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European Journal of Pharmacology 481 (2003) 197–201
www.elsevier.com/locate/ejphar

Effect of drugs active at adenosine receptors upon chronic stress-induced hyperalgesia in rats

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Received 17 September 2003; accepted 23 September 2003

Abstract

Hyperalgesia and altered activities of enzymes involved in nucleotide hydrolysis are observed after exposure to repeated restraint in rats. Here, we investigated the effect of an adenosine A_1 receptor agonist, N^6-cyclopentyladenosine (CPA, 3.35 mg/kg, i.p.), adenosine A_1 receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 0.8 mg/kg, i.p.) as well the effect of an adenosine reuptake blocker, dipyridamole (5 mg/kg, i.p.), on nociception in chronically stressed and control rats. We repeatedly submitted rats to restraint for 40 days. Nociception was assessed with a tail-flick apparatus. The control group presented increased tail-flick latencies after administration of CPA and dipyridamole, but this effect was not observed in the stressed group. DPCPX by itself had no effect on nociception. The analgesic effect of CPA and dipyridamole observed in the control group was reverted by DPCPX. These results indicate the involvement of adenosine A_1 receptor in the antinociception observed in control animals and suggest that the pain signaling induced by chronic stress presents a different modulation involving the adenosinergic system.

Keywords: Hyperalgesia; Chronic stress; Adenosine; CPA; N6-cyclopentyladenosine; Dipyridamole; Tail-flick

1. Introduction

Acute exposure to a variety of stressors produces immediate analgesia in several pain tests (Watkins and Mayer, 1986; Bodnar, 1986). In addition, some studies have reported that, under some experimental conditions, both acute and chronic stress can elicit hyperalgesia instead of analgesia (Satoh et al., 1992; Quintero et al., 2000). Previous data from our laboratory showed decreased pain thresholds after exposure to repeated restraint stress in male rats (Gamaro et al., 1998; Torres et al., 2001a). Unlike stress-induced analgesia, the mechanisms involved in the stress-induced hyperalgesia are less known.

Adenosine is one of the main neuromodulators associated with cell stress (Cunha, 2001). Indeed, it is known that expression of adenosine A_1 receptors is increased by glucocorticoids (Svenningsson and Fredholm, 1997). Increase in extracellular adenosine concentration has been observed following stressful challenges (Latini and Pedata, 2001), including exposure to inescapable shock (Minor et al., 2001). Extracellular adenosine can be released as such through bidirectional non-concentrative adenosine transporters (Cass et al., 1998) or can originate from the extracellular catabolism of released ATP through the ecto-nucleotidase pathway (Cunha, 2001; Zimmermann, 1996). ATPase, ADPase and 5′ -nucleotidase activities in the blood serum were increased by acute restraint stress (Böhmer et al., in press). A previous work showed that chronically stressed male rats exhibit a decreased ADP hydrolysis in synaptosomes from the spinal cord (Torres

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doi:10.1016/j.ejphar.2003.09.045

et al., 2002a), with no effect when acute stress was utilized. Additionally, we also showed that only hydrolysis of ADP was altered in the blood serum of chronically stressed male rats (Torres et al., 2002b). In contrast, when the animals were submitted to acute stress, opposite effect was observed, i.e., an increase in the nucleotidase pathway in rat serum (Böhmer et al., in press), which agrees with previous studies using acute stress models (see reviews of Cunha, 2001; Latini and Pedata, 2001). Chronically stressed animals do not experience all the hormonal consequences that animals exposed to one single stress episode (Hashiguchi et al., 1997; Torres et al., 2001b), and the phenomenon of adaptation to chronic stress may be reflected in several biochemical and physiological processes, including those regulating adenosine availability.

Extracellular nucleotides can be hydrolyzed by a variety of enzymes that are located on the surface, or may be soluble in the interstitial medium or within body fluids (Zimmermann, 2001). Members of several families of ecto-nucleotidases can contribute to the extracellular hydrolysis of nucleotides. Nucleoside 5′-tri- and diphosphates (NTP and NDP) may be hydrolyzed by members of the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family, ectonucleoside pyrophosphatase/phosphodiesterase (E-NPP) family and by alkaline phosphatases (Zimmermann, 2001). These ecto-nucleotidases, together with 5′-nucleotidase, control the availability of ligands (ATP, ADP, AMP and adenosine) for both nucleotide and nucleoside receptors and, consequently, the duration and extent of receptor activation (Chen and Guidotti, 2001). Therefore, this cascade formed by ecto-nucleotidases and 5′-nucleotidase is an enzymatic pathway with a double function of removing a signal of ATP and generating a second one, adenosine. These enzymes may also have a protective function by keeping extracellular ATP/ADP and adenosine levels within physiological conditions (Agteresch et al., 1999).

It has been proposed that extracellular adenosine is involved in physiological pain control at the spinal cord level and in opioid antinociception (Sawynok and Liu, 2003). Animal studies have demonstrated adenosine-mediated inhibitory influences on presumed nociceptive reflex responses (Sawynok, 1998), possibly through the adenosine A_1 receptors (Keil and DeLander, 1996). Adenosine analogs have antinociceptive properties in experimental and clinical situations, including neuropathic pain, where pain-signaling mechanisms have been altered (Sawynok, 1998; Jarvis and Kowaluk, 2001).

In this study, we investigated the effect of administration of an adenosine A_1 receptor agonist, N^6-cyclopentyladenosine (CPA) and antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), as well the blockade of adenosine uptake with dypiridamole on chronic stress-induced hyperalgesia in rats, to explore the role of adenosine in this process.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (200–230 g) from our breeding stock were used. Experimentally naive animals were housed in groups of five in home cages. They were maintained on a standard 12-h dark/light cycle (lights on 7:00 a.m.) at room temperature (22 ± 2 °C). The rats had free access to food and water, except during the period of exposure to the stressor. The Institutional Research Committee approved all animal procedures, and measures were taken to minimize pain and discomfort.

2.2. Chronic restraint stress procedure

The animals were stressed by restraint 1 h daily, 5 days/week for 40 days (Ely et al., 1997). Restraint was carried out by placing the animal in a 25 × 7-cm plastic tube and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1-cm hole in the far end for breathing. The control group was not submitted to stress and the animals were kept in their home cages. The immobilization procedure was always performed between 10:00 a.m. and 1:00 p.m.

2.3. Tail-flick measurement

Nociception was assessed with the tail-flick apparatus (D'Amour and Smith, 1941). Rats were wrapped in a towel and placed on the apparatus. The light source positioned below the tail was focused on a point 2–3 cm rostral at the tip of the tail. Deflection of the tail activated a photocell and automatically terminated the trial. The tail-flick latency represented the period of time (s) from the beginning of the trial to the tail deflection. Light intensity was adjusted so to obtain baseline tail-flick latencies of 3–4 s (0.7 mA). A cut-off time of 10 s was used to prevent tissue damage. After the last session of the treatment (40 days) and 24 h before injection of the drugs, the animals were exposed to the tail-flick apparatus to familiarize them with the procedure, since the novelty of the apparatus can itself induce antinociception (Netto et al., 1987).

2.4. Drugs administration

The drugs used were CPA (3.35 mg/kg), DPCPX (0.8 mg/kg) and dipyridamole (5 mg/kg). CPA was dissolved in 0.9% NaCl, DPCPX in 5% dymethyl sulfoxide + 1.25% NaOH 1 M and dipyridamole was dissolved in 0.9% NaCl, pH 4.0. All drugs were administered i.p. in a volume of 1.0 ml/kg, 24 h after last session of stress. Tail-flick latencies were measured before (basal measure), at 30 and 60 min after the injection, depending on the drug tested. The protocol of associated administration of

Table 1
Nociceptive response to CPA (3.35 mg/kg) or vehicle after chronic stress

Drugs	Time	Control group	Stressed group
	Basal measure (s)	4.22 ± 1.92 (22)	2.93 ± 1.18 (28)[a]
Vehicle	30 min (Δ)	− 0.80 ± 0.63 (11)	0.45 ± 0.14 (14)
	60 min (Δ)	− 1.67 ± 0.54 (11)	0.20 ± 0.23 (14)
CPA	30 min (Δ)	0.92 ± 1.14 (11)	0.05 ± 0.28 (14)
(3.35 mg/kg)	60 min (Δ)	2.72 ± 1.40[b] (11)	0.29 ± 0.39 (14)

Values are mean ± S.E.M. Number of animals per group in parenthesis. Δ(s)=[post-drug latency (30 or 60 min) − pre-drug latency (basal measure)].
[a] Significant difference compared to the control group (Student's *t*-test, $P = 0.009$).
[b] Significant difference compared to the latency of the respective vehicle group (Student's *t*-test, $P < 0.02$).

CPA or dipyridamole plus DPCPX consists of a first injection of CPA or dipyridamole, immediately followed by an injection of DPCPX, at similar doses previously tested.

2.5. Statistical analysis

Data were expressed as mean ± S.E.M. of Δ(s)=[post-drug latency (30 or 60 min) − pre-drug latency (basal measure)]. Basal measure was expressed in seconds. Statistical significance was determined by Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

In all experiments using control and stressed animals, the basal measures were compared and this revealed a significant difference between the groups. The stressed group showed a hyperalgesic effect in all experiments (Student's *t*-test, $P < 0.05$; (Tables 1, 2 and 4)).

3.1. Effect of CPA and DPCPX on the tail-flick test

CPA (3.35 mg/kg, i.p.) produced a significant analgesic effect in the control group at 60-min group after the drug administration (Student's *t*-test, $P < 0.02$). CPA had no significant effect upon nociception at any time after the drug administration (30 and 60 min) in stressed rats (Student's *t*-test, $P > 0.05$). Results are presented in Table 1.

DPCPX (0.8 mg/kg), at 30 and 60 min, did not promote significant changes in the nociception in the tail-flick test for the control and stressed group (Student's *t*-test, $P > 0.05$; Table 2).

To confirm the involvement of adenosine A_1 receptors in the analgesic action of CPA, we tested the associated administration of CPA plus DPCPX in the control group. CPA plus DPCPX did not show effects in the tail-flick test, suggesting that DPCPX reverted the analgesic effect induced by CPA in the control rats (Student's *t*-test, $P > 0.05$; Table 3).

Table 2
Nociceptive response to DPCPX (0.8 mg/kg) or saline after chronic stress

Drugs	Time	Control group	Stressed group
	Basal (s)	5.99 ± 1.75 (16)	4.15 ± 1.21[a] (14)
Vehicle	30 min (Δ)	0.57 ± 0.43 (10)	3.42 ± 1.15 (6)
	60 min (Δ)	0.41 ± 0.58 (10)	0.20 ± 0.23 (6)
DPCPX	30 min (Δ)	0.26 ± 0.97 (6)	0.05 ± 0.28 (8)
(0.8 mg/kg)	60 min (Δ)	0.40 ± 0.60 (6)	0.29 ± 0.39 (8)

Values are mean ± S.E.M. Number of animals per group in parenthesis. Δ(s)=[post-drug latency (30 or 60 min) − pre-drug latency (basal measure)].
[a] Significant difference compared to the control group (Student's *t*-test, $P = 0.003$).

Table 3
Nociceptive response to CPA (3.35 mg/kg) + DPCPX (0.8 mg/kg) or saline + vehicle in control group

Drugs	Time	Control Group
Saline + vehicle	30 min (Δ)	0.03 ± 0.61 (8)
	60 min (Δ)	0.78 ± 0.77 (8)
CPA (3.35 mg/kg) +	30 min (Δ)	0.52 ± 0.59 (10)
DPCPX (0.8 mg/kg)	60 min (Δ)	− 0.57 ± 0.74 (10)

Values are mean ± S.E.M. Number of animals per group in parenthesis. Δ(s)=[post-drug latency (30 or 60 min) − pre-drug latency (basal measure)].

3.2. Effect of dipyridamole on the tail-flick test

Administration of dipyridamole (5 mg/kg, i.p.) produced an analgesic effect at 30 min (Student's *t*-test, $P < 0.0001$) and 60 min (Student's *t*-test, $P < 0.05$) after drug administration in the control group. Dipyridamole had no significant effect upon nociception at any time after drug administration (30 and 60 min) in stressed rats (Student's *t*-test, $P > 0.05$). Results are presented in Table 4.

To confirm the involvement of adenosine in the analgesic action of dipyridamole, we tested the associated administration of dipyridamole plus DPCPX in the control group. Dipyridamole plus DPCPX did not show effects in the tail-flick test, suggesting that DPCPX reverted the analgesic

Table 4
Nociceptive response to dipyridamole (5 mg/kg) or saline after chronic stress

Drugs	Time	Control	Stressed
	Basal (s)	4.54 ± 2.42 (22)	3.36 ± 1.46[a] (28)
Vehicle	30 min (Δ)	− 0.42 ± 0.43 (13)	0.71 ± 0.39 (13)
	60 min (Δ)	0.78 ± 0.43 (13)	1.24 ± 0.46 (13)
Dipyridamole	30 min (Δ)	3.25 ± 0.69[b] (9)	0.68 ± 0.43 (15)
(5 mg/kg)	60 min (Δ)	2.97 ± 0.90[b] (9)	0.69 ± 0.62 (15)

Values are mean ± S.E.M. Number of animals per group in parenthesis. Δ(s)=[post-drug latency (30 or 60 min) − pre-drug latency (basal measure)].
[a] Significant difference compared to the control group (Student's *t*-test, $P < 0.05$).
[b] Significant difference compared latency of the respective vehicle group (30 min: Student's *t*-test, $P = 0.0001$; 60 min: Student's *t*-test, $P < 0.05$).

Table 5
Nociceptive response to dipyridamole (5 mg/kg) + DPCPX (0.8 mg/kg) or saline + vehicle in control group

Drugs	Time	Control Group
Saline ± vehicle	30 min (Δ)	− 0.05 ± 0.52 (6)
	60 min (Δ)	− 0.47 ± 0.87 (6)
Dipyridamole (5 mg/kg) ± DPCPX (0.8 mg/kg)	30 min (Δ)	− 1.61 ± 0.7 (6)
	60 min (Δ)	− 0.70 ± 0.77 (6)

Values are mean ± S.E.M. Number of animals per group in parenthesis. Δ(s)=[post-drug latency (30 or 60 min) − pre-drug latency (basal measure)].

effect induced by dipyridamole in the control rats (Student's *t*-test, $P > 0.05$; Table 5).

4. Discussion

In this study, we observed an analgesic effect of CPA and dipyridamole in control animals, but no effect in chronically stressed rats. Additionally, the effect of CPA and the effect of dipyridamole on nociception in control rats were prevented by DPCPX, an antagonist of A_1 receptors, suggesting that the antinociception induced by CPA or dipyridamole is mediated through these receptors. The absence of effect observed on chronically stressed animals indicates a different modulation of pain signaling induced by chronic stress involving the adenosinergic system.

Previous studies have demonstrated that the manipulation of endogenous adenosine levels induces antinociception in the mouse tail-flick test (Keil and DeLander, 1994). The rat tail-flick test used in the present study involves a spinal nociceptive reflex, and it is thus suitable for studying the adenosinergic influence. In addition, i.t. administration of adenosine receptor antagonists induces thermal hyperalgesia in the tail-flick test under certain conditions (Sawynok et al., 1986). These results indicated that an endogenous purinergic system might be active at spinal sites modulating nociceptive neurotransmission. Facilitation of this system would be expected to induce antinociception, whereas its inhibition would result in facilitated nociceptive neurotransmission.

Chronically stressed male rats exhibit a decreased tail-flick latency just after an exposure to restraint, indicating a hyperalgesic response (Gamaro et al., 1998). Spinal systems, including opioid and adenosine purinergic systems, modulate nociceptive neurotransmission in the dorsal horn (Yaksh and Malmberg, 1994). Furthermore, evidence indicates that chronic restraint stress induces a decrease in the sensitivity to morphine (Torres et al., 2003). Several studies support the hypothesis that adenosine is involved in opioid-induced antinociception (Sawynok and Liu, 2003). In this study, whilst CPA, an adenosine A_1 receptor agonist, induced an analgesic response in the control group, no effect was observed when it was administrated in chronically stressed animals. It is important to consider that the drugs tested were administered intraperitoneally, being possible a partial contribution of peripheral adenosine A_1 receptors in the analgesic effects observed. Our results suggest that alterations in adenosine A_1 receptors might be involved in the hyperalgesia observed in stressed rats.

Nucleoside transport process may play a role in regulating endogenous levels of the adenosine in the central nervous system. Equilibrative nucleoside transporters (ENT) carry nucleosides across cell membranes in either direction according to their concentration gradients. Two ENT subtypes accepting both purine and pyrimidine nucleosides as well as a number of synthetic nucleoside analogs have been cloned and termed ENT1 and ENT2. ENT1 and ENT2, and present a wide cellular and regional distribution in rat and human brain (Anderson et al., 1999a,b). ENT1 is differentiated from ENT2 by its sensitivity to inhibition by nanomolar concentrations of the nucleoside analog nitrobenzylthioinosine, but both are inhibited by dipyridamole (Cass et al., 1998). The nucleoside transporter inhibition can significantly increase basal extracellular adenosine concentrations, probably due to inhibition of nucleotide-derived adenosine reuptake. Inhibition of this uptake could cause greater synaptic adenosine levels and subsequently increased activation of extracellular adenosine receptors, and these effects can induce antinociception (Sweeney et al., 1993). Furthermore, morphine has been demonstrated to release adenosine per se from primary afferent neurons, mediated by nucleoside transporters, sensitive to the inhibitor dipyridamole but insensitive to nitrobenzylthioinosine (Sweeney et al., 1993). In agreement with previous studies (Zarrindast et al., 1993), the administration of nucleoside transport inhibitors induced an antinociceptive effect in naive rats. On the other hand, this effect was not observed in stressed rats. Extracellular ADP hydrolysis has been demonstrated to decrease in stressed rats (Torres et al., 2002a,b), which could induce decreased levels of extracellular adenosine. According to the present results, inhibition of adenosine reuptake by dipyridamole in stressed rats is not enough to compensate this possible reduction of extracellular adenosine levels. Previous studies from our laboratory have demonstrated that acute stress induces an increase in the nucleotidase pathway in rat serum (Böhmer et al., in press). Repetitive exposure to restraint stress could induce an adaptative response in chronically stressed animals, which could lead to a desensitization of adenosine receptors. Previous work showed that mice lacking the adenosine A_1 receptor showed hyperalgesic responses (Johansson et al., 2001). Therefore, the lack of effect of the drugs tested in the stressed animals can be due to: (1) decreased effectiveness of adenosine A_1 receptors or (2) stress-induced augmentation of the extracellular levels of adenosine that would saturate adenosine A_1 receptors (Cunha, 2001; Latini and Pedata, 2001). These results further support the hypothesis that adenosinergic modulation is altered in chronically stressed animals.

In summary, we demonstrated an absence of the adenosine antinociceptive effect in chronically stressed rats. Future

studies concerning the mechanisms of stress-induced hyperalgesia may be relevant for the research into the etiology of chronic pain disorders.

Acknowledgements

This work was supported by National Research Council of Brazil (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior from Brazil (CAPES). ILS Torres was recipient of CAPES fellowship.

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