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Chloramine-T Decreases Capsaicin-activated Currents in Cultured Dorsal Root Ganglion Neurons from Neonatal Rats

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ABSTRACT

Capsaicin-activated channel has a key role in the sensation of thermal and inflammatory pain. During inflammation, redox state of cells will be changed by increased production of reactive oxygen species. Oxidation of amino acid residues in proteins can be caused by a variety of oxidizing agents produced in pathophysiological conditions. In the present study, we investigated the effect of chloramine-T, which preferentially oxidizes cysteine and methionine residues, on the activity of capsaicin channel using a a whole-cell patch clamp technique in cultured dorsal root ganglion (DRG) neurons from neonatal rats. Co-application of chloramine-T (3μM) with capsaicin (0.3μM) reversibly blocked capsaicin-activated inward current (I_{cap}) in a dose-dependent manner. Dithiothreitol (DTT, 10 mM), a reducing agent, reversed the blocking effect of chloramine-T on I_{cap}. These results suggest that chloramine-T could decrease I_{cap} by oxidation of cysteine residue rather than methionine in the extracellular surface of capsaicin channel in small DRG neurons, which may play an important role in the regulation of inflammatory pain transmission.

Key words: Capsaicin receptor, chloramine-T, oxidation, DRG neuron

INTRODUCTION

Oxidation of amino acid residues in proteins is known to drastically affect their functional properties (Stadtman, 1993). Ion channel proteins are often target of oxidation induced diverse array of physiological factors, including those oxidants involved in intracellular signaling, such as nitric oxide (NO) (Suzuki et al., 1997; Kourie, 1998). Although all amino acids can be oxidized, cysteine, methionine, histidine, tyrosine, and tryptophan are readily oxidized by biologically relevant oxidants such as H_2O_2, hydroxyl radicals and hypochlorite (Vogt, 1995). It has been reported that oxidation of cysteine residues by reactive oxygen species affect ligand-gated and voltage-dependent channels in a variety of different cells (Aizenman et al., 1989; DiChiara and Reinhart, 1997; Amato et al., 1999; Fearon et al., 1999; Pan et al., 2000). In addition to cysteine, methionine is easily oxidized to form methionine sulfoxide [Met(O)] by the addition of an oxygen to the sulfur atom (Stadtman, 1993; Vogt, 1995). Methionine oxidation has been shown to modulate N-type inactivation of a *Drosophila* transient A-type

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K^+ channel (ShC/B) (Ciorba et al., 1997), P/C type inactivation in voltage-gated K^+ channel (Chen et al., 2000), and large conductance Ca^{2+}-activated K^+ channel (Tang et al., 2001).

Capsaicin, the pungent ingredient of hot peppers, produces burning pain or neurogenic inflammation through excitation of small sensory neurons (Bevan and Szolcsany, 1990; Szallasi and Blumberg, 1999). In cultured dorsal root ganglion (DRG) neurons, capsaicin activates a ligand-gated, nonselective cation channel (Bevan and Szolcsany, 1990; Oh et al., 1996). VR1, cloned capsaicin channel, is also activated by noxious heat and extracellular acid (Caterina et al., 1997; Tominaga et al., 1998). VR1-deficient mice showed that VR1 is essential for mediating thermal hyperalgesia induced by inflammation (Caterina et al., 2000; Davis et al., 2000). During inflammation, increased generation of reactive oxygen species affecting the redox state in the injured tissues. Furthermore, VR1 contains multiple methionine as well as cysteine residues at various locations of channel protein. Because methionine and cysteine residues are readily susceptible to oxidation, activities of the capsaicin channel could be regulated by redox state of the cell. Furthermore, it has been reported that cooperation of subunits of capsaicin receptor was subject to redox modulation (Szallasi et al., 1993). Thus, we examined the effects of mild oxidant chloramine-T on the whole-cell membrane currents induced by capsaicin (I_{cap}) in DRG neurons isolated from neonatal rats. The results show that oxidation of cysteine residues by chloramine-T blocked the I_{cap} without any effect on the activation time course, suggesting that sulfhydryl oxidation could act as an important functional regulator of capsaicin channel.

MATERIALS AND METHODS

Cell culture

Primary cultures of DRG neurons isolated from 2 d-old rats were used for recording whole cell currents as described previously (Oh et al., 1996). Briefly, DRG from all levels of spinal cord of neonatal rats were collected in cold DMEM/F-12 (Life Technologies, Grand Island, NY). Ganglia were then incubated at 37°C for 30 min in culture medium containing collagenase (1 mg/ml, type II, Worthington, Freeshold, NJ). Ganglia were then washed with HBSS and incubated in HBSS containing 2.5 mg/ml trypsin (Life Technologies) at 37°C for 30 min followed by a 10 min centrifugation at 1,000 rpm. The pellet was resuspended in the culture medium by gentle trituration. Suspended cells were plated on the pieces of coverslips pretreated with 0.04 mg/ml polyethylenemine (Sigma, St Louis, MO). Cells were incubated at 37°C in 95% air and 5% CO_2 and used 1～3 days after plating.

Electrophysiology

Recordings were made from small DRG neurons at a holding potential of -60 mV in the whole-cell configuration and pipette resistances were ～5 MΩ. To record whole-cell currents, gigaseals were formed first, and then membrane was ruptured by gentle suction. After a whole-cell was formed, the capacitative transients were canceled. Currents were recorded with an EPC 7 amplifier (HEKA, Lambrecht, Germany) connected via a TL-1 interface to IBM compatible computer. In most cases, data were filtered at 3 kHz using low-pass Bessel filter and acquired at a sample frequency of 1 kHz. Data were analyzed using pClamp 6 software (Axon instruments, Union City, CA).

Solutions and chemicals

All experimental solutions were adjusted to pH 7.4. The Ca^{2+}-free bath solution contained (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 HEPES, glucose and 10 EGTA. The pipette solution contained (in mM): 110 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 11 BAPTA-K_3, 2 Mg-ATP, and 0.1 Na-GTP. Capsaicin was dissolved and stored as 10 mM stock solution in absolute ethanol. Chloramine-T was freshly diluted to appropriate concentrations at the time of experiments. DTT was prepared in the bath solution at the concentration indicated before use. All chemicals were obtained from Sigma (St. Louis, MO).

Statistics

All values are expressed as mean±SEM. For statistical comparisons paired student's t-test was performed. A difference was considered to be significant when $P < 0.05$.

RESULTS

Effect of chloramine-T on the whole-cell currents induced by capsaicin

DRG neurons were used 2～3 days after culture. Because capsaicin affected primarily the small-diameter neurons (Wood et al., 1988), capsaicin-activated inward currents (I_{cap}) were recorded in medium to small DRG neurons isolated from neonatal rats. Under whole-cell configuration, the membrane potential of a DRG neuron was held at -60 mV. After the holding current stabilized, DRG neurons were perfused with 0.3μM capsaicin for 20 s and then washed out for more than 2 min before next application. In about 70% of DRG neurons, capsaicin induced I_{cap}. Most of the current activated by extracellular application of capsaicin reached their peak amplitude within 20 s and ranged from 50 pA to >4 nA (n=48). The current activated by the second application to capsaicin was usually smaller than produced by first application, presumably desensitization (Docherty et al., 1996; Koplas et al., 1997). To avoid this tachyphylaxis, we used Ca^{2+}-free extracellular bath solution as well as 11 mM BAPTA containing pipette solution and waited for 5 min after forming whole-cell patch throughout experiments.

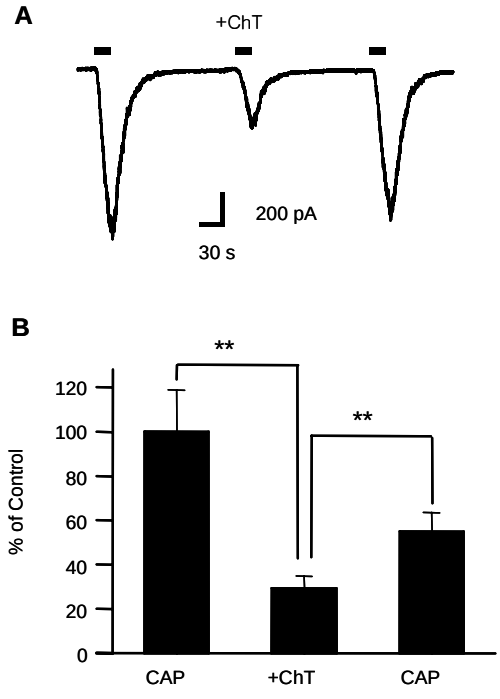

Fig. 1. Effects of chloramine-T, mild oxidizing agent, on I_{cap} in DRG neurons (A) Application of chloramine-T (3μM) with capsaicin (0.3μM) to the bath solution caused significant decrease in I_{cap}. Effects of chloramine-T on I_{cap} were partially reversible after washout. Bars over the representative trace indicated application of 0.3μM capsaicin. Holding potential was -60 mV. (B) Summary of the decreasing effect of chloramine-T on I_{cap} (n=4). *Astericks* indicate a significant difference ($P < 0.01$).

In a previous study, we have shown that sulfhydryl oxidant thimerosal decreased I_{cap} by oxidation of extracellular cysteine residues (Kwak et al., 2002). Addition to cysteine, methionine residue in protein is susceptible to oxidation. So we examined regulatory effect of chloramine-T on I_{cap} in small DRG neurons isolated from neonatal rats. As shown in Fig. 1, chloramine-T at 3μM co-applied with capsaicin (0.3μM) decreased I_{cap} to 29.3±5.5% of the control response at a holding potential of -60 mV (n=4, $P < 0.01$). The blocking effect of chloramine-T on I_{cap} was spontaneously reversible because I_{cap} with 54.8±8.8% magnitude of control was observed when the capsaicin was applied after wash out of the chloramine-T. To examine whether chloramine-T affects the kinetics of I_{cap}, the open and close time constants for I_{cap} were determined. Neither open time constant (τ_o) nor close time constant (τ_c) of I_{cap} was not changed by co-application of chloramine-T (Table 1). From these results, it seems likely that oxidation of capsaicin channel by chloramine-T decreased the peak amplitude of I_{cap} without any effect on gating properties of the channel.

As shown in Fig. 2A, increasing concentration of chloramine-T applied to the extracellular side of cell containing capsaicin channels already activated by 0.3μM capsaicin decreased I_{cap} dose-dependently. This result indicates that effect of chloramine-T was not results from desensitization of I_{cap}. Various

Table 1. Effect of chloramine-T on the open and close time constants of I_{cap} in DRG neurons

	Open time constant (τ_o)	Close time constant (τ_o)
Control (capsaicin)	15.9±1.0 s	16.1±1.0 s
Chloramine-T	16.0±1.3 s	25.1±8.1 s

Values are mean±SE. Time constants of whole-cell currents activated by capsaicin could be fitted well by two exponential functions by Clampex software.

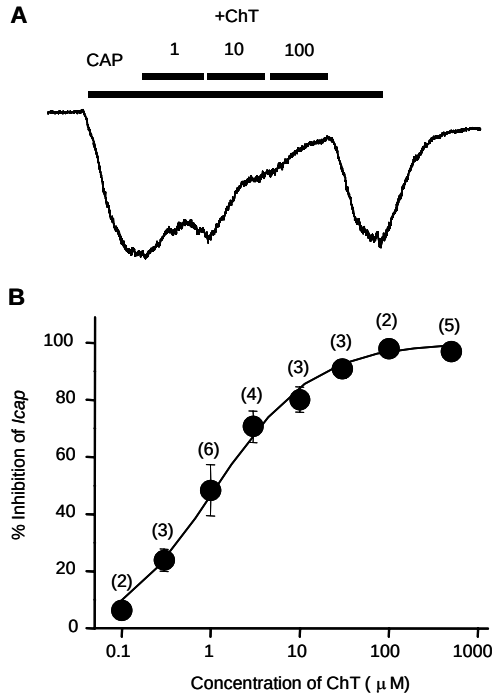

Fig. 2. Dose-dependent decrease of I_{cap} by chloramine-T. (A) As shown in the representative trace, application of chloramine-T (1, 10, and 100μM) to the activated channel in the continuous presence of capsaicin (0.3μM) decreased I_{cap} reversibly. Holding potential was -60 mV. (B) Chloramine-T inhibited I_{cap} in concentration-dependent manner. The mean±SEM values of the % block of I_{cap} by chloramine-T at various concentrations (0.1～1000μM) were plotted. Numbers on top of the symbol represent number of neurons tested. Bars represent mean±SEM.

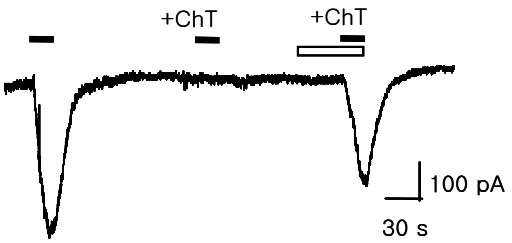

Fig. 3. Effect of dithiothreitol (DTT) on the decrease of I_{cap} induced by chloramine-T in DRG neurons. Representative trace shows that pretreatment of DTT (10 mM) for 1 min before co-application of chloramine-T and capsaicin (0.3μM) prevented decreasing effect of chloramine-T on I_{cap}. Closed and open bars over the representative trace indicate application of capsaicin and DTT, respectively.

doses of chloramine-T from 0.1μM to 1 mM applied with capsaicin (0.3μM) to the bath solution decreased I_{cap} in a dose-dependent manner. The half-maximal inhibitory concentration (IC_{50}) was 1.04 μM. Chloramine-T by itself did not evoke any membrane currents in DRG neurons at a holding potential of -60 mV. From these results, it can be suggested that oxidation of methionine or cysteine by chloramine-T may be act as negative modulators of the capsaicin channel in DRG neurons.

Sulfhydryl reducing agent DTT reverses the chloramine-T-induced decrease of I_{cap}

Chloramine-T oxidizes SH-containing amino acid residues such as cysteine and methionine of the channel proteins (Shechter et al., 1975). Oxidized cysteine is readily reduced back by the specific reducing agent, DTT (Clenand, 1963). In contrast to cysteine, Met(O), oxidized methionine, is reduced back to methionine by methionine sulfoxide reductase (MsrA) using thioredoxin *in vivo* or DTT *in vitro* (Rahamn et al., 1992; Moskovitz et al., 1996; Kuschel et al., 1999). To test whether the observed chloramine-T-induced changes in I_{cap} were due to oxidation of cysteine or methionine, we examined the effects of chloramine-T on I_{cap} in the presence of DTT. If the effect of chloramine-T was produced by methionine oxidation, decreasing effect on I_{cap} might be resistant to reduction of sulfhydryl group by DTT. As shown in Fig. 3, pretreatment with 10 mM DTT for 1 min prevented the blocking effects of chloramine-T on I_{cap} (Fig. 3). From the results that DTT reversed the effect of chloramine-T on I_{cap}, it can be suggested the possibility that cysteine oxidation is involved. However, point mutation studies will be necessary to identify the exact molecular mechanism related to the oxidative modulation of I_{cap} by chloramine-T.

DISCUSSION

Capsaicin-activated channels that exist in a group of small sensory neurons are believed to be involved in the generation of multiple forms of nociceptive neural signals and are now considered as molecular pain transducer for various noxious stimuli (Kress and Zeilhofer, 1999; Szallasi and Blumberg, 1999; Caterina et al., 2000; Davis et al., 2000). Thus study of the mechanisms by which the capsaicin channel is modulated by various factors

will greatly aid in understanding the cellular processes underlying nociception. In the present study, we investigated the effect of oxidizing agent chloramine-T on I_{cap} in cultured DRG neurons from rat.

Oxidation of proteins as a result of reactions with various biological oxidants is important in many physiological and pathological conditions (Dean et al., 1993). It has been shown that intrathecal administration of redox agents can influence pain perception, putatively by interaction with spinal NMDA receptors (Laughlin et al., 1998). Furthermore, reducing agents including DTT and the endogenous amino acid L-cysteine promote cutaneous thermal and mechanical hyperalgesia. Conversely, the oxidizing agent DTNB produces analgesia to cutaneous thermal stimuli by blockade of T-type Ca^{2+} channel (Todorovic et al., 2001). These results suggest that redox agents are able to modulate nociceptive transmission by regulating ion channel. Recently, it has been reported that sulfhydryl reducing agent DTT (30 mM) facilitated I_{cap} and heat-evoked current in DRG neurons isolated from rat (Vyklicky et al., 2002). And we showed previously that sulfhydryl-oxidizing agents such as thimerosal reversibly blocked I_{cap} by oxidation of cysteine residues (Kwak et al., 2002). Although cysteine oxidation has been generally suspected, increasing evidence suggest that oxidation of methionine may function as a general antioxidant mechanism and also as an important physiological regulator of many proteins (Levine et al., 1996).

In the present study, we studied the oxidative effect of chloramine-T on I_{cap} in DRG neurons. Chloramine-T decreased I_{cap} reversibly and dose-dependently (Fig. 1, 2). Such a down regulation of I_{cap} by chloramine-T was likely to be involving methionine oxidation, because chloramine-T preferentially oxidizes methionine to Met (O). Oxidation of methionine is unique in that the reaction is reversible and enzymatically controlled. That is, Met (O) is reduced back to methionine by MsrA using thioredoxin *in vivo* or DTT *in vitro* (Moskovitz et al., 1996; Kuschel et al., 1999). In our study, chloramine-T was able to decrease I_{cap} in cultured DRG neurons, but its effect was reversed by pretreatment of DTT without MsrA. One possible mechanism can account for the reversible effect of chlroamine-T on I_{cap} may be existence of MsrA in DRG neurons. Combined with thioredoxin system, MsrA enzyme is thought to be present in neurons throughout nervous system (Moskovitz et al., 1996) as membrane-associated and soluble forms (Spector et al., 2003). Although little has been known about the existence of MsrA in peripheral neurons, it is possible that endogenous MsrA was expressed in DRG neurons and might reduce Met(O) using DTT as a cofactor. It is also possible that in our experimental condition, chloramine-T oxidized cysteine or both cysteine and methionine of capsaicin channel. Oxidized cysteines could be readily reduced by DTT, but oxidized methionine remained as Met(O). However, it is not likely that methionine oxidation to Met(O) is involved, because the effect of chloramine-T on I_{cap} was partially reversed by simple washout of the drug from the bath (Fig. 1). Although point mutation study will be needed to ascertain the mechanism related to oxidative modification induced by chloramine-T, it can be suggested that chlormaine-T regulates I_{cap} by oxidation of cysteine rather than methionine residues of channel proteins.

In general, effects of reducing or oxidizing agent on biologically active proteins are reversed by addition of agents that exert opposite response. In case of capsaicin channel, the effect of chloramine-T on I_{cap} was reversed by washout of drugs from bath solution (Fig. 1A, 2A). From this reversibility, it can be argued that blockade of I_{cap} by chloramine-T is not resulted from oxidation but direct modification of channel protein. However, other sulfhydryl oxidants such as 5'5'-dithio-bis-(2-nitro-benzoic acid) (DTNB), thimerosal, hydrogen peroxide (H_2O_2) also showed spontaneous reversibility of their effects on I_{cap} recorded in DRG neurons (Kwak et al., 2002). Furthermore, similar reversibility was reported for redox modulation of T-type Ca^{2+} channel in DRG and behavioral study *in vivo* (Todorovic et al., 2001). From these results, spontaneous recovery from the oxidizing effects of chloramine-T and other sulfhydryl oxidants may imply the existence of an unknown endogenous reducing factor in DRG neurons.

In summary, we showed here that chloramine-T could decrease I_{cap} via cysteine oxidation of channel protein. It can be suggested that cysteine oxidation of capsaicin channel may have a role in

integration of the information during inflammatory signal transduction. Taken together these results, it can be suggested that endogenous or exogenous redox agents have potential role in the modulation of pain sensation and sulfhydryl oxidizing agents could represent new classes of drugs to treat chronic pain associated with thermal hyperalgesia.

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