Role of the Na\(^+\)-K\(^+\)-2Cl\(^-\) Cotransporter in the Development of Capsaicin-Induced Neurogenic Inflammation

Sandra Valencia-de Ita, Nada B. Lawand, Qing Lin, Gilberto Castañeda-Hernandez, and William D. Willis

1Seccion Externa de Farmacologia, Centro de Investigacion y de Estudios Avanzados del Instituto Politécnico Nacional, Mexico, D.F.; and 2Department of Neurosciences and Cell Biology, University of Texas Medical Branch, Galveston, Texas

Submitted 17 October 2005; accepted in final form 8 March 2006

INTRODUCTION

The inflammatory response induced by substances released from peripheral terminals of sensory nerve fibers is called neurogenic inflammation and is characterized by arteriolar vasodilation, plasma extravasation, and hyperalgesia (Holzer 1998; Lin et al. 1999, 2003; Szolcsányi et al. 1988, 1996a; Willis 2001; see Willis 1999 for review). Intradermal administration of capsaicin (CAP), the main active ingredient in hot chili peppers, produces neurogenic inflammation by activating primary afferent depolarization (PAD) (see Price et al. 2005; Willis 1999 for review), which results from the opening of Cl\(^-\) channels and efflux of Cl\(^-\) ions from primary afferent neurons. The high gradient of Cl\(^-\) in primary afferent neurons is maintained by the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter (NKCC) (Alvarez-Leefmans et al. 1988, 1998; O’Grady et al. 1987a,b) and determination of the effect of bumetanide on the increased DRR activity, arteriolar vasodilatation in the skin, hindpaw edema, and mechanical secondary hyperalgesia and allodynia induced by intradermal CAP in male Sprague-Dawley rats.

METHODS

Experimental animals

Male Sprague-Dawley rats (n = 6 per group, weighing 250–300 g) were used in this study. The rats for DRRs and vasodilation studies were from Harlan Indianapolis, IN, whereas rats from Harlan, Mexico City, Mexico, were used for experiments on edema and behavior. All experimental protocols were approved by the Animal Care and Use Committee and were in accordance with the guidelines of the National Institutes of Health and the International Association for the Study of Pain. All experimental animals were housed and maintained in accordance with the guidelines of the University of Texas Medical Branch Animal Care and Use Committee and by the Institutional Animal Care and Use Committee of the Centro de Investigación y de Estudios Avanzados, México, DF, México.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: W. D. Willis, Dept. of Neurosciences and Cell Biology, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-1069 (E-mail: wdwillis@utmb.edu).
Drugs

Bumetanide (Sigma) and artificial cerebrospinal fluid (ACSF) were administered through an intrathecal catheter. Bumetanide was dissolved in ACSF. Therefore ACSF was used as vehicle control for all the experiments. Because bumetanide is soluble in alkaline solutions (Reynolds 1996), it was dissolved in ACSF that had a pH = 10 before being bubbled with 95% CO\textsubscript{2}-5% O\textsubscript{2}. Once bumetanide was completely dissolved, this solution was bubbled for 15 min with the conditions before stated, and the pH of this solution decreased from 10 to 7.2–7.4. The ACSF contained (in mM) 1.3 CaCl\textsubscript{2} 2H\textsubscript{2}O, 2.6 KCl, completely dissolved, this solution was bubbled for 15 min with the conditions before stated, and the pH of this solution decreased from 10 to 7.2–7.4. The ACSF contained (in mM) 1.3 CaCl\textsubscript{2} 2H\textsubscript{2}O, 2.6 KCl, 0.9 MgCl, 21.0 NaHCO\textsubscript{3}, 2.5 Na\textsubscript{2}HPO\textsubscript{4}, 125.0 NaCl, and 3.5 glucose and was bubbled with 95% CO\textsubscript{2}-5% O\textsubscript{2}, pH 7.2–7.4. The CAP (1%, 50 μl) was injected into the plantar surface (heel area) of the hindpaw of rats in behavioral and edema experiments. In the blood-flow experiments, CAP was injected near the rats’ toes. CAP (Fluka, Ec No. 2069698) was dissolved in Tween 80 (7%), alcohol (20%), and saline. Alcohol was evaporated before use.

Placement of intrathecal catheter

Male rats were anesthetized with pentobarbital sodium (50 mg/kg ip). After anesthesia, a catheter (PE-10, OD 0.61 mm) was inserted into the subarachnoid space (Storkson et al. 1996) through a guide cannula connected to a 20-gauge needle, which punctured the dura at the level of the cauda equina. The catheter was then carefully implanted rostrally, aiming its tip at the lumbar enlargement. The external part of the catheter was secured in place using a suture. Animals were then allowed to recover for 3 days before any test was conducted and were checked for sensory and motor deficits. Those showing any deficit were excluded from the study. Control experiments were performed to demonstrate that the intrathecal injection of 10 μl of ACSF did not modify the CAP-induced vasodilatation, edema, or secondary mechanical allodynia and hyperalgesia.

Cutaneous blood flow measurement

Rats implanted with an intrathecal fiber were anesthetized with pentobarbital sodium (50 mg/kg ip). The jugular vein was cannulated to allow the continuous flow of anesthesia by infusion of pentobarbital sodium (5–8 mg · kg\textsuperscript{-1} · h\textsuperscript{-1}) in a saline solution. The level of anesthesia was monitored by frequent examination of pupillary size and responses to stimulation, and the absence of a flexion reflex. The rat was placed over a heating blanket with the abdomen down. A rectal temperature probe was inserted and body temperature was maintained at 37°C by a servo-controlled heating blanket. Once a stable level of surgical anesthesia was reached, blood flow measurements were recorded as blood cell flux by a laser Doppler flowmeter and processed by a computer analysis system (CED 1401 plus, with Spike2 software). Blood flow was shown as a voltage level. Two probes were placed on the plantar surface of the foot, one on the area of primary hyperalgesia (~0.5 cm from CAP injection, zone 1) and the second on the area of presumed secondary hyperalgesia (~2 cm from CAP injection, zone 2) (Kinman and Levine, 1995; Sun et al. 2003).

Blood flow changes were measured before and after intradermal injection of 1% CAP (50 μl). To test the involvement of NKCC in the CAP-induced neurogenic inflammation, bumetanide (10 μl) was administered through the intrathecal catheter before or after CAP injection.

PRETREATMENT STUDIES. In three groups of rats (n = 6), 10 μl of bumetanide at concentrations of 1, 10, or 100 μM was administered through the intrathecal fiber onto the spinal cord. After taking a baseline measurement of the blood flow, an intradermal CAP injection was made 5 min after intrathecal bumetanide administration. Changes in blood flow after CAP injection were recorded for 90 min. As a control, ACSF was given via the same route as bumetanide. Post-treatment studies: in three groups of rats (n = 6), 10 μl of bumetanide at concentrations of 1, 10, or 100 μM was given after taking a baseline measurement and 5 min after CAP injection into the paw. In other animals, ACSF (as a control) was injected intrathecally. Finally, dose response curves for the effect of bumetanide given either as a pretreatment or as a posttreatment on regional blood flow changes were plotted.

Hindpaw edema measurement

Paw volume (to assess edema) was determined by measuring volume displacement using a commercially available plethysmometer (Ugo Basile). The hindpaw was immersed in a chamber containing electrolyte solution up to the boundary between hairy and nonhairy skin, and the volume displacement was determined electronically. Measurements were performed in duplicate.

PRETREATMENT STUDIES. At the outset of an experiment, rats with an intrathecal catheter received an intradermal injection of 50 μl of 1% CAP into the plantar surface of the left hindpaw. To determine the effect of bumetanide on hindpaw edema formation, rats were given intrathecally 10 μl of ACSF or increasing concentrations of bumetanide (1, 10, and 100 μM) 10 min prior to intradermal injection of CAP. Before administration of either drug or vehicle, baseline hindpaw volumes were determined. The paw volume was determined 1 min prior to CAP injection and 15, 30, 60, 90, and 120 min after CAP injection. Posttreatment studies: To evaluate the effect of bumetanide on the maintenance of CAP-induced hindpaw edema, rats were intradermally injected with CAP. Thirty minutes later (this time was based on our pilot experiments which showed that maximal edema was seen 30 min after CAP injection; in contrast changes in blood flow were seen immediately after CAP injection) rats were given ACSF or increasing concentrations of bumetanide (1, 10, and 100 μM) intrathecally. The paw volume was then determined 15, 30, 40, 60, 90, and 120 min after CAP injection. For both pre- and posttreatment studies, each animal was used only once, and the investigator was blinded as to which drugs were injected with CAP. At the end of the experiment, rats were killed with CO\textsubscript{2}.

DRR recordings

Rats were anesthetized with pentobarbital sodium (50 mg/kg ip). The jugular vein was cannulated to maintain a continuous flow of anesthesia. When the level of surgical anesthesia was stable, rats were paralyzed with pancuronium (0.3–0.4 mg/h iv), and the trachea was intubated for artificial respiration. The CO\textsubscript{2} level was monitored throughout the experiment, and the core body temperature was maintained at 37°C using a heating blanket. Laminitectomy was done to expose the spinal cord between the L\textsubscript{4} and S\textsubscript{1} segments. The dura mater was cut, and the exposed spinal cord was then covered with mineral oil that was kept warm using a heating element. Dorsal roots (L\textsubscript{4}–S\textsubscript{1}) were separated, isolated, and cut distally near the dorsal root ganglia. Multitunit DRRs were recorded by placing a teased filament on a platinum unipolar electrode. Digitized signals were processed by an interface (CED 1401), fed into a computer and analyzed by spike 2 waveform software. Waves and peristimulus time histograms were recorded to analyze the spikes and to estimate the firing rate, respectively. The DRR activity was measured in response to mechanical stimulation of the receptive field (brush, 10 and 200 mN of Frey filaments). After baseline activity was recorded, CAP was injected into the foot, far from the receptive field (~2 cm from CAP-injection). Bumetanide (10 μl; 1, 10, 100 μM) was then administered intrathecally onto the surface of the spinal cord 20 min after CAP injection. Discharges were continuously monitored over 50 min after CAP injection.
Behavioral tests

Rats with an implanted intrathecal catheter were used in this study. Animals were tested for hindpaw-withdrawal responses (PWRs) to application of von Frey filaments of different bending forces. Animals were placed in plastic cages on an elevated screen and allowed to acclimate to the testing environment for 30 min. Baseline measurements were taken first. Two von Frey filaments (bending forces of 10 and 200 mN) were applied 10 times in each testing set at the base of the third toe on the plantar surface of the foot 2 cm away from the CAP injection site. Three trials were performed to get an average of the number of PWR to ten applications of von Frey filaments of different bending forces. The responses were recorded at each time point (Wu et al. 2001). A force of 10 mN does not activate cutaneous nociceptors (Leem et al. 1993), so it was considered innocuous and the appearance of responses to this filament as an indicator of allodynia. On the other hand, a force of 200 mN activates cutaneous nociceptors and thus was considered a noxious stimulus. Increased responses to this stimulus were regarded as an indicator of hyperalgesia. Therefore enhanced responses to 10 and 200 mN von Frey filaments at a distance of ~2 cm from the CAP-injection site were assumed to reflect secondary mechanical allodynia and hyperalgesia, respectively, because the CAP should not have affected nociceptors that ended 2 cm away.

Pretreatment study, 10 μl of bumetanide at a concentration of 1, 10 or 100 μM was administered through the intrathecal cannula 10 min before CAP injection. Posttreatment study, 10 μl of bumetanide was given 30 min after CAP injection. This time was chosen based on pilot studies showing that the maximal nociceptive response occurred at this time. ACSF was also used as a control substance in both pre- and posttreatment studies.

Data analysis

All data were expressed as means ± SE. Baseline blood flow level was expressed as 100% and percentage changes after CAP injection were compared for different groups of animals. All rat hindpaw volumes were expressed as percentage of the baseline value. Time courses of the antiinflammatory response after each treatment were constructed by plotting the mean percentage increase as a function of time. For behavioral experiments, the mean responses to 10 applications of the von Frey filaments were obtained and these were expressed as a function of time. From all time-course plots, the total area under the curve (AUC) was calculated. Statistical differences between groups were determined by one-way ANOVA followed by the Dunnett’s analysis or by Dunn’s analysis when data did not have a normal distribution. Evoked DRR activity after CAP injection was expressed as a percentage of baseline, with baseline set at 100%.

R E S U L T S

Effect of bumetanide on initiation of cutaneous vasodilation induced by intradermal CAP injection

To evaluate the effect of bumetanide on the cutaneous blood flow in sites both near and remote from the CAP injection, one laser probe was placed close to the CAP injection site (zone 1, Fig. 1A) and another laser probe was put ~2 cm distally (zone 2, Fig. 1C). CAP was injected once baseline blood flow was considered stable. CAP injection resulted in a rapid enhancement in blood flow in both sites on the foot (zone 1 and zone 2, Fig. 1, A and C). However, cutaneous vasodilation evoked by CAP in zone 2 was higher and lasted longer than that recorded in zone 1 (Fig. 1, A–D). Pretreatment of the spinal cord with bumetanide 5 min before CAP injection reduced the CAP-induced vasodilation in zone 2 in a dose-dependent man-
ner (Fig. 1D). However, intrathecal bumetanide only in concentrations 10 and 100 μM reduced significantly the vasodilatation at sites remote to the CAP-injection. In contrast, the CAP-induced vasodilation at sites near the injection was not modified by spinal bumetanide (Fig. 1, A and B). No significant changes in blood flow in the contralateral paw were observed after any treatment (data not shown).

Effect of bumetanide on maintenance of cutaneous vasodilation induced by intradermal CAP injection

For evaluating the possible involvement of NKCC in maintenance of vasodilation induced by CAP injection, baseline measurements were taken and then either bumetanide at different concentrations (1, 10, and 100 μM) or ACSF were given intrathecally 5 min after CAP injection. Figure 2, A and C, shows an increase in the cutaneous blood flow in zones 1 and 2 after CAP injection. Rats treated with ACSF after CAP injection showed a peak increase in the blood flow at 15 min for zone 1 and 25 min for zone 2 after CAP injection. The blood flow peak increase evoked by CAP in the zone 2 (25 min) but not in zone 1 (15 min) was reduced after spinal bumetanide (10 and 100 μM; Fig. 2, A and C). Posttreatment of the spinal cord with bumetanide (1, 10, and 100 μM) did not significantly modify the CAP-induced vasodilation in zone 1 during the 90 min recording period (Fig. 2, A and B). On the contrary, spinal posttreatment with bumetanide decreased the cutaneous vasodilation induced by CAP in sites remote to the injection in a dose dependent fashion (Figs. 2C and 2D). No significant changes in blood flow in the contralateral paw were observed after any treatment (data not shown).

**Effect of bumetanide on initiation of the hindpaw neurogenic edema induced by intradermal CAP**

To assess the possible involvement of NKCC in the development of the neurogenic edema induced by CAP, intrathecal bumetanide was injected before CAP injection. The CAP injection resulted in edema in the ipsilateral hindpaw but not in the contralateral paw (data not shown). A maximum increase in the volume of the rat hindpaw was observed 15 min after CAP injection (Fig. 3A). Pretreatment of the spinal cord by intrathecal administration of ACSF did not affect the increase in the hindpaw volume. However, when the spinal cord was pretreated with bumetanide at 10 or 100 μM, a partial decrease in the CAP-evoked hindpaw edema was observed (Fig. 3, A and B). Pretreatment of the spinal cord with bumetanide (1 μM) did not significantly affect hindpaw edema.

**Effect of bumetanide on maintenance of the neurogenic edema induced by intradermal CAP injection**

Posttreatment of the spinal cord by intrathecal administration of bumetanide only at the highest concentration (100 μM) significantly reduced the hindpaw edema produced by intradermal CAP (Fig. 4B).

**Effects of posttreatment of the spinal cord with bumetanide on enhanced DRR activity after CAP injection**

Pretreatment studies for DRR recordings were not performed because not all fibers are sensitized after capsaicin. If
we gave bumetanide prior to CAP or simultaneously with CAP, the effect obtained could be the result of bumetanide or the lack of CAP effect.

DRRs were recorded from the cut central ends of isolated filaments of dorsal roots (L3–L4) in response to mechanical stimulation of the receptive field before and after intradermal CAP injection (1%; 50 μl). Bumetanide (1, 10, and 100 μM) was then applied onto the spinal cord to determine the contribution of NKCC to the DRR activity. CAP injection produced a significant increment in mechanically evoked DRR activity, which persisted for 1 h. The peak increase in DRR activity was seen at 20 min after CAP injection, and this was significantly diminished only when 10 μM bumetanide was injected (Fig. 5). Posttreatment of the spinal cord with ACSF at 20 min after CAP injection did not affect DRR activity during the time evaluated. In contrast, the increased DRR activity evoked mechanically after CAP injection was significantly reduced after administration of the bumetanide (1 and 10 μM) compared with the values at 20 min after CAP injection in the same group (Fig. 5). This effect was seen when DRR activity was evoked by applying von Frey filaments (10 and 200 mN) and brushing to the “receptive field” on the foot.

Effects of bumetanide on initiation of CAP-induced secondary mechanical hyperalgesia and allodynia

For evaluating the effect of bumetanide (1, 10, and 100 μM; 10 μl) on secondary mechanical hyperalgesia and allodynia induced by CAP (1%; 50 μl), bumetanide was injected intrathecally onto the spinal cord 10 min before CAP injection. In both pre- and posttreatment studies, mechanical allodynia and hyperalgesia was evaluated with von Frey filaments with bending forces of 10 and 200 mN, respectively. In all animals, CAP injection produced an increase in the number of PWR to mechanical stimulation with graded von Frey filaments (10 and 200 mN). These results indicated that CAP injection resulted in secondary mechanical allodynia and hyperalgesia (Fig. 6, A and C). Pretreatment of the spinal cord with bumetanide (1, 10, and 100 μM) reduced markedly the number of responses to von Frey filament stimulation with bending forces of 10 and 200 mN compared with the responses of the control group (rats treated with ACSF; Fig. 6, A and C). Figure 6, B and D, shows clearly that bumetanide decreased the total number of responses to von Frey filaments (10 and 200 mN) applied to the rat hindpaw. All concentrations of bumetanide that were evaluated showed both anti-allodynic and anti-hyperalgesic effects, as the AUC from bumetanide-treated rats was significantly smaller than that from ACSF-injected rats.
DISCUSSION

The present study suggests that intrathecal application of bumetanide blocks NKCC in intraspinal primary afferents, and this results in inhibition of neurogenic inflammation induced by intradermal capsaicin injection. In agreement with this hypothesis, spinal bumetanide inhibited the development and maintenance of vasodilation, edema, secondary mechanical allodynia, and hyperalgesia induced by an intradermal CAP injection. Furthermore, electrophysiological studies show that increased DRR activity evoked by CAP is diminished by intrathecally administered bumetanide.

It has been suggested that intradermal injection of CAP results in a local vasodilatation, increased plasma extravasation (PE), and hyperalgesia through release of neuropeptides from peripheral primary afferent terminals (Brain 1996; Holzer 1998; Louis et al. 1989; Willis 1999). Several studies suggest that substance P and CGRP are the major peptides involved in PE (Gamse et al. 1980; Khalil and Helme 1989) and vasodilation (Holzer 1998; Hughes and Brain 1991), respectively.

It is thought that local changes in blood flow after CAP injection (i.e., changes at zone 1, 5 mm away from the CAP injection), result mainly from the interaction of CAP with nociceptors and from local axon reflexes (Szelcánhyi 1996b), whereas more distant changes (i.e., those 15–20 mm away from the CAP injection) are mediated by DRRs via central actions (Lin et al. 1999).

The mechanisms by which DRRs are triggered after peripheral inflammation (CAP injection) have yet to be determined. It has been suggested that the DRRs are generated by an enhanced PAD sufficient to reach threshold for action potential generation in intraspinal primary afferents fibers (see Willis 1999 for review). It is thought that PAD results from the opening of Cl⁻ channels and efflux of Cl ions after GABA₂ receptors on the central terminals of primary afferents are activated (see Willis 1999 for review). Several lines of evidence support this view. First, intradermal injection of CAP produces an increase in the excitability of C-fibers in the capsaicin injection site (Szallasi and Blumberg 1999; Szelcánhyi 1996b for review), which in turn, might result in augmented DRR activity (Lin et al. 1999, 2000). Second, spinal administration of a GABA₂ receptor antagonist, bicuculline, prevents the triggering of DRRs induced by the injection of either intra-articular kaolin/carrageenan (Rees et al. 1995) or intradermal CAP (Lin et al. 1999; Willis 1999).

It is widely accepted that the Cl⁻ gradient in afferent neurons is maintained by an electroneutral bumetanide-sensitive cotransporter, NKCC, which is expressed in these neurons (Alvarez-Leefmans et al. 1988, 2001). Thus an increased activity and/or upregulation of this cotransporter might be a mechanism underlying an excessive PAD, which could lead to triggering of DRRs that are conducted to the periphery and that might induce a release of neuropeptides that cause vasodilation, edema, and hyperalgesia after CAP injection (Lin et al. 1999; Willis 1999).

In this study, spinal application of bumetanide decreased significantly DRR activity evoked by noxious mechanical stimulation after CAP injection, suggesting a pivotal role of the NKCC in the triggering of DRRs after CAP injection. Furthermore, we have found that intrathecal bumetanide (10 and 100 μM) administered before or after CAP injection decreased or
reversed the vasodilation in sites far from the injection (zone 2). In contrast, spinal bumetanide failed to reduce the CAP-induced vasodilation in sites near the injection (zone 1). This finding is consistent with previous results from our laboratory (Lin et al. 1999) in which the CAP-induced vasodilation in zone 2, but not in zone 1, was blocked by spinal GABA<sub>A</sub>, N-methyl-D-aspartate (NMDA), and non-NMDA receptor antagonists. Furthermore, cutting either the sciatic nerve or dorsal}

**FIG. 6.** BUM retards the generation of secondary mechanical allodynia (A) and hyperalgesia (B) induced by injection of CAP. The time course of the mean number of paw-withdrawal responses to 10 applications of von Frey filaments of 2 different bending forces 10 (A) and 200 mN (C). Rats received 1st the intrathecal injection of either BUM (1, 10, and 100 μM) or the vehicle followed 5 min later with CAP injection (denoted by an arrow at time 0). BUM decreased the total responses to 10 mN (B) and 200 mN (D) filaments when applied onto the secondary hyperalgesia area of the paw. *P < 0.05 vs. ACSF injection, ANOVA 1-way followed by Dunn’s test.

**FIG. 7.** BUM inhibits the secondary mechanical allodynia and hyperalgesia induced by injection of CAP. The time course of the mean number of paw withdrawal responses to 10 applications of von Frey filaments of 2 different bending forces 10 (A) and 200 mN (C). Rats were 1st injected with CAP intradermally followed 30 min later with an intrathecal injection of either BUM (1, 10, and 100 μM) or the vehicle (denoted by an arrow). When compared with ACSF, BUM produced a decrease in total responses to stimulation with both 10 mN (B) and 200 mN (D) filaments. *P < 0.05 vs. ACSF injection, ANOVA 1-way followed by Dunn’s test (B) or by Dunn’s test (D).
roots blocked the vasodilation induced by Aβ fibers seen outside the area of sural nerve innervation after intradermal CAP (García-Nicas et al. 2001). Taken together these observations suggest that vasodilation at sites far from the CAP injection is due to increased DRR activity. Moreover, the evidence presented here suggests a key role for NKCC in the triggering of DRRs and subsequent peripheral vasodilation seen in the secondary zone in response to CAP injection.

We also show that spinal bumetanide partially blocked the development and maintenance of the hindpaw edema induced by intradermal CAP. Thus it may be suggested that CAP-induced hindpaw edema not only results from the release of neuropeptides from CAP-sensitive peripheral fibers (Lin et al. 1999) at the site of injection, but also from the release of neuropeptides from peripheral afferents located away from the CAP injection site. These latter fibers could be C or Aβ fibers as DRRs have been recorded in both type of fibers after CAP injection (Lin et al. 2000; Wang et al. 2004).

It is assumed that pain hypersensitivity may be modulated by neurogenic inflammation (Dong et al. 2002). Thus CAP-induced neurogenic inflammation could result in increased nociception. In this study, we evaluated the possible role of NKCC in the development and maintenance of mechanical hyperalgesia and allodynia induced by intradermal CAP at sites far from the injection, as CAP-induced secondary mechanical allodynia and hyperalgesia are mainly attributed to spinal mechanisms (Willis 1999). The results show that pre- and posttreatment with spinal bumetanide resulted in decreased PWRs to both innocuous and noxious mechanical stimulation at a site away from the receptive fields of nociceptive fibers, which have an area of only ~6 mm² (Bharali and Lisney 1992). These findings suggest that DRRs conducted to the periphery produced vasodilation and edema, which in turn, increased the excitability of nociceptors present at sites remote to the CAP injection. Furthermore, the spinal blockade of NKCC reduced the DRR activity, neurogenic inflammation, mechanical allodynia and hyperalgesia at secondary sites. As shown in Figs. 1–5, spinal bumetanide had dose-dependent effects on CAP-evoked flare, edema, and DRRs. Also, bumetanide had a dose-dependent effect on CAP-evoked allodynia and hyperalgesia when it was administered after CAP (Fig. 7) but not before CAP injection (Fig. 6). In these experiments, the lowest dose used might already have had a maximal effect, and so larger doses might result in no further change. The lack of a dose-dependent change could thus be a ceiling effect. Another explanation might be that a high dose of bumetanide could have a separate nonspecific action in addition to a dose-dependent action on NKCC.

Our observations are consistent with those of Granados-Soto et al. (2005) showing that intrathecal bumetanide and other NKCC inhibitors (furosemide and piretanide) decreased formalin-induced nociceptive responses. Furthermore, evidence for a pro-nociceptive role of this cotransporter comes from studies using NKCC1 knockout mice. These animals are less sensitive to thermal nociceptive stimuli than wild-type animals as shown by an increased withdrawal latency in the hot plate test (Sung et al. 2000). Also, NKCC1 knockout mice showed increased tail-flick latencies and a reduction of the duration of pain behavior induced by intradermal CAP applications (Laird et al. 2004). Taken together, these studies suggest that NKCC1 has a pro-nociceptive role and its blockade results in analgesia.

Plausible mechanisms by which NKCC affects DRR activity after intradermal CAP are unknown. However, an increase in the activity and/or expression of the NKCC in primary afferent neurons could account for our findings. It is well established that activity of this cotransporter may be modulated by phosphorylation, as Ser/Thr protein kinase-mediated NKCC phosphorylation stimulates cotransporter activity (for review, see Flatman 2002). Moreover, stimulation of NKCC in rat cortical neurons induced by activation of group-I metabotropic glutamate receptors was blocked by inhibiting a protein kinase, calcium/calmodulin-dependent protein kinase II (CaM II) (Schomberg et al. 2001). On the other hand, intradermal CAP injection results in increased expression of both CaM II and phosphorylated CaM II in the spinal cord (Fang et al. 2002). Based on these findings, it is plausible to suggest that a CaM-II-mediated NKCC phosphorylation results in increased NKCC activity and subsequently triggering of DRRs. Moreover, recent observations show that a fast and transient phosphorylation of NKCC1 follows intracolonic capsaicin administration (Galan and Cervero 2005). On the other hand, enhanced functional NKCC1 expression in primary afferent neurons may explain the increased DRR activity after intradermal capsaicin. In fact, a recruitment of NKCC1 to the plasma membranes of spinal cord neurons after intracolonic capsaicin has recently been shown (Galan and Cervero 2005).

In summary, this study provides evidence not only that spinal bumetanide blocked the increased DRR activity but also that the spinal administration of bumetanide inhibited the allodynia and hyperalgesia associated with neurogenic inflammation produced by intradermal CAP. Therefore targeting drugs that inhibit NKCC activity may be a novel therapeutic approach for controlling edema, vasodilation, and pain produced by neurogenic inflammation.

GRANTS
This work was supported by National Institute of Neurological Disorders and Stroke Grants NS-09743 to W. D. Willis and NS-40723 to Q. Lin and by Consejo Nacional de Ciencia y Tecnologia Grant 38940-M to G. Castañeda-Hernandez. S. Valencia-de Ita is a CONACYT fellow from Mexico.

REFERENCES
BUMETANIDE BLOCKS NEUROGENIC INFLAMMATION

J Neurophysiol  •  VOL 95  •  JUNE 2006  •  www.jn.org


