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Potentiation of formalin-evoked adenosine release by an adenosine kinase inhibitor and an adenosine deaminase inhibitor in the rat hind paw: a microdialysis study

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Abstract

The present study examined the effects of local subcutaneous administration of formalin on adenosine release from the rat hind paw, and the effects of inhibitors of adenosine metabolism on such release. Microdialysis probes were inserted into the subcutaneous tissue of the plantar surface of rat hind paws. Samples were collected every 10 min at a perfusion rate of 2 μl/min and high performance liquid chromatography was used to measure adenosine levels. At lower concentrations of formalin (0.5–2.5%), a significant increase in adenosine levels was observed in the first 10 min after formalin injection, while at the highest concentration of formalin (5%), the increase in adenosine release was observed over 60 min. Co-administration of the adenosine kinase inhibitor 5′-amino-5′-deoxyadenosine (100 nmol) with formalin, significantly increased adenosine release evoked by 0.5–1.5% formalin, but did not produce a further enhancement of release evoked by 5% formalin. The adenosine deaminase inhibitor 2′-deoxycoformycin (100 nmol) significantly increased adenosine levels at 5% formalin but had no effect at lower concentrations of formalin. In confirmation of previous studies, subcutaneous injection of formalin (0.5–5%) produced a characteristic biphasic concentration-related expression of nociceptive behaviours and an increase in paw volume. This study directly demonstrates that formalin can evoke a concentration-dependent local release of adenosine from the rat hind paw. The ability of an adenosine kinase inhibitor and an adenosine deaminase inhibitor to modulate this release is dependent on substrate adenosine concentrations.

Keywords: Adenosine; Adenosine kinase; Adenosine deaminase; Formalin; Microdialysis; Pain; Inflammation

1. Introduction

Both clinical and animal studies demonstrate that adenosine, an endogenous neuromodulator, has an important role in pain modulation (Segerdahl and Sollevi, 1998; Sawynok, 1998). Studies in humans show that low-dose adenosine infusion increases pain thresholds (Ekblom et al., 1995), reduces post-operative analgesic requirements (Segerdahl et al., 1997) and reduces pain evoked by both nerve injury (Belfrage et al., 1995, 1999; Segerdahl et al., 1995; Sollevi et al., 1995) and inflammatory injury (Sjölund et al., 1999). A decrease in adenosine concentrations in plasma and cerebrospinal fluid in patients with neuropathic pain has even been suggested to contribute to the pathophysiology of neuropathic pain (Guieu et al., 1996). In rodent studies, tonic antinociceptive influences of endogenous adenosine mediated by adenosine A_1 receptors have been demonstrated, both peripherally (Doak and Sawynok, 1995) and centrally (Sawynok et al., 1986; Keil and DeLander, 1996).

Besides, its effects on pain, adenosine also has been suggested to be an autocoid modulating inflammation, primarily acting on adenosine A_{2A} receptors (Cronstein, 1994; Sullivan and Linden, 1998). The anti-inflammatory effects of certain agents used clinically, such as methotrexate and sulfasalazine, are mediated by an increase in extracellular adenosine concentrations (Cronstein et al., 1993; Gadangi et al., 1996; Morabito et al., 1998).

There are at least four key factors that are important in adenosine metabolism. Adenosine is formed from ATP via

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5′-nucleotidase, metabolised by adenosine kinase and adenosine deaminase to 5′-AMP and inosine, respectively, and transported bidirectionally by adenosine transporters on the cell surface (Geiger et al., 1997). The net effect of formation, degradation and transportation determines intracellular and extracellular levels of adenosine. Modulation of extracellular adenosine concentrations by inhibitors of adenosine metabolism can produce antinociceptive and anti-inflammatory effects. Thus, behavioural studies demonstrate that adenosine kinase inhibitors produce a peripherally mediated antinociceptive effect in the formalin-induced persistent pain model (Sawynok et al., 1998), and an anti-inflammatory effect in the carrageenan-induced inflammation model (Poon and Sawynok, 1999). These effects can be blocked by adenosine receptor antagonists, indicating an activation of adenosine receptors.

The formalin test has been widely used to study persistent pain with inflammation (Tjølsen et al., 1992). Behavioural studies suggest that there is a tonic antinociceptive effect of endogenous adenosine (Doak and Sawynok, 1995) and that this effect can be modulated by an adenosine kinase inhibitor (Sawynok et al., 1998), but there has been no direct evidence indicating that adenosine levels are increased by formalin. Microdialysis provides a method to continuously measure the extracellular level of substances in vivo. The aim of the present study was to use the microdialysis technique to examine (1) whether adenosine is released subcutaneously after formalin injection, (2) whether such release can be modulated by an adenosine kinase inhibitor and an adenosine deaminase inhibitor and (3) whether the released adenosine and the modulation of adenosine by enzyme inhibitors correlates with previously reported antinociceptive effects and anti-inflammatory effects produced by adenosine indirectly acting agents.

2. Materials and methods

Male Sprague–Dawley rats (Charles River, Quebec, Canada) 120–160 g (formalin test) or 250–300 g (microdialysis study) were used. Rats were housed in pairs and allowed free access to food and water on a 12/12 h light/dark cycle at 21 ± 1°C. Rats were used only once. Procedures were approved by the University Committee on Laboratory Animals.

2.1. Subcutaneous microdialysis and adenosine measurement

Rats were anaesthetized throughout the procedure with sodium pentobarbital (45 mg/kg, i.p. for induction, 10 mg/kg, i.p. per 30 min for maintenance) and body temperature was maintained with an electric warming pad. The microdialysis probe (LM-5 linear probe, 5-mm active membrane length, 320-μm OD, 35 kDa wt. cut-off; BAS, USA) was planted into the subcutaneous area of the plantar surface of the rat hind paw (Fig. 1). An introducer needle was inserted through the tissue and the microdialysis probe was pulled gently through the needle. After withdrawal of the needle, the probe was adjusted so that the entire membrane window was totally covered under tissue. The inlet and outlet tubing were then fixed at the

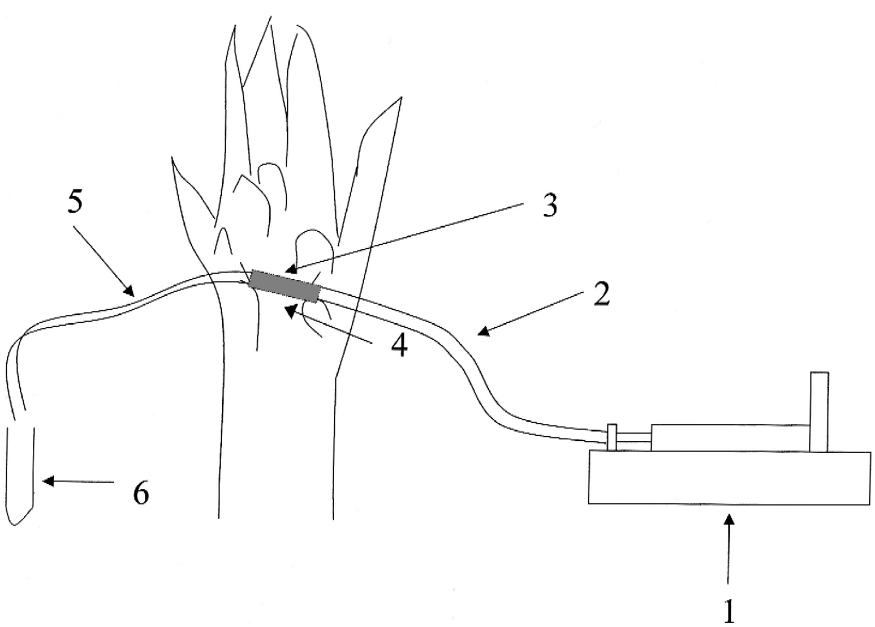

Fig. 1. Schematic illustration of the microdialysis system in the rat hind paw. The probe was placed into the subcutaneous space of the rat hind paw through an introducer needle and then perfused with standard Krebs–Henseleit solution by a microsyringe pump. (1) Microsyringe pump, (2) inlet tubing, (3) active dialysis membrane, (4) injection site of formalin or formalin and drug combination (3 mm parallel to the probe), (5) outlet tubing, (6) collecting tube.

entry and exit points using tissue glue. The inlet tubing was then connected with a microsyringe pump (Harvard/22 USA) through FEP Teflon tubing (26-cm length, 650-μm OD, BAS). Following implantation, the probe was perfused with standard Krebs–Henseleit solution (mM NaCl, 111; $NaHCO_3$, 26.2; NaH_2PO_4, 1.2; KCl, 4.7; $CaCl_2$, 1.8; MgCl, 1.2, at pH 7.4) at a rate of 2 μl/min and dialysate was collected at 10-min intervals in a 1.5-ml microtube containing 10 μl $ZnSO_4$ (0.15 M) at room temperature. The probe was flushed for at least 2 h to achieve a steady basal level (Fig. 2). After two baseline samples were collected, a 50-μl volume of either formalin or a combination of formalin and drugs was injected into the plantar subcutaneous area approximately 3 mm parallel to the dialysis membrane using a 30-gauge needle (Fig. 1). Post injection dialysate collection was started with a 1.5-min delay because of the dead space of the outlet tubing.

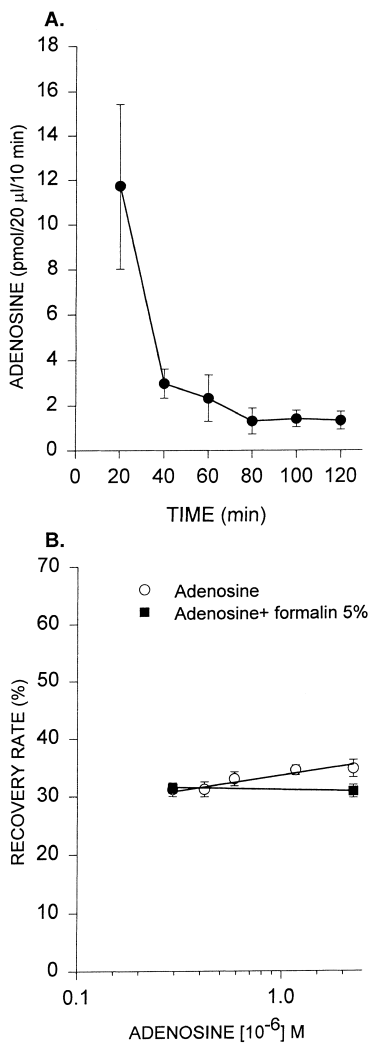

Fig. 2. (A) Time course of changes in measured adenosine levels following the microdialysis probe implantation at time zero. Samples were collected immediately after probe implantation. (B) In vitro recovery rate of adenosine at different concentrations of adenosine in the absence and presence of 5% formalin (means ± S.E.M., $n = 8$).

Adenosine was measured as described previously (Wang and White, 1998). Samples were deproteinated with $Ba(OH)_2$ and $ZnSO_4$ immediately after collection and then derivatized with chloroacetaldehyde to form 1-N^6-ethenoadenosine. Samples were then stored at −4°C before being assayed by high performance liquid chromatography with fluorescence detection. Adenosine standards were prepared in appropriate drug-containing Krebs–Henseleit solutions and processed identically to dialysate samples. Adenosine peaks were identified by using standard adenosine to spike the peak or using adenosine deaminase (10 units/ml for 10 min at room temperature) to eliminate the peak. Adenosine content was quantitated by peak height compared with the standards and was expressed as pmol/20 μl/10 min. Total evoked adenosine release was determined as the cumulative amount of evoked release during a 60-min interval (individual time point release minus the average basal release) and expressed as pmol/120 μl/60 min.

2.2. Formalin test

The formalin test was performed as previously described (Sawynok et al., 1998). Rats were placed in a 28 × 28 × 28 cm Plexiglas observation chamber for an initial 20 min to allow acclimatization to the testing environment. Formalin (0.5–5%) or formalin/drug combinations were injected subcutaneously in a volume of 50 μl into the dorsal aspect of the hind paw. Following injections, rats were returned to the observation chamber and monitored for flinching behaviours (lifting, shaking and overt flinching with a ripple over the haunch) and biting/licking time. Two rats in adjacent chambers were observed at one time, with observations occurring in alternate 2-min bins. Recorded episodes were not corrected, thus values represent about half of the total behaviours expressed.

2.3. Paw volume

Paw volumes were measured as previously described (Sawynok et al., 1999) using a commercially available plethysmometer (Ugo Basile). The hind paws were immersed to the junction of the hairy and non-hairy skin, and volumes were read from a digital display. Measurements were performed in triplicate at 30-min intervals for up to 180 min following injections. Values were standardised by expression as a percentage of individual preinjection volumes to accommodate the variations in body weights.

2.4. Data analysis and statistics

Microdialysis data was expressed as a time course of adenosine levels in individual dialysate (10 min) or total evoked release (60 min). Formalin test data was expressed

as the number of episodes (flinches) or total time (biting/licking) in 2 min bins or cumulative over phase 1 (0–12 min) or phase 2 (16–60 min). Data were analyzed using one-way analysis of variance (one-way ANOVA) followed by Student–Neuman–Keuls test. The level of significance was set at 0.05.

2.5. Drugs

Adenosine, 5′-amino-5′-deoxyadenosine and adenosine deaminase were obtained from Sigma (St. Louis, MO), 2′-deoxycoformycin from Parke-Davis Pharmaceutical Research Division of Warner-Lambert (Ann Arbor, MI), and formalin (37% formaldehyde) from British Drug Houses (Toronto, Ontario). Adenosine was dissolved in Krebs–Henseleit when used as standards, all other drugs were dissolved in saline.

3. Results

3.1. Basal adenosine levels in the subcutaneous space of the rat hind paw

Fig. 2A shows the time course of changes in adenosine levels in the subcutaneous space after implantation of the microdialysis probe. Samples were taken with a flow rate of 2 μl/min from the start of perfusion and collected at 10-min intervals. Adenosine concentrations were initially high, but fell to a steady state level within 80–100 min after probe implantation. Basal adenosine levels were thus measured 120 min after probe implantation. The basal subcutaneous adenosine level was 1.50 ± 0.10 pmol/20μl/10 min (mean ± S.E.M., $n = 76$). To estimate the adenosine concentration in vivo, and to find out if formalin or different concentrations of adenosine had an effect on recovery, the in vitro recovery rate was determined for dialysis probes. There was no significant difference in probe recovery rate at different adenosine concentrations

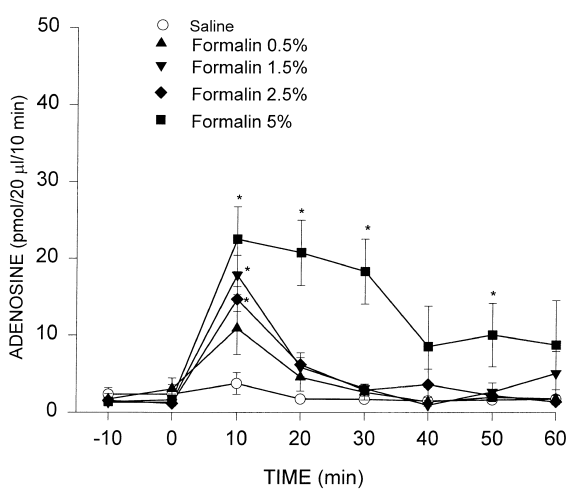

Fig. 3. Time course of measured adenosine levels in microdialysate samples following saline or formalin 0.5–5% injection. * $P < 0.05$ compared to saline group, one-way ANOVA followed by Student–Neuman–Keuls test ($n = 6$–8 per group).

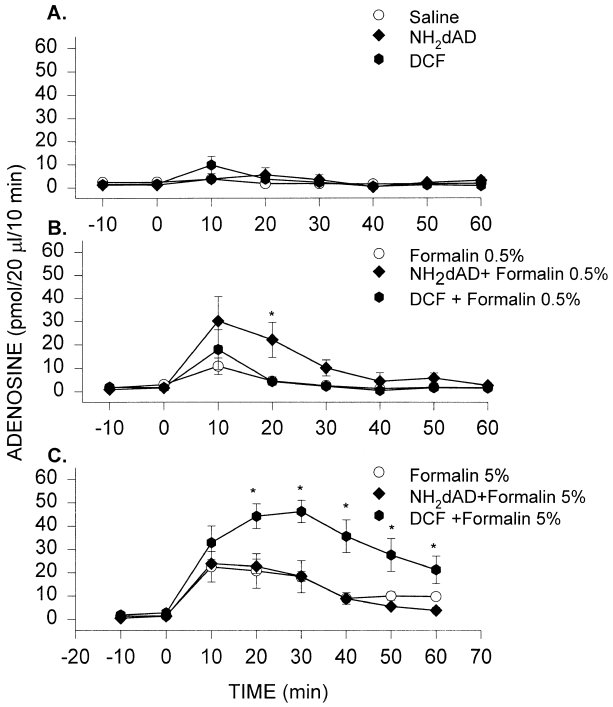

Fig. 4. Time course of adenosine levels in microdialysate samples following injection of the adenosine kinase inhibitor 5′-amino-5′-deoxyadenosine (NH_2dAD) 100 nmol, and the adenosine deaminase inhibitor 2′-deoxycoformycin (DCF) 100 nmol with saline (A), 0.5% formalin (B), 5% formalin (C). * $P < 0.05$ compared to corresponding control group, one-way ANOVA followed by Student–Neuman–Keuls test ($n = 6$–8 per group).

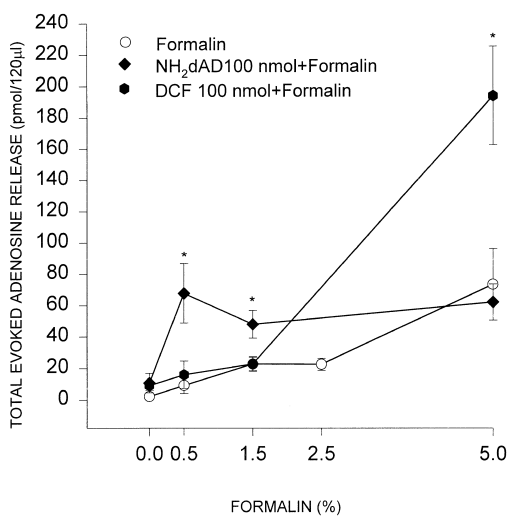

Fig. 5. Total evoked adenosine release over 60-min interval following formalin, formalin with 5′-amino-5′-deoxyadenosine (NH_2dAD) and formalin with 2′-deoxycoformycin (DCF). * $P < 0.05$ compared to corresponding formalin group, one-way ANOVA followed by Student–Neuman–Keuls test ($n = 6$–8 per group).

(Fig. 2B), and formalin (5%) did not change the recovery rate (Fig. 2B). The average recovery of adenosine from the microdialysis probes was $32.7 \pm 1.4\%$ (Fig. 2B). After correction for the in vitro recovery rate, the in vivo basal adenosine concentration in rat hind paw subcutaneous tissues was estimated to be 0.23 ± 0.02 μM (means ± S.E.M.).

3.2. Effect of formalin on adenosine release

Injection of saline (50 μl) did not alter adenosine levels (Fig. 3). Injection of formalin (0.5–5%) increased adenosine release in a concentration-dependent manner (Figs. 3 and 5). During the first 10 min after injection, all concentrations of formalin increased adenosine release; 1.5–5% formalin values were significantly different from saline with the highest level of 22.52 ± 4.32 pmol/20 μl/10 min evoked by 5% formalin. The increase in adenosine produced by 0.5–2.5% formalin was transient, with no significant increase being measured after the first 10 min, while the increase evoked by 5% formalin was long-lasting, persisting for up to 60 min after injection (Fig. 3).

3.3. Effect of the adenosine kinase inhibitor 5′-amino-5′-deoxyadenosine on formalin-evoked adenosine release

Injection of the adenosine kinase inhibitor, 5′-amino-5′-deoxyadenosine at 100 nmol, did not alter basal adenosine

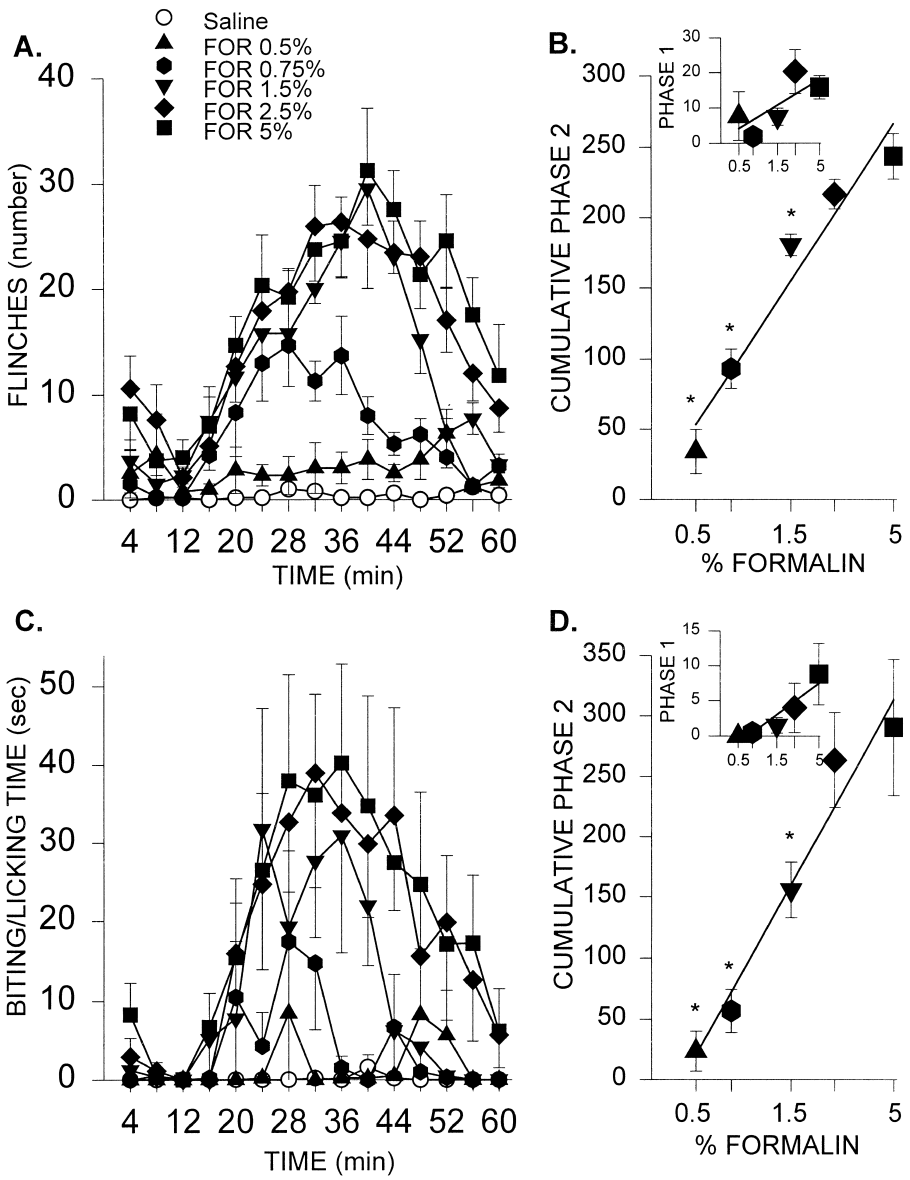

Fig. 6. Time course and cumulative changes over 60 min produced by different concentrations of formalin with respect to flinches (A and B) or biting/licking (C and D) behaviours. * $P < 0.05$ compared to 5% formalin group, one-way ANOVA followed by Student–Neuman–Keuls test ($n = 5$–7 per group).

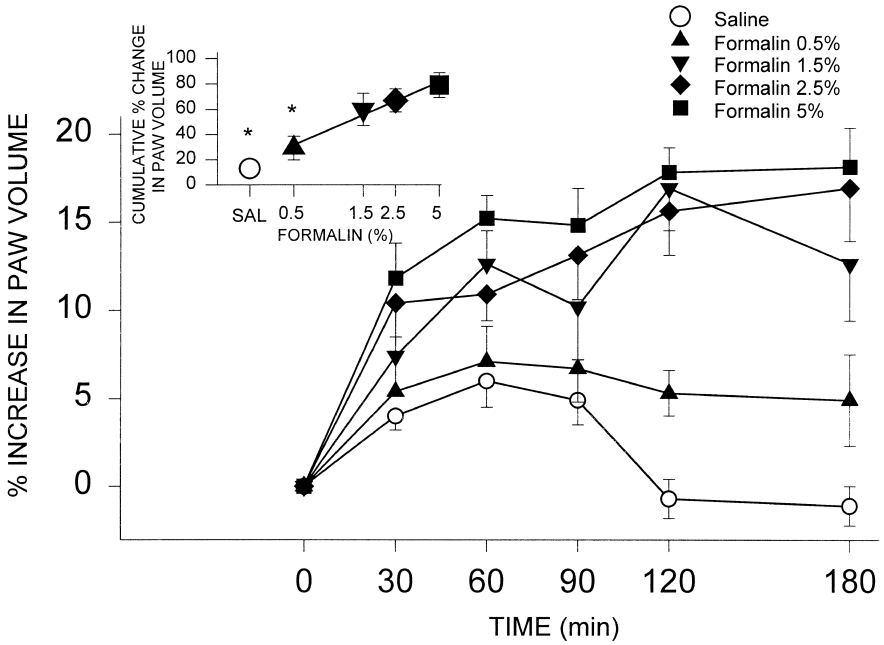

Fig. 7. Time course and cumulative changes over 180 min produced by different concentrations of formalin with respect to paw volume. * $P < 0.05$ compared to 5% formalin group, one-way ANOVA followed by Student–Neuman–Keuls test ($n = 5–7$ per group).

levels (Fig. 4A). Co-administration of 5′-amino-5′-deoxyadenosine with 0.5% formalin increased adenosine release during the initial 20 min (Fig. 4B). The total evoked adenosine release produced by the combination of 5′-amino-5′-deoxyadenosine with 0.5% formalin was 67.8 ± 18.9 pmol/120 μl/60 min, which was significantly higher than 0.5% formalin alone (11.8 ± 4.7 pmol/120 μl/60 min), and comparable to release evoked by 5% formalin (Fig. 5). With a higher concentration of formalin (1.5%), a significant increase in total evoked adenosine release was observed but this increase was not greater than the 5′-amino-5′-deoxyadenosine plus 0.5% formalin group (Fig. 5). There was no significant increase in evoked adenosine release when 5′-amino-5′-deoxyadenosine was co-administered with 5% formalin (Figs. 4C and 5).

3.4. Effect of the adenosine deaminase inhibitor 2′-deoxycoformycin on formalin-evoked adenosine release

Injection of the adenosine deaminase inhibitor 2′-deoxycoformycin at 100 nmol did not alter basal adenosine levels (Fig. 4A), and co-administration of 2′-deoxycoformycin with 0.5–1.5% formalin did not increase formalin-evoked adenosine release (Figs. 4B and 5). However, co-administration of 2′-deoxycoformycin with 5% formalin markedly increased evoked adenosine levels during the entire 60 min time course (Fig. 4C). Under these conditions, adenosine levels increased to a maximum of 46.30 ± 4.77 pmol/20 μl/10 min, which is much higher than 5% formalin alone (18.31 ± 6.91 pmol/20 μl/10 min) and 5′-amino-5′-deoxyadenosine plus 5% formalin (18.19 ± 2.14 pmol/20 μl/10 min, Fig. 4C). The total amount of adenosine released by the combination of 2′-deoxycoformycin with 5% formalin was 193.88 ± 31.22 pmol/120 μl/60 min, which is much higher than 5% formalin alone (73.6 ± 22.9 pmol/120 μl/60 min) or the combination of 5′-amino-5′-deoxyadenosine with 5% formalin (62.18 ± 11.59 pmol/120 μl/60 min, Fig. 5).

3.5. Pain behaviours and increase in paw volume induced by different concentrations of formalin

Subcutaneous injection of 0.5–5% formalin produced a dose-related biphasic behavioural response (Fig. 6). Phase 2 (16–60 min) flinches and biting/licking time induced by 5% formalin were significantly higher than those induced by lower concentrations (0.5–1.5%), but there was no significant difference between 2.5% and 5% formalin (Fig. 6A–D). Phase 1 (0–12 min) behaviours exhibited more variability and there were no significant differences in flinches and biting/licking times at different concentrations of formalin (Fig. 6B and D insets). Formalin (0.5–5%) increased paw volume in a dose-related manner, and the increase was significantly higher at 5% formalin than at 0.5% (Fig. 7).

4. Discussion

Microdialysis has previously been used to measure purine release in both brain (Porkka-Heiskanen et al., 1997; Bell et al., 1998; Britton et al., 1999) and peripheral tissues (Lönnroth et al., 1989; Blay et al., 1997; MacLean et al., 1998; Manthei et al., 1998). In the present study,

using this technique, we demonstrate that the basal adenosine concentration in the subcutaneous area of the rat hind paw was about 0.23 ± 0.02 μM. This is comparable to basal adenosine concentrations in subcutaneous tissue of canine (0.32 ± 0.04 μM, Fredholm and Sollevi, 1981) and human (0.13 ± 0.03 μM, Lönnroth et al., 1989) subjects. The present study also demonstrates that the adenosine recovery rate was relatively constant and independent of the surrounding adenosine concentration gradient or the presence of formalin. Thus, subcutaneous microdialysis appears to be a valid method to measure local adenosine levels in the presence of formalin.

4.1. Concentration-related adenosine release evoked by formalin

Injection of formalin into the rat hind paw produces two distinct phases of pain behaviours. Phase 1 (0–12 min) behaviours are generally considered to result from direct nociceptor activation, while phase 2 (16–60 min) behaviours result from the combination of peripheral inflammation with central sensitization (Tjølsen et al., 1992; Puig and Sorkin, 1996). Nociceptive behaviour and inflammation produced by formalin are dependent on formalin concentrations. High concentrations of formalin produced more prominent nociceptive behaviour and paw edema than lower concentrations. Formalin-evoked adenosine release was also dependent on formalin concentrations. Lower concentrations of formalin (0.5–2.5%) produced an increase in adenosine release, which was predominantly observed during the phase 1 period (0–10 min). There was a plateau between 1.5% and 2.5% formalin, and 5% formalin evoked a release that was both higher in peak release and longer in duration (observed in both phase 1 and phase 2).

At lower concentrations (0.5–2.5%), as the released adenosine was observed primarily during the first 10 min, it is likely that neuronal activation plays a major role in this release, although a non-specific release resulting from tissue injury cannot be excluded. An electrophysiological study shows that the magnitude of neuronal activity in primary afferent fibers is much higher in phase 1 than phase 2 following formalin (Puig and Sorkin, 1996). Nociceptor activation can release ATP from peripheral endings of primary sensory neurons (Holton and Holton, 1954), and sympathetic nerve stimulation can induce ATP release from sympathetic nerve terminals as a co-transmitter with noradrenaline and neuropeptide Y (Fredholm and Sollevi, 1981). This released ATP is largely converted to extracellular adenosine by ecto-5′-nucleotidases or by the nucleotidases released following neuronal activity (Todorov et al., 1997). It is thus likely that at lower concentrations of formalin, a concentration-related increase in adenosine release reflects the intensity of peripheral nerve terminal activity.

It is interesting to note that adenosine release evoked by 5% formalin was quite different from release evoked by 2.5% formalin, although the nociceptive behaviours produced by the two concentrations were comparable. It is likely that 1.5–2.5% formalin produces a maximal activation of neuronal activity. At 5% formalin, an additional release of adenosine due to tissue injury, cell necrosis and platelet aggregation may occur, and this non-neuronal release may contribute a major source of adenosine in the latter phase of the formalin test. Five percent formalin produces a more pronounced tissue damage (Rosland et al., 1990) with an involvement of multiple inflammatory mediators (Damas and Liégeois, 1999). With 5% formalin, combinations of both neurogenic and non-neurogenic inflammatory mediators including neuropeptides, prostanoids, 5-hydroxytryptamine, histamine (Damas and Liégeois, 1999) glutamate (Omote et al., 1998, 1999) and nitric oxide (Omote et al., 1999) are involved. The various inflammatory mediators may act on neuronal tissues to promote adenosine release (Sweeney et al., 1990; Fallahi et al., 1996; Cahill et al., 1997; Conway et al., 1997; Conway and Yaksh, 1998). Non-neuronal tissues, such as endothelial cells (Ager and Gordon, 1984), neutrophils (Cronstein et al., 1983), mast cells (Marquardt et al., 1984) and subcutaneous adipose tissue (Fredholm and Sollevi, 1981) can also release adenosine, secondary to neuronal activity or inflammatory mediator stimulation. The release of adenosine by these inflammatory mediators may contribute substantially to the adenosine release evoked by 5% formalin, especially in the latter phase when inflammation has developed.

4.2. Potentiation of formalin-evoked adenosine release by 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin

The present study directly demonstrates that inhibition of adenosine kinase and adenosine deaminase have no effect on basal adenosine release, but can increase formalin-evoked adenosine release in a manner that correlates with the kinetics of the two enzymes. In the absence of formalin, neither the adenosine kinase inhibitor 5′-amino-5′-deoxyadenosine nor the adenosine deaminase inhibitor 2′-deoxycoformycin increased basal adenosine release. This concurs with the previous study in brain (White, 1996) demonstrating that the augmentation of adenosine levels by inhibiting these enzymes is event-specific, and that the enhancement becomes substantial only in conditions where basal adenosine levels are increased.

In the present study, the adenosine kinase inhibitor 5′-amino-5′-deoxyadenosine increased adenosine levels at lower concentrations of formalin, but not at 5% formalin, while the adenosine deaminase inhibitor 2′-deoxycoformycin increased release at 5% but not lower concentrations of formalin. These results reflect the different kinetics of the two enzymes. Adenosine kinase has a higher affinity (K_m: 0.8–2.0 vs. 17–47 μM; Arch and New-

sholme, 1978; Phillips and Newsholme, 1979) and a lower maximum activity (V_{max}: 60–136 vs. 174–1430 nmol/min/g fresh tissue; Arch and Newsholme, 1978) than adenosine deaminase for metabolising adenosine in most tissues. The most significant increase of adenosine by the adenosine kinase inhibitor occurred 10–20 min after 0.5% formalin injection. This indicates that the optimal concentration of adenosine for adenosine kinase in rat paw subcutaneous tissue is around 0.93 μM in vivo (after correction for the recovery rate). In the presence of 5% formalin, when more adenosine is released, there is no increase in adenosine levels by inhibiting adenosine kinase. It is likely substrate inhibition occurs under this condition, as adenosine kinase exhibits substrate inhibition at high adenosine levels (Arch and Newsholme, 1978). On the other hand, at lower concentrations of formalin, inhibition of adenosine deaminase did not increase adenosine levels, as adenosine levels were lower than the required concentration for metabolism by this lower affinity enzyme. In the presence of 5% formalin, when more adenosine is released at all time intervals, the modulation by adenosine deaminase became significant, and there was an increase in adenosine levels by 2′-deoxycoformycin throughout the entire 60 min. Previous neurochemical studies in brain (Lloyd and Fredholm, 1995; White, 1996; Hebb and White, 1998) and spinal cord (Golembiowska et al., 1996) also demonstrate that adenosine kinase exerts a predominant role in adenosine metabolism in most tissues. When adenosine concentrations are elevated substantially, such as in tissues that are under severe energy depletion (Lloyd and Fredholm, 1995), adenosine deaminase inhibition produces a substantial effect. The current study clearly demonstrates that adenosine kinase is the more important enzyme modulating adenosine levels at low to moderate levels of inflammation, while adenosine deaminase becomes more important at higher levels of inflammation, when large amounts of adenosine are released.

4.3. Correlation of formalin-evoked adenosine release with behavioural and paw volume studies

The concentration-related adenosine release correlates with the tonic adenosine receptor activity revealed in previous behavioural studies. Thus, at 2.5% formalin, an adenosine A_1 receptor antagonist augments, while an A_{2A} receptor antagonist reduces the early part of phase 2 flinching behaviours, but no such modulation occurs at 0.5% formalin (Doak and Sawynok, 1995; Sawynok et al., 1998). These studies indicate that adenosine released by 0.5% formalin is not sufficient to activate peripheral adenosine receptors, but at higher concentrations, such as 2.5% formalin, the released adenosine can produce a tonic activation of both adenosine A_1 and A_{2A} receptors, which mediate antinociceptive and pronociceptive effects, respectively (Karlsten et al., 1992; Sawynok, 1998). In the present study, the increase in adenosine levels caused by low concentrations of formalin is significant only within 10 min after injection, which corresponds to phase 1 behaviours, but the tonic nociceptive effect revealed by adenosine receptor antagonists is predominately observed in the early part of phase 2 behaviours (14–36 min, Doak and Sawynok, 1995). Thus, there is a delayed expression of adenosine effects on behaviour. A dissociation of neurochemical and behavioural effects has also been demonstrated for formalin-induced glutamate release. Hence, while formalin evokes spinal glutamate and aspartate release predominately in phase 1 (Malmberg and Yaksh, 1995), spinal antagonists acting on glutamate receptors have a predominant antinociceptive effect on phase 2 behaviours (Haley et al., 1990; Yamamoto and Yaksh, 1992).

The different abilities of adenosine kinase inhibitors and adenosine deaminase inhibitors to modulate adenosine levels also accord with previous behavioural studies. Thus, while the antinociceptive effect of adenosine kinase inhibitors has been demonstrated both peripherally (Sawynok et al., 1998) and spinally (Keil and DeLander, 1992, 1996; Poon and Sawynok, 1995, 1998), inhibition of adenosine deaminase does not produce an intrinsic antinociceptive effect, although it does enhance the effect of adenosine kinase inhibitors in all instances. However, when substrate adenosine levels are substantially elevated, such as in the presence of exogenous adenosine, inhibition of adenosine deaminase produces antinociceptive effects (Keil and DeLander, 1994).

In view of the marked enhancement of adenosine released by local co-administration of 2′-deoxycoformycin with 5% formalin, we investigated if 2′-deoxycoformycin could alter nociceptive responses or the change in paw volume produced by 5% formalin. However, we did not observe any antinociceptive effect of 2′-deoxycoformycin on either flinching or biting/licking behaviours at 5% formalin (data not shown). One explanation is that the adenosine amounts released under these conditions were high enough to activate not only adenosine A_1 receptors producing antinociceptive effects, but also adenosine A_{2A} and A_3 receptors producing pronociceptive effects (Sawynok, 1998) and thus the antinociceptive effects were obscured. Another possibility is that, at 5% formalin, so many nociceptive mediators were released (cf. Tjølsen et al., 1992) that the effect of adenosine was obscured amongst the multiple effects.

The lack of effect of 2′-deoxycoformycin on paw volume at 5% formalin over the 3-h time course at 30-min intervals (data not shown) reflects a similar involvement of multiple mediators in generating this effect at 5% formalin (Damas and Liégeois, 1999). Curiously, no modulatory effect on paw volume was observed with 5′-amino-5′-deoxyadenosine at either 0.5% or 1.5% formalin over this time course (data not shown). (Earlier time intervals at 10 and 20 min, considered more relevant to the neurochemical observations, could not be examined, as the stress of

repeated handling at the short time intervals prevented formalin from increasing the paw volume). In other paradigms of inflammation, systemic inhibition of adenosine kinase produced anti-inflammatory actions (Cronstein et al., 1994; Firestein et al., 1994; Rosengren et al., 1995). The lack of effect of the inhibition of adenosine kinase in this study may indicate (a) that paw volume alone reflects only a limited aspect of the inflammatory response, and that other anti-inflammatory endpoints are not reflected in this paradigm, or that (b) when adenosine kinase inhibitors are administered systemically, a component of their activity is mediated at spinal, rather than at peripheral, sites (cf. Bong et al., 1996). It is likely that the influence of inhibiting adenosine kinase on inflammation is dependent on degree of inflammation and substrate adenosine concentration.

5. Conclusion

In summary, this study demonstrates that subcutaneous formalin evokes adenosine in the rat hind paw. The pattern of adenosine release is dependent on formalin concentration, which suggests that at different levels of inflammation, different mechanisms mediate this release. The ability of inhibitors of adenosine kinase and adenosine deaminase to modulate this release accords with the kinetics of the two enzymes.

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