Nitric oxide directly activates calcium-activated potassium channels from rat brain reconstituted into planar lipid bilayer

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Received 30 July 1997; revised version received 4 September 1997

Abstract Using the planar lipid bilayer technique, we tested whether NO directly activates calcium-activated potassium (Maxi-K) channels isolated from rat brain. We used streptozotocin (STZ) as NO donor, and the NO release was controlled with light. In the presence of 100–800 μM STZ, the Maxi-K channel activity increased up to 3-fold within several tens of seconds after the light was on, and reversed to the control level several minutes after shutting off the light. Similar activation was observed with other NO donors such as *S*-nitroso-*N*-acetylpenicillamine and sodium nitroprusside. The degree of activity increase was dependent upon the initial open probability (P_{init}). When the P_{init} was lower, the activity increase was greater. These results demonstrate that NO can directly affect the Maxi-K channel activity, and suggest that the Maxi-K channel might be one of the physiological targets of NO in brain.

Key words: Calcium-activated potassium channel; Nitric oxide; Lipid bilayer; Streptozotocin

1. Introduction

Nitric oxide (NO) is one of the biological messengers. It is synthesized in vivo by NO synthase (NOS) from L-arginine, and has many roles in various tissues including cytotoxic effects of activated macrophages [1], cytokine- and endotoxin-evoked decreases in cardiac contractility [2], and the regulation of vascular tone [3]. NO has also been suggested to be a neurotransmitter in brain [4]. The signalling pathway of NO seems very complicated [5,6]. One of the mechanisms for NO action is to activate guanylate cyclase, which increases the concentration of cGMP, and cGMP activates various enzymes either directly or indirectly via the action of cGMP-dependent protein kinase. Some channels have been identified as the target of this kinase [7,8].

Direct action of NO on many channel proteins has also been reported [9–12]. Bolotina et al. [10] observed that NO applied to the excised patches of vascular smooth muscle cells activated calcium-dependent potassium (Maxi-K) channels, and concluded that NO directly affects the channel activities. However, it is still arguable that the Maxi-K channel is activated indirectly since there could be some components in the patch membrane which might affect the channel activity through NO.

We investigated the question whether NO directly affects the activities of the Maxi-K channels using the lipid bilayer reconstitution method. This method has some advantages over the patch clamping method. Firstly, since the method utilizes artificial lipid bilayer, it provides a relatively clean environment, minimizing the possibility of NO affecting the channel via other proteins in the bilayer. Secondly, the chances of obtaining single channel activity are far greater than with patch clamping. Through the kinetic analysis of the single channel recording, it is then possible to suggest a mechanism of NO action at the molecular level.

In this paper, we studied the Maxi-K channel isolated from rat brain, and found that NO directly activates the Maxi-K channels reconstituted into the lipid bilayer. In brain, the Maxi-K channel current participates in action potential repolarization and fast after-hyperpolarization, thereby affecting the amount of neurotransmitter release. The results from this paper strongly suggest that the Maxi-K channel is one of the physiological targets of NO in brain.

2. Materials and methods

2.1. Materials

Phosphatidylethanolamine (PE) and phosphatidylserine (PS) were purchased from Avanti Polar Lipid (Alabaster, AL), *S*-nitroso-*N*-acetylpenicillamine (SNAP) from LC Laboratories (Woburn, MA), and sodium nitroprusside (SNP) from Alexis Corporation (Lüfelfingen, Switzerland). Streptozotocin (STZ) and all other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Preparation of rat brain plasma membrane vesicles

Rat plasma membrane vesicles were prepared as described [13]. Briefly, rat brain cortex was excised and immediately homogenized in an ice-cold isotonic sucrose buffer (10 ml/g tissue) containing 0.25 M sucrose, 2.5 mM KCl, 0.1 mM EGTA, 0.1 mM dithiothreitol (DTT), and 20 mM HEPES (pH 7.2). The homogenate was centrifuged at $1000\times g$ 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 (pH 8.2), and allowed to stand on ice for 20 min. The membrane was then re-homogenized, and centrifuged at $100\,000\times g$. The pellet was layered under a discontinuous step gradient of Percoll (25, 18, 10, and 0%, v/v). The gradient was centrifuged at $40\,000\times g$ for 2 min, and the membrane fraction at the 0–10% interface was collected. Percoll was removed by centrifugation at $100\,000\times g$ for 50 min. The membrane was stored at −70°C until use.

2.3. Single channel recording using planar lipid bilayer reconstitution

We obtained single channel records by incorporating these plasma membrane vesicles into planar lipid bilayers. Two compartments of the recording chamber were filled with 150 mM KCl, 1 mM EGTA, 1.05 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.2) for the cytosolic (or cis) solution, and 5 mM KCl, 0.1 mM EGTA, 10 mM HEPES (pH 7.2) for the extracellular (or trans) solution. The bilayer with a capacitance of >200 pF was made around a 250 μM diameter hole by painting with a mixture of PE and PS (3:1) dissolved in *n*-decane (20 mg/ml) [13].

Maxi-K channels were identified by two criteria. First, when reconstituted into lipid bilayer, a Maxi-K channel has a single channel

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PII S0014-5793(97)01144-7

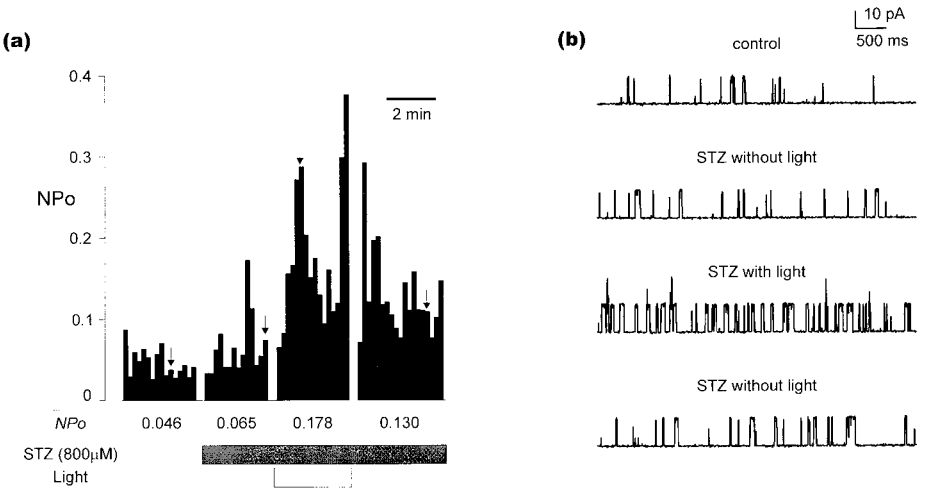

Fig. 1. STZ increases the open probability of the Maxi-K channel upon illumination. a: The open probabilities, NP_o, of the Maxi-K channels in the lipid bilayer were sequentially plotted. In this case, the number of channels in the bilayer was three. Each box represents NP_o of 5-s recordings. They are grouped into four different categories according to the presence and absence of 800 μM STZ (gray box) and light (open box). $\overline{NP_o}$ is the average of NP_os within each group. b: Plotted are representative recordings of 5-s periods as indicated by arrows in (a). $[Ca^{2+}]_{cis}$ was 1.50 μM and the holding potential was +20 mV.

conductance of around 220 pS using K^+ as a charge carrier [13]. Second, its activity is dependent upon the calcium concentration in the cis ($[Ca^{2+}]_{cis}$) [13,14]. Initially, the $[Ca^{2+}]_{cis}$ was set at 56.54 μM. When a Maxi-K channel activity is observed, we routinely lower the $[Ca^{2+}]_{cis}$ and the transmembrane osmotic gradient by increasing the $[K^+]_{trans}$ in order to prevent further fusion and lower the open probability.

The current was amplified with an Axopatch 200A amplifier (Axon Instruments, Inc., Foster City, CA), 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 a pClamp 6 software (Axon Instruments, Inc.). The activity of more than one Maxi-K channel was described by NP_o which is defined as

$$\sum_{k=1}^{n} kP_k$$

where P_k is the probability of a total of k channels being open. NP_o was calculated using the software 'NPo' developed by Drs. Logothetis and Sui at Mount Sinai School of Medicine.

2.4. NO application using NO donors

STZ is an antibiotic, and its rate of NO release increases in the presence of white light [15]. We performed all experiments in the dark, and the flash light was illuminated when needed. Specifically, a fiber-optic light guide (Olympus, Japan) with a 150 W halogen bulb was placed 1 cm from the camber. The flash light was always at its maximum intensity. Other NO donors (SNAP and SNP) were applied to the solution at around their effective concentration.

3. Results and discussion

Fig. 1a is a sequential plot of the open probability (NP_o) of the brain Maxi-K channels in the lipid bilayer. NP_o was calculated for every 5-s interval. While adding 800 μM STZ to the cis chamber in the dark did not appear to affect the Maxi-K channel activity significantly, the open probability of the channel increased significantly from 0.046 to 0.178 (287% increase, $P < 0.001$ with Student's t-test) upon illumination. Representative recordings of 5-s period as indicated by arrows in Fig. 1a are plotted in Fig. 1b. Similar activation was observed in 10 other experiments, summarized in Table 1. In these experiments, we did not observe any significant changes in the single channel conductance after STZ application.

In Fig. 1a, the maximum increase was observed as early as 25 s after the light was turned on. However, the channel activity did not fully return to the control level and remained increased for 2 min after shutting off the light. Fig. 2 is an open probability (P_o) plot of single channel recordings from other experiment. It shows that the NO effect reverses 5 min after the termination of the light.

In these experiments, there is a possibility that the by-product of STZ, not NO, is responsible for the activation. To rule out this possibility, we performed the following experiments. First, a 'non-active' STZ solution was prepared by providing illumination to the STZ solution for 2 h before application to the cis chamber. Both with and without illumination, it failed to induce an increase of the channel activities (3 out of 3, data

Table 1
Summary of effects by various NO donors on the Maxi-K channel activities

Drug	Conc. (mM)	Open probability		Increase (%)
		before	after	
STZ	0.11	0.204	0.574	181.37
	0.27	0.050	0.158	219.35
	0.54	0.127	0.173	36.22
	0.54	0.303	0.341	12.54
	0.54	0.065	0.193	195.11
	0.54	0.201	0.225	11.94
	0.54	0.060	0.314	423.33
	0.54	0.341	0.733	114.96
	0.81	0.032	0.134	321.77
	0.81	0.076	0.140	85.19
			mean ± S.D.	160.18 ± 136.36
SNAP	0.19	0.010	0.023	136.08
	0.19	0.096	0.164	70.30
	0.19	0.287	0.401	39.72
			mean ± S.D.	82.03 ± 49.24
SNP	1.00	0.049	0.077	58.20
	1.00	0.072	0.108	50.21
	1.00	0.019	0.028	49.20
			mean ± S.D.	52.53 ± 4.93

not shown). However, when 'fresh' STZ was further added to the chamber, channel activation was observed upon illumination. Second, we tried other kinds of NO donors such as SNAP and SNP, and observed similar activation, summarized in Table 1. These results strongly support that NO is responsible for the activation of the Maxi-K channels.

As summarized in Table 1, the degree of activity increase by STZ ranges widely from 11.94% to 423.33%. This variation does not correlate with the concentration of applied STZ. However, when each value of the activity increase is plotted against its initial P_o (P_{init}) as in Fig. 3, there appears to be a correlation between the P_{init} and the degree of activity increase. The activity increase is greater when the P_{init} is lower, and vice versa. A similar tendency was also observed in the experiments using SNAP (data not shown). One possible explanation for this tendency is that NO might shift the P_o vs. calcium curve to the left as in the inset of Fig. 3. Therefore, the degree of the activity increase (ΔP) could depend on the P_{init} as we observed in the experiments.

In this paper, we demonstrated that NO directly increases the activity of the rat brain Maxi-K channels reconstituted into the lipid bilayer. When reconstituted, proteins from microsome will reside in an infinitely diluted environment of phospholipid. Therefore, it is very unlikely that some other components separate from the channel mediate NO effects [16]. However, there is a possibility that some components are closely associated with the Maxi-K channel protein and mediate NO effects.

The onset and offset of the activation by NO have different time courses. Whereas the onset of NO activation was observed within 30 s, the recovery to the P_{init} took as long as 5 min. One possible explanation is that the steady-state level of released NO is far above the effective concentration for the activation. When the light is turned on, the concentration begins to rise and soon reaches the activation range. However, even though the NO level falls after the termination of light, it will still be high enough for the activation. In Fig. 2, there is indeed a plateau lasting about 2 min after the termination of light. Considering that the half-life of NO in aqueous solution is around a few tens of seconds [17], the falling phase after the plateau is much too slow to be explained by the decrease of

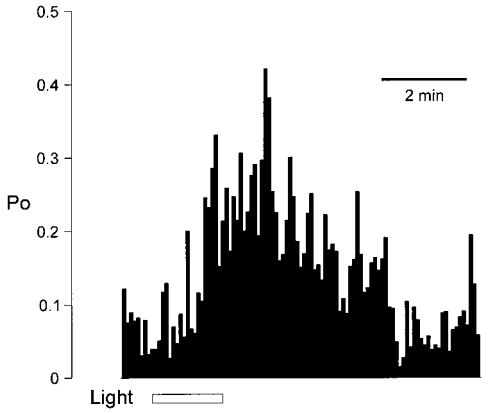

Fig. 2. Time course of recovery from STZ effect. The open probability of a single channel activity was plotted as in Fig. 1. Each box represents the P_o of a 5-s recording. The light was turned on during the period depicted as an open box. $[Ca^{2+}]_{cis}$ was 2.29 μM and the holding potential was +10 mV.

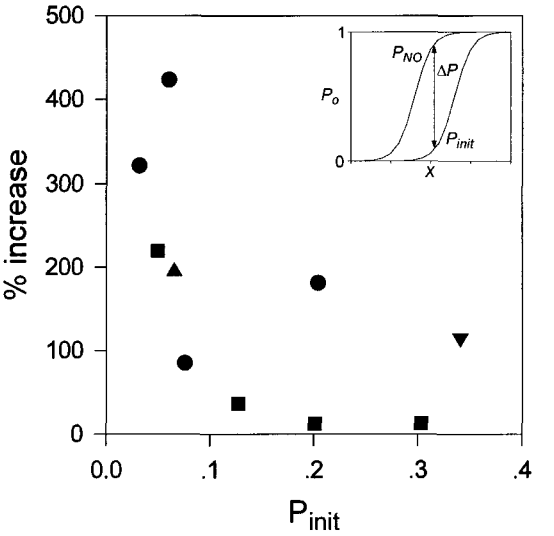

Fig. 3. The degree of the activity increase by NO is dependent upon the initial P_o. Each value of the activity increase from STZ experiments was plotted against its P_{init}. Data points were marked according to the number of channels in the bilayer: $n=1$ (●), $n=2$ (■), $n=3$ (▲), and $n=4$ (▼). In the inset two hypothetical P_o curves are plotted before (P_{init}) and after (P_{NO}) NO application. We assumed that the P_o is a function of some independent variable such as calcium, x: $P_{init}=(x^n)/(x^n+a^n)$, where n is a Hill coefficient and a is an effective concentration, and that the P_o vs. calcium curve shifts to the left after NO application with no change in n. ΔP corresponds to the degree of activity increase.

NO concentration. It is believed that one of the possible mechanisms for the direct NO action is through nitrosylation on cysteine residues of target proteins [18,19]. Possibly, the deactivation process, denitrosylation, takes place at a much lower rate [12].

Negative results of NO effects on the Maxi-K channels have been reported. Koh et al. [20] investigated the NO effects on the Maxi-K channels from canine colonic smooth muscle and found that 10 μM of SNAP increases the activity of the channels in cell-attached patches but not the channels in inside-out patches. Haburcák et al. [21] also reported that the Maxi-K channels from cultured human endothelial cells are not directly activated by NO. One possible reason for this discrepancy is the type and concentration of NO donors. For example, the SNAP concentration used by Koh et al. [20] was 20-fold lower than in our experiments. The other possibility is that the NO effects on the Maxi-K channels from various tissues may be different. Cloning of the Maxi-K channel from mouse and human revealed that the Maxi-K channel α-subunits have many splicing variants [22,23], suggesting that a number of subtypes of the Maxi-K channel may be expressed in different tissues and respond differently to NO.

The Maxi-K channels are widely distributed in brain [24]. Physiological roles of the Maxi-K channel in brain include action potential repolarization and fast after-hyperpolarization, thereby affecting the amount of neurotransmitter release [25]. The channel activation by NO as presented in this paper strongly suggests that the Maxi-K channel is one of the physiologic targets of NO in brain.

Acknowledgements: We thank Dr. Chul-Seung Park for critical comments on the manuscript. This work was supported by the 1996 year program of the Inha University research funding (to J.H.S.) and the

Academic Research Fund of the Ministry of Education, Republic of South Korea (to S.C.).

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