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Title Page

Acetazolamide, a carbonic anhydrase inhibitor, reverses inflammation-induced thermal hyperalgesia in rats.

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Running Title Page

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Abbreviations: CA = carbonic anhydrase; ACTZ = acetazolamide; PWL= paw withdrawal latency; ANOVA = analysis of variance; SEM = standard error of mean

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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. Further, 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 induction of inflammation. In separate studies, pH was measured in the inflamed and non-inflamed 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 to 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 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 inhibition of ion channels and/ or inhibition of spinally located CAs contribute to the observed antihyperalgesia.

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Introduction

A reduction in extracellular pH in muscle tissue produces pain in a pH dependant manner (Issberner et al., 1996) possibly through activation of acid sensing ion channels (ASICs) and/or transient receptor potential vanilloid (TRPV) channels (Krishtal 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; Häbler, 1930; Cummings and Nordby, 1966). One of the major biochemical 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 isoform, CA IV, that is found extracellularly on the sarcolemma (Geers et al., 1985; Decker et al., 1996; Geers and Gros, 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 subpopulation of mechanoheat sensitive "polymodal" C-units in rat

skin-nerve preparation (Steen et al., 1992) which are inhibited by the application of the carbonic anhydrase inhibitor, ACTZ. Thus, CAs generate protons in the tissues from carbon dioxide resulting in increased primary afferent fiber activity. In humans, referred pain experienced after abdominal laparoscopic surgery under carbon dioxide insufflation is reduced by systemic ACTZ (Woehlck et al., 2003), supporting a role for CAs 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 CA (Bohlmeyer 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 inflamed muscle. In an effort to elucidate alternative sites of action for ACTZ-induced antihyperalgesia, we tested the central effects of ACTZ also, by administering ACTZ spinally.

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Methods

Animals

Male Sprague-Dawley rats weighing 250 – 300 g (n = 115) (Harlan, St. Louis, Missouri, USA) were housed in 12h 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 pre-approved 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 cubicles 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 5 readings was taken as the PWL for each time. The intensity of the heat source was kept constant in all experiments with a constant voltage-power supply to obtain baseline response times between 12 – 16 s. Cut-off time was set to 25 s to prevent damage to the skin. The

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validity and test-retest reliability of this method was previously established (Hargreaves et al., 1988; Sluka et al., 1999). A decrease in withdrawal latency is interpreted as heat hyperalgesia for the purpose of this study.

Mechanical testing

Animals were kept in a Lucite cubicle on an elevated platform with a mesh wire top. Threshold to mechanical stimuli was tested using von Frey filaments with increasing bending forces as described elsewhere (Sluka et al., 2001). Briefly, the filament with the lowest threshold was applied perpendicularly to the plantar surface of the hind paw two times and observed for a withdrawal. If there was no response, the next higher force filament was tested. The value of the lowest force filament causing a withdrawal of the paw was taken as the mechanical threshold. This was confirmed by applying filaments one level up and one level down (up and down method). 350 mN was set as the cut-off. The following bending forces were used: 8, 12, 16, 32, 44, 56, 75, 104, 162, 350 mN. The reliability of this testing method was previously established (Gopalkrishnan and Sluka, 2000). A decrease in withdrawal threshold compared to baseline is interpreted as mechanical allodynia in this study.

Induction of inflammation

The hindpaw skin overlying the gastrocnemius muscle of the rats was shaved and cleaned with alcohol prep-pads. Rats were injected with 100 µl of 3% carrageenan suspension percutaneously into the left gastrocnemius muscle belly under halothane anesthesia (2-4 % in oxygen). Animals were returned to their cages and left for 24 h for

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the inflammation to develop. At 24 h, the circumference of the inflamed and the non-inflamed gastrocnemius muscles were measured over the skin using a measuring tape to confirm the development of inflammation.

Measurement of tissue pH in the gastrocnemius muscle

Animals were anesthetized with halothane and kept on a heating pad (37° C) to keep the body temperature stable. The skin above the gastrocnemius muscle was cleaned with an alcohol prep-pad and an incision was made to expose the gastrocnemius muscle. A calibrated needle pH electrode (16G, ORION), connected to a pH meter (210 A+, ORION), was placed about 0.5 cm deep into the muscle, taking care not to puncture any major blood vessels. If bleeding occurred during insertion, then the needle was removed and inserted into a new spot. The reading on the pH meter was allowed to stabilize and recorded. The pH needle was calibrated before each set of measurements using pH 4.0 and pH 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 G 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 shaved and cleaned with Betadine® solution. A 2 cm incision was made at the iliac crest. A 32 G polyethylene catheter (Recathco LLC, PA) was introduced into the lumbar space between L4-L5 with the help of a 23 G guide needle and advanced to a length of 3.0 - 3.5 cm rostrally. The

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catheter was fixed in place and the tip 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 5-7 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 (i) intraperitoneally (saline, n=6; ACTZ 10 mg/kg, n=4; ACTZ 100 mg/kg, n=7; ACTZ 200 mg/kg, n=6) (ii) locally in the inflamed muscle (saline, n=5; ACTZ 1 mg, n=6; ACTZ 5 mg, n=7) or (iii) intrathecally (saline, n=4; ACTZ 1 nmol, n=5; ACTZ 10 nmol, n=4; ACTZ 100 nmol, n=5). To control for potential systemic effects of local i.m. injection of ACTZ, 5 mg ACTZ (n=3) was injected into the contralateral gastrocnemius muscle. In separate groups of non-inflamed 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; ACTZ 100 mg/kg, n=4; ACTZ 200 mg/kg, 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, 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

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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 inflammation. The experimenter was blinded to treatment groups after collection of preliminary data.

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 i) 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), 200 mg/kg (n=4); ii) 15 min, 30 min, 45 min and 1 h after intrathecal administration of saline (n=3) or ACTZ (100 nmol, n=5). The experimenter was blinded to treatment groups after collection of preliminary data.

Drugs

Lambda - carrageenan (type IV) was obtained from Sigma Chemical Company, USA and ACTZ for injection USP (Ben Venue Laboratories, Inc., 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 7.8 – 8.0. Therefore the saline for control injections was adjusted to a pH of 8.0 using 0.1 N sodium hydroxide solution.

Statistical analysis

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PWL to heat, and pH values are presented as mean ± SEM. Mechanical withdrawal thresholds are presented as median with 25^{th} and 75^{th} percentiles. The PWLs and pH values on the contralateral and ipsilateral sides were compared independently across time (baseline, post-inflammation, post-treatment) for drug effects (saline, different doses of ACTZ) using a multivariate ANOVA followed by a Tukey's posthoc test between groups. Mechanical withdrawal thresholds were analyzed using a Kruskal-Wallis ANOVA. Thresholds of ipsilateral and contralateral sides were compared separately for drug effects (saline, different doses of ACTZ) at each time point (baseline, post-inflammation, 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.

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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 to the contralateral non-inflamed 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 1B, 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 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

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ACTZ significantly reversed (p=0.002, 100 mg/kg; p=0.0001, 200 mg/kg, i.p.) the carrageenan-induced decrease in PWL to heat on the inflamed limb compared to the i.p. saline treated group 30 min after drug administration (Fig. 2A). The dose of 200 mg/kg ACTZ significantly increased (p=0.048) the PWL of the contralateral limb above baseline (18.21±0.49 s after ACTZ compared 14.01±0.29 s after saline control).

In non-inflamed animals, intraperitoneal injection of ACTZ at the dose of 200 mg/kg, but not saline or ACTZ 100 mg/kg, significantly increased the paw withdrawal latency to heat on both hindlimbs (right paw =12.11 ± 0.55 s, p=0.016; left paw =12.65 ± 0.45 s, p=0.006) compared to baseline (right paw =9.10 ± 0.56 s; left paw =10.24 ± 0.25 s). Thus ACTZ, apart from reversing hyperalgesia, also possesses antinociceptive activity at a higher dose. The increases in PWL after ACTZ (200 mg/kg, i.p.) from baseline in normal non-inflamed animals (right paw = 3.0±0.6; left paw = 2.4±0.38) were significantly less than in animals with inflammation (contralateral paw = 5.9±0.61, p=0.016, ipsilateral paw = 6.63±1.02, p=0.018).

To test the site of action, ACTZ was injected locally into the inflamed 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 to intramuscular saline controls (Fig. 2B). A lower dose of 1 mg did not show any effect on the PWL. To test if 5 mg 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).

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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 to the PWL after intrathecal saline (Fig. 2 C). Thus systemic, local, and intrathecal administration of ACTZ reversed the secondary heat hyperalgesia caused by carrageenan inflammation of the muscle.

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Effect of ACTZ on mechanical allodynia

Systemically administered ACTZ (10-200 mg/kg., i.p.) had no effect on the reduced mechanical thresholds induced by carrageenan inflammation (Fig. 2 D). 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 (Figs. 2 E, 2 F). Thus ACTZ has no effect on mechanical allodynia produced by carrageenan inflammation. Mechanical withdrawal thresholds were also unaffected by systemic ACTZ in normal non-inflamed 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 to 7.04±0.14 cm and 6.96±0.16 cm, 30 min and 1 h after ACTZ respectively.

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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 (24 h post injection) 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 muscle (7.16±0.03), 24 h after carrageenan injection, compared to the contralateral non-inflamed muscle (7.21±0.03) or to saline-injected muscle in control animals (7.16±0.04) (Fig. 3 A). ACTZ injection (10 mg/kg, 100 mg/kg, or 200 mg/kg, i.p.) increased the muscle pH bilaterally 15 min and 30 min after ACTZ compared to saline controls. The increases in pH were significant in the inflamed muscle at 15 min and in both muscles at 30 min (Fig. 3A & B). The change in pH was not dose-dependent with 10 mg/kg producing similar increases in pH as 100 and 200 mg/kg doses (Fig. 3 A & B). In animals injected with 200 mg/kg ACTZ, pH was measured at two additional time points – 45 min and 1 h after ACTZ or saline injections. The pH on both left and right muscles stayed significantly higher in ACTZ treated animals (7.33±0.05 on the contralateral, $p=0.03$; 7.27±0.01 on the ipsilateral, $p=0.049$) compared to saline controls (contralateral = 7.17±0.02; ipsilateral = 7.18±0.04) at 45 min, but not at 1 h.

Local injection of ACTZ (5 mg) into the muscle increased the muscle pH which remained increased up to 30 min compared to saline controls (Fig. 4). Intrathecal administration of ACTZ (100 nmol) did not affect the muscle pH, observed for 30 min after the injection (Fig. 4). Thus the intrathecal ACTZ produces antihyperalgesia independent of changes in muscle pH.

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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.

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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 non-inflamed animals. However this increase was not as pronounced when compared to the inflamed animals suggesting an increased efficacy of ACTZ after inflammation. Further, injection of ACTZ (5 mg) into the inflamed muscle reverses the heat hyperalgesia. Injection of the same effective dose of ACTZ into the contralateral non-inflamed muscle had no effect on the hyperalgesia, ruling out systemic effects as well as local effect in non-inflamed 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 (Bohlmeyer 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 sarcolemma (Decker et al., 1996). Further, CAs are found in medium to large DRG (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.

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Central antihyperalgesic effects of ACTZ

Since ACTZ crosses the blood-brain barrier (Hanson et al., 1981), it 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, with doses above 100 nmol causes side effects while 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 $GABA_A$ receptors caused by outward flux of bicarbonate ions (HCO_3^-) instead of the normally occurring influx of Cl^- 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., 2004).

Alternative antinociceptive mechanisms of CA inhibitors

It is possible that ACTZ is acting on other targets which could also contribute to its antinociceptive activity. One such possibility is the opening of calcium- activated K^+ channel by ACTZ (Pickkers et al., 2001). Opening of calcium activated potassium channels in the periphery is antinociceptive in formalin test (Ortiz et al., 2003). Another possibility is blockade of calcium channels, specifically α_{1E} Ca^{2+} channels, by carbonic anhydrase inhibitors (McNaughton et al., 2004). These channels are important in mediating inflammatory pain, and formalin-induced pain behaviors (Sluka, 1997, 1998; Saegusa et al., 2000).

Differential effects of ACTZ on heat hyperalgesia vs. 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 co-activation 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-Br-cAMP to the spinal cord, produces mechanical allodynia simultaneously with heat hypoalgesia (Sluka 2002). Both the effects are reversed by spinal blockade of adenylate cyclase or protein kinase A (PKA) suggesting cAMP involvement in the spinal cord differentially mediates heat and mechanical responsiveness. In mice lacking the TRPV1 channel and in rats administered with resiniferatoxin, heat, but not mechanical allodynia is reduced in animals 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 (Häbler, 1929; Häbler, 1930; Geborek et al., 1989;

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Cummings and Nordby, 1966). However, 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 (Häbler et al., 1929).

The current study measured pH with a large needle electrode (16 G) 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 (Wetzel 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, Wetzel 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. However, systemic and local ACTZ administration increase muscle pH and reverse hyperalgesia, suggesting a change in CA activity within the muscle maintains hyperalgesia.

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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 isoform(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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Footnote

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Legends for Figures

Figure 1

Bar graphs show paw withdrawal latency (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 to baseline. Values are mean ± SEM for PWL and median with 25^{th} and 75^{th} percentiles for mechanical threshold. *, significantly different from baseline values, $p<0.05$.

Figure 2

Bar graphs show the effect of different doses of ACTZ or saline administered intraperitoneally (A, D), locally into the inflamed muscle (B, E), or intrathecally (C, F), on carrageenan-induced reduction in PWL to heat (A, B, C), and reduction in mechanical withdrawal thresholds (D, E, F). Intraperitoneal (100 and 200 mg/kg), local (5 mg), or intrathecal (100 nmol) injection of ACTZ, significantly reversed the carrageenan-induced heat hyperalgesia compared to the saline group (A, B, C). ACTZ administration by the same routes had no effect on the mechanical hyperalgesia (D, E, F). Values for heat hyperalgesia (A, B, C) are mean ± SEM, and for mechanical hyperalgesia (D, E, F) are median with 25^{th} and 75^{th} percentiles. *, significantly different from saline controls, $p<0.05$.

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Figure 3

Graph A shows the effect of saline or ACTZ (10, 100 and 200 mg/kg) injected intraperitoneally, on the pH of the ipsilateral inflamed gastrocnemius muscle. All doses of ACTZ significantly increased the pH of the ipsilateral muscle 15 min and/ or 30 min after the injection. Graph B shows the effect of saline or ACTZ (10, 100 and 200 mg/kg) injected intraperitoneally, on the pH of the contralateral non-inflamed gastrocnemius muscle. All doses of ACTZ significantly increased the pH of the ipsilateral muscle 15 min and/ or 30 min after the injection. Values are mean ± SEM. *, significantly different from saline controls, $p<0.05$.

Figure 4

Graph shows the effect of saline or ACTZ (5 mg) injected into the inflamed gastrocnemius muscle; or ACTZ (100 nmol) or saline injected intrathecally, on pH of inflamed gastrocnemius muscle. ACTZ injected into the inflamed gastrocnemius muscle significantly increased the pH of the inflamed muscle 15 min and 30 min after the injection. There were no changes in pH in the muscle saline injected animals or in the intrathecal groups. Values are mean ± SEM. *, significantly different from saline controls, $p<0.05$.

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Figure 1

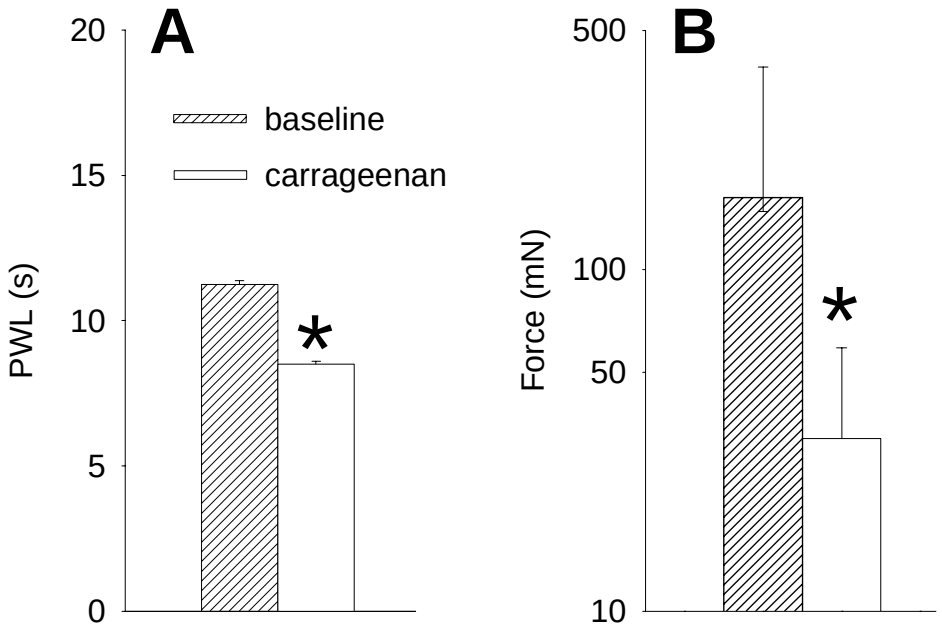

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Figure 2

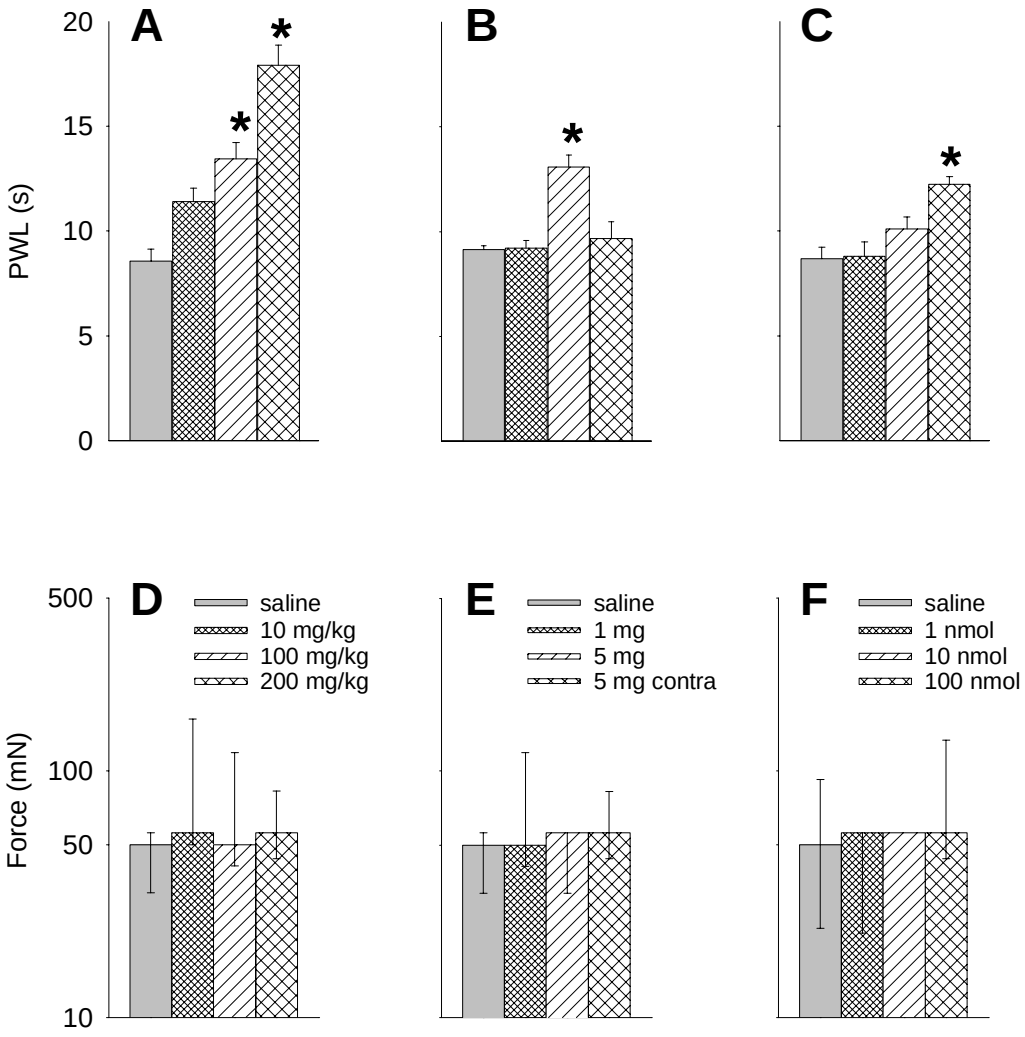

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Figure 3

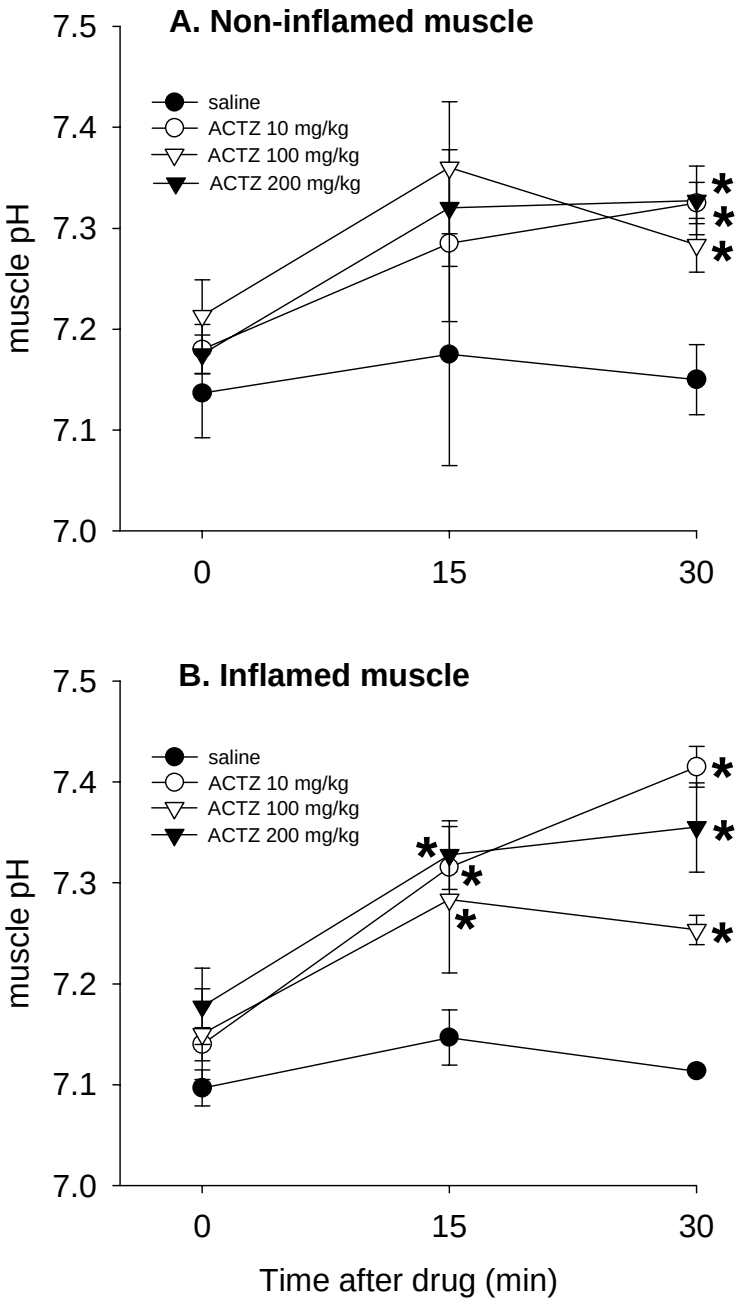

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Figure 4