Journal of Biochemistry and Molecular Biology, Vol. 39, No. 1, January 2006, pp. 91-96

JOURNAL OF Biochemistry and Molecular Biology

Dual Effects of Nitric Oxide on the Large Conductance Calcium-activated Potassium Channels of Rat Brain

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Received 13 September 2005, Accepted 25 November 2005

Previously, we have shown that nitric oxide (NO) directly activates the Maxi-K channels. In the present study, we have investigated whether NO has prolonged effects on the Maxi-K channels reconstituted in lipid bilayer. Application of *S*-nitroso-*N*-acetyl-D, L-penicillamine (SNAP), a NO donor, induced an immediate increase of open probability (*Po*) of Maxi-K channel in a dose-dependent manner. When SNAP was removed from the cytosolic solution, the *Po* did not simply returned to, but irreversibly decreased to a level lower than that of the control *Po*. At 0.2 mM, (Z)-[N-(3-Ammoniopropyl)-N-(n-propyl)amino] diazen-1-ium-1,2-diolate (PAPA-NO), another NO donor, produced a similar increase of *Po* and decrease of *Po* upon washout. The increasing effects of SNAP on *Po* were not blocked by either 50 U/ml superoxide dismutase (SOD) or 2 mM N-ethylmaleimide (NEM) pre-treatments. However, NEM appears to be ineffective when applied after SNAP. These results suggest that NO can modulate Maxi-K channel via direct interaction and chemical modification, such as S-nitrosylation in the brain.

Keywords: Lipid bilayer, Maxi-K channel, Nitric oxide, PAPA-NO, SNAP

Introduction

Nitric oxide (NO) is a neurotransmitter (Dawson and Snyder, 1994) but its mechanism of action differs from that of other conventional ones (Dawson *et al*., 1994). Firstly, NO is not released from pre-packaged synaptic vesicles but synthesized by activated NO synthase (NOS) whose activity is regulated by intracellular calcium. Secondly, since NO is membrane-permeable, its target is not confined to receptors on the cell membrane, but extends to the diffusion-limited intracellular bio-molecules. One of the major signaling pathways of NO is mediated by activation of guanylate cyclase. Activation of this enzyme leads to increase the concentration of cGMP, which in turn activates many enzymes and ion channel proteins either directly or indirectly through activation of cGMP-dependent kinase (Pineda *et al*., 1996). However, direct action of NO on many channel proteins has also been reported (Lei *et al*., 1992; Bolotina *et al*., 1994; Fangi and Bockaert, 1996).

Large conductance calcium-activated potassium (Maxi-K) channels are distributed in different types of cells and tissues, and play important roles in repolarization of the action potential in neurons (Lancaster *et al*., 1991) and also regulation of arterial tone in smooth muscles (Nelson *et al*., 1995). In brain, at least two types of Maxi-K channels have been characterized (Reinhart *et al*., 1989). The channels are suggested to be composed of two subunits, a and b. The a subunit, *Slo*, was first cloned in Drosophila (Atkinson *et al*., 1991) and subsequently in other organisms (Butler *et al*., 1993; Tseng-Crank *et al*., 1994). Recently, Ha *et al*. (2000) cloned full-length cDNAs of Maxi-K channel a subunit from rat brain (rslo) and found that some brain regions such as habenula, hippocampus and neocortex express high levels of rslo transcript. Knaus *et al.* (1996) investigated tissue expression and distribution of the channels in rat brain by *in situ* hybridization, and found that Slo immunoreactivity was highly concentrated in presynaptic area, which implicates an important role of the Maxi-K channel in neurotransmission. NO is thought to play an important role in the induction of synaptic plasticity (Gally *et al*., 1990; Hawkins *et al*., 1998) and has been suggested as a candidate for retrograde messenger which can diffuse from post- to presynaptic terminals to enhance neurotransmitter release from presynaptic terminal in the hippocampus (Malen and Chapman, 1997; Bon and Garthwaite, 2001, 2003). Therefore, presynaptic Maxi-K channel can be a good target of NO.

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Previously, we have shown that NO directly increases the Maxi-K channel activity using streptozotocin (STZ) (Shin *et al*., 1997). In this paper, we investigated whether the effects of NO derived from SNAP and PAPA-NO on Maxi-K channel are reversible upon wash-out of these NO-donors. Furthermore, we have also determined whether the effects of NO donors are affected by pre-treatment with thiol-modifying agent, N-ethylmaleimide (NEM).

Materials and Methods

Materials. *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) was purchased from TOCRIS, and (Z)-[N-(3-Ammoniopropyl)-N-(n-propyl)amino] diazen-1-ium-1,2-diolate (PAPA-NO) from Alexis Biochemicals. All other chemicals were purchased from Sigma. SNAP was dissolved in dimethyl sulfoxide (DMSO), and applied to lipid-bilayer at concentrations ranging up to 1 mM. The application of DMSO vehicle without SNAP up to1% DMSO did not affect the Maxi-K channel activities.

Preparation of rat brain plasma membrane vesicles. Rat brain plasma membrane vesicles were prepared as described (Shin *et al*., 1997). Briefly, rat brain cortex was excised and homogenized immediately in an ice-cold isotonic sucrose buffer (10 ml/gram of tissue) containing 0.25 M sucrose, 2.5 mM KCl, 0.1 mM ethylene glycol bis (β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.1 mM dithiothreitol (DTT), and 20 mM N-Z-hydroxylethyl-piperazine-N-Z-ethanesulfonic acid (HEPES) at pH 7.2. The homogenate was centrifuged at $1,000 \times g$ for 2 min, and the supernatant was centrifuged at $13,000 \times g$ for 10 min. The pellet was resuspended in a hypotonic lysis buffer containing 0.1 mM DTT, 2.5 mM KCl, 5 mM Tris-HCl, 0.1 mM EGTA, and 0.1 mM EDTA at pH 8.2, and allowed to stand on ice for 20 min. The obtained membrane fraction was then re-homogenized, and centrifuged at $100,000 \times g$ for 50 min. The pellet was layered under a discontinuous Percoll gradient (25, 18, 10, and 0%; v/v). The gradient was centrifuged at $40,000 \times g$ for 2 min, and the membrane fraction present at 0 to 10% Percoll gradient interface was collected. Percoll was removed by centrifugation at $100,000 \times g$ for 50 min. The obtained membrane fraction was stored at −70°C until use.

Channel recording using planar lipid bilayer reconstitution. We obtained Maxi-K channel records by incorporating the plasma membrane vesicles into planar lipid bilayer. The recording chamber was filled with cytosolic (or *cis*) solution (150 mM KCl, 1 mM EGTA, 1.05 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.2) and the extracellular (or *trans*) solution (5 mM KCl, 0.1 mM EGTA, 10 mM HEPES, pH 7.2). The bilayer with a capacitance of >200 pF was made around a 250 mm diameter hole by painting with a 3 : 1 mixture of phosphatidylethanolamine (PE) and phosphatidylserine (PS), dissolved in n-decane (20 mg/ml). When a single or multiple number of Maxi-K channels were reconstituted into lipid bilayer, we routinely perfused the *cis* chamber with 10-volume of the *cis* solution to remove excess vesicles and prevent further fusion. The composition of the perfused solution was the same as the *cis*-solution described above except that the concentration of EGTA was raised to 1.135 mM. During experiments, the free calcium concentration ($[Ca^{2+}]$) in the *cis* solution was adjusted by adding a calculated volume of either 100 mM $CaCl_2$ or 50 mM EGTA. $[Ca^{2+}]$ was estimated using software developed by Dr. Schoenmakers at University of Nijmegen. The current was amplified by an Axopatch 200A amplifier (Axon Instruments, Inc.), and filtered at 1 kHz. Both the current and the voltage were stored using a digital tape recorder DTR 1204 (Biologic, France). Later, recorded data were played back, and digitized using an interface Digidata 1200 (Axon Instruments, Inc.) and a software, Axotape (Axon Instruments, Inc.) at 5 kHz sampling rate. The channel activities were analyzed using the pClamp 6 software (Axon Instruments, Inc.). NPo was calculated using software 'NPo' developed by Drs. Logothetis and Sui at Mount Sinai School of Medicine.

Results and Discussion

Effects of SNAP on the Maxi-K channel activities. When SNAP was added to the *cis* solution, it increased the open probability (*Po*) of the Maxi-K channel in a dose-dependent

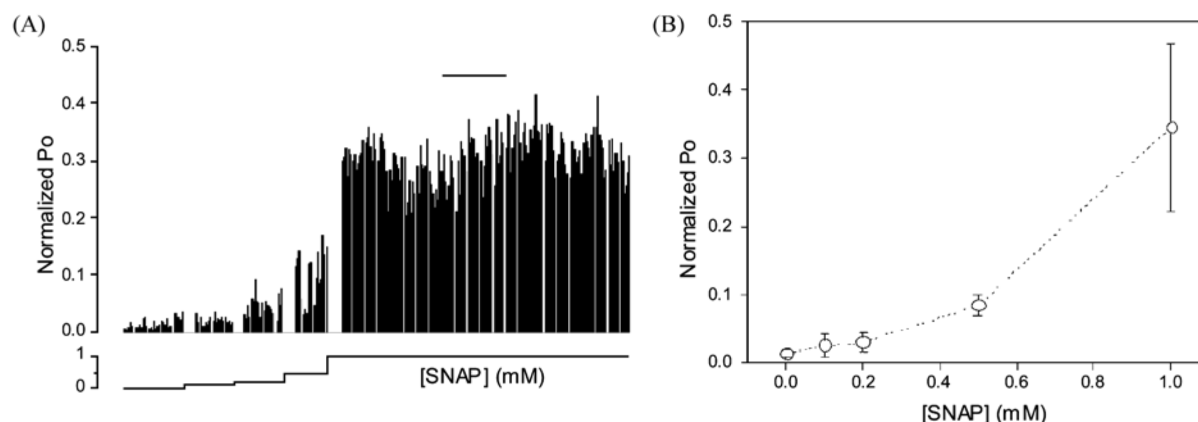

Fig. 1. Dose-dependent activation of Maxi-K channel activity by SNAP. (A) *Po*'s calculated for every 5-second are plotted. The concentration of SNAP was sequentially raised to 0.1, 0.2, 0.5, and 1 mM as indicated.

Table 1. Summary of effects on the Maxi-K channel activity by NO-donor application and washout. Data are presented as means ± SD of the number of experiments indicated in parentheses

Compound	Po (% of control)	
	+ compound	washout
SNAP		
0.1 mM	160.21 ± 38.84 (8)**	38.04 ± 20.77 (8)**
1 mM	428.31 ± 475.54 (9)*	77.76 ± 44.57 (7)
SNAP (0.1 mM) w/ 50 U/ml SOD	212.38 ± 146.80 (3)	52.60 ± 37.47 (3)
PAPA (0.2 mM)	145.74 ± 64.32 (3)	53.29 ± 33.36 (3)
NEM (2 mM)	28.79 ± 18.76 (9)**	-
SNAP (1 mM) after NEM (2 mM)	421.08 ± 385.42 (3)	79.10 ± 38.35 (4)
NEM (2 mM) after SNAP (1 mM)	86.53 ± 38.59 (4)	-

*$p < 0.05$; **$p < 0.005$

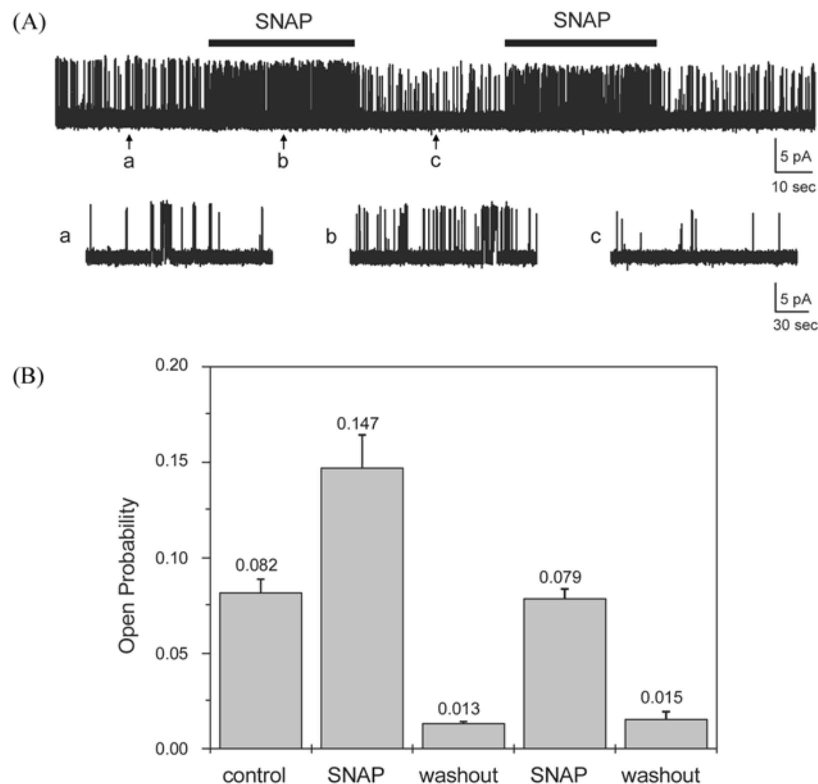

Fig. 2. Effects of 0.1 mM SNAP application and washout. 0.1 mM SNAP was added to the *cis* solution and removed by perfusion. (A) Representative trace shows single channel activity and the upward deflection represents the channel opening. Selected portion of the trace are expanded at the bottom. (B) The average *Po*'s with standard error are plotted as bars. The $[Ca^{2+}]_i$ was 3.82 μM and the holding potential −10 mV.

manner as shown in Fig. 1A. The *Po* remained increased as long as SNAP was present. A similar dose-dependency of SNAP was observed in two other experiments as summarized in Fig. 1B.

When SNAP was removed by superfusing the *cis* solution with 10-volume of the control solution, the activity was suppressed below the control level (15.8% of the control) as shown in Fig. 2. Re-application of SNAP at this point still increased the *Po*, but further suppression after the 2nd washout was not observed. In other experiments, we observed that the suppression of *Po* after 1st washout was maintained as long as the recording continued.

The activation and suppression effects of SNAP are summarized in Table 1. In a total of 8 experiments, 0.1 mM SNAP induced a significant increase (160.2 ± 38.8% of the control, n = 8) and decrease (38.0 ± 20.8% of the control, n = 8) after wash-out. 1 mM SNAP induced much larger increase (428.3 ± 475.5% of the control, n = 9) as expected.

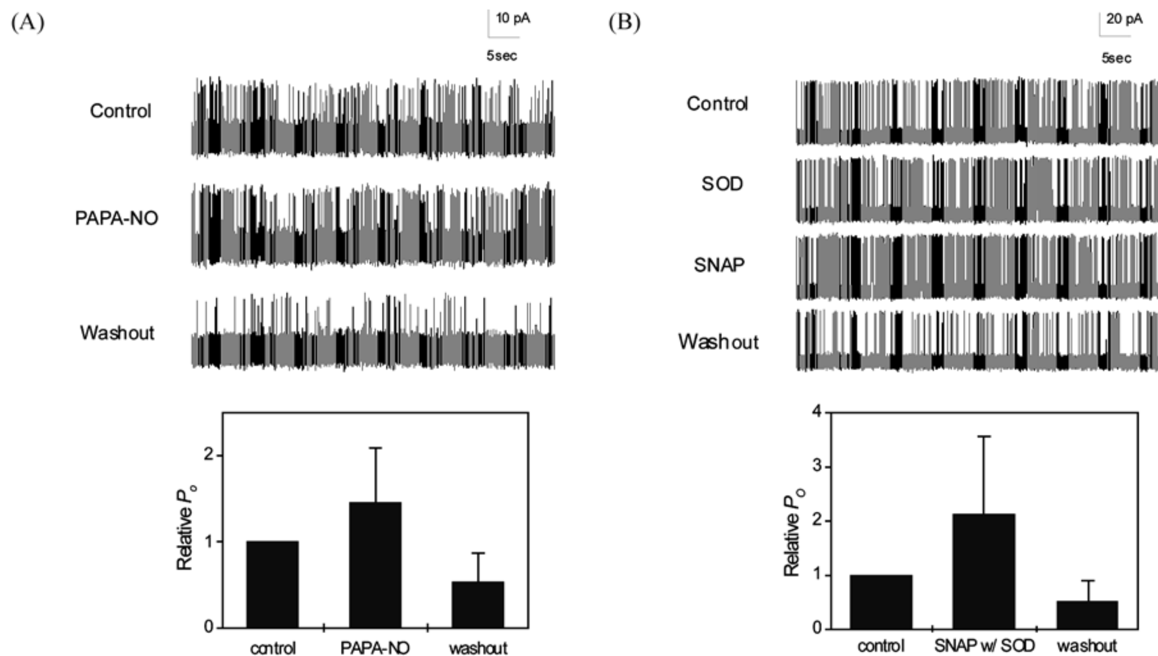

Fig. 3. Effects of 0.2 mM PAPA-NO and 50 U/ml SOD. (A) Three representative traces of 1-minute long are plotted: control (*upper*), after application of 0.2 mM PAPA-NO (*middle*), and after washout (*lower*). Bar graph indicates the relative *Po*. The $[Ca^{2+}]_i$ was 3.82 mM and the holding potential 0 mV. (B) Four representative traces of 1-minute long are plotted: control (*first row*), application of 50 U/ml of SOD (*second*), further addition of 0.1 mM SNAP (*third*), and washout of SNAP (*fourth*). Bar graph indicates the relative *Po.* The $[Ca^{2+}]_i$ was 3.82 μM and the holding potential +15 mV.

However, the degree of suppression was less than 0.1 mM SNAP (77.8 ± 44.8% of the control, n = 7). This may be due to a residual SNAP since we also observed from other experiments that additional perfusion further decreased the channel activities.

Since the increasing effect of SNAP is reversible, it is likely that the activation is due to a direct binding of NO on the channel protein rather than chemical modification such as nitrosylation of sulfhydryl groups. The suppression after SNAP washout is not due to 'run-down' since the channel activities were observed to be stable without a decrease for a period of as long as 20 minutes, which is usually longer than the time for application and washout of SNAP in the experiments described above. The suppression did not reverse to the control level as long as the recording continued. This suggests that the suppression after washout is due to chemical modification on the channel proteins.

Effects of PAPA-NO on the Maxi-K channel activities. Gbadegesin *et al.* (Fagni and Bockaert, 1996) claimed that PAPA-NO is one of the 'true' NO donors whereas other NO donors such as 3-morpholinylsydnoneimine chloride/5-amino-3-morpholinyl-1,2,3-oxadiazolium chloride (SIN-1) can release superoxide anion as well as NO, thereby producing peroxynitrite ($ONOO^-$). Therefore, it is possible that $ONOO^-$ not NO acts on the target protein as an oxidant. We tested PAPA-NO as a NO donor, and found that 0.2 mM PAPA-NO also induces the activation (145.7±64.3% of control, n=3) and suppression (53.3±33.4% of control, n=3) of the Maxi-K channel activities as SNAP (Fig. 3A).

We also tested whether SOD blocks the SNAP effect. While the application of 50 U/ml SOD did not significantly affect the channel activities, 0.1 mM SNAP still induces the activation (212.4 ± 146.8% of control, n = 3) and suppression (52.6 ± 37.5% of control, n = 3) in the presence of SOD (Fig. 3B). From these experiments, we can exclude the possibility that the SNAP effects are through $ONOO^-$.

Effects of NEM pre-treatment on SNAP effects. Application of NEM irreversibly suppresses the Maxi-K channel activity (Wang *et al*., 1997), presumably by alkylating thiol residues on the protein. We have examined whether the pre-treatment of NEM affects the SNAP effects. When 2 mM NEM was applied, the channel activity decreased irreversibly to 28.8 ± 18.8% of the control (n = 9, $p < 0.005$) (Fig. 4A). At this point, further application of NEM did not induce more suppression, implying the NEM effects reach saturation when treated with this concentration. However, subsequent application of 1 mM SNAP after removing NEM from the *cis* solution still induced the reversible activation (421.0 ± 385.4% of control, n = 3) and also the irreversible further suppression (79.1 ± 38.3% of control, n = 4). In consistent with our results, Jeong *et al*., (2001) also reported that NEM did not block the SNAP effect on Maxi-K channel from rat brain expressed either in HEK 293 cells or *Xenopus* oocytes. These results are different from the findings of Bolotina *et al.* (1994). They

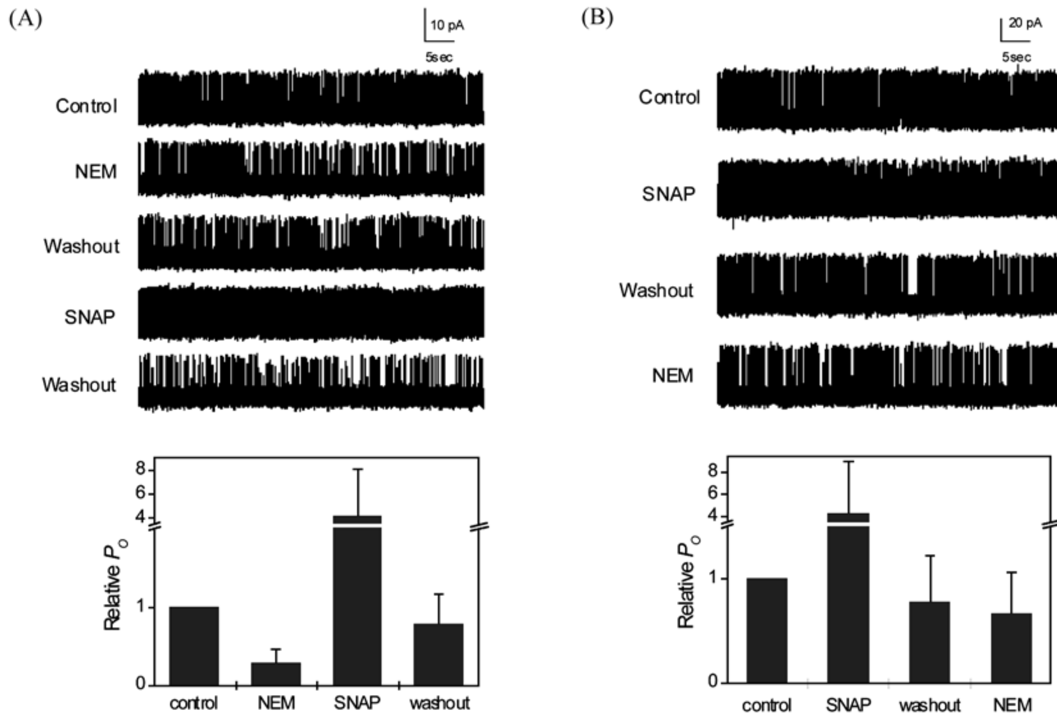

Fig. 4. Effects of NEM application on SNAP effects. (A) Five representative traces of 1-minute long are plotted: control (*first row*), application of 0.2 mM NEM (*second*), after washout of NEM (*third*), application of 1 mM SNAP (*fourth*), and after washout of SNAP (*fifth*). Bar graph indicates the relative *Po.* The $[Ca^{2+}]_i$ was 3.82 μM and the holding potential 0 mV. (B) Four representative traces of 1-minute long are plotted: control (*first row*), application of 1mM SNAP (*second*), after washout of SNAP (*third*), and application of NEM (*fourth*). Bar graph indicates the relative *Po*. The $[Ca^{2+}]_i$ was 3.82 μM and the holding potential +10 mV.

observed that pre-treatment with NEM blocked the SNAP effects on the Maxi-K channels in vascular smooth muscle. This discrepancy may be from the heterogeneity of Maxi-K channels from various tissues or the different properties between native channel and reconstituted one in lipid bilayer preparation. On the other hand, when we reversed the order of application of the compounds, the suppression effects by NEM was significantly smaller (86.5 ± 38.6%, n = 4) than when NEM was added without the SNAP treatment ($p < 0.05$) (Fig. 4B). This may be due to a residual SNAP or pre-occupation of thiols responsible to NEM by SNAP pretreatment.

As stated above, the activating effect of SNAP on Maxi-K channel was reversible and followed by prolonged suppression of *Po* after washout. Since activating effect was not blocked by NEM which alkylates free sulfhydryl groups, NO released from SNAP may directly interact with the channel. In contrast, partial blockade of NEM-induced inhibition by SNAP pre-treatment suggests that NEM and NO decrease Maxi-K channels by chemical modification. Although further studies may be needed, prolonged suppression of *Po* by SNAP may have common mechanism with NEM. From these results, we can suggest that NO may modulate Maxi-K channel by dual mechanisms, direct interaction and chemical modification.

Acknowledgments This work was supported by a grant No. 981-0706-044-2 from the Basic Research program of the Korea Science & Engineering Foundation (to JHS) and .by a Science Research Center grant from Korea Science and Engineering Foundation to the Nitric Oxide Radical Toxicology Research Center (to CKS).

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