Spinal Cord Adenosine Receptor Stimulation in Rats Inhibits Peripheral Neutrophil Accumulation

The Role of \(N\)-methyl-\(D\)-aspartate Receptors

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Abstract

The effect of spinal adenosine receptor ligation on peripheral leukocyte accumulation was studied in two rat models of inflammation. Neutrophil infiltration into dermal inflammatory sites was significantly reduced by adenosine \(A_1\) receptor agonists injected through intrathecal catheters. These effects were mimicked by \((\pm)\)-2-amino-5-phosphonopentanoic acid (AP-5), a glutamate NMDA receptor antagonist. Peripheral adenosine levels, as measured in air pouch exudates, decreased markedly in inflamed pouches but remained near normal after intrathecal treatment with AP-5. Moreover, the antiinflammatory effects of intrathecal \(A_2\) receptor agonists and AP-5 were reversed by an adenosine \(A_2\) receptor antagonist administered intraperitoneally. Hence, central NMDA receptor activity can regulate neutrophil accumulation in peripheral inflammatory sites by reducing local levels of adenosine, an antiinflammatory autacoid which inhibits neutrophil function through \(A_2\) receptor activation. This represents a previously unknown pathway by which the central nervous system influences inflammatory responses. (J. Clin. Invest. 1996. 98:2779–2785.) Key words: spinal cord • neutrophil • inflammation • glutamate • adenosine

Introduction

Acute peripheral inflammation is associated with a series of well-defined events in the spinal cord, including release of the excitatory amino acid glutamate and aspartate (1) after afferent C-fiber activation. In addition, spinal cord neuron responses to innocuous stimuli are enhanced by peripheral inflammation, and the receptive fields of these neurons become enlarged (2). This hyperexcitability of neurons, as well as the resulting hyperalgesia, is dependent on activation of glutamate receptors of the N-methyl-D-aspartate (NMDA)\(^1\) subtype, and is blocked by intrathecal administration of NMDA receptor antagonists (3–6). While the effects of peripheral noxious stimulation on spinal cord events and nociception are well documentated, there is little or no information the reverse situation, i.e., how spinal cord events can, in turn, modulate the peripheral inflammatory response itself.

To investigate whether the spinal cord can regulate peripheral inflammation, we studied the effect of several agents administered intrathecally (i.t.) in two rat models of peripheral inflammation. These experiments focused on the role of two potential mediators. Glutamate receptor stimulation was examined due to its aforementioned role in nociception. We also studied the role of adenosine as a central antiinflammatory agent, since the hyperalgesia induced by noxious stimuli, such as inflammation, can be inhibited by adenosine \(A_1\) receptor stimulation (7). This is thought to be achieved in part through inhibition of glutamate and aspartate release (8, 9). Furthermore, adenosine raises the NMDA receptor activation threshold, leading to a lower sensitivity of the receptor (reviewed in reference 10).

Our studies demonstrate that central \(A_1\) adenosine receptor stimulation suppresses neutrophil accumulation in dermal inflammation. This is mediated through deactivation of the NMDA receptor signalling pathway, which in turn, modulates tissue levels of adenosine at inflamed peripheral sites. Enhanced local endogenous adenosine release inhibits neutrophil influx by activating the \(A_2\) receptor on neutrophils. These surprising results represent the first demonstration that events in the spinal cord can modify neutrophil migration at peripheral sites.

Methods

Reagents. Adenosine and glutamate receptor agonists and antagonists were purchased from Research Biochemical International (Natick, MA). They included the nonselective adenosine agonist \(N\)-ethylcarboxyadenosine (NECA) and CGS-21680 which are agonists to \(A_1\) and \(A_2\), respectively; the adenosine \(A_2\) antagonist 1,3-dimethyl-7-propargylxanthine (DMPX); the glutamate agonist NMDA; and antagonists to the \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) (d-\(\gamma\)-glutamyl-ammoniumethanesulfonic acid, GAMS), AMPA/kainate (6-cyano-7-nitroquinoxaline-2,3-dione, CNQX), metabotropic (L(+)-2-amino-3-phosphonopropionic acid, AP-3), and NMDA ((\(\pm\))-2-amino-5-phosphonopentanoic acid, AP-5) classes of glutamate receptors. CNQX was obtained from Gensia, Inc., San Diego, California 92121.

\(^1\) Abbreviations used in this paper: AMPA, \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; AP-3, L(+)-2-amino-3-phosphonopropionic acid; AP-5, (\(\pm\))-2-amino-5-phosphonopentanoic acid; CHA, cyclohexyladenosine; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DMPX, 1,3-dimethyl-7-propargylxanthine; GAMS, d-\(\gamma\)-glutamyl-ammoniumethanesulfonic acid; i.t., intrathecal; MPO, myeloperoxidase; NECA, \(N\)-ethylcarboxyadenosine; NMDA, \(N\)-methyl-\(D\)-aspartate; ZAP, zymosan-activated plasma.
as a hydroxypropyl-cyclodextrin complex to enhance solubility. All other reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Inflammatory stimuli included 1% carrageenan (Sigma Chemical Co.) in PBS, or rat plasma treated with zymosan as a source of activated complement (zymosan-activated plasma, ZAP) (11). Additionally, a reverse passive Arthus reaction was induced by an intravenous injection of BSA followed immediately by an intradermal (i.d.) injection of a rabbit antiserum against BSA (Sigma Chemical Co.), as previously described in (12).

Animals. All animals were handled in accordance with USDA guidelines. Male rats of type SA (Simonsen Laboratories, Gilroy, CA) or type CD (Charles River Laboratories, Wilmington, MA) were housed for at least 1 wk before use. Halothane inhalation anesthesia was used for all surgical procedures and injections. All animals were implanted with an intrathecal catheter placed in the spinal subarachnoid space using a modification of the method described in reference 13. After a 6-d recovery period, all animals except those that appeared emaciated, dehydrated, or had motor abnormalities (< 5% of the total number cannulated) were used for experiments. For intrathecal administration, 10 μl of drug or vehicle followed by 10 μl of PBS was injected through the catheter. This was immediately followed by the injection of inflammatory stimuli into skin lesions or a preformed air pouch (see below).

Skin lesion model. Catheterized male SA rats were briefly anesthetized, and the caudal dorsal area was shaved and injected intradermally with 100 μl of inflammatory stimuli or PBS. Each rat received up to three intradermal injections. 1–8 h later, the injection sites were biopsied and homogenized in 0.5% mixed alkal methylammonium bromide, and levels of myeloperoxidase (MPO) were quantified in homogenates as in reference 12 as an indication of neutrophil accumulation. In general, the concentration of MPO in carrageenan-injected skin was 100–150 U/gram.

Air pouch model. Catheterized male CD rats were lightly anesthetized and 3 ml of sterile filtered air was injected subcutaneously into the back, forming a pocket. This procedure was repeated 2 and 4 d later to form a mature, stable pouch (14). After intrathecal drug administration, 3 ml of carrageenan or PBS was injected into each pouch. After 4 h, the animals were killed and 3 ml of sterile filtered air was injected subcutaneously into the pouch adenosine level was usually 400–800 nM after injection of 6 ml of fluid over the course of the experiment.

Results

Both NECA, a nonselective adenosine receptor agonist, and CHA, a selective adenosine A2 receptor agonist (16), inhibited dermal neutrophil accumulation in response to carrageenan in a dose-dependent fashion when administered intrathecally (Fig. 1). Significant effects were seen at 3–5 μg/kg, and near complete inhibition was obtained at 30 μg/kg. On the other hand, the A2 selective receptor agonist CGS-21680 (17) did not affect neutrophil infiltration even at the highest dose tested, 100 μg/kg (Fig. 1). Neither NECA nor CHA had any effect on neutrophil accumulation when administered intraperitoneally at 30 μg/kg (NECA, 103±16%; CHA, 106±4% of vehicle).
The effect of intrathecal NECA persisted for at least 5 h after carrageenan injection (Fig. 2). The inhibition of neutrophil infiltration by intrathecal adenosine agonists was not limited to carrageenan-induced inflammation, since NECA also inhibited neutrophil accumulation induced by zymosan-activated plasma, a source of activated complement (Fig. 2), or by a reverse passive Arthus’ reaction (units MPO/gram tissue at 3 h: vehicle, 74.6±8.3; NECA, 41.5±8.2). These data suggest that activation of central adenosine A\(_1\) receptors results in potent inhibition of peripheral neutrophil migration.

Adenosine receptor stimulation down-regulates glutamate release in response to a variety of noxious stimuli (see Discussion). To investigate whether this might account for the anti-inflammatory effect of intrathecal adenosine agonists, the glutamate receptor agonist, NMDA, was coadministered with NECA. This led to a complete reversal of the inhibition of neutrophil accumulation by NECA (Fig. 3) which indicates an involvement of the excitatory amino acid glutamate in the regulation of peripheral neutrophil migration. Further support for this hypothesis was provided by the fact that AP-5, an antagonist to the NMDA class of glutamate receptors (18), profoundly inhibited carrageenan-induced neutrophil infiltration (Fig. 4). On the other hand, antagonists to other classes of glutamate receptors were ineffective (Fig. 4). They included AP-3, GAMS, and CNQX, which are antagonists to the metabotropic, AMPA, and AMPA/kainate receptors, respectively (18, 19). The inhibition by intrathecal AP-5 was not due to gross hemodynamic changes since no effect on heart rate or blood pressure was seen for at least 5 h after intrathecal administration of AP-5 (data not shown). In addition, intrathecal AP-5 had no effect on the peripheral blood leukocyte count in the air pouch model (see Table I). These results indicate that intrathecal adenosine agonists decrease peripheral neutrophil accumulation in response to a local inflammatory stimulus through the down-regulation of NMDA receptor activation.

To investigate whether the peripheral inflammation-induced spinal NMDA receptor activation affects levels of pro- or anti-inflammatory mediators in the inflammatory site, an air pouch model that produced inflammatory exudates was used. In this model, intrathecal administration of AP-5 at 100 μg/kg inhibited carrageenan-induced leukocyte migration into the pouch by > 70% (Fig. 5A). Cell-free exudates were then assayed for various autacoids including substance P, TNF-α, and adenosine. Substance P was undetectable in uninflamed pouches but appeared after carrageenan injection (141.8±7.3 pg/ml). Intrathecal AP-5 had no effect on substance P levels in air-pouches injected with carrageenan (146.4±13.1 pg/ml). TNF-α, a proinflammatory cytokine, was also increased by carrageenan from 203±26 to 1150±128 pg/ml. Intrathecal AP-5 modestly decreased TNF-α levels to 908±121 pg/ml, but the difference did not reach statistical significance. On the other hand, AP-5 administration significantly affected adenosine levels in inflamed pouches. Adenosine, present at 570±85 nM in normal saline-injected pouches, largely disappeared after the addition of carrageenan (two had levels under the limit of detection at 90 nM, and the remaining seven had levels of 110±5 nM). In AP-5 treated animals, however, levels were 447±113 nM in the presence of carrageenan (Fig. 5B). In time

**Table I. Effect of Intrathecal AP-5 on Peripheral Blood Leukocyte Count in the Rat Air Pouch Model**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>i.t. Vehicle</th>
<th>i.t. AP-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.7±1.0*</td>
<td>9.7±1.0</td>
</tr>
<tr>
<td>30</td>
<td>9.0±0.7</td>
<td>9.2±1.2</td>
</tr>
<tr>
<td>240</td>
<td>11.3±0.9</td>
<td>11.8±1.0</td>
</tr>
</tbody>
</table>

Rats were treated with intrathecal (i.t.) vehicle or AP-5 (100 μg/kg). Air pouches were then injected with carrageenan. Air pouch exudates were analyzed at baseline (t = 0), 30, and 240 min after injection. *10^6 leukocytes/ml; n = 6/group.
course experiments, the decrease in air pouch adenosine concentration occurred at the same time as the influx of leukocytes (2–4 h after carrageenan injection) (see Table II). Adenosine could still be detected at expected levels in standard solutions mixed with carrageenan, indicating that the carrageenan itself did not affect the ability to detect adenosine in the exudates. These data indicate that tissue adenosine levels normally decrease in inflamed sites, through a spinal cord NMDA-receptor mediated mechanism.

Because adenosine inhibits peripheral neutrophil migration, its preservation in the inflamed tissue after intrathecal AP-5 administration offered a possible mechanism for the spinal antiinflammatory action of this compound, as well as that of spinal A₁ receptor stimulation. This possibility was investigated using an A₂ receptor antagonist, DMPX (20), administered intraperitoneally (10 mg/kg). DMPX, administered at the time of i.t. treatment and again 2 h later, reverses the inhibition of leukocyte migration by AP-5 in the air pouch model. Data are presented as mean±SEM of number of leukocytes. *Significant difference from vehicle group. n = 5–10/group. (B) DMPX reverses the antiinflammatory effects of intrathecal NECA, CHA, and AP-5, but not those of systemic dexamethasone, in the skin lesion model. Doses are as follows: CHA (5 μg/kg i.t.), NECA (3 μg/kg i.t.), AP-5 (100 μg/kg i.t.), and dexamethasone (Dex, 0.2 mg/kg i.p. 30 min before intradermal injections). DMPX (striped bars) or its vehicle (solid bars) was administered once at the time of treatment. Data are presented as percent of control (mean±SEM). *Significant difference between DMPX and vehicle groups. n = 5–10/group.

Table II. Effect of Intrathecal AP-5 on Air Pouch Adenosine and Leukocyte Accumulation

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>i.t. Vehicle</th>
<th>i.t. AP-5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenosine (nM)</td>
<td>Leukocyte* (×10⁶/ml)</td>
</tr>
<tr>
<td>30</td>
<td>961±84</td>
<td>2.6±0.3</td>
</tr>
<tr>
<td>60</td>
<td>585±104</td>
<td>3.2±0.3</td>
</tr>
<tr>
<td>120</td>
<td>407±33</td>
<td>4.0±0.2</td>
</tr>
<tr>
<td>240</td>
<td>107±7¹</td>
<td>12.2±1.9¹</td>
</tr>
</tbody>
</table>

Rats were treated with intrathecal (i.t.) vehicle or AP-5 (100 μg/kg). Air pouches were then injected with carrageenan. Air pouch exudates were analyzed at various times after injection. *10⁶ leukocytes/ml; n = 6/group. ¹P < 0.01 compared with i.t. AP-5.
alone had no effect on leukocyte accumulation (Fig. 6A) and it did not reverse the anti-inflammatory effect of a systemically administered corticosteroid, dexamethasone (Fig. 6B). Intrathecal administration of DMPX (100 μg/kg) did not reverse inhibition of neutrophil accumulation by central adenosine receptor stimulation (data not shown). Systemic DMPX did not alter adenosine concentrations in saline-injected air pouches (vehicle 1.728±971 nM; DMPX 1.540±497 nM). Finally, coinjection of adenosine deaminase (ADA; 3 U/ml) with carrageenan into air pouches reversed the effect of intrathecal adenosinergic stimulation (i.t. vehicle = 4.4±1.2 × 10⁶ leukocytes/ml; i.t. adenosinergic stimulation = 1.6±0.3 × 10⁷; i.t. adenosinergic stimulation+ADA = 4.1±0.9 × 10⁶; P < 0.05, n = 5/group). Lower concentrations of ADA (1 U/ml) were ineffective (data not shown). This indicates that adenosine agonists and NMDA antagonists administered intrathecally exert their antiinflammatory effects by maintaining adenosine levels in inflammatory sites.

**Discussion**

The induction of inflammation by injection of noxious stimuli into a peripheral site, such as a knee joint, is followed by a series of well-defined events in the spinal cord. These include changes in spinal fluid potassium concentration (21), induction of c-fos mRNA (22), biosynthesis of dynorphin (23), release of substance P and neurokinin A (24, 25), glutamate release (1) and hyperexcitability of neurons (2). These events have traditionally been associated with pain perception and the development of hyperalgesia; however, the present findings indicate that some of these events can influence the inflammatory response itself. This report is the first to demonstrate a direct effect of spinal cord activity on neutrophil infiltration into a peripheral site in response to a locally administered inflammatory stimulus.

Injection of adenosine analogs into the spinal fluid led to marked inhibition of dermal neutrophil infiltration in response to a variety of stimuli. The relative potency of the various agonists indicated that the inhibition was due to activity at the adenosine A₂ receptor in the central nervous system. Adenosine and its analogs can also reduce neutrophil infiltration directly by activating adenosine receptors on the neutrophil (26, 27); however it is unlikely that the antiinflammatory action in the present study was due to leakage of agonists out of the central nervous system, dispersal throughout the body, and direct action on neutrophils, for several reasons. First, the agonists were ineffective when administered into a peripheral site at the same doses that were highly effective after intrathecal administration. Second, the direct effect of adenosine on neutrophils is due to action at the adenosine A₂ receptor (28, 29) rather than at the A₁ receptor. Hence, the explanation for the inhibitory effect of adenosine agonists after intrathecal administration must lie elsewhere, and must be related to a direct effect inside the central nervous system.

Adenosine has several well-documented effects on nervous tissue, one of which is the inhibition of the release of several different classes of neurotransmitters through A₁ receptor activation (reviewed in reference 30), including the excitatory amino acids, glutamate and aspartate (8, 9). Glutamate induces hyperexcitability of neurons after inflammation (3, 4), and its levels are elevated in the spinal cord during a peripheral inflammatory event (1). Thus, reduced glutamate release might be one reason for the inhibitory effect of intrathecal adenosine agonists on peripheral neutrophil migration. In support of this notion, glutamate receptor antagonists to the NMDA receptor subclass also inhibited infiltration of neutrophils, whereas antagonists to other subclasses of glutamate receptors were ineffective.

To elucidate the connection between glutamate inhibition in the spinal cord and reduced peripheral neutrophil infiltration, the airpouch model was used. When carrageenan was injected into airpouches, increased levels of several inflammatory mediators were detected, in line with earlier reports regarding TNF-α (31) and substance P (32). Neither of these were decreased after intrathecal NMDA antagonist injection. However, carrageenan injection also induced a marked reduction in puch adenosine levels. Surprisingly, when NMDA antagonists were injected intrathecally before carrageenan administration, adenosine levels in the pouch were essentially maintained at baseline levels. The inhibitory effect of adenosine on neutrophil adhesion and migration through action on neutrophil adenosine A₂ receptors is well documented (26–29, 33). The changes in peripheral adenosine concentrations after an inflammatory stimulus and/or intrathecal administration of AP-5 have thus far been documented only for carrageenan. Although intrathecal therapy of the reverse passive Arthus reaction or intradural ZAP injection has pharmacology similar to carrageenan, the effect of these stimuli on peripheral adenosine levels has not been examined. Hence, one can not necessarily extrapolate these data to all mechanisms of peripheral inflammation.

Since leukocyte influx occurs at the same time as the decrease in adenosine, it is possible that the preservation of adenosine in AP-5–treated animals contributes to the antiinflammatory effect. To investigate this possibility, rats were treated intraperitoneally with the adenosine A₂ receptor antagonist, DMPX, using a dosing regimen that was earlier found to reverse the antiinflammatory effects of an orally administered adenosine kinase inhibitor known to elevate peripheral adenosine levels (12, 34). This treatment reversed the inhibitory effects on neutrophil migration by NMDA antagonists as well as those of intrathecal adenosine agonists. The effects of DMPX were specific, as inhibition of neutrophil infiltration by dexamethasone remained intact, and was not due to leakage into the central nervous system since DMPX administered centrally was ineffective. Finally, direct injection of adenosine deaminase into the air pouches to metabolize adenosine also reversed the antiinflammatory effects of intrathecal therapy.

Decreased neutrophil accumulation from adenosine could be due to changes in local hemodynamic parameters. However, macroscopic studies of blood pressure and heart rate did not detect significant alterations. Also, adenosine acts as a microvascular vasodilator and local application of adenosine to microvascular beds leads to increased blood flow (35). It is unlikely that adenosine decreased peripheral neutrophil accumulation due to lower flow, although intravital microscopy studies are required to determine the impact of intrathecal adenosine agonists on the local microcirculation.

The exact mechanisms underlying the changes in peripheral adenosine levels during inflammation and the restoration of these levels through spinal NMDA receptor antagonist remains undetermined. The disappearance of adenosine in inflamed sites is not likely due to degradation of adenosine to inosine by adenosine deaminase, since HPLC tracings showed...
a similar reduction in inosine levels (data not shown). With regards to the antiinflammatory effects of intrathecal glutamate receptor antagonists, others have reported that CNQX reduces carrageenan-induced knee edema whereas NMDA receptor antagonists were ineffective (36). This has been ascribed to CNQX-sensitive dorsal root reflexes generated in response to acute arthritis and leading to peripheral substance P release (37, 38), which would potentiate edema formation. It is unlikely that a similar mechanism underlies the present findings, since (a) we saw no effects of intrathecal CNQX, and (b) substance P levels remained unchanged in animals treated with NMDA antagonists even though potent antiinflammatory effects were seen.

In conclusion, spinal NMDA receptor activity regulates neutrophil accumulation during acute peripheral inflammation (see Fig. 7). Adenosine and its analogs can influence this chain of events at two distinct sites, one central and one peripheral. Stimulation of central A1 receptors leads to a decrease in postsynaptic glutamate receptor stimulation. When carrageenan is the inflammatory stimulus, this results in the preservation of adenosine levels at peripheral inflammatory sites through decreased NMDA receptor activity, which subsequently inhibits neutrophil migration through action on A2 receptors. These findings illustrate a previously unknown link between the central nervous system and the immune system, and introduce the possibility of developing novel antiinflammatory therapies.

Acknowledgments

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References


