Reaction of nitric oxide with superoxide inhibits basolateral K⁺ channels in the rat CCD

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Lu, Ming, and Wen-Hui Wang. Reaction of nitric oxide with superoxide inhibits basolateral K⁺ channels in the rat CCD. Am. J. Physiol. 275 (Cell Physiol. 44): C309–C316, 1998.—We previously demonstrated that nitric oxide (NO) stimulates the basolateral small-conductance K⁺ channel (SK) via a cGMP-dependent pathway [M. Lu and W. H. Wang. Am. J. Physiol. 270 (Cell Physiol. 39): C1336–C1342, 1996]. Because NO at high concentration has been shown to react with superoxide (O₂⁻) to form peroxynitrite (ONOO⁻) [W. A. Pryor and G. L. Squadrato. Am. J. Physiol. 268 (Lung Cell. Mol. Physiol. 12): L699–L722, 1995 and M. S. Wolin. Microcirculation 3: 1–17, 1996], we extended our study to examine, using patch-clamp technique, the effect of high concentrations of NO on SK in cortical collecting duct (CCD) of rat kidney. Addition of NO donors [100–200 µM S-nitroso-N-acetyl-penicillamine (SNAP) or sodium nitroprusside (SNP)] reduced channel activity, defined as the product of channel number and open probability, to 15 and 25% of the control value, respectively. The inhibitory effect of NO was completely abolished in the presence of 10 mM Tiron, an intracellular scavenger of O₂⁻. NO donors, 10 µM SNAP or SNP, which stimulate channel activity under control conditions, can also inhibit SK in the presence of an O₂⁻ donor, pyrogallol, or in the presence of an inhibitor of superoxide dismutase, diethyldithiocarbamic acid. The inhibitory effect of NO is still observed in the presence of exogenous cGMP, suggesting that the NO-induced inhibition is not the result of decreased cGMP production. We conclude that the inhibitory effect of NO on channel activity results from an interaction between NO and O₂⁻.

peroxynitrite; potassium transport; guanosine 3',5'-cyclic monophosphate; patch clamp; cortical collecting duct

The cortical collecting duct (CCD) plays an important role in K⁺ secretion and hormone-regulated Na⁺ reabsorption (27, 30). The basolateral K⁺ channels in the CCD participate in generating the cell membrane potential and are involved in K⁺ recycling across the basolateral membrane. Because Na⁺ reabsorption and K⁺ secretion are electrogenic in the CCD, alteration in cell membrane potential is expected to have an effect on Na⁺ and K⁺ transport. Inhibition of basolateral K⁺ conductance by Ba²⁺ reduced Na⁺ reabsorption in the CCD, an effect presumably related to the Ba²⁺-induced depolarization (28). At least three types of K⁺ channels, small conductance (28 pS), intermediate conductance (85 pS), and large conductance (145 pS), have been identified in the basolateral membrane of the CCD (11, 33). However, a previous study showed that the small-conductance K⁺ channel (SK) is involved in determining the cell membrane potential (17).

The constitutive nitric oxide synthase (NOS) has been identified in the kidney (32) and plays an important role in regulation of kidney function (13). Reaction of nitric oxide (NO) in a variety of cells includes stimulation of guanylate cyclase and interaction with superoxide (O₂⁻) (3, 13, 35). We previously found that NO stimulates the basolateral SK in the rat CCD (17) and is involved in protein kinase C-induced activation of the SK (18). The effect of NO on the SK is mediated by a cGMP-dependent pathway, since a cGMP analog mimics the effect of NO (17). In addition, activation of a cGMP-dependent pathway has been shown to hyperpolarize the cell membrane in the CCD (12). On the other hand, reaction of NO with O₂⁻ to form peroxynitrite (ONOO⁻) has been shown to oxidize a variety of molecules such as thiols (24, 25, 29). Redox mechanisms have been found to modulate several ion channels, including L-type Ca²⁺ channel (6) and Ca²⁺-dependent K⁺ channel (4). In the present study we examine the effect of NO and O₂⁻ on the SK in the basolateral membrane of the CCD.

**METHODS**

Preparation of rat CCD. The CCD was isolated from kidneys of pathogen-free Sprague-Dawley rats (Taconic, Germantown, NY) and transferred onto a 5 × 5-mm cover glass coated with Cell-Tak (Collaborative Research, Bedford, MA) to immobilize the tubules. The cover glass was placed in a chamber (1,000 µl) mounted on an inverted microscope (Nikon), and the tubules were superfused with HEPES-buffered NaCl solution. The method for exposing the basolateral membrane was previously described (33). The temperature of the chamber was maintained at 37 ± 1°C by circulating warm water surrounding the chamber.

Patch-clamp technique. Patch-clamp electrodes were pulled with a vertical pipette puller (model 700C, David Kopf Instruments, Tujunga, CA) using glass capillaries (Degan, Minneapolis, MN) and had resistances of 4–6 MΩ when filled with 140 mM KCl. An Axon 200A patch-clamp amplifier was used to record channel activity. The output of the amplifier was low-pass filtered at 1 kHz using an eight-pole Bessel filter (902LPF, Frequency Devices, Haverhill, MA) and was digitized at a sampling rate of 44 kHz using a modified Sony PCM-501ES pulse code modulator and stored on videotape (Sony SL-2700). For analysis, the data stored on the tape were replayed and collected by an IBM-compatible 486 computer (Gateway 2000) at a rate of 5 kHz and analyzed using the pCLAMP software system 6.02 (Axon Instruments, Burlingame, CA).

We used NP₀ as an index of channel activity and made no efforts to examine whether the alteration of channel activity was due to a change in channel number (N) or in channel open probability (Pₒ). The NP₀ was calculated from data samples of 1-min duration at the steady state. However, if NP₀ decreased to zero for 20–30 s, we changed the bath solution immediately after a 1-min duration.
Table 1. Effect of 10 µM SNAP on intracellular cGMP level

<table>
<thead>
<tr>
<th>Control</th>
<th>SNAP</th>
<th>Increase, %</th>
<th>n</th>
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<tr>
<td>44 ± 15</td>
<td>92 ± 20*</td>
<td>108</td>
<td>8</td>
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Values are means ± SE of cGMP concentrations measured using ELISA; n, no. of determinations. SNAP, S-nitroso-N-acetyl-penicillamine. *Significantly different from control value.

...to increase the P_o and restore channel activity. Accordingly, only 30-s data were collected for calculation of NP_o. We used the following equation to obtain NP_o

\[ NP_o = \sum (t_1 + t_2 + \cdots + t_n) \]

where the maximum number of superpositions of current level seen in the patch is taken as N, and t is the fractional open time spent at each of the observed current levels (1 ton).

cGMP and protein concentration assay. The CCDS (total length of 10 mm) were collected and incubated in a Ringer solution (300 µl) in the presence of 1 mM IBMX at 37°C for 15 min. After addition of either 10 µM S-nitroso-N-acetylpenicillamine (SNAP) or vehicle solution (DMSO) to the tubule suspension for 2 min at 37°C, experiments were terminated by addition of 0.7 ml ice-cold ethanol. The sample was frozen in liquid nitrogen and dried in a speed vacuum concentrator. The residues were resuspended in 100 µl of phosphate buffer and acetylated. cGMP content was measured with specific ELISA (Cayman Chemical). The Pierce protein assay reagent was used to measure protein concentration. This assay is based on the competition between free cGMP and a cGMP tracer (cGMP linked to an acetylcholine-ester molecule) for a limited number of cGMP-specific rabbit antiserum binding sites.

Experimental solution. The bath solution was composed of (in mM) 140 NaCl, 5 KCl, 1.8 CaCl_2, 1.8 MgCl_2, and 10 HEPES (pH 7.40). The composition of the pipette solution was (in mM) 140 KCl, 1.8 MgCl_2, and 10 HEPES (pH 7.4). Sodium nitroprusside (SNP), pyrogallol, 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron), diethylthiocarbamic acid, and 8-bromo-cGMP (8-BrcGMP) were purchased from Sigma (St. Louis, MO). SNAP was obtained from Calbiochem (La Jolla, CA) and was dissolved in pure ethanol. The final concentration of ethanol in the bath was 0.1% and had no effect on channel activity. The chemicals were added directly to the bath to reach the final concentration. After addition of chemicals, experiments were carried out in a standing bath. The response of the channel to a given agent is the same in experiments performed either in running bath or in standing bath.

Statistics. Data are shown as means ± SE. Paired Student’s t-tests were used to determine the significance of differences between the control and experimental periods. Statistical significance was taken as P < 0.05.

RESULTS

We confirmed the previous finding that addition of 10 µM SNAP increased channel activity (17). The notion that the effect of 10 µM SNAP is mediated by a cGMP-dependent pathway is further supported by experiments in which 10 µM SNAP significantly increased cGMP concentration from 44 to 92 fM/µg (Table 1). In addition to stimulation of soluble guanylate cyclase (13), it has been suggested that increasing NO production can enhance the formation of OONO⁻ by facilitating the interaction between NO and O₂⁻ (24, 35). OONO⁻ has been shown to modulate several types of ion channels (4–6). Therefore, we extended our study to investigate the effects of a high concentration of NO on the SK. Figure 1 is a representative experiment recorded from a cell-attached patch showing the effect of 100–200 µM SNAP on channel activity. It is apparent that application of 100 µM SNAP reversibly inhibited the SK, and channel activity in nine such experiments was reduced by 85 ± 10%. Because several NO donors have been shown to directly modify channel activity (4, 5, 10), we examined the effect of 100 µM SNAP on the SK in inside-out patches (Fig. 2). From inspection of Fig. 2, it is clearly demonstrated that 100 µM SNAP has no direct effect on the SK in inside-out patches within 100–120 s. Because the activity of the SK in inside-out patches decreased progressively, it is beyond the technical limitation to maintain channel activity in a steady state for >5 min. Thus we are unable to...
examine the effect of SNAP on channel activity in inside-out patches for >2 min without channel rundown. However, it is believed that the direct effect of NO donors should occur within 1 min. Thus it is safe to conclude that SNAP has no direct effect on the SK.

To exclude the possibility that the high concentrations of SNAP decreased channel activity by a mechanism other than release of NO, we examined the effect of another NO donor, SNP. Figure 3 shows that addition of 100–200 µM SNP also blocked channel activity in cell-attached patches. The inhibitory effect averaged 75 ± 10% (n = 8) and was fully reversible. Thus the results suggested a biphasic effect of NO on the SK: low concentrations of NO stimulate the SK, whereas high concentrations of NO inhibit the SK. This view has been further tested by examining the dose-response curve of the SK to SNAP in the presence of 100 µM nitro-L-arginine methyl ester (L-NAME) to block the endogenous NO production. Figure 4 summarizes the results from such experiments. It is apparent that application of 10 mM Tiron has no significant effect on channel activity (n = 8). However, the inhibitory effects of 100–200 µM SNP were completely abolished in the presence of Tiron. Furthermore, in the presence of Tiron, addition of 100–200 µM SNP significantly increased channel activity by 30 ± 7% (Fig. 5).

These results strongly suggest that the inhibitory effect of NO is the result of interaction between O₂ and NO. Because the formation of OONO⁻ can be enhanced by increasing either NO or O₂ concentration (35), we examined the effect of NO in the presence of an O₂ donor, pyrogallol (19). Addition of 50–100 µM pyrogallol decreased channel activity by 45 ± 8%, and further application of 10 µM SNAP, which increased channel activity under control conditions, resulted in a complete inhibition of channel activity (Fig. 6). The effect of pyrogallol and 10 µM SNAP was partially reversible (Fig. 6). Further support that OONO⁻ mediates the inhibitory effect of NO was obtained from experiments in which the channel activity was inhibited by addition of 10 µM SNAP in the presence of 1 mM diethyldithiocarbamic acid to block superoxide dismutase. Figure 7 is a typical channel recording made in a cell-attached patch showing that, after blockade of superoxide dismutase, addition of 10 µM SNAP reduced channel activity by 95 ± 10% (n = 6) and that the effect was partially reversed by washout.

Because the activity of SK is stimulated by a cGMP-dependent pathway, we next examined whether the
inhibitory effect of NO resulted from an inhibition of guanylate cyclase and decreased cGMP production. Figure 8 is a channel recording made in a cell-attached patch showing the effect of SNAP and pyrogallol in the presence of a cGMP analog, which has been shown to stimulate the SK (17). Addition of 10 µM SNAP and pyrogallol inhibited channel activity. However, application of 100 µM 8-Br-cGMP failed to restore the channel activity, suggesting that the inhibitory effect of NO is not the result of a decrease in cGMP production ($n = 6$).

**DISCUSSION**

At least three types of $\text{K}^+$ channels, large-conductance channels (145 pS), intermediate-conductance channels (85 pS), and SK (28 pS), have been identified in the basolateral membrane of the CCD (11, 33). Several lines of evidence indicate that the biophysical properties and regulation of the basolateral SK are different from those in the apical membrane. 1) The basolateral SK is not sensitive to ATP, whereas the apical SK is inhibited by ATP. 2) NO and cGMP have no effect on the apical SK in either cell-attached or inside-out patches (unpublished observations), whereas they activate the basolateral SK. 3) Protein kinase A stimulates the apical SK in both cell-attached and inside-out patches but has no effect on basolateral SK in inside-out patches (unpublished observations). 4) Previous studies (33) showed that the SK in the basolateral membrane has two closed states (0.5 and 10 ms) and two open states (3 and 35 ms). In contrast, the apical SK has only one open state (20 ms) and one closed state (1 ms). Figure 9 shows two recordings of the basolateral SK and the apical SK, respectively. The recordings were obtained from the same cell, in which the activity of the apical $\text{K}^+$ channels was first recorded and then the lateral membrane was patched. Both patches have the same channel number (confirmed by application of Ba$^{2+}$ after formation of an inside-out patch). It is apparent that the channel kinetics of the two $\text{K}^+$ channels are different.

Hirsch et al. (10–12) have found that the intermediate-conductance $\text{K}^+$ channel has a high open probability and was abundant in their preparation. On the other hand, we have observed that the SK is predominant and could be important for determination of membrane potential. Although we do not know what causes this discrepancy regarding the $\text{K}^+$ channel population, it is possible that the difference may be the result of the animals' dietary conditions. We used CCD from rats on a high-$\text{K}^+$ diet, whereas Hirsch et al. (10–12) obtained tubules from rats on a low-$\text{Na}^+$ diet. Further experiments are required to explore this possibility. Although we have found that the SK is predominant under our experimental conditions, addition of either L-NAME (15) or high concentrations of SNAP (unpublished observations), both maneuvers that inhibited the SK by 80%, did not proportionally decrease cell membrane potential. It is possible that the contribution of the SK to the overall cell membrane potential is limited and depends on the cell membrane potential. We have observed that, if cell membrane potential is higher than $-70 \text{ mV}$, addition of either L-NAME or 100 µM SNAP caused a more than 10-mV depolarization (unpublished observations). On the other hand, when cell membrane potential is below $-50 \text{ mV}$, neither L-NAME nor 100 µM SNAP has a significant effect on cell membrane potential (unpublished observations). This suggests that other basolateral ionic conductances may be major factors under such circumstances. That the basolateral membrane potential is controlled by several types of $\text{K}^+$ channels is important to safeguard the cell function, since both $\text{K}^+$ secretion and $\text{Na}^+$ reabsorption are electrogenic processes and thus are affected by alteration of cell membrane potential (23).

The main finding of this study is that increasing NO concentration or raising $\text{O}_2$ concentration blocks the SK in the CCD. Thus we have shown that NO has a biphasic effect on the SK: low concentrations of NO stimulate the SK via a cGMP-dependent pathway, and high concentrations of NO inhibit the SK via a cGMP-independent pathway. Hirsch et al. (10) have demonstrated that SNP directly stimulated the intermediate-
conductance K⁺ channel. However, we did not observe the direct effect of SNAP/SNP on the SK in inside-out patches. Therefore, it is possible that the effects of NO on the intermediate-conductance K⁺ channel and on the SK are different. NO has been shown to elicit a number of different responses in a variety of cells, including stimulation of guanylate cyclase and reaction with O₂⁻ (13, 35). Three lines of evidence suggest that the inhibitory effect of high NO concentrations on the SK results from an interaction between NO and O₂⁻. First, application of Tiron, which scavenged O₂⁻, abolished the inhibitory effect of NO. Second, addition of 10 µM SNAP, which has been shown to stimulate the SK under control conditions, inhibits the channel activity in the presence of pyrogallol. Finally, inhibition of superoxide dismutase can also reverse the stimulatory effect of NO to an inhibitory effect.

Three types of NOS have been found in the kidney (1, 20, 32). The constitutive NOS has been shown to be expressed in the CCD (32). Our unpublished observations have also confirmed that neuronal NOS is expressed in the CCD. A large body of evidence indicates that NO plays an important role in regulation of renal blood flow (2), renin secretion (9), tubuloglomerular feedback (34), and tubular transport (17, 31). In previous studies, we showed that NO activated the SK and accordingly led to hyperpolarization of the cell membrane (15, 17). The effect of NO is mediated by a
cGMP-dependent process, since addition of 8-BrcGMP mimics the effect of NO. In the present investigation, we observe that a high concentration of NO can also cause inhibition of channel activity.

The present study shows that O₂ production is important for determining the effects of NO on basolateral K⁺ channels. O₂ is produced by mitochondria and several oxidases such as NAD(P)H oxidases (35). Under normal conditions, the O₂ is degraded by superoxide dismutase. However, when NO concentration increases, NO competes with superoxide dismutase and enhances the formation of OONO⁻ (35). Alternatively, when O₂ levels increase under conditions such as ischemia, an excessive amount of O₂ can also react with NO. OONO⁻ is a highly active oxidant that can react with a variety of molecules (3, 24). Although OONO⁻ has been attributed to NO-induced cell injury (14), it could also have a potentially important role in signal transduction mechanisms (29, 35). For instance, several types of ion channels have been shown to contain thiol groups in their structures, and thiol oxidation has been suggested to be an important mechanism through which OONO⁻ can directly affect channel activity (4–6).

Although our data support the notion that the inhibitory effect of high concentrations of NO is the result of an interaction between NO and O₂, the mechanism by which the NO-O₂ product inhibits the SK is not completely understood. It is unlikely that the inhibition is the result of a decrease in cGMP production, since addition of cGMP analogs failed to reverse the inhibitory effect of NO. The second possibility is that OONO⁻ may inhibit the SK by thiol nitrosylation or by oxidation of the SK or its associated proteins. Thiol nitrosylation has been shown to play an important role in the regulation of the Ca²⁺-dependent K⁺ channel in smooth muscle cells (4), L-type Ca²⁺ channels of myocytes (6), and the “minK” channel expressed in Xenopus oocytes (5). Further experiments will be needed to determine whether thiol nitrosylation or oxidation is the mechanism by which high concentrations of NO inhibit the SK.

The experimental results indicate that NO has a dual effect on the activity of the SK: NO increases cGMP production and accordingly activates the SK, and NO reacts with O₂ to form OONO⁻, which blocks the SK. Interestingly, intracellular Ca²⁺ has also been shown to have a dual effect on the basolateral K⁺ channels. We recently demonstrated that raising intracellular Ca²⁺ from 10 to 100 nM activates the SK (16). On the other
hand, Hirsch et al. (10) reported that a high concentration of \( \text{Ca}^{2+} \) (>100 nM) inhibits the intermediate-conductance \( \text{K}^+ \) channel. It is possible that the dual effect of NO might be related to \( \text{Ca}^{2+} \). Further experiments are required to explore this hypothesis.

The dual regulatory mechanisms may have important physiological and pathophysiological relevance. Increasing \( \text{Na}^+ \) influx across the luminal membrane stimulates the \( \text{Na}^+ \text{-K}^+ \text{-ATPase} \) (7). To maintain the activity of the \( \text{Na}^+ \text{-K}^+ \text{-ATPase} \), the basolateral \( \text{K}^+ \) conductance must increase to cope with the turnover rate of \( \text{Na}^+ \text{-K}^+ \text{-ATPase} \). Because an increase in \( \text{Na}^+ \) influx diminishes the driving force of the \( \text{Ca}^{2+}/\text{Na}^+ \) exchanger, intracellular \( \text{Ca}^{2+} \) concentration increases. As the activity of neuronal NOS is stimulated by elevation in intracellular \( \text{Ca}^{2+} \) (21, 22), NO formation is enhanced and accordingly activates the basolateral \( \text{K}^+ \) channels. However, if the increase in intracellular \( \text{Ca}^{2+} \) is continued and sustained, as during ischemia, the concentration of NO could increase further so that NO reacts with \( \text{O}_2 \) to form OONO (36). As OONO- reduces channel activity via oxidation of the basolateral \( \text{K}^+ \) channels or their closely related proteins, it prevents an excessive \( \text{K}^+ \) leakage. In proximal tubules it was demonstrated that cell damage evidenced by DNA breakdown and lactate dehydrogenase leakage during ischemia is closely related to the \( \text{K}^+ \) leakage (26), since inhibition of the basolateral \( \text{K}^+ \) channel partially reversed the ischemia-induced cell damage.

We conclude that NO can have dual effects on the basolateral \( \text{K}^+ \) channel. The stimulatory effect of NO is mediated by a cGMP-dependent mechanism, whereas the inhibitory effect of NO is mediated by interaction between NO and \( \text{O}_2 \).

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