

# A Potentiating Effect of Endogenous NO in the Physiologic Secretion from Airway Submucosal Glands

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It is known that several second messengers, such as  $\text{Ca}^{2+}$  or cAMP, play important roles in the intracellular pathway of electrolyte secretion in tracheal submucosal gland. However, the participation of cGMP, and therefore nitric oxide (NO), is not well understood. To investigate the physiologic role of NO, we first examined whether tracheal glands can synthesize NO in response to acetylcholine (ACh), and then whether endogenous NO has some effects on the ACh-triggered ionic currents. From the experiments using the NO-specific fluorescent indicator 4,5-diaminofluorescein diacetate salt (DAF-2DA), we found that a physiologically relevant low dose of ACh (100 nM) stimulated the endogenous NO synthesis, and it was almost completely suppressed in the presence of the nonspecific NO synthase (NOS) inhibitor *N* $\omega$ -Nitro-L-arginine Methyl Ester Hydrochloride (L-NAME) or the neuronal NOS (nNOS)-specific inhibitor 7-Nitroindazole (7-NI). Patch-clamp experiments revealed that both the NOS inhibitors (L-NAME or 7-NI) and cGK inhibitors (KT-5823 or Rp-8-Br-cGMP) partially decreased ionic currents induced by 30 nM of ACh, but not in the case of 300 nM of ACh. Our results indicate that NO can be synthesized through the activation of nNOS endogenously and has potentiating effects on the gland secretion, under a physiologically relevant ACh stimulation. When cells were stimulated by an inadequately potent dose of ACh, which caused an excess elevation in  $[\text{Ca}^{2+}]_i$ , the cells were desensitized. Therefore, due to NO, gland cells become more sensitive to calcium signaling and are able to maintain electrolyte secretion without desensitization.

**Keywords:** cholinergic receptor; calcium; chloride secretion; Clca; Kca

The airway mucosa is always exposed to various microbes or microscopic inhaled foreign bodies (1, 2), but the airway is protected against infection. This is because both a nonspecific and a specific defense system always work in the airway. The airway submucosal gland secretes mucin, various enzyme proteins, and electrolytes (therefore, water), which serve as a nonspecific airway defense mechanism. Moreover, the submucosal gland secretes immunoglobulins to neutralize and/or eliminate microbes or foreign substances, serving as a specific airway defense mechanism (2). Concerning electrolyte or water secretion, it is well known that the airway surface liquid is regulated quantitatively (the volume of mucus or water) (3, 4) and qualitatively (osmolality or pH, etc.) (5–8) to maintain a suitable environment for the airway mucosal defense systems. Cystic fibrosis (CF) is caused by mutations of CF transmembrane conductance regulator (CFTR),

## CLINICAL RELEVANCE

Nitric oxide works as one of the second messengers in the physiologic electrolyte secretion from airway submucosal glands. This pathway may become a new therapeutic target for not only hypersecretion of chronic bronchitis or bronchial asthma, but also dehydration of airway mucosa in cystic fibrosis.

which acts as a cAMP-activated chloride channel in the airway epithelium (9). Mutations of this chloride channel alter the transport of chloride and associated liquid, resulting in an impairment of the lung defenses (8). This pathogenesis reveals that the airway chloride secretion plays a very important role in the airway defense. Because human airway epithelium is likely to be primarily absorptive (1, 10, 11), a major fraction of the airway fluid seems to be derived from the submucosal glands (12, 13). We have reported that human, feline, and swine tracheal gland acinar cells generated ionic currents in response to relative low doses of cholinergic and  $\alpha$ -adrenergic stimuli, and that the currents were activated by the intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) raised by these neurotransmitters (14–17).

Nitric oxide (NO) is produced by the activation of nitric oxide synthase (NOS) and plays multiple roles in physiologic processes in the airways such as bronchodilation (18), pulmonary vasodilatation (19), the modulation of mucin secretion (20), and so on. (for review see Ref. 21) To date three isoforms of NOS have been identified, namely neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). All three isoforms are able to synthesize NO and L-citrulline from L-arginine and molecular oxygen. nNOS and eNOS are constitutively expressed in the airways, and their activities are dependent on  $[\text{Ca}^{2+}]_i$ , whereas the expression of iNOS is induced by cytokines and lipopolysaccharides in chronic inflammatory airways and is  $[\text{Ca}^{2+}]_i$ -independently activated (22). Studies evaluating the mechanism of action of NO indicate that the soluble guanylyl cyclases (sGC) activated by NO catalyze the formation of cGMP (23), which in turn activates cGMP-gated ion channels, cGMP-dependent phosphodiesterases, and cGMP-dependent protein kinases (cGK) (24).

Various exocrine gland acinar cells are well known to be able to stimulate the production of intracellular cGMP after cholinergic stimulation (25). Concerning airway secretion, NO has been shown to stimulate glycoconjugate secretion from human tracheal submucosal glands (20). But there is hardly any information about the physiologic role of NO in airway electrolyte secretion.

In this study we investigated whether tracheal glands synthesize NO when stimulated by acetylcholine (ACh) and whether endogenous NO is involved in the physiologic signaling pathway in tracheal gland secretion. Our results suggest that ACh- or  $[\text{Ca}^{2+}]_i$ -dependent NO/cGMP/cGK signaling is involved in the

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physiologic signaling pathway in the airway electrolyte secretion, and that endogenous NO potentiates the ACh-induced Cl<sup>-</sup> secretion.

## MATERIALS AND METHODS

### Cell Preparation

Swine tracheas were obtained at a local slaughterhouse immediately after the animals had been killed and were transported to our laboratory in an ice-cold extracellular solution. The external surface of the trachea was cleaned of fat and connective tissues, and cut into rings 3–4 cm long. The posterior (membranous) strip of the tracheal wall was then excised longitudinally, leaving attached to both sides ~1 cm width of the cartilaginous portion, and was fixed by pins in the extracellular solution with the external wall side up. The outermost layer and thick smooth muscle layer were carefully removed. The submucosal gland could then be easily distinguished from the surrounding connective tissue under a stereoscopic microscope with the light shed horizontally. Fresh, unstained submucosal glands were isolated using two pairs of tweezers and microscissors (14–17, 26). The isolated glands were further dispersed enzymatically by incubating them with enzyme solution containing collagenase (200 U/ml), DL-dithiothreitol (0.31 mg/ml), and trypsin inhibitor (1 mg/ml) for 30 min at 37°C. After dispersion and a wash with centrifugation at 180 × g the cells were resuspended in a standard extracellular solution until use. Although DTT is a strong reducing reagent that interacts with free radicals, because DTT is not stable in a standard ring solution and was washed out repeatedly before use, the artifacts of DTT on the synthesis and the biological activity of NO can be practically ignored in our study.

### Intracellular NO Imaging with DAF-2DA

A highly specific fluorescent NO indicator, 4,5-diaminofluorescein (DAF-2), is known to be useful to estimate the amount of NO produced in the cytosol (27). The diacetate salt DAF-2DA is membrane permeable and is soon hydrolyzed at the ester bonds by the intracellular esterase, resulting in the membrane-impermeable and relatively nonfluorescent compound DAF-2. In the presence of oxygen, NO combines with DAF-2 and forms the highly fluorescent triazolofluorescein DAF-2T. Peak excitation and emission wavelengths for DAF-2T occur at 495 and 515 nm, respectively. The fluorescence intensity shows a linear correlation with the concentration of DAF-2T, which is in parallel with the cellular production of NO. In the present study, freshly isolated swine tracheal glands were preloaded with 10 μM DAF-2DA for 40 min or more at 37°C so that the cytosol was saturated with DAF-2. At the beginning of each experiment, we adjusted the fluorescent intensity so that little fluorescence would be observed in control and nonstimulated conditions. All experiments were performed under the same conditions. To prevent the fluorescence signaling from becoming attenuated, the fluorescence intensity was measured for 5 s every 5 or 10 min. The solutions were the same compositions as used in the patch-clamp experiments (*see below*), and were superfused over the cells continuously at 2 ml/min at 37°C with or without 100 nM of ACh. Then, the solutions were gassed with 100% O<sub>2</sub> during the measurements. The gray levels in the areas of gland cells in the captured microfluorographs were determined by a digital image processor (NIH Image software, version 1.62/ Macintosh G3/100 AV, National Institutes of Health, Bethesda, MD). In this way, we detected the increase in the amount of intracellular NO per unit area of gland cells at each measurement time. NO productions were shown as a fold increases over the basal fluorescent intensity.

### Electrical Recordings

Ionic currents were measured with a patch-clamp amplifier (EPC9; HEKA Electronic, Lambrecht/Pfalz, Germany), low-pass filtered at 2.9 kHz, and monitored on both a built-in software oscilloscope and a thermal pen recorder (RECTI-HORIZ-8K; Nippondenki Sanei, Tokyo, Japan). Patch pipettes were made of borosilicate glass capillary with an outer diameter of 1.5 mm using a vertical puller (PP-83; Narishige Scientific Instruments, Tokyo, Japan), and had a tip resistance of 2 to 6 MΩ. The junction potential between the patch-pipette and bath solution was nulled by the amplifier circuitry. After establishing a high-resistance (1 GΩ), tight seal, the whole cell configuration was obtained

by rupturing the patch membrane with negative pressure applied to the pipette tip. Membrane currents were monitored at two different holding potentials (Hps), 0 and -80 mV, which roughly corresponded to the Cl<sup>-</sup> and K<sup>+</sup> equilibrium potential, respectively, under the present electrolyte conditions. The double current monitoring (i.e., alternate recording of the ionic currents corresponding to Hp of 0 and -80 mV) was accomplished by applying 200 ms voltage pulses of -80 mV at a frequency of 2 Hz to the pipette voltage of 0 mV (14–17, 26). The upward or downward deflection of the current tracing represents outward (*I<sub>o</sub>*) or inward current (*I<sub>i</sub>*), respectively. Using proper channel inhibitors and ion substitution experiments, we have reported that the ACh-induced *I<sub>o</sub>* and *I<sub>i</sub>* were carried mainly by K<sup>+</sup> and Cl<sup>-</sup>, respectively, which were dependent on [Ca<sup>2+</sup>]<sub>i</sub> (14, 17, 26). The solutions employed were of the following compositions (in mM): extracellular (bath) solution, 120 NaCl, 4.7 KCl, 1.13 MgCl<sub>2</sub>, 1.2 CaCl<sub>2</sub>, 10 glucose, and 10 Hepes; and intracellular (pipette) solution, 120 KCl, 1.13 MgCl<sub>2</sub>, 0.5 EGTA, 1 Na<sub>2</sub> adenosine triphosphate (ATP), 10 glucose, and 10 Hepes. The fluids were superfused over the cells by hydrostatic pressure-driven application (20–30 cm H<sub>2</sub>O) through polyethylene tubes. All solutions were at pH 7.2, and all experiments were performed at room temperature (22–25°C).

### Quantification Procedure

The procedure to estimate the ionic responses was also applied in our previous reports (5, 26).

We first measured the area circumscribed with the current trace (*I<sub>o</sub>* or *I<sub>i</sub>*) and baseline for 20 s (= Area Under Curve<sub>20</sub>) by using a digital planimeter (PLACOM, KP-92N; Koizumi, Tokyo, Japan). This area shows the net electric charge movements of 20 s duration in each condition. Next, the mean magnitudes of ionic currents for 20 s were expressed by the calculation below.

$$I_{\text{mean}} = \text{Area-Under-Curve}_{20} / 20 \text{ (pQ/s)}$$

The effect of Nω-Nitro-L-arginine Methyl Ester Hydrochloride (L-NAME) on the ACh-stimulated response was estimated by comparing just before and after treatment with L-NAME and was expressed as a percentage of the pretreatment control values.

$$\begin{aligned} \%I_{\text{mean}}(\text{ACh} + \text{L-NAME}) \\ = \{I_{\text{mean}}(\text{ACh} + \text{L-NAME}) / I_{\text{mean}}(\text{ACh})\} \times 100 (\%) \end{aligned}$$

The effect of L-arginine on the responses induced by both ACh and L-NAME was expressed as a percentage of *I<sub>mean</sub>*(ACh+L-NAME).

$$\begin{aligned} \%I_{\text{mean}}(\text{ACh} + \text{L-NAME} + \text{L-arginine}) \\ = \{I_{\text{mean}}(\text{ACh} + \text{L-NAME} + \text{L-arginine}) / I_{\text{mean}}(\text{ACh} + \text{L-NAME})\} \\ \times 100 (\%) \end{aligned}$$

In bar graphs, %*I<sub>mean</sub>*(ACh+L-NAME+L-arginine) was modified to express the percentage of *I<sub>mean</sub>*(ACh+L-NAME+L-arginine) to *I<sub>mean</sub>*(ACh). In some experiments, L-NAME was replaced by 7-NI, and L-arginine was replaced by NOC-5.

### Statistics

The data were expressed as means ± SE; *n* is the number of experiments on different cells. Data were analyzed by Wilcoxon's signed rank test, and significance was accepted at *P* < 0.05 and indicated by *asterisks* in all figures.

### Reagents

Hepes and NOC-5 were purchased from Dojindo Co. Ltd. (Kumamoto, Japan). DAF-2DA was from Daiichi Pure Chemicals Co. Ltd. (Ibaraki, Japan). Collagenase was from Wako Pure Chemicals (Osaka, Japan), and KT-5823 and Rp-8-Br-cGMP were from Calbiochem (La Jolla, CA). All other chemicals used were purchased from Sigma (St. Louis, MO).

## RESULTS

### ACh-Induced NO Synthesis in Tracheal Glands Acinar Cells

DAF-2 is often used to measure the NO production in neural cells (28), cardiomyocytes (29), vascular endothelial cells (30),

and parotid acinar cells (31), but not that in tracheal gland acinar cells. Therefore, we confirmed first whether the fluorescence intensity obtained from tracheal gland cells specifically indicates the total amount of intracellular NO or not. A well-established NO donor NOC-5 is widely used for DAF-2DA fluorescent experiments to detect NO (32). When 10  $\mu$ M of NOC-5 were added to the DAF-2-preloaded cells, the fluorescence intensity increased rapidly, and then scaled out over 4-fold. In the case of 0.5  $\mu$ M of NOC-5, the fluorescent intensity increased linearly and slopingly (Figure 1A). These findings indicated that DAF-2DA in the cytosol of tracheal gland acinar cells combined with NO and that the increase of fluorescence intensity reflected the total amounts of intracellular NO. We therefore confirmed that NO measurement in tracheal gland cells also worked well, and then continued several protocols described below.

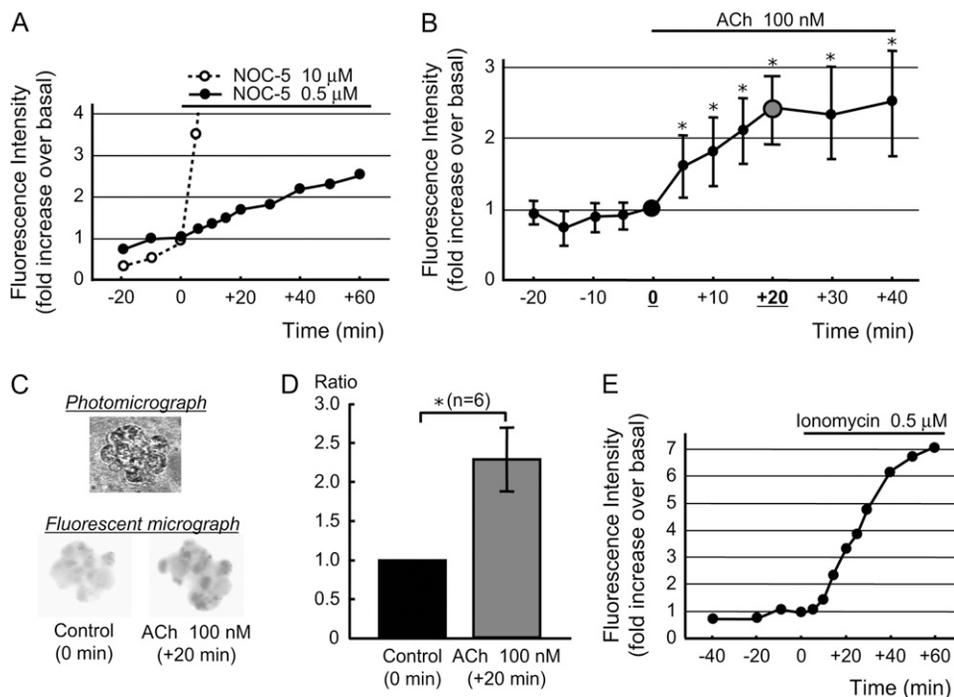
When cells were stimulated by 100 nM of ACh, the DAF-2T fluorescence intensities were increased linearly in a time-dependent manner as in the case of 0.5  $\mu$ M of NOC-5 (Figures 1B and 1C). At 20 min after the stimulation of ACh, the fluorescence intensity significantly increased as much as 2.3-fold as compared with that at 0 min (1.0 for prestimulated control value and  $2.32 \pm 0.43$  for ACh-stimulated value,  $P < 0.05$ ;  $n = 6$ , Figure 1D). Because the fluorescence intensity represents the integral amount of accumulated NO, the inclination of this increasing curve line indicates the amount of synthesized NO per unit time. Interestingly, tracheal glands continued to synthesize NO for  $\sim 20$  min after ACh stimulation, but after that ceased to produce NO even in the presence of ACh.

In tracheal glands, ACh activates, via a G protein coupled with muscarinic  $M_3$  receptor, the plasma membrane phospholipase C, generating inositol 1,4,5-trisphosphate ( $IP_3$ ).  $IP_3$  binds to its specific receptor on the cytosolic  $Ca^{2+}$ -storage compart-

ment to release  $Ca^{2+}$  into the cytosol, thereby initiating the  $Ca^{2+}$  signal. Ionomycin, a calcium ionophore, can increase  $[Ca^{2+}]_i$  directly without the activation of muscarinic receptors on the cell surface. To confirm the involvement of  $Ca^{2+}$  in the endogenous NO synthesis, we investigated the effect of Ionomycin. When 0.5  $\mu$ M of Ionomycin was added to DAF-2 preloaded cells, the fluorescence intensity increased linearly during the stimulation (Figure 1E). These findings demonstrate that ACh is able to increase endogenous NO synthesis and that the elevation in  $[Ca^{2+}]_i$  plays a pivotal role in the NO synthesis.

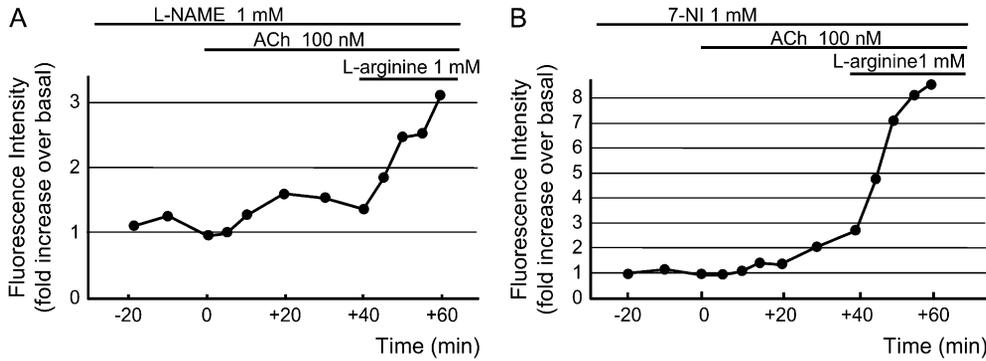
### Effects of NOS Inhibitors on NO Synthesis

Among the three isoforms of NOS, nNOS and eNOS are known to be constitutively expressed in the airways and their activities are dependent on  $[Ca^{2+}]_i$  (22). Therefore, we next investigated the involvement of NOS in tracheal gland NO synthesis. We used two different types of NOS inhibitors, L-NAME and 7-Nitroindazole (7-NI). L-NAME is a nonspecific NOS inhibitor, and 7-NI is a specific nNOS inhibitor. Both of these inhibitors competitively block the binding of NOS with L-arginine, and these inhibitory effects are reversible by the subsequent addition of L-arginine (33). Before ACh stimulation, cells were preloaded with 1 mM of L-NAME or 7-NI for 1 h. In the presence of L-NAME, the addition of ACh (100 nM) caused no obvious increase in the fluorescence intensity. However, the intensity distinctly increased after the subsequent addition of L-arginine (1 mM) (Figure 2A). In the presence of 7-NI, ACh (100 nM) made no obvious increase in the fluorescence intensity, but after the subsequent addition of L-arginine (1 mM), the intensity rapidly and greatly increased (Figure 2B). These findings suggested that the activation of NOS, especially nNOS, would be essential to the ACh-stimulated NO synthesis in tracheal glands.



**Figure 1.** Detection of endogenous NO synthesis induced by ACh-stimulation in the swine tracheal acinar cells. (A) Changes in fluorescence intensity from DAF-2T in the presence of NOC-5. The fluorescence intensity indicates the integrated amount of NO in the cytosol and is displayed by changes from the baseline (indicated as 0 min), which represents the intensity in the unstimulated condition (see MATERIALS AND METHODS). NOC-5 was added at the time 0. *Open circles*, the changes in the presence of 10  $\mu$ M of NOC-5; *solid circles*, the changes in the presence of 0.5  $\mu$ M of NOC-5. (B) Changes in endogenous NO-associated fluorescent intensities in the presence of ACh (100 nM) ( $n = 6$ ). It is of note that the fluorescence intensity shows the integrated amount of synthesized NO in the cytosol. Therefore, NO synthesis appears to have discontinued at 20 min (C) Representative photomicrograph (*upper panel*) and fluorescent micrograph (*lower panel*) of tracheal gland acinus used for the NO imaging. These clusters of cells easily adhere and are so hard to detach

from the surface of the slide glass that one can observe the change in fluorescence intensity under the same condition even if extracellular solutions flow over the cells during each experiment. The fluorescence intensity was observed within the cytosol area only. (D) Summary of a comparison in fluorescence intensities between just before ACh stimulation (control) and 20 min after stimulation (ACh 100 nM). The fluorescence intensity was calculated by the intensities per unit area and compared by estimating the mean intensities of pre ACh stimulation as 1.0. ACh (100 nM) significantly increased the endogenous NO synthesis ( $n = 6$ ).  $*P < 0.05$ . (E) Representative traces of the changes in NO fluorescence in the presence of Ionomycin. Ionomycin (0.5  $\mu$ M) markedly increased the fluorescent intensity.

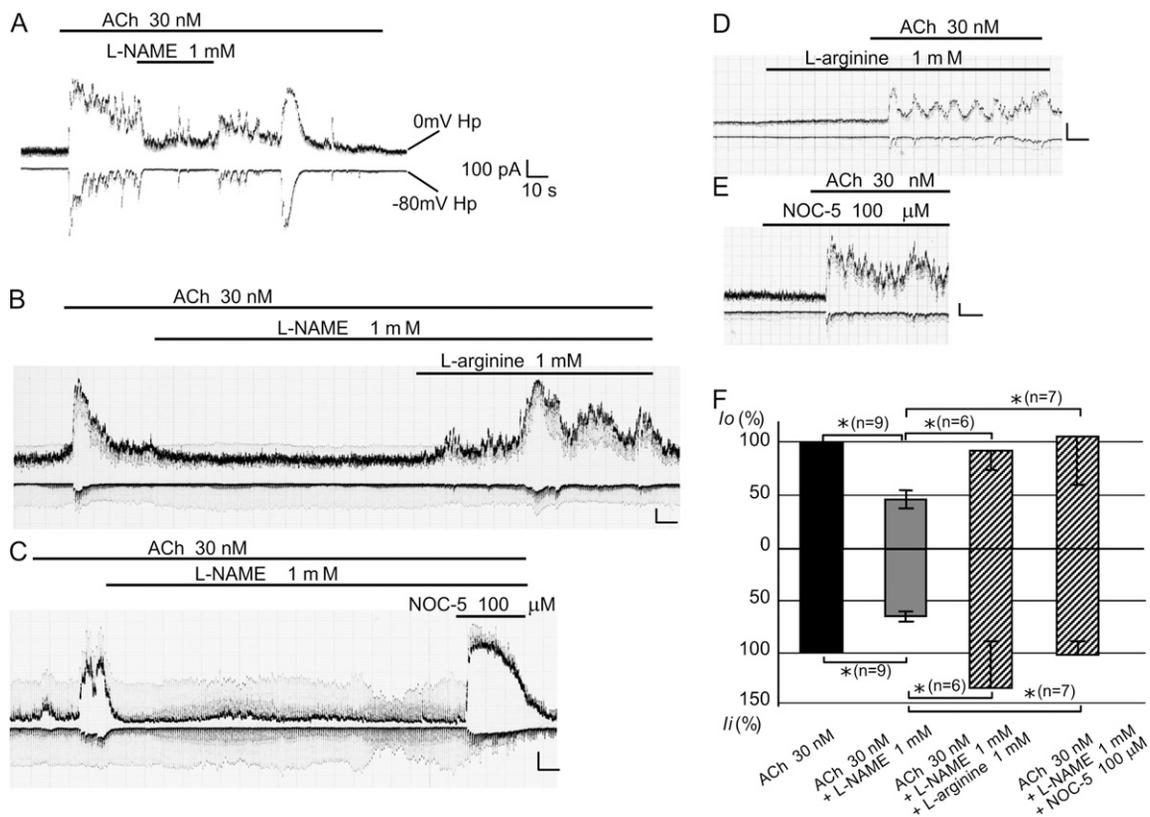


**Figure 2.** Representative traces showing the effects of NOS inhibitors on endogenous NO synthesis. (A) L-NAME (1 mM), a competitive NOS inhibitor, inhibited the ACh-mediated NO synthesis. The fluorescence intensities were elevated after the subsequent addition of L-arginine. (B) 7-NI (1 mM), a specific inhibitor of nNOS, also inhibited the ACh-mediated NO synthesis, which was recovered by the subsequent addition of L-arginine.

### Effects of NOS Inhibitors on ACh-Induced Ionic Currents

We investigated whether the endogenous NO has some effects on the ACh evoked ionic currents. Because endogenous NO synthesis was almost completely suppressed in the presence of NOS inhibitors (Figure 2), we could observe the ACh-evoked ionic currents without the effects of endogenous NO using NOS

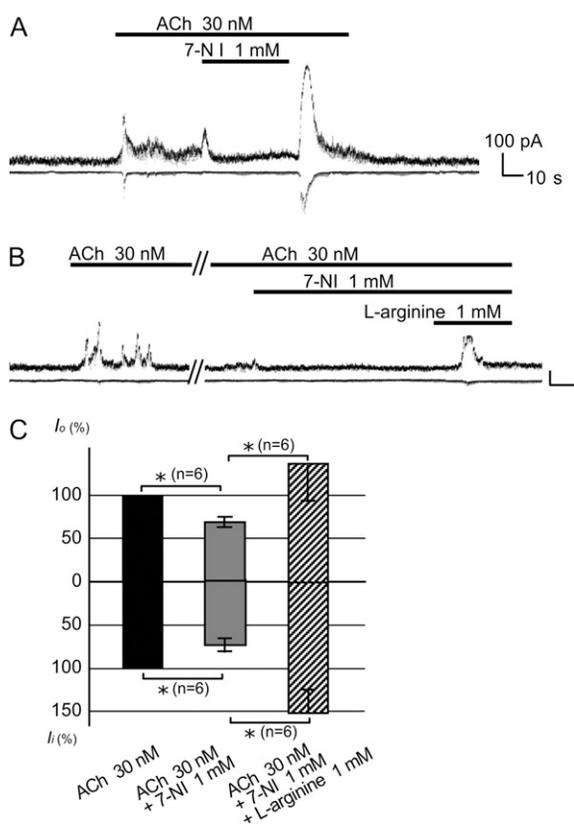
inhibitors. As shown in Figure 3, in the presence of L-NAME (1 mM), both  $I_o$  and  $I_i$  induced by ACh (30 nM) were decreased to two-fifths ( $175.5 \pm 33.5$  pQ/s for ACh and  $65.6 \pm 12.7$  for ACh/L-NAME,  $P < 0.05$ ;  $n = 9$ ) and to two-thirds ( $22.2 \pm 3.6$  versus  $13.5 \pm 1.7$  pQ/s,  $P < 0.05$ ;  $n = 9$ ) of the pre-L-NAME control values, respectively (Figure 3A). Further, either the



**Figure 3.** Representative original recordings showing the effect of L-NAME on ACh-induced ionic currents. The upward or downward deflection of the current tracing represents outward ( $I_o$ ) or inward current ( $I_i$ ), respectively. Using proper channel inhibitors and ion substitution experiments, we have reported that the ACh-induced  $I_o$  and  $I_i$  were carried mainly by  $K^+$  and  $Cl^-$ , respectively, which were dependent on  $[Ca^{2+}]_i$  (14, 17, 26). As described in Ref. 26, cells sometimes showed a simultaneous activation of both oscillatory  $I_o$  and  $I_i$ , but sometimes exhibited  $I_o$  alone with a minimal activity of  $I_i$ . Even if the responses in  $I_i$  are less robust, these responses are suitable for meaningful comparisons, as long as they are sustained and showed the same effect by the same agent. (A) L-NAME (1 mM) partially inhibited the ACh-induced ionic currents. After the removal of L-NAME, the ionic response recovered to the pre-inhibitory levels. (B) This inhibitory effect was also reversibly abolished after the subsequent addition of L-arginine (1 mM). (C) An exogenous NO donor, NOC-5 (100 μM), also reversibly abolished the inhibitory effect of L-NAME on the ionic currents. (D) L-arginine (1 mM) alone did not cause any ionic responses. Because the subsequent addition of ACh generated ionic currents in the same cell, it is considered that the cell viability was conserved. (E) NOC-5 (100 μM) also caused no ionic response from unstimulated cells, which generated ionic currents after the subsequent addition of ACh. (F) Summary of the inhibitory effects of L-NAME on ACh-induced  $I_o$  and  $I_i$  currents. The electric charge movements of 20 s duration just before and after introducing L-NAME or other agents were compared by estimating the mean values of the L-NAME responses as 100%, to exclude the artifacts from the scattering of the membrane capacitance in each cell. L-NAME significantly inhibited both  $I_o$  and  $I_i$ . \* $P < 0.05$ .

removal of L-NAME or the subsequent addition of L-arginine reversibly abolished the inhibitory effect of L-NAME (Figures 3A and 3B). When L-arginine was introduced on ACh/L-NAME, both  $I_o$  and  $I_i$  recovered to the pre L-NAME control values ( $I_o$ :  $83.3 \pm 14.9$  pQ/s for ACh/L-NAME and  $156.1 \pm 30.4$  for ACh/L-NAME/L-arginine,  $P < 0.05$ ;  $n = 6$ ,  $I_i$ :  $15.0 \pm 1.8$  versus  $27.4 \pm 5.8$  pQ/s,  $P < 0.05$ ;  $n = 6$ ) (Figure 3B). The exogenous NO donor NOC-5 also reversibly abolished the inhibitory effect of L-NAME on the ionic currents ( $I_o$ :  $111.8 \pm 28.7$  pQ/s for ACh/L-NAME and  $147.6 \pm 20.6$  for ACh/L-NAME/NOC-5,  $P < 0.05$ ;  $n = 7$ ,  $I_i$ :  $32.4 \pm 10.0$  versus  $44.1 \pm 11.1$  pQ/s,  $P = 0.09$ ;  $n = 7$ ) (Figure 3C). L-arginine or NOC-5 alone did not raise any electrical response (Figures 3D and 3E). These findings suggest that the endogenous NO takes part in the generation of ACh-evoked currents and has a potentiating effect on these currents in the presence of physiologic doses of ACh.

In the presence of 7-NI (1 mM), both  $I_o$  and  $I_i$  induced by ACh decreased to two-thirds ( $102.9 \pm 17.4$  pQ/s for ACh and  $73.3 \pm 17.1$  for ACh/7-NI,  $P < 0.05$ ;  $n = 6$ ) and to three-quarters ( $11.1 \pm 2.4$  versus  $7.8 \pm 1.2$  pQ/s,  $P < 0.05$ ;  $n = 6$ ) of the pre 7-NI control values, respectively (Figure 4A). The removal of 7-NI and the subsequent addition of L-arginine also reversibly abolished the inhibitory effect of 7-NI ( $I_o$ :  $99.8 \pm 31.2$  pQ/s for ACh/7-NI and  $163.7 \pm 50.3$  for ACh/7-NI/L-arginine,  $P < 0.05$ ;  $n = 6$ ,  $I_i$ :  $19.2 \pm 5.7$  versus  $31.7 \pm 8.0$  pQ/s,  $P < 0.05$ ;  $n = 6$ ) (Figures 4A and 4B).



**Figure 4.** Representative original recordings showing the effect of 7-NI on ACh-induced ionic currents. (A) 7-NI (1 mM) partially inhibited the ACh-induced ionic currents. After the removal of 7-NI, the ionic response recovered to the pre inhibitory levels. (B) This inhibitory effect was also reversibly abolished after the subsequent addition of L-arginine (1 mM). (C) Summary of the inhibitory effects of 7-NI on  $I_o$  and  $I_i$ . 7-NI inhibited significantly both  $I_o$  and  $I_i$ . \* $P < 0.05$ .

The electrical responses obtained in our present study originated in different sizes of cells. In other words, since each responding cell has different membrane capacitances, for a strict estimation the electrical responses should be compared by the change rate between the times before and after introduction of the materials in each cell. Again, the data were compared by estimating the values of the pre L-NAME response as 100% and are summarized in Figure 3F and Figure 4C.

### Effects of cGK Inhibitors on ACh-Induced Ionic Currents

To assess whether cGK activation is necessary for the ACh-induced ionic currents, we investigated the effects of two different types of membrane-permeable cGK inhibitors: KT-5823 and Rp-8-Br-cGMP. KT-5823 has been reported to be a potent cGK I inhibitor only in cell-free systems and not in intact cells (34). However, because of some findings described in DISCUSSION, we believe that KT-5823 works as a specific cGK inhibitor in tracheal gland cells. We think that 1  $\mu$ M cGK I inhibitors may be sufficient to inhibit cGK I activity in the very delicate ionic responses, without other unexpected effects.

In the presence of KT-5823, both  $I_o$  and  $I_i$  induced by ACh decreased to three-fifths ( $233.3 \pm 44.2$  pQ/s for ACh and  $134.0 \pm 21.8$  for ACh/KT-5823,  $P < 0.05$ ;  $n = 9$ ) and to two-thirds ( $28.0 \pm 9.3$  versus  $18.3 \pm 4.6$  pQ/s,  $P < 0.05$ ;  $n = 9$ ) of the pre-KT-5823 control values, respectively (Figures 5A and 5B). After the removal of KT-5823, the inhibitory effect was reversibly abolished within several seconds (Figure 5A).

In the case of Rp-8-Br-cGMP, both  $I_o$  and  $I_i$  showed a tendency to decrease ( $I_o$ :  $323.6 \pm 131.8$  versus  $274.4 \pm 108.4$  pQ/s,  $P < 0.05$ ;  $n = 8$ ,  $I_i$ :  $62.3 \pm 18.2$  versus  $44.0 \pm 12.0$  pQ/s,  $P = 0.14$ ;  $n = 8$ ) (Figure 5C). When the values are compared by estimating the mean values of the pre-cGK inhibitor responses as 100% to exclude the scatter from the differences in membrane capacitance of each cell, the inhibitory effects in  $I_o$  and  $I_i$  in the case of both KT-5823 and Rp-8-Br-cGMP were statistically significant (Figure 5D). These findings suggest that NO activates cGK and plays an important role in the maintenance of ionic currents induced by physiologically relevant doses of ACh in tracheal gland cells.

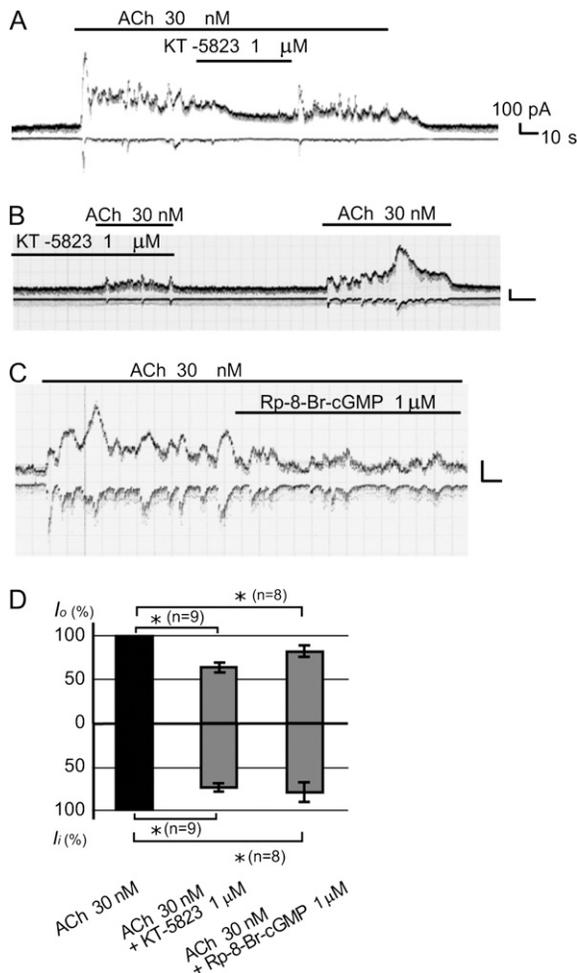
### No Effect of NOS Inhibitor on Ionic Currents Induced by High Dose of ACh

In the case of a high dose (300 nM) of ACh, the response of ionic currents is very large and desensitized before long, compared with that in the case of low dose (30 nM) of ACh (Figures 6A and 6B). Under this physiologically irrelevant robust stimulation, the inhibitory effect of L-NAME could not be observed, in the case of both pre- and post-treatment with L-NAME (Figure 6B). These findings suggest that NO can potentiate the ionic currents only when cells are moderately stimulated by ACh, but not when the stimulation by ACh is too strong.

## DISCUSSION

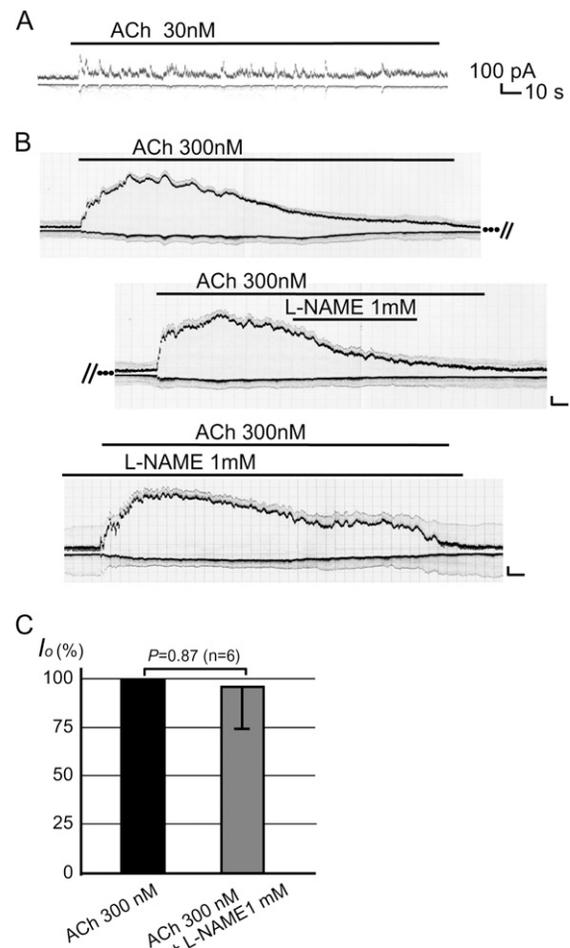
In the present study, we revealed that endogenous NO has a role in the intracellular signaling pathway involved in tracheal submucosal gland secretion stimulated by a physiologically relevant dose of ACh.

NO imaging experiments revealed that a relatively low concentration of ACh (100 nM) stimulated endogenous NO synthesis in swine tracheal gland acinar cells. In general, the endogenous NO synthesis is dependent on the activation of NOS. NOS is known to have at least three isoforms as follows: eNOS expressed in vascular endothelium (35) or pulmonary epithelium (36); nNOS expressed in airway nerves (37) or various exocrine acinar cells (31, 38, 39); and iNOS, an inducible isoform expressed



**Figure 5.** Representative original recordings showing the effects of cGK inhibitors on ACh-induced ionic currents. One of the main pathways in which NO exerts its biological effects is to activate soluble guanylate cyclase (sGC) and increase cGMP, resulting in the activation of cGK (see Figure 7). (A) KT-5823 (1  $\mu$ M), a membrane permeable and specific cGK inhibitor, showed a partial inhibition on  $I_o$  and  $I_i$ . After the removal of KT-5823, the ionic currents recovered to the pre inhibition level. (B) When the cells were preloaded with KT-5823 (1  $\mu$ M), the ACh-induced ionic responses were much smaller than those observed in the absence of KT-5823. (C) Rp-8-Br-cGMP (1  $\mu$ M), another membrane permeable cGK inhibitor, also partially inhibited  $I_o$  and  $I_i$ . (D) Summary of the inhibitory effects of cGK inhibitors on  $I_o$  and  $I_i$ . Both KT-5823 and Rp-8-Br-cGMP significantly inhibited both  $I_o$  and  $I_i$ . \* $P < 0.05$ .

in the airway epithelium (22) and in alveolar macrophages during cytokine stimulation (40). Both eNOS and nNOS depend on  $Ca^{2+}$  transients, whereas iNOS is  $Ca^{2+}$  independent. Our study demonstrated that ACh-induced NO synthesis was mimicked by Ionomycin and inhibited by NOS inhibitors. Although we did not use BAPTA or other calcium-chelating agents owing to the fragile cell viability, these findings suggest that tracheal submucosal glands generate NO by the activation of  $Ca^{2+}$ -dependent constitutive NOS (nNOS or eNOS). It was previously reported that nNOS is expressed in various exocrine cells such as pancreatic acinar cells (38), submandibular glands (39), parotid glands (31), and lacrimal glands (38). Therefore, we speculated that nNOS might be localized and play some physiologic roles in tracheal acinar cells. To identify whether nNOS takes part in the NO synthesis induced by ACh, we investigated the effects of the nonspecific NOS inhibitor L-NAME and the specific nNOS



**Figure 6.** Representative original recordings showing no effect of L-NAME on the high dose ACh-induced ionic currents. (A) When cells were stimulated by 30 nM of ACh, the cells usually generated oscillatory currents imposed on sustained currents in  $I_o$  and  $I_i$  during the stimulation. (B) A robust stimulation by high dose (300 nM) of ACh generated large and transient currents and desensitization even in the presence of ACh. L-NAME (1 mM) had no apparent inhibitory effect on these large currents, as in the case of pretreatment with L-NAME. (C) Summary of the effects of L-NAME on  $I_o$  in the presence of 300 nM of ACh. L-NAME (1 mM) did not inhibit the ionic currents raised by 300 nM of ACh, in contrast to what was observed in the case of 30 nM of ACh (Figure 3).

inhibitor 7-NI. Both L-NAME and 7-NI completely inhibited the ACh-induced NO synthesis, and this inhibitory effect was reversibly abolished by the subsequent addition of L-arginine. Further, Ionomycin mimicked the ACh-induced NO synthesis. These findings indicated that the activation of constitutive NOS, probably mainly nNOS, is essential for endogenous NO synthesis by ACh in tracheal gland cells.

In this study, we employed a fluorescent NO indicator, DAF-2DA. A fluorescent form, DAF-2, has the ability to bind with NO easily and specifically in the presence of oxygen, resulting in the highly fluorescent form DAF-2T (27). Because the cells were fully saturated with DAF-2 beforehand and gassed with 100%  $O_2$  during all experiments, it is not likely that either DAF-2 or oxygen were insufficient and cause a limited of NO synthesis. In addition, it is widely known that many cells have as much as 100  $\mu$ M to 2 mM of L-arginine in the cytosol (41) and that L-arginine is hardly exhausted. Therefore, in our study the fluorescent intensity of DAF-2T is thought to be in proportion to the total amount of synthesized NO. Furthermore, under stimulation

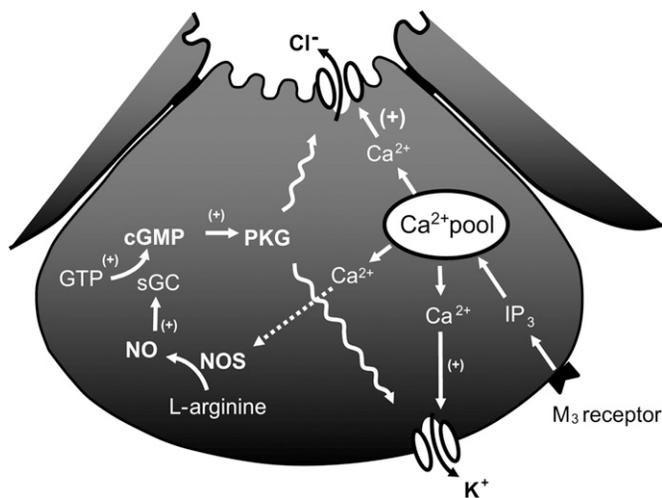
of Ionomycin, the increase in the fluorescence intensity was much stronger than that observed in the case of ACh 100 nM. These findings indicated that the tracheal gland cells had enough L-arginine to produce NO in the cytosol and that the plateau phase in the case of ACh-stimulation did not indicate the exhaustion of L-arginine. In the present study, we could not measure the concentration of NO in the tracheal glands precisely, but we can speculate on its concentration from our results. The intensity of fluorescence under the stimulation of 100 nM of ACh was between that in the presence of 0.5  $\mu$ M of NOC-5 and that of 10  $\mu$ M of NOC-5. The theoretical NO release (two NO per NOC-5) was calculated using the half-life time for NOC-5 of 25 min in PBS (pH 7.4) at 37°C (Calbiochem). We could determine that the intracellular NO concentration after stimulation of 100 nM of ACh should be around several  $\mu$ M in tracheal gland cells. These values were in good agreement with those in other cells (12–37  $\mu$ M in polymorphonuclear leukocytes (42) or 2–4  $\mu$ M in brain (43).

Articles concerning the physiologic properties of freshly isolated tracheal submucosal gland are quite limited in number (5, 14, 16, 17, 26, 44), and most studies investigating the submucosal gland electrophysiology made use of cells in culture (6, 12, 45–47). This is probably because the number of cells available is anatomically very small, and because small experimental animals including rat, mouse, rabbit, and goose have few tracheobronchial glands, if any (48). The submucosal gland bioelectric properties in fresh- and cultivated-cell preparations are similar in terms of the presence of  $[\text{Ca}^{2+}]_i$ -activated  $\text{Cl}^-$  secretion in response to cholinergic agents (5, 14, 16, 17, 26, 44). However, conflicting observations have been reported concerning the responses to some agents such as  $\beta$ -adrenergic agonists (12, 15, 49). Nevertheless, the electrophysiologic properties of fresh tracheal gland are quite similar to those of other freshly isolated exocrine glands, including lacrimal, salivary, and exocrine pancreas when investigated by a patch-clamp methodology. Moreover, an *in vivo* study demonstrated that cholinergic agents were much more potent stimulators of gland secretion than were adrenergic agonists, when estimated using hillock formations of a powdered tantalum layer coating the airway surface (50). Therefore, we used ACh as cholinergic agonists, which activate  $[\text{Ca}^{2+}]_i$ -induced ionic currents in freshly isolated submucosal preparations. Using proper channel inhibitors and ion substitution experiments, we previously reported that the ACh-induced  $I_o$  and  $I_i$  were carried mainly by  $\text{K}^+$  and  $\text{Cl}^-$ , respectively, and that these currents were dependent on  $[\text{Ca}^{2+}]_i$ , because the cells did not generate any ionic currents in the presence of a  $\text{Ca}^{2+}$  chelator, such as BAPTA (5, 14, 16, 17, 26). The  $I_i$  in exocrine acinar cells corresponded to the  $\text{Cl}^-$  secretion from the acini, which have been shown to parallel the water secretion physiologically. We have observed that when swine tracheal gland cells were stimulated by a high concentration of ACh (300 nM or more), large currents were transiently observed and decreased gradually to the pre-ACh levels (26). After such strong stimulation, the cells were desensitized and showed no apparent response for several minutes (Figure 6B). Such stimulations do not seem to be physiologically relevant. On the other hand, when the cells were stimulated by ACh at a very low concentration (10 nM or less), most of the cells showed no apparent response (26). From these observations, we assumed that the physiologically relevant ACh concentrations should be between 30 and 100 nM. Therefore, we used 30 nM of ACh for cells to generate physiologic electric responses (Figures 3–5). Indeed, cells usually generated oscillatory currents imposed on sustained currents in  $I_o$  and  $I_i$  under this stimulation *in vitro*. As described in our previous report (26), the magnitudes of ionic current induced by ACh 30 nM are dependent on the sensitivity or membrane capacity of each cell. It is impossible to make

these magnitudes completely even. Even if the responses are less robust, we think that the responses are suitable for meaningful comparisons, as long as they are sustained and showed the same effect by the same agent. Therefore, when we analyzed the data statistically, each ionic response was estimated by comparing the net electric charge movements of 20 s duration just before and after treatment with some agents such as L-NAME in the same cell. We also reported that there were two distinct types of response in porcine tracheal glands, when cells were stimulated by 30 nM of ACh. One showed a simultaneous activation of both oscillatory  $I_o$  and  $I_i$  (called the type I response in that manuscript, as shown in Figure 3A), and the other exhibited  $I_o$  alone with a minimal activity of  $I_i$  (called the type II response, as shown in Figures 3B or 3C). In that report, we described that, among the 116 successful whole cells tested, 45 cells (38.8%) showed the type I response, 62 cells (53.4%) showed type II, and the rest of the cells (nine cells, 7.8%) exhibited no response to 30 nM ACh. The unstained dispersed acinar cells showed no discernible difference in their appearance under a light microscope. Even in the type II cells, however,  $I_i$  became manifest with increasing concentrations of ACh. Our patch-clamp experiments revealed that both NOS inhibitors (L-NAME or 7-NI) and cGK inhibitors (KT-5823 and Rp-8-Br-cGMP) partially decreased the ACh-activated ionic currents, which were recovered by the removal of these inhibitors and the subsequent addition of L-arginine or NOC-5. KT-5823 has been reported to be a potent cGK I inhibitor only in cell-free systems and not in intact cells (34). However, we believe that KT-5823 works as a specific cGK inhibitor in tracheal gland cells, because (i) Carabelli and coworkers mention that KT-5823 acts as a selective cGK inhibitor in bovine chromaffin cells (51), (ii) Kwan and coworkers describe that KT-5823 prevents the inhibitory effect of 8-Br-cGMP on ACh-induced calcium oscillations in human bladder epithelial cells (52), (iii) both Rp-8-Br-cGMP and KT-5823 showed similar effects on ionic currents, and (iv) it is reported that KT-5823 does not act as a PKA inhibitor in the secretory response of tracheal submucosal glands. Because L-arginine or NOC-5 alone did not cause any ionic currents (Figures 3D and 3E), our findings suggest that NO/cGMP/cGK signaling acts as a potentiator of the electrolyte secretions in tracheal glands. Interestingly, this potentiating effect was observed only when cells were moderately stimulated by 30 nM of ACh, but not in the case of 300 nM of ACh (Figure 6B). It appeared that ACh-induced ionic currents in a physiologic situation may be maintained by two intracellular second messengers, that is,  $[\text{Ca}^{2+}]_i$  and NO. Indeed, a proper rise in  $[\text{Ca}^{2+}]_i$  is needed to secrete electrolytes, but a potent elevation in  $[\text{Ca}^{2+}]_i$  causes desensitization which would be disadvantageous in electrolyte secretion. Therefore, endogenous NO may act as a key molecule to cause sufficient electrolyte secretion without an excess rise in  $[\text{Ca}^{2+}]_i$ , resulting in the prevention of desensitization. In other words, NO/cGMP/cGK signaling plays a defensive role against desensitization by an excessive rise in  $[\text{Ca}^{2+}]_i$ .

Concerning the effect of NO against human airway anion secretions, Duszyk reported that NO by itself could activate transepithelial anion secretion via a cGMP-dependent pathway in Calu-3 cells and speculated that cGK activation would cause an activation of both  $\text{Ca}^{2+}$ -activated potassium (Kca) and  $\text{Ca}^{2+}$ -activated chloride (Clca) channels (46). However, our findings showed some differences from his. First, NO by itself did not generate any ionic currents in our study (Figure 3E). Second, we revealed that NO has potentiating effects on  $I_o$  and  $I_i$  only under the stimulation by a physiologically relevant low dose of ACh, but not in the case of an excessively high dose of ACh. We think these two points are original and essential for our study. It is widely known that many cells have enough L-arginine

in the cytosol (41). We believe that tracheal gland cells also contain enough L-arginine. However, gland cells never generate ionic currents in unstimulated conditions. Therefore, we think it is reasonable that L-arginine has no effect *per se* on ACh-induced ionic currents, unless NOS is inhibited. We revealed that NO has potentiating effects on  $I_o$  and  $I_i$  under the stimulation by such a low dose of ACh, but not in the case of both an excessive stimulation by high dose of ACh and nonstimulated control condition. Therefore, we think it is also reasonable that NOC-5 has no effect on ionic currents when cells are not stimulated by ACh. The main purpose of Figures 3D and 3E is to show that both L-arginine and NOC-5 do not induce any ionic currents *per se*, but has an effect to remove the inhibition by L-NAME on ACh-induced ionic currents, as shown in Figures 3B and 3C. These points are very important and different from other reports, which described that GSNO or other NO donors induced  $\text{Cl}^-$  secretion from airway epithelial cells (46, 53, 54). We do not have a clear explanation for these discrepancies. But we consider that one possibility may be differences between cultured cells and freshly isolated cells. A major advantage of our study was that we could use freshly isolated tracheal submucosal gland cells and observe the electrical responses stimulated by a physiologically relevant low dose of ACh. In the case of cultured cells, the cells were usually stimulated by a very high dose of agonist to get the maximum responses. We believe that the former responses are more physiologic than the latter. However, such considerations are still controversial.



**Figure 7.** A drawing delineating the proposed pathways of NO/cGMP/cGK signaling in electrolyte secretion in tracheal submucosal glands acinar cells. ACh-induced  $I_o$  and  $I_i$  were carried mainly by  $\text{K}^+$  and  $\text{Cl}^-$ , respectively, which were dependent on  $[\text{Ca}^{2+}]_i$ . The  $I_i$  in exocrine acinar cells corresponded to the  $\text{Cl}^-$  secretion from the acini, which has been shown to parallel the water secretion physiologically. In the present study, we revealed that ACh could also activate NOS and stimulate NO synthesis simultaneously. Endogenously synthesized NO could potentiate the ACh-induced ionic currents via the activation of cGK only in the case of a physiologically relevant low dose of ACh. When ACh stimulation was excessively robust, cells became desensitized and the NO effects disappeared. Indeed, a proper rise in  $[\text{Ca}^{2+}]_i$  is needed for electrolyte secretion, but a potent elevation results in desensitization and should be even considerably cytotoxic. Thus, the endogenous NO may act as a key molecule to cause sufficient electrolyte secretion without an excess rise in  $[\text{Ca}^{2+}]_i$ , resulting in the preservation of the electrolyte secretion and prevention from desensitization by an excessive increase in  $[\text{Ca}^{2+}]_i$  during electrolyte secretion.

Concerning the intracellular signaling pathways of physiologic secretions, the target molecule of cGK phosphorylation is still unclear. As mentioned by Duszyk (46), we also speculate that the effects of cGK phosphorylation may be to enhance the sensitivity of  $\text{Kca}$  and  $\text{Clca}$  against  $[\text{Ca}^{2+}]_i$  (see Figure 7). In other exocrine glands including pancreas (38), submandibular (39), and parotid glands (31), the same mechanisms appeared to be involved in the effects of NO on secretion. To confirm these speculations, further investigations will be needed. We think that one of the intracellular mechanisms of the potentiating effect of NO is a pathway via the activation of cGK, especially cGK I. Vaandrager and coworkers reported that the membrane localization of cGK II was important to activate CFTR in intestinal epithelium, but the soluble type I cGK did not work in the activation of CFTR (55). It is known that there are two major pathways in the chloride secretion from tracheal submucosal glands. One is characterized by the CFTR chloride channel, which is mainly activated by cAMP, and partially by cGMP. The other is caused by the  $\text{Ca}^{2+}$ -activated chloride channel. In our study, we focused on the latter one only and revealed that cGK I contributes to this pathway. Concerning the involvement of cGK II, it should be further investigated in the future.

In summary, the results of our study suggest that NO/cGMP/cGK signaling is involved in the physiologic signaling pathway of airway electrolyte secretion. It is known that NO synthesis is up-regulated in the airways in patients with bronchial asthma (56) and down-regulated in patients with cystic fibrosis (57).

These findings support the conclusion that an abnormal up-regulation of NO may have some influence on the pathophysiology of various chronic hypersecretory airway diseases, including chronic bronchitis or bronchial asthma, whereas the down-regulation of NO may contribute to dehydration of the airway surface liquid in chronic airway infectious diseases such as cystic fibrosis (4).

**Conflict of Interest Statement:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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