The Role of Calcium in the Desensitization of Capsaicin Responses in Rat Dorsal Root Ganglion Neurons

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Capsaicin (Cap) is a pungent extract of the Capsicum pepper family, which activates nociceptive primary sensory neurons. Inward current and membrane potential responses of cultured neonatal rat dorsal root ganglion neurons to capsaicin were examined using whole-cell and perforated patch recording methods. The responses exhibited strong desensitization operationally classified as acute (diminished response during constant Cap exposure) and tachyphylaxis (diminished response to successive applications of Cap). Both acute desensitization and tachyphylaxis were greatly diminished by reductions in external Ca\textsuperscript{2+} concentration. Furthermore, chelation of intracellular Ca\textsuperscript{2+} by addition of either EGTA or bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid to the patch pipette attenuated both forms of desensitization even in normal Ca\textsuperscript{2+}. Release of intracellular Ca\textsuperscript{2+} by caffeine triggered acute desensitization in the absence of extracellular Ca\textsuperscript{2+}, and barium was found to effectively substitute for calcium in supporting desensitization. Cap activated inward current at an ED\textsubscript{50} of 728 nM, exhibiting cooperativity (Hill coefficient, 2.2); however, both forms of desensitization were only weakly dependent on [Cap], suggesting a dissociation between activation of Cap-sensitive channels and desensitization. Removal of ATP and GTP from the intracellular solutions resulted in nearly complete tachyphylaxis even with intracellular Ca\textsuperscript{2+} buffered to low levels, whereas changes in nucleotide levels did not significantly alter the acute form of desensitization. These data suggest a key role for intracellular Ca\textsuperscript{2+} in desensitization of Cap responses, perhaps through Ca\textsuperscript{2+}-dependent dephosphorylation at a locus that normally sustains Cap responsiveness via ATP-dependent phosphorylation. It also seems that the signaling mechanisms underlying the two forms of desensitization are not identical in detail.

Key words: capsaicin; sensory neurons; patch clamp; desensitization; calcium; nociceptors; dorsal root ganglion

The ability of neurons to adapt to specific stimuli is crucial for the normal physiological operation of the nervous system. Through the process of desensitization, a neuron can diminish its overall response to a particular chemical, physical, or electrical signal. For example, the inactivation of glutamate-dependent ion channels within the CNS seems to play a protective role during a prolonged exposure to glutamate by limiting potentially cytotoxic calcium overload in the neurons (Choi, 1988). Since the early characterization of the desensitization of acetylcholine responses at the frog neuromuscular junction by Katz and Thesleff (1957), receptors in a wide range of excitable tissues have demonstrated desensitization and provided model systems to investigate the possible mechanisms underlying this change in sensitivity to agonists.

The desensitization of sensory neurons is particularly important considering their crucial role in the physiological perception of and reaction to the external environment. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is the active ingredient in hot peppers that selectively targets polymodal nociceptive and warmth-sensitive thermoreceptor fibers of the C and A\textsubscript{\delta} classes (Jancso et al., 1967; Bevan and Solcsanyi, 1990; Dray, 1992; Dray and Dickenson, 1993). Capsaicin excites afferent neurons through the activation of a nonselective cation channel, leading to depolarization and release of neurotransmitters, including substance P, from sensory nerve terminals (Bevan and Forbes, 1988; Holzer, 1988). The capsaicin response exhibits a pronounced desensitization that functionally inactivates nociceptive neurons subsequent to the initial excitation (Jancso et al., 1967). This selective inactivation of nociceptive neurons by capsaicin has generated extensive research on the possible therapeutic effectiveness of capsaicin as a clinical analgesic tool (Bernstein, 1987; Maggi, 1991; Wallengren, 1991; Breneman et al., 1992; Campbell et al., 1993; Epstein and Macroe, 1994).

In the present study we investigate the mechanisms of desensitization of the capsaicin-activated current. Desensitization exhibited by capsaicin can be divided into two phenomenological categories: (1) “acute desensitization,” an inactivation of the current during a prolonged application of capsaicin; and (2) “tachyphylaxis,” a diminution of the maximal current amplitude during successive deliveries of the same capsaicin concentration. It has been observed previously that tachyphylaxis of capsaicin-activated currents is affected by changes in extracellular calcium (Santicioli et al., 1987; Yeats et al., 1992; Cholewinski et al., 1993), but there are no comparable observations concerning the acute desensitization of capsaicin responses. Although examination of the published data suggests that acute desensitization occurs during extended applications of capsaicin in calcium-containing external solutions (Marsh et al., 1987; Petersen and LaMotte, 1991; Bevan...
et al., 1992), this phase of desensitization has not been evaluated in detail.

The experiments described in this paper were designed to characterize the acute desensitization and tachyphylaxis of the capsaicin-activated channel in cultured neonatal rat dorsal root ganglion (DRG) neurons and to evaluate the role of divalent cations in these processes. In addition to confirming the earlier reports of extracellular calcium dependence, we demonstrate that the locus of this sensitivity is a rise in intracellular Ca$^{2+}$. Furthermore, we show that desensitization can be supported by other divalent cations, including Ba$^{2+}$.

**MATERIALS AND METHODS**

**Dissociations and cultures of dorsal root ganglion neurons.** Sprague Dawley rat pups between the ages of 2 and 8 d were used for preparation of the DRG cultures. After the DRGs were collected into a Petri dish containing Ca- and Mg-free HBSS (CMFH), a scalpel was used to trim the remaining nerve roots and connective tissue from the ganglia. All cleaned ganglia were collected into a sterile 15 ml polyethylene centrifuge tube containing approximately 2 ml of CMFH with 0.01% added trypsin (Sigma, St. Louis, MO) (type III, bovine pancreas) and incubated at 37°C for 6–10 min in 5% CO$_2$. After the tryptic digestion, the DRGs were rinsed twice in 1–2 ml of growth medium and triturated with a fire-polished siliconized glass pipette. The dissociated neurons were plated in 12-well dishes containing plastic coverslips previously exposed to ultraviolet radiation for 15 min and coated with 0.04 mg/ml sterile poly-D-lysine. After the neurons were returned to the incubator for 30–60 min to allow proper adherence to the coverslips, 1 ml of growth medium containing 50 ng/ml nerve growth factor (Sigma) (nerve growth factor 2.5S, mouse submaxillary gland) was added to each well. The final density of the plated cells was relatively high, because cell density seemed to affect both the success rate of patch-clamp recordings and the amplitude of capsaicin responses. The experiments were conducted exclusively on cells cultured for only 20–30 d.

**Electrophysiological solutions.** All experimental solutions were adjusted to pH 7.4 and osmolarity of ~300 mOsm. The standard external solution (SES) contained (in mM): 145 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, and 10 glucose. The calcium-free external solution (0 Ca-ES) contained no added calcium and 1 mM EGTA to chelate ambient calcium. The standard internal solution (SIS) consisted of (in mM): 130 potassium aspartate, 20 KCl, 10 HEPES, and 0.10 GTP; in addition, SIS contained a nucleotide-regenerating system consisting of (in mM): 2 mM ATP, 2 MgATP, and 5 mM creatine phosphate, and 20 μM creatine phosphokinase (Sigma). In a subset of experiments, EGTA or bis(2-aminophenoxy)ethane-N,N',N''-tetra-acetic acid (BAPTA) was included in the internal solution (EGTA-IS or BAPTA-IS, respectively) at a concentration of 10 mM.

For all of the experiments, drug solutions were delivered to the cells via a U tube perfusion system with a delivery response time on the order of milliseconds (Oxford and Wagoner, 1989). To confirm the proper delivery of all solutions throughout the course of an experiment, each solution was returned to the incubator for 30–60 min to allow proper adherence to the coverslips, 1 ml of growth medium containing 50 ng/ml nerve growth factor (Sigma) (nerve growth factor 2.5S, mouse submaxillary gland) was added to each well. The final density of the plated cells was relatively high, because cell density seemed to affect both the success rate of patch-clamp recordings and the amplitude of capsaicin responses. The experiments were conducted exclusively on cells cultured for only 20–30 d.

**RESULTS**

**Desensitization of capsaicin responses**

We first characterized and verified the capsaicin responses in our DRG neuron cultures because culture conditions and plating substrate can have a substantial impact on cellular phenotype. A typical current activated by 1 μM capsaicin is shown in Figure 1. Capsaicin activated an inward current carried predominately by sodium ions in the bath solution with a small additional contribution from external calcium ions (data not shown). In neurons voltage clamped at a holding potential of ~60 mV, 1 μM capsaicin activated inward currents that ranged in peak amplitude from 50 pA to >19 nA (producing saturation of the patch-clamp amplifier). The mean current amplitude for a representative set of neurons was 2.17 ± 0.33 nA (n = 30). As seen in Figure 1 (inset), there was a delay between the initial exposure to capsaicin and activation of the inward current. In all experiments, the duration of this activation interval was quite variable and ranged from 1 to 5 sec. The delay was not the result of a time required for the drug to reach the cell, because the U tube perfusion system has a delivery response time on the order of
20–30 msec (Oxford and Wagoner, 1989). Rather, the slow activation of the capsaicin-dependent channels suggests that a coupling mechanism involving other membrane-associated proteins or cellular messengers is the rate-limiting step in the current activation. Alternatively, a slow diffusion of capsaicin through the plasma membrane to an intramembrane or intracellular site of action may be responsible for the delay in activation.

The capsaicin-activated current exhibits two types of desensitization, both of which are illustrated in Figure 1. We have defined acute desensitization as the decrease in inward capsaicin-activated current during an extended application of capsaicin. We specifically distinguish this from tachyphylaxis, which refers to the diminution of the current amplitude observed during repeated applications of the same capsaicin concentration. Overall, the acute desensitization and tachyphylaxis of capsaicin responses throughout this study were quite heterogeneous in that some currents displayed complete desensitization, whereas other capsaicin responses only partially desensitized. One explanation for this variability is that the mechanisms underlying acute desensitization and tachyphylaxis result in the inactivation of a fraction or subpopulation of the channels. The additional variability in activation kinetics of capsaicin-dependent currents may also reflect the contribution of subtypes of capsaicin-gated channels. The existence of capsaicin receptor channel subtypes has previously been suggested by Liu and Simon (1994) based on the rapid and slow inward currents activated by capsaicin in rat trigeminal neurons.

Calcium dependence of desensitization

We next sought to determine whether external calcium was necessary for and therefore integral to capsaicin-induced acute desensitization and tachyphylaxis in our culture preparation of DRG neurons. Figure 2 illustrates consecutive capsaicin responses recorded in the absence and presence of extracellular calcium. In the 0 Ca-ES solution, negligible desensitization was observed during the first capsaicin challenge. After switching to the SES containing 2 mM external calcium, the capsaicin-activated current desensitized to only 15% of the maximal current amplitude. The peak current magnitude decreased very little between the first two applications, indicating a lack of tachyphylaxis in the absence of Ca²⁺. In contrast, a pronounced tachyphylaxis was evident in the third response, presumably because of the presence of extracellular calcium during the second activation of the capsaicin-dependent current. The channels were not completely inactivated,
in that a subsequent challenge with a higher concentration of capsaicin (5 μM) stimulated a substantial inward current.

**Concentration dependence of calcium-facilitated desensitization**

In an effort to quantify the relationship between external calcium and desensitization, the external calcium concentration was varied within the range of 0–10 mM, and both the acute desensitization and tachyphylaxis of capsaicin responses were assessed. We also manipulated the relative concentration of free internal Ca\(^{2+}\) by including Ca\(^{2+}\) chelators in the pipette solution. BAPTA and EGTA were both tested, because these compounds differ in their efficiency of calcium chelation. Although each chelator is highly selective for Ca\(^{2+}\) over Mg\(^{2+}\), BAPTA mediates a faster Ca\(^{2+}\) chelation with no pH sensitivity, because protons are not released during the binding of Ca\(^{2+}\) (Tsien, 1980). The three internal solutions tested were: (1) control, with no added chelator (SIS); (2) 10 mM BAPTA (BAPTA-IS); and (3) 10 mM EGTA (EGTA-IS).

The experimental protocol performed to test acute desensitization used a 60 sec challenge with 1 μM capsaicin. For the tachyphylaxis protocol, a series of successive 15–20 sec applications of 1 μM capsaicin with interdose intervals of 2 min was delivered. Experiments testing the effect of the interdose interval on tachyphylaxis indicated that there was no significant difference in the extent of tachyphylaxis observed for interval durations ranging from 30 sec to 20 min (results not shown). This finding agrees with the results of Cholewinski et al. (1993), who observed only limited recovery of capsaicin-stimulated increases in intracellular Ca\(^{2+}\) at 25 min.

**Acute desensitization**

Overall, the results indicated that both the rate and degree of acute desensitization were dependent on the concentration of external calcium. Representative traces recorded in various external Ca\(^{2+}\) concentrations with either SIS or BAPTA-IS are shown in Figure 3, A and B, respectively. It is evident that the capsaicin responses displayed little acute desensitization in the absence of external calcium. As the external calcium was increased, the rate and degree of acute desensitization was enhanced. The presence of internal chelation capacity seemed to reduce the acute desensitization at lower Ca\(^{2+}\) concentrations. This can be seen from a comparison of the acute desensitization observed for the 1 mM Ca\(^{2+}\) concentration in SIS (Fig. 3A) and BAPTA-IS (Fig. 3B).

The summary of the calcium dependence of the degree of acute desensitization is presented in Figure 3C. For 0 Ca-ES and all three internal solutions, the acute desensitization of the capsaicin responses was absent or minimal in the 55 cells tested. As the external calcium concentration was raised, a larger percentage of cells demonstrated acute desensitization, and the degree of desensitization also increased. Furthermore, at the low Ca\(^{2+}\) concentration of 1 mM, desensitization was inhibited by internal Ca\(^{2+}\) buffering by either EGTA or BAPTA.

Statistical analysis of the results for the 0, 1, and 2 mM external Ca\(^{2+}\) solutions indicated a significant effect of external calcium concentration (p < 0.0005), internal solution (p = 0.055), and a
significant interaction between these two variables ($p = 0.010$, two-way ANOVA). Results of the posthoc analysis indicated that the inclusion of BAPTA ($p = 0.014$) significantly reduced the degree of desensitization for the 1 mM external Ca$^{2+}$ condition relative to control, whereas the addition of EGTA had an effect of only marginal significance ($p = 0.105$). There was no significant difference for the three internal solutions in the 0 mM ($p > 0.455$) and 2 mM ($p > 0.2$) Ca$^{2+}$ solutions.

The dependence of acute desensitization on calcium concentration raises the question of whether the site for calcium modulation of acute desensitization is extracellular or intracellular. The sensitivity of acute desensitization to internal chelation suggests that intracellular calcium concentration is the important variable for activation of acute desensitization processes. To further explore the possibility that intracellular calcium is the key effector in acute desensitization, we investigated the action of caffeine on capsaicin responses in the absence of extracellular calcium. Rat DRG and other neurons have been shown to release calcium from internal stores in response to a caffeine challenge (Holliday et al., 1991; Benham et al., 1992; Schmigol et al., 1994). The presence of caffeine-sensitive calcium stores in our cultures of DRG neurons supports the idea that this rise in internal calcium is capable of supporting acute desensitization processes in the absence of external calcium and calcium influx.

Tachyphylaxis

In general, the degree of tachyphylaxis increased with increases in external calcium concentration as seen for acute desensitization.
An example of the tachyphylaxis behavior recorded under control conditions (SIS internal) in an external Ca\(^{2+}\) concentration of 2 mM is shown in Figure 5A. It is evident that the tachyphylaxis proceeds with successive capsaicin applications. A summary of the tachyphylaxis for all three internal solutions is shown in Figure 5B. The initial maximal current amplitude (\(I_{\text{max}}\)) corresponds to the maximal current amplitude observed during the initial delivery of 1 mM capsaicin. The results are presented as the percentage of this initial capsaicin-activated current calculated for each response elicited by four consecutive capsaicin applications. It should be noted that in contrast to the reduced currents typically observed during successive applications of capsaicin, an increase in the peak current amplitude was observed for three of seven cells during dialysis with BAPTA-IS. This increase in current amplitude is similar to the potentiation phenomenon reported by Yeats et al. (1992), who observed an increase in capsaicin responses in the presence of external barium and internal EGTA chelation. In our experiments, the potentiation was occasionally observed under a variety of solution conditions but was most pronounced in solutions containing external barium and internal BAPTA (see Fig. 7B below). At present, the mechanism underlying this increase in current is not known.

The mean tachyphylaxis observed for the second capsaicin application across the entire range of external Ca concentration is compared between SIS and BAPTA-IS internals solutions in Figure 5C. It is evident that the actual calcium concentration at which the tachyphylaxis of the second capsaicin response reached 50–75% changed markedly with calcium chelation. Statistical analysis of these data revealed a significant effect of the internal solution\((p = 0.001)\), the external calcium concentration\((p < 0.0005)\), and an interaction between these two independent variables\((p = 0.066,\) two-way ANOVA). A further comparison of specific pairs of solution conditions indicated the following: (1) at 0 mM external Ca\(^{2+}\), there was no significant difference in the tachyphylaxis
observed in SIS and BAPTA-IS ($p = 0.31$); (2) for 1 mM external Ca$^{2+}$, BAPTA-IS had significantly less tachyphylaxis than SIS ($p = 0.03$), and BAPTA-IS and EGTA-IS (data not shown) did not differ significantly ($p = 0.61$); and (3) for 5 mM external Ca$^{2+}$, there was no significant difference in tachyphylaxis among the three internal solutions ($p > 0.64$). All of the above $p$ values are unadjusted values resulting from a post hoc analysis of individual group means.

**Desensitization and capsaicin concentration**

We examined the acute desensitization and tachyphylaxis behavior as a function of capsaicin concentration (0.05–5 $\mu$M) in the SES and SIS solutions, as summarized in Figure 6. The degree of acute desensitization to single applications of capsaicin ranging from 50 nM to 5 $\mu$M was assessed as the fraction of peak current, which declined to a steady state level during the application (Fig. 6, open circles). The degree of tachyphylaxis was assessed as the fraction of peak current response to a brief initial capsaicin application that was absent during a second application 20 sec later (Fig. 6, open triangles). Statistical analysis of the data reveal that the degree of acute desensitization was not dependent on capsaicin concentration ($p = 0.32$, one-way ANOVA). Likewise, although a substantial decrease in the current amplitude occurred between the first and second capsaicin applications, there was little difference evident in the extent of current reduction among the capsaicin concentrations examined ($p = 0.18$, one-way ANOVA). Both types of desensitization were also tested across this capsaicin concentration range for the 0 Ca SES/BAPTA-IS condition (results not shown). Although there was a pronounced reduction in the overall degree of acute desensitization and tachyphylaxis recorded in the absence of extracellular calcium, there was no significant effect of capsaicin concentration on the desensitization behavior.

The absence of agonist concentration dependence for desensitization stands in sharp contrast to the concentration dependence of inward current activation. For comparison, the dose-response relationship for capsaicin-induced currents is shown in Figure 6 (filled circles), which reveals a steep dependence of current activation on agonist concentration. The Hill coefficient of 2.2 suggests possible cooperativity between two capsaicin molecules to activate a conductance increase. Consequently, it seems that the activation of additional receptors by higher concentrations of capsaicin and the corresponding larger inward currents fail to produce a significantly greater degree of acute desensitization or tachyphylaxis.

**Barium and desensitization**

The ability of barium to substitute for calcium in various biological processes is often thought to reveal fundamental insights into the mechanisms of divalent cation regulation. We thus examined the desensitization of capsaicin-activated currents (acute and tachyphylaxis) with an external solution containing 2 mM Ba$^{2+}$ in place of calcium (Ba-ES) and three different internal solutions (SIS, EGTA-IS, and BAPTA-IS). In general, the capsaicin responses recorded in Ba-ES were smaller in amplitude than the currents observed in SES, supporting the notion that calcium permeates the capsaicin-gated channels more readily than does barium. Specifically, the mean current amplitude of 0.61 ± 0.13 nA ($n = 36$) measured in Ba-ES was significantly less than the mean values for both the SES condition (2.17 ± 0.33 nA; $n = 30$) and the 0 Ca SES condition (3.55 ± 0.30 nA; $n = 22$; $p < 0.0001$, one-way ANOVA).

For all three internal conditions (SIS, EGTA-IS, and BAPTA-IS), the capsaicin-activated currents exhibited substantial declines during agonist application in Ba-ES, demonstrating that this divalent can effectively support acute desensitization (Fig. 7A, open bar). Although there was no statistical difference in the level of acute desensitization observed in Ba-ES among the three internal solutions ($p = 0.25$, one-way ANOVA), the mean level of desen-
The capsaicin-activated currents recorded in external barium was less during dialysis with BAPTA-IS (data not shown).

The capsaicin-activated currents recorded in external barium also exhibited tachyphylaxis. It can be seen in Figure 7B (filled squares) that substantial tachyphylaxis occurs with consecutive applications of capsaicin in the Ba-ES and SIS solutions. As mentioned previously, an anomalous current potentiation occurred occasionally in the presence of external barium during the tachyphylaxis experiments. This increase in amplitude was observed with all three internal chelation conditions; however, with BAPTA-IS, the potentiation of the capsaicin-activated current was the exclusive response, because none of the cells showed tachyphylaxis (Fig. 7B, open diamonds; n = 5). This is in contrast to the acute desensitization observed with barium under the same conditions.

**Comparison of desensitization in calcium, barium, nominal calcium, and calcium-free solutions**

The observation that acute desensitization and tachyphylaxis are supported in the barium-containing solution raised the question of whether residual calcium rather than barium actually mediates this desensitization. Because no external EGTA chelation was present in the Ba-ES solution, contaminating calcium from the water and stock solutions could potentially reach micromolar concentrations. To address this issue, we assessed acute desensitization and tachyphylaxis in an nominal Ca external solution (Nom-Ca-ES).

The desensitization behavior for the four external solutions (SES, Ba-ES, nom-Ca-ES, and 0 Ca-ES) and the SIS internal solution are compared in Figure 7. Figure 7A indicates that the degree of acute desensitization was highest in SES, reduced in Ba-ES, and lowest in the nominal and 0 Ca solutions. There was significantly greater acute desensitization in SES and Ba-ES when compared with the two external solutions containing no added divalent cation [p < 0.001, multivariate ANOVA (MANOVA), Tukey–Kramer]. Moreover, there was no significant difference in the acute desensitization observed for the SES and Ba-ES solutions (p > 0.05, MANOVA, Tukey–Kramer).

Figure 7B shows that the degree of tachyphylaxis was most pronounced in external calcium, reduced in barium, and smallest in the nominal and 0 Ca external solutions, consistent with the observations on acute desensitization. A statistical analysis of the tachyphylaxis observed for the second capsaicin application indicated that there was no significant difference among the four solutions (p = 0.059, one-way ANOVA), suggesting that a calcium-independent process may also contribute to the reduction in current amplitude. Nonetheless, comparison of the third and fourth applications clearly indicates that external barium does support greater tachyphylaxis than observed in the nominal and 0 Ca conditions.

**Desensitization and nucleotide analogs**

Intracellular calcium can modulate the process of desensitization through a variety of mechanisms. Ligand- and voltage-gated ion channels that conduct inward calcium currents can be desensitized or inactivated by mechanisms involving kinases and phosphatases, calcium, and voltage (Inoue et al., 1986; Kalman et al., 1988; Chen et al., 1990; Clark et al., 1990). For example, the inactivation of high voltage activated calcium channels and GABAA receptors can be regulated by a Ca- and calmodulin-dependent dephosphorylation event (Chad and Eckert, 1986; Armstrong and Eckert, 1987; Chen et al., 1990).

In the case of capsaicin responses, the only intracellular manipulation thus far reported to affect tachyphylaxis is an inhibition of the specific calcium- and calmodulin-dependent phosphatase calcineurin (Yeats et al., 1992; Docherty et al., 1996). Based on this suggestion that phosphorylation reactions are possibly involved in desensitization, we examined the influence of changing intracellular nucleotides on the acute desensitization and tachyphylaxis to capsaicin. To examine the potential contribution of GTP-binding proteins to desensitization, we compared capsaicin-activated currents recorded in internal solutions containing no added GTP, 100
μM GTP, or 500 μM GTPγS. The nucleotide ATP is necessary for intracellular phosphorylation events, as well as energy-dependent pumps and transport systems. To assess the role of ATP-dependent mechanisms, capsaicin responses were recorded in internal solutions that contained 2 mM MgATP, 2 mM ATPγS, or no added ATP. In all of these experiments, SES was the external solution, and the internal solution was identical to SIS except for the absence or presence of the respective ATP and GTP analogs. The nucleotide regenerating system was included in all internal solutions except for the conditions with no added ATP or GTP.

Tachyphylaxis was assessed using successive exposures to 1 μM capsaicin as described previously. A summary of the tachyphylaxis results for the various internal solutions is shown in Figure 8A as the percentage of reduction in the I_max for the second capsaicin challenge. There was no significant difference in the level of current reduction for the internal solutions containing combinations of ATP and GTP analogs (p > 0.05, MANOVA, Tukey–Kramer). In contrast, a dramatically larger tachyphylaxis was observed for the internal solution with no added nucleotides (p < 0.01). This result implicates a role for ATP and/or GTP in the tachyphylaxis processes regulating the desensitization state of the capsaicin-activated channels.

Data is that the significant enhancement of tachyphylaxis results from the absence of intracellular calcium chelation by ATP itself. The exclusion of ATP from an internal solution containing no other Ca chelator might be expected to result in higher concentrations of intracellular calcium after a capsaicin response, consequently mediating a larger tachyphylaxis compared with ATP-containing internal solutions. To test this possibility, calcium chelation was restored to the internal solution containing no added nucleotides by the addition of 10 mM BAPTA. If calcium chelation by ATP is replaced by BAPTA, one would expect to reverse the enhanced degree of tachyphylaxis seen in the absence of nucleotides. In contrast to this expectation, the mean level of current reduction for the second capsaicin application was similar for internal solutions containing no added nucleotides regardless of the absence or the presence of BAPTA (Fig. 8A). The enhanced tachyphylaxis observed in the absence of nucleotides is illustrated by comparing the current traces recorded in one neuron dialyzed with BAPTA-IS containing ATP and GTP (Fig. 8B) with those obtained in another neuron dialyzed with BAPTA-IS and no added nucleotides (Fig. 8C). Overall, these results suggest that ATP is not simply acting as a calcium chelator but may...
support a phosphorylation event that normally limits the degree of tachyphylaxis.

In contrast to the effects on tachyphylaxis, the various manipulations of ATP and GTP nucleotides had no effect on the acute desensitization of the capsaicin-activated currents (results not shown). A dramatic acute desensitization (90%) occurred in all internal solutions tested, but the absence or presence of the various analogs had no significant effect ($p = 0.70$, one-way ANOVA). These results suggest that the processes responsible for acute desensitization of capsaicin-activated channels are independent of ATP-dependent phosphorylation and GTP-binding proteins.

**Desensitization in current clamp**

The primary effect of capsaicin on sensitive DRG neurons is excitation reflected by depolarization and the generation of action potentials. Although a decline in capsaicin-activated cation currents during exposure to agonist under voltage-clamp conditions provides a clear indication of desensitization, the consequence of this phenomenon to the responsiveness of normal neurons is not clear. To determine whether the desensitization observed under voltage-clamp conditions influenced neuronal excitability under more physiological conditions, we examined voltage responses to continuous or repeated applications of capsaicin under current-clamp conditions.

A typical membrane potential response to 1 μM capsaicin recorded in SES and SIS solutions is shown in Figure 9A. After an initial delay, the neuron depolarized and fired a single action potential. The response exhibited acute desensitization as the membrane potential returned almost completely to the resting potential. Acute desensitization of the capsaicin response was observed in all seven cells tested with 1 μM capsaicin. Tachyphylaxis was also present, because only a minimal depolarization was produced by the second capsaicin application (Fig. 9A).

Capsaicin was also tested at a concentration of 30 nM. As seen in Figure 9B, there was a minimal acute desensitization of the capsaicin-induced depolarization. Although the failure of this neuron to continuously fire action potentials after the initial train of spikes suggests that some acute desensitization may have occurred, an alternative explanation is that the voltage-gated Na channels responsible for the spikes became inactivated. Acute desensitization occurred in only two of five cells challenged with 30 nM capsaicin, suggesting that lower capsaicin concentrations produce less acute desensitization under current-clamp conditions. In contrast, tachyphylaxis was observed in all five cells tested with 30 nM capsaicin. An example of a capsaicin application series is shown in Figure 10A. The first capsaicin challenge produced an initial train of action potentials and an extended depolarization. During the subsequent capsaicin deliveries, no action potentials were observed, and the depolarization of the membrane potential was reduced.

Because the whole-cell configuration necessitates a disruption of the plasma membrane, possibly disrupting calcium-buffering
capacity and signaling components critical for cellular responses, we also investigated the desensitization of capsaicin responses with the perforated patch technique. In general there was no difference in the desensitization of responses to 30 nM capsaicin recorded with the perforated patch technique versus the whole-cell recording mode. Acute desensitization was observed for three of eight cells, and tachyphylaxis occurred in five of six cells tested (e.g., Fig. 10B). These data suggest that the desensitization of capsaicin responses is not substantially different in intact versus dialyzed neurons. This conclusion is supported by results from voltage-clamp experiments completed with amphotericin B (results not shown) in which there was no significant difference in desensitization of capsaicin-activated currents in neurons recorded with perforated patch and whole-cell techniques.

**DISCUSSION**

The precise mechanisms underlying desensitization of capsaicin responses are not known. Our results, however, provide additional clues linking the phenomena to calcium-dependent processes. In summary, our data demonstrate that both acute desensitization and tachyphylaxis of the capsaicin-activated current are dependent on calcium concentration and independent of capsaicin concentration. There was no correlation between the overall extent of acute desensitization and tachyphylaxis within a given cell, suggesting that these two desensitization processes operate independently to modulate capsaicin receptors. Our divalent cation substitution experiments indicate that barium can successfully support both acute desensitization and tachyphylaxis, although less effectively than does calcium. However, during intracellular divalent chelation barium still supports acute desensitization while inducing a potentiation of current during consecutive capsaicin applications. This again suggests separable processes for the two forms of desensitization. The reduced ability of barium to stimulate the desensitization machinery within the cell may reflect a lower affinity or slower activation of divalent-dependent mechanisms by barium. Both types of desensitization were also observed for a subset of capsaicin responses in the absence of external calcium or barium, indicating that calcium-independent processes can mediate some acute desensitization and tachyphylaxis (see Figs. 3C and 5C).

**Acute desensitization**

The dependence of acute desensitization on both the external calcium concentration and the presence and identity of an internal chelating agent suggests that a critical calcium concentration must be established on the intracellular membrane surface for activation of the acute desensitization process. At the lower external calcium concentration of 1 mM, the acute desensitization was not only sensitive to the presence of an intracellular chelator but was differentially affected by EGTA and BAPTA, which exhibit varying speeds of calcium chelation. Above a 2 mM external Ca²⁺ concentration the acute desensitization process was insensitive to the presence of internal chelators at relatively high concentrations.
A major question regarding the mechanism of acute desensitization is whether the calcium critical for activation of the process enters the cell through capsaicin-dependent channels or is released from internal calcium stores by calcium-induced calcium release or other mechanisms such as inositol triphosphate-dependent release. Calcium-imaging experiments (Oxford et al., 1995) revealed a capsaicin-dependent increase in intracellular calcium concentration that was maintained throughout a 1 min capsaicin application. Thus an elevation of internal calcium is maintained for the same interval in which the capsaicin-activated current exhibits acute desensitization. From the results of the caffeine experiments, an increase in calcium concentration at the intracellular surface of the channel is sufficient to enhance acute desensitization in the absence of external calcium and calcium influx. Although a nonspecific block of the capsaicin-activated current by caffeine would produce a similar result, a pharmacological block of ion current has not been reported as a mechanism for caffeine action (Nehlig et al., 1992; Sawynok and Yaksh, 1993).

The limited observations of single capsaicin-gated channels also suggest that acute desensitization requires the presence of some intracellular factor. In the report of Forbes and Bevan (1988), there was no mention of any desensitization of single channel activity in cell-free membrane patches. Similarly, little acute desensitization is evident in the single channel records of Dray et al. (1990). Such continuous single channel activity in the excised patches exposed to capsaicin and calcium suggests that the calcium-dependent mechanism necessary for the acute desensitization of capsaicin-gated channels is not expressed within the isolated membrane patch and may require some cytoplasmic component.

Our observations that acute desensitization depends on the internal calcium concentration represent the first mechanistic evidence on the process of acute capsaicin desensitization. Our internal nucleotide experiments indicate that acute desensitization most likely does not involve ATP- or GTP-dependent mechanisms. This conclusion assumes that the endogenous nucleotides were sufficiently replaced or eliminated within the 5 min cytoplasmic dialysis before agonist application. In addition, the possible role of various intracellular signaling components should be tested, including phosphatases, phospholipases, and cytoskeletal components. In this regard, it has been recently proposed that ATP and calcium modulate the activity of NMDA channels by affecting the state of actin polymerization in the cytoskeleton (Rosenmund and Westbrook, 1993). A series of detailed single channel experiments would allow the investigation of the cellular machinery necessary and sufficient to support acute desensitization of capsaicin-activated channels.

Tachyphylaxis

Similar to acute desensitization, tachyphylaxis of capsaicin-activated currents is sensitive to the concentration of free internal calcium. The apparent dose–response curve for the external calcium dependence of capsaicin tachyphylaxis is shifted toward higher calcium levels by adding intracellular divalent chelators EGTA and BAPTA (see Fig. 5). The differential success of EGTA and BAPTA chelation at blocking tachyphylaxis in 2 mM external Ca\(^{2+}\) (see Fig. 5B,C) suggests that the internal calcium necessary for tachyphylaxis must reach a critical concentration within a particular interval to trigger tachyphylaxis successfully. However, we cannot yet establish the critical calcium concentration required to trigger tachyphylaxis, because we lack experimental data with various fixed concentrations of intracellular calcium.

The intracellular mechanism responsible for tachyphylaxis of the capsaicin-dependent current is activated during the initial capsaicin response. The interval between the first and second capsaicin applications can be varied over a wide range from 30 sec to 20 min with no change in the level of tachyphylaxis observed for the second capsaicin response (results not shown). This extreme time window for the tachyphylaxis process suggests at least two possibilities for the type of cellular mechanism involved: (1) a specific enzymatic modification of the capsaicin receptor or associated protein responsible for inactivation of the channels is relatively “irreversible” for at least 20 min; or (2) the tachyphylaxis of capsaicin-activated channels is mediated by a process activated during the first capsaicin response and continuously operating for an extended period. One possible event that could continuously activate the tachyphylaxis process would be a prolonged elevation of internal calcium levels after a capsaicin challenge. In the calcium-imaging experiments of Cholewinski et al. (1993), intracellular calcium responses to 0.1 \(\mu\)M capsaicin remained elevated after the removal of capsaicin and exhibited a tachyphylaxis that required 40 min for a full recovery. Using imaging of fluo-3 responses we have confirmed this observation, indicating that calcium elevations triggered by capsaicin can outlive agonist application by a considerable period. Thus calcium-dependent enzymatic processes may be prolonged because of the time course of calcium elevation.

A limited amount of information regarding the cellular mechanisms involved in tachyphylaxis can be derived from the nucleotide experiments in which the reduction of current was significantly enhanced in the absence of ATP and GTP in the internal solution. The important nucleotide seems to be ATP, because the exclusion of GTP alone had no effect on tachyphylaxis. It is possible, however, that internal ATP may have been converted to GTP by transphosphorylating enzymes, thereby maintaining a GTP supply within the cytoplasm. The increased tachyphylaxis observed in the absence of added ATP is not because of a simple removal of ATP-dependent calcium chelation, because the addition of BAPTA failed to reverse the effect. This result suggests that an ongoing ATP-dependent event actually limits the extent of tachyphylaxis experienced by the capsaicin-activated channels. Possibly, a kinase-mediated phosphorylation of the capsaicin receptor–channel complex or associated protein interferes with modulation by the tachyphylaxis machinery; alternatively, a simple dephosphorylation of a critical protein may be responsible for tachyphylaxis. In support of a phosphorylation role, Yeats et al. (1992) observed an increase in the extent of tachyphylaxis in the presence of nonspecific kinase inhibitors. Furthermore, the inhibition of the calcium- and calmodulin-dependent phosphatase calcineurin has been the only intracellular manipulation reported to block tachyphylaxis successfully (Yeats et al., 1992; Docherty et al., 1996). Moreover, evidence has been presented that barium (which supports desensitization) can activate calcineurin-dependent dephosphorylation independently of calcium and calmodulin (Verhage et al., 1995), consistent with our observations that barium can support desensitization in the absence of calcium.

Summary

The results presented in this paper contribute to understanding how calcium feeds back to desensitize capsaicin-activated channels. The importance of calcium in the desensitization of capsaicin responses is not surprising considering the role of calcium in the desensitization of other receptors, including the NMDA class of the glutamate receptor family (Clark et al., 1990; Legendre et al.,
REFERENCES


Forscher P, Oxford GS (1985) Modulation of calcium channels by nor-