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Purinergic regulation of bradykinin-induced plasma extravasation and adjuvant-induced arthritis in the rat

(neurogenic inflammation/sympathetic postganglionic neuron/synovial joint/ATP/adenosine)

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ABSTRACT **We assessed the contribution of ATP and adenosine (*i*) to a major sign of acute inflammation, plasma extravasation (PE), in the rat knee joint and (*ii*) to the severity of joint injury in adjuvant-induced experimental arthritis, a chronic inflammatory disease. PE induced by local infusion of bradykinin, which we have previously shown to depend on the sympathetic postganglionic neuron terminal, was markedly enhanced by coinfusion of either ATP or the adenosine A_2-receptor agonist 2-[4-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine. Bradykinin-induced PE was inhibited by coinfusion of the ATP receptor antagonist adenosine 5′-[α,β-methylene]triphosphate, the A_2-receptor antagonist 3-(5*H*-thiozolo[2,3b]quinazolin-3-yl)phenol monohydrochloride, or the adenosine A_1-receptor agonist N^6-cyclopentyladenosine. The joint injury associated with experimental arthritis, which is reduced in severity in sympathectomized rats, was also markedly attenuated by daily administration of either ATP (40% reduction) or adenosine (55% reduction). These results demonstrate that the purines ATP and adenosine (acting at the A_2 receptor), cotransmitters in the sympathetic postganglionic neuron terminal, enhance bradykinin-induced sympathetic postganglionic neuron terminal-dependent PE but inhibit the joint injury of arthritis. These opposing purinergic effects on PE and joint injury suggest that enhanced PE protects against joint injury.**

A contribution of the peripheral limb of the sympathetic nervous system to inflammation has been demonstrated in a variety of animal models (1, 2) and also in patients with inflammatory diseases, such as rheumatoid arthritis (3, 4). For example, sympathetic nerve stimulation increases vascular permeability, even though it also produces vasoconstriction (5). Consistent with these data, surgical (6, 7) or chemical (8–10) sympathectomy strongly inhibits noxious stimulus-evoked plasma extravasation (PE), a major sign of acute inflammation. Sympathectomy also markedly reduces inflammation and joint injury in rats with adjuvant-induced arthritis (11).

We have recently shown that the PE produced by bradykinin (BK), a potent inflammatory agent, is mediated via an action on the sympathetic postganglionic neuron (SPGN) terminal (12). Specifically, sympathectomy significantly reduced PE evoked by infusion of BK into the knee joint of the rat. Although many SPGN neurotransmitters have been identified, our results suggested that release of the known sympathetic "cotransmitters" (i.e., norepinephrine, neuropeptide Y, and ATP) could not alone account for the PE produced by SPGN-terminal stimulation. Thus, norepinephrine inhibited PE, and neuropeptide Y had no effect on PE. Although ATP increased PE, its effect, even at the highest doses, was smaller in amplitude and much shorter in duration than that produced by BK or by acute activation of SPGN terminals with 6-hydroxydopamine (12). Because indomethacin, a prostaglandin synthesis inhibitor, markedly reduced the PE produced by BK and because SPGNs synthesize prostaglandin E_2 (PGE_2) (13), we proposed that PGE_2 released from the SPGN terminal contributes to the BK-evoked PE. In support of this hypothesis, we observed that in the presence of indomethacin, PGE_2 reconstituted BK-induced PE. PGE_2 by itself, however, produces little PE. This fact suggests that additional factor(s) contribute to BK-induced PE (12). In fact, because the PE induced by the combination of BK and PGE_2 is reduced by sympathectomy, these other factors appear to be SPGN derived or dependent.

Recent literature provides increased evidence for the importance of purines (i.e., ATP and adenosine) as cotransmitters with norepinephrine in SPGNs (14–16), and both ATP and adenosine are released from frog sympathetic ganglia (17). Norepinephine and ATP are coreleased from sympathetic perivascular nerves to act on blood vessels to vasoconstrict most of them (18–20). In addition, ATP and adenosine can modulate SPGN function by inhibiting norepinephrine release (14, 21). Because of the extensive evidence for potentiation of BK-mediated PE by PGE_2 (12), we asked whether ATP and adenosine could enhance BK-induced PE. Since we had shown (22) an inverse relationship between magnitude of SPGN-dependent PE and severity of joint injury in experimental arthritis in rat, we assessed the contribution of ATP and adenosine to the severity of tissue injury seen in the arthritic rat model, a disease to which the SPGN contributes significantly (11).

METHODS

The experiments were done on male Sprague–Dawley rats (Bantin & Kingman, Fremont, CA), weighing 300–380 g for the PE studies and 300–320 g for the arthritis experiments. The rats were housed in a temperature- and humidity-controlled environment and had a 12-hr light/dark cycle (lights on at 06:00). Number of rats in each experiment is indicated in the figure legends.

PE. The technique used to measure PE into the perfused knee joint has been described in detail (12). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg) and then given a tail-vein injection of Evans blue dye at 50 mg/kg (dye volume was 2.5 ml/kg). Rate of perfusion inflow was regulated with a syringe pump that infused fluid through a 30-gauge needle placed into the joint cavity; the outflow perfusate was withdrawn through a 25-gauge needle by a

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Abbreviations: PE, plasma extravasation; BK, bradykinin; SPGN, sympathetic postganglionic neuron; CGS21680C, 2-[4-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine; CPA, N^6-cyclopentyladenosine; HTQZ, 3-(5*H*-thiozolo[2,3b]quinazolin-3-yl)phenol monohydrochloride; PGE_2, prostaglandin E_2.

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second syringe pump. The perfusing fluid was injected and withdrawn at a constant rate of 200 μl/min. Perfusate samples were collected every 5 min for a period of 60 min. Samples were analyzed for the amount of Evans blue dye, by spectrophotometric measurement of absorbance at 620 nm, which is linearly related to its concentration.

The knee joint was continuously perfused with saline, and after establishment of baseline levels of PE, BK (160 nM) was added to the perfusing fluid. ATP, adenosine, or their analogs were also added to the fluid either alone or with BK. In these latter groups of rats, after a 15-min baseline period of perfusion with vehicle, the ATP or adenosine analogue was added to the perfusion fluid for an additional 15 min. This procedure was followed by BK addition to the perfusion fluid (160 nM) for the remainder of the experiment.

The following drugs were used in these experiments: sodium pentobarbital injection (Anthony Products, Arcadia, CA); BK triacetate; dipyridamole; α,β-methylene adenosine 5′-triphosphate (Sigma); (2-[4-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS21680C, provided by CIBA–Geigy); N^6-cyclopentyladenosine (CPA, Research Biochemicals, Natick, MA); deoxycoformycin and 3-(5*H*-thiozolo[2,3b]quinazolin-3-yl)phenol monohydrochloride (HTQZ, provided by Warner–Lambert/Parke–Davis, Ann Arbor, MI); and rolipram (provided by Berlex Laboratories, Cedar Knolls, NJ). Rolipram was used because cAMP is the second messenger for adenosine in many tissues, and thus the effect of adenosine should be enhanced by rolipram. The pharmacological effects of the drugs used in these studies are summarized in Table 1.

With two exceptions, all drugs were dissolved in saline. HTQZ was first dissolved in dimethyl sulfoxide (Sigma) and then further diluted with saline to the appropriate concentration (final dimethyl sulfoxide concentration, 0.04%). Rolipram was dissolved in 10% Cremophor EL (Sigma) before further dilution with saline (final Cremophor EL concentration, 0.003%).

Experimental Arthritis. Arthritis was induced by an intradermal injection in the tail of 0.1 ml of a 10 mg/ml suspension of *Mycobacterium butyricum* in mineral oil (23). Arthritic rats were bedded on soft wood shavings, and food and water were placed within easy reach inside their cages. Approximately 10–14 days after injection of the adjuvant, rats developed a symmetric distal polyarthritis. Twenty-eight days after injection, rats were anesthetized, and x-rays were taken to assess the severity of arthritis radiographically (24). A skeletal radiologist (Clyde Helms, Division of Skeletal Radiology, University of California at San Francisco), who was unaware of treatment group, evaluated and scored the radiographs for each hind paw, according to the 0–3 grading scale of Ackerman and colleagues (24). This scale assesses the following radiographic signs: soft-tissue swelling, decreased bone density (osteoporosis), narrowing of the joint-space (loss of cartilage), destruction of bone (erosions), and formation of periosteal new bone. Radiographic scores correlate well with scores from histological sections of joints and periarticular tissues (24).

Table 1. Pharmacological effects of purinergic agents

Drug	Action
CPA	Adenosine A_1-receptor agonist
CGS21680C	Adenosine A_2-receptor agonist
HTQZ	Adenosine A_2-receptor antagonist
ATP	ATP P_2-receptor agonist
α,β-Methylene ATP	ATP P_2-receptor antagonist
Dipyridamole	Adenosine uptake inhibitor
Deoxycoformycin	Adenosine metabolism inhibitor
Rolipram	Phosphodiesterase inhibitor

Three treatment groups were used in the arthritis study: the first group received daily ATP injections (2 mg/kg in 8–9 ml of saline i.p.); the second group received daily adenosine injections (5 mg/kg in 8–9 ml of saline i.p.); and the third group received daily saline injections (8–9 ml i.p.). The injections started 1 day before injection of *Mycobacterium* and continued throughout the experiment for a total of 28 days. This method was adapted from Rapaport and Fontaine (25), who noted that this method of administering ATP in large volumes of saline produced long-lasting increase in erythrocyte ATP pools and sustained increase in plasma ATP levels for ≈6–8 hr after i.p. ATP injection. Drugs were dissolved in saline, and pH was adjusted to 6.2.

Statistics. Differences in PE between experimental groups were assessed by repeated measures of analysis of variance. For arthritis-severity scores, analysis of variance followed by *post hoc* test (Fisher protected least-squares difference) was used to determine significant differences.

RESULTS

PE. When BK (160 ng/ml) was added to the vehicle, it produced an increase in PE of rapid onset, which peaked at 20 min and continued until the end of the experiment, another 25 min (Fig. 1). Addition of ATP (0.5 mg/ml) to the vehicle by itself produced only small and transient (duration <15 min) increase in PE; adenosine (1 μM) or the selective A_2-receptor agonist CGS21680C alone did not affect PE (data not shown). When either ATP, adenosine, or CGS21680C was coinjected with BK, however, PE was enhanced (Figs. 1, 2, and 3) over that produced by BK alone. Combination of the inhibitor of adenosine metabolism deoxycoformycin (10 μM) and the inhibitor of adenosine uptake dipyridamole (50 μM) also enhanced BK-induced PE; however, this enhancement was not statistically significant (Fig. 2). Both the ATP-receptor antagonist α,β-methylene ATP (Fig. 1), as well as the adenosine A_2-receptor antagonist HTQZ and the A_1-receptor agonist CPA (Fig. 3) inhibited the PE induced by BK. Finally, BK-induced PE was significantly enhanced by adding the phosphodiesterase inhibitor rolipram (50 μM)

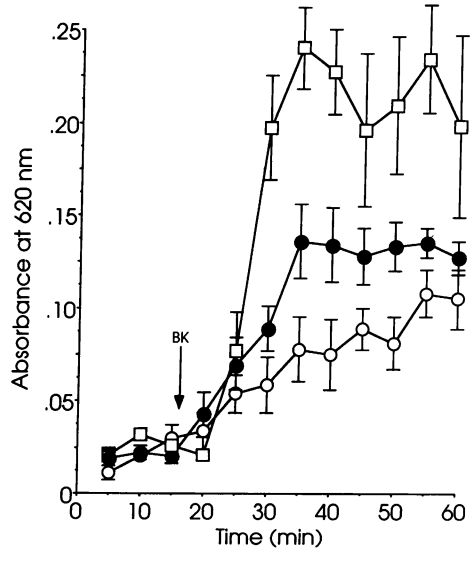

FIG. 1. Effect of ATP and α,β-methylene ATP on BK-induced PE. Time course of the effect of BK (160 ng/ml) infusion into the rat knee joint: ●, BK alone (mean ± SEM, $n = 8$); □, BK coinfused with ATP (0.5 mg/ml; $n = 8$); ○, BK coinfused with α,β-methylene ATP (1 μM; $n = 6$). Ordinate indicates PE as measured by light absorption at 620 nm. Repeated-measures analysis of variance revealed the following differences: BK vs. ATP, $P < 0.01$; BK vs. α,β-methylene ATP, $P = 0.05$.

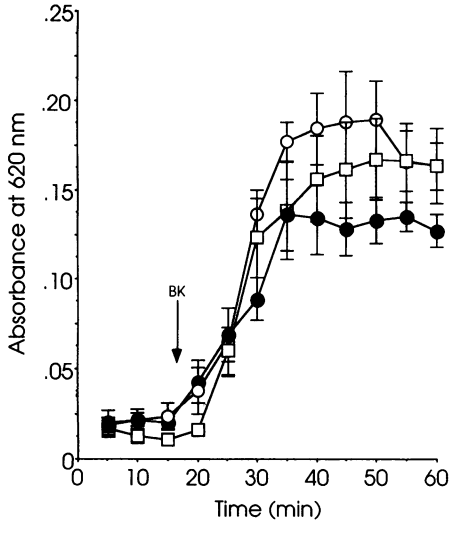

FIG. 2. Effect of adenosine and deoxycoformycin plus dipyridamole on BK-induced PE. Time course of the effect of BK (160 ng/ml) infusion into the rat knee joint: ●, BK alone (mean ± SEM, $n = 8$); ○, BK coinfused with adenosine (1 μM; $n = 6$); □, BK coinfused with deoxycoformycin plus dipyridamole (10 μM and 50 μM, respectively; $n = 6$). Ordinate indicates PE as measured by light absorption at 620 nm. Repeated-measures analysis of variance revealed the following differences: BK vs. adenosine, $P < 0.05$; BK vs. deoxycoformycin plus dipyridamole, P = not significant.

(Fig. 4). Vehicle alone neither stimulated PE nor potentiated BK-induced PE (data not shown).

Experimental Arthritis. The injection of *Mycobacterium* into the tail of a control (saline-treated) group of rats produced a distal symmetric polyarthritis first apparent 10–14 days later and yielded a mean day-28 radiographic joint injury score of 2.25 ±0.26 ($\bar{X}$ ± SEM, $n = 16$). Animals administered ATP (2 mg/kg i.p.) or adenosine (5 mg/kg i.p.), once daily throughout the experimental period, had a markedly attenuated severity of joint injury when compared with control animals that received saline (Fig. 5). Compared with

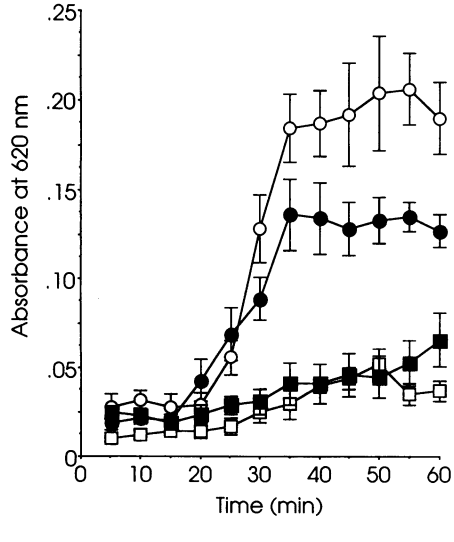

FIG. 3. Effect of CGS21680C, CPA, and HTQZ on BK-induced PE. Time course of the effect of BK (160 ng/ml) infusion into the rat knee joint: ●, BK alone (mean ± SEM, $n = 8$); ○, BK coinfused with CGS21680C (1 μM; $n = 6$); □, BK coinfused with CPA (10 nM; $n = 8$); ■, BK coinfused with HTQZ (1 μM; $n = 8$). Ordinate indicates PE as measured by light absorption at 620 nm. Repeated measures of analysis of variance revealed the following differences: BK vs. CGS21680C, $P < 0.05$; BK vs. CPA, $P < 0.0001$; BK vs. HTQZ, $P < 0.0001$.

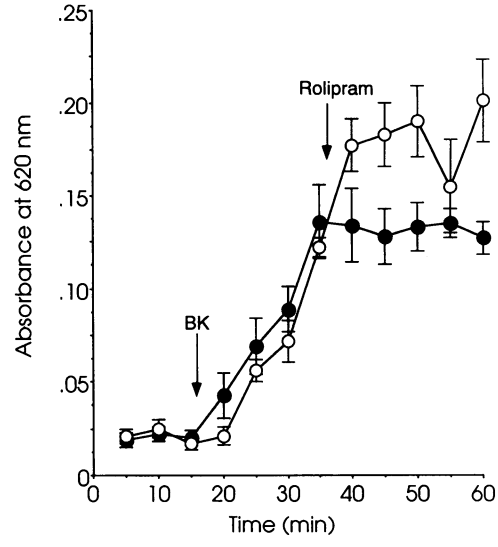

FIG. 4. Effect of rolipram on BK-induced PE. Time course of the effect of BK (160 ng/ml) infusion into the rat knee joint: ●, BK alone (mean ± SEM, $n = 8$); ○, BK coinfused with rolipram (50 μM; $n = 7$). Ordinate indicates PE as measured by light absorption at 620 nm. Repeated-measures analysis of variance revealed the following differences: BK vs. rolipram, $P < 0.01$.

the saline controls, ATP reduced arthritis score by 40% ($p < 0.005$); adenosine reduced the score by 55% ($p < 0.0001$).

DISCUSSION

In this study we investigated the contribution of purinergic mechanisms to the PE evoked by BK, an inflammatory mediator that produces PE via action on SPGN terminals (12). Using selective ATP and adenosine agonists and antagonists, we showed that, although adenosine by itself did not produce significant PE and although high ATP concentrations only produced a small transient PE (12), ATP or the adenosine A_2-receptor agonist significantly enhanced the PE induced by BK. Consistent with that result, the adenosine A_2-receptor antagonist HTQZ significantly reduced the PE induced by BK. Finally, the combination of deoxycoformycin, an adenosine deaminase inhibitor, and dipyridamole, an adenosine uptake inhibitor, elevated PE over that produced by BK alone—although this increase was not statistically significant. Lack of significant effect may be from adenosine

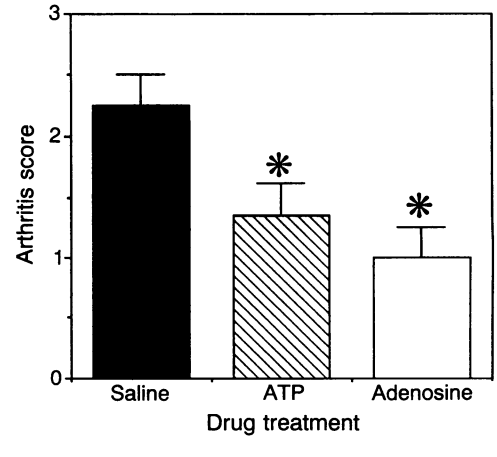

FIG. 5. Effect of ATP and adenosine on experimental arthritis 28 days after arthritis induction. Severity of arthritis was scored on 0–3 scale (see text). Data show a significant reduction in arthritis scores, compared with saline controls, in those rats receiving either daily ATP injections ($P < 0.05$) or daily adenosine injections ($P < 0.001$).

being phosphorylated to AMP by adenosine kinase, which was not inhibited in this protocol.

Because the specific ATP receptor antagonist α,β-methylene ATP suppressed BK-induced PE (Fig. 1), ATP, as well as adenosine, probably contributes, at least in part, to the BK-induced, SPGN terminal-dependent PE. On the other hand, because ATP is rapidly metabolized to adenosine, some ATP action could be mediated through its metabolism to adenosine.

Studies have demonstrated that endogenous adenosine probably modulates blood flow and vascular permeability (26) via its action on the microvasculature (27). Adenosine vasodilates vascular beds by means of an action at A_2-receptors on vascular smooth muscle (28–30). Because the phosphodiesterase inhibitor rolipram enhanced BK-induced PE, our results show that BK-induced PE is mediated through an increase in cAMP levels. This result is consistent with the proposed action of adenosine at A_2-receptors because A_2 receptors have consistently been linked to increased cAMP production (31, 32).

The site at which adenosine and ATP act to increase PE is unknown. Adenosine may act directly on postcapillary venules or may have an indirect action—e.g., on polymorphonuclear leukocytes, which we have shown contribute to BK-induced PE (34). In fact, polymorphonuclear leukocytes have recently been shown to possess both A_1 and A_2 receptors (33).

Because sympathectomy decreases BK-evoked PE, we have evaluated the SPGN-dependent factors involved in PE. We have previously demonstrated that BK-evoked PE is inhibited by indomethacin but can be reconstituted by coinfusion of BK with PGE_2, indicating that PGE_2 contributes to BK-evoked PE. Our present data indicate that purines also contribute to BK-evoked PE. Coinfusion of PGE_2 and purines, however, does not reproduce the effect of BK; only a small and transient increase in PE is obtained (unpublished results). We conclude that additional factor(s) must contribute to BK-induced SPGN-dependent PE. This other factor(s) could include either BK itself or other factors the release of which is induced by BK.

Because PE is a component of the inflammatory process and may, therefore, influence the course of tissue injury that develops in inflammatory diseases, such as arthritis, we also evaluated whether ATP or adenosine affects arthritis severity. The relationship between PE and arthritis severity is of particular interest, as we have previously reported that a β_2-receptor antagonist or an α_2-receptor agonist enhanced BK-evoked PE in rat knee joint, whereas these drugs significantly reduce joint injury in rats with experimentally induced arthritis (22). This inverse relationship between PE and severity of arthritis was also seen with our present data; both ATP and adenosine enhanced BK-evoked PE but decreased severity of joint injury in rats with experimentally induced arthritis. Taken together, these data are compatible with the suggestion that PE contributes not to tissue injury but to tissue repair, perhaps by dilution and clearance of inflammatory mediators.

In conclusion, these data suggest that BK acts on the SPGN terminal to produce PE via mechanisms that include an adenosine A_2 receptor and/or ATP action. Activation of the adenosine A_2-receptor enhances the PE response to BK, probably through the cAMP second-messenger system. As expected, A_1-receptor activation, which decreases cAMP, inhibits BK-induced PE. The ameliorative effect of adenosine and ATP on joint injury suggests that the purines are important in controlling tissue damage in inflamed joints and, thus, suggests other therapeutic approaches to the treatment of arthritis.

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