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# Cellular Mechanisms in Activation of Na-K-Cl Cotransport in Nasal Gland Acinar Cells of Guinea Pigs

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**Abstract.** The cellular regulation mechanism of Na-K-Cl cotransport was studied in dispersed acinar cells of the guinea pig nasal gland by a microfluorimetric imaging method using the $Na^+$-sensitive dye sodium-binding benzofuran isophthalate. Addition of 1 μM acetylcholine (ACh) induced an immediate increase in intracellular $Na^+$ concentration ($[Na^+]i$) by 36.7 ± 9.9 mM, which was almost completely abolished by the addition of atropine. The increased $[Na^+]i$ after cholinergic stimulation was due to the external $Cl^-$-dependent cotransport system (about 80% of the total $Na^+$ influx) and the dimethyl amiloride-sensitive $Na^+$-$H^+$ exchange system (of about 20%). The ACh-induced increase in $[Na^+]i$ was dependent on extracellular $Ca^{2+}$ and was prevented by pretreatment with 8-(N, N-diethylamino)octyl-3,4,5-trimethoxybenzoate or O-O′-bis(2-aminophenyl)ethyleneglycol-N, N, N′, N′-tetraacetic acid tetraacetoxymethyl ester. Addition of 1μM ionomycin mimicked the ACh-induced increase in $[Na^+]i$ which was dependent on external $Cl^-$. Moreover, both a calmodulin antagonist trifluoperazine and a myosin light chain kinase inhibitor ML-7 reduced the ACh-induced response in $[Na^+]i$. However, the following treatment did not affect the basal $[Na^+]i$ nor the ACh-induced increase in $[Na^+]i$: (i) addition of dibutyryl cAMP, 8-Br-cGMP, or phorbol 12-myristate 13-acetate, (ii) pretreatment of protein kinase inhibitors, H-89, H-8, H-7 or chelerythrine, (iii) prevention of cytosolic $Cl^-$ efflux by the addition of diphenylamine-2-carboxylic acid or, (iv) prevention of cytosolic $K^+$ efflux by the addition of charybdotoxin. The present results suggest that the ACh-induced increase in $[Na^+]i$, mainly responsible for activation of Na-K-Cl cotransport, is mediated by a $Ca^{2+}$/calmodulin-dependent phosphorylation.

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**Key words:** Na-K-Cl cotransport — Nasal gland — Acetylcholine — Intracellular Na — Intracellular Ca — Calmodulin

## Introduction

Cholinergic stimulation to the mammalian nasal cavity evokes secretory responses, mainly originating from the mammalian nasal gland acinar cells distributed in the submucosal layer (Gawin et al., 1991). In the nasal gland serous acinar cells dissociated from the guinea pig nasal septum, we demonstrated that application of acetylcholine (ACh) increased the intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]i$), leading to augmentation of both $K^+$ and $Cl^-$ currents (Sunose et al., 1994; Ikeda et al., 1995*a*; Wu et al., 1994). Applying a recent exocrine acinar cell model Petersen (1992) and Nauntofte (1992) demonstrated that agonist-induced NaCl secretion is induced by $Cl^-$ exit via the apical cell membrane, accompanied by paracellular Na movement (Frömter & Diamond, 1972). Intracellular $Cl^-$ is maintained at a high level by Na-K-Cl cotransport and the combined $Na^+$-$H^+$ and $Cl^-$-$HCO_3^-$ exchange, which results in the increase in intracellular $Na^+$ concentration ($[Na^+i]$). Thus, Na uptake mechanisms of both Na-K-Cl cotransport and $Na^+$-$H^+$ exchange contribute to a continuous secretion induced by secretagogues. In the nasal gland acini, the Na-K-Cl cotransport is mainly responsible for the net $Na^+$ influx induced by ACh in a $HCO_3^-$-free solution (Ikeda et al., 1995*b*).

The cellular and molecular regulation of the Na-K-Cl cotransport varies considerably between different tissues (Geck & Heinz, 1986; O'Grady et al., 1987; Haas, 1989). In the exocrine glands, the $K^+$ and $Cl^-$ losses inside the cell resulting from stimulation favor the overall chemical gradient for the cotransport (Nauntofte, 1992). On the other hand, current studies suggest that

the cotransport is directly phosphorylated by an adenylate cyclase/cyclic AMP system, a $Ca^{2+}$/diacylglycerol-mediated pathway, and multiple protein kinase/phosphate systems (Torchia et al., 1992; Paulais & Turner, 1992; Lytle & Forbush, 1992).

In the present study, we present evidence that ACh-induced activation of the Na-K-Cl cotransport results from a $Ca^{2+}$/calmodulin-dependent mechanism in the nasal gland acini.

## Materials and Methods

### Preparation of Isolated Nasal Gland Acinar Cells

Healthy albino guinea pigs weighing 200 to 300 g were anesthetized by inhalation of diethyl ether. After decapitation, the nasal septum was quickly removed and suspended in an oxygenated cell-storage solution. We developed a dissociation procedure to prevent the contamination of the surface epithelium. The septal mucosa was separated from the cartilage beneath the cartilaginous membrane and inverted it on a Petri dish containing the cell-storage solution. Following the removal of the cartilaginous membrane, the area containing a large amount of nasal glands was meticulously dissected using fine forceps and ophthalmic scissors without injury of the epithelial lining and then minced into cubes varying the size from 0.2 to 0.5 mm. The pooled fragments were suspended in an isolation solution and incubated for 20–30 min at 37°C in a shaking water bath. The isolation solution was made by removing $Ca^{2+}$ from the standard solution and adding 1 mM ethylene glycol-bis(β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA), 0.2% bovine serum albumin, and 100 U/ml collagenase. The digested tissue was dissociated by flushing it 10–20 times through a siliconized Pasteur pipette, and then the filtered clusters of the cells were washed and centrifuged (1000 rpm, 1 min) three times and the resulting sediment was resuspended in a fresh cell-storage solution without collagenase. The final cell preparation was mainly composed of nasal gland acini as revealed by microscopic and ultrastructural observations previously reported (Sunose et al., 1994, Figs. 3 and 4).

### Fluorescent Dye Loading

The dispersed acini were incubated with 5 μM acetoxymethyl ester of sodium-binding benzofuran isophthalate (SBFI) at 37°C for 50 to 60 min. The ester was dissolved in dimethyl sulphoxide at a concentration of 5 mM and mixed with an equal volume of 10% w/v Pluronic F-127. After dye loading the acini were washed once with the cell-storage solution and stored under a light-free condition until use. The coverslip coated with a natural cell adhesive, Cell Tak, was put in the Petri dish containing enough humidity for 20 min to allow the immobilization of the cells. The coverslip was then placed in a superfusion chamber with a bath capacity of 0.2 ml which was mounted on the stage of an inverted epifluorescence microscope. During the experiments, acinar cells were continuously superfused with the desired solutions oxygenated and warmed to 37°C at a rate of 1.6 ml/min ensuring the exchange of the bathing solution within several seconds.

Instrumentation used for digital imaging has been reported previously (Ikeda et al., 1991; Ikeda et al., 1992*a*, Ikeda et al., 1992*b*). Briefly, the cells mounted on the epifluorescence microscope were alternately illuminated by 340 nm and 380 nm excitation lights from an arc lamp through neutral density filters, 10 nm bandpass filters, a 440 nm dichroic mirror and an objective lens (DPlan Apo 10 × UV, N.A.: 0.40, Olympus). Emitted light path included the objective, the dichroic mirror and a 510 nm bandpass filter. Images were focused on a silicon intensifier target camera (SIT camera C-2400-8, Hamamatsu Photonics, Hamamatsu, Japan) and analyzed by a digital image processor (ARGUS-50, Hamamatsu Photonics). The viewfield of the SIT camera consisted of 512 × 483 picture elements (pixels) and one pixel corresponding to a 1.0 × 1.0 $\mu m^2$ area of the object. All the images were obtained for paired excitation radiations by averaging 16 frames per 0.53 sec for each excitation wavelength usually at every 10-sec interval. The excitation radiation was transmitted only during the data-collection cycle. After background subtraction, the images of fluorescence ratio were obtained by dividing, pixel by pixel, at 340 nm and 380 nm excitations ($F_{340}$ and $F_{380}$ respectively).

### In Situ Calibration and Calculation of $[Na^+]i$

Calibration of the fluorescence intensity ratio in terms of $[Na^+]i$ was accomplished *in situ* (Harootunian et al., 1989). Exposure of the cells to various mixture of isotonic NaCl and KCl solutions containing 1.0 mM $Ca^{2+}$, 1.0 mM $Mg^{2+}$, and 5.0 mM N-2-hydroxymethylpiperazine-N′-2-ethanesulfonic acid (HEPES)/tris-(hydroxymethyl)aminomethane (TRIS) mixture for 15 min in the presence of ionophores, 2 μM gramicidin D, 5 μM monensin and 5 μM nigericin, permits calibration of $F_{340}/F_{380}$ ratios for $[Na^+]i$. The correlation of the fluorescence ratios and logarithm of $[Na^+]i$ were then analyzed via linear regression analysis, and the equation derived in this manner was used to calculate $[Na^+]i$ (Ikeda et al., 1992*b*).

### Solutions and Chemicals

The standard solution contained (in mM unless otherwise stated: 139.2 $Na^+$, 4.69 $K^+$, 2.56 $Ca^{2+}$, 1.13 $Mg^{2+}$, 136.1 $Cl^-$, 4.91 pyruvate$^-$, 5.38 fumarate$^-$, 4.92 glutamate$^-$, 2.8 glucose, and 5.0 HEPES/TRIS mixture with pH adjusted to 7.4 at 37°C. The cell-storage solution was prepared by adding 0.2% bovine serum albumin in the standard solution, and $NO_3^-$ solution was prepared by replacing all $Cl^-$ with $NO_3^-$. SBFI acetoxymethyl ester and Pluronic F-127 were from Molecular Probes (Eugene, OR), Cell-Tak from Collaborative Research (Bedford, MA), dimethyl amiloride and 8-(diethylamino)octyl-3, 4, 5-trimethoxybenzoate hydrochloride (TMB-8) and charybdotoxin (ChTX) from Research Biochemicals (Natick, MA), ACh, ouabain, atropine, furosemide, collagenase (type IV), dibutyryl cAMP (db-cAMP), 3-isobutyl-1-methylxanthine (IBMX), forskolin, 8-Br-cGMP, phorbol 12-myristate 13-acetate (PMA), trifluoperazine, gramicidin D, monensin, and nigericin from Sigma (St. Louis, MO), vasoactive intestinal peptide (VIP) from Peptide Institute (Osaka, Japan), O-O′-bis(2-aminophenyl)-ethyleneglycol-N,N,N′,N′-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM) from Dojindo Laboratories (Kumamoto, Japan), ionomycin from Calbiochem (La Jolla, CA), chelerythrine chloride from LC laboratories (Woburn, MA), and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), N-2-(methylamino)-ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-8), N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) and 1-[N,O-bis(1, 5-isoquinolinesulfonyl)-N-methyl-L-tyrosy]-4-phenylpipezazine (KN-62), and 1-(5-iodonaphthalene-1-sufonyl)-1H-hexahydro-1, 4-diazepine hydrochloride (ML-7) from Seikagaku Kogyo (Tokyo, Japan). Other chemicals were purchased from Wako Junyaku (Tokyo, Japan).

### Statistics

The experimental values are given as the mean ± SD. Statistical significance of the data was analyzed by paired or unpaired *t*-test and *P* values less than 0.05 were accepted as significant.

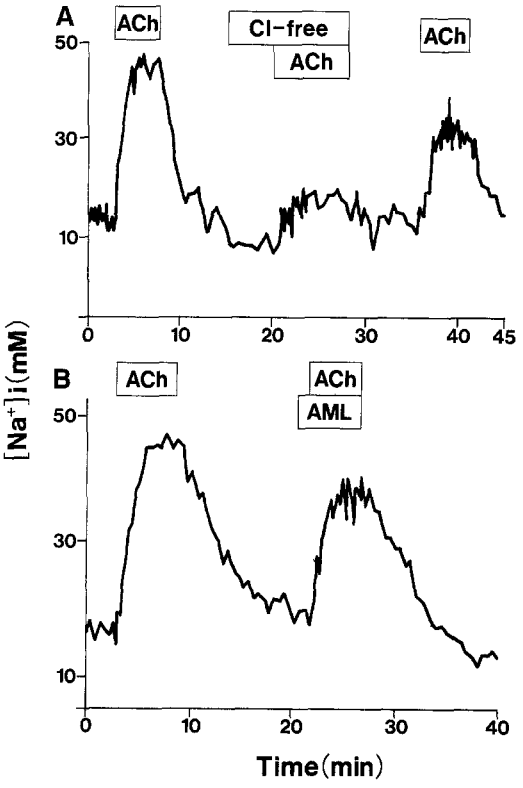


**Fig. 1.** Effect of superfusion of $Cl^-$-free $NO_3^-$ solution (*A*) and addition of dimethyl amiloride (AML, 50 μM, *B*) on ACh-induced increase in $[Na^+]i$.

## Results and Discussion

### Muscarinic Activation of Na-K-Cl Cotransport

Under the unstimulated condition, the values of $[Na^+]i$ were distributed in the range between 10 mM and 20 mM with a mean of 13.3 ± 5.8 mM (n = 104), which consisted well with our previous report (Ikeda et al., 1994*b*). Addition of 1 μM ACh to the standard solution caused an immediate increase in the $[Na^+]i$, which reached a peak value within 3 min. The peak magnitude of the increase in $[Na^+]i$ ($\Delta[Na^+]i$) was 36.7 ± 9.9 mM (n = 104). Washout of ACh resulted in a gradual decrease in $[Na^+]i$ to the control value. Addition of 10 μM atropine almost completely inhibited ACh-induced increase in $[Na^+]i$ (−1.5 ± 2.4 mM from the baseline, n = 9), indicating that the increase in $[Na^+]$ is activated by a muscarinic cholinergic receptor stimulation. The contribution of the two transport processes, Na-K-Cl cotransport and $Na^+$-$H^+$ exchange, to the increase in $[Na^+]i$ was examined (Fig. 1). Removal of $Cl^-$ in the standard solution remarkably inhibited the increase in $[Na^+]i$ induced by ACh as compared with the control solution ($\Delta[Na^+]i$ = 28.6 ± 3.2 mM in the control versus 6.2 ± 1.5 mM in the $Cl^-$-free condition, n = 5). Readmission of the standard NaCl solution restored the ACh responses in the acinar cells, which corresponded to 82.6 ± 18.0% of the initial increase in $[Na^+]i$ by ACh. A similar result was obtained by the addition of 0.5 mM furosemide, a relatively specific inhibitor of Na-K-Cl cotransport (82.5 ± 3.5% inhibition, n = 5). Addition of 10 μM bumetanide, a more potent and specific inhibitor of the cotransport than furosemide, also inhibited ACh-induced increase in $[Na^+]i$ by 72.3 ± 10.9% (n = 4). Addition of 50 μM dimethyl amiloride, a specific inhibitor of the $Na^+$-$H^+$ exchange, caused a small but irreversible inhibition of ACh-induced increase in $[Na^+]i$ ($\Delta[Na^+]i$ = 33.6 ± 5.2 mM in the control *vs.* 26.2 ± 4.3 mM in the presence of dimethyl amiloride, n = 5). The ACh-induced increase in $[Na^+]i$ mainly arises from the activation of the Na-K-Cl cotransport since the cotransport contribute to about 80% of the total $Na^+$ influx and to only 20% of the $Na^+$-$H^+$ exchange. However, other transport pathways such as nonselective cation channels and metabolic substrate-dependent $Na^+$ influx cannot be ruled out.

The present study was designed to elucidate the intracellular route of signal transduction mediating the activation of the Na-K-Cl cotransport by ACh. Major pathways have been proposed: (i) cAMP-protein kinase A (PKA) pathway (O'Grady et al., 1987; Haas, 1989) and cGMP system (O'Donnell & Owen, 1986). (ii) alterations in chemical gradients for $K^+$ and $Cl^-$ (Petersen & Maruyama, 1984; Petersen 1992) and a decrease in intracellular $Cl^-$ concentration ($[Cl^-]i$) (Breitwieser, Altamirano & Russell, 1990; Haas & McBrayer, 1994) (iii) cytosolic $Ca^{2+}$ (Brock et al., 1986; Smith & Smith, 1987), and (iv) protein kinase C (Paris & Pouyssegur, 1986).

### Effects of Cyclic AMP and GMP Systems

The stimulatory effects of the cAMP-PKA system on the Na-K-Cl cotransport has been demonstrated in a variety of cells (O'Grady et al., 1987; Haas, 1989). In the present experiments, we examined the effects of activations of the cAMP-PKA system using db-cAMP (a membrane permeable analogue of cAMP), forskolin/IBMX (an inhibitor of phosphodiesterase) and VIP, as well as the inhibition using H-89. Addition of db-cAMP (1 mM) did not affect the $[Na^+]i$ in the acinar cells in which an ACh-induced increase in $[Na^+]i$ was demonstrated. A similar result was obtained by the addition of 10 μM forskolin/100 μM IBMX (Table 1). VIP (1 μM) which was reported to raise intracellular levels of cAMP in other exocrine gland (Stoff et al., 1979; Torchia et al., 1992) and to evoke glandular fluid secretion in human nasal mucosa (Baraniuk et al., 1990), had no effect on the $[Na^+]i$ nor on the ACh-induced increase in the $[Na^+]i$ (Table 1). Na-K-Cl cotransport in the avian salt gland (Torchia et al., 1992) and in the shark rectal gland (Lytle

**Table 1.** Effects of activators and inhibitors of cAMP and cGMP systems on intracellular $Na^+$ concentration ($[Na^+]i$)

| | Increase in basal $[Na^+]i$ (mM) | ACh-induced change in $[Na^+]i$ (mM) |
|---|---|---|
| Dibutyryl cAMP (1 mM) | 0.5 ± 0.1 (8) | |
| Forskolin (10 μM)/ IBMX (100 μM) | 0.3 ± 0.2 (5) | |
| VIP (1 μM) | 0.1 ± 0.2 (14) | |
| 8-Br-cGMP (1 mM) | 0.3 ± 0.4 (14) | |
| None (control) | | 33.5 ± 4.5 (22) |
| H-89 (10 μM) | 1.1 ± 1.5 (14) | 35.2 ± 8.5 (14) |
| H-8 (10 μM) | 2.2 ± 0.9 (14) | 33.8 ± 6.5 (14) |

Changes in the basal $[Na^+]i$ were determined after incubation of each drug for 10 min. Acetylcholine (ACh) at a concentration of 1 μM was added to cells and induced changes denote the difference between $[Na^+]i$ prior to the peak 5 min after addition of ACh. Data are shown in mean ± SD. Number of observations is given in the parentheses. IBMX, 3-isobutyl-1-methyl xanthine; VIP, vasoactive intestinal peptide; H-89, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; H-8, N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride.

**Table 2.** Effects of activators and inhibitors of protein kinase C on intracellular $Na^+$ concentration ($[Na^+]i$)

| | Increase in basal $[Na^+]i$ (mM) | ACh-induced change in $[Na^+]i$ (mM) |
|---|---|---|
| None (control) | | 37.5 ± 8.2 (15) |
| PMA (1 μM) | 2.4 ± 1.5 (7) | 34.5 ± 7.2 (8) |
| H-7 (50 μM) | −1.1 ± 2.5 (7) | 35.2 ± 9.5 (7) |
| Chelerythrine (10 μM) | 1.5 ± 3.8 (14) | 34.0 ± 7.5 (14) |

Changes in the basal $[Na^+]i$ were determined after incubation of each drug for 10 min. Acetylcholine (ACh) at a concentration of 1 μM was added to cells and induced changes denote the difference between $[Na^+]i$ prior to the peak 5 min after addition of ACh. Data are shown in mean ± SD. Number of observations is given in the parentheses. PMA, phorbol 12-myristate 13-acetate; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride.

& Forbush, 1992) has been demonstrated to be regulated by direct phosphorylation of the cAMP-dependent PKA pathway. The effect of 10 μM H-89, known as a selective inhibitor of PKA (Chijiwa et al., 1990), on the basal $[Na^+]i$ was negligibly small. When ACh was added to the cells pretreated with H-89, a similar increase induced by ACh alone was observed (Table 1). This result provides further evidence denying the contribution of cAMP-dependent phosphorylation to upregulate the cotransport.

In vascular smooth muscle cell (O'Donnell & Owen, 1986), activation of the cotransport by atrial natriuretic peptide is mediated by cGMP-dependent protein kinase, whereas the cotransport in winter flounder intestine (DeJonge et al., 1985) is inhibited by cGMP-dependent phosphorylation. The addition of a membrane permeable cGMP analogue 8-Br-cGMP (1 mM) to the standard solution did not affect the $[Na^+]i$ in the nasal gland acinar cells (Table 1). H-8 (10 μM), a protein kinase inhibitor of both cGMP- and cAMP-dependent protein kinase (Hagiwara, Inagaki & Hidaka, 1987), showed no significant effect on the $[Na^+]i$ response induced by ACh (Table 1). These findings indicate that cAMP- and cGMP-dependent protein phosphorylation are unlikely to regulate the Na-K-Cl cotransport stimulated by ACh.

### Effects of $Cl^-$ and $K^+$ Efflux

Based on a recent cell model for exocrine gland transport (Petersen, 1992; Nauntofte, 1992), the activation of the cotransport resulting from muscarinic cholinergic receptor stimulation is secondary to the activation of apical $Cl^-$ or basolateral $K^+$ channels. In addition to a possible gradient effect, it has now been well documented in a number of tissues that a decrease in $[Cl^-]i$ activates Na-K-Cl cotransport independently from a gradient effect or an effect of cell volume (Breitwieser et al., 1990; Haas & McBrayer, 1994). The opening of both channels can decrease each cellular content, leading to a favorable chemical gradient for a net uptake of the Na-K-Cl cotransport. In the nasal gland acinar cells of the guinea pig, cholinergic stimulation produces a large augmentation of the $Cl^-$ current followed by a slight increase in the $K^+$ current (Wu et al., 1994). Therefore, the increase in $Cl^-$ and $K^+$ permeability induced by ACh may favorably alter the driving force of the cotransport. In a whole-cell clamp study (Wu et al., 1994) ACh-evoked $Cl^-$ current was almost completely inhibited by the addition of diphenylamine-2-carboxylic acid (DPC). Addition of 1 mM DPC during ACh application slightly inhibited the increased $[Na^+]i$ by 7.8 ± 6.1% (n = 6), (Fig. 2*A*). The increased $[Na^+]i$ was inhibited only by 15.9 ± 7.4% (n = 8) by exposure to DPC prior to and during the superfusion of ACh (Fig. 2*B*). Removal of external $Cl^-$ almost completely suppressed the increase of $[Na^+]i$ ($\Delta[Na^+]i = 31.9 \pm 4.8$ mM, n = 8) induced by ACh in the presence of DPC to the basal value ($\Delta[Na^+]i = 2.0 \pm 6.4$ mM, n = 8), indicating that ACh-induced $[Na^+]i$ increase nearly insensitive to DPC is totally due to the $Cl^-$-coupled mechanism. The slight but significant inhibition by DPC might represent a direct effect on the cotransporter (Wittner et al., 1987). The potent inhibitor of $Ca^{2+}$-activated $K^+$ channels, ChTX (0.1 μM), inhibited only 2.5 ± 1.3% (n = 5) of the $[Na^+]i$ increase induced by ACh. These data suggest that changes in the driving as well as a decrease in $[Cl^-]i$ contributed slightly to the activation mechanism in the cotransport.

### Activation by Intracellular $Ca^{2+}$

Activation of a muscarinic receptor on the plasma membrane, which leads to phosphoinositide breakdown gen-

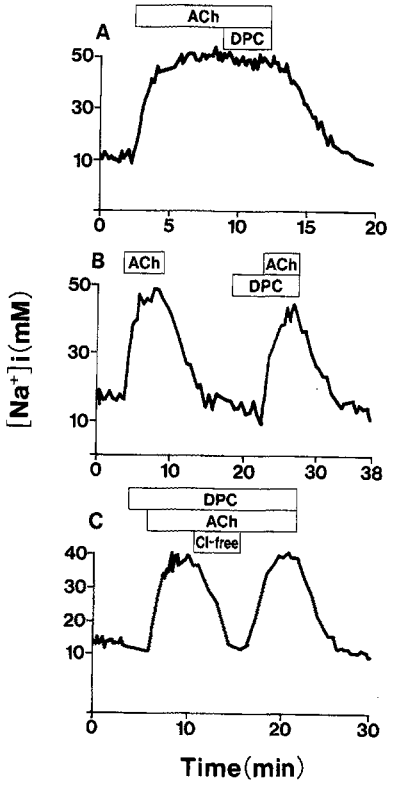


**Fig. 2.** Effect of diphenylamide-2-carboxylic acid (DPC, 1 mM) on ACh-induced increase in $[Na^+]i$. During application of ACh, DPC was added to the superfusate (*A*). The effect of pretreatment of DPC on ACh-induced response in $[Na^+]i$ was examined (*B*). During exposure to DPC, effect of removal of external $Cl^-$ replaced by $NO_3^-$ on ACh-induced $[Na^+]i$ response was tested (*C*).

erating diacylglycerol and inositol 1, 4, 5-trisphosphate ($IP_3$) in exocrine glands, results in the release of $Ca^{2+}$ from internal $Ca^{2+}$ stores (Berridge & Irvine, 1989). In the guinea pig nasal gland acinar cells, muscarinic cholinergic stimulation produces a biphasic increase in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]i$); an initial peak arising from a release of $Ca^{2+}$ from the internal pools followed by a sustained plateau arising from $Ca^{2+}$ entry from extracellular space (Ikeda et al., 1995*a*). Stimulatory effect of cytosolic $Ca^{2+}$ on the cotransport activity is not surprising in light of signaling transduction processes.

In the absence of external $Ca^{2+}$, the increased $[Na^+]i$ ($\Delta[Na^+]i$ = 38.6 ± 11.2 mM, n = 7) induced by ACh returned to the basal level and reached 12.1 ± 5.2 mM (n = 7) in 5 min (Fig. 3*A*). In the presence of $Ca^{2+}$, ACh obtained a peak value of $[Na^+]i$ to 44.1 ± 11.2 mM (n = 7), which was not significantly different from that in the absence of external $Ca^{2+}$. In the presence of external $Ca^{2+}$, however, it reached 38.7 ± 14.0 mM (n = 7) in 5 min, which was significantly greater than that in the absence of $Ca^{2+}$ (18.3 ± 8.4 mM, $P < 0.01$, paired *t*-test, n = 7) (Fig. 3*A*). Thus, the ACh-induced increase in $[Na^+]i$ was more prolonged in the presence than in the absence

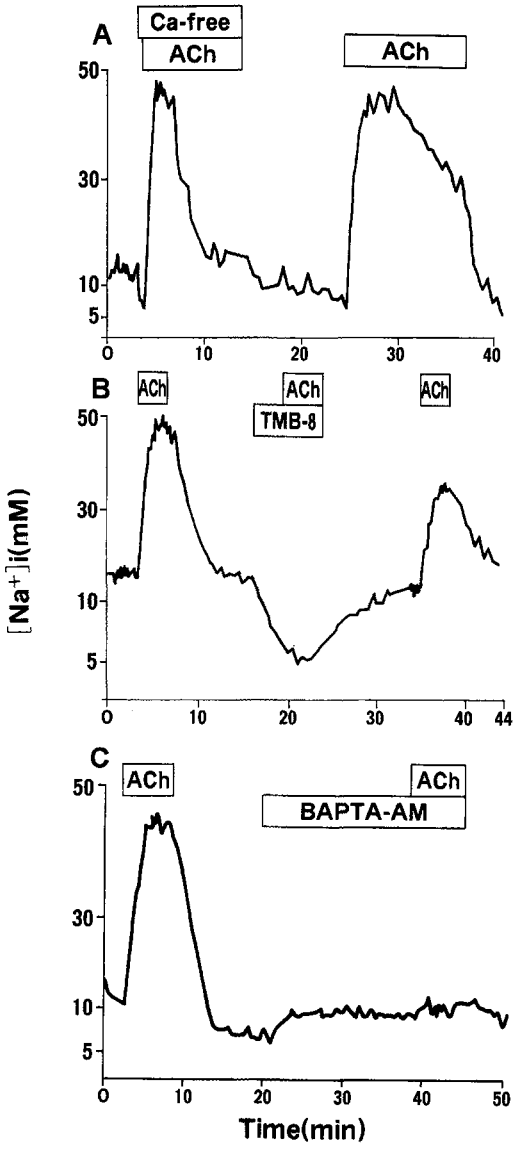


**Fig. 3.** Effect of intracellular and extracellular $Ca^{2+}$ on ACh-induced increase in $[Na^+]i$. ACh was applied in the absence and presence of external $Ca^{2+}$ (*A*). The effect of TMB-8 in the $Ca^{2+}$-free solution was examined (*B*). ACh-induced $[Na^+]i$ response was examined by pretreatment of BAPTA-AM in the $Ca^{2+}$-free solution (*C*). $Ca^+$-free solution contained 0.2 mM EGTA.

of external $Ca^{2+}$. We have already reported that the addition of TMB-8, intracellular $Ca^{2+}$ antagonist (Chiou & Malagodi, 1975), almost completely suppressed the mobilization of $Ca^{2+}$ from the internal stores in the nasal gland acinar cells (Ikeda et al., 1995*a*). In the absence of external $Ca^{2+}$, addition of 50 μM TMB-8 significantly reduced the basal $[Na^+]i$ and completely prevented the ACh-induced increase in $[Na^+]i$ which reached 8.3 ± 2.4 mM (n = 11) below the basal value (Fig. 3*B*). Pretreatment with 50 μM BAPTA-AM, intracellular $Ca^{2+}$ chelator, 20 min before and during ACh application resulted in

a remarkable reduction in ACh-induced increase in $[Na^+]i$ by 93.5 ± 6.8% (n = 8) of the control (Fig. 3*C*). Based on these findings, intracellular $Ca^{2+}$ plays a central and critical role in the ACh-induced increase in $[Na^+]i$ and a rise in $[Ca^{2+}]i$ is required for activation of $Na^+$-$H^+$ exchange as well as Na-K-Cl cotransport.

To confirm further the significant role of internal $Ca^{2+}$ in regulating the cotransport, $Ca^{2+}$ ionophore was tested. Addition of 1 μM ionomycin increased the $[Na^+]i$ by 25.4 ± 7.5 mM (n = 7), which corresponded to 76.7 ± 25.0% of that induced by 1 μM ACh. Ionomycin-induced increase in $[Na^+]i$ was significantly reduced by 59.1 ± 19.6% (n = 7) by removal of external $Cl^-$. The addition of 50 μM dimethyl amiloride in the $Cl^-$-free solution almost completely suppressed the ionomycin-induced increase in $[Na^+]i$ (89.5 ± 23.6% inhibition, n = 5). The increased $[Ca^{2+}]i$ caused by ionomycin activates $Na^+$ influx through Na-K-Cl cotransport (Brock et al., 1986; Smith & Smith, 1987) and $Na^+$-$H^+$ exchange (Manganel & Turner, 1989; Okada et al., 1991; Zhang, Gragoe & Melvin, 1992).

To investigate further the cellular transduction pathway stimulating Na-K-Cl cotransport activity, the effect of calmodulin antagonist was examined. The rise in $[Na^+]i$ ($\Delta[Na^+]i$ = 40.3 ± 12.1 mM, n = 12) induced by ACh was significantly inhibited by pretreatment of 50 μM trifluoperazine for 2 min, causing the $[Na^+]$ to decrease by 35.2 ± 8.8 mM (n = 12). The $[Ca^{2+}]i$ measured by fura-2 was not influenced by addition of trifluoperazine (*data not shown*). These findings suggest that Na-K-Cl cotransport is mediated by $Ca^{2+}$/calmodulin-dependent mechanism. In the next series of experiments, two types of inhibitors of $Ca^{2+}$/calmodulin-dependent protein kinase were tested. KN-62 (5 μM), a selective and potent inhibitor of $Ca^{2+}$/calmodulin-dependent protein kinase II (Tokumitsu et al., 1990), had no influence on ACh-induced increase in $[Na^+]i$ (6.5 ± 10.5% inhibition, n = 10, Fig. 4*A*). On the other hand, exposure to 50 μM ML-7, an inhibitor specific for smooth muscle myosin light chain kinase (Saitoh et al., 1987), prior to and during ACh application significantly and irreversibly inhibited the increase in $[Na^+]i$ induced by ACh by 64.7 ± 7.4% (n = 14) (Fig. 4*B*). Both protein kinase inhibitors showed no change in the increase in $[Ca^{2+}]i$ induced by ACh (*data not shown*).

In the endothelial cell (Brock et al., 1986; Smith & Smith, 1987; O'Donnell, 1991) and in the vascular smooth muscle cells (Smith & Smith, 1987; Owen & Ridge, 1989), Na-K-Cl cotransport activity was reduced by addition of TMB-8, and the removal of external $Ca^{2+}$, under both stimulated and unstimulated conditions, and was increased by the addition of $Ca^{2+}$ ionophore. Similar results were obtained in the nasal gland acinar cells. Since TMB-8 treatment to prevent the mobilization of $Ca^{2+}$ from internal stores reduces cotransport activity and ionomycin treatment to increase the $[Ca^{2+}]i$ augments its

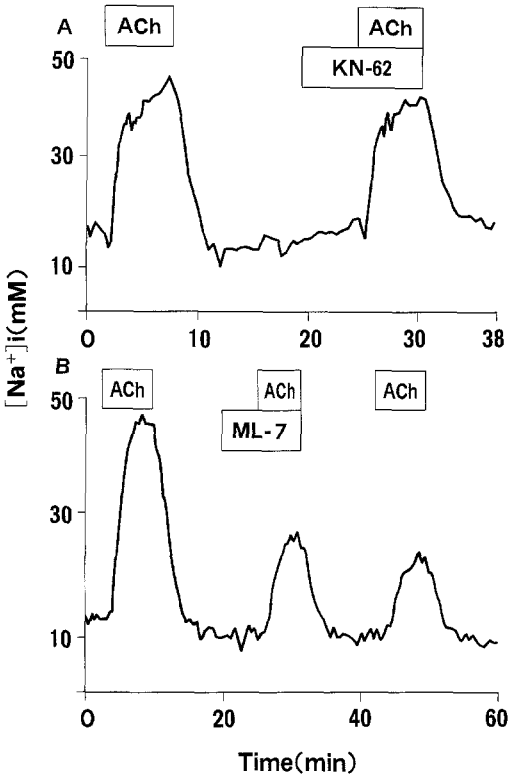


**Fig. 4.** Effect of protein kinase inhibitors, 5 μM KN-62 (*A*) and 50 μM ML-7 (*B*), on ACh-induced increase in $[Na^+]i$.

activity, they both indicate that the activity is dependent on intracellular $Ca^{2+}$. Treatment of endothelial cells with calmodulin antagonist W-7 reduces the cotransport activity activated by angiotensin II, vasopressin, or bradykinin (O'Donnell, 1991). In the guinea pig nasal gland acinar cells, addition of trifluoperazine significantly suppresses the ACh-induced increase in Na-K-Cl cotransport activity, suggesting that stimulation of the cotransport involves phosphorylation by a $Ca^{2+}$/calmodulin-dependent kinase. Further pharmacological experiments suggest that the cotransport is activated by phosphorylation by myosin light chain kinase. Current studies on the cotransport in the avian salt glands provide evidence suggesting that an endogenous membrane bound protein closely related to chloride secretion is phosphorylated by the carbachol-stimulation (Torchia et al., 1991) and that cotransport is directly phosphorylated via a $Ca^{2+}$/diacylglycerol-mediated pathway (Torchia et al., 1992).

### Effect of Protein Kinase C

It is well accepted that stimulation by ACh leads to the activation of PKC (Berridge, 1984; Nishizuka, 1984). Exposure of the endothelial cell (O'Donnell, 1991) and the ocular ciliary body (Crook, von Brauchitsch, Polansky, 1992) to phorbol ester reduces Na-K-Cl cotransport.

We evaluated whether this enzyme is involved in mediating the cotransport or regulating the ACh-induced activation of the cotransport.

Superfusion with 1 μM PMA did not significantly change the basal level of $[Na^+]i$ ($\Delta[Na^+]i = 2.4 \pm 1.6$ mM, n = 7). Preincubation of 1 μM PMA for 10 min did not influence the ACh-induced increase in $[Na^+]i$ either (Table 2). The increase in $[Na^+]i$ induced by ACh was not affected by the pretreatment with PKC inhibitor H-7 (Kawamoto & Hidaka, 1984) at 50 μM for 10 min (Table 2). Recently available PKC inhibitor showing more potent and selective action than H-7 (Herbert et al., 1990), 10 μM chelerythrine, also resulted in an insignificant effect on the increase in $[Na^+]i$ induced by ACh (Table 2). Thus, it is predicted that the Na-K-Cl cotransport in the guinea pig nasal gland acinar cell has no direct effect on the PKC pathway.

### Regulatory Mechanism of Na-K-Cl Cotransport

The role of intracellular $Ca^{2+}$ in the stimulatory regulation of the Na-K-Cl cotransport has been proposed in endothelial cells (Brock et al., 1986; Smith & Smith, 1987; O'Donnell, 1989,1991), vascular smooth muscle cells (Smith & Smith, 1987; Owen & Ridge, 1989), and the avian salt gland (Torchia, 1992). The present study showed activation of the Na-K-Cl cotransport mediated by cytosolic $Ca^{2+}$ based on the following evidences: (i) activation of the cotransport by ACh was abolished when ACh-induced elevation of $[Ca^{2+}]i$ was suppressed by elimination of extracellular $Ca^{2+}$ and addition of TMB-8 or BAPTA-AM, (ii) an increase in $[Ca^{2+}]i$ induced by ionomycin activated the cotransport and (iii) treatment of anticalmodulin antagonist inhibited cotransport activity. On the other hand, the role of cAMP/PKA, cGMP, or PKC pathway in ACh-induced activation of the Na-K-Cl cotransport can be neglected since activators and inhibitors of these pathways failed to affect the $[Na^+]i$ itself and the ACh-induced increase in $[Na^+]i$. Furthermore, the activation of cotransport by ACh was not blocked by pharmacological inhibitors of $Cl^-$ or $K^+$ channels.

A muscarinic cholinergic stimulation to the guinea pig nasal gland acinar cells leads to release of $Ca^{2+}$ from internal stores followed by a continuous $Ca^{2+}$ influx. Increased $[Ca^{2+}]i$ activates the opening of both $K^+$ and $Cl^-$ channels, resulting in rapid loss of intracellular $K^+$ and $Cl^-$. Simultaneously, intracellular $K^+$ and $Cl^-$ are provided by activation of the Na-K-Cl cotransport mediated by increased $[Ca^{2+}]i$, presumably involving phosphorylation by $Ca^{2+}$/calmodulin-dependent myosin light chain kinase. Thus, the rise in $[Ca^{2+}]i$ plays a central role in the regulation of stimulus-secretion coupling in nasal gland acinar cells.

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