Nitric Oxide Activates Leak K⁺ Currents in the Presumed Cholinergic Neuron of Basal Forebrain

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1Departments of Neuroscience and Oral Physiology and 4Removable Prosthodontics, Osaka University Graduate School of Dentistry, Suita, Osaka; and 2The Research Institute of Personalized Health Science, 3Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, 5Department of Removable Prosthodontics, School of Dentistry, Health Sciences University of Hokkaido, Hokkaido, Japan

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Kang Y, Dempo Y, Ohashi A, Saito M, Toyoda H, Sato H, Koshino H, Maeda Y, Hirai T. Nitric oxide activates leak K⁺ currents in the presumed cholinergic neuron of basal forebrain. J Neurophysiol 98: 3397–3410, 2007. First published October 10, 2007; doi:10.1152/jn.00536.2007. Learning and memory are critically dependent on basal forebrain cholinergic (BFC) neuron excitability, which is modulated profoundly by leak K⁺ channels. Many neuromodulators closing leak K⁺ channels have been reported, whereas their endogenous opener remained unknown. We here demonstrate that nitric oxide (NO) can be the endogenous opener of leak K⁺ channels in the presumed BFC neurons. Bath application of 1 mM S-nitroso-N-acetylpenicillamine (SNAP), an NO donor, induced a long-lasting hyperpolarization, which was often interrupted by a transient depolarization. Soluble guanylyl cyclase inhibitors prevented SNAP from inducing hyperpolarization but allowed SNAP to cause depolarization, whereas bath application of 0.2 mM 8-bromo-guanosine-3',5'-cyclic monophosphate (8-Br-cGMP) induced a similar long-lasting hyperpolarization alone. These observations indicate that the SNAP-induced hyperpolarization and depolarization are mediated by the cGMP-dependent and -independent processes, respectively. When examined with the ramp command pulse applied at −70 mV under the voltage-clamp condition, 8-Br-cGMP application induced the outward current that reversed at K⁺ equilibrium potential (E_K) and displayed Goldman-Hodgkin-Katz rectification, indicating the involvement of voltage-independent K⁺ current. By contrast, SNAP application in the presumed BFC neurons either dialyzed with the GTP-free internal solution or in the presence of 10 μM Rp-8-bromo-β-phenyl-1-N₂-ethenoguanosine 3',5'-cyclic monophosphorothioate sodium salt, a protein kinase G (PKG) inhibitor, induced the inward current that reversed at potentials much more negative than E_K and close to the reversal potential of Na⁺-K⁺ pump current. These observations strongly suggest that NO activates leak K⁺ channels through cGMP-PKG-dependent pathway to markedly decrease the excitability in BFC neurons, while NO simultaneously causes depolarization by the inhibition of Na⁺-K⁺ pump through ATP depletion.

INTRODUCTION

Based on studies of Alzheimer’s disease (AD) (Bartus et al. 1982; Coyle et al. 1983), basal forebrain cholinergic (BFC) neurons are implicated in the essential processes of learning and memory (Dunnett and Fibiger 1993). In the basal forebrain, neurons containing nitric oxide synthase (NOS) and/or nicotinamide adenine dinucleotide phosphate-diphosphorase (NADPH-D, a histochemical marker for NOS) have been found among cholinergic as well as noncholinergic neurons (Pasqualetto and Vincent 1991; Sugaya and McKinney 1994). Nitric oxide (NO) is known to play crucial roles in learning and memory (Hawkins et al. 1998; Susswein et al. 2004) presumably by acting as a neuromodulator and/or retrograde messenger (O’Dell et al. 1991; Schuman and Madison 1991), which activates postsynaptic and/or presynaptic cGMP-coupled targets (Arancio et al. 1996; Zhuo et al. 1994). Nevertheless, little is known about the roles of NO in the basal forebrain. Therefore it is very important to address if NO affects the excitability of BFC neurons.

On the other hand, NO has also been implicated in neuronal degeneration and cell death (Beckman 1991). The mechanisms of NO neurotoxicity have been proposed to include the nicotinamide adenine dinucleotide (NAD⁺) and ATP depletion (Zhang et al. 1994), the glutamate excitotoxicity, the inhibition of mitochondrial respiration (Hewett et al. 1994), and the upregulation of pro-apoptotic factors, such as neurogranin that enhance calcium-mediated signaling (Gui et al. 2007). Because of high susceptibility of BFC neurons to NO toxicity among other brain neurons (Fass et al. 2000), roles of NO in the AD pathogenetic mechanism or putative links between NO and AD are beginning to be recognized (Law et al. 2001). Therefore it is very important to examine whether NO plays any physiological roles in spite of its own toxicity in BFC neurons.

In the present study, we aimed to investigate the effects of NO on BFC neurons using the techniques of whole cell patch-clamp and the optical measurements of NO concentration by diaminofluorescein-2 (DAF-2). We found that NO activates leak K⁺ channels through cGMP-dependent mechanism, thereby markedly decreasing the excitability of BFC neurons. This effect was accompanied by an inhibition of ATP production, which was revealed as the membrane depolarization caused by inhibition of Na⁺-K⁺ pump, especially when NO concentration was high.

METHODS

Slice preparation

Experiments were performed using slice preparations obtained from 7 to 15 days postnatal Wistar rats of both sexes (Charles River...
Breeders, Osaka, Japan). Under diethyl ether anesthesia, the brain was quickly removed from the skull and immersed in cold (1–4°C) extracellular solution. Slices of 250–300 μm thickness were cut coronally from the basal forebrain containing the nuclei of medial septum/diagonal band (MS/DB).

**Electrophysiological recording**

Whole cell patch-clamp recording method was essentially similar to our previous studies (Kang et al. 2004, 2007; Saito et al. 2006). Axopatch 200B (Molecular Devices, Foster City, CA) was used for recording from MS/DB neurons. The extracellular solution had the following composition (in mM): 124 NaCl, 1.8 KCl, 2.5 CaCl₂, 1.3 MgCl₂, 26 NaHCO₃, 1.2 KH₂PO₄, and 10 glucose, bubbled with mixture of 95% O₂-5% CO₂. The standard internal solution (Std-InS) had the following composition (in mM): 123 K-gluconate, 18 KCl, 10 NaCl, 2 MgCl₂, 2 ATP-Na₂, 0.3 GTP-Na₃, 10 HEPES, and 0.1 EGTA; the pH was adjusted to 7.3 with KOH. As a modified internal solution (Mod-InS), the low-ATP/high-Na solution was prepared to examine if [ATP]i would not decrease so quickly removed from the skull and immersed in cold (1–4°C) extracellular solution. Slices of 250–300 μm thickness were monitored with a membrane-impermeant fluorescence indicator of diaminofluorescein-2 (DAF-2, Sigma-Aldrich) or dimethylsulfoxide (DMSO) and added to the extracellular or intracellular solution. Relative changes of NO concentration in the extracellular solution or in the intracellular space were monitored with a membrane-impermeant fluorescence indicator of diaminofluorescein-2 (DAF-2, Daichi Pure Chemicals, Tokyo, Japan), which was dissolved in dimethylsulfoxide (DMSO) and added to the extracellular or intracellular solutions at a dilution 1:1,000 to give a final concentration of 2 mM and 10 μM, respectively. DAF-2 reacts with NO to form NO-imaging techniques

Relative changes of NO concentration in the extracellular solution or in the intracellular space were monitored with a membrane-impermeant fluorescence indicator of diaminofluorescein-2 (DAF-2, Daichi Pure Chemicals, Tokyo, Japan), which was dissolved in dimethylsulfoxide (DMSO) and added to the extracellular or intracellular solutions at a dilution 1:1,000 to give a final concentration of 2 mM and 10 μM, respectively. DAF-2 reacts with NO to form a low-threshold Ca²⁺ spike (LTS) from which a fast action potential was triggered (→, Fig. 1Ab), whereas the same type I neuron did not display LTS at –70 mV due to its inactivation (Fig. 1Aa). The type II neuron was characterized by a late-spiking (→, Fig. 1Bb) due to A-like K⁺ current in response to depolarizing current pulses applied at a hyperpolarized holding potential of −96 mV. This neuron showed a regular spiking at −72 mV where A-like K⁺ current should be considerably inactivated (Fig. 1Ba). Because cholinergic neurons in MS/DB are well established to display either A-like K⁺ current or late-spiking (Griffith and Matthews 1986; Markram and Segal 1990), type II neurons were subsequently presumed to be cholinergic. Neurons classified as type III displayed neither LTS nor late spiking (Fig. 1C). Among 135 healthy MS/DB neurons that displayed either stable resting MPs (<−60 mV) or positive baseline currents (>0 pA) at −70 mV, 113 MS/DB neurons were identified as type II neurons, and 15 and 7 neurons were identified as type I and III neurons, respectively.
oxidation of NO, irreversibly forming fluorescent DAF-2 triazole (DAF-2T).1 Fluorescent images of DAF-2T were acquired every 20 s with a digital CCD camera (C4742-95-12ER, Hamamatsu Photonics, Hamamatsu, Japan), using an imaging system (Aqua-cosmos, Hamamatsu Photonics).

Drug application

S-nitroso-N-acetylpenicillamine (SNAP, an NO donor; Wako Pure Chemicals, Osaka, Japan), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, a soluble guanylyl cyclase inhibitor; Sigma-Aldrich, St. Louis, MO), 6-anilinoquinoline-5,8-quinone (LY83583, a soluble guanylyl cyclase inhibitor; Calbiochem, Darmstadt, Germany), and tolbutamide (TLB, a K\textsubscript{ATP} channel blocker; Wako Pure Chemicals) were dissolved in DMSO for preparing respective stock solutions. They were bath-applied at a dilution >1:1,000 to give a final concentration of 0.5–1 mM (SNAP), 50 μM (ODQ), 5 μM (LY83583), and 0.1 mM (TLB). SNAP was added together with DAF-2 in the extracellular solution 20–30 min before its bath application to release NO gas sufficiently. It can be considered that 0.5 and 1 mM SNAP release 0.50 and 0.55 μM NO, respectively, based on the previous studies (Borson et al. 1999; Han et al. 2006). We assumed that BFC neurons can generate ~0.55 μM NO under physiological condition because NO concentration in the somatosensory cortex is known to increase up to ~0.6 μM after increases in neuronal activity (Buerk et al. 2003). 8-Bromoguanosine-3′,5′-cyclic monophosphate (8-Br-cGMP, a membrane-permeable cGMP analogue; Sigma-Aldrich), Rp-8-bromo-β-phenyl-1,2-N\textsubscript{2}-ethenoguanosine 3′,5′-cyclic monophosphorothioate sodium salt (Rp-8-Br-PET-cGMP, a membrane-permeable protein kinase G inhibitor; Sigma-Aldrich), and ouabain (a Na\textsuperscript{+}–K\textsuperscript{+}-ATPase inhibitor; Sigma-Aldrich) were bath-applied at 0.1–0.4 mM, 10 μM, and 50 μM, respectively. These drugs were applied usually after confirming that the MP remained almost constant at least for 1 min after full “run-up” of K\textsubscript{ATP} channel activity (Allen and Brown 2004).

Data analysis

Results are expressed as the means ± SD. The statistical significance was assessed using paired or unpaired Student’s t-test or using ANOVA followed by Fisher’s PLSD (protected least significant difference) post hoc test.

RESULTS

The data presented in the following text were based on whole cell recordings obtained from 113 type II neurons in the nuclei of MS/DB (see methods), which displayed well-established characteristics of cholinergic neurons in the MS/DB nuclei (Griffith and Matthews 1986; Markram and Segal 1990).

Two opposing effects of SNAP on type II neurons

Bath application of SNAP for 5 min usually induced a biphasic response consisting of a hyperpolarization followed by a transient depolarization (Fig. 2A). With an increase in the DAF-2T FI after 1 mM SNAP application (Fig. 2Aa), the MP was hyperpolarized more, reaching the maximum level 80 s after starting the SNAP application (upward arrow, Fig. 2Ab), which in turn led to a gradual depolarization. This depolarization also increased with an increase in the fluorescence intensity (FI), but reached the maximum level about 20–40 s later than the timing of the peak FI in the extracellular solution (compare open and filled arrowheads placed in Fig. 2Aa and b, respectively). This time lag is consistent with the time difference in achieving the peak concentration between extracellular and intracellular spaces (compare open arrowheads, Fig. S1, Aa and Ab, in supplementary information). During washout of SNAP, the depolarization decayed in association with the decay of the FI, consequently disclosing the underlying hyperpolarization that lasted even after the washout of SNAP (asterisks, Fig. 2Aa). The long-lasting property of SNAP-induced hyperpolarization was clearly seen when there was no apparent or much less interrupting depolarization (Fig. 2Bb). In such a case, there was no marked difference in the peak time between the FI and hyperpolarization (compare open arrowhead and upward arrow, Fig. 2B, a and b), in contrast to a large difference in the peak time between the FI and the hyperpolarizing component of the biphasic response (open arrowheads and upward arrow, Fig. 2A, a and b).

Thus the simultaneous measurements of DAF-2T FI and MP clearly revealed that the SNAP-induced hyperpolarization lasted long even after the DAF-2T FI completely decayed (asterisks, Fig. 2B) although it was occasionally interrupted by a transient depolarization (Fig. 2Aa) presumably depending on the basal level of endogenous NO. This transient depolarization that was always preceded by hyperpolarization became apparent at higher DAF-2T FI and disappeared first during washout of SNAP, leaving behind the hyperpolarization (Figs. 2Aa and 2Ab). Therefore it is likely that the hyperpolarizing effect alone could be seen at a low concentration of NO, whereas at higher concentrations of NO, the depolarizing effects might have emerged in addition to the hyperpolarizing one. Such a hyperpolarizing effect of SNAP was cumulative in terms of the duration and amplitude as shown by the paired application of 1 mM SNAP (Fig. 2C).

In 8 of 12 type II neurons dialyzed with the Std-InS, 1 mM SNAP applications at the resting MP (−74.3 ± 5.3 mV) induced the biphasic response with the mean maximum amplitude of the hyperpolarizing component of −3.5 ± 1.5 mV (P < 0.001, filled circles, Fig. 2Da). It was difficult to determine the amplitude of the depolarizing component of biphasic responses. However, in the remaining four type II neurons, 1 mM SNAP application at artificially hyperpolarized MPs (−83.8 ± 9.9 mV) induced only the depolarizing response with the mean maximum amplitude of 3.1 ± 0.6 mV (P < 0.02, filled circles, Fig. 2Db).

Because NO depletes intracellular ATP (Lizasoain et al. 1996; Radi et al. 1994; Zhang et al. 1994), it was also examined if [ATP], decrease is involved in SNAP-induced MP changes by using a Mod-InS (see methods). There was no significant difference (P > 0.5) in the resting MP between type II neurons dialyzed with Std-InS (−73.9 ± 7.2 mV, n = 48) and those dialyzed with Mod-InS (−74.9 ± 5.0 mV, n = 23). This is presumably due to the abundant presence of mitochondria (Albers et al. 2002; Bereiter-Hahn 1990), which would provide a high concentration of ATP around ATP-sensitive K\textsuperscript{+} (K\textsubscript{ATP}) channels and Na\textsuperscript{+}–K\textsuperscript{+} pump, subsequently eliminating the difference in effective [ATP], on K\textsubscript{ATP} channels or Na\textsuperscript{+}–K\textsuperscript{+} pumps between the two groups of type II neurons dialyzed differently.

In five type II neurons dialyzed with the Mod-InS, 1 mM SNAP application at the resting MPs (−75.0 ± 4.1 mV) induced the biphasic responses with the mean maximum amplitudes of hyperpolarizing component of −9.6 ± 2.7 mV (P < 0.001, filled circles, Fig. 2Dc).

1 The online version of this article contains supplemental data detailing intracellular NO measurement using DAF-2.
A constant at least for 1 min following the "run-up" process of (Fig. 3). SNAP was applied after the MP remained almost A by extracellular Cs

Separation of the two opposing effects of SNAP in the MPs (see DISCUSSION).

0.001, open circles, Fig. 2Da), which was 2.7 times larger (P < 0.05, 2-way ANOVA) than that observed using the Std-InS (n = 8). On the other hand, 1 mM SNAP application at artificially hyperpolarized MPs (−85.8 ± 6.2 mV, n = 4) in similarly dialyzed type II neurons caused mono-phasic depolarizing responses with the mean maximum amplitudes of 7.2 ± 2.4 mV (P < 0.002, open circles, Fig. 2Db). The SNAP-induced depolarization obtained using the Mod-InS (7.2 ± 2.4 mV, n = 4) was significantly (P < 0.01, 2-way ANOVA) larger in amplitude than that obtained with the Std-InS (3.1 ± 0.6 mV, n = 4). Thus SNAP exerted the two opposing effects on the MP presumably through two different mechanisms, irrespective of the difference in the internal solution. However, both the effects were similarly enhanced when neurons were dialyzed with Mod-InS, suggesting involvements of [ATP], decrease in the SNAP-induced changes in the MPs (see DISCUSSION).

Separation of the two opposing effects of SNAP by extracellular Cs

The SNAP-induced hyperpolarization might be brought about by activation of K⁺ channels through the action of NO. First, we examined the effects of Cs⁺ on the SNAP-induced hyperpolarization. To monitor changes in the input resistance (IR) and the threshold for evoking spikes, a short hyperpolarizing current pulse (100-ms duration) followed by a long depolarizing current pulse (1-s duration) was applied every 10 s (Fig. 3Da, top) before, during, and after SNAP application (Fig. 3A). SNAP was applied after the MP remained almost constant for at least 1 min following the “run-up” process of K̅ₐₚ channel activity (Allen and Brown 2004) (asterisk, Fig. 3B, see METHODS). The first application of 1 mM SNAP induced a hyperpolarization from −79 to −87 mV (Fig. 3B, b), which was completely reversed to −76 mV by the bath application of 5 mM, but not by 2 mM, Cs⁺ (Fig. 3B, c). The hyperpolarization was accompanied by decreases in the IR (Fig. 3C, b and c). Contrary to the first SNAP application, the second SNAP application in the presence of 5 mM Cs⁺ caused a marked depolarization from −76 to −62 mV (Fig. 3B). This depolarization promptly decayed after washout of SNAP, leaving a small irreversible depolarizing component. This brief time course is in contrast to the SNAP-induced long-lasting hyperpolarization shown in Fig. 2B. The washout of Cs⁺ resulted in the further hyperpolarization beyond the initial resting MP to −86 mV (Fig. 3B, d), similar to that caused by the first SNAP application, consequently disclosing the long-lasting nature of SNAP-induced hyperpolarization.

Thus Cs⁺ clearly revealed the presence of two opposing responses to SNAP application between which there was a marked difference in the decay time course as revealed following washout of SNAP. SNAP-induced hyperpolarization was completely eliminated by 5 mM Cs⁺ application in eight neurons examined. In the presence of 5 mM Cs⁺, SNAP never induced hyperpolarization but caused a depolarization (5.2 ± 4.0 mV, n = 8, P < 0.003), without significant changes (P > 0.5) in the IR when applied at −69.5 ± 7.1 mV. These observations suggest that SNAP might have induced hyperpolarization by opening Cs⁺-sensitive K⁺ channels through the action of NO.

Effects of SNAP in the presence of soluble guanylyl cyclase inhibitors

One of the possible mechanisms underlying the hyperpolarizing effects of NO on type II neurons may be the activation of
soluble guanylyl cyclase (sGC) and the subsequent production of cGMP (Hanafy et al. 2001). The involvement of cGMP in the SNAP-induced hyperpolarization was evaluated by using sGC inhibitors, ODQ and LY83583. The slices were preincubated with 50 μM ODQ or 5 μM LY83583 for 20 min. In the presence of TTX and either of these sGC inhibitors, 1 mM SNAP application at the resting MP never caused hyperpolarization (n = 100). Instead, SNAP caused a small depolarization (Fig. 4A, a and b), which was accompanied by increases in the IR, but never by decreases in the IR (Fig. 4Ac). In the two groups of five type II neurons examined in the presence of ODQ and in the presence of LY83583, 1 mM SNAP application invariably caused small depolarizations (5.2 ± 3.1 and 6.6 ± 4.5 mV, respectively) accompanied by IR increases (32 ± 23 and 48 ± 54%, respectively; Fig. 4A, d and e). Thus sGC inhibitors completely prevented SNAP from inducing hyperpolarization but allowed SNAP to cause depolarization. Therefore the SNAP-induced hyperpolarization seemed to be mediated by cGMP, whereas the SNAP-induced depolarization was independent of cGMP. In the next experiment, we examined if 8-Br-cGMP causes a similar hyperpolarization.

**Hyperpolarizing effects of 8-Br-cGMP**

At the resting MP, bath application of a membrane-permeable cGMP analogue, 8-Br-cGMP (0.2 mM) for 5 min induced a long-lasting hyperpolarization accompanied by decreases in the IR in a type II neuron (Fig. 4Bd). The MP and IR returned to their original levels >25–30 min after washout of 8-Br-cGMP (Fig. 4B, b and c). The maximum amplitude of the 8-Br-cGMP-induced hyperpolarization measured from the rest-
ing MPs (–70.4 ± 3.4 mV, n = 6) of type II neurons dialyzed with the Std-InS was –9.1 ± 3.1 mV (P < 0.002, Fig. 4Bd). Comitantly, the IR decreased by 32 ± 13% (P < 0.04, Fig. 4Be). The hyperpolarization and concomitant IR decrease caused by 8-Br-cGMP were quite similar to those induced by SNAP application (Figs. 2B and 3), and 8-Br-cGMP never induced depolarization in these six type II neurons examined. Taken together with the effects of sGC inhibitors, SNAP-induced hyperpolarization is likely to be mediated by the activation of NO-cGMP pathway.

**Ba**<sup>2+</sup>, but not TLB, antagonizes the hyperpolarizing effect of 8-Br-cGMP

Because BFC neurons are known to express K<sub>ATP</sub> channels (Allen and Brown 2004) and these channels are reported to be activated also through cGMP pathway (Baker et al. 2001; Deka and Brading 2004), effects of TLB, a blocker of K<sub>ATP</sub> channels, on 8-Br-cGMP-induced hyperpolarization were examined in comparison with those of Ba**<sup>2+</sup>, a less specific leak K<sup>+</sup> channel blocker. Ba**<sup>2+</sup> application (50 μM) caused a significant (P < 0.04) depolarization of 1.6 ± 1.0 mV from the resting MP (–74.4 ± 7.2 mV) in five type II neurons examined (Fig. 5A, a, b, and d). Under this depolarized condition in the presence of Ba**<sup>2+</sup>, 8-Br-cGMP application for 5 min caused no apparent hyperpolarization (Fig. 5A, a and b). There were no significant differences both in the MP (P > 0.4) and in the IR (P > 0.8) obtained before and 5 min after application of 8-Br-cGMP in the presence of Ba**<sup>2+</sup> (Fig. 5A, d and e). Nevertheless, after the simultaneous washout of 8-Br-cGMP and Ba**<sup>2+</sup>, the MP was significantly (P < 0.001) hyperpolarized by –7.2 ± 1.7 mV when measured from the resting MP, and the IR was also significantly (P < 0.001) decreased from 458 ± 84 to 230 ± 46 mV beyond the control value at the resting MP (347 ± 58 mV). 8-Br-cGMP induced a significant hyperpolarization from the resting MP (–70.4 ± 3.4 mV) to –79.5 ± 2.7 mV, IR decreased from 421 ± 178 to 276 ± 86 mV. ***: P < 0.002; †: P < 0.04.

Thus the action of 0.1 mM 8-Br-cGMP was completely antagonized by 0.1 mM Ba**<sup>2+</sup> but not by 0.1 mM TLB, indicating that the K<sup>+</sup> channels responsible for the 8-Br-cGMP-induced hyperpolarization are sensitive to Ba**<sup>2+</sup> but not to the K<sub>ATP</sub> channel blocker. Therefore it is likely that Ba**<sup>2+</sup>-sensitive K<sup>+</sup> channels, but distinct from K<sub>ATP</sub> channels, are responsible for the hyperpolarization induced by SNAP or 8-Br-cGMP application.
Goldman-Hodgkin-Katz rectification of 8-Br-cGMP-induced current

To further investigate the ionic mechanism underlying the 8-Br-cGMP-induced hyperpolarization accompanied by conductance increases, voltage-clamp experiments using a ramp command pulse from −135 to −55 mV (ramp rate: 80 mV/s) were done. Membrane currents were obtained in response to the ramp command pulse before and during application of 8-Br-cGMP (0.1 mM; Fig. 6, Ab and Ba, gray and black traces, respectively). The current-voltage (I-V) relationships of the 8-Br-cGMP-induced responses in 3 and 10 mM [K+], (Fig. 6, Ab and Bb) were obtained, respectively, after subtraction of the control responses from the current responses recorded during 8-Br-cGMP application (Fig. 6, Aa and Aa). The 8-Br-cGMP-induced current exhibited an outward rectification when evoked in 3 mM [K+]o (Fig. 6Ab), whereas it displayed much less apparent rectification when evoked in 10 mM [K+]o (Fig. 6Bb) as revealed by interrupted straight lines. A similar outward rectification was consistently observed in 10 type II neurons when evoked in 3 or 5 mM [K+]o, and less rectified or linear I-V relationship was also consistently observed in four type II neurons when evoked in 10 mM [K+]o. In these pooled data, five type II neurons were not included because their I-V relationships in the control condition were largely contaminated by inward-rectifier and/or delayed rectifier K+ currents, and such contamination became much less clear after 8-Br-cGMP application, presumably due to the reduced performance on space clamp by the increased leak conductance (inverse of IR). Consequently, in these excluded five type II neurons, the I-V relationships of the 8-Br-cGMP-induced currents obtained by subtraction were not rectified outwardly.

Although there were exceptions due to contamination of other K+ currents, 8-Br-cGMP-induced current usually rectified outwardly depending on the magnitude of the concentration gradient between intra- and extracellular K+, indicating the nature of Goldman-Hodgkin-Katz (GHK) rectification. As illustrated in Fig. 6C, I-V relationships of the 8-Br-cGMP-induced currents shown in Fig. 6, Ab and Bb, were fitted by GHK equation and extrapolated to a more positive voltage range to show the GHK-type outward rectification (interrupted curves). 8-Br-cGMP-induced current never displayed inward rectification, in spite of the presence of free Mg2+ (≥0.5 mM, estimated by using WEBMAXC) in Mod- or Std-InS, which was much higher than the minimum concentration of free Mg2+ (~20 μM) (Hille 2001) required for inward-rectifier K+ (Kir) channels to display a strong inward rectification.

The reversal potentials (Erev) of 8-Br-cGMP-induced currents obtained in 3 and 10 mM [K+]o, in two different type II neurons were −96 and −66 mV, respectively (Fig. 6, Ab and Bb). These values were close to the respective calculated K+ equilibrium potentials (EK). The K+ selectivity of the underlying channels was further confirmed by measuring the shift in the Erev of the 8-Br-cGMP-induced current after increases in [K+]o. The relationship between [K+]o and the mean Erev values for 3, 5, and 10 mM [K+]o (Fig. 6D) yielded a predicted shift of 61.4 mV for a 10-fold change in [K+]o. This value is...
consistent with what would be predicted if the underlying channels were highly selective for K⁺ ions. Taken together, SNAP- or 8-Br-cGMP-induced hyperpolarization is likely to be brought about by the activity of K⁺ channels displaying GHK-type rectification, most likely the leak K⁺ channel.

Irreversible depolarization evoked in the presumed cholinergic neurons by SNAP

SNAP usually induced a biphasic response consisting of a hyperpolarization followed by a transient depolarization (Fig. 2A), whereas it predominantly induced membrane depolarization in type II neurons in the presence of ODQ or LY83583 (Fig. 4). Therefore, SNAP-induced hyperpolarization and depolarization are likely to be generated through the cGMP-dependent and -independent processes, respectively. In the following series of the experiments, we further characterized the SNAP-induced membrane depolarization by examining the effects of SNAP on type II neurons that were dialyzed with the GTP-free Mod-InS in which cGMP production by NO would largely be inhibited. In addition, the MP was artificially hyperpolarized to enhance the depolarizing response to SNAP application.

In a type II neuron displaying the characteristic late spiking at an artificially hyperpolarized MP (–90 mV, Fig. 7A), the first application of SNAP (1 mM) induced a depolarization, which promptly decayed after washout of SNAP (Fig. 7C, a and b). However, the second SNAP (0.5 mM) application induced an irreversible depolarization, which did not decay after washout of the SNAP (Fig. 7C, a and b). The third and fourth applications of SNAP (1 and 0.5 mM, respectively) caused similar irreversible depolarizations, which were summated almost in a stepwise manner (Fig. 7B). A similar observation has been made in four type II neurons, in which the first application of SNAP (1 mM) caused a membrane depolarization of 10.3 ± 3.9 mV from the artificially hyperpolarized MPs (–85.5 ± 5.0 mV), and the repetitive applications of SNAP invariably induced the irreversible depolarization, which might lead to cell death (see DISCUSSION). To investigate the ionic mechanisms underlying the SNAP-induced depolarization, we examined the effects of SNAP on voltage-clamped type II neurons that were dialyzed with the GTP-free Mod-InS in the next series of experiments.

SNAP-induced depolarization is mediated by inhibition of an outward current

Under the voltage-clamp condition at a holding potential of –70 mV, the ramp command pulse was repetitively applied at every 20 s before and during bath application of SNAP in a type II neuron dialyzed with the GTP-free Mod-InS. Following SNAP application, the baseline current was shifted inwardly (Fig. 8A) in agreement with the membrane depolarization observed under the current-clamp condition (Fig. 7C), and the current response to the ramp pulse was also changed to have a less steep slope in comparison with those observed before application of SNAP (compare black and gray traces in Fig. 8B). As revealed by the subtraction of the control current response (Fig. 8, A and B, 1) from the current response obtained during SNAP application (Fig. 8, A and B, 2), SNAP-induced current was an inward current that decreased with membrane hyperpolarization and appeared to reverse at a potential much more negative than the calculated K⁺ equilibrium potential (Eₖ; Fig. 8C). The reversal potential was estimated by linear extrapolation as –132 ± 1 mV (n = 4), which...
is far more hyperpolarized than \( E_K \) \((-97 \text{ mV})\) and rather close to the reversal potential of \( \text{Na}^+-\text{K}^+ \) pump \((-150 \text{ mV})\) (Chapman et al. 1983). These observations strongly suggests that SNAP indirectly induced the inward current through the inhibition of the outward current carried by \( \text{Na}^+-\text{K}^+ \) pump (gray trace in Fig. 8C). In the next series of experiments, this possibility was further addressed by examining the effects of SNAP on the ouabain-sensitive outward current.

**FIG. 7.** Irreversible depolarization caused by repetitive applications of SNAP. A: late spiking pattern in a type II neuron that was dialyzed with GTP-free Mod-InS. B: sample fluorescence images showing different fluorescence intensities of DAF-2T in the extracellular solution obtained in response to repetitive applications of SNAP (0.5 and 1 mM). C: simultaneously measured changes in the FI \( (a) \) and MP \( (b) \) in the same type II neuron as shown in A, which were plotted against time. The duration of bath application of SNAP are indicated with horizontal bars. Note that the depolarizing response became irreversible after the 2nd application of SNAP. The sample fluorescence images used to measure FI at the respective times indicated with serial numbers of 1–8 are shown in B with the labels of the corresponding numbers.

**FIG. 8.** SNAP-sensitive outward current. A: continuous recording of current responses to repetitively applied ramp voltage pulses at –70 mV under the voltage-clamp condition obtained before, during, and after application of 1 mM SNAP, as indicated with a black horizontal bar. B top: ramp command pulse. Bottom: superimposed sample current traces obtained before \((1)\) and during \((2)\) SNAP (1 mM) application (black and red traces, respectively), as indicated with 1 and 2 in A. C: \( I-V \) relationships of SNAP-induced current (red trace) and its mirror image current as SNAP-sensitive current (gray trace). The SNAP-induced current was obtained by subtraction of the control current \((1\ \text{in} \ B)\) from that obtained after SNAP application \((2\ \text{in} \ B)\). Note that the SNAP-induced inward current decreased with membrane hyperpolarization. This indicates that the inward current induced by SNAP was brought about by the inhibition of the outward current that reversed at a potential much more negative than \( E_K \), representing the SNAP-sensitive current (gray trace).
Ouabain abolished SNAP-sensitive outward current

In a type II neuron dialyzed with the GTP-free Mod-InS, a brief bath application of SNAP (1 mM) caused a transient inward shift of the baseline current (Fig. 9A), which was accompanied by an inhibition of the ramp current response (Fig. 9Ba). The SNAP-sensitive outward current was revealed by subtraction of the current response recorded during SNAP application from the control response to have an I-V relationship (Fig. 9Bb) similar to that seen in Fig. 8C (gray trace). After the baseline current returned to the original level following washout of SNAP, bath application of ouabain (50 μM) caused a similar but larger inward shift of the baseline current (Fig. 9A), which was also accompanied by the inhibition of current responses to the ramp pulse (Fig. 9Ca). The I-V relationship of the ouabain-sensitive current was almost linear, and its reversal potential was estimated by linear extrapolation of the ouabain-sensitive current was almost linear, which was also accompanied by the inhibition of the ramp current response (Fig. 9Ba). The SNAP-sensitive outward current was revealed by subtraction of the response recorded during application of SNAP (2) from the control (1), by subtraction of that recorded during application of ouabain (4) from the control (3), and by subtraction of that recorded during application of SNAP in the presence of ouabain (5) from the response recorded in the presence of ouabain alone (4), respectively. Note the presence of SNAP-sensitive (B) and ouabain-sensitive outward currents (C), and the absence of SNAP-sensitive current in the presence of ouabain (D).

FIG. 9. Occlusion between the effects of SNAP and ouabain. A: continuous recording of current responses to repetitively applied ramp voltage pulses at –70 mV under the voltage-clamp condition obtained before, during, and after the 1st application of SNAP (1 mM, black horizontal bar) alone, and before, during, and after the 2nd SNAP (1 mM, black horizontal bar) application in the presence of ouabain (50 μM, gray horizontal bar). B–D: superimposed traces of the 2 current responses to the ramp pulse obtained at the times indicated with 1 and 2, with 3 and 4, and with 4 and 5 in A, respectively (Ba, Ca, and Da). The I-V relationships of the respective currents (Bb, Cb, and Db) obtained by subtraction of the response recorded during application of SNAP (2) from the control (1), by subtraction of that recorded during application of ouabain (4) from the control (3), and by subtraction of that recorded during application of SNAP in the presence of ouabain (5) from the response recorded in the presence of ouabain alone (4), respectively. Note the presence of SNAP-sensitive (B) and ouabain-sensitive outward currents (C), and the absence of SNAP-sensitive current in the presence of ouabain (D).

SNAP-sensitive K⁺ current versus SNAP-induced K⁺ current revealed by a protein kinase G inhibitor

A similar SNAP-sensitive outward current was observed in the presence of 10 μM Rp-8-Br-PET-cGMPS, a potent protein kinase G (PKG) inhibitor. A brief bath application of SNAP (1 mM) in a type II neuron in the presence of 10 μM Rp-8-Br-PET-cGMPS caused a transient inward shift of the baseline current (Fig. 10A, a and b), which was accompanied by an inhibition of the outward current (Fig. 10Ba). As revealed by subtraction of the current response recorded during SNAP application (Fig. 10, Ab and Ba, 2) from the control response (Fig. 10, Ab and Ba, 1), this SNAP-sensitive outward current displayed a linear I-V relationship and reversed its polarity at
–134 ± 10 mV (n = 6; Fig. 10Bb). The conductances of SNAP-sensitive outward currents measured between –120 and –70 mV in the presence of Rp-8-Br-PET-cGMPS were 0.47 ± 0.14 nS (n = 6), similar to those obtained with the GTP-free internal solution. These observations indicate that the PKG inhibitor prevented SNAP from enhancing leak K⁺ current but allowed SNAP to inhibit the activity of Na⁺-K⁺ pump, as was the case with the GTP-free internal solution (Fig. 9).

However, after simultaneous washout of SNAP and Rp-8-Br-PET-cGMPS, the baseline current was markedly shifted in the outward direction (Fig. 10Aa, a and b). This was accompanied by the enhancement of leak K⁺ current (Fig. 10Cb, 3 – 1), as revealed by the subtraction of the current response obtained in the presence of Rp-8-Br-PET-cGMPS before SNAP application (Fig. 10, Ab and Ca, 1) from that obtained after the simultaneous washout of Rp-8-Br-PET-cGMPS and SNAP (Fig. 10, Ab and Ca, 3). This is presumably because the cGMP produced and accumulated in the type II neuron by SNAP application slowly activated PKG following washout of intracellular PKG inhibitor. Thus NO-cGMP-PKG pathway is likely to be involved in enhancing leak K⁺ current, whereas such pathway is unlikely to be involved in the inhibition of Na⁺-K⁺ pump.

**DISCUSSION**

**Involvement of NO-cGMP-PKG pathway in generating hyperpolarization**

In the present study, type II neurons, which were presumed to be BFC neurons based on their electrophysiological membrane properties (Fig. 1), displayed a long-lasting hyperpolarization following bath application of NO donor (Fig. 3). The NO-induced hyperpolarization was completely suppressed by preincubation with sGC inhibitors (Fig. 4A) and mimicked by cGMP analogue (Fig. 4B). Under the voltage-clamp condition, 8-Br-cGMP-induced currents reversed at the predicted E_K (Fig. 6D). These observations clearly indicate that NO activates K⁺ channels through the cGMP-dependent pathway. Furthermore, SNAP-induced hyperpolarization was significantly larger when recorded with Mod-InS than with the Std-InS (Fig. 2Da). This observation is also consistent with the involvement of sGC-cGMP pathway in generating hyperpolarization by SNAP because sGC responsiveness to NO is inhibited dose dependently by intracellular ATP with a Kᵢ of 1–2 mM (Ruiz-Stewart et al. 2004), whereas there would be no difference in the activity of K_ATP channels between 0.5 and 2 mM [ATP]ᵢ in intact BFC neurons (Kᵢ ≈ 6 mM) (Allen and Brown 2004). Finally, a PKG inhibitor prevented SNAP from activating K⁺ channel opening.
current (Fig. 10). Taken together, it is strongly suggested that NO-cGMP-PKG pathway activates K⁺ current in the presumed BFC neurons.

It has recently been reported that NO exerted the facilitatory and inhibitory effects on the activity of delayed rectifier K⁺ channels in neocortical neurons through activation of NO-cGMP and redox pathways, respectively (Han et al. 2006). Because delayed rectifier K⁺ channels are closed at the resting MP, NO would not cause any changes in the resting MP in neocortical neurons, unlike in the presumed BFC neurons, which were found to be modulated by NO through the same two mechanisms in the present study. To the best of our knowledge, only one study reported that NO application induced membrane hyperpolarization in noradrenergic neurons in the locus coeruleus, although its ionic mechanism remained unknown (Xu et al. 1998). It is noteworthy that neurons in the locus coeruleus were later found to express TASK1 channels (Sirois et al. 2000), although NO is not yet proved to activate TASK1 channels in this neuron.

Expression of leak K⁺ channels in presumed BFC neurons

Apart from the responsibility for evoking the slow hyperpolarization in response to NO-cGMP pathway activation, there are three candidates of tonically active K⁺ channels in BFC neurons: Kᵢᵥ (Stanfield et al. 1985), Kᵢₐ₅ (Allen and Brown 2004), and leak K⁺ channels. Because the hyperpolarizing action of 0.1 mM 8-Br-cGMP was not antagonized by 0.1 mM TLB (Fig. 5B), Kᵢₐ₅ channels are not likely to be involved in the slow hyperpolarization. Although Kᵢᵥ currents or channels are well known to be sensitive to Ba²⁺, the outward current in comparison with the inward current flowing through Kᵢᵥ channels was much less sensitive to Ba²⁺ at the concentration of 50–100 μM in cultured BFC neurons (Yamaguchi et al. 1990), as in other types of Kᵢᵥ channels (Hagiwara 1983; Nichols and Lopatin 1997). By contrast, the NO-cGMP-induced hyperpolarization was completely sensitive to 50–100 μM Ba²⁺ in the present study (Figs. 5 and 6). Furthermore, depending on changes in [K⁺], 8-Br-cGMP-induced current displayed GHK-type outward rectification in spite of the presence of free Mg²⁺ (≥0.5 mM) in Mod- or Std-InS (Fig. 4), which is far more than enough for Kᵢᵥ channels to display the inward rectification (<20 μM) (Hille 2001). Thus the Ba²⁺ sensitivity and Mg²⁺ independence of the outward K⁺ current concerned are not compatible with Kᵢᵥ channels. Therefore leak K⁺ channels, which have been defined as displaying a GHK-type rectification of I-V relationship without voltage and time dependency (Goldstein et al. 2001; Patel and Honore 2001), are most likely to be involved in generating the long-lasting hyperpolarization in BFC neurons in response to SNAP or 8-Br-cGMP application. Our preliminary data indicated that the leak K⁺ current responsible for the SNAP-induced long-lasting hyperpolarization was sensitive to acidification of pH from 7.3 to 6.3 (Toyoda et al. 2007).

Manipulation of intracellular ATP

We hypothesized that the [ATP]ᵢ is not necessarily dominated by the [ATP] in the internal solution of patch pipettes. It is reported that no difference was observed in the resting MP between the two groups of intact cortical pyramidal neurons dialyzed with 2 and 5 mM ATP internal solutions, whereas there was a clear difference in the resting MP between the two groups of mechanically injured pyramidal neurons dialyzed differently with 2 and 5 mM ATP internal solutions (Tavalin et al. 1997). This difference in the resting MP was attributed to the difference in the activity of Na⁺–K⁺ pump due to the differential internal ATP concentration (Tavalin et al. 1997). This may be because there are numerous mitochondria in the cytoplasm (Bereiter-Hahn 1990; Kovacs et al. 2005), which may be especially abundant at sites where ATP demands are high, such as a site close to active transporters (Alberts et al. 2002; Bereiter-Hahn 1990). If this is the case, [ATP], affecting active transporters would be effectively dominated by the [ATP] in the internal solution only when the ATP production in mitochondria is largely impaired by the mechanical injury or NO. Then, in response to SNAP application in neurons dialyzed with the Mod-InS (0.5 mM ATP, 22 mM Na⁺), a larger [ATP], decrease would occur under the condition of the increased baseline activity of Na⁺–K⁺ pump, in comparison with the case in neurons dialyzed with Std-InS (2 mM ATP, 15 mM Na⁺). Indeed the SNAP-induced depolarization obtained using Mod-InS was significantly larger in amplitude than that obtained with the Std-InS (Fig. 2Db), whereas there was no significant difference in the resting MP between type II neurons dialyzed with Std-InS and those dialyzed with Mod-InS.

On the other hand, these presumed decreases in [ATP], ranging between 0.5 and 2 mM following SNAP application would not cause marked additional activation of Kᵢₐ₅ channels, because Kᵢₐ₅ channels are already almost fully run-up as Kᵢᵥ is ~6 mM in the BFC neuron due to the presence of PIP2 that reduces the binding affinity of ATP to Kᵢₐ₅ channels (Allen and Brown 2004).

Susceptibility of BFC neurons to NO toxicity revealed by membrane depolarization

It has been reported in a variety of cells that NO activates Na⁺–K⁺ pump (Gupta et al. 1994, 1995; Nathanson et al. 1995; William et al. 2005). However, it is also reported in cortical neurons that NO may cause membrane depolarization through the downregulation of mitochondrial ATP production (Tavalin et al. 1997). The present study clearly showed the involvement of Na⁺–K⁺ pump inhibition in generating the membrane depolarization by NO (Fig. 9). It is well established that NO and peroxynitrite (ONOO⁻) causes reversible and irreversible impairments of mitochondrial respiration involved in ATP production, respectively (Lizasoain et al. 1996; Radi et al. 1994; Zhang et al. 1994). SNAP-induced depolarization became irreversible during repetitive application of SNAP, presumably due to the slow production of peroxynitrite by the reaction of NO with superoxide (O₂⁻). Cholinergic neurons in the basal forebrain are highly susceptible to NO. It is reported that superoxide dismutase (SOD) mRNA levels in BFC neurons were relatively lower than those in cholinergic neurons in the laterodorsal tegmental nucleus (LDTN) and the pedunculopontine nucleus (PPN) (Kent et al. 1999). In agreement with this low SOD level, BFC neurons have been reported to be 300 times more susceptible to NO toxicity than those in LDTN/PPN (Fass et al. 2000). In this regard, it is of great interest that NO-induced depolarization became irreversible after repetitive application of SNAP. This
irreversible depolarization might be responsible for and/or indication of subsequent neuronal death, presumably because the irreversible membrane depolarization would increase [Ca^{2+}], which in turn causes various effects such as activation of calpain (Nixon 2000; Saito et al. 1993) and facilitation of amyloid-β accumulation (Isaacs et al. 2006; Pierrot et al. 2006), leading to a cell death.

Putative linkage between learning/memory and dementia

NO application (Pitsikas et al. 2005) or NO-sGC activation (Chien et al. 2005) causes memory improvement, whereas NOS inhibition (Kopf et al. 2001) or neuronal NOS knockout (Weitzdoerfer et al. 2004) causes memory impairment. Thus it has generally been believed that NO enhances learning and memory. Apparently inconsistent with these views, NO-induced hyperpolarization in the presumed BFC neurons by activating leak K⁺ channels in the present study. However, this hyperpolarizing effect would increase the signal/noise (S/N) ratio by depressing the background noise. This is similar to the GABAAergic inhibitory action triggered by cholinergic or noradrenergic inputs, which could increase the cortical S/N ratio (Guo 2003). Not only synaptically plastic but also increases in S/N ratio in the basal forebrain may be involved in memory and learning.

In conclusion, BFC neurons may need NO to open leak K⁺ channels for the better performance of memory and learning, at the risk of their high susceptibility to NO toxicity (Fass et al. 2000; Kent et al. 1999). Crucial roles of NO in the AD pathogenic mechanism are beginning to be recognized (Law et al. 2001). Our present study would provide a cellular basis for the putative link between learning/memory and dementia.

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