Identification of in Vivo Disulfide Conformation of TRPA1 Ion Channel

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Background: The TRPA1 ion channel is activated by electrophilic compounds and plays a role in chemical nociception. Results: Four in vivo disulfide bonds were detected following electrophilic treatment. Conclusion: Channel activation by electrophiles could involve the formation of disulfide bonds in the N terminus of TRPA1. Significance: Understanding the activation mechanism of TRPA1 is important for elucidating its role in chemical nociception.

TRPA1 (transient receptor potential ankyrin 1) is an ion channel expressed in the terminal of sensory neurons and is activated in response to a broad array of noxious exogenous and endogenous thiol-reactive compounds, making it a crucial player in chemical nociception. A number of conserved cysteine residues on the N-terminal domain of the channel have been identified as critical for sensing these electrophilic pungent chemicals, and our recent EM structure with modeled domains predicts that these cysteines form a ligand-binding pocket, allowing for the possibility of disulfide bonding between the cysteine residues. Here, we present a comprehensive mass spectrometry investigation of the in vivo disulfide bonding conformation and in vitro reactivity of 30 of the 31 cysteine residues in the TRPA1 ion channel. Four disulfide bonds were detected in the in vivo TRPA1 structure: Cys-666—Cys-622, Cys-666–Cys-463, Cys-622–Cys-609, and Cys-666–Cys-193. All of the cysteines detected were reactive to N-methylmaleimide (NMM) in vitro, with varying degrees of labeling efficiency. Comparison of the ratio of the labeling efficiency at 300 μM versus 2 mM NMM identified a number of cysteine residues that were outliers from the mean labeling ratio, suggesting that protein conformational changes rendered these cysteines either more or less protected from labeling at the higher NMM concentrations. These results indicate that the activation mechanism of TRPA1 may involve N-terminal conformation changes and disulfide bonding between critical cysteine residues.

Chemical nociception enables an organism to sense damaging chemical compounds and reduce its exposure to harmful environments. The TRPA1 (transient receptor potential ankyrin 1) ion channel, expressed in a subset of sensory neurons, provides sensing and transducing functions relative to a wide variety of chemical stimuli (1, 2). The channel is composed of four identical subunits, each containing six transmembrane segments and a large cytoplasmic domain composed of its N and C termini (Fig. 1). Exogenous pungent chemicals and endogenous inflammatory mediators, such as allicin, cinnamaldehyde, allyl isothiocyanate, iodoacetamide (IA),3 N-methylmaleimide (NMM), nitric oxide (NO), hydrogen peroxide (H2O2), 4-hydroxynonenal, and 15-deoxy-Δ12,14-prostaglandin J2 (1–6), are sensed by and activate the channel. The resulting cation influx through the central pore depolarizes the neuron, producing an action potential that ultimately leads to the perception of pain in mammal organisms (7).

Recent studies on the activation mechanism of TRPA1 by electrophilic agonists have revealed that conserved cysteine residues on the N terminus of the channel are key mediators of the response to electrophilic ligands, including Cys-415, Cys-422, Cys-622, Cys-642, and Cys-666 and, more recently, Cys-174, Cys-193, Cys-634, and Cys-859 (for simplicity, numbering for mouse TRPA1 will be used throughout) (4–6, 8, 9). The specific cysteines involved in activation seem to vary according to the nature of the electrophilic agonist and perhaps according to the TRPA1 species-specific orthologs (4–6, 8–10). Additionally, some ambiguity remains over whether some of the implicated cysteines (i.e. Cys-415 and Cys-422) play structural, functional, or dual roles (5, 6, 8, 9). Covalent modification of the cysteine residues by electrophilic compounds has been proposed as the mode of activation for at least some of the activators, such as allyl isothiocyanate, IA, and NMM, although other modes of action, such as disulfide bond formation, have also been proposed (6, 9, 11). To provide a deeper understanding of the TRPA1 channel activation mechanism by these compounds, determination of the structure and dynamics of all cysteine residues is required.

In our recently resolved EM structure of the TRPA1 channel, we placed I-TASSER (iterative threading assembly refinement)-generated models of the N terminus of the channel and observed a pocket of cysteines proposed to be important for

1 Received for publication, December 2, 2011, and in revised form, December 28, 2011 Published, JBC Papers in Press, December 29, 2011, DOI 10.1074/jbc.M111.329748
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3 The abbreviations used are: IA, iodoacetamide; NMM, N-methylmaleimide; BisTris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; FT, Fourier transform.
electrophilic activation (12). The proximity of these cysteines to one another led us to hypothesize that crosstalk or disulfide interactions could be occurring as part of the channel activation mechanism. To follow up on these results, we present here a comprehensive mass spectrometry study revealing the in vivo disulfide bonding status and the in vitro reactivity of each cysteine residue in mouse TRPA1. We detected four disulfide bonds in the TRPA1 structure: Cys-666–Cys-622, Cys-666–Cys-463, Cys-622–Cys-609, and Cys-666–Cys-609. Because Cys-666 and Cys-622 have previously been reported to be involved in electrophilic activation of the channel and have multiple disulfide partners, this disulfide bonding pattern could correspond to a variety of TRPA1 intermediate states in an activating or desensitizing channel. We also present evidence for ligand-induced conformational changes that alter the susceptibility of portions of the channel’s N terminus to covalent modification. These results provide a structural and regulatory framework for understanding the activation of the TRPA1 channel in response to electrophilic and oxidizing agonists.

**EXPERIMENTAL PROCEDURES**

**In Vivo Labeling and Protein Purification for Analysis—**

Mouse TRPA1 tagged with a 1D4 epitope was overexpressed in a 30-liter culture of yeast (*Saccharomyces cerevisiae*) as described previously (12, 13). The resulting 105 g of cells were resuspended in 100 ml of 25 mM Tris (pH 8.0), 300 mM sucrose, 5 mM EDTA, and 1 mM PMSF, and 1 ml of yeast protease inhibitor mixture (Sigma) was added. NMM (Sigma) was added to a final concentration of 20 mM, and one-half the total volume of 0.5-mm glass beads (BioSpec Products) was added. Cells were disrupted by five cycles of 5 min of vortexing alternated with 5 min on ice. After centrifugation at 4000 × g for 15 min, 15 mM Fos-Choline-12 was added to the supernatant, and the mixture was solubilized by stirring at 4 °C for 1 h. After ultracentrifugation (45 min at 100,000 × g), TRPA1 was purified by 1D4 chromatography as described previously (12), except that DTT was omitted from the buffers. Following purification, TRPA1 was concentrated, denatured in 8 M urea, and labeled with 100 mM IA (Sigma). Samples were electrophoresed on 3–8% Tris acetate gels (Invitrogen) in the absence of DTT (Sigma) or any reducing agents.

**NMM in Vitro Labeling of Isolated TRPA1—**

Mouse TRPA1 protein was overexpressed and purified using 1D4 chromatography and gel filtration chromatography as described previously (12), except that DTT was used at a concentration of 300 μM. Purified TRPA1 was modified by incubation with 0, 300 μM, or 2 mM NMM at 37 °C for 10 min. The reaction was quenched with 30 mM DTT, and gel electrophoresis on a 4–12% BisTris gel (Invitrogen) was used to separate TRPA1/NMM from detergent and other small molecules.

**In-gel Digestions—**

SDS-polyacrylamide gels from the in vivo and in vitro labeling studies were stained with Coomassie Blue to visualize protein gel bands and washed in distilled water to remove excess background stain. Protein bands were excised and destained with 50 mM ammonium bicarbonate and 50% acetonitrile solution for 2–8 h. Acetonitrile and 100 mM NH₄HCO₃ were then used alternatively to dehydrate and rehydrate the gel pieces three times. For the in vitro cysteine reactivity test, 10 mM DTT was added to reduce disulfide bonds, and free cysteines were alkylated with 55 mM IA. 50 μl of freshly prepared 20 ng/μl trypsin (Promega sequencing grade modified) or chymotrypsin (Roche Applied Science sequencing grade) in 25 mM NH₄HCO₃ was added to the samples and digested at room temperature overnight.

For disulfide bond detection, no DTT or IA was added to the gel bands. Following the dehydration/rehydration cycles, 50 μl of freshly prepared 20 ng/μl trypsin (Promega sequencing grade modified), chymotrypsin (Roche Applied Science sequencing grade), or a combination of trypsin and Glu-C (Roche Applied Science sequencing grade) in 25 mM NH₄HCO₃ was added to the samples and digested at room temperature overnight. Digested peptides were extracted with 60% acetonitrile and 5% formic acid, dried, and dissolved in 10 μl of 0.1% formic acid solution for MS analysis.

**Nanoelectrospray Ionization-MS/MS—**

MS experiments were carried out using a Velos LTQ-FT mass spectrometer (Thermo Finnigan). Nano-reverse phase liquid chromatography separations were performed on a nanoACQUITY Ultra high pressure LC-MS/MS system (Waters) with a BEH130 C18 column (2.5 mm × 75 μm; Waters) directly connected to a 10-μm nanospray emitter (New Objectives). Chromatography was performed using mobile phases A (0.1% formic acid in water) and B (80% acetonitrile and 0.04% formic acid in water) with a linear gradient of 1%/min, starting with 100% of mobile...
phase A at a flow rate of 0.3 μl/min. All data were acquired in positive-ion mode. Collision-induced dissociation was used to fragment peptides in Fourier transform (FT)-MS. In these experiments, full MS scans (m/z 300–2000) were followed by eight subsequent MS/MS scans on the top five most abundant peptide ions from full MS scan or inclusion mass lists using a normalized collision energy of 35%. For the cysteine reactivity test, high mass accuracy FT-MS was performed only for detecting precursor ions (resolution, 60,000; mass accuracy, 5 ppm); product ions were detected in ion trap with relative low mass accuracy (1 Da). For disulfide bond detection, high mass accuracy FT-MS was performed for detecting both precursor ions (resolution, 60,000; mass accuracy, 5 ppm) and product ions (resolution, 15,000; mass accuracy, 0.02 Da).

**RESULTS**

Identification of Disulfide Bonds in TRPA1 in Vivo by LC-MS/MS—To identify the ground-state conformation of critical TRPA1 cysteines in vivo, we used an *S. cerevisiae* expression system to produce functional TRP channels for biophysical and structural analysis (13, 17–20). The yeast expression system is ideal for this experiment because 1) TRP channels can be overexpressed in amounts adequate for obtaining a high sequence coverage in MS experiments, 2) the system preserves the function of TRP channels similar to those seen for mammalian cell lines and is thus appropriate for probing activation and gating mechanisms (19, 20), 3) *S. cerevisiae* cells have significant homology to mammalian cells with regard to redox signaling and regulation and have been widely used as a model system for redox-related studies (21–25), and 4) structural analysis of the TRPV1 (transient receptor potential vanilloid 1) and TRPA1 ion channels by electron microscopy has confirmed the structural integrity of the proteins after expression in yeast (12, 26).

To determine the *in vivo* disulfide bonding pattern of the cysteine residues, functional TRPA1 protein was overexpressed in yeast and treated before cell lysis with excessive amounts of NMM to label surface-accessible reduced cysteines and to prevent oxidation or disulfide bond interchange during processing (14). Following purification, buried cysteines were labeled with IA under denaturing conditions, and the TRPA1 proteins were separated from detergents and other small molecules by SDS-PAGE in the absence of reducing reagents. TRPA1 migrated on the nonreducing gel in a single band at a molecular weight expected for a monomer, ruling out the possibility of intersubunit disulfide bonds. In-gel proteolytic digestions using trypsin, chymotrypsin, and trypsin/Glu-C were performed in parallel, and the digests were separated by nano-LC and analyzed using a high mass accuracy FT-ion cyclotron resonance/ion-trap hybrid mass spectrometer. A total sequence coverage of 95.0% was obtained by combining data from the three digestion methods, allowing us to detect all of the 31 cysteines except Cys1118. Specifically, the trypsin, chymotrypsin, and trypsin/Glu-C digestions yielded 79 peptides (72.5% sequence coverage), 222 peptides (84.2% sequence coverage), and 129 peptides (72.7% sequence coverage), respectively (supplemental Fig. 1A).

Peptides with intact disulfide bonds are often difficult to identify by MS/MS for two main reasons. 1) regions inaccessible to proteolytic cleavage or ionization in peptides with intrachain disulfide bonds can create blind spots that prevent detection of a complete set of signature products, and 2) peptides with interchain disulfide bonds can have product ions clustered at both low and high *m/z* regions, which complicates MS data analysis. To overcome these challenges, we combined cleavage with multiple proteases and high mass accuracy FT-MS to detect both precursor ions (within 5 ppm) and product ions (within 0.02 Da). By improving the mass accuracy of fragment ions, we have dramatically increased the detection sensitivity for true positive identification of disulfide bonds. After manually verifying possible hits from MassMatrix MS database search results, we identified four intrachain disulfide bonds with high confidence (MassMatrix pp score of >30): Cys-666–Cys-622, Cys-666–Cys-463, Cys-666–Cys-193, and Cys-622–Cys-609 (Table 1). MS/MS was used to sequence the peptides forming disulfide bonds, and the high accuracy FT mass spectrum of the precursor ions allows unambiguous assignment (Fig. 2A and supplemental Fig. 2A–C).

Assessment of Thiol Status and Reactivity of Cysteines in Isolated TRPA1—To gain a better understanding of the activation-dependent conformational changes in TRPA1, the reactivity of each cysteine was quantified. The TRPA1 channel was purified as reported previously (12) and modified with various concentrations of NMM, and gel electrophoresis was used to separate labeled TRPA1 from small molecules and detergent. Disulfide bonds were reduced with 10 mM DTT, and incubation with 55 mM IA labeled the resulting free cysteines in the denatured state. In-gel digestion was performed using trypsin or chymotrypsin, and LC-MS/MS was used to analyze the TRPA1 digests. The total sequence coverage between the two digests was 97%, with 101 unique peptides identified from the trypsin digests (73% sequence coverage) and 284 unique pep-
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**TABLE 1**

*In vivo* disulfide bonds identified in TRPA1

TRPA1 was overexpressed in yeast and treated with 20 mM NMM before cell lysis to label surface-accessible reduced cysteines and to prevent oxidation or disulfide bond interchange during processing. Following purification, buried cysteines were labeled with 100 mM IA under denaturing conditions (6 M urea). TRPA1 proteins were separated from detergents and other small molecules by SDS-PAGE in the absence of reducing reagents. In-gel proteolytic digestions were performed in parallel, and the digests were separated by nano-LC and analyzed using a high mass accuracy FT-ion cyclotron resonance/ion-trap hybrid mass spectrometer. Hits from MassMatrix MS database search results were verified manually, and four intrachain disulfide bonds were assigned high confidence (MassMatrix pp score of >30).

| Disulfide                  | Peptide sequence                          | m/z (charge state) | Mass accuracy (ppm) |
|----------------------------|-------------------------------------------|--------------------|---------------------|
| **Trypsin/Glu-C digests**  |                                           |                    |                     |
| Cys-666–Cys-622            | **603**YLCPSL**M**C621 603                   | 602.2715 (+3)       | 0.61                |
| Cys-666–Cys-463            | **603**YLCPSL**M**C621 603, **603**YLCPSL**M**C621 | 483.4848 (+4)       | 0.52                |
| Cys-666–Cys-193            | **603**YLCPSL**M**C621 603, **603**YLCPSL**M**C621 | 520.5925 (+3)       | <0.01               |
| Cys-622–Cys-609            | **603**YLCPSL**M**C621 603, **603**YLCPSL**M**C621 | 527.7394 (+3)       | 0.47                |
| **Trypsin digests**        |                                           |                    |                     |
| Cys-666–Cys-193            | 603YLCPSL**M**C621 603 603, **603**YLCPSL**M**C621, **603**YLCPSL**M**C621 | 520.5917 (+3)       | 1.6                 |
| Cys-666–Cys-463            | 603YLCPSL**M**C621 603 603, **603**YLCPSL**M**C621, **603**YLCPSL**M**C621 | 483.4851 (+4)       | 0.16                |

tides detected from the chymotrypsin digests (86% sequence coverage) (supplemental Fig. 1B).

Because labeling with NMM and IA was performed in the folded *versus* denatured states, respectively, the relative quantities of these two cysteine modifications were used to determine the surface accessibility and reactivity of the various cysteine residues in isolated TRPA1. Peptides containing the two chemical modifications were detected by MS and identified by incorporating the expected mass shifts into the database search (111.0320 Da for NMM and 57.0516 Da for IA). Fig. 2 shows the extracted chromatographic peak of peptide 663–672 for Cys-666 labeled with IA (Fig. 2B) or with NMM (Fig. 2C). Identical fragmentation patterns, aside from the expected mass shifts of the product ions starting from y7, confirmed that these mass spectra are of the same peptide with different modifications (Fig. 2, *insets* in B and C). To assess TRPA1 cysteine residue reactivity, NMM labeling was calculated by dividing the peak area of the NMM-modified peptides by the sum of the areas for both IA-modified and NMM-modified peptides (NMM-modified/total-modified). For each digestion method, we calculated in triplicate the NMM labeling for each TRPA1 cysteine at 300 μM and 2 mM NMM, excluding Cys-1118, which was not detected (supplemental Table 1). These results are summarized in Fig. 3A and provide a comprehensive picture of cysteine residue reactivity in the TRPA1 channel. Our data are consistent with the previously reported analysis of the reactivity of some TRPA1 cysteines identified in a mass spectral study with 30–40% total sequence coverage (4). In agreement with that study, we found Cys-415, Cys-422, and Cys-622 to be labeled by NMM (supplemental Fig. 3).

The base-line expectation for this experiment is that an increased concentration of NMM label at a fixed time of reaction should result in increased labeling. For most of the TRPA1 cysteines, this expectation was fulfilled, as the *in vitro* labeled fraction increased when NMM was increased from 300 μM to 2 mM, with a mean labeling ratio of 0.864 ± 0.185 (Fig. 3A). However, the histogram of data shown in Fig. 3B reveals the existence of two types of outliers from the average behavior. The low [NMM] to high [NMM] ratios of labeling for Cys-214, Cys-259, and Cys-729 are significantly smaller than the mean ratio (0.2–0.6), and the labeling ratios of Cys-31, Cys-274, and Cys-622 are significantly greater than the mean ratio (1.1–1.2). For these Cys residues, the data indicate that the incubation of the protein at 2 mM NMM results in a change in protein conformation and thus a change in reactivity of the relevant Cys residues compared with incubation at 300 μM NMM. Specifically, this conformational switch increases the labeling of some Cys residues relative to the mean (Cys-214, Cys-259, and Cys-729) while decreasing the labeling of others (Cys-31, Cys-274, and Cys-622).

**DISCUSSION**

Using both *in vivo* and *in vitro* approaches, we examined the disulfide bonding and relative reactivity of each cysteine within the TRPA1 channel. We examined the *in vivo* status of the TRPA1 protein after NMM activation and identified key cysteine residues participating in disulfide bonds (Fig. 1). The results revealed four disulfide bonds that could possibly represent a variety of TRPA1 conformations correlating to different intermediate states, with altered sensing characteristics playing a role in an activating or desensitizing channel. In addition, evidence for ligand-induced conformational change comes from the *in vitro* experiments, which revealed altered reactivity to NMM for certain cysteines when incubated at low versus high concentrations of NMM (Fig. 3, A and B). More specifically, at higher concentrations of NMM, Cys-214, Cys-259, and Cys-729 became more reactive, potentially in a more open, accessible state, whereas Cys-31, Cys-274, and Cys-622 were less reactive compared with the mean behavior of the Cys residues as a whole, indicating that they may become protected conformationally. Interestingly, high concentrations of NMM elicited a conformational change that might be the result of electrophile-evoked channel activation or desensitization. Among the conformationally dynamic cysteines, Cys-214 and Cys-259, located on the distal region of the N terminus, showed dramatic increases in labeling (2–4 times higher than the mean), indicating that this portion of the channel could be undergoing an extensive structural change that leaves the distal N terminus
more open and accessible to small molecules. This conformation could be indicative of either an activated or a desensitized channel.

Using in vivo labeling and MS, we detected disulfide bonds within the TRPA1 channel (Table 1). The data suggest that Cys-666 can be alternatively involved in one of three disulfide bonds (Cys-666–Cys-622, Cys-666–Cys-463, or Cys-666–Cys-193) and that a fourth disulfide bond can form between Cys-622 and Cys-609. There is precedent for such complex disulfide patterns in the protein-tyrosine phosphatases (27), which are regulated through disulfide switch/exchange mechanisms involving reversible $S$-glutathionylation or so-called...
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“A backdoor cysteines.” Protein-tyrosine phosphatases are involved in a wide array of intracellular signaling pathways and are understood to have tight regulation of their activities. One mechanism for transient inactivation of these phosphatases involves the oxidation of the cysteine residue to a sulfenic acid group, which is readily converted to the S-glutathionylated mixed disulfide (28). This effectively turns off the phosphatase activity of the protein until glutaredoxin reactivates it by reducing glutathionylation. However, the sulfenic acid intermediate renders this Cys residue susceptible to further oxidation that can irreversibly inactivate the phosphatase activity. Besides reversible glutathionylation to protect against irreversible oxidation, the catalytic cysteine may undergo intramolecular disulfide bonding with backdoor cysteines, making it resistant to irreversible oxidation. The backdoor cysteines in turn undergo disulfide bonding with alternate cysteine residues, thereby replenishing the reduced catalytic cysteine and reactivating the phosphatase activity (see Refs. 29–32). Our results indicate that Cys-666 is capable of forming disulfide bonds with Cys-622, Cys-463, and Cys-193. Cys-622 can in turn form a disulfide bond with Cys-609. These results present a network of disulfide bonds, perhaps representing different conductance states or redox “set points” of the channel.

Supporting our hypothesis is the fact that many of the cysteines identified as participants in the potential disulfide cascade are critical for the response of the channel to electrophilic agonists or oxidizing agents. Point mutation of Cys-622 and/or Cys-666 diminishes responses to electrophilic activators, such as NMM, allyl isothiocyanate, 15-Δ-prostaglandin J2, 4-hydroxynonenal, H2O2, chlorodantoin, disulfiram, 2-aminoethyl methanethiosulfonate hydrobromide, and diallyl disulfide (4–6, 33). Additionally, Takahashi et al. (9) recently found that mutation of Cys-666 and Cys-193 (among others) significantly suppressed the responses of the channel to both hyperoxia and diallyl disulfide. The authors suggested that these cysteines may be protecting the reactivity of other cysteines (Cys-634 and Cys-856), which they propose are directly oxidized/modified by those agonists. This interpretation is consistent with our proposed network of disulfide bonds, which could play a protective or regulatory role in the activation or desensitization mechanism of TRPA1.

The TRPA1 channel is a predicted tetramer, with each of its subunits containing a transmembrane domain and cytoplasmic N and C termini. The N terminus accounts for ~60% of the channel sequence and is composed of a large ankyrin repeat domain followed by a more flexible region linking to the transmembrane segments. A high resolution structure of the TRPA1 channel has yet to be elucidated; however, our recent EM structure and N-terminal model of mouse TRPA1 (12) gives a framework for understanding the implications of the mass spectral results presented here. Cys-666 and Cys-622, two of the residues we found to be involved in a disulfide bond, are located in close proximity in the linker region between the N-terminal ankyrin repeat domain and the transmembrane domain of the channel. The other two cysteines forming disulfide bonds with Cys-666 (Cys-463 and Cys-193) are within the ankyrin repeat domain and the transmembrane domain of the channel. The structure was reconstructed under reducing conditions in the absence of ligand, it likely reflects the closed conformation of the channel. Because NMM is a TRPA1 activator, our mass spectral results presented in this work reflect either an activated or a desensitized channel. Cordero-Morales et al. (10) have recently used TRPA1 channel ortholog chimeras to demonstrate the importance of the ankyrin repeat domain in fine-tuning the activation response to electrophilic agonists, suggesting that there is crosstalk between these regions. For the ankyrin repeat domain to be involved in electrophilic activation by means of a disulfide network as discussed above, substantial conformation changes would have to occur for the cysteines to form disulfide bonds. As described above, our data suggest that conformation changes do occur in the N-terminal region of the TRPA1 channel, and substantial conformation changes are also predicted from molecular modeling (12, 34). In conclusion, our mass spectral analysis has revealed that the TRPA1 channel
structure is dynamically mediated by electrophilic activation and contains an in vivo network of disulfide bonds that potentially plays a role in the activation and/or desensitization mechanism of the channel.

Acknowledgment—We thank Dr. John Mieyal for critical reading of this manuscript.

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