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IONIC CURRENTS EVOKED BY ACETYLCHOLINE IN ISOLATED ACINAR CELLS OF THE GUINEA PIG NASAL GLAND

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Summary: The patch-clamp whole cell recording was used to demonstrate activation of membrane conductance to K^+, Cl^- and cations induced by acetylcholine (ACh) in the isolated acinar cells of the guinea pig nasal gland. A small outward K^+ current at 0 mV and a large transient and sustained inward current at -90 mV were evoked by ACh and ACh-evoked reversal potential was about -3 mV nearly to Cl^- equilibrium potential in 140 mM KCl in the pipette and physiological saline in the bath. The ionic substitutional experiments indicated that ACh-evoked inward currents were carried by both Cl^- and cations. Both outward and inward currents evoked by ACh were almost completely abolished by removal of external Ca^{2+} and mimicked those evoked by a calcium ionophore A23187. These findings indicate that ACh-evoked membrane conductances are mediated by an increase in intracellular Ca^{2+}.

A cholinergic stimulation to the nasal cavity induces glandular secretory responses in an in-vivo guinea pig model (2) and isotonic glandular secretion rich in NaCl in ferret lateral nasal glands (11). In exocrine glands, acinar cells transfer ions from the blood to the acinar lumen by a Ca^{2+}-dependent process. Ca^{2+}-dependent channels, the Na^+-K^+ pump, and a Na^+-K^+-$2Cl^-$ cotransport system have been proposed to contribute this transport (8,12,14). However, no cellular mechanism involving electrolyte and fluid transport in the mammalian nasal gland has been investigated. Recently, the patch-clamp recording technique has been applied to acinar cells of a number of mammalian exocrine glands enzymatically dispersed (10,13). Current models of fluid and electrolyte secretion in exocrine gland

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emerged by patch-clamp studies (12,14) revealed that agonist-induced increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) activates both the basolateral K^+ channels and luminal Cl^- channels. To preserve electroneutrality, Na^+ ions move into the lumen through paracellular spaces.

In the present study, we applied patch-clamp method to demonstrate activation of membrane conductance to K^+, Cl^- and cations induced by acetylcholine (ACh), dependent on intracellular Ca^{2+}, in isolated acinar cells of the nasal gland of the guinea pig.

Materials and Methods

Preparation of dispersed 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 oxygenated cell-storage solution. The septal mucosa was separated from cartilage beneath the cartilaginous membrane and inverted on a Petri dish containing the cell-storage solution. Following removal of the cartilaginous membrane, the area containing a large amount of nasal glands was meticulously dissected using fine forceps and ophthalmic scissors and then minced into cubes from 0.2 to 0.5 mm. The pooled fragments were suspended in an isolation solution and incubated for 20 to 30 min at 37°C . The isolation solution was made by removing Ca^{2+} from the standard solution and adding 1 mM ethylene glycol-bis (ß-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 and then filtered clusters of cells were washed and centrifuged (1000 rpm, 1 min) three times and resulting sediment was resuspended in a fresh cell-storage solution.

Patch-clamp procedure: Acinar cells were distinguished from other cells under the microscope on the basis of morphological hallmarks, i.e., the formation of acini and the absence of cilia according to Sunose et al (16) and the conventional whole-cell techniques were employed (3) after the cells were allowed to settle and adhere on the bottom of the recording chamber. Pipette electrodes with a resistance of 2 to 5 MΩ were pulled from hematocrit tubes with a PP-83 vertical puller. After establishing a high resistance tight-seal, rupture of the patch membrane led to a whole-cell configuration. Successful whole-cell preparation rapidly achieved a stable negative potential with referece to the bathing solution. Membrane currents were recorded using a CEZ-2300 patch-clamp amplifier and low pass filtered at 0.5 kHz. Electrical stimulus was generated by SET-1201 step pulse generator. Current output was recorded on a RTA-1100 thermal array recorder, digitized using a PCM-501ES modified digital processor and stored on videocassette tape for later analysis. Liquid junctional potentials were measured by means of 3 M KCl filled electrodes and appropriate corrections were applied to the data. Current-voltage relationships were fit to second order polynomials by regression analysis to obtain the reversal potential. All experiments were performed at 35-37°C.

Solutions and chemicals: The standard external solution contained (in mM) 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 N-2-hydroxymethylpiperazine-N'-2-ethanesulfonic acid (HEPES)/tris-(hydroxymethyl)aminomethane (TRIS) mixture and pH was adjusted to 7.4 at 37° C. The cell-storage solution was prepared by addition of 0.2 % bovine serum albumin in the standard external solution, low-Na^+ solution by

replacing 125 mM Na^+ with 125 mM N-methyl-D-glucamine ($NMDG^+$), K^+-free NaCl solution by replacing all K^+ with Na^+, Ca^{2+}-free solution by eliminating all Ca^{2+} and adding 0.2 mM EGTA. The standard pipette solution contained (in mM) 140 K^+, 1.13 Mg^{2+}, 142.26 Cl^-, 1 K_2ATP, 1 EGTA, 1 HEPES and pH was adjusted to 7.2 with KOH. K^+-free solution was prepared by replacing all K^+ with Na^+ and K_2ATP with Na_2ATP. Low-Cl^- pipette solution was prepared by substituting all K^+ with Na gluconate and K_2ATP with Na_2ATP. ACh, calcium ionophore A23187, diphenylamine-2-carboxylic acid (DPC), collagenase (type IV) were from Sigma (St. Louis, Mo.). Other chemicals were purchased from Wako Junyaku (Tokyo, Japan).

All results are the means±S.D. with n equal to the number of cells. Statistical significant of data was analyzed by Student's t-test and p-value < 0.05 was accepted as significant.

Results

Whole-cell recording from individual nasal gland acinar cells equilibrated with high-K^+ solution containing 1 mM EGTA provided resting membrane potentials averaging -41.7±8.7 mV (n=37). The hyperpolarizing and depolarizing pulse protocol used in the present study was designed to independently obtain changes in both K^+ and Cl^- conductances. The membrane potential was fixed at -40 mV nearly to the resting potential, and K^+ currents were measured at 0 mV, the reversal potential for symmetrical Cl^- solutions; and Cl^- currents at -90 mV, the reversal potential for K^+ equilibrium potential. Addition of 10^{-7} M ACh evoked an immediate and remarkable increase in inward currents at -90 mV, which reached a peak 2-3 sec followed by a gradual decay (Fig.1a). In a latent period of 2-3 sec between the start of ACh application and onset of the response, a small and gradual increase in outward current was observed. At the holding potential of -40 mV near to the resting potential, ACh produced an outward current to reach a maximum of 0.6 nA and subsequently declined to a steady-state level of 0.2 nA. Removal of ACh caused a prompt return to prestimulatory levels. The relationship between the amplitude of the ACh-evoked current and the membrane potential obtained from the representative experiment was shown in Fig.1b. The ACh-evoked reversal potential (E_{ACh}) obtained by subtracting the value in the absence of ACh from that in the presence of the drug was -2.6±4.5 mV (n=11), which was close to the estimated Cl^- reversal potential (E_{Cl}) of 1.2 mV rather than the estimated K^+ reversal potential (E_K) of -90 mV. Although Cl^- channels contribute to a large extent to the ACh-evoked current, other carriers such as K^+ through K^+ channels and Na^+ through non-selective cation channels are not ruled out when physiological ionic gradients exist across the membrane.

The Ca^{2+}-dependent non-selective cation channels have been reported in the mouse parotid acinar cells (9). ACh-evoked inward currents are likely to involve the increase in Na^+ conductance through

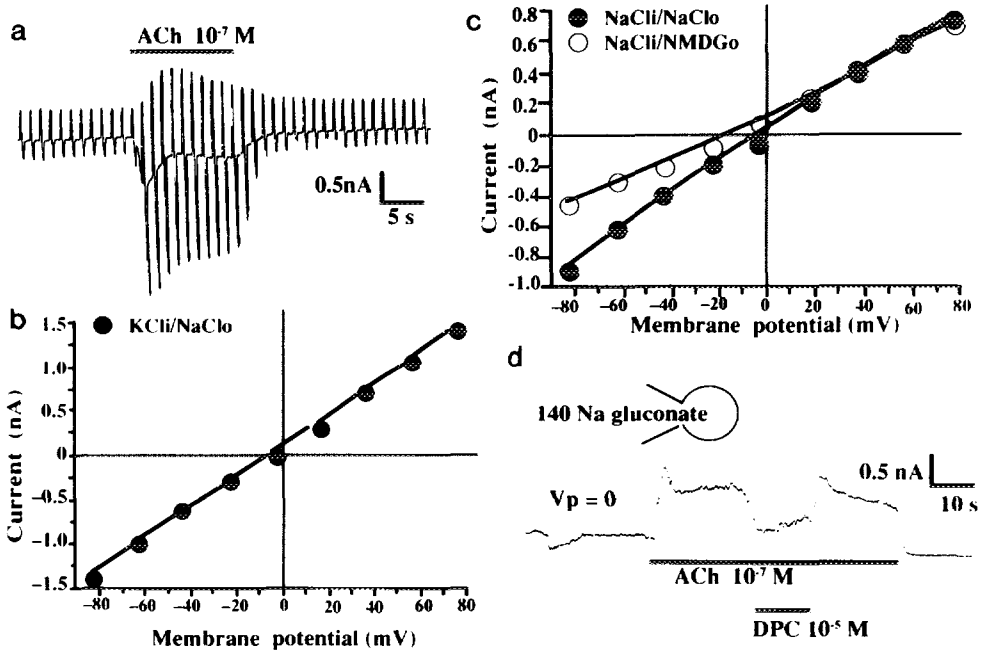

Fig. 1. Effect of ACh on the whole-cell currents of the nasal gland acinar cell. a: The typical response was elicited by the application of ACh (10^{-7} M) in the patch clamp whole-cell current recording. The membrane potential of the cell was clamped at -40 mV, and serial 40 mV and -50 mV voltage jumps of 100 msec were applied every 500 msec. The potential of 0 mV and -90 mV corresponded to equilibrium potentials of Cl^- and K^+, respectively. Application of ACh evoked both an inward current at -90 mV and a slight outward current at 0 mV. Removal of ACh caused a prompt return to the prestimulating levels. b: The current-voltage relationship for ACh-evoked whole-cell currents in the standard pipette and external solutions. The amplitude of the apparent instantaneous ACh-evoked current at any test potential was measured by subtracting the value in the absence of ACh from that in the presence of the drug. In this and subsequent figures the major constituents in the external and internal are indicated by subscripts o and i, respectively. c: The current-voltage relationship for ACh-evoked response in the symmetrical K^+-free NaCl solutions. ACh-evoked whole-cell currents reversed at 0 mV. E_{ACh} was shifted to negative direction when Na^+ was replaced by $NMDG^+$ in the external solution. d: Effect of non-specific anionic channel inhibitor on Cl^- current. The pipette solution was Na gluconate and the external solution did not contain potassium. A distinct Cl^- current was isolated when the membrane potential was clamped at 0 mV. After eliciting a control response to ACh, the cell was exposed to 10^{-5} M DPC for 20 sec, then DPC was removed during the application of ACh.

non-selective cation channels. A response to ACh application was examined in K^+-free symmetrical NaCl solution. In this case, E_{ACh} was -0.2±0.2 mV (n=4) (Fig.1c). When external Na^+ was replaced by $NMDG^+$ in the same cell, E_{ACh} was -17.5±2.7 mV (n=4) which is statistically different from that in the external NaCl solution ($p<0.05$) and the negative shift in E_{ACh} can be explained by the increase in Na^+ conductance resulting from activation of non-selective cation channels.

In order to isolate the Cl^- current, responses to ACh were recorded with a Na gluconate-filled pipette in the K^+-free NaCl bath solution. When the patch-pipette is clamped at 0 mV, the outward current is solely carried by Cl^-. The application of ACh to the cell induced an initial transient of 1.3±0.3 pA (n=9) which declined to a sustained level. A non-specific anionic channel inhibitor, DPC (1mM), suppressed ACh-evoked Cl^- current (Fig. 1d) by 77.7±9.6 % (n=4).

Both outward and inward currents induced by ACh were inhibited when external Ca^{2+} was eliminated, in spite of the presence of ACh (Fig. 2a). For example, ACh-evoked inward currents at -90 mV, with average magnitudes of 2.5±0.3 pA (n=5) in the presence of external Ca^{2+}, were reduced by Ca^{2+}-free solution containing 0.2 mM EGTA to 0.9±0.3 pA, which was corresponding to 129.6±16.2 % (n=5) of the unstimulated level.

Application of Ca^{2+} ionophore A23187 (10^{-5} M) in the KCl pipette solution increased both outward and inward current. The amplitude of currents started to increase about 2-3 sec after the onset of the ionophore application and reached the plateau within 5 sec without a transient peak of the outward current (Fig. 2b). The different pattern of the inward current between ACh and A23187 with respect of

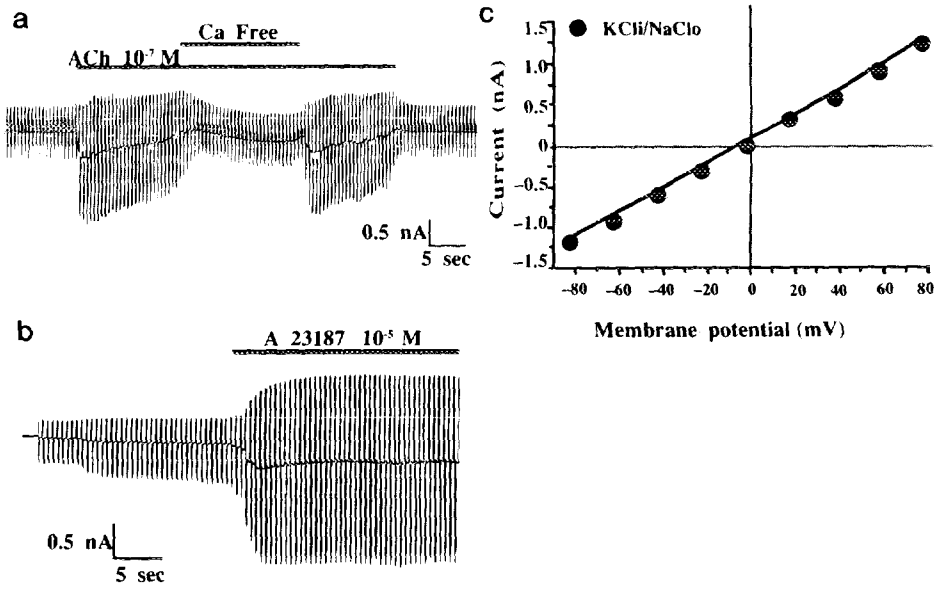

Fig. 2. Dependence of whole-cell currents on intracellular Ca^{2+}. a: ACh-evoked inward and outward currents were abolished by removal of extracellular Ca^{2+} during ACh application. b: Effect of calcium ionophore A23187 on whole-cell current of nasal gland acini. The amplitudes of inward and outward currents started to increase about 2-3 sec after the onset of Ca^{2+} ionophore A23187 application and reached to plateau within 5 sec. c: The current-voltage relationship of recording obtained from Fig. 2b.

the transient peak may be due to the different change in $[Ca^{2+}]_i$; a transient peak was followed by a sustained level with addition of ACh and a gradual and sustained response was induced by A23187 (5). Instantaneous ionophore-evoked currents were obtained by subtracting the control traces from the records. The reversal potential of A23187-evoked currents (E_{A23187}) was -4.6±1.7mV (n=3), close to E_{Cl} (Fig. 2c). This implies that Cl^- ions dominantly carry A23187-evoked inward currents.

Discussion

The present study demonstrates that muscarinic receptor stimulation by ACh activates the membrane conductance to K^+, Cl^- and non-selective cations by three distinct pathways in the guinea pig nasal gland acinar cells. ACh induced a small outward K^+ current and a large transient and sustained inward current. ACh-evoked reversal potential was close to that of Cl^-. When external Na^+ was replaced by $NMDG^+$ in symmetrical NaCl solution, E_{ACh} was shifted to negative direction. This finding indicates that ACh-evoked inward currents were produced by an increase in both a Cl^- and non-selective cation conductances. Thus ACh evoked a complex conductance increase but the part of Cl^- and non-selective conductances are more prominent than K^+ conductance, which is similar to rodent exocrine pancreas (7) and is different from salivary and lacrimal glands (1,6,8,9).

Three types of conductances induced by ACh are activated by increasing level of intracellular Ca^{2+} by the following evidences; i) ACh-evoked sustained responses were dependent on the presence of extracellular Ca^{2+}, ii) removal of external Ca^{2+} abolished the increase in $[Ca^{2+}]_i$ induced by ACh (5), iii) increased Ca^{2+} inside the cell by application of A23187 mimicked ACh-evoked responses, and iv) an initial large transient of the ACh-evoked inward current corresponded to an initial transient in $[Ca^{2+}]_i$ originated from the release of Ca^{2+} from internal stores (5). Thus an increase in $[Ca^{2+}]_i$ seems to be a primary trigger for opening ACh-evoked currents although various kinds of Ca^{2+} release mechanisms from internal stores and Ca^{2+} entry mechanisms are predicted as the source of increased $[Ca^{2+}]_i$. Furthermore, a stimulant-evoked increase in $[Ca^{2+}]_i$ and its spatial spread, presumably providing with mechanisms of acinar fluid secretion, remains unknown in the nasal gland acinar cells. Another possible mechanism in regulating the outward K^+ current is voltage-dependency. We observed voltage-dependent activation of K^+-single channel currents in the acinar cells (unpublished data). When the cell voltage was clamped at -40 mV near the zero-current level, ACh immediately evoked a strong inward current, which corresponds to the membrane depolarization recorded by current-clamp mode (16). The ACh-induced depolarization would result in further activation of the K^+ channel.

In exocrine acinar cells agonist stimulation is proposed to induce activation of both the basolateral K^+ conductance and the luminal Cl^- conductance (15). On the basis of the present study , ACh indirectly activates both Cl^- and K^+ channels by raising $[Ca^{2+}]_i$. Subsequent membrane depolarization resulting from Cl^- efflux via Cl^- channels in the luminal membrane leads to further activation of basolateral K^+ channel and a favorable electrochemical gradient for K^+ efflux. Both K^+ and Cl^- enter the cell by operation of the Na^+-K^+ ATPase and Na^+-K^+-$2Cl^-$ cotransport (4). The resultant luminal negativity draws Na^+ into the lumen via the paracellular pathway. The role of non-selective channel is still unknown, but might partly contribute to Na^+ influx and K^+ efflux.

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