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Regulation of voltage-dependent K^+ channels by methionine oxidation: effect of nitric oxide and vitamin C

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Abstract Methionine oxidation is known to alter functional properties of a transient A-type potassium channel expressed in *Xenopus* oocytes. We show here that nitric oxide (NO) slows down the K^+ channel inactivation time course by oxidizing a critical methionine residue in the inactivation ball domain of the channel protein. We also demonstrate that the channel protein is protected from methionine oxidation by the enzyme methionine sulfoxide reductase and the antioxidant vitamin C.

Key words: Methionine oxidation; Nitric oxide; Antioxidant; A-type potassium channel; *Xenopus* oocyte

1. Introduction

Functional properties of many proteins are post-translationally modified in a variety of ways. Phosphorylation/dephosphorylation markedly alters protein activity and its involvement in numerous cellular signal transduction cascades is well documented. Oxidation of amino acid residues is also known to affect protein function and protein oxidation has been implicated in many physiological processes. Although all amino acids can be oxidized, the oxidization of cysteine and methionine occurs reversibly, suggesting a possible regulatory role in cellular processes. In the case of methionine, the thiol ether group can be reversibly oxidized to methionine sulfoxide (Met(O)), decreasing the side chain flexibility and increasing hydrophilicity [1,2]. Reduction of Met(O) in proteins back to methionine is unique in that the reaction is specifically catalyzed by an enzyme, peptide methionine sulfoxide reductase (MsrA) [3,4], which has been cloned from bacteria [5], plants [6], and bovine adrenal gland [4]. MsrA is present in many organs including brain, kidney, and retina [7]. The observation that reduction of Met(O) to methionine in proteins is enzymatically controlled by MsrA suggests that methionine oxidation/reduction could act as a cellular regulatory mechanism.

Physiological roles of methionine oxidation and reduction mediated by MsrA have been suggested in several different systems. The oxidation of a single methionine residue of α1-proteinase inhibitor destroys its ability to inactivate elastase and is thought to contribute to smoking-induced emphysema [8] and adult respiratory distress syndrome [9]. The inactive oxidized protein can be reactivated when incubated with MsrA [9,10]. Cataract formation may also involve methionine oxidation since about 45% of the methionine residues in lens proteins are oxidized [11,12]. DNA binding of a *Drosophila* protein is regulated by methionine oxidation [13] and the conformational stability of calmodulin is also influenced by methionine oxidation [14]. Previous studies using MsrA-deficient *Escherichia coli* and yeast strains suggest that MsrA may function as a ROS defense mechanism [15,16]. In a previous paper [17], using a heterologous expression system we demonstrated that methionine oxidation/reduction functions as a regulator of voltage-dependent A-type K^+ channels. We showed that N-type inactivation mediated by the ball and chain mechanism in the ShC/B channel is slowed when a critical N-terminal methionine (amino acid 3) is oxidized to Met(O) and that coexpression of MsrA enhances the inactivation process by promoting the reduction of Met(O) to methionine.

Oxidation of methionine to Met(O) can be induced by many biologically synthesized free radical species with unpaired electrons, including hydrogen peroxide, hydroxyl radicals, hypochlorous acid, and chloramines [1]. These free radicals are thought to play crucial roles in oxidative stress and tissue injury. Nitric oxide (NO) and its related species are involved in regulation of many different physiological processes such as vascular tension, synaptic transmission, macrophage function and apoptosis [18]. In addition, NO has been suggested to contribute to methionine oxidation either directly or indirectly through other free radical species. Some neurotoxic effects of NO may be mediated by the oxidant peroxynitrite (ONOO), which is formed by the reaction of NO with the superoxide anion ($O_2^{-\bullet}$) [19,20]. ONOO in turn has been shown to oxidize methionine to Met(O) in α1-proteinase inhibitor [21]. Biosynthesis of NO is mediated by NO synthase (NOS) from L-arginine [22] and it is present in at least three different isoforms, nNOS, eNOS, and iNOS [23].

In this study, we used the ShC/B voltage-dependent K^+ channel expressed in *Xenopus* oocytes as a model system to examine which NO cellular signals could regulate oxidation of methionine. Since oxidation of a critical methionine residue to Met(O) in the N-terminus of the ShC/B channel slows down the inactivation process [17], we hypothesized that any treatment that generates oxidants capable of oxidizing methionine to Met(O) should slow down the inactivation time course and that MsrA and antioxidant agents should accelerate the inactivation time course.

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2. Materials and methods

2.1. Expression in oocytes

The ShC/B and ShB channels were expressed in *Xenopus* oocytes by RNA injection as described [17]. Each oocyte was injected with about 40 nl of the RNA solution diluted to give the desired expression levels and incubated in a modified ND96 solution (96 mM NaCl, 15 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, 2.5 mM Na pyruvate, 0.5% penicillin/streptomycin, pH 7.6). The rat nNOS cDNA [24] was linearized with *Xba*I and the RNA was synthesized using T7 polymerase (Ambion, Austin, TX, USA). The NOS activity was assayed as described [25]. The oocytes were incubated at 17°C in the dark.

2.2. Electrophysiology

Whole-oocyte currents were recorded using a two-electrode voltage-clamp amplifier (OC725B, Warner Instrument, Hamden, CT, USA). When filled with 3 M KCl, the electrode resistances were 0.3–0.8 MΩ. Macro-patch data were obtained using an AxoPatch 200B amplifier (Axon, Foster City, CA, USA) in the inside-out configuration. The output of the amplifier was digitized using an ITC-16 (Instrutech, Port Washington, NY, USA) attached to an Apple Macintosh computer. The data acquisition and analysis were performed using PULSE/PULSEFIT (HEKA, Lambrecht, Germany) and IGORPRO (WaveMetrics, Lake Oswego, OR, USA). Leak and capacitative currents were subtracted using the *P/n* protocol as implemented in PULSE. The external solution contained (in mM): 132 NaCl, 10 KCl, 2 $CaCl_2$, 10 HEPES (*N*-methylglucamine), pH 7.2. The internal solution in the excised patch experiments contained (in mM): 140 KCl, 11 EGTA, 2 $MgCl_2$, 10 HEPES (*N*-methylglucamine), pH 7.2. Experiments were typically performed 1–2 days after RNA injection at room temperature.

Statistical comparisons were made using the results obtained from the same batch of oocytes and they are given as mean ± S.E.M.

2.3. Reagents

ONOO was synthesized according to the protocol provided by the University of Iowa Free Radical Research Institute, which is derived from that by Beckman et al. [26]. The synthesized ONOO was filtered and concentrated by placing at −20°C. The concentration was estimated by measuring the absorbance in the 250–300 nm range. For some experiments, peroxynitrite was also purchased from Calbiochem (San Diego, CA, USA).

DEANO [27] was obtained from Molecular Probes (Eugene, OR, USA) and NOR-3 [28] was obtained from ICN (Costa Mesa, CA, USA). To examine the effects of DEANO or NOR-3, the oocytes were typically incubated in the presence of the drug (0.5 mg/ml DEANO; 2 mg/ml NOR3) for 1 h in an air-tight container in the ND96 solution aerated with O_2 and CO_2 (95%/5%). The control cells were incubated in the similarly aerated ND96 without any drug for 1 h. After the incubation, the cells were allowed to recover in fresh ND96 for 1–2 h before data collection. The incubation in the aerated ND96 solution alone did not induce any noticeable change in the measured ShC/B channel properties.

Antioxidant vitamins, vitamin C (L-ascorbic acid), vitamin A (synthetic *trans*-β-carotene), and vitamin E ((+)-δ-tocopherol acetate) were obtained from Sigma (St. Louis, MO, USA). Cells were incubated in the presence of 10 mM ascorbic acid. To maintain the proper osmolarity, ascorbic acid (10 mM) replaced the equimolar NaCl in the ND96 solution so that the final Na concentration in the cell incubating solution was 86 mM. Cells were incubated in the vitamin A or E solution overnight. Vitamins A and E were dissolved in a solution containing approximately 97% ethanol and 0.025% Triton NP-40 and mixed with a vortex. The final concentrations of ethanol and Triton in the ND96 solution were 1.5% and 0.004%, respectively.

3. Results and discussion

3.1. Peroxynitrite slows the inactivation time course of the ShC/B channel

N-type inactivation of the ShC/B channel is regulated by the oxidation of one methionine residue at position 3 in the N-terminus (M3) of the protein [17]. When this methionine is oxidized to Met(O), the inactivated state becomes unstable, producing the slow exponential component in the macroscopic inactivation time course. Peroxynitrite (ONOO) has been implicated in mediating protein modification during reperfusion injury [29], and it has been postulated that it promotes methionine oxidation [1,30] especially at low CO_2 concentrations [30]. Thus, we examined whether ONOO could oxidize M3 in the ShC/B channel to slow the inactivation time course. If ONOO promotes methionine oxidation, the inactivation time course of the ShC/B channel should become slower. Fig. 1A shows the ShC/B channel inactivation before and after addition of ONOO directly to the patch. The ONOO addition to the patch markedly slowed the inactivation time course. Systematic investigations of the ONOO effect on the inactivation time course were difficult as the ONOO application often resulted in a rapid loss of the tight seal.

The effect of ONOO to slow the ShC/B inactivation time course is very likely mediated by its action on the N-terminus ball domain of the channel. The ShC/B ball peptide, corresponding to the distal N-terminal amino acid sequence of the ShC/B channel, restores inactivation when applied to the ShBΔ6–46:T449V channel that does not normally show N-type inactivation [17,31]. The peptide is much less effective in inducing inactivation when M3 is replaced with Met(O) [17]. We hypothesized that if peroxynitrite promotes methionine oxidation in the N-terminus ball domain, the ONOO-treated ShC/B peptide should be less effective in inducing inactivation. The effects of the control and ONOO-treated ShC/B peptides are compared in Fig. 1B. Consistent with the hypothesis, the inactivation time course of the ShBΔ6–46:T449V channel current recorded at +50 mV was markedly slower with the ONOO-treated peptide than with the control peptide. These results suggest that ONOO promotes methionine oxidation in the N-terminus of the ShC/B channel to slow down the inactivation time course.

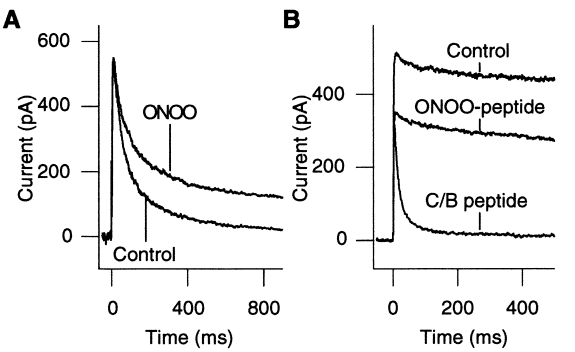

Fig. 1. A: Peroxynitrite (ONOO) slows the ShC/B channel inactivation time course. The ShC/B macroscopic currents were recorded in the inside-out configuration in response to pulses to +50 mV from the holding voltage of −100 mV. ONOO (4 mM) was applied to the patch directly. B: ONOO-treated ShC/B ball peptide (MQMILVAGGSLPKLSS) is not effective in inducing inactivation. Macroscopic K^+ currents were recorded from the ShBΔ6–46:T449V channels in the inside-out configuration at +50 mV. This channel has a large deletion in the N-terminus to disrupt N-type inactivation and a point mutation to drastically slow C-type inactivation. The ShC/B peptide was treated with ONOO (1.5 mM) in the standard internal solution for 1 h at room temperature and then applied to the cytoplasmic side.

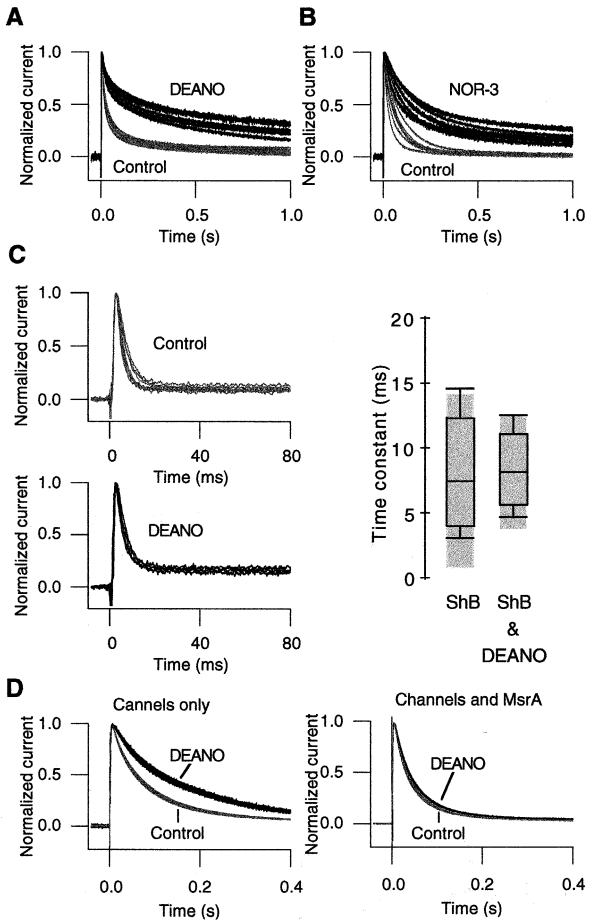

Fig. 2. DEANO and NOR-3 slow the time course of the ShC/B channel inactivation. A: Whole-oocyte currents recorded at +30 mV from seven cells in the control group (light sweeps) and six cells in the DEANO-incubated group (dark sweeps) are scaled and shown superimposed. The mean peak current amplitudes in the control and DEANO groups were 5.8 ± 0.4 μA and 3.9 ± 0.7 μA, respectively. B: Whole-oocyte currents recorded at +30 mV from seven cells before (light sweeps) and after (dark sweeps) incubation in NOR-3 for 1 h. The currents from the cells were measured (control), incubated in the presence of NOR-3 (2 mg/ml) for 1 h, and then measured again. C: DEANO does not affect the time course of the ShB channel inactivation. Whole-oocyte currents recorded at +30 mV from eight cells in the control group (top) and four cells in the DEANO-incubated group (bottom) are scaled and shown. The inactivation time course was fitted with a single exponential and the time constant values are compared using boxplots (right). The shaded area represents the 95% confidence interval of the median. The average currents are also shown (right). The mean peak current amplitudes in the control and DEANO groups were 7.9 ± 1.4 μA and 5.4 ± 1.1 μA, respectively. D: MsrA protects the ShC/B channel from oxidation induced by DEANO. The average whole-oocyte currents recorded at +30 mV from the oocytes expressing the ShC/B channels alone (left) and MsrA and the ShC/B channels together (right) are compared. In each panel, the currents from the control and DEANO-incubated groups are compared. The DEANO incubation protocol is described in Section 2. Each line represents an average response from >20 cells and the line width indicates S.E.M. The mean peak current amplitudes in the ShC/B DEANO, ShC/B control, ShC/B-MsrA DEANO, and ShC/B-MsrA groups were 13.9 ± 1.2, 7.8 ± 0.8, 7.2 ± 0.8 and 11.7 ± 0.6 μA, respectively.

3.2. NO donors slow the inactivation time course of the ShC/B channel

ONOO is formed when NO reacts with superoxide anion ($O_2^{-\bullet}$). Thus, NO should in turn promote methionine oxidation to slow the inactivation time course of the ShC/B channel. This hypothesis was tested by examining the effects of NO donors. The ShC/B K^+ currents recorded from the oocytes incubated with NO donors were compared with the currents recorded from the control cells. Fig. 2A shows that DEANO (0.5 mg/ml), a NO donor with a half-life of 2 min, markedly slowed the ShC/B channel inactivation time course. Another NO donor, NOR-3 with a half-life of 40 min, also slowed the ShC/B inactivation time course (Fig. 2B). Similar effects of DEANO and NOR-3 were observed using four other batches of oocytes.

The ShB channel differs from the ShC/B channel only in the amino-terminus and it does not contain any additional methionine in the ball domain. N-type inactivation of the ShB channel is not regulated by methionine oxidation [17]. If the effect of the NO donors to slow the ShC/B inactivation time course is mediated by methionine oxidation in the N-terminus, these NO donors should not alter the ShB inactivation time course. Consistent with this prediction, incubation of the cells expressing the ShB channels with DEANO (0.5 mg/ml) did not markedly alter the inactivation time course (Fig. 2C; 2-sample t-test, $P = 0.65$). In some cells, however, greater steady-state currents were observed, suggesting that the NO donors may have other targets. The differential effects of NO donors on the ShB and ShC/B channels suggest that NO slows the ShC/B inactivation time course by promoting methionine oxidation in the N-terminus.

MsrA catalyzes reduction of Met(O) to methionine [3,4]. We showed previously that MsrA can accelerate the inactivation time course of the ShC/B channel by promoting reduction of Met(O) to methionine at position 3 in the ball domain [17]. We hypothesized that MsrA should protect the channel from the oxidizing influence of DEANO. Thus, we compared the

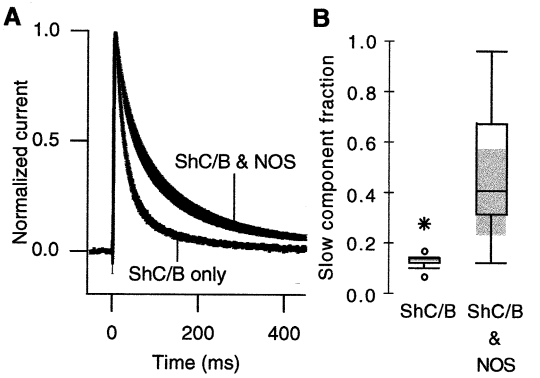

Fig. 3. NOS coexpression slows the ShC/B channel inactivation time course. A: Average scaled whole-oocyte currents recorded from the cells injected with the ShC/B RNA only and from the cells injected with the ShC/B and nNOS RNAs together. The currents were recorded from 11 cells in each group in response to pulses to 0 mV. Line width represents S.E.M. The cells in both the control and the ShC/B-NOS experimental groups were incubated in the presence of the Ca^{2+} ionophore A23187 (10 μM) for 1 h and allowed to recover in ND96 solution for >2 h before the recording. The mean peak current amplitudes in the control and coexpression groups were 2.0 ± 0.1 μA and 5.8 ± 1.7 μA, respectively. B: Relative fraction of the slow inactivation is increased by NOS coexpression. The ShC/B inactivation time course was fitted with a sum of two exponentials and the relative fraction of the slow component is compared using boxplots. The shaded areas represent the 95% confidence intervals of the median.

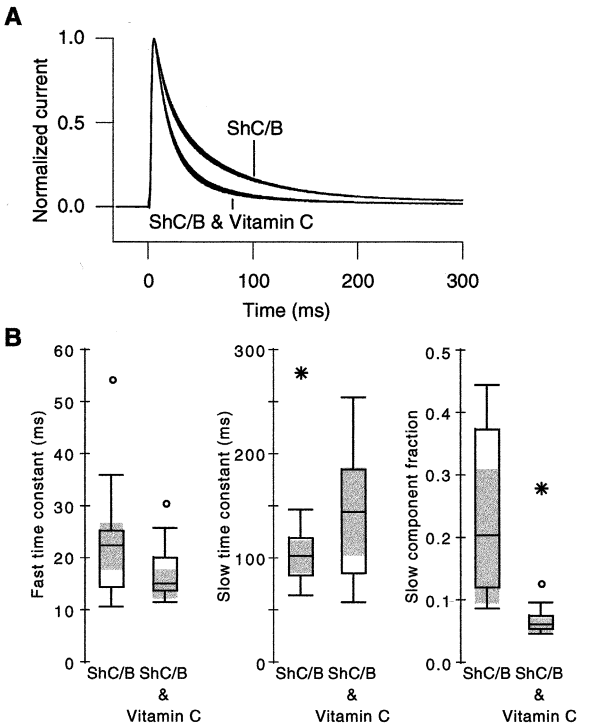

Fig. 4. Vitamin C accelerates the ShC/B channel inactivation time course. A: Average scaled whole-oocyte currents recorded at 0 mV from the control cells and the cells incubated with vitamin C. The currents recorded from 10 cells in the control and 14 cells in the experimental groups in response to pulses to 0 mV were scaled and averaged. Line width represents S.E.M. The average currents are also shown (right). The mean peak current amplitudes in the control and vitamin C groups were 17.1 ± 2.0 μA and 14.0 ± 2.5 μA, respectively. B: The ShC/B inactivation time course was fitted with a sum of two exponentials and the fit parameter values are compared using boxplots. The shaded areas represent the 95% confidence intervals of the median.

effects of DEANO on the oocytes expressing the ShC/B channels alone and those expressing the channels and MsrA together. As predicted by the hypothesis, DEANO was much less effective in slowing the inactivation time course in the oocytes expressing both the channels and MsrA (Fig. 2D) than in the oocytes expressing the channels alone, showing that MsrA can protect the channels from the NO-mediated oxidative stress. Similar results were observed using two other batches of oocytes.

3.3. nNOS coexpression slows the inactivation time course of the ShC/B channel

We further tested whether NO could promote methionine oxidation to slow the ShC/B channel inactivation time course by coexpression of the channels with nNOS [24]. Injection of the nNOS RNA into *Xenopus* oocytes followed by an incubation with the Ca^{2+} ionophore A23187 (10 μM; Sigma, St. Louis, MO, USA) enhanced the NOS activity of the cell extract by approximately 300% from 50 nmol/mg protein to 155 nmol/mg protein using the method of Xie et al. [25]. The mean ShC/B K^+ current recorded at +30 mV from the cells injected with the channel RNA alone and treated with A23187, and the mean current from the cells injected with the channel and nNOS RNAs together and treated with A23187 are compared in Fig. 3. The average inactivation time course was markedly slower in the cells expressing both the channels and nNOS. The effect of the nNOS coexpression was specific in that it selectively enhanced the relative fraction of the slow inactivation component without markedly altering the time constant values of the two components (Fig, 3B; 2-sample *t*-test, $P = 0.06$ for the fast time constant value, $P = 0.64$ for the slow time constant value and $P = 0.0002$ for the slow time constant fraction). The results suggest that the increase in NOS activity could cause methionine oxidation in the channel and regulate the ShC/B channel kinetics.

3.4. Vitamin C accelerates the inactivation time course

Since methionine oxidation slows the ShC/B channel inactivation time course, it was hypothesized that antioxidants should accelerate the ShC/B channel inactivation time course. We examined the effects of antioxidant vitamin A, vitamin C, and vitamin E on the ShC/B channel. The mean ShC/B K^+ currents from the control cells and from the cells incubated with vitamin C are compared in Fig. 4. Incubation with vitamin C (10 mM) accelerated the inactivation time course in a statistically significant manner. The incubation specifically decreased the fraction of the slow inactivation component without affecting the time constant values of the two inactivation components (2-sample *t*-test, $P = 0.13$ for the fast time constant, $P = 0.20$ for the slow time constant and $P = 0.0009$ for the slow component fraction). Incubation with vitamin A or vitamin E (1 mM) did not produce consistent effects on the ShC/B K^+ currents ($n = 50$). Since the N-terminal ball domain of the Shaker channel is cytoplasmic and these vitamins are lipid soluble, their inability to regulate the ShC/B channel may be caused by their preferential distribution in the plasma membrane away from the ball domain.

3.5. Methionine oxidation as a regulator of cellular excitability

The results presented show that a variety of treatments, such as ONOO, NO donors and nNOS coexpression, promote methionine oxidation and slow the inactivation time course of the ShC/B channel. Such a role of NO in methionine oxidation has been speculated, but there has been little direct evidence previously to support this idea [1]. It is likely that NO induces methionine oxidation indirectly via ONOO [1,21]. ONOO is a strong oxidant formed by the reaction of superoxide with NO and it is implicated in a variety of tissue and cellular injury phenomena [32]. The observation that the ShC/B inactivation time course is slowed by direct application of ONOO as well as by NO donors and NOS coexpression suggests that ONOO may mediate the action of NO in the ShC/B channel although other mechanisms cannot be totally excluded [33]. Furthermore, MsrA protects the ShC/B channel from slowing of the inactivation time course induced by a NO donor. The Shaker and NOS proteins could physically interact with the postsynaptic density protein PSD95 via their PDZ domains [34,35], suggesting that these proteins may preferentially co-localize. A recent study suggests that at low L-arginine concentrations NOS could also produce superoxide, leading to formation of ONOO, which then contributes to cytotoxicity and cellular injury [36]. Although peroxynitrite has a short half-life under physiological conditions [37], the potential proximity of the Shaker and NOS proteins may allow NO-triggered methionine oxidation to serve as an important regulator of cellular excitability.

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