Nitric oxide inhibits lung sodium transport through a cGMP-mediated inhibition of epithelial cation channels

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Jain, Lucky, Xi-Juan Chen, Lou Ann Brown, and Douglas C. Eaton. Nitric oxide inhibits lung sodium transport through a cGMP-mediated inhibition of epithelial cation channels. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L475–L484, 1998.—We used the patch-clamp technique to study the effect of nitric oxide (NO) on a cation channel in rat type II pneumocytes [alveolar type II (AT II) cells]. Single-channel recordings from the apical surface of AT II cells in primary culture showed a predominant cation channel with a conductance of 20.6 ± 1.1 (SE) pS (n = 9 cell-attached patches) and Na⁺-to-K⁺ selectivity of 0.97 ± 0.07 (n = 7 cell-attached patches). An NO donor, S-nitrosoglutathione (GSNO; 100 µM), inhibited the basal cation-channel activity by 43% [open probability (Po); control 0.28 ± 0.05 vs. GSNO 0.16 ± 0.03; P < 0.001; n = 16 cell-attached patches], with no significant change in the conductance. GSNO reduced the Po, by reducing channel mean open and increasing mean closed times. GSNO inhibition was reversed by washout. The inhibitory effect of NO was confirmed by using a second donor of NO, S-nitroso-N-acetylpenicillamine (100 µM; Po, control 0.53 ± 0.05 vs. S-nitroso-N-acetylpenicillamine 0.31 ± 0.04; −42%; P < 0.05; n = 5 cell-attached patches). The GSNO effect was blocked by methylene blue (a blocker of guanylyl cyclase; 100 µM), suggesting a role for cGMP. The permeable analog of cGMP, 8-bromo-cGMP (8-BrcGMP; 1 mM), inhibited the cation channel in a manner similar to GSNO (Po, control 0.38 ± 0.06 vs. 8-BrcGMP 0.09 ± 0.02; P < 0.05; n = 7 cell-attached patches). Pretreatment of cells with 1 µM KT-5823 (a blocker of protein kinase G) abolished the inhibitory effect of GSNO. The NO inhibition of channels was not due to changes in cell viability. Intracellular GSNO was found to be elevated in AT II cells treated with NO (control 13.4 ± 3.6 vs. GSNO 25.4 ± 4.1 fmol/mi; P < 0.05; n = 6 cell-attached patches). We conclude that NO suppresses the activity of an Na⁺-permeant cation channel on the apical surface of AT II cells. This action appears to be mediated by a cGMP-dependent protein kinase.

Guanosine 3′,5′-cyclic monophosphate; nonselective cation channel; alveolar type II cells; S-nitrosoglutathione; sodium channel; single-channel recording; amiloride

DISTAL LUNG EPITHELIUM plays a critical role in maintaining normal alveolar fluid balance (1, 9, 19, 20) and in the adaptation of newborn lungs to air breathing (9, 22). The alveolar walls, lined by type I and type II cells, regulate the fluid to keep the alveoli moist while avoiding excessive buildup of fluid. Transepithelial fluid movement appears largely to be a result of active salt transport, which drives the osmotic movement of water. Recent patch-clamp studies show that Na⁺ channels, located on the apical surface of alveolar type II (AT II) cells, allow vectorial transport of Na⁺ from the alveolar space into the cell, with subsequent extrusion into the interstitium by Na⁺-K⁺-ATPase located on the basolateral membrane (7, 17, 19, 20). The interstitial fluid is then taken up by the flow vessels and lymphatics. The exact mechanism by which the lung epithelial cells control fluid reabsorption and prevent pulmonary edema is not clear, although disruption of this process has been implicated in several disease states.

The use of inhaled nitric oxide (NO) is currently being evaluated in a variety of lung disorders including pulmonary hypertension (25), acute respiratory distress syndrome (27), and high-altitude pulmonary edema (28). Studies done in vitro and in vivo suggest that NO may have an effect on lung fluid dynamics, although the mechanism underlying the NO effect is largely unknown. Because alveolar epithelial cells are exposed to high concentrations of NO during inhaled NO treatment, it is possible that NO may alter lung epithelial Na⁺ and water transport. In the kidney, NO has been shown to inhibit Na⁺ reabsorption by cultured cortical collecting duct cells (29). In the lung, Compeau et al. (4) have shown that endotoxin-stimulated alveolar macrophages impair distal lung epithelial ion transport by inactivating amiloride-sensitive, nonselective cation (NSC) channels. This inhibition was dependent on NO synthesis by the macrophage, suggesting that NO may promote lung edema formation by inhibiting cation channels in the AT II cells. However, other investigators have shown that NO prevents pulmonary edema formation in the isolated rat lung (8) and in humans prone to high-altitude pulmonary edema (28). The reasons for the discrepancy in the findings of these investigators remain to be elucidated.

The objective of this study was to examine the effect of NO on lung epithelial Na⁺ transport and to determine its mechanism of action. We used the patch-clamp technique to study the effect of NO on an amiloride-sensitive, Na⁺-permeable cation channel on the apical surface of rat AT II cells. Our results show that NO inhibits these cation channels (and, presumably, Na⁺ reabsorption) by AT II cells and that this inhibition is mediated by intracellular cGMP acting through a cGMP-dependent protein kinase (PK).

METHODS AND PROCEDURES

Type II pneumocyte isolation and culture. AT II cells were isolated by enzymatic digestion of lung tissue from adult Sprague-Dawley rats (200–250 g) with published techniques (2). Briefly, the rats were anesthetized with pentobarbital sodium and heparinized (100 units/kg). AT II cells were digested by tracheal installation of elastase (0.4 mg/ml). Lung tissue was minced in DNase (1 mg/ml) and filtered sequentially through 100- and 20-µm nylon mesh. Purification was based on the differential adherence of cells to dishes coated with rat IgG. Nonadherent AT II cells were collected, centrifuged, and seeded onto glass coverslips (~2 × 10⁵ cells/cm²) in...
Dulbecco's modified Eagle's medium-F-12 medium containing 5% FCS and antimicrobial agents and supplemented with L-glutamine and Na⁺ bicarbonate. Cells were incubated in 90% air-10% CO₂ and used for patch-clamp studies between 24 and 96 h after harvest. No significant difference in Na⁺-channel activity was observed within this time frame. Cell viability (90%) and purity (95%) associated with this isolation procedure have been validated in our laboratory (2). Solutions and drugs. All solutions were made with deionized water and then passed through a 0.2-µm filter (Gelman Sciences, Bedford, MA) before use. The bath and pipette solutions used in the cell-attached mode contained (in mM) 140 NaCl, 1 MgCl₂, 1 CaCl₂, 5 KCl, and 10 HEPES, pH 7.4 with 2 N NaOH. In the inside-out recordings, the pipette solution was the same, but the bath solution was changed to (in mM) 5 NaCl, 140 KCl, 4 CaCl₂, 5 EGTA, 1 MgCl₂, and 10 HEPES, pH 7.4 with 2 N KOH. The contents of the bathing and pipette solutions were varied as appropriate for specific protocols. All chemicals were obtained from Sigma (St. Louis, MO) except 8-bromo-cGMP (8-BrcGMP) and KT-5823 that were from Calbiochem.

Procedure for single-channel recordings. Patch-clamp experiments were carried out at room temperature. The pipettes were pulled from filamented borosilicate glass capillaries (TW-150, World Precision) with a two-stage vertical puller (Narishige, Tokyo, Japan). The pipettes were coated with Sylgard (Dow Corning) and fire polished (Narishige). The resistance of these pipettes was 5–8 MΩ when filled with pipette solution. We used the cell-attached configuration for most of our studies because, in this configuration, the cytoplasmic constituents remain intact, thus allowing us to study the role of cytoplasmic second messengers in the regulation of ion-channel activity. Inside-out patches were also used to determine the selectivity of the channel and to determine whether the effects of agents were directly on the channel or mediated by a signaling cascade. After formation of a high-resistance seal (~50 GΩ) between the pipette and the cell membrane, channel currents were sampled at 5 kHz with a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA) and filtered at 1 kHz with an eight-pole, low-pass Bessel filter. Data were recorded by a computer with pCLAMP 6 software (Axon Instruments, Foster City, CA). Current-amplitude histograms were made from stable continuously recorded data, and the open and closed current levels were determined from least square fitted Gaussian distributions. We used the product (NP₀) of the number of channels (N) times the open probability (P₀) as a measure of the activity of the channels within a patch. This product could be calculated from the single-channel record without making any assumptions about the total N in a patch or the P₀ of a single channel

\[
NP_0 = \sum_{n=0}^{N} \frac{n t_n}{T}
\]

where T is the total record time, n is the number of channels open, and t₀ is the record time during which n channels are open. Current-amplitude histograms provided the clearest demonstration of multiple current levels. The total N in a patch was estimated by observing the number of peaks in a current-amplitude histogram over the entire duration of the recording period. The P₀ of the channels was calculated with FETCHAN in pCLAMP 6. Single-channel conductance was determined by recording the conductivity of the channels.

To determine whether changes in NP₀ were due to a change in P₀, the mean open (τ₀) and closed (τ₉) times were determined. τ₀ and τ₉ are experimental measures that can provide information as to the average duration in all open and closed states. The mean τ₀ and τ₉ for N observed channels can be calculated from the following equations

\[
\tau_0 = \frac{T \cdot N P_0}{n/2}
\]

\[
\tau_9 = \frac{T \cdot (N - N P_0)}{n/2}
\]

where n is the total number of transitions between states during T and N and NP₀ were calculated as described above. The mean open time calculated in this manner is not the same as the mean open time for a single channel or, for that matter, the mean time in any particular kinetic state of the channel but is rather a reflection of the average open or closed time for all channel states. As such, the mean open and closed times provide a mechanism for distinguishing whether the effects of an experimental maneuver that alters the P₀ of multiple channels are caused by a change in the duration of open intervals or a change in the duration of closed intervals. Determinations of mean open and closed times were made, and interval histograms were generated with locally developed software (18).

Procedure for cGMP estimations. Intracellular cGMP levels were measured in cultured AT II cells with an enzyme immunoassay (Biotra EIA, Amersham, Arlington Heights, IL). Briefly, cells were treated with S-nitrosogluthathione (GSNO), S-nitroso-N-acetylpenicillamine (SNAP), and carbacol for 20 min, and the reaction was stopped by removal of the incubation medium and addition of 65% ice-cold ethyl alcohol. The intracellular cGMP extracted into the supernatant was measured by enzyme immunoassay in duplicate.

Methods for statistical analysis. Statistical analysis for the changes in the P₀ of channels and the biochemical estimations were performed with SPSS for Windows. Statistical significance between two groups was determined by paired or unpaired t-tests as appropriate. When the comparison between more than one group was required, statistical significance was usually determined by one-way ANOVA followed by pairwise comparisons with a Bonferroni t-test to determine significant differences between each group. A P value < 0.05 was regarded as significant. Because of variability in the mean open and closed times of control cells, the effects of GSNO were determined with a Kruskal-Wallis one-way ANOVA on ranks and Dunn's method to determine statistically significant differences from control values.

RESULTS

All cells used for the present experiments had lamellar bodies and other phenotypic features of AT II cells. The predominant Na⁺-permeant channel seen in apical cell-attached patches is shown in Fig. 1A. This channel had a linear current-voltage (I-V) relationship (Fig. 1B) with a conductance of 20.6 ± 1.1 pS (n = 9 cell-attached patches) with 140 mM NaCl in the bath and pipette. No rectification of the I-V curve was observed (Fig. 1B). The pipette potential at which current polarity reversed was estimated to be −37 mV. Because the resting membrane potential of alveolar epithelium has previously been shown to be approximately −30 to −40 mV, the reversal potential appears to be close to 0 mV, which would be expected for an NSC channel (20). Ion selectivity was determined with inside-out recording
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and solutions of varying ionic compositions. The channel had a similar permeability to Na\(^+\) and K\(^+\) (Na\(^+\)-to-K\(^+\) permeability = 0.97 ± 0.07; n = 7 cell-attached patches). The channel P\(_o\) was decreased by amiloride (0.1–1 µM) applied to the extracellular side (i.e., in the micropipette; P\(_o\) control 0.31 ± 0.01 vs. amiloride 0.03 ± 0.01; P < 0.01; n = 7 cell-attached patches). More than one current level was observed in 85.7% of active patches. Thus this channel was very similar in characteristics to the NSC channel described by Orser et al. (24) and Marunaka (17).

NO reduces the P\(_o\) of apical NSC channels. To investigate the acute effects of NO on NSC channel activity, we used two agents that are known to release NO. GSNO (100 µM) was added to the bath. Figure 2A shows the typical time course of NSC channel activity after exposure to GSNO in the bath. With each cell-attached patch acting as its own control, channel activity, measured as P\(_o\), consistently decreased from a mean control value of 0.28 ± 0.05 to a mean treated value of 0.16 ± 0.03 (−43%; P < 0.001; n = 16 cell-attached patches; Fig. 2B). The effect was immediate in onset and was sustained for up to 30 min of recording in stable patches. It was reversible, with a return to control levels after washout (P\(_o\); control 0.16 ± 0.05 vs. 100 µM GSNO 0.062 ± 0.03 vs. washout 0.21 ± 0.06; Fig. 2C). There was no change in the conductance of the channel. An alternate donor of NO, SNAP (100 µM), caused a decrease similar to that of GSNO in the P\(_o\); of the channel (control 0.53 ± 0.05 vs. SNAP 0.31 ± 0.04; −42%; P < 0.05; n = 5 cell-attached patches; Fig. 3). This effect was not seen when GSH, the carrier of NO in GSNO, was used in a 100 µM concentration [P\(_o\); control 0.35 ± 0.11 vs. GSH 0.27 ± 0.07; P = not significant (NS); n = 8 cell-attached patches; Fig. 4]. Taken together, these experiments indicate that NO released by the NO donors suppresses basal NSC channel activity in apical cell-attached patches in AT II cells.

NO reduces mean open time and increases mean closed time of NSC channels. NO could reduce P\(_o\) either by reducing the mean open time of the channels or by increasing the mean closed time. The difficulty in examining changes in channel kinetics is the large variability from cell to cell in the mean open and closed times. Nonetheless, by using each cell as its own control, we were able to make appropriate comparisons. GSNO usually caused a decrease in mean open time (from 157 ± 48.2 ms in untreated cells to 49.3 ± 20.2 ms after GSNO; n = 19 cell-attached patches) and always caused an increase in mean closed time (from 495 ± 334 ms in untreated cells to 2,080 ± 639.2 ms after GSNO; n = 19 cell-attached patches; Fig. 5). For three patches that, based on their amplitude histograms, only had single channels, we generated interval histograms (Fig. 6). Examination of the histograms suggests that there is one predominant open state of the channel (with a mean duration of 10.0 ± 0.861 ms), although there are occasional long openings that might represent a second long open state (with a mean duration of 313 ± 29.5 ms). On the other hand, the closed interval histogram clearly consists of at least two populations of events: short closures (with a mean duration of 2.15 ± 0.507 ms) and long closures (with a mean duration of 381 ± 48.6 ms). To emphasize the effects of GSNO, we superimposed the open and closed interval histograms obtained in the absence and presence of GSNO. An examination of the histograms shows that there are two predominant effects of GSNO. First, GSNO increases the number and causes an almost sevenfold reduction in the mean duration of short open events (from a mean of 10.0 ± 0.861 ms in untreated cells to 1.45 ± 0.583 ms after GSNO), with little effect on the number or duration of long open events (from a mean of 313 ± 29.5 ms in untreated cells to 143 ± 21.3 ms after GSNO). Second, GSNO increases the number and...
causes more than a threefold increase in the mean duration of long closed events (from a mean of 381 ± 48.6 ms in untreated cells to 1,130 ± 199 ms after GSNO), with no effect on the number or duration of short closed events (from a mean of 2.15 ± 0.507 ms in untreated cells to 2.56 ± 0.300 ms after GSNO). Thus the effect of GSNO is to change the kinetics of the channels in such a way as to favor less time in open states and more time in closed states.

NO acts via a cGMP-mediated pathway. We next tried to elucidate the mechanism of action of NO. To see whether NO was acting via a cGMP-mediated pathway, we studied the effect of methylene blue (MeB; an inhibitor of guanylyl cyclase) added to the bath before application of GSNO. MeB (100 µM) resulted in an increase in basal channel activity (Pₒ, control 0.17 ± 0.03 vs. MeB 0.34 ± 0.04; P < 0.04; n = 7 cell-attached patches). GSNO (100 µM) was then added to the bath. MeB (100 µM) blocked the effect of 100 µM GSNO (Pₒ, MeB 0.36 ± 0.07 vs. MeB + GSNO 0.37 ± 0.07; P = NS; n = 6 cell-attached patches), suggesting a role for cGMP (Fig. 7). This was further confirmed by application of a permeable analog of cGMP, 8-Br-cGMP (1 mM), to the bath solution; 8-Br-cGMP decreased the Pₒ of channels in cell-attached apical patches (Pₒ, control 0.38 ± 0.06 vs. 8-Br-cGMP 0.09 ± 0.02; −76%; P < 0.05; n = 7 cell-attached patches; Fig. 8).

cGMP action is mediated via a PK. To determine whether cGMP action on NSC channels is direct or is mediated via a PK, we pretreated cells with KT-5823 (a PKG inhibitor; 1 µM) before applying 100 µM GSNO to the bath. Prior application of KT-5823 did not alter basal channel activity (Pₒ, control 0.30 ± 0.05 vs. KT-5823 0.35 ± 0.04; P = NS; n = 13 cell-attached patches). However, KT-5823 blocked the action of GSNO (Pₒ, KT-5823 0.41 ± 0.06 vs. KT-5823 + GSNO 0.49 ± 0.06; P = NS; n = 5 cell-attached patches), suggesting that PKG was necessary for the action of NO (Fig. 9).

cGMP is elevated in cells treated with NO. Figure 10 shows the results of intracellular cGMP levels in cells treated with NO. cGMP measurements by ELISA 20 min after exposure of cells to two donors of NO (100 µM GSNO and 100 µM SNAP) and to 100 µM carbachol (positive control) showed that AT II cells were capable of increasing cGMP levels in response to NO.

DISCUSSION

Research during the past several years utilizing a variety of approaches has underscored the physiological importance of active Na⁺ transport by alveolar epithelium. Disruption of this process has been impli-
cated in a number of disease states. Because several pharmacological agents, especially those applied topically to the lung epithelium, have the potential for altering epithelial ion and water transport, their effect on lung fluid balance warrants detailed study before they are inducted into the clinical armamentarium.

The major findings of this study are that NO inhibits an NSC channel in AT II cells and that this action is mediated by a cGMP-activated PK. This is the first study to report the effect of NO on cation channels in the distal lung epithelia. We believe that this effect may be beneficial in some situations and may be harmful in others. A classic example of the former situation is cystic fibrosis in which increased Na\(^+\)-channel activity results in viscous secretions because of excessive salt and water reabsorption from the alveolar spaces. Agents inhibiting Na\(^+\) transport by the lung epithelium would have a beneficial role in cystic fibrosis, and aerosolized amiloride has been employed with some benefit. Our observation that GSNO inhibits amiloride-sensitive Na\(^+\) transport in the lung points to a novel role for this compound with its potential bronchodilator, antimicrobial, and vasoregulatory properties. However, our study suggests that lung conditions accompanied by pulmonary edema could potentially be worsened by NO treatment. This is especially important if the lung edema is not associated with pulmonary hypertension. Because cation channels with characteristics similar to those reported in this paper have been reported from a variety of tissues, as has the ability to produce NO locally, our results could have a greater general significance.

**Lung epithelial cation channels.** A complete understanding of the role of NSC channels in the physiology of lung water has yet to be achieved. Our study examined a 20.6-pS NSC channel recorded from apical cell-attached patches of AT II cells in primary culture. When grown under the conditions described in METHODS AND PROCEDURES, this was the predominant cation-permeable channel in AT II cells. The presence of NSC channels in alveolar epithelial cells has been shown by several investigators (7, 18, 24). Orser et al. (24) studied fetal distal lung epithelial cells from 20-day-gestation rat fetuses cultured on collagen-coated coverslips. Using symmetrical solutions and inside-out recording, the investigators observed single channels with a conductance of 23 ± 1.1 pS and an Na\(^+\)-to-K\(^+\) permeability of 0.9. These channels were blocked by amiloride applied to the apical side of the membrane. Marunaka (17) described an NSC channel with a linear I-V relationship and a single-channel conductance of 20.6 pS in rat alveolar epithelial cells. This channel was sensitive to amiloride, and its conductance was inhibited by the PK inhibitor H89 (17). However, the presence of NSC channels in the lung has not been widely reported, and their role in lung water balance remains unclear.

**Fig. 3.** Effect of 100 µM S-nitroso-N-acetylpenicillamine (SNAP) on cation channel. A: acute exposure to SNAP causes inhibition of channel as seen with GSNO. Arrows, closed state. B: summary of results (means ± SE; † on left and right) from 5 cell-attached patch experiments. Each symbol represents a different patch; lines connect data points from the same patch. *SNAP decreased P\(_o\) by 42% from control level, P < 0.05.

**Fig. 4.** Exposure of cells to 100 µM glutathione (GSH) did not have a significant effect on channel activity. A: single-channel recordings show no change in channel activity. Arrows, closed state. B: summary of results (means ± SE; † on left and right) from 8 cell-attached patch experiments shows no significant change in P\(_o\) after exposure to GSH. Each symbol represents a different patch; lines connect data points from the same patch. Each cell-attached patch served as its own control.
26.9 ± 0.8 pS in the fetal distal lung epithelium. Feng et al. (7) recently described a similar NSC channel observed in apical cell-attached and inside-out patches from rat AT II cells. Like the channels observed by us, these channels are nonselective (Na\textsuperscript{+}-to-K\textsuperscript{+} permeability = 1), voltage independent, and inhibited by amiloride. A wide variety of single-channel properties has been reported for amiloride-sensitive cation channels, including single-channel conductances ranging from 1 to over 50 pS (7, 11). It has been proposed that different combinations of the various subunits comprising the channel (α, β, and γ) could produce channels with varying unitary conductances (3, 11, 31). Kizer et al. (13) recently showed that expression of the α-subunit of the epithelial Na\textsuperscript{+} channels from osteoblasts into a null cell line (LM TK\textsuperscript{−}) resulted in an NSC channel (Na\textsuperscript{+}-to-K\textsuperscript{+} permeability = 1.1 ± 0.1) and a conductance of 24.2 ± 1.0 pS. Alternatively, the conductance could reflect the ionic conditions and membrane composition in the tissue, which determine the physical state of the membrane (11). We have also observed considerable variability in the \( P_o \) of these channels. Such variability has also been observed in single-channel recordings of amiloride-sensitive channels in cultured Xenopus renal cells, human lymphocytes, rat osteoclasts, and rat colonic epithelial cells. The exact physiological role for these NSC channels is unclear, although Tohda et al. (30) showed that these channels may play a role in the increased reabsorption of fluid by alveolar epithelia in response to β-agonist stimulation.

Effect of NO on apical NSC channels. The inhibition of NSC channels by NO suggests that NO may play a role in the regulation of alveolar fluid and edema formation. The inhibitory effect of NO on NSC channels is consistent with studies by Stoos et al. (29), who showed that NO inhibits Na\textsuperscript{+} reabsorption in the isolated cortical collecting duct, and Koivisto and Nedergaard (14), who found that NO donors block NSC channel activity in rat brown adipose tissue. Compeau et al. (4) showed that endotoxin-stimulated alveolar...

Fig. 5. GSNO reduces mean open time and increases mean closed time. In 7 cell-attached patch experiments, GSNO usually produced a decrease in mean open time (A). Significant decrease between control and GSNO, \( P < 0.05 \). In the 3 cases in which GSNO was washed off cell, mean open time always increased, and there was no statistically significant difference between control patches and patches after GSNO was washed off. In the same patches, there was always an increase in mean closed time in GSNO and a decrease when GSNO was subsequently washed off (B). There was a significant GSNO-induced increase in mean closed time compared with control level (\( P < 0.05 \)) but no significant difference after washout.

Fig. 6. GSNO alters both open (A) and closed (B) interval histograms. Solid bars, untreated cells; open bars, after GSNO. There is a significant shift of short open events to a population of even shorter duration events. At the same time, GSNO induces a change in closed interval histogram. Untreated cells have populations of both short-duration events and long-duration events. After GSNO, there is a major increase only in population of long-duration events.
macrophages impair distal lung epithelial ion transport by inactivating amiloride-sensitive NSC channels. These investigators showed a 60% reduction in amiloride-sensitive short-circuit current and a 60% decrease in the density of 25-pS NSC channels on the apical membrane of epithelium exposed to endotoxin and macrophages. This effect was blocked by N\(^6\)-monomethyl-L-arginine, suggesting an NO effect. These studies are in contrast to studies by Guidot et al. (8), who used isolated perfused rat lungs to show that inhaled NO prevents a neutrophil-mediated, oxygen radical-dependent leak in isolated perfused rat lungs. The investigators reported a modest reduction in pulmonary arterial pressure 30 min after NO exposure but felt that NO prevented an oxygen radical-dependent leak in the lungs. The question of whether NO increases or decreases the propensity for pulmonary edema is yet to be resolved. It is possible that in in vivo studies where pulmonary hypertension is contributing to pulmonary edema formation, inhaled NO may act by reducing the hydrostatic pressure and hence alveolar fluid formation. In situations where pulmonary vasoconstriction is not a major player and in vitro, NO appears to worsen pulmonary edema by impeding epithelial ion transport. NO may also have an effect on Na\(^+\)-K\(^+\)-ATPase, but we have not addressed this in our study. The answer to these questions is important because inhaled NO is currently undergoing clinical trials in a variety of lung disorders.

The effects of GSNO and SNAP are generally attributed to the release of NO. The fact that two biochemically different but specific NO-releasing compounds, GSNO and SNAP, were equipotent in their effect on the NSC channel suggests a common mechanism of action. In this study, GSH (carrier of NO in GSNO) did not affect the NSC channels, suggesting that GSNO was acting via release of NO (3). We were able to demonstrate that the GSNO effect can be reversed by washout. One puzzling observation was the apparent stimulating effect of washout on patches previously exposed to GSNO. Possible mechanisms for this phenomenon may include suppression of endogenous NO and/or cGMP production by exogenous GSNO. Once the inhibitory effect of GSNO was washout, there was an increase in channel activity attributable to the lower level of endogenous inhibition.

The concentration of NO donors used in this study is higher than the range of NO concentrations encountered in the physiological state (10). However, because...
NO has a short half-life and needs to diffuse inside the cell for its action, the actual effective concentration of NO inside the cell may have been lower than the donor concentration used. Ichinomiya et al. (10) found that 100 µM SNAP generated a stable concentration of 0.1 µM NO at 25°C, a concentration that is well within the physiological range.

The interval histograms are consistent with a minimum model of the NSC channel that has one short-duration and one long-duration open state and two similar closed states. Such a model can be represented by the following kinetic scheme:

\[
\text{closed}_1 \rightleftharpoons \text{closed}_2 \rightleftharpoons \text{open}_1 \rightleftharpoons \text{open}_2
\]

The simplest interpretation of the results is that GSNO produces an overall change in the rate constants of the model above that favors a shift in the equilibrium toward the closed states on the left side of the equation. This idea is consistent with the decrease in the number of long-duration open events, an increase in the number of short-duration open events, and a significant increase in the long-duration closed events (Fig. 6).

NO acts via cGMP-dependent activation of a PK. Guanylate cyclase stimulation is believed to be responsible for many of the physiological and pathological effects of NO (21). We hypothesized that NO was acting on NSC channels via a guanylate cyclase-mediated increase in cGMP. We found that a permeable analog of cGMP (BcGMP) had a virtually identical effect on NSC channels as did GSNO and SNAP. To further examine the role of cGMP in the inhibition of NSC channels, we utilized MeB to block soluble guanylate cyclase. We found that MeB abolished the effect of NO, suggesting that the inhibitory effect of GSNO on NSC channels in AT II cells is largely mediated by cGMP.

These studies show that NO acts on NSC channels via a cGMP-dependent mechanism. This is clear from the fact that cGMP analogs mimic and MeB blocks the action of NO. Furthermore, AT II cells respond to NO by production of cGMP. These findings are consistent with other studies (4, 5, 31) that have suggested a role for intracellular second messengers as modulators for ion-channel activity. Rocha and Kudo (26) showed that, in the kidney, hormones such as atrial natriuretic factor that increase cGMP levels result in inhibition of Na+ reabsorption. Light et al. (16) confirmed this with patch-clamp studies in which they showed that cGMP inhibits ion channels both directly and through a cGMP-dependent PK. We have also shown that cGMP acts via activation of PKG. It is possible that NO may have additional effects through the tyrosine kinase pathway, or the G protein-coupled receptor, or release of cytokines or other second messengers. The physiological regulation of epithelial Na+ channels appears to be complex because, in addition to the pathway discussed, channel activity is also modulated by methylation, arachidonic metabolites, and interactions with the cytoskeleton (15, 16).

There is considerable indirect evidence that cGMP action on renal epithelial Na+ channels is mediated via activation of PKG, leading to phosphorylation of cation channel or some related protein (5, 6). In the present study, we used KT-5823, a blocker of PKG (12), to study

![Fig. 9](http://ajplung.physiology.org/)

Fig. 9. Inhibition of protein kinase G with KT-5823 blocked effect of GSNO. Cells were pretreated with KT-5823 for 15 min before exposure to GSNO. A: Single-channel recordings show no effect of 100 µM GSNO on cells pretreated with 1 µM KT-5823. Arrows, closed state. B: Summary of results (means ± SE; ■ on left and right) from 5 cell-attached patch experiments. Each symbol represents a different patch; lines connect data points from the same patch.

Fig. 10. AT II cells respond to nitric oxide donors (100 µM GSNO and 100 µM SNAP) with a rise in cGMP as measured by ELISA 20 min after exposure in 6 cell-attached patch experiments. Carbachol (100 µM) served as a positive control. *Significant difference between control and each treatment group, P < 0.05.
whether the NO-cGMP-induced inhibition of the channels was mediated by PKG. We found that KT-5823 blocked the effect of NO, suggesting a role for PKG in the observed effect of NO on the cation channels. It is possible that higher concentrations of KT-5823 may inhibit other PKs, which can then affect the channel under study or other related proteins (32).

Other mechanisms may contribute, in part, to the observed inhibition of NSC channels. These include a direct cGMP effect on the membrane and a phosphodiesterase (PDE)-mediated fall in cAMP levels because cAMP is known to stimulate epithelial Na⁺ channels (23). To invoke this mechanism, one would assume that elevated cGMP levels lead to an increase in PDE concentration that then lowers the cellular cAMP concentration. Whether there is a role for PDE in the NO-mediated inhibition of NSC channels is not clear.

Exposure of AT II cells to NO does not cause cell death. It is possible that at the concentrations used in this study, NO may be toxic to epithelial cells. We did not find any difference in the viability of the cells after exposure of AT II cells to NO donors under the conditions used in our patch-clamp protocols. Furthermore, reversibility of NO effect after washout confirms that the inhibition of NSC channels was not related to any permanent changes in the cell and/or due to spontaneous “rundown” of channels in the patches being examined.

In summary, our study suggests that NO inhibits cation channels on the apical surface of AT II cells via a cGMP-mediated action. This suggests that NO may have a regulatory role in lung epithelial Na⁺ transport. We speculate that pharmacological modalities, which can then affect the channel under study or other related proteins (32).

REFERENCES


