLC with fluorescence detection, from rat spinal cord synaptosomes. Although increasing the concentration of extracellular Ca^{2+} reduces the total amount of adenosine detected extrasynaptosomally, the component derived from the release of adenosine *per se* is actually augmented. This release of adenosine occurs from dorsal but not ventral spinal cord synaptosomes and appears to originate from capsaicin-sensitive nerve terminals. The Ca^{2+} ionophore A23187 also releases adenosine, but this release is due to the extrasynaptosomal conversion of released nucleotide(s) to adenosine, as it is markedly reduced by ecto-5'-nucleotidase inhibitors. Release of adenosine by A23187 occurs from both the dorsal and ventral spinal cord, and is not capsaicin-sensitive. Ethanol, used as a vehicle for the ionophore, releases adenosine which is a mixture of adenosine and nucleotide from both dorsal and ventral spinal cord synaptosomes. These observations provide direct support for behavioural studies which demonstrate that methylxanthines block antinociception produced by intrathecal administration of Ca^{2+} .

Ca^{2+} is known to be important in the modulation of opioid antinociception. A number of studies have demonstrated that intracerebroventricular (i.c.v.) administration of Ca^{2+} and agents which increase Ca^{2+} availability inhibit opioid antinociception. Thus, i.c.v. Ca^{2+} induces hyperalgesia (1, 2, 3) and antagonizes the antinociceptive effects of morphine (1, 4), as do the Ca^{2+} ionophores X-537 A (5), and A23187 (6). Similarly, voltage-sensitive Ca^{2+} channel agonists, following either peripheral (7, 8) or i.c.v. administration (2, 5, 6, 9), antagonize morphine-induced antinociception. Conversely, agents which reduce extracellular Ca^{2+} , such as the Ca^{2+} chelator EGTA, produce antinociception following i.c.v. administration (4, 5, 10). Ca^{2+} channel antagonists have been shown to potentiate the effects of morphine and other opioids (3, 11, 12, 13), as well as to induce antinociception by themselves (14, 15).

Correspondence: Jana Sawynok, Ph.D., Department of Pharmacology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

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In contrast to supraspinal administration, the intrathecal (i.t.) or spinal administration of Ca^{2+} potentiates the spinal antinociceptive action of morphine (16, 17), and at higher doses produces an intrinsic antinociceptive action (16, 17). Conversely, antinociception produced by the i.t. administration of morphine can be blocked by agents which inhibit Ca^{2+} flux such as Ca^{2+} channel antagonists and the Ca^{2+} chelator EGTA (16).

The mechanism by which i.t. administration of Ca^{2+} produces antinociception is not clearly understood. It has been proposed that i.t. Ca^{2+} results in the release of endogenous opioids (16, 18). Spinal release of adenosine also appears to be involved in this action of Ca^{2+} , as i.t. administration of methylxanthine adenosine receptor antagonists inhibits antinociception by Ca^{2+} (17, 18). This adenosine appears to originate from small diameter primary afferent fibres because i.t. pretreatment with capsaicin eliminates the antinociceptive action produced by Ca^{2+} (17), but not that produced by adenosine analogs (19).

In the present study, we examined the release of adenosine from spinal cord synaptosomes in the presence of various Ca^{2+} concentrations and agents which alter Ca^{2+} availability. Manipulations used included increasing the extracellular CaCl_2 concentration in the medium and introducing the Ca^{2+} ionophore A23187 which increases the intracellular Ca^{2+} concentration independently of voltage-sensitive Ca^{2+} channels (20). Low Ca^{2+} conditions were achieved by eliminating Ca^{2+} from the medium and by the addition of the Ca^{2+} chelating agent EGTA. Characterization of the adenosine released by different Ca^{2+} conditions was performed by examining release from the dorsal and ventral spinal cord, the nature of the adenosine released (i.e. adenosine *per se* versus nucleotide which is converted to adenosine extrasynaptosomally by ecto-5'-nucleotidases) and the sensitivity of release to an i.t. pretreatment with the sensory afferent neurotoxin capsaicin.

Methods

Release of adenosine from spinal cord synaptosomes. Male Sprague-Dawley rats (250-300 g, Charles River, Quebec) were used, and synaptosomes were prepared from dorsal and ventral portions of the spinal cord as previously described (21). The crude P2 synaptosomal fraction was suspended in 5 ml Krebs-Henseleit medium (NaCl 111 mM, KCl 4.7 mM, CaCl_2 1.8 mM, MgCl_2 1.2 mM, NaHCO_3 26.2 mM, glucose 11 mM, gassed with 95% O_2 /5% CO_2 to pH 7.4) and incubated at 37°C for 30 minutes. Following centrifugation for 10 min at room temperature, the synaptosomal pellet was resuspended in 5-6 ml Krebs-Henseleit medium to yield a protein concentration ranging from 2-2.5 mg/ml. The synaptosomal suspension (350 μl) was added to microfuge tubes containing the drugs to be investigated and incubated at 37°C for 15 min. In all cases release was terminated by centrifugation (11,600 g for 4 min) followed by deproteination of an aliquot of supernatant with ZnSO_4 and $\text{Ba}(\text{OH})_2$. One tube was immediately centrifuged prior to the 15 min drug incubation to determine the amount of adenosine released during the preparation of the synaptosomes. A second tube was incubated in the absence of drugs, and the difference in values between these was used to determine the basal release of adenosine. The deproteinated supernatant was derivatized by the addition of chloroacetaldehyde and boiled to form an etheno derivative of adenosine which was then quantitated by High Performance Liquid Chromatography (HPLC) with fluorescent detection (22). Adenosine release was expressed as picomoles per milligram of protein per 15 minutes.

When examining the release of adenosine under various Ca^{2+} concentrations and Ca^{2+} free conditions, synaptosomes were prepared in Ca^{2+} free medium and the appropriate concentration of Ca^{2+} (as CaCl_2) or EGTA (1 mM buffered with 1 N NaOH to pH 7.4) added during the incubation step.

Pretreatment with capsaicin. Adult rats (250-300 g) were anaesthetized with halothane and placed in the earbars of a stereotaxic apparatus. Volumes of 20 μ l capsaicin (60 μ g) or vehicle (60% v/v dimethylsulfoxide in saline) were injected into the spinal subarachnoid space via an acutely inserted i.t. catheter which was allowed to remain in place for 10 min after injection prior to removal (23). In our laboratory, this procedure reduced substance P determined immunocytochemically in the dorsal horn of the spinal cord (19). Animals were permitted to recover for 7 to 10 days after surgery before use in neurochemical experiments.

Expression of results. Basal adenosine release over the 15 min incubation period was calculated by subtracting release at 0 min from release in the absence of drugs. The 0 min value was then subtracted from all incubations. When multiple concentrations of Ca^{2+} were examined, a basal adenosine release value was calculated for each dose of Ca^{2+} . Evoked values were then calculated by subtracting the basal value from the total release in the presence of drugs. For determining the evoked release of adenosine by the Ca^{2+} ionophore A23187, the adenosine released by the vehicle (1.02 % v/v ethanol) was subtracted from the total release in the presence of A23187.

Statistics. Comparisons between different treatments on the same synaptosomal suspension were made using the randomized block analysis of variance followed by the Student Newman Keuls post hoc test.

Drugs. Drugs were obtained from the following sources: A23187, EGTA (ethylene glycol-bis(β -aminoethyl ether) N,N,N',N'-tetraacetic acid), chloroacetaldehyde, 5'-AMP, GMP, α,β -methylene ADP and capsaicin (Sigma Chemical Co., St. Louis, MO), absolute ethanol (Consolidated Alcohols Ltd., Toronto, Ontario), dimethylsulfoxide (BDH Chemicals, Toronto, Ontario).

Results

Effects of Ca^{2+} on basal and evoked release of adenosine. The effect of varying Ca^{2+} concentrations on adenosine release from dorsal spinal cord synaptosomes is illustrated in Fig. 1 (panels A and B). In the Ca^{2+} free Krebs-Henseleit medium, adenosine release was significantly increased compared to the total amount of adenosine released in Krebs-Henseleit medium containing 1.8 mM Ca^{2+} . The addition of 1 mM EGTA did not further enhance the total amount of adenosine release compared with that released by the Ca^{2+} free medium. In contrast to the augmented release of adenosine by the Ca^{2+} free condition, higher concentrations of Ca^{2+} depressed the total amount of adenosine released compared to release under normal Ca^{2+} concentrations. Both experiments in Fig. 1 demonstrate that the total amount of adenosine released is increased in the absence of Ca^{2+} , whereas increasing the extracellular Ca^{2+} concentration decreased the total amount of adenosine release.

The ionophore A23187 dose-dependently released adenosine in the presence of 1.8 mM Ca^{2+} (Fig. 2). The ionophore was prepared in a solution of ethanol (final concentration 1.02 % v/v at all concentrations of A23187). This concentration of ethanol produced an intrinsic release of adenosine (see inset Fig. 2), and this was subtracted from the total adenosine released by A23187 to yield the release evoked by the ionophore.

The adenosine released by A23187 and ethanol in the presence of various Ca^{2+} concentrations is presented in Table I. In the absence of Ca^{2+} , adenosine release was increased compared to release by 1.8 mM Ca^{2+} , whereas higher concentrations of Ca^{2+} significantly attenuated the release of adenosine (cf. Fig. 1). Ethanol evoked release was Ca^{2+} -independent. In contrast,

release of adenosine evoked by A23187 was Ca^{2+} -dependent, being significantly reduced in the Ca^{2+} free medium (by 44%) and further diminished in the presence of EGTA (reduced by 90%). Curiously, adenosine release by the Ca^{2+} ionophore was also decreased in the presence of a higher (7.2 mM) extracellular Ca^{2+} concentration.

FIG. 1

Effects of elevated Ca^{2+} concentrations and Ca^{2+} free conditions on the release of adenosine from dorsal spinal cord synaptosomes. Panels A and B represent two sets of experiments which span different ranges of Ca^{2+} -free and high Ca^{2+} conditions. Statistical comparisons are made against release in 1.8 mM Ca^{2+} (normal Krebs-Henseleit Ca^{2+} concentration). * $p < 0.05$, *** $p < 0.001$. Values in this figure and all other figures represent mean adenosine release $\pm$ s.e.m. ($n=6$).

FIG. 2

The effect of the Ca^{2+} ionophore A23187 on the release of adenosine from dorsal spinal cord synaptosomes in the presence of 1.8 mM Ca^{2+} . Statistical analyses are made for ethanol versus basal, and A23187 versus ethanol * $p < 0.05$, *** $p < 0.001$ ($n=6$).

TABLE I

Effects of Ethanol and A23187 on the Release of Adenosine from Dorsal Spinal Cord Synaptosomes in the Presence of Varying Concentrations of Extracellular Ca^{2+} .

	Basal	Ethanol (1.02%)	A23187 (100 μM)
Ca^{2+} -free + EGTA	199.6 $\pm$ 7.7 †††	242.0 $\pm$ 19.9 <i>42.2 $\pm$ 3.4</i>	250.2 $\pm$ 16.3 <i>12.1 $\pm$ 10.7 ***</i>
Ca^{2+} -free	216.7 $\pm$ 13.8 †††	242.7 $\pm$ 12.0 <i>26.6 $\pm$ 5.4</i>	310.0 $\pm$ 17.1 <i>67.7 $\pm$ 3.3 ***</i>
Ca^{2+} 1.8 mM	144.9 $\pm$ 6.3	171.5 $\pm$ 17.4 <i>27.9 $\pm$ 6.3</i>	297.5 $\pm$ 13.1 <i>126.4 $\pm$ 2.2</i>
Ca^{2+} 7.2 mM	105.6 $\pm$ 13.5 †††	136.2 $\pm$ 13.6 <i>30.7 $\pm$ 10.1</i>	195.3 $\pm$ 14.5 <i>59.8 $\pm$ 5.8 ***</i>

Numbers in the body of the table represent total adenosine release under the indicated conditions, while numbers in italics represent the evoked release of adenosine under each condition (pmol/mg protein/15 min). For ethanol, these were obtained by subtracting basal values, while for the ionophore, these were obtained by subtracting the ethanol vehicle value. ††† $p < 0.001$ compared to adenosine release by 1.8 mM Ca^{2+} . *** $p < 0.001$ compared to evoked release of adenosine by 1.8 mM Ca^{2+} ($n=6$).

Characterization of adenosine released from dorsal and ventral spinal cord synaptosomes.

In order to characterize the nature of the adenosine released, synaptosomes were incubated with inhibitors of ecto-5'-nucleotidase (0.5 mM α,β -methylene ADP and 5 mM GMP) which converts 5'-AMP to adenosine. The efficiency of the inhibitors in preventing the conversion of 5'-AMP to adenosine was determined by the addition of a control tube containing 1 μM 5'-AMP. At all Ca^{2+} concentrations, a similar amount of 5'-AMP was converted to adenosine, and inhibition of ecto-5'-nucleotidase was greater than 90% (data not shown). In the absence of Ca^{2+} , adenosine release was increased in both ventral and dorsal synaptosomes compared to release by 1.8 mM Ca^{2+} (Fig. 3). In dorsal spinal cord synaptosomes, increasing concentrations of Ca^{2+} decreased the total adenosine released (cf. Fig. 1 and Table I); this reduction was not observed in synaptosomes prepared from the ventral spinal cord. Release of adenosine *per se* from dorsal spinal cord synaptosomes was increased with increasing concentrations of extracellular Ca^{2+} , and at 18 mM Ca^{2+} , adenosine accounted for approximately 70% of the total adenosine detected (Fig. 3A). In contrast, release of adenosine *per se* from ventral spinal cord synaptosomes did not vary with changes in the extracellular Ca^{2+} concentration (Fig. 3B).

The nature of the purine released by ethanol and A23187 is reported in Table II. Release of adenosine in Ca^{2+} free medium was comparable from both the dorsal and ventral spinal cords (cf. Fig. 3), but all of this release appeared to originate as nucleotide(s) which is (are) converted to adenosine extracellularly as release was no longer observed in the presence of the ecto-5'-nucleotidase inhibitors. Ethanol (1.02%) also released similar amounts of adenosine from dorsal and ventral synaptosomes. The ethanol-evoked release of adenosine from both dorsal and ventral synaptosomes appeared to be a mixture of adenosine *per se* (30-50%) and nucleotide(s) which is (are) converted to adenosine extracellularly. A23187 released similar amounts of adenosine from dorsal and ventral spinal cord synaptosomes, and this release originated primarily as nucleotide (approximately 90%).

FIG. 3

Characterization of adenosine release from dorsal and ventral spinal cord synaptosomes. Bargraph values (open and hatched bars) represent mean total adenosine release. * $p < 0.05$, *** $p < 0.001$ compared to release with 1.8 mM Ca^{2+} . Values in hatched bars represent release of adenosine *per se* (i.e. in the presence of ecto-5'-nucleotidase inhibitors). ** $p < 0.01$, *** $p < 0.001$ are compared to release with 1.8 mM Ca^{2+} . Open columns represent nucleotide component of release ($n=6$).

TABLE II

Release of Adenosine Evoked by Ethanol, A23187 and Ca^{2+} -Free Conditions from both Dorsal and Ventral Spinal Cord Synaptosomes in the Absence and Presence of 5'-Nucleotidase Inhibitors (α, β -methylene ADP 0.5 mM and GMP 5 mM) (pmol/mg prot/15 min).

	Without Inhibitors	With Inhibitors	% Adenosine
Dorsal			
Ca^{2+} -free	155.4 $\pm$ 15.3	-15.2 $\pm$ 6.4	0
1.02 % Ethanol	71.0 $\pm$ 8.1	36.5 $\pm$ 7.4	51
100 μM A23187	147.6 $\pm$ 23.6	20.4 $\pm$ 5.2	13
Ventral			
Ca^{2+} -free	107.0 $\pm$ 12.8	-14.9 $\pm$ 3.9	0
1.02 % Ethanol	61.9 $\pm$ 19.7	18.4 $\pm$ 12.0	29
100 μM A23187	140.7 $\pm$ 16.1	17.5 $\pm$ 2.3	12

Basal values (1.8 mM Ca^{2+}): dorsal = 234 ± 12 and ventral = 249 ± 16 pmol/mg protein/15 min. Conversion of 5'-AMP to adenosine was inhibited by approximately 90% in the presence of inhibitors ($n=5$).

Effect of pretreatment with capsaicin on the release of adenosine. In order to determine whether the adenosine released by Ca^{2+} originates from small diameter primary afferents, release was examined following i.t. pretreatment with capsaicin. While the total release of adenosine by each Ca^{2+} condition was comparable in both the vehicle and capsaicin pretreated groups, the release of adenosine *per se* (hatched columns) was no longer augmented by elevated extracellular Ca^{2+} levels (release was in fact reduced) in the capsaicin pretreated group (Fig.4).

FIG. 4

Effects of intrathecal capsaicin pretreatment on the release of adenosine by different Ca^{2+} concentrations from the dorsal spinal cord. Values represent mean adenosine release $\pm$ s.e.m. expressed as pmol/mg protein/15 min in the absence and presence of ecto-5'-nucleotidase inhibitors. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to release with 1.8 mM Ca^{2+} . Conversion of 5'-AMP to adenosine was inhibited by 90% and this inhibition was independent of the extracellular concentration ($n=6$).

The effect of i.t. capsaicin pretreatment on the evoked release of adenosine by ethanol and A23187 also was examined. There was no difference between the evoked release from the vehicle and capsaicin treated groups for either agent (ethanol: vehicle 54 ± 14 , capsaicin 78 ± 9 pmol/mg protein/15 min; A23187: vehicle 190 ± 19 , capsaicin 164 ± 16 pmol/mg protein/15 min, $n=6$). The adenosine released by ethanol (mixture of adenosine/nucleotide) and A23187 (primarily nucleotide) does not appear to originate from capsaicin-sensitive small diameter primary afferent neurons.

Discussion

The present study was undertaken to determine whether high Ca^{2+} concentrations would result in the release of adenosine from the spinal cord, which could contribute to the antinociceptive actions produced by i.t. administration of Ca^{2+} (17). When Ca^{2+} is administered into the subarachnoid space, it produces an intrinsic antinociceptive action (higher doses) while at lower doses it potentiates the analgesic actions of morphine (16, 17) and noradrenaline (17). Both the intrinsic effect of Ca^{2+} and augmentation of the action of noradrenaline are blocked by i.t. administration of methylxanthines (17). Ca^{2+} is an important mediator of the release of a variety of neurotransmitters, and it seemed likely that the i.t. administration of Ca^{2+} might produce its antinociceptive action by facilitating the release of one or more endogenous neurotransmitters or neuromodulators (e.g. adenosine) involved in eliciting an antinociceptive effect.

This study demonstrates that although the total release of adenosine at the high external Ca^{2+} concentrations was diminished, the release of adenosine *per se* from dorsal spinal cord synaptosomes was enhanced. The absolute amount of adenosine *per se* released by the high Ca^{2+} concentration (~ 90 pmol/mg protein/15 min, Fig. 3) is comparable to that released by morphine (21, 22, 24, 25), and this adenosine release is considered to contribute to spinal antinociception by morphine (26). The enhanced release of adenosine by high extracellular Ca^{2+}

is specific to the dorsal spinal cord and appears to originate from capsaicin-sensitive small diameter primary afferent neurons, findings which are consistent with the observations that adenosine is released from this source by other agents (24).

Behavioral studies have demonstrated that i.t. administration of A23187, a Ca^{2+} ionophore, can also produce antinociception in mice (27), and it has been suggested that A23187 mediates this action by a mechanism similar to that produced by high concentrations of external Ca^{2+} (27). The present study demonstrated that A23187 dose-dependently releases adenosine from spinal cord synaptosomes by a Ca^{2+} -dependent mechanism. This adenosine originates primarily as nucleotide, occurs from both dorsal and ventral spinal cord, and is unaltered by i.t. capsaicin pretreatment. These results indicate that high extracellular Ca^{2+} concentrations and the Ca^{2+} ionophore do not produce identical patterns of adenosine release from the spinal cord. The difference in these neurochemical effects may stem from differences in the mechanism by which these two conditions lead to an increased intracellular Ca^{2+} availability. Thus, following exposure to high external Ca^{2+} concentrations, Ca^{2+} may enter the synaptosome via Ca^{2+} or cation channels, while the ionophore induces Ca^{2+} entry independently of these channels and will bypass mechanisms involved in regulation of Ca^{2+} entry via such channels (20). In view of the difference in characteristics of release of adenosine by the ionophore, adenosine release may not account for antinociception by A23187 as behavioural relevance of adenosine release requires a capsaicin-sensitive form of release from the dorsal spinal cord (cf. morphine, serotonin, 24, 28).

Recently, the release of adenosine from the spinal cord by a number of agents such as K^+ , capsaicin, serotonin and morphine (21, 22, 24, 28) has been shown to be dependent on the extracellular Ca^{2+} concentration. Thus, release is markedly attenuated when synaptosomes are incubated in a Ca^{2+} free medium containing EGTA. However, exposure of spinal cord synaptosomes to the Ca^{2+} free medium results in an increased level of basal adenosine compared to release in the presence of Ca^{2+} (Figs 1,3 and 4, cf. 22, 24). A similar increase in endogenous adenosine release by Ca^{2+} free conditions has also been shown for brain synaptosomes (29, 30, 31), and brain slices (32). This report demonstrates that essentially all of the adenosine released from spinal cord synaptosomes by Ca^{2+} free conditions originates as nucleotide (Table II). The nature of the adenosine released from synaptosomes by low Ca^{2+} conditions has not previously been examined. This increased release of nucleotide occurs from synaptosomes prepared from both the dorsal and ventral spinal cord in comparable quantities. Moreover, i.t. pretreatment with capsaicin had no effect on the enhanced release of nucleotide(s) produced by the Ca^{2+} free medium, indicating that the nucleotide(s) probably does not originate from small diameter primary afferent neurons. The nature of the nucleotide(s) released is not known, but it should be noted that the nucleotide(s) may well differ from that released by capsaicin and 5-HT, as in these cases release does originate from capsaicin-sensitive neurons (24, 28). It is possible that release of nucleotide in Ca^{2+} -free conditions results from a redistribution of intracellular Ca^{2+} stores, or that Ca^{2+} -free conditions increase sensitivity of Na^+ channels to voltage changes so that small depolarizations can increase axonal excitability (33) and promote the release of some neuroactive active substances (34).

The effect of ethanol (1.02%) on adenosine release also was examined in this series of experiments, as ethanol was used as a solvent for the Ca^{2+} ionophore. This study demonstrates that ethanol releases adenosine from spinal cord synaptosomes, confirming a previous report (25). The adenosine released by ethanol originates in approximately equal proportions as nucleotide and adenosine *per se* (c.f. 25). Ethanol releases adenosine from both dorsal and ventral spinal cord synaptosomes and is unaffected by i.t. pretreatment of capsaicin, indicating that it might be a relatively non-specific effect. Ethanol previously has been shown to release

adenosine from cerebellar synaptosomes (35), S49 lymphoma cells (36) and hepatocytes (37), and this has been attributed to an inhibition of the uptake of adenosine by interaction with a nucleoside carrier (35, 36, 37). In support of this, dipyridamole, a nucleoside transport inhibitor, produces a similar augmentation of adenosine release from this preparation (38). The increase in the release of adenosine from the spinal cord produced by ethanol in the present study may result from inhibition of reuptake of that adenosine generated extrasynaptosomally from nucleotide(s) released under basal conditions.

In summary, high concentrations of extracellular Ca^{2+} release adenosine *per se* from capsaicin-sensitive primary afferents in the dorsal spinal cord. The Ca^{2+} ionophore A23187 also releases adenosine but this is derived from a released nucleotide, occurs from ventral as well as dorsal spinal cord synaptosomes and does not appear to originate from capsaicin-sensitive afferents. Removal of Ca^{2+} from the medium induces a non-specific release of nucleotide which is not capsaicin-sensitive. These results support the hypothesis that the i.t. administration of high Ca^{2+} concentrations releases adenosine, and that this adenosine may contribute to spinal antinociception.

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