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# Gramicidin-Perforated Patch Analysis on $HCO_3^-$ Secretion Through a Forskolin-Activated Anion Channel in Rat Parotid Intralobular Duct Cells

**C. Hirono[1], T. Nakamoto[2], M. Sugita[1], Y. Iwasa[1], Y. Akagawa[2], Y. Shiba[1]**
[1]Department of Oral Physiology, Hiroshima University School of Dentistry, Hiroshima 734-8553, Japan
[2]Department of Removable Prosthodontics, Hiroshima University School of Dentistry, Hiroshima 734-8553, Japan

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**Abstract.** Forskolin-induced anion currents and depolarization were investigated to clarify the mechanism of $HCO_3^-$ secretion in the intralobular duct cells of rat parotid glands. Anion currents of the cells were measured at the equilibrium potential of $K^+$, using a gramicidin-perforated patch technique that negligibly affects intracellular anion concentration. The forskolin-induced anion current was sustained and significantly (54%) suppressed by glibenclamide (200 μM), a blocker of the cystic fibrosis transmembrane conductance regulator (CFTR) $Cl^-$ channel. The anion current was markedly suppressed by addition of 1 mM methazolamide, a carbonic anhydrase inhibitor, and removal of external $HCO_3^-$. Forskolin depolarized the cells in the current-clamp mode. Addition of methazolamide and removal of external $HCO_3^-$ significantly decreased the depolarizing level. These results suggest that activation of anion channels (mainly the CFTR $Cl^-$ channel located in luminal membranes) and production of cytosolic $HCO_3^-$ induce the inward anion current and resulting depolarization. Inhibition of the $Na^+$-$K^+$-$2Cl^-$ cotransporter and the $Cl^-$-$HCO_3^-$ exchanger had no significant effect on the current or depolarization, indicating that the uptake of $Cl^-$ via the $Na^+$-$K^+$-$2Cl^-$ cotransporter or the $Cl^-$-$HCO_3^-$ exchanger is not involved in the responses. Taken together, we conclude that forskolin activates the outward movement (probably secretion) of $HCO_3^-$ produced intracellularly, but not of $Cl^-$ due to lack of active $Cl^-$ transport in parotid duct cells, and that the gramicidin-perforated patch method is very useful to analyze anion transport.

**Key words:** Gramicidin-perforated patch — Parotid duct cell — Forskolin — Bicarbonate secretion — Cystic fibrosis transmembrane conductance regulator — Methazolamide

*Correspondence to:* C. Hirono

## Introduction

The functions of salivary ducts are saliva modifications, i.e., reabsorption of $Na^+$ and $Cl^-$ ions secreted from acinar cells, secretion of $K^+$ and $HCO_3^-$ ions and resulting production of low osmotic saliva according to the two-stage hypothesis of fluid secretion in mammalian salivary glands [8, 29]. These functions were confirmed by microperfusion studies on the main excretory ducts of rat submandibular glands [22]. The reabsorption mechanism has been well investigated by electrophysiological studies in mouse submandibular duct cells, using whole-cell patch-clamp techniques [7]. On the contrary, the mechanism of $K^+$ and $HCO_3^-$ secretion is still unclear. This is the first report to electrophysiologically reveal the mechanism of $HCO_3^-$ secretion from salivary duct cells.

Patch-clamp studies, including whole-cell recording, have greatly contributed to the progress in analysis of the mechanism of ion transport and characterization of ion channels in salivary glands [7]. However, the conventional whole-cell configuration washes out some second messengers in the signal transduction system, e.g., cAMP and $Ca^{2+}$, and changes the intracellular concentrations of ions, e.g., $Cl^-$ and $HCO_3^-$, to those of the pipette solutions. To analyze the physiological movement of $HCO_3^-$ and $Cl^-$ through ion channels, it is necessary to preserve the intracellular concentration of the ions. Recently, the gramicidin-perforated patch-clamp, a method that avoids the problems associated with whole-cell recording, has been developed [12]. In this configuration, although the intracellular concentration of small single cations, such as $K^+$ and $Na^+$, is restricted by the

ion concentration in pipette solutions, the intracellular concentration of anions remains independent of that in pipette solutions, since anions are almost completely impermeable to gramicidin-perforated membranes. Accordingly, it is possible to analyze the movement of anions actively transported into the cells by measuring the anion current and membrane potential of cells which have the physiological composition of intracellular anions [12].

In this paper, we investigated forskolin-stimulated outward movement of anions from rat parotid duct cells through anion channels, such as the CFTR $Cl^-$ channel, measuring the ionic current and membrane potential via gramicidin-perforated patch recording. The results suggest that $HCO_3^-$ ions are secreted from the duct cells in the steady state during stimulation by forskolin and that $Cl^-$ ions are not actively transported in the cell, but may passively descend the transcellular electrochemical gradient.

## Materials and Methods

The external solution of cells throughout preparation and experiments, except for a $HCO_3^-$-free solution and a $Cl^-$-free solution, was a modified Krebs-Henseleit Ringer (KHR) solution containing (in mM): 103 NaCl, 4.7 KCl, 2.56 $CaCl_2$, 1.13 $MgCl_2$, 2.8 glucose, 4.9 sodium pyruvate, 2.7 fumaric acid disodium salt, 4.9 L-glutamic acid monosodium salt, 12.5 HEPES-NaOH (pH 7.4), 25 $NaHCO_3$, and 1.15 $NaH_2PO_4$. The solution was gassed with 95% $O_2$ + 5% $CO_2$. $NaHCO_3$ was replaced with sodium gluconate in the $HCO_3^-$-free solution, which was not gassed. Chloride was replaced with gluconate in the $Cl^-$-free solution. Parotid glands were removed from male Wistar rats (260–350 g), which were anesthetized with pentobarbital sodium (Nembutal, 70 mg/kg), and digested with 0.2% collagenase (type S-1, Nitta Gelatin, Osaka, Japan) for 30 min at 37°C. After gentle pipetting and centrifugation, pellets were dispersed with KHR solution, and the suspension was poured into a 90 mm dish to allow ducts to attach tightly to the dish. The dish was then washed with KHR solution several times to remove acini that were not attached tightly. Ducts on the dish were collected with 0.05% trypsin (Nacalai, Kyoto, Japan) dissolved in phosphate-buffered saline containing 0.016% EDTA and then washed with KHR solution. The ducts were digested again with 0.2% collagenase + 0.4% trypsin inhibitor (Sigma, St. Louis, MO) for 30 min at 37°C. Dispersed cells were washed three times and placed on coverslips with a diameter of 5 mm. The coverslips with attached cells were placed in a 0.2-ml chamber which was constantly perfused at a rate of 1 ml/min with KHR solution. Patch-clamp pipettes were pulled from borosilicate glass capillaries (Cat. No. G75-1511, Warner Instrument, Hamden, CT). First, we tried to record the ionic currents of parotid cells in a conventional whole cell configuration, but only a few cells responded to stimulation with forskolin + IBMX, probably due to the washing out of important factors for current activation [1]. Dinudom et al. [11] found a high incidence of submandibular duct cells that responded to forskolin in a nystatin-perforated patch configuration, although they did not use the method in the anion selectivity experiments because of the low permeability of nystatin pores to anions. In the present study, our purpose was not a detailed analysis of channel properties and we used a gramicidin-perforated patch to avoid washing out and to preserve anions of physiological concentration. We made an assumption that the gramicidin-perforated patch technique did not affect the intracellular anion concentration in parotid cells, as is the case for neurons [12]. Gramicidin D (Sigma) was dissolved in methanol at 10 mg/ml and diluted 100 times with a standard KCl-rich perforated patch solution containing (in mM): 150 KCl, 10 HEPES adjusted to pH 7.4 by KOH. Pipette tips were placed for a few seconds into the gramicidin-free pipette solution. The pipettes were then back-filled with the gramicidin-containing pipette solution. A patch/whole-cell clamp amplifier CEZ-2400 (Nihon Kohden, Tokyo, Japan) was used to measure ionic currents in the perforated patch configuration. The reference electrode was a Ag-AgCl electrode, which was placed in the bath when the external $Cl^-$ concentration was not changed, or connected to the bath via 100 mM KCl-containing agar bridge in experiments in which the external $Cl^-$ concentration was changed. Parotid duct cells were distinguishable from those of acini by their shape [14], size and membrane capacitance, as is the case for submandibular duct cells [32]. The resting potential of the duct cells, measured with the patch/whole-cell clamp amplifier in the current-clamp mode of the perforated patch configuration, was −75 ± 1 mV (mean ± SE, $n = 68$) which is close to the equilibrium potential of $K^+$ (about −80 mV). The resting potential was not changed significantly by application of amiloride (10 μM), which was reported to inhibit $Na^+$ conductance in mouse mandibular duct cells [10, 19]. This suggests that the current through the epithelial sodium channel is negligible and that almost exclusively potassium channels are open in the resting state. Therefore, we set the holding potential of the voltage clamp at −80 mV to measure only the anion current. The ionic currents and membrane potential averaged for 30 sec in the steady state 4–6 min after the application of drugs were used for statistical analyses. The series resistance (about 30 MΩ or less) was not compensated. Forskolin, 3-isobutyl-1-methylxanthine (IBMX), glibenclamide, methazolamide, bumetanide, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), amiloride and 5-(*N*,*N*-dimethyl)-amiloride (DMA) were all obtained from Sigma. All measurements were performed at room temperature (24–27°C).

## Results

### An Anion Current Measured at a Holding Potential of −80 mV in a Gramicidin-Perforated Patch Configuration and the Effect of Glibenclamide on the Current

To clarify stimulated secretion of anions from parotid duct cells, we studied anion currents induced by two cyclic AMP-increasing agents, forskolin and IBMX. We used a gramicidin-perforated patch method to keep cells in physiological condition and measured the anion current in the voltage-clamp mode at a holding potential of −80 mV, at which other current components were negligible, as described in Materials and Methods. Simultaneous application of 10 μM forskolin and 100 μM IBMX (forskolin + IBMX) induced a sustained inward anion current during the application of the stimulants (Fig. 1). The steady-state amplitude of the current measured 4–6 min after the application of forskolin + IBMX was 76 ± 4 pA (mean ± SE, $n = 65$). The current response to forskolin + IBMX was observed in 90% of the duct cells ($n = 105$), while no marked current response was detected in acinar cells of rat parotid glands ($n = 10$).

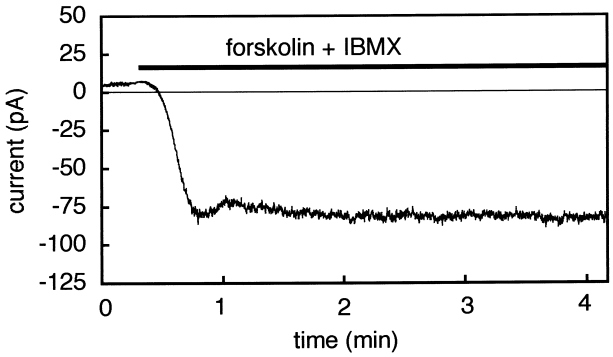


**Fig. 1.** Time course of an anion current induced by simultaneous application of 10 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX). The current was measured with the gramicidin-perforated patch method at −80 mV in a rat parotid duct cell.

In duct cells of mouse and rat submandibular glands, forskolin and isoproterenol activate an anion current, probably mediated by the CFTR $Cl^-$ channel [7, 11, 32]. To clarify channels responsible for the forskolin-induced anion current in rat parotid duct cells, we studied the effect of the CFTR channel blocker, glibenclamide, on the current. Addition of 200 μM glibenclamide during the application of forskolin + IBMX suppressed the current from the control level of 74 ± 7 pA to 34 ± 7 pA (mean ± SE, $n = 10$) (Fig. 2). This supports the idea that at least 54% of the anion current is very likely mediated by the CFTR $Cl^-$ channel (*see* Discussion).

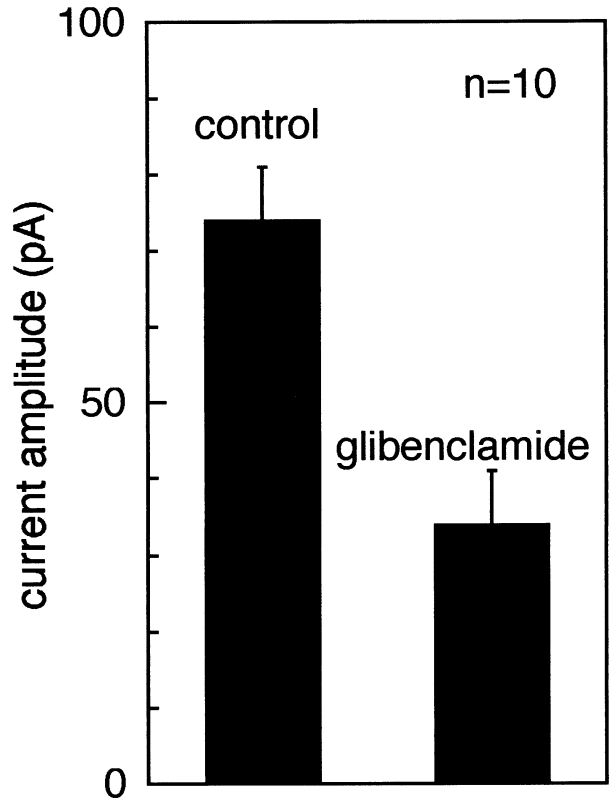


**Fig. 2.** Effects of 200 μM glibenclamide on an anion current induced by simultaneous application of 10 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX). The current was measured with the gramicidin-perforated patch method at −80 mV. Glibenclamide added during the steady state of the current at least 4 min after the application of forskolin and IBMX significantly suppressed the current ($P < 0.001$ by Student's *t*-test).

### Effects of Methazolamide and Removal of $HCO_3^-$ Ions on the Anion Current

In a conventional whole-cell patch-clamp, the pipette solution does not contain $HCO_3^-$ ions and the intracellular $Cl^-$ concentration ($[Cl^-]_i$) is equal to the $Cl^-$ concentration in the pipette, which is often much higher than physiological $[Cl^-]_i$. Therefore, the anion current is mainly made up of the $Cl^-$ current. In this experiment, we studied which anion was dominant, $Cl^-$ or $HCO_3^-$, in the steady state of the anion current measured at −80 mV in a gramicidin-perforated patch with a $HCO_3^-$-containing external solution (KHR solution).

First, we examined the effect of a reduction in the cytosolic $HCO_3^-$ production rate on the current. The anion current at −80 mV was suppressed from the control level of 75 ± 11 pA to 13 ± 1 pA (mean ± SE, $n = 9$) within 4–6 min by the addition of 1 mM methazolamide, a carbonic anhydrase inhibitor, which suppresses $HCO_3^-$ secretion in salivary glands [3, 30] and the colon [9], during the application of forskolin + IBMX (Fig. 3*A*). This result suggests that intracellular $HCO_3^-$ ions may be dominant in ions that carry the anion current at −80 mV and/or that $HCO_3^-$ ions may contribute to the anion current via exchange of intracellular $HCO_3^-$ ions with external $Cl^-$ ions, which may become the carriers of the anion current after the exchange.

We also examined the contribution of external $HCO_3^-$ ions as potential source of $CO_2$ to produce intracellular $HCO_3^-$ ions. When external $HCO_3^-$ ions were removed by replacement of $HCO_3^-$ with gluconate, the anion current decreased from the control level of 79 ± 12 pA to 32 ± 5 pA (mean ± SE, $n = 11$) (Fig. 3*B*), suggesting that intracellular $HCO_3^-$ is produced from $CO_2$, which is at least partially supplied from $HCO_3^-$ in the external solution.

### Effect of DMA on the Anion Current

The production of intracellular $HCO_3^-$ is accompanied by an increase in intracellular $H^+$ ions which are extruded from cells for continuous $HCO_3^-$ production. We examined the mechanism of $H^+$ extrusion. Application of 5-(*N*,*N*-dimethyl)-amiloride (DMA) (20 μM), a $Na^+$-$H^+$

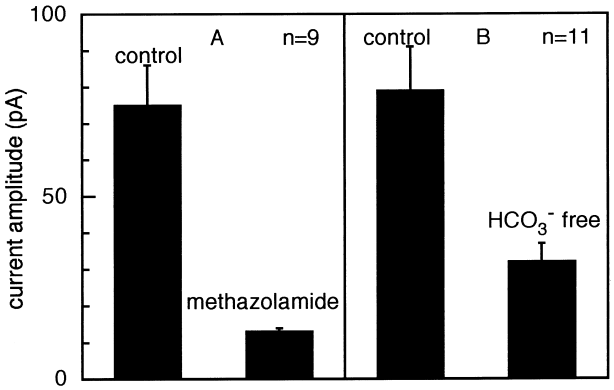


**Fig. 3.** Effects of 1 mM methazolamide (*A*) and removal of $HCO_3^-$ by replacing $HCO_3^-$ with gluconate (*B*) on the anion current induced by simultaneous application of 10 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX). The current was measured with the gramicidin-perforated patch method at −80 mV. Methazolamide added during the steady state of the current at least 4 min after the application of forskolin and IBMX significantly suppressed the current (*A*; $P < 0.001$ by Student's *t*-test). Removal of external $HCO_3^-$ ions also significantly suppressed the current (*B*; $P < 0.01$).

exchanger blocker, suppressed forskolin-induced anion current from the control level of 83 ± 9 pA to 25 ± 3 pA (mean ± SE, $n = 11$) (Fig. 4). This result suggests that $H^+$ ions are transported from the cytosol to the extracellular region mainly by the $Na^+$-$H^+$ exchanger.

### Effects of Bumetanide, DIDS and Removal of $Cl^-$ Ions on the Anion Current

We then investigated the contribution of $Cl^-$ ions to the anion current. Figure 5*A* shows the effect of a reduction in the uptake rate of $Cl^-$ by the blockage of a $Na^+$-$K^+$-$2Cl^-$ cotransporter on the anion current. Addition of 500 μM bumetanide, an inhibitor of the $Na^+$-$K^+$-$2Cl^-$ cotransporter, reduced the anion current from the control level of 71 ± 9 pA to 58 ± 8 pA (mean ± SE, $n = 14$), but not significantly, indicating that $Cl^-$ ions transported by the $Na^+$-$K^+$-$2Cl^-$ cotransporter are not the dominant source of anions for the anion current. Moreover, removal of external $Cl^-$ ions by replacing the KHR solution with the $Cl^-$ free solution had no effect on the steady-state amplitude of the anion current (Fig. 5*B*), indicating the absence of $Cl^-$ transporters, such as the $Na^+$-$K^+$-$2Cl^-$ cotransporter, in the cells.

Another possible mechanism supplying anions as the carriers of the current may be based on the $Cl^-$-$HCO_3^-$ exchanger, as described above. Therefore, we examined the effect of DIDS, an inhibitor of the $Cl^-$-$HCO_3^-$ exchanger, on the anion current. Addition of 100 μM DIDS did not significantly suppress the anion current (Fig. 5*C*), suggesting that external $Cl^-$ ions are not exchanged with intracellular $HCO_3^-$ ions. This idea is also suggested by the lack of an effect of the $Cl^-$-free external solution on the anion current shown in Fig. 5*B*. Taken together, all results shown in Figs. 3 and 5 suggest that $Cl^-$ ions are not actively transported into the cell and that $HCO_3^-$ ions, not $Cl^-$ ions, are the carriers of the anion current.

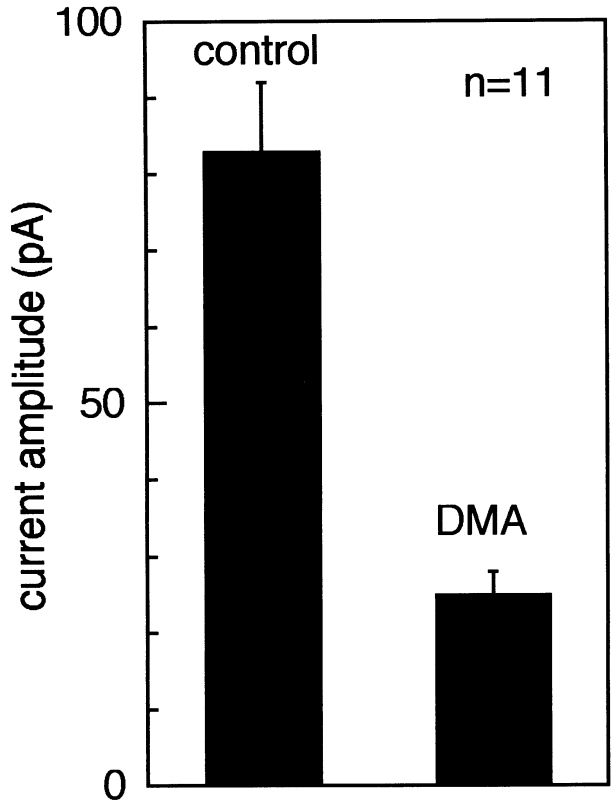


**Fig. 4.** Effects of 20 μM 5-(*N*,*N*-dimethyl)-amiloride (DMA) on the anion current induced by simultaneous application of 10 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX). The current was measured with the gramicidin-perforated patch method at −80 mV. Addition of DMA during the steady state of the current at least 4 min after the application of forskolin and IBMX significantly suppressed the current ($P < 0.001$ by Student's *t* test).

### Membrane Potential Measured in the Current-Clamp Mode of a Gramicidin-Perforated Patch Configuration and Effects of Glibenclamide on Membrane Potential

In the physiological condition, a large inward anion current induces membrane depolarization, which changes the driving forces of anion and cation currents. Currents measured in the voltage-clamp mode do not directly correspond to the physiological movement of ions. The amount and direction of ion fluxes through the cell membrane in the physiological condition are closer to those in

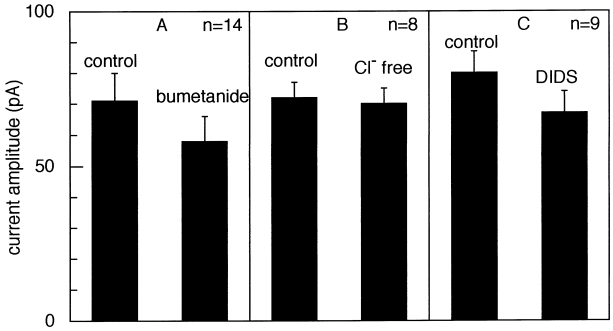


**Fig. 5.** Effects of addition of 500 μM bumetanide (*A*), removal of external $Cl^-$ ions by replacing $Cl^-$ with gluconate (*B*) and addition of 100 μM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) (*C*) on the anion current induced by simultaneous application of 10 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX). The current was measured with the gramicidin-perforated patch method at −80 mV. Bumetanide or DIDS added during the steady state of the current at least 4 min after the application of forskolin and IBMX did not significantly suppress the current (*A*,*C*). Neither did removal of external $Cl^-$ ions (*B*).

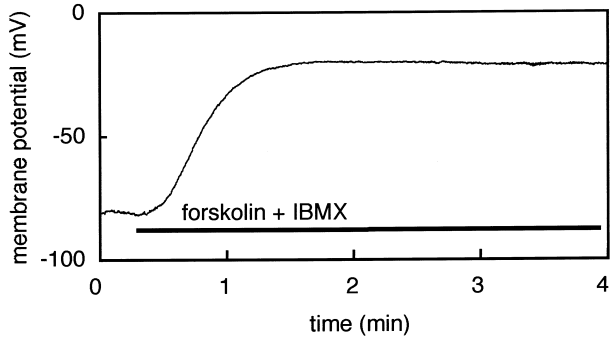


**Fig. 6.** Time course of membrane depolarization induced by simultaneous application of 10 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX). The membrane potential of a rat parotid duct cell was measured under the current-clamp condition of a gramicidin-perforated patch configuration in a rat parotid duct cell.

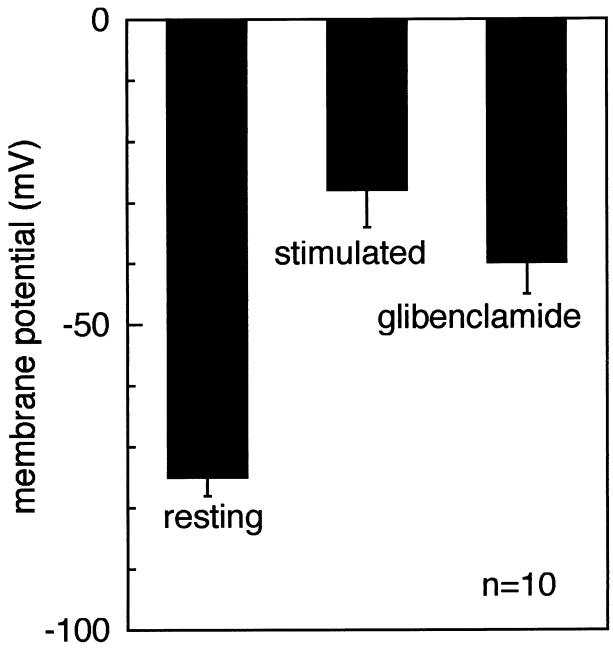


**Fig. 7.** Effects of 200 μM glibenclamide on depolarization induced by application of 10 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX). The membrane potential was measured under the current-clamp mode of the gramicidin-perforated patch recording and the depolarization evoked by the application of forskolin and IBMX was slightly, but not significantly, suppressed by glibenclamide which was added at least 4 min after the application of the forskolin and IBMX.

the current-clamp mode than those in the voltage-clamp mode. We thus studied membrane potential changes in the cells in the current-clamp mode (zero current) of a gramicidin-perforated patch to confirm that $HCO_3^-$ was actually secreted in the physiological condition.

The resting potential measured in the current-clamp mode in KHR was −75 ± 1 mV (mean ± SE, $n = 68$), which is close to the $K^+$ equilibrium potential as described in Materials and Methods. The application of forskolin + IBMX depolarized the cells, and the membrane potential reached a steady-state level of −26 ± 2 mV (mean ± SE, $n = 68$) within 4–6 min (Fig. 6). These results indicate that forskolin + IBMX induces a large inward anion current which markedly depolarizes the cells. At the depolarized level around −26 mV, the inward anion current is thought to be canceled by an outward current which is probably carried by $K^+$ ions (*see* Discussion) and has the same amplitude as that of the inward anion current.

Depolarization of the cells by forskolin + IBMX was slightly suppressed by glibenclamide from the control level of −28 ± 6 mV to −40 ± 5 mV (mean ± SE, $n = 10$), but the suppression was not significant (Fig. 7), suggesting that the unsuppressed part of the channel conductance is still large enough to keep the membrane potential close to the depolarized level. The difference between the effects of glibenclamide on the anion current (Fig. 2) and the membrane potential (Fig. 7) may be due to the voltage dependency of the blockage [26], that is, the blockage of the CFTR $Cl^-$ channel by glibenclamide is less potent at the depolarized level than at a holding potential of −80 mV.

### Effects of Methazolamide and Removal of $HCO_3^-$ Ions on Membrane Potential

The addition of methazolamide significantly changed the depolarized level from −26 ± 3 mV to −50 ± 4 mV (mean ± SE, $n = 11$) within 4–6 min (Fig. 8*A*). These results

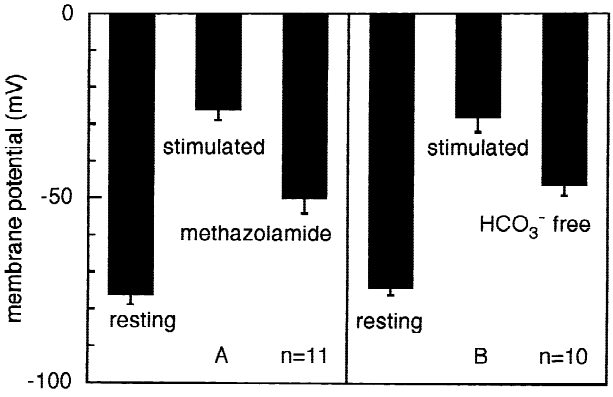


**Fig. 8.** Effect of addition of 1 mM methazolamide (*A*) and removal of external $HCO_3^-$ by replacing $HCO_3^-$ with gluconate (*B*) on depolarization induced by application of 10 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX). The membrane potential was measured under the current-clamp mode of the gramicidin-perforated patch recording. Methazolamide was added at least 4 min after the application of forskolin and IBMX. Methazolamide significantly suppressed depolarization by the stimuli ($P < 0.001$ by Student's *t*-test). Removal of external $HCO_3^-$ ions also significantly suppressed depolarization ($P < 0.01$).

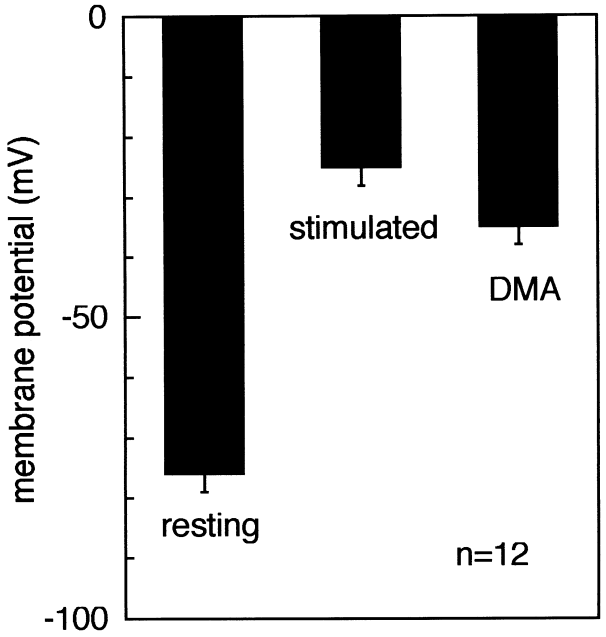


**Fig. 9.** Effect of 20 μM 5-(*N*,*N*-dimethyl)-amiloride (DMA) on depolarization induced by application of 10 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX). The membrane potential was measured under the current-clamp mode of the gramicidin-perforated patch recording. DMA was added at least 4 min after the application of forskolin and IBMX. DMA significantly suppressed depolarization by the stimuli ($P < 0.05$ by Student's *t*-test).

suggest that the reduction in the production rate of intracellular $HCO_3^-$ ions due to methazolamide decreases the inward anion current, which induces depolarization in the current-clamp mode, and that the inward anion current is carried by the efflux of $HCO_3^-$ ions or $Cl^-$ ions exchanged with intracellular $HCO_3^-$ ions. Therefore, these results are consistent with those of the effect of methazolamide on the inward anion current at −80 mV shown in Fig. 3*A*.

We then examined the effects of removal of external $HCO_3^-$ ions on membrane potential. Removal of external $HCO_3^-$ ions did not significantly change the resting potential of the cell, but significantly suppressed the depolarization induced by forskolin + IBMX from −28 ± 4 mV to −46 ± 3 mV (mean ± SE, $n = 10$) (Fig. 8*B*), probably due to inhibition of the inward anion current by the removal of $HCO_3^-$, which is consistent with the effect of the removal of external $HCO_3^-$ ions on the anion current shown in Fig. 3*B*.

### Effect of DMA on Membrane Potential

We examined the effects of DMA on forskolin-induced depolarization. DMA (20 μM) significantly reduced the depolarization from −25 ± 3 mV to −35 ± 3 mV (mean ± SE, $n = 12$) (Fig. 9), but the effect is unexpectedly rather small, compared to that on the current (Fig. 4) (*see* Discussion).

### Effects of Bumetanide, DIDS and Removal of External $Cl^-$ Ions on Membrane Potential

The effect of blockage of $Cl^-$ transport on forskolin + IBMX-induced depolarization was examined. Bumetanide (500 μM) did not change the depolarized level of the membrane potential during the application of forskolin + IBMX (Fig. 10*A*). This result again indicates almost no $Cl^-$ uptake by the $Na^+$-$K^+$-$2Cl^-$ cotransporter. We also showed that removal of external $Cl^-$ ions had no effect on the steady-state level of depolarization (Fig. 10*B*). Moreover, addition of 100 μM DIDS did not significantly suppress the depolarization (Fig. 10*C*). These results suggest the absence of active $Cl^-$ transport, including $Cl^-$-$HCO_3^-$ exchange, in the cell as is the case with the anion current shown in Fig. 5.

All membrane potential results shown in Figs. 8 and 10 suggest that $HCO_3^-$ ions, not $Cl^-$ ions, carry the inward anion current which induces membrane depolarization. They also suggest that $Cl^-$ ions move passively through open $Cl^-$ channels, changing $[Cl^-]_i$ during a rising phase of membrane potential response evoked by forskolin + IBMX (Fig. 6), and soon stop the movement at an equilibrium concentration in the steady state of the response.

## Discussion

This is the first attempt to perform electrophysiological studies on ion transport in salivary duct cells using the

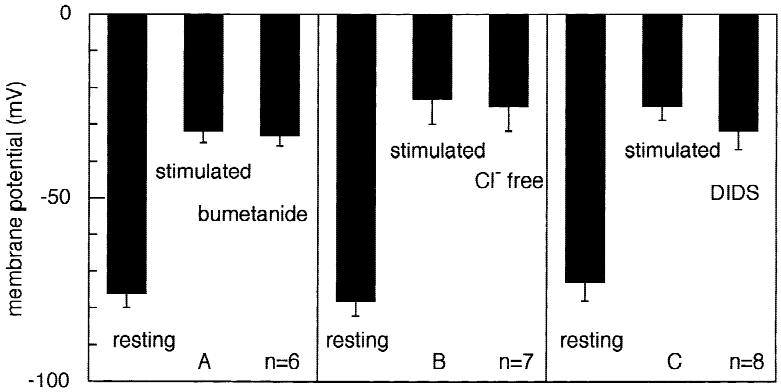


**Fig. 10.** Effects of addition of 500 μM bumetanide (*A*), removal of external $Cl^-$ ions by replacing $Cl^-$ with gluconate (*B*) and addition of 100 μM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) (*C*) on depolarization induced by application of 10 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX). The membrane potential was measured under the current-clamp mode of the gramicidin-perforated patch recording. Bumetanide and DIDS added during the steady state of the current at least 4 min after the application of forskolin and IBMX did not significantly suppress depolarization (*A* and *C*). Neither did removal of external $Cl^-$ ions (*B*).

gramicidin-perforated patch technique and the results obtained show that the technique is very useful in measuring anion secretion and transport without disturbing the physiological anion concentration of the cell.

It was shown that addition of the carbonic anhydrase inhibitor, methazolamide, and removal of external $HCO_3^-$ ions markedly suppressed forskolin-induced anion current and depolarization and that neither an inhibitor of the $Na^+$-$K^+$-$2Cl^-$ cotransporter, bumetanide, an inhibitor of the $Cl^-$-$HCO_3^-$ exchanger, DIDS, nor removal of external $Cl^-$ ions significantly affected the current and depolarization. These results strongly suggest that forskolin induces the efflux of $HCO_3^-$ ions, which are continuously produced in rat parotid duct cells, but not the secretion of $Cl^-$ ions, which are not actively transported into the cells.

The greater effectiveness of methazolamide compared to $HCO_3^-$ removal is explained as follows: Bicarbonate is produced from $H_2O$ and $CO_2$, and $CO_2$ is generated intracellularly and/or diffused into the cell through cell membrane from the external solution. Methazolamide suppresses the reaction of $H_2O$ with $CO_2$, supplied from both routes, inhibiting carbonic anhydrase. On the other hand, $HCO_3^-$ removal reduces only $CO_2$ supply from the external solution and does not suppress intracellular $CO_2$ production. Therefore, the effect of methazolamide should be larger than that of $HCO_3^-$ removal.

Forskolin-induced anion current and depolarization were significantly suppressed by DMA, indicating the presence of $Na^+$–$H^+$ exchange. However, the reduction of the depolarizing level was smaller compared to the effect on the current. This discrepancy may be due to unknown pathways of voltage-dependent and DMA-insensitive $H^+$ extrusion. It is likely that $H^+$ produced in $HCO_3^-$ synthesis process is extruded at least partially by DMA-sensitive $Na^+$-$H^+$ exchangers.

The counter ions of $HCO_3^-$ are probably $K^+$ ions, because the resting potential was close to the $K^+$ equilibrium potential (about −80 mV) in our present study, suggesting that during depolarization, $K^+$ ions move outward through $K^+$ channels that are always open in the cells. Amiloride, an epithelial $Na^+$ channel blocker, did not change the resting potential of parotid duct cells (*see* Materials and Methods), indicating that the $Na^+$ current mediated by the channel is negligible in the resting state, compared to $K^+$ currents. In forskolin-stimulated cells, activation of CFTR further suppresses epithelial $Na^+$ channels [20].

The most likely candidate for the forskolin-activated anion channel in parotid duct cells is the CFTR $Cl^-$ channel, since many properties similar to those of the CFTR $Cl^-$ channel were observed in the forskolin-activated anion channel in the present study as follows: (i) The channel was activated by forskolin which increases intracellular cAMP level, as the CFTR $Cl^-$ channel is [20], (ii) and was blocked by glibenclamide at a membrane potential more negative than the reversal potential, the same as the CFTR $Cl^-$ channel [26]. (iii) $HCO_3^-$ ions are permeable through the CFTR $Cl^-$ channel [5, 25]. Moreover, histochemical studies showed the existence of the CFTR in luminal membranes of rat parotid and submandibular duct cells [18, 32].

We used glibenclamide at a concentration of 200 μM which blocked the forskolin-induced anion current by 54%. This inhibitory effect of glibenclamide in rat parotid duct cells is smaller than that on a forskolin and isoproterenol-activated $Cl^-$ current of the submandibular duct cells, in which 100 μM glibenclamide inhibits the current by about 80% [32]. The discrepancy between the effects of glibenclamide in the two studies may be due to the difference between the two patch-clamp techniques, i.e., the gramicidin-perforated and conventional whole-cell patch recording methods, rather than the difference between parotid and submandibular glands. We have no direct evidence of this hypothesis, because the incidence of current response in the conventional whole-cell patch recording was too small to get enough data to compare to those obtained in this study, as described in Materials and Methods. However, it is reasonable to explain the discrepancy as follows: The current measured by the conventional whole-cell patch recording has only one rate-limiting step, i.e., ion permeability of the channel.

On the other hand, there are two possible rate-limiting steps in the anion current measured by the gramicidin-perforated patch technique. One of them is the anion supply process (anion uptake via transporters or anion production by enzyme) and the other is anion permeability of the channel. In this study, $HCO_3^-$ production by carbonic anhydrase from $CO_2$ and $H_2O$ is probably the rate-limiting step rather than anion permeability of the channel, since the instantaneous current just after the stepwise change of membrane potential from depolarizing level to −80 mV was much larger than the steady-state current at −80 mV without marked conductance change (*data not shown*). In other words, the anion channel is probably open enough, but the $HCO_3^-$-generating rate is not large enough to induce a full scale current. Accordingly, if 80% of the channel permeability is suppressed by glibenclamide, the remaining part of the permeability (20%) still keeps the current rather large, i.e., about 50% of the control, in the gramicidin-perforated patch recording.

Removal of $Cl^-$ did not affect the anion current and depolarization, which seems to argue against the idea that $Cl^-$ and $HCO_3^-$ share anion channels (e.g., the CFTR $Cl^-$ channel) to pass through. In the presence of $Cl^-$, $Cl^-$ may compete with $HCO_3^-$ in the channel pore, since $Cl^-$ is permeable to the channel but not a source of the current due to the lack of $Cl^-$ uptake. Removal of $Cl^-$ may reduce the competition, resulting in an increase of the anion current. However, this effect is probably negligible in the gramicidin-perforated patch recording, because, as described above, the rate-limiting step of the anion current is $HCO_3^-$ production.

The above three discussions suggest that the major part of the forskolin-activated anion current in rat parotid duct cells is probably mediated by the CFTR $Cl^-$ channel. Nevertheless, there is still a possibility that $HCO_3^-$ passes through a $HCO_3^-$-selective ($Cl^-$-impermeable) pathway. If the channel responsible for forskolin-induced anion current is not the CFTR $Cl^-$ channel located in luminal membranes, but an unknown channel, the anion current should correspond to $HCO_3^-$ efflux that could be $HCO_3^-$ secretion. Further investigations are required to clarify channels responsible for the anion current.

Secretion of $HCO_3^-$ is mediated by luminal $Cl^-$-$HCO_3^-$ exchange in rat submandibular ducts [33], mouse submandibular intralobular ducts [21] and, at least partially, pancreatic ducts [15, 21, 23], while very low activity of the exchange was found in mouse submandibular main ducts [4]. In the intralobular ducts of rat parotid glands, it is unlikely that $HCO_3^-$ secretion is due to exchangers and/or cotransporters, because blockage of the $Cl^-$-$HCO_3^-$ exchanger and the $Na^+$-$K^+$-$2Cl^-$ cotransporter did not significantly affect forskolin-induced anion current and depolarization in this study, no evidence of $Cl^-$-$HCO_3^-$ exchange was found [24] and $K^+$ and $HCO_3^-$ secretions are not due to cotransport of the ions in the duct [24].

We showed that the cyclic AMP-increasing agents, forskolin and IBMX, induced an anion current probably via the CFTR $Cl^-$ channel in rat parotid duct cells. β-Adrenergic secretagogues, which also increase cyclic AMP levels [13, 27], induce $HCO_3^-$ secretion [31] in rat submandibular ducts, and forskolin or forskolin + isoproterenol evokes an anion current via the CFTR $Cl^-$ channel in rat and mouse submandibular duct cells [11, 32]. On the other hand, in the acinar cells of rat parotid and submandibular glands, neither the β-adrenergic secretagogue, isoproterenol at low concentrations (e.g., 0.1 μM), forskolin nor cAMP induces any marked current [14, 16, 28], while microiontophoretic application of isoproterenol induces $Na^+$-, $K^+$- and $Cl^-$-sensitive depolarization in mouse parotid acinar cells [17], and bath application of isoproterenol at high concentrations (1–2 μM) evokes ionic currents in a $Ca^{2+}$-dependent manner in rat submandibular secretory cells [6]. The currents in the two reports [6, 17] are not likely mediated by activation of the CFTR $Cl^-$ channel. Taken together, cyclic AMP-increasing agents activate the CFTR $Cl^-$ channel in salivary duct cells, but not in acinar cells.

Our results suggest $HCO_3^-$ secretion in forskolin-stimulated parotid duct cells, which is consistent with results obtained by microperfusion studies in isoproterenol-stimulated submandibular ducts [22, 31]. Intracellular pH and luminal volume measurements in pancreatic ducts suggest secretion of $HCO_3^-$ ions accompanying volume secretion [15]. Since secretion of $HCO_3^-$ ions may induce movement of counter ions (probably $K^+$ ions), the resulting osmolarity change may induce transcellular water movement. Further investigations are required to demonstrate water movement with $HCO_3^-$ secretion in salivary ducts.

There is probably no driving force for $Cl^-$ ions in the steady state as discussed above. In intact ducts, however, $HCO_3^-$ secretion can induce a transcellular potential difference (lumen-negative) [2], which may drive the absorption of $Cl^-$ ions through $Cl^-$ channels if the $Cl^-$ absorption in rat parotid ducts is mediated by $Cl^-$ channels in the same way as in mouse submandibular ducts [7]. This means that the anion channel may have two opposing functions, i.e., simultaneous absorption and secretion.

In summary, intracellular $HCO_3^-$ ions are created by carbonic anhydrase from $CO_2$ and $H_2O$ and are probably secreted from parotid duct cells through forskolin-activated anion channels, mainly the CFTR $Cl^-$ channel, which is present in luminal membranes. $Cl^-$ ions are not actively transported into the cell and do not contribute to anion secretion, and/or $Cl^-$ ions may be absorbed through the channel via the transcellular potential difference induced by $HCO_3^-$ secretion.

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