Acetazolamide, a Carbonic Anhydrase Inhibitor, Reverses Inflammation-Induced Thermal Hyperalgesia in Rats

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ABSTRACT

Inflammatory pain is linked to reduction in tissue pH. Tissue proton generation is mainly mediated by carbonic anhydrases (CAs). We therefore hypothesized that inhibition of CAs with acetazolamide (ACTZ) increases the tissue pH and reverses inflammation-induced pain. CAs are also present in the central nervous system and control anion concentrations. Furthermore, ACTZ has direct effects on ion channels involved in nociception. In the current study, responses to heat and mechanical stimuli (von Frey filaments) of the paw were assessed before and after carrageenan-induced muscle inflammation and after treatment with ACTZ in rats. ACTZ was administered systemically, locally, or intrathecally 24 h after the induction of inflammation. In separate studies, pH was measured in the inflamed and noninflamed muscles and after administration of ACTZ. Carrageenan injection to the gastrocnemius muscle produced heat hyperalgesia and mechanical allodynia of the paw. Systemic ACTZ reversed the heat hyperalgesia but not mechanical allodynia. Similarly, injections of ACTZ into the inflamed muscle or intrathecally reversed the heat hyperalgesia but not mechanical allodynia. Surprisingly, the pH in the inflamed muscle was not reduced compared with noninflamed muscle. Thus, the current data do not support our hypothesis that ACTZ reduces inflammatory hyperalgesia by raising the reduced pH in muscle. Although the possibility of pH changes and the role of CAs in the microenvironment cannot be ruled out, the mechanism of ACTZ-induced antihyperalgesia is not clear from this study. It is possible that the inhibition of ion channels and/or the inhibition of spinally located CAs contribute to the observed antihyperalgesia.

A reduction in extracellular pH in muscle tissue produces pain in a pH-dependent manner (Issberner et al., 1996), possibly through the activation of acid-sensing ion channels and/or transient receptor potential vanilloid channels (Krish- tal and Pidoplichko, 1980; Waldmann et al., 1997; Tominaga et al., 1998). Extracellular tissue pH is reduced in an isolated perfused heart preparation during ischemia (Jacobus et al., 1977) in the paw or gastrocnemius muscle after incision (Woo et al., 2004) in periarticular soft tissue during different phases of antigen-induced arthritis (Andersson et al., 1999) and in exudates from inflammatory conditions (Häbler, 1929, 1930; Cummings and Nordby, 1966). One of the major bio-chemical reactions involved in proton generation in tissues at rest is through reversible hydration of carbon dioxide, mediated by carbonic anhydrase (CA) enzymes (Chen and Chesler, 1992). It is proposed that CAs are involved in the extracellular control of pH in muscle (Wetzel et al., 2001). In the muscle, most of the subtypes of CAs (CA II, III, IV, and V) are found intracellularly, although there is at least one iso- form, CA IV, that is found extracellularly on the sarcolemma (Geers et al., 1985; Decke and Decke, 1996; Geers and Gru, 2000). There is significant carbonic anhydrase activity in the extracellular space in the brain in rat hippocampal slice preparations (Chen and Chesler, 1992). Carbonic anhydrase inhibition by acetazolamide (ACTZ) in hippocampal slices enhances the alkaline shift in the extracellular space produced by neuronal activation with glutamate (Chen and Chesler, 1992). However, the role of CAs in the regulation of pH within the interstitial space, where the nociceptor terminals are located, is not known. Saturated carbon dioxide solution is a source of protons and activates a distinct sub- population of mechanohot-sensitive “polymodal” C-units in rat skin-nerve preparation (Steen et al., 1992), which are inhibited by the application of the carbonic anhydrase inhib- itor ACTZ. Thus, CAs generate protons in the tissues from carbon dioxide, resulting in increased primary afferent fiber activity. In humans, referred pain experienced after abdom- inal laparoscopic surgery under carbon dioxide insufflation is

ABBREVIATIONS: CA, carbonic anhydrase; ACTZ, acetazolamide; PWL, paw withdrawal latency.
reduced by systemic ACTZ (Woehlck et al., 2003), supporting a role for Ca₂⁺ in nociception. However, ACTZ also inhibits ion channels (Pickkers et al., 2001; McNaughton et al., 2004) that are known to be involved in pain transmission. Thus, ACTZ could have effects peripherally and/or centrally to reduce hyperalgesia by acting on carbonic anhydrases and/or ion channels.

Based on the above-mentioned findings, and taking into consideration the fact that muscle injury increases the levels of Ca₂⁺ (Bohmeyer et al., 1994), we hypothesized that inhibition of carbonic anhydrases in the muscle reduces the generation of protons in the extracellular space and reduces inflammation-induced hyperalgesia. In the current study, although ACTZ reversed the inflammation-induced heat hyperalgesia, the tissue pH did not reduce during inflammation. This observation was contrary to our original hypothesis that ACTZ reduces inflammatory hyperalgesia by raising the reduced pH in inflated muscle. In an effort to elucidate alternative sites of action for ACTZ-induced antihyperalgesia, we also tested the central effects of ACTZ by administering ACTZ spinaly.

**Materials and Methods**

**Animals.** Male Sprague-Dawley rats weighing 250 to 300 g (n = 115) (Harlan, St. Louis, MO) were housed in 12-h dark/light cycle with free access to standard rat chow and water. Animals were brought to the behavioral testing room the day before to acclimatize them to the testing environment. All behavioral tests were done during the light cycle of the day. Experiments were preapproved by University of Iowa Animal Care and Use Committee and were carried out according to the guidelines of the International Association for the Study of Pain and National Institute of Health (Zimmermann, 1983).

**Heat Testing.** On the day of testing, rats were kept in Lucite cubes on an elevated platform with a clear glass top for about 30 min for acclimatization. A high-intensity radiant heat source was used as the stimulus. The heat source was positioned below the plantar skin of the hind paw, and the beam was switched on, simultaneously starting a built-in timer. When the animal withdrew the paw abruptly to heat stimulus, the heat source and the timer were stopped. The duration in seconds from the start of heat application to the paw withdrawal was taken as the paw withdrawal latency (PWL). PWLs were determined five times bilaterally, with an interval of 5 min between each test, and the mean of five readings was kept constant in all experiments with a constant voltage-power supply of 5 min between each test, and the mean of five readings was calculated for each experiment using pH 4.0 and 7.0 standard buffers and checked for accuracy before each measurement using pH 7.0 buffer. Measurements were recorded bilaterally.

**Intrathecal Cannulation.** For intrathecal drug administration, a 32-gauge polyethylene catheter was placed intrathecally as described before (Storkson et al., 1996; Pogatzki et al., 2000). Briefly, rats were anesthetized with 2% halothane, and the dorsal surface of the spine was shaved and cleaned with Betadine solution. A 2-cm incision was made at the iliac crest. A 32-gauge polyethylene catheter (ReCathCo LLC, Allison Park, PA) was introduced into the lumbar space between L4 and L5 with the help of a 23-gauge guide needle and was advanced to a length of 3.0 to 3.5 cm rostrally. The catheter was fixed in place, and the tip was connected to a sterile saline-filled PE10 tube, which was externalized dorsally between the scapulas. The tip of the catheter was sealed, and the animal was allowed to recover for 5 to 7 days.

**Experimental Protocol.** Animals were tested for withdrawal latency to heat and mechanical threshold before and 24 h after injection of carrageenan. Vehicle or ACTZ was then administered: 1) intraperitoneally (saline, n = 6; 10 mg/kg ACTZ, n = 4; 100 mg/kg ACTZ, n = 7; 200 mg/kg ACTZ, n = 6), 2) locally in the inflamed muscle (saline, n = 5; 1 mg of ACTZ, n = 6; 5 mg of ACTZ, n = 7), 3) intrathecally (saline, n = 4; 1 nmol of ACTZ, n = 5; 10 nmol of ACTZ, n = 4; 100 nmol of ACTZ, n = 5). To control for potential systemic effects of local i.m. injection of ACTZ, 5 mg of ACTZ (n = 3) was injected into the contralateral gastrocnemius muscle. In separate groups of noninflamed animals, withdrawal latency to heat and mechanical thresholds were determined before and after intraperitoneal administration of saline or ACTZ to study the effects of ACTZ on the nociceptive thresholds in normal animals (saline, n = 4; 100 mg/kg ACTZ, n = 4; 200 mg/kg ACTZ, n = 4). Animals treated with ACTZ or vehicle were tested for paw withdrawal latency to heat and mechanical withdrawal thresholds as follows: 1) 30 min after intraperitoneal administration, 2) 10 min after local or intrathecal administration. In pilot experiments, 300 mg/kg systemic ACTZ caused hematuria. Higher doses of intrathecal ACTZ (1000, 500, and 200 nmol) caused untoward effects like shivering and increased locomotor activity. Thus, 200 mg/kg i.p. and 100 nmol i.t. were the maximum systemic and intrathecal doses, respectively, used in the current study that did not cause any visible side effect. In the animals treated with 200 mg/kg ACTZ intraperitoneally, the circumference around the inflamed gastrocnemius muscle was measured before and 30 min and 1 h after ACTZ to examine the effect of ACTZ on the nociceptive thresholds in normal animals.
on inflammation. The experimenter was blinded to treatment groups after the collection of preliminary data.

The measurement of pH was done bilaterally in the gastrocnemius muscle in separate groups of animals 24 h after carrageenan injection in the muscle (n = 12) or saline injection in the muscle (n = 5). In the group with inflammation, muscle pH was measured 1) 15 min, 30 min, and in some cases 45 min and 1 h after intraperitoneal administration of saline (n = 4), 10 mg/kg ACTZ (n = 4), 100 mg/kg ACTZ (n = 4), or 200 mg/kg (n = 4); 2) 15 min, 30 min, 45 min, and 1 h after intrathecal administration of saline (n = 3) or 100 nmol of ACTZ (n = 5). The experimenter was blinded to treatment groups after the collection of preliminary data.

Drugs. A-Carrageenan (type IV) was obtained from Sigma-Aldrich (St. Louis, MO), and ACTZ for injection USP (Ben Venue Laboratories, Inc., Bedford, OH) was obtained from the University of Iowa Hospitals and Clinics Pharmacy. Carrageenan and ACTZ were dissolved in sterile saline. The minimum pH at which ACTZ goes into solution was adjusted to a pH of 8.0 using 0.1 N sodium hydroxide solution. Solutions were 7.8 to 8.0. Therefore, the saline for control injections was prepared as mean ± S.E.M. Mechanical withdrawal thresholds are presented as median with 25th and 75th percentiles. The PWLs and pH values on the contralateral and ipsilateral sides were compared independently across time (baseline, postinflammation, and post-treatment) for drug effects (saline and different doses of ACTZ) using a multivariate analysis of variance followed by a Tukey’s post hoc test between groups. Mechanical withdrawal thresholds were analyzed using a Kruskal-Wallis analysis of variance. Thresholds of ipsilateral and contralateral sides were compared separately for drug effects (saline and different doses of ACTZ) at each time point (baseline, postinflammation, and post-treatment). If a difference was observed, Wilcoxon signed ranks test was used to examine differences between groups. The level of statistical significance was set at p < 0.05. Statistical analysis was performed using SPSS version 11.5.0 software (SPSS Inc., Chicago, IL).

Results

Effect of Carrageenan Injection in the Muscle. Injection of 3% carrageenan into the left gastrocnemius muscle produced visible inflammation of the muscle at 24 h. The circumference of the inflamed gastrocnemius muscle was significantly greater (7.02 ± 0.24 cm) compared with the contralateral noninflamed muscle (5.75 ± 0.14 cm). The inflammation was accompanied by a significant ipsilateral reduction in withdrawal latency to heat and mechanical withdrawal threshold measured from the plantar aspect of the paw (Fig. 1, A and B, respectively). For the purpose of this study, we define these consistent and statistically significant reductions in withdrawal latency to heat and mechanical withdrawal threshold as heat hyperalgesia and mechanical allodynia, respectively.

Dose Titration and Side Effects of ACTZ. By titration, 200 mg/kg was found to be the highest systemic dose, and 100 nmol (22.24 μg) was the highest intrathecal dose that did not cause visible side effects. A dose of 300 mg/kg, i.p. produced hematuria in the animals; however, this dose did not produce abnormal gait or signs of motor dysfunction in a placement reflex test. Intrathecal administration of ACTZ at doses of 200, 500, and 1000 nmol caused side effects in the rats, such as shivering and increased locomotion inside the cubicle.

Effect of ACTZ on the Heat Hyperalgesia. ACTZ significantly reversed heat hyperalgesia (p = 0.002, 100 mg/kg; p = 0.0001, 200 mg/kg, i.p.) on both hindlimbs (right paw 6.63 ± 0.11 s; left paw 6.12 ± 0.18 s). The in-flamed muscle or intrathecally into spinal cord. ACTZ (5 mg in 100 μl) injected directly into the inflamed muscle significantly reversed the inflammation-induced reduction in PWL (p = 0.0001) ipsilaterally when compared with intramuscular saline controls (Fig. 2B). A lower dose of 1 mg did not show any effect on the PWL. To test whether 5 mg of ACTZ i.m. produced a systemic effect, the same dose of ACTZ (5 mg in 100 μl) was injected into the contralateral gastrocnemius muscle. Contralateral ACTZ injection had no effect on the ipsilaterally decreased PWL produced by muscle inflammation or on the baseline PWL of the injected limb, supporting the conclusion that ACTZ in muscle produces a local effect (Fig. 2B). The effective local dose (5 mg) was selected by trial and error, which is less than the lowest effective systemic dose (100 mg/kg). Administration of ACTZ to the spinal cord (100 nmol = 22.24 μg, i.t.) also significantly reversed (p = 0.006) the decreased PWL to heat compared with the PWL after intrathecal saline (Fig. 2C). Thus, systemic, local, and intrathecal administration of ACTZ reversed the secondary heat hyperalgesia caused by carrageenan inflammation of the muscle.

Effect of ACTZ on Mechanical Allodynia. Systemically administered ACTZ (10–200 mg/kg, i.p.) had no effect on the

Fig. 1. Bar graphs show PWL to radiant heat (A) or withdrawal threshold to von Frey filaments (B) before (hatched bar) and after (open bar) induction of inflammation with carrageenan. There were significant decreases in PWL (A, p < 0.0001, Student’s t test) and mechanical withdrawal threshold (B, p < 0.0001, Wilcoxon signed ranks test) after carrageenan injection when compared with baseline. Values are mean ± S.E.M. for PWL and median with 25th and 75th percentiles for mechanical threshold. *, significantly different from baseline values, p < 0.05.
reduced mechanical thresholds induced by carrageenan inflammation (Fig. 2D). Similarly, direct injection of ACTZ (1–5 mg) into the inflamed muscle or intrathecal injection into the spinal cord (1–100 nmol) had no effect on the reduced mechanical threshold produced by carrageenan (Fig. 2, E and F). Thus, ACTZ has no effect on mechanical allodynia produced by carrageenan inflammation. Mechanical withdrawal thresholds were also unaffected by systemic ACTZ in normal noninflamed animals (data not shown).

Effects of ACTZ on Inflammation. The highest systemic dose of ACTZ (200 mg/kg, i.p.) used in behavior studies had no effect on muscle inflammation. The circumference around the inflamed gastrocnemius muscle before ACTZ was 6.92 ± 0.15 cm compared with 7.04 ± 0.14 and 6.96 ± 0.16 cm, 30 min and 1 h after ACTZ, respectively.

pH Changes in the Muscle after ACTZ. In animals injected with carrageenan in the left gastrocnemius muscle, the pH was 7.16 ± 0.03 on the ipsilateral side and 7.21 ± 0.03 on the contralateral side 24 h after injection. In animals injected with saline in the gastrocnemius muscle instead of 3% carrageenan, the pH was 7.16 ± 0.04 (ipsilateral) and 7.10 ± 0.07 (contralateral) 24 h after injection. There was no significant difference in the pH of the inflamed gastrocnemius muscle before ACTZ was 6.92 ± 0.15 cm compared with 7.04 ± 0.14 and 6.96 ± 0.16 cm, 30 min and 1 h after ACTZ, respectively.

In pilot experiments, to determine the validity of the pH measurement technique, we measured pH in the left gastrocnemius muscle during ischemia induced by partial blockade of left common iliac artery. The pH of the left gastrocnemius muscle dropped from 7.15 to 6.5, whereas the pH of the right muscle remained unchanged (from 7.1 to 7.2) 30 min after induction of ischemia.

Discussion

Peripheral Antihyperalgesic Effects of ACTZ. In the current study, systemic ACTZ (100 and 200 mg/kg, i.p.) reverses secondary heat hyperalgesia induced by muscle inflammation. The highest systemic dose of ACTZ (200 mg/kg) also increased the basal nociceptive threshold in normal noninflamed animals. However, this increase was not as pronounced when compared with the inflamed animals, suggest-
ing an increased efficacy of ACTZ after inflammation. Furthermore, the injection of 5 mg of ACTZ into the inflamed muscle reverses the heat hyperalgesia. Injection of the same effective dose of ACTZ into the contralateral noninflamed muscle had no effect on the hyperalgesia, ruling out systemic effects as well as local effect in noninflamed muscle for this dose. Therefore, the antihyperalgesic activity of ACTZ has a peripheral site of action in inflamed muscle.

During tissue damage and inflammation, the levels of CAs increase (Bohlmeier et al., 1994), which could increase proton formation. Most isoforms of CAs are found intracellularly, but at least one form (CA IV) is found on the sarclemma (Decker et al., 1996). Furthermore, CAs are found in medium to large dorsal root ganglia (Wong et al., 1987), suggesting that CAs are found on peripheral terminals of primary afferents. Although CAs play a major role in tissue proton generation, in the current study, inflammation did not reduce the pH in the muscle. It should be noted, however, that ACTZ did increase the pH in the muscle after systemic or local injection. One possibility is that inflammation reduces pH in the interstitial space in the muscle, which could not be detected with our methodology and that ACTZ could reverse the interstitial pH reduction caused by inflammation.

Central Antihyperalgesic Effects of ACTZ. Since ACTZ crosses the blood-brain barrier (Hanson et al., 1981), it is possible that the ACTZ has a central site of action. The current study shows that very low doses of intrathecal ACTZ reduces inflammation-induced heat hyperalgesia, confirming a role for CA in pain processing in the central nervous system. However, the therapeutic window for intrathecal ACTZ is very narrow; doses above 100 nmol cause side effects, whereas 10 nmol is ineffective. Importantly, the intrathecal injection of 100 nmol did not affect the pH in the inflamed muscle. Thus, the reversal of heat hyperalgesia by intrathecal ACTZ is independent of its effect on local tissue pH.

Our findings are supported by a prior study that showed spinal blockade of carbonic anhydrase prevents the decrease in the withdrawal latency to heat produced by drugs acting at GABA receptors (pentobarbital and midazolam) (Wang et al., 2003). The enhancement of withdrawal latency is suggested to occur through an “anion shift” at GABA \textsubscript{A} receptors caused by outward flux of bicarbonate ions (\text{HCO}_3^-) instead of the normally occurring influx of Cl\textsuperscript{-} ions (Archer and Roth, 1999). This shift in anion produces a depolarization of the postsynaptic cell, causing excitation rather than inhibition. Since CAs mediate the production of bicarbonate ions, one could hypothesize that blockade of CAs in the spinal cord reduces production of bicarbonate ions (Wang et al., 2003). It
follows that ACTZ, by inhibiting production of bicarbonate ions, reduces excitation of GABA_A receptors in the spinal cord, thereby attenuating hyperalgesia. In support, a similar shift in the anion gradient occurs in spinal neurons after peripheral nerve injury (Coull et al., 2003). Clinically, carbonic anhydrase inhibitors are used to treat epilepsy, where central neuronal excitation plays an important role in the etiology. Carbonic anhydrase enzymes in the central nervous system, specifically CA VII, are suggested as a putative target of CA inhibitors when used as antiepileptic drugs (Rivera et al., 2005).

Alternative Antinociceptive Mechanisms of CA Inhibitors. It is possible that ACTZ is acting on other targets that could also contribute to its antinociceptive activity. One such possibility is the opening of calcium-activated K^+ channels by ACTZ (Pickkers et al., 2001). The opening of calcium-activated potassium channels in the periphery is antinociceptive in the formalin test (Ortiz et al., 2003). Another possibility is blockade of calcium channels, specifically α_{EL} Ca^{2+} channels, by carbonic anhydrase inhibitors (McNaughton et al., 2004). These channels are important in mediating inflammatory pain and formalin-induced pain behavior (Sluka, 1997, 1998; Saegusa et al., 2000).

Differential Effects of ACTZ on Heat Hyperalgesia versus Mechanical Allodynia. In the current study, ACTZ reverses heat hyperalgesia, but not mechanical allodynia, produced by inflammation. The lack of effect on mechanical allodynia is surprising, and we currently have no explanation for this observation. However, differences between development of heat hyperalgesia and mechanical allodynia, or effects of drugs on heat hyperalgesia and mechanical allodynia, have been observed in numerous studies (Meller et al., 1993; Ossipov et al., 1999; Caterina et al., 2000; Sluka, 2002; Neubert et al., 2003; Vogel et al., 2003; Walker et al., 2003). Activation of ionotropic NMDA receptors alone in the spinal cord produces thermal hyperalgesia, but coactivation of ionotropic AMPA and metabotropic glutamate receptors is required to produce mechanical allodynia (Meller et al., 1993). Peripheral injection of capsaicin, or administration of 8-BrcAMP to the spinal cord, produces mechanical allodynia simultaneously with heat hyperalgesia (Sluka, 2002). Both the effects are reversely by spinal blockade of adenylate cyclase or protein kinase A, suggesting cAMP involvement in the spinal cord differentially mediates heat and mechanical responsiveness. In mice lacking the transient receptor potential vanilloid-1 channel and in rats administered with resinsiferatoxin, heat, but not mechanical allodynia, is reduced with peripheral inflammation (Caterina et al., 2000) or nerve injury (Ossipov et al., 1999), respectively. Thus, there are a number of peripheral and central factors that differentially mediate heat and mechanical allodynia.

Inflammation and Tissue pH. Although it is generally believed that pH decreases during inflammation, in the current study we did not observe a decrease in pH of the inflamed muscle. This is an important and surprising finding. To our knowledge, there are no reports in the literature showing reductions in extracellular pH following muscle inflammation. However, reduction in pH in ischemic tissues, arthritic joints, and incisional wounds have been reported in humans and animals (Jacobus et al., 1977; Andersson et al., 1999; Woo et al., 2004), including in exudates from patients with inflammatory conditions, such as arthritis, mastitis, and bursitis (Habler, 1929, 1930; Cummings and Bordby, 1966; Geborek et al., 1989). Furthermore, there is a wide range of pH decreases observed in the literature with reductions ranging from 0.1 pH unit in inflamed tissue (Andersson et al., 1999) to as great as 2.0 pH units in exudate (Habler, 1929).

The current study measured pH with a large needle electrode (16-gauge) placed within the gastrocnemius muscle. Since extracellular fluid and circulating blood contribute to the observed pH with this method, it is likely that the measured pH does not reflect interstitial pH where nociceptors are located. However, inhibition of sarcolemmal CAs reduces interstitial pH in an in vitro muscle preparation (Geers and Gros, 2000). In contrast, lactate increased surface pH, and the increase in pH was enhanced by a CA inhibitor in a single muscle fiber in vitro, measured using microelectrode (Wetzell et al., 2001). Previously, we measured pH after injection of acidic saline into the gastrocnemius muscle using the same electrode as in the current study (Sluka et al., 2001). The decrease after pH 4.0 saline injection averaged pH 6.5 and was completely reversed by 7 min after injection, showing the rapid buffering capacity of the muscle (Sluka et al., 2001). Thus, in muscle, where blood flow is high and CAs are present, detection of reduction in pH as a result of inflammation may be difficult.

The current study was designed using a poorly cell-permeable CA inhibitor (ACTZ; Wetzell et al., 2001) to confine the inhibition to membrane CAs that presumably modulate interstitial pH; however, from the present data, it is difficult to conclude whether inflammatory hyperalgesia observed in this model is mediated by reduced pH in the microenvironment. It may be noted that systemic and local ACTZ administration increases muscle pH and reverse hyperalgesia, suggesting a change in CA activity within the muscle maintains hyperalgesia.

In conclusion, ACTZ reduces inflammation-induced heat hyperalgesia, probably acting both peripherally and centrally. Identifying the specific target(s) of ACTZ action and the carbonic anhydrase isofrom(s) involved in causing hyperalgesia in inflammation will be pivotal in discovering more selective CA inhibitors useful in treating pain with reduced side effects.

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