Modulation of $I_A$ Currents by Capsaicin in Rat Trigeminal Ganglion Neurons

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Liu, L., and S. A. Simon. Modulation of $I_A$ currents by capsaicin in rat trigeminal ganglion neurons. J Neurophysiol 89: 1387–1401, 2003; 10.1152/jn.00210.2002. When capsaicin, the pungent compound in hot pepper, is applied to epithelia it produces pain, allodynia, and hyperalgesia. We investigated, using whole cell patch clamp, whether some of these responses induced by capsaicin could be a consequence of capsaicin blocking $I_A$ currents, a reduction in which, such as occurs in injury, increases neuronal excitability. In capsaicin-sensitive (CS) rat trigeminal ganglion (TG) neurons, capsaicin inhibited $I_A$ currents in a dose-dependent manner. $I_A$ currents were reduced 49% by 1 $\mu$M capsaicin. In capsaicin-insensitive (CIS) rat TG neurons, or small-diameter mouse VR1−/− neurons, 1 $\mu$M capsaicin inhibited $I_A$ currents 9% and 3%, respectively. These data suggest that in CS neurons the vast majority of the capsaicin-induced inhibition of $I_A$ currents occurs as a consequence of the activation of vanilloid receptors. Capsaicin (1 $\mu$M) did not alter the $I_A$ conductance-voltage relationship but shifted the inactivation-voltage curve about 15 mV to hyperpolarizing voltages, thereby increasing the number of inactivated $I_A$ channels at the resting potential. $I_A$ currents were relatively unaffected by 1 mM CTP-cAMP or 500 nM phorbol-12,13-dibutyrate (a protein kinase C agonist) but were inhibited by 20–30% with either 1 mM CTP-cGMP or 25 $\mu$M N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCl (a calcium-calmodulin kinase inhibitor). In the presence of 0.5 $\mu$M KT8823, an inhibitor of protein kinase G (PKG) pathways, 1 $\mu$M capsaicin inhibited $I_A$ by only 26%. In summary, in CS neurons, capsaicin decreases $I_A$ currents through the activation of vanilloid receptors. That activation, partially through the activation of cGMP-PKG and calmodulin-dependent pathways should result in increased excitability of capsaicin-sensitive nociceptors.

INTRODUCTION

Capsaicin, the pungent ingredient in chili peppers, is used in various medicinal treatments to alleviate pain, even though its initial application can cause pain and inflammation (Sterner and Szallas 1999; Szelesanyi 1977; Waddell and Lawson 1989). The initial painful sensation after capsaicin application arises from the selective activation of vanilloid receptors in capsaicin-sensitive nociceptors that leads to depolarization and the generation of action potentials (APs) (Baumann et al. 1991; Gold et al. 1996a; Heyman and Rang 1985; LaMotte et al. 1991; Williams and Ziegglranberger 1982). Our goal is to investigate mechanisms by which capsaicin can initiate hyperalgesia in rat trigeminal ganglion (TG) neurons. Here, we investigate its effects on $I_A$ currents of capsaicin-sensitive (CS) and capsaicin-insensitive (CIS) TG neurons. $I_A$ currents are involved in modulating the action potential shape, threshold, and the regulation of the interspike interval (Yoshimura et al. 1996; Yost 1999).

Primary sensory neurons contain several types of voltage-gated potassium channels (VGPCs), which include those that activate rapidly and inactivate slowly ($I_K$) and those that activate and inactivate rapidly ($I_A$) (Gold et al. 1996b; Kostyuk et al. 1991; McCleskey and Gold 1999; Petersen et al. 1987; Rasband et al. 2001; Ricco et al. 1996; Seifert et al. 2001; Stansfeld et al. 1986; Woolf and Costagin 1999; Yoshimura et al. 1996). VGPCs of various types are distributed among different types of neurons (Cardenas et al. 1995; Djouhri and Lawson 1999; Harper and Lawson 1985). In one study, $I_A$ currents were found only in CS neurons (Cardenas et al. 1995), but in another similar study, they were found in both CS and CIS DRG neurons (Gold et al. 1996b).

There have been numerous studies reporting the effects of capsaicin on VGPCs. In an earlier study of the effect of capsaicin on $I_A$ currents, Petersen et al. (1987) found that 30 $\mu$M capsaicin reduced the amplitude of $I_A$ and accelerated their inactivation in all chick and guinea pig neurons. Capsaicin blocked $I_K$ currents in Schwann cells with an IC$_{50}$ (=$K_{1/2}$) of 8.7 $\mu$M (Baker and Ritchie 1994) and spinal neurons from Xenopus embryos with a $K_{1/2}$ = 21 $\mu$M (Kuenzi and Dale 1996). Capsaicin inhibited VGPCs in nodose ganglion neurons with a $K_{1/2}$ = 20 $\mu$M (Bielefeldt 2000), and in a similar study in rat DRG neurons, capsaicin inhibited both $I_K$ and $I_A$ currents with a $K_{1/2}$ = 8 $\mu$M (Atkins and McCleskey 1993). In recordings from cardiac muscle, capsaicin blocked $I_K$ with a $K_{1/2}$ = 11.5 $\mu$M (Castle 1992), and $I_K$ currents were blocked with a $K_{1/2}$ = 17.4 $\mu$M in melanotrophs (Kehl 1994). In a study where various Shaker-type K channels were expressed in cell lines and subsequently exposed to capsaicin, the $K_{1/2}$ ranged from 26 $\mu$M (for $K_{V_{1.3}}$) to 158 $\mu$M (for $K_{V_{1.1}}$) (Grissmer et al. 1994). This wide range of $K_{1/2}$ indicates that capsaicin must directly interact with these channels rather than produce some nonspecific membrane effect. Although there is a great range in the $K_{1/2}$, in all these cases, the inhibition occurred at concentrations much larger than that required to activate vanilloid receptors ($K_{V_{1.3}}$ = 0.68 $\mu$M) in CS TG neurons (Liu and Simon 1996). Here, we found that 1 $\mu$M capsaicin inhibited $I_A$ currents.

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rents by 49% in CS neurons and that most of this inhibition is a consequence of the activation of vaniloid receptors.

**METHODS**

**Cell culture**

TG neurons from both adult Sprague-Dawley rats and from VR1−/− mice, the gift of Dr. David Julius of the University of California at San Francisco, were cultured as described previously (Liu and Simon 1996). Briefly, trigeminal ganglia were dissected aseptically and collected in modified Hank’s balanced salt solution (mHBSS). After washing in mHBSS, the ganglia were diced into small pieces and incubated in mHBSS for 30–50 min at 37°C in 0.1% collagenase (Type XI-S). Individual cells were dissociated by triturating the tissue through a fire-polished glass pipette, followed by a 10-min incubation at 37°C in 10 μg/ml DNase I (Type IV) in F-12 medium (Life Technologies, Gaithersburg, MD). After washing three times with F-12, the cells were cultured in DMEM supplemented with 10% fetal bovine serum. The cells were plated on poly-D-lysine glass coverslips (15 mm diam) and cultured overnight at 37°C. The composition of this solution was (in mM) 80 choline Cl, 80 TEACl, 5 KCl, 2.0 CaCl₂, 1.0 MgCl₂, 10 HEPES, 10 d-glucose, and 1 CdCl₂, adjusted to pH 7.4. CdCl₂ was included to block voltage-gated calcium channels, choline-Cl and TEACl were included to reduce currents from voltage-gated sodium channels, I_K currents, hyperpolarization-activated cation channels, and capsaicin-induced inward currents. Only neurons without or with short processes were used. All experiments were carried out at room temperature (22–25°C).

Care of animals conforms to standards established by the National Institutes of Health. All animal protocols were approved by the Duke University Institutional Animal Care and Use Committee.

**Patch-clamp recording**

In these experiments, we used glass pipettes (R-6 borosilicate, Drummond Scientific, Broomall, PA) with resistances ≤2 MΩ. The pipette solution was (in mM) 120 K-aspartate, 20 KCl, 1.0 CaCl₂, 2.0 MgCl₂, 10 HEPES, 10 d-glucose, and 1 CdCl₂, adjusted to pH 7.4. CdCl₂ was included to block voltage-gated calcium channels, choline-Cl and TEACl were included to reduce currents from voltage-gated sodium channels, I_K currents, hyperpolarization-activated cation channels, and capsaicin-induced inward currents. Only neurons without or with short processes were used. All experiments were carried out at room temperature (22–25°C).

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**Chamber/solution delivery**

The recording chamber had a volume of 370 μl and was continuously perfused. Capsaicin, KT5323 and KN-93 were dissolved in dimethylsulfoxide (DMSO) to form stock solutions that were further diluted before delivering into the recording chamber using a multibarrelled electrode (Adams and List Associated, Westbury, NY) placed about 50 μm from the cell. N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCl (W-7) was dissolved in the solution used for the external buffer. Event markers associated with the opening or closing of valves (Parker Hannifin, Fairfield, NJ) signaled the onset and removal times of the various stimuli.

**RT-PCR**

Cellular total RNA was prepared from the TGs of six individual adult Sprague-Dawley rats. TG was removed and treated with Trizol Reagent (Gibco BRL. No. 15596-026; 100 mg tissue/1 ml Trizol Reagent). The tissues were then quickly homogenized. After treating with ethanol, 20% chloroform, 50% isopropanol, and cold ETOH, the samples were centrifuged and dried under vacuum. The total RNA concentrations were calculated from the absorbance (A) at 260 nm and the purity determined by A260/A280 (the ratio >1.7). To eliminate any residual DNA, all total RNA samples were treated with amplification grade DNase I (Gibco BRL). The cDNA was synthesized by first-strand cDNA synthesis kit for RT-PCR (Boehringer Mannheim). The PCR primers that were used in the present experiment (see Table 1) were synthesized by Gibco BRL. PCR amplification was performed with a PCR core kit (Boehringer Mannheim) with 32 reaction cycles in a Thermal Cycler 3200 (Perkin Elmer, Foster City, CA). Preliminary experiments revealed that under these conditions we were not in the plateau region. The first PCR cycle consisted of a 5-min denaturation time and the last cycle had a 7-min extension time. The other PCR cycles included 1 min of denaturation at 94°C, 1 min of primer annealing at 57°C, and 1 min of extension/synthesis at 72°C. The PCR reaction (10 μl) was separated, and a 2 μl gel loading solution was added to each tube. Agarose gels (2% agarose gel containing 0.6-μg/ml ethidium bromide) were electrophoresed. The gels were digitized using a Foto/analyst TM image analysis system (Fotodyne, Hartland, WI), and the optical density was measured by Image I (Universal Imaging, West Chester, PA.). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to verify the quality and determine the quantity of RNA. Two controls were used in this experiment. One negative control was performed with all the reactions except primers, and another was run with distilled water to test for possible DNA contamination.
Statistics and curve fitting

Data were analyzed and fitted using pClamp (Axon Instruments) or SigmaPlot (SPSS, Chicago IL) software. Dose-response curves were fitted to a modified Hill equation $Y = Y_0 + A \text{Cap}^n/(K_{1/2}^n + \text{Cap}^n)$ characterized by $K_{1/2}$ (concentration of stimulus producing a half-maximal inhibition), $n$, the cooperative index and where the constants $A$ and $Y_0$ were always taken at the maximum current, which was at the maximum depolarized potential (+50 mV).

Data were analyzed for statistical significance using the paired and unpaired (as indicated in the text), Student’s $t$-test, and presented as the means ± SD. For the dose-response curves, the percent inhibition of the $I_A$ currents, at the same voltage, was calculated by subtracting the inhibited current from the control. From these data, the mean of the differences between these two values gave the mean of the percentage inhibited.

Chemicals

W-7, KN-93, and KT5823 were purchased from Calbiochem (La Jolla, Ca). Unless stated otherwise, all other chemicals came from...
The potassium current was separated into $I_A$ and $I_K$ currents by using TEA to inhibit $I_K$ currents. In Fig. 2A, the $I_K$ current did not markedly inactivate, whereas the $I_A$ current inactivated in an exponential manner. At the maximum peak current, $I_{A_{\text{Ap}_{\text{max}}}}$, the inactivation rate constant ($\tau$) was 94 ms. In another type of response shown in Fig. 2B, the total outward current did not markedly inactivate because of the compensation of the slower inactivation times of $I_A$ and the slower activation times of $I_K$. In a third type of response, the total outward current also did not inactivate, the $I_K$ current activated slowly and the $I_A$ current exhibited both a rapid and a slow inactivating component (Fig. 2C).

$I_A$ currents in CS and CIS neurons

Figure 3 shows a recording from a CS neuron. After obtaining an $I_A$ current, 1 $\mu$M capsaicin evoked a slowly activating and desensitizing inward current ($V_c = -80$ mV). Because the external solution had the impermeant cations choline and TEA, the maximal inward current was small $-1.2 \pm 0.6$ nA ($n = 13$), relative to the current obtained in a NaCl-containing buffer, $-4.5$ nA (Liu and Simon 1996). After applying capsaicin continuously for 1–3 min, the current desensitized to a near steady state value of $-0.39 \pm 0.21$ nA ($n = 12$). At this time, compared with control values, the membrane resistance was lower, and the $I_A$ currents were reduced $52.4 \pm 18.0\%$ [from $10.4 \pm 5.3$ nA (control) to $5.6 \pm 6.7$ nA ($n = 12$)]. Because some of this inhibition could arise as a consequence of the capsaicin-induced leak current, we performed leak subtraction (see Fig. 3, box). This correction was calculated from the membrane resistance at $-80$ mV, and, with this correction, $I_A$ was inhibited by $55.3 \pm 18.2\%$ ($n = 12$). However, because the $I_V$ relation for capsaicin-induced currents is outwardly rectifying, the leak correction may not have been fully compensated at more positive potentials. To obtain a better estimate of the “true” inhibition of $I_A$ by capsaicin, the cell was washed until the membrane resistance returned to its control value. That is before capsaicin application, the membrane resistance was $770.4 \pm 129$ M$\Omega$ ($n = 12$) and after about a 30-s wash, it was $763 \pm 137$ M$\Omega$ ($n = 12$). Using this criterion, the $I_A$ currents after the washout were inhibited by $49.2 \pm 17.5\%$ ($n = 12$). This latter procedure was used to calculate the inhibition of $I_A$ by capsaicin (see Fig. 6). Under these conditions, the percentage blockage is not dependent on the voltage (see $I_{A_{\text{Ap}}}$-V plots) even though the $I_V$ relation of capsaicin-activated currents is strongly voltage dependent (Liu and Simon 2000), suggesting that the capsaicin-induced currents do not markedly contribute to the blockage.

Finally, after washing these cells for another 3–6 min, the $I_A$ currents recovered ($78.7 \pm 21.2\% n = 12$) to their precapsaicin values.

Figure 3 also shows that capsaicin did not markedly affect the time to peak (at $I_{A_{\text{Ap}_{\text{max}}}}$) but significantly increased $\tau$ (in bottom corner, compare the “control” and “normalized” traces). In the presence of 1 $\mu$M capsaicin, $\tau$ the inactivation rate constant at $I_{A_{\text{Ap}_{\text{max}}}}$ increased from 141 $\pm 58$ to 231 $\pm 47$ ms ($n = 12$, $P < 0.01$, paired t-test). The percentage inactivation of $I_A$ current was estimated by the equation $(I_{A_{\text{Ap}_{\text{max}}}} -}$
Capsaicin-insensitive neurons (CIS)

Rat TG neurons. In CIS neurons, the inhibitory effect of capsaicin, even at 30 μM, was small relative to CS neurons at 1 μM capsaicin. For the \( I_A \) recordings shown in Fig. 4, \( I_{A_{\text{max}}} \) was reduced by 17.2 and 32.7% by 1 and 30 μM capsaicin, respectively. Figure 4 also shows the \( I-V \) relations at these two different concentrations. In the presence of 30 μM capsaicin, the \( G-V \) relation did not differ from the control. On average, the \( G-V \) curves were unaffected by either 1 or 30 μM capsaicin (control: \( V_{0.5} = -3.1 \pm 9.1 \) mV, \( k = 18.3 \pm 4.9 \); 1 μM capsaicin: \( V_{0.5} = -3.1 \pm 8.1 \) mV, \( k = 18.5 \pm 4.6 \); \( n = 6 \)); (control: \( V_{0.5} = -3.2 \pm 4.3 \) mV, \( k = 15.8 \pm 1.6 \); 30 μM capsaicin: \( V_{0.5} = -3.3 \pm 3.7 \) mV, \( k = 15.7 \pm 1.4 \); \( n = 11 \)). The maximal \( I_A \) current was reduced by 9.3 ± 6.1% (\( n = 5 \)) in the presence of 1 μM capsaicin. At \( I_{A_{\text{max}}} \), \( \tau \) was not significantly altered being 89.2 ± 66.1 ms for the control and 91.4 ± 59.5 ms in the presence of 1 μM capsaicin (\( P = 0.52 \), \( n = 7 \) paired \( t \)-test).

Mouse TG neurons from VR1−/− mice

To eliminate the possibility that the subset of rat CIS neurons that we tested did not resemble nociceptors, we chose a population of mouse VR1−/− TG neurons that have soma diameters between 15 and 22 μm. Neurons in this size range have long-duration action potentials, TTX-resistant sodium currents (unpublished observation) and other receptors that indicate that they are nociceptors (Caterina et al. 1999). The inhibitory effects of 1 and 10 μM capsaicin on \( I_A \) currents, obtained from two different neurons, are seen in Fig. 5. On average, 1 and 10 μM capsaicin inhibited \( I_A \) currents by 3.0 ± 6.9 (\( n = 6 \)) and 29.7 ± 11.9 (\( n = 13 \)), respectively. The \( G-V \)
relationship significantly was not altered at either of these concentrations (data not shown).

**Concentration dependence of capsaicin inhibition of $I_A$**

Figure 6 shows the concentration dependence of capsaicin’s inhibition of $I_{Ap_{max}}$ in rat CS and CIS neurons. In CS neurons, the total $I_A$ inhibition was concentration dependent, ranging from 5.0 ± 4.6% ($n = 4$) at 0.1 μM to 72.0 ± 17.7% ($n = 7$) at 3 μM capsaicin (with 10 μM, the leak resistance was too high to compensate). In CIS neurons, the inhibition of $I_A$ currents increased linearly from 6.4 ± 5.4% ($n = 7$) at 1 μM capsaicin to 24.6 ± 9.7% ($n = 11$) at 30 μM capsaicin. To obtain an estimate of the inhibition that can be attributed to the activation of vanilloid receptors (- - -), the CIS inhibition was subtracted from the total inhibition, and the data were fit to the Hill equation and yielded the following parameters: $K_{1/2} = 0.67$ μM, $n = 1.3$ and a maximal inhibition equal to 67.8%. These data show a clear distinction between the responses of these two types of neurons to capsaicin and suggest that the activation of vanilloid receptors can result in a marked inhibition of $I_A$ currents in CS neurons.

**Effects of capsaicin on $I_A$ on inactivation-voltage curves**

To obtain a better understanding of how capsaicin may inhibit $I_A$ currents, we explored its effects in CS (Fig. 7A) and CIS (Fig. 7B) neurons on the inactivation-voltage parameters. Figure 7A shows the response of a CS neuron, in which 1 μM capsaicin reduced the amplitude of $I_{Ap_{max}}$ by 41% and shifted the midpoint of the inactivation curve toward hyperpolarizing voltages by 17 mV. Note that for depolarizing voltages greater than −40 mV, the current decreased about 90%. In contrast, in rat CIS TG neurons, 1 μM capsaicin did not produce a change in the inactivation-voltage relation (Fig. 7B). Interestingly, in many of these neurons the current could only be maximally inactivated by about 50%. Gold et al. (1996b) saw a similar behavior in studies using DRG neurons.

In CS neurons, the mean conductance-voltage ($G/G_{max}$) and inactivation-voltage relations for the $I_A$ currents in the presence and absence of 1 μM capsaicin are shown in Fig. 8. The $G-V$ curve was not significantly changed [$P = 0.43$; control: $V_{0.5} = −13.3 ± 14.3$ mV, $k = 16.6 ± 2.7$ mV ($n = 10$); 1 μM capsaicin: $V_{0.5} = −11.1 ± 15.4$ mV, $k = 19.1 ± 3.5$; $n = 10$], but the inactivation-voltage relation shifted to hyperpolarizing

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**FIG. 4.** Effects of capsaicin on $I_A$ currents in capsaicin-insensitive neurons. $I_{Ap_{max}}$ was reduced from 12.2 nA (control) to 10.1 and 8.2 nA, on exposure to 1 and 30 μM capsaicin, respectively (see $I_{Ap_{max}}$-V relationships). After a 3-min wash, the current slightly recovered from exposure to 30 μM capsaicin. The $G-V$ curve was not altered in the presence of 30 μM capsaicin (solid line, fits to the Boltzmann equation: control: $V_{0.5} = −2.7$, $k = 16.7$; 30 μM capsaicin: $V_{0.5} = −0.2$ mV and $k = 17.2$). Neither the activation nor inactivation rate constants were markedly altered by 1 μM capsaicin. Bars indicate duration of capsaicin-application. Holding potential = −80 mV.
voltages by 14.7 mV (control: $V_{0.5} = -66.1 \pm 4.9$ mV, $k = -6.0 \pm 1.1$, $C = 0.14 \pm 0.04$; 1 µM capsaicin, $V_{0.5} = -80.8 \pm 8.1$ mV, $k = -7.4 \pm 0.4$, $C = 0.08 \pm 0.04$; $n = 10$). Although no significant differences were found for the parameter, $k$, the shift in $V_{0.5}$ and the values for $C$ were significantly different ($P < 0.05$; paired $t$-test). For CIS neurons, the inactivation-voltage relation was not significantly different from control in the presence of capsaicin (control: $V_{0.5} = 51.6 \pm 8.5$ mV, $k = 6.2 \pm 1.03$, $C = 0.35 \pm 0.15$; 1 µM capsaicin: $n = 5$; $P = 0.13, 0.36$ and 0.23 for $V_{0.5}$, $k$, and $C$, respectively).

Use-dependent block

It is well known that voltage-gated ion channels can be inhibited by a process named “use-dependent block” (Hille 1993). To test whether capsaicin blocks $I_A$ in a use-dependent manner, the cells were held at $-80$ mV and in the presence and absence of 1 µM capsaicin, a sequence of depolarizing pulses to 50 mV were delivered every second. For nine CS neurons, the ratio between the first and last of 16 pulses was 0.77 to 0.74 ($n = 9$, mean SD) in the absence and presence of 1 µM capsaicin (data not shown). For five CIS neurons, the ratio between the first and last of 16 pulses was 0.92 to 0.90 in the absence and presence of 1 µM capsaicin (data not shown). These differences are not significantly different.

Effects of cAMP, cGMP, and PDBu on $I_A$ currents

CTP-cAMP. Because capsaicin appears to modulate $I_A$ currents, in part through the activation of vanilloid receptors, we have
The presence and absence of 1 were hard to analyze. Consequently, 10 neurons were analyzed in which IA was almost completely blocked because the IA amplitude was reduced the the inactivation-voltage curve was shifted by about 17 mV in the hyperpolarizing direction. The inactivation curves were fitted to a modified Boltzmann equation having the parameters: (control: \( V_{0.5} = -54.8 \text{ mV, } k = -6.5, C = 0.11; 1 \mu M \text{ capsaicin: } V_{0.5} = -72.1 \text{ mV, } k = -9.9, C = 0.09 \)).

The application of 1 M CPT-cAMP did not produce large changes in IA currents in either CS (\( n = 4 \), see Fig. 9A) or CIS neurons (\( n = 3 \), data not shown). CPT-cAMP (1 M) did not markedly alter \( I_{A_{\text{Apmax}}} \) (5.5 ± 5.6%; \( n = 7 \); range 0 to 16%; Fig. 9A), the G-V relation (control: \( V_{0.5} = -3.1 ± 6.8 \text{ mV, } k = 16.9 ± 2.5; 1 \text{ M CTP-cAMP, } V_{0.5} = 0.0 ± 10.8 \text{ mV, } k = 17.6 ± 2.9; n = 5 \)), and the inactivation rate constants (control: \( \tau = 105.2 ± 60.2; 1 \text{ M CTP-cAMP, } \tau = 106.3 ± 57.2 \text{ ms; } n = 5 \)).

We next investigated the effects of 500 nM PDBu, a PKC agonist, on IA currents. In neither CS nor CIS neurons did 500 nM PDBu alter IA currents. As seen in Fig. 9B (in a CS neuron), a 3-min application of PDBu did not alter the amplitude of the IA current. The G-V curve (data not shown) and activation and inactivation time courses were also not altered. On average, 500 nM PDBu reduced the amplitude of IA current by 0.3 ± 6.4% (\( n = 5 \)). PDBu (500 nM) did not alter either the G-V curves (control: \( V_{0.5} = -4.5 ± 15.2, k = 13.4 ± 2.6; 1 \mu M \text{ capsaicin: } V_{0.5} = 5.3 ± 13.0 \text{ mV, } k = 14.2 ± 2.7; n = 5 \)), or the inactivation rate constants (control: 195.5 ± 91.0; 1 \mu M capsaicin: 193.7 ± 83.8).

The application of 1 M CPT-cGMP to TG neurons reduced IA in both CS (Fig. 10) and CIS neurons with no major differences between them. In the CS neuron shown in Fig. 10, a 3-min exposure of 1 M CPT-cGMP reduced \( I_{A_{\text{Apmax}}} \) by 33% (also see \( I_{A_{\text{Ap-V}}} \) curve) and after 3-min wash, the current partially recovered. The application of CPT-cGMP (1 M) did not shift the G-V curve, but the inactivation rate constant decreased from 226 to 193 ms.

The presence of 1 M CPT-cGMP decreased the amplitude of IA current 32.0 ± 13.8% (\( n = 8 \)). It did not, however,
significantly alter the $G-V$ relation (control: $V_{0.5} = 9.8 \pm 9.9$ mV, $k = 15.2 \pm 3.1$; 1 mM CPT-cGMP: $V_{0.5} = 6.5 \pm 8.8$ mV, $k = 15.9 \pm 3.1$, $n = 8$; paired $t$-test $P = 0.09$), the inactivation-voltage parameters (control: $V_{0.5} = -64.2 \pm 5.4$, $k = -6.34 \pm 0.77$, $C = 0.27 \pm 0.14$; 1 mM CTP-cGMP: $V_{0.5} = -67.0 \pm 6.6$ mV, $k = -7.04 \pm 1.11$, $C = 0.28 \pm 0.14$) and inactivation rate constant (control: 165 $\pm$ 9.9 ms; 1 mM CTP-cGMP: 202 $\pm$ 71 ms; $P = 0.16$).

**KT5823, a PKG inhibitor, partially reverses the inhibitory effect of capsaicin**

We have shown that, by itself, capsaicin can inhibit $I_A$ currents (Fig. 3) and have also shown that $I_A$ currents are inhibited by cGMP (Fig. 10). Given this information, we determined whether capsaicin would be a less effective inhibitor if the cGMP-PKG pathway was inhibited. We therefore tested whether the presence of a PKG inhibitor, KT5823, would reduce the extent that capsaicin inhibits $I_A$ currents.

We used 0.5 $\mu$M KT5823 to test capsaicin effect because it virtually abolished the effects of 8-Br-cGMP on N-type calcium channels (D’Ascenzo 2002). A representative tracing, seen in Fig. 11A, shows that a 3-min incubation of 5$\mu$M KT5823 caused a small 8.9 $\pm$ 11.7% ($n = 8$) increase in the $I_A$ current. In the presence of 0.5 $\mu$M KT5823, 1 $\mu$M capsaicin activated an inward current that desensitized in a few minutes to a value $<$0.3 nA. At this time, $I_A$ currents were recorded and, after wash, when the membrane resistance recovered, the $I_A$ current was inhibited by 19%. After a 3-min wash, the $I_A$ current almost completely recovered (Fig. 11B). Whereas, 1 $\mu$M capsaicin inhibits the total $I_A$ by 49% (Fig. 6), in the presence of 0.5 $\mu$M KT5823, it inhibits by 24.8 $\pm$ 13.6% ($n = 8$, $P < 0.01$).

**Calmodulin-dependent pathways**

**RESTING CONDITIONS. W-7: a Ca$^{2+}$-calmodulin inhibitor.** Because the activation of vanilloid receptor-mediated second-messenger pathways permits the entry of calcium and because calcium associates with calmodulin (CaM), we investigated the effects of W-7 (25 $\mu$M), a calcium-calmodulin-inhibitor (Fig. 12). This concentration was chosen because it has previously been shown to be an inhibitor of VGPCs (Peretz et al. 2002). Figure 11 shows that 25 $\mu$M W-7 inhibited $I_{A_{\text{apmax}}}$ by about 30% and that the inhibition was partially reversible after a 3-min wash. On average, $\tau$ was significantly decreased (control: 100 $\pm$ 44.7 ms; 25 $\mu$M W-7: 63.2 $\pm$ 36.9 ms; $n = 8$, $P = 0.009$), but the $G-V$ curve was not altered significantly (control: $V_{0.5} = 6.86 \pm 9.07$ mV, $k = 17.9 \pm 4.23$; 25 $\mu$M W-7: $V_{0.5} = 7.49 \pm 9.9$ mV, $k = 18.32 \pm 4.77$; $P = 0.53$). Finally, W-7 produced a 5-mV hyperpolarizing shift in the voltage dependence of inactivation (control: $V_{0.5} = -66.31 \pm 10.2$ mV, $k = -8.2 \pm 1.3$, $C = 0.26 \pm 0.22$ 25 $\mu$M W-7: $V_{0.5} = -71.86 \pm 13.2$ mV, $k = -10.6 \pm 2.9$, $C = 0.20 \pm 0.14$, $n = 7$; $P = 0.0065$).

**KN-93: a CaMKII inhibitor.** Because the CaMKII pathway is activated by calcium-calmodulin, we tested under resting conditions, whether 5 $\mu$M KN-93, a CaMKII inhibitor, would also modulate $I_A$ currents. This concentration was chosen because it has previously been shown to inhibit VGPCs (Peretz et al. 2002). Figure 13 shows a representative recording of the effect of 5 $\mu$M KN-93 on $I_A$ currents. The application of 5 $\mu$M KN-93 reduced both $I_{A_{\text{apmax}}}$ and the inactivation rate constant. On average, after a 3- to 4-min incubation with 5 $\mu$M KN-93, $I_{A_{\text{apmax}}}$ decreased 15.5 $\pm$ 10.6% ($n = 8$) without either altering the $G-V$ curve (control: $V_{0.5} = -8.97 \pm 4.57$ mV, $k = 15.3 \pm 1.93$; 5 $\mu$M KN-93: $V_{0.5} = -12.2 \pm 5.93$ mV, $k = 16.01 \pm 1.04$, $n = 7$, $P = 0.42$) or the inactivation-voltage parameters (control: $-62.5 \pm 4.14$, $k = -5.38 \pm 0.78$, $C = 0.18 \pm 0.07$; 5 $\mu$M KN-93: $V_{0.5} = -64.9 \pm 4.4$ mV, $k = -7.20 \pm 2.15$, $C = 0.126 \pm 0.05$; $P = 0.18$, 0.037 and 0.054 for $V_{0.5}$, $k$, and $C$, respectively). There was, however, a very large and significant decrease in the inactivation rate constant (control: $\tau = 134.8 \pm 38.7$ ms; 5 $\mu$M KN-93: $\tau = 28.6 \pm 7.4$ ms, $P < 0.00001$).
STIMULATED CONDITIONS. Because the activation of vanilloid receptors increases intracellular calcium, which will then associate with calmodulin, it is expected that the CaMKII concentration would increase which could result in a decrease in $I_A$ currents. We therefore investigated whether inhibiting CaMKII (with KN-93) would decrease capsaicin's ability to inhibit $I_A$ currents.

Figure 14 shows that in a CS neuron $1 \mu M$ capsaicin in the presence of $5 \mu M$ KN-93, only inhibited $I_{A_{\text{pmax}}}$ by 12%. Whereas $1 \mu M$ capsaicin inhibits $I_A$ on by $49.2 \pm 17.5\%$, in the presence of KN-93 $I_A$ is inhibited $23 \pm 13.5\%$ ($n = 7$). Thus it appears that CaMKII pathways are involved in capsaicin's inhibition of $I_A$ currents.

DISCUSSION

It is well known that nociceptors that are activated by capsaicin can become sensitized (Baumann et al. 1991; Green 1989). To determine whether $I_A$ currents may contribute to this behavior, we have investigated how capsaicin modulates $I_A$ currents in TG neurons. We studied $I_A$ currents because inhibiting them, through nerve injury, or pharmacologically, will lead to hyperexcitability and hyperalgesia (Nashmi and Fehlings 2001; Pearce and Duchen 1994; Rasband et al. 2001). In CS neurons, we found that the activation of vanilloid receptors evokes intracellular cascades that will inhibit $I_A$ currents. Although pathways involving PKC and cAMP do not appear to be involved, the activation of cGMP-PKG and CaMKII pathways contributes to capsaicin-mediated inhibition of $I_A$ currents.

$I_A$ currents in TG neurons

The RT-PCR experiments revealed that rat TG neurons, like DRG neurons, contain many types of $\alpha$ (Kv1.2–1.6) and $\beta$ (Kv1.1, 1.2, 1.6) VGPC subunits (Fig. 1) (Ishikawa et al. 2001; Seifert et al. 2001). In addition, TG neurons have been found to contain other VGPC subunits (Kv1.1, 2.4, 2.2) (Seifert et al. 2001).
2001). Some α subunits alone, or when associated with β subunits give rise to characteristic $I_{K}$ or $I_{A}$-type currents (Dolly and Parcej 1996, Grissmer et al. 1994; Rasband et al. 2001; Sheih et al. 2000). The electrophysiological data reveal that TG or DRG neurons contain multiple types of functional VGPCs, including more than one type of $I_{A}$ current (Dolly and Parcej 1996, Grissmer et al. 1994; Rasband et al. 2001). In this study, we did not distinguish between different subunits (Rasband et al. 2001) as well as their phosphorylation state (An et al. 2000).

Capsaicin has been shown to produce a small use-dependent block of VGPCs (about 5% after 15 cycles and 10% after 30 cycles of 0.5 Hz). This blockage was interpreted to arise from capsaicin preferentially binding to and blocking an open state of the channel (Bielefeldt 2000; Kehl 1994). We did not observe a statistically significant difference in use-dependent blockage after 16 cycles at 1 Hz in either CIS or CS neurons albeit at a lower capsaicin (1 μM) concentration. It is likely that some of the cells tested contained vanilloid receptors (e.g., Atkins and McCleskey 1993).

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**Capsaicin inhibits $I_{A}$ currents**

Non-specific inhibition. In CIS neurons, we found that the magnitude of the $I_{A}$ current decreased linearly with increasing capsaicin concentration such that at 30 μM it reached 24.6% (Fig. 6). In TGs from VR1−/− mice that were obtained from mice having characteristics of nociceptors, 10 μM capsaicin inhibited $I_{A}$ currents 29% (Fig. 5). Both these values are much less than the 49% inhibition by 1 μM capsaicin in CS neurons (Fig. 6). Capsaicin has previously been shown to inhibit $I_{A}$ currents at $K_{12}$ ranging from 6 to 158 μM in a manner that depends on the cell and/or the particular subtype of receptor (Castle 1992, Grissmer et al. 1994). In all cases, except for what we found in CS neurons (Fig. 6), these values are much higher than the concentration needed to activate vanilloid receptors (0.7 μM) (Caterina et al. 1997). In many of these studies, especially those where the $K_{12}$ were comparatively low (6–10 μM), it is likely that some of the cells tested contained vanilloid receptors (e.g., Atkins and McCleskey 1993).

CS neurons: inhibition of $I_{A}$ through the activation of vanilloid receptors

We have shown that in CS neurons that capsaicin inhibits $I_{A}$ currents largely through the activation of vanilloid receptors (Figs. 3–6). For 1 μM capsaicin, 43% of the blockage of $I_{A}$ currents can be attributed to the activation of vanilloid receptors (Figs. 6 and 15). That the inhibition is a consequence of the activation of vanilloid receptors is supported by the following evidence: the blockage of $I_{A}$ currents in CS neurons occurs at concentrations comparable to the activation of vanilloid receptors (Fig. 6), the inhibition persists even after the decrease in

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**FIG. 11.** Inhibitory effect of capsaicin on $I_{A}$ current is partially reversed by KT 5823. $A$: $I_{A}$ current was recorded in capsaicin-sensitive neuron (data not shown). After a 3-min preincubation of 0.5 μM KT5823, $I_{A}$min, was reduced to 10.2 nA from 10.5 nA. On the addition of 1 μM capsaicin to 0.5 μM KT5823, only 18% of the $I_{A}$ current was blocked. The $I_{A}$ current recovered after 3-min wash. $B$: a histogram showing the percentage inhibition of $I_{A}$ current by 1 μM capsaicin (49.2 ± 17.5%, n = 12) and 5 μM KT5823 + 1 μM capsaicin (24.8 ± 13.6%, n = 8, mean ± SD).
resistance produced by capsaicin has recovered (Fig. 3), and at 1 μM capsaicin in small diameter VR1−/− TG neurons, the inhibition is small (Fig. 5).

If the channel \( I_A \) conductance is unaffected by capsaicin, then reduction in the current may be due to a decrease in the open state probability, perhaps as a consequence of the hyperpolarizing shift in the inactivation-voltage relation (Dubois 1982).

**\( I_A \) currents are modulated through capsaicin-activated intracellular pathways**

It is well known that potassium channels, like other voltage-gated ion channels, are modulated by intracellular compounds that regulate their degree of phosphorylation (Ishikawa et al. 2001; Nicol et al. 1997; Wickman and Clapham 1995; Zhang et al. 2001). Because the activation of vanilloid receptors activates a variety of pathways, it is to be expected that some of them would also modulate \( I_A \) currents.

A summary of the effects of all the compounds tested on \( I_A \) currents is presented in Fig. 15. The data clearly show that the ability to modulate \( I_A \) currents is very dependent on which pathways are activated. They also indicate that by themselves, none of these pathways inhibit the current to as great an extent as 1 μM capsaicin (49 or 43% attributed to the activation of vanilloid receptors). In DRGs and/or TG neurons, it has been found that the activation of vanilloid receptors induces increases in cAMP, cGMP, and PKC (Harvey et al. 1995; Liu et al. 2001, Wood et al. 1989). Consequently, we tested whether the activation of these three pathways can modulate \( I_A \) currents. Neither 1 mM CTP-cAMP nor 500 nM PDBu (a PKC activator) had a marked effect on \( I_A \) currents (Figs. 9, A and B, and 15). We therefore can eliminate these two pathways as those that contribute to capsaicin’s inhibition of \( I_A \).

The data that we have obtained provides good evidence that vanilloid receptor activation of the cGMP-PKG pathway, perhaps through the production of NO (Fazekas et al. 1994), is involved in the capsaicin-induced inhibition of \( I_A \) currents. The
The evidence is as follows. In CS neurons, the application of capsaicin increases intracellular Ca$^{2+}$ (Cholewinski et al. 1993) and consequently the calcium-dependent cGMP concentration (Wood et al. 1989). We showed that the application of 1 mM CTP-cGMP to TG neurons reduced the $I_{A}$ currents by 30% (Fig. 11). Another argument for a role of the cGMP-PKG pathway is that the ability of capsaicin to inhibit $I_{A}$ currents was significantly reduced in the presence of the PKG antagonist KT5823 (Fig. 11). In addition, $I_{A}$ currents were increased by KT5823. Finally, in the absence of external stimuli, W-7, a CaM inhibitor, also decreased $I_{A}$ currents. The inhibitory effect of W-7 may, at least partially be attributed to its ability to increase cGMP concentrations (Parfenova et al. 1993).

However, it is evident that by itself, the cGMP-PKG pathway cannot account for the magnitude of capsaicin-mediated inhibition or the 16 mV shift in the inactivation-voltage curve (Fig. 8). This suggests that other mechanisms (pathways) must be contributing to the inhibitory effect of capsaicin on the $I_{A}$ currents.

The other obvious pathway that we explored is the CaMKII pathway. Because the activation of vanilloid receptors permits the entry of calcium (Cholewinski et al. 1993) and because calcium associates with calmodulin (CaM), the activation of VR1 receptors should increase the CaMKII concentrations. If a direct connection between the activation of VR1 receptors and increases in CaMKII was present, then decreasing the CaMKII concentration with KN-93 would result in an increase in $I_{A}$ currents. This is exactly what was found (Fig. 15). That is, by itself 1 μM capsaicin inhibits $I_{A}$ 49% and in the presence of 5 μM KN-93, capsaicin inhibited $I_{A}$ currents by 23%. These data suggest that $I_{A}$ channels have phosphorylation sites that could be modulated by CaMKII-mediated pathways.

In summary we have shown that cGMP-PKG and CaMKII pathways contribute to capsaicin’s ability to inhibit $I_{A}$ currents. However, individually they cannot account for all of capsaicin's effects, such as producing a hyperpolarizing large shift in the inactivation-voltage curves (Fig. 8). Clearly, these pathways acting together, or other pathways must contribute to capsaicin’s inhibitory effects.
Physiological implications

The application of capsaicin to epithelia produces many physiological responses. Depending on its concentration, length of application, and interstimulus interval, it can produce sensitization, hyperalgesia, or desensitization (Baumann et al. 1991; Dessirier et al. 2000; Green 1989). Capsaicin’s activation of nociceptors arises from the depolarization produced by the opening of vanilloid receptors (whose \( K_{1/2} \) approximately 1 \( \mu \)M at physiological pH). Capsaicin-induced sensitization of nociceptors can arise from many factors, including peptide and transmitter release or changes in the properties of ion channels. Under physiological conditions, \( I_A \) currents contribute to the suppression of neuronal excitability so that a reduction in them, whether by nerve injury or pharmacological methods such as capsaicin, results in increased neuronal excitability. Given that at a resting potential of \(-60 \) mV, about 57% of the \( I_A \) channels are inactivated, whereas in the presence of 1 \( \mu \)M capsaicin about 83% of \( I_A \) currents (in CS neurons) are inactivated, we propose that the inhibitory effect of capsaicin on \( I_A \) currents will cause CS nociceptors to become more excitable and in this manner may contribute to its sensitizing effects.

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