J. Membrane Biol. 190, 189–196 (2002)
DOI: 10.1007/s00232-002-1036-2

The Journal of
Membrane
Biology

Forskolin-Induced Clearance of the Fluorescent Dye Sulforhodamine from Rat Parotid Intralobular Duct Lumen: Visualization of the Secretory Function under a Confocal Laser Scanning Microscope

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Received: 1 April 2002/Revised: 23 July 2002

Abstract. Cyclic AMP evokes fluid secretion with bicarbonate in exocrine ducts. Clearance of fluorescent dyes from rat parotid intralobular ducts by forskolin was visualized as a fluorescence change in the duct luminal space by optical sectioning under a confocal laser scanning microscope to clarify the secretory function in the ducts. When the isolated rat parotid intralobular duct segments were superfused with membrane-impermeable fluorescent dyes during the experimental period, fluorescent dyes were passively moved into the duct space. Forskolin and isobutylmethylxanthine decreased the fluorescence of anionic dye, sulforhodamine B, and neutral dye, dextran tetramethyl-rhodamine, in the duct space, suggesting that the forskolin-induced clearance of fluorescent dyes might be the result of fluid secretion in the ducts. Methazolamide inhibited a forskolin-induced sustained decrease in duct fluorescence and intracellular acidification. Low concentrations of external Cl^-, DIDS, bumetanide and amiloride did not markedly inhibit a forskolin-induced decrease in duct fluorescence. These findings suggest that a major portion of the steady decrease in duct fluorescence by forskolin was related to intracellular HCO_3^- production, not the uptake mechanism of external Cl^-. Glibenclamide, NPPB, DPC and DMA inhibited the forskolin-induced decrease. Forskolin evokes the clearance of fluorescent dyes from duct space possibly due to fluid secretion in rat parotid ducts, associated with secretion through CFTR and DPC-sensitive anion channels of carbonic anhydrase-dependent bicarbonate linked with the Na^+/H^+ exchange mechanism.

Key words: Fluid movement — Rat parotid ducts — Forskolin — Confocal laser scanning microscope — Sulforhodamine

Introduction

Fluid secretion is observed at the duct openings of the exocrine glands and the cut ends of perfused glands. The fluid secretion mechanism has been investigated by studying ion channels and ion transporters at the cellular level, and it was found that transcellular transport of anion ions contributes to the fluid secretion in the acinar cells [20, 24, 29, 36]. Cyclic AMP evokes fluid secretion with bicarbonate in the pancreatic ducts [14] and also induces bicarbonate release in rat parotid ducts [11]. However, the role of the ducts in the fluid secretion remains obscure, since the fluid secretion is not measured at the tissue and cell levels. A new method for the detection of fluid movement will be required for the clarification of the fluid secretion at tissue and cellular levels.

The secretion process in the exocrine acinar cells has been visualized using fluorescent dye [13, 32–34]. Secretory stimulation induces luminal membrane changes including vesicle invagination and omega structures under a confocal laser scanning microscope [30]. The secretion process of bicarbonate in the exocrine ducts has also been visualized with pH-sensitive fluorescent dye [6, 14]. Dye clearance from ducts with acini is observed under a fluorescent microscope, suggesting that dye clearance is due to fluid secretion [13]. It is likely that dye clearance from isolated duct

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segments can be visualized by optical sectioning at the level of the duct lumen under a confocal microscope. This technique would then contribute to helping identify how the cyclic AMP-increasing agent forskolin stimulates fluid secretion with bicarbonate in the salivary ducts.

In this study, we investigated the fluorescent dye exclusion from rat parotid intralobular ducts by forskolin to clarify the production route and secretion pathway of bicarbonate. We visualized the secretory function of the cyclic AMP-increasing agent in the parotid intralobular ducts under a confocal laser scanning microscope. Based on experiments using channel inhibitors, we suggest that activation of CFTR and anion channels is responsible for the secretion of bicarbonate produced with carbonic anhydrase by the cyclic AMP-increasing agent.

Materials and Methods

Tissue Preparation

Male Wistar rats (about 300 g) were anesthetized by intraperitoneal injection of sodium pentobarbital. The minced parotid glands were digested for 20 min by collagenase (260 U/ml; type S-1, Nitta Gelatin, Osaka, Japan) dissolved in Krebs-Henseleit Ringer (KHR) solution. KHR solution contained in mM: 103 NaCl, 4.7 KCl, 2.56 $CaCl_2$, 1.13 $MgCl_2$, 25 $NaHCO_3$, 1.15 $NaHPO_4$, 2.8 glucose, 4.9 sodium pyruvate, 2.7 sodium fumarate, 4.9 sodium glutamate, and 0.1% bovine serum albumin, buffered with 12.5 mM HEPES at pH 7.4. The solution was thoroughly aerated with 95% O_2 and 5% CO_2 before each experiment. The digestives, vigorously dispersed by pipetting, were filtrated through a 200-μm filter to remove undigested tissues. The remnant in the KHR solution was suspended in KHR solution and was allowed to attach to a cover glass coated with poly-L-lysine. Then the intralobular ducts (about 40 μm in diameter) on the cover glass without the acini were observed under a confocal laser scanning microscope (LSM-410, Carl Zeiss; Germany) or an inverted epifluorescence microscope (Nikon IMT2-RFL, with a Xenon lamp) equipped with an image analysis system, ARGUS-HiSCA (Hamamatsu Photonics, Hamamatsu, Japan).

Chemicals

Forskolin and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (St. Louis, MO) and Houei Yakukou (Osaka, Japan), respectively. Diphenylamine-2-carboxylate (DPC) was purchased from Wako Pure Chemical (Osaka, Japan). Methazolamide, bumetanide and 4,4′-diisothioctanostilbene-2,2′-disulfonic acid (DIDS) were purchased from Sigma. Sulforhodamine B and dextran tetramethyl-rhodamine were purchased from Eastman Fine Chemicals (Rochester, NY).

Observation of Isolated Ducts Using a Confocal Laser Microscope

The isolated ducts on the cover glass under the confocal laser microscope were superfused with KHR solution containing membrane-impermeable fluorescent dye, dextran tetramethyl-rhodamine (2×10^{-6} M), or sulforhodamine B (5×10^{-6} M) at 3.5 ml per min. The fluorescent dye was allowed to enter into the duct lumen from the superfusion solution and the fluorescence images in the duct lumen with the largest diameter were observed, after 5 min superfusion, by optical sectioning of 0.875 μm depth (40 × oil-immersion objective lens). The isolated ducts were superfused with KHR solution containing the fluorescent dye throughout the experiment. Quantitative fluorescence images were obtained by maintaining the eight-bit digitized signal at less than 235-gray-level and controlling laser intensity to avoid fluorescence saturation. Images were obtained every 15 sec at the same focus level and at the same control levels of contrast and brightness determined at the initial stage of the experiment. Images consisted of 256 × 256 pixels. Since forskolin induced enlargement of the inside diameter of the ducts, but not of the outside diameter, the optical sectioning level at the duct lumen did not change with forskolin application.

Changes in fluorescence intensity at 3–5 points (1.5 × 1.5 μm area (8 × 8 pixels) and 0.875 μm depth) in the duct lumen after forskolin application were monitored at an excitation of 543 nm (He Ne laser). Each fluorescence change in the duct lumen was expressed as relative to that before stimulation. The mean fluorescence intensities in the duct lumen at 2 min, 1 min 45 sec and 1 min 30 sec before forskolin addition were regarded as 100%. Fluctuations in fluorescence due to light-power changes were compensated by measurement of the concomitant fluorescence change in the external superfused solution, monitored at 3 points. Relative fluorescent intensities at 3–5 points in the duct lumen, after compensation for light power fluctuations, were calculated to obtain the mean in one experiment. The results in this study were expressed as means ± SE from typically more than 3 experiments. Fluorescent intensity of sulforhodamine B showed a linear relation with the concentration changes, but no pH-dependency.

The inhibitory effects of inhibitors for channels and transporters were expressed as the percentage of fluorescence recovery. The percentage of fluorescence recovery was calculated as the net fluorescence increase at 5 min after inhibitor addition relative to net decrease at 5 min after forskolin and IBMX addition without inhibitors.

Measurements of Intracellular pH (pH_i) by BCECF in Isolated Duct Cells

The isolated ducts were incubated with 2×10^{-6} M 2′,7′-bis (carboxyethyl)-5(6)-carboxyfluorescein-AM (BCECF-AM; purchased from Dojindo; Kumamoto, Japan) for 15 min and then attached to cover slips that had been coated with poly-L-lysine. The ducts were perfused continuously with KHR solution under an inverted epifluorescence microscope (Nikon IMT2-RFL, with a Xenon lamp). BCECF fluorescence images upon emission at 450 nm and 490 nm were analyzed with an image analysis system, ARGUS-HiSCA (Hamamatsu Photonics, Hamamatsu, Japan). Fluorescence ratios (F490/F450) were calculated simultaneously using the measurements from seven cells or regions in the ducts.

Results

Visualization of fluid secretion in the exocrine glands has been achieved using fluorescent dyes [13, 30, 32–34]. When the isolated rat parotid ducts were superfused with modified KHR solution with membrane-impermeable fluorescent dyes, fluorescent dyes were passively moved into the duct space. By optical sectioning under a confocal laser scanning microscope at the level of the duct lumen with maximum diameter, ducts were observed as dark areas without fluores-

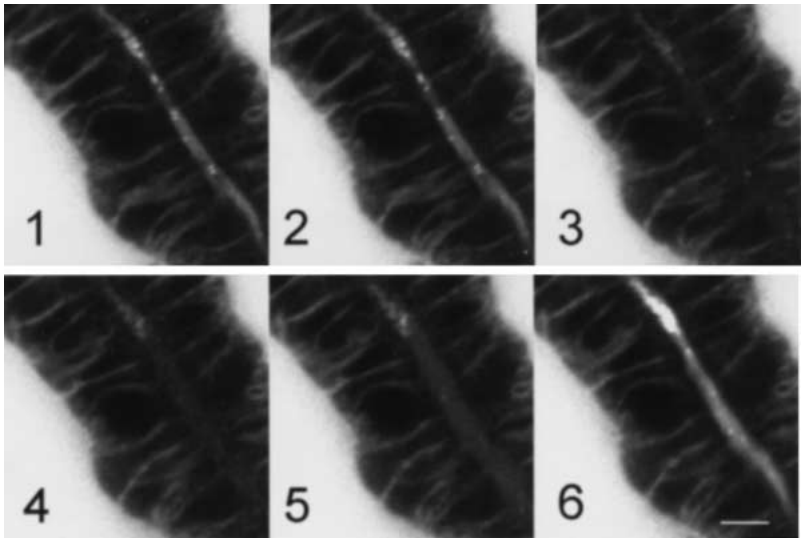

Fig. 1. Effect of forskolin and isobutylmethylxanthine (IBMX) on the change in fluorescence of anionic dye, sulforhodamine, in rat parotid duct lumen, visualized under a confocal laser scanning microscope. The isolated duct was superfused with Krebs-Henseleit solution containing 5 μM sulforhodamine. The isolated duct was treated for 15 min with 10 μM forskolin and 100 μM IBMX. The fluorescence in the duct lumen was observed 1 min before (*1*), immediately before (*2*), 2 min (*3*), 15 min (*4*) after addition of forskolin and IBMX, and 3 min (*5*) and 7 min (*6*) after removal of forskolin. Bar indicates 10 μm.

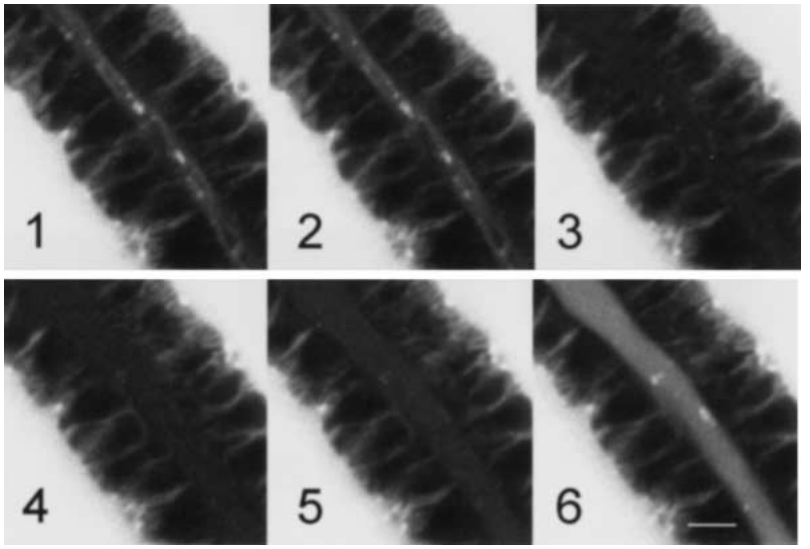

Fig. 2. Effect of forskolin and isobutylmethylxanthine (IBMX) on the change in fluorescence of neutral dye, dextran tetramethyl-rhodamine, in rat parotid duct lumen, visualized under a confocal laser scanning microscope. The isolated duct was superfused with Krebs-Henseleit solution containing 2 μM dextran tetramethyl-rhodamine. The isolated duct was treated for 5 min with 10 μM forskolin and 100 μM IBMX. The fluorescence in the duct lumen was observed 1 min before (*1*), immediately before (*2*), 2 min (*3*), 5 min (*4*) after addition of forskolin and IBMX, and 3 min (*5*) and 7 min (*6*) after removal of forskolin. Bar indicates 10 μm.

cence and the duct lumen exhibited the fluorescence (Fig. 1). The addition of forskolin and isobutylmethylxanthine (IBMX) to a superfusion of the fluorescent dye sulforhodamine B decreased the fluorescence of the anionic dye in the duct space in addition to enlarging the duct's inside diameter (Fig. 1). Forskolin stimulation did not induce morphological changes in the luminal portion in the duct cells. Upon the removal of forskolin, restoration of the fluorescence intensity in the duct space was rapid compared with the restoration of duct space (Fig. 1). Neutral dye, dextran tetramethyl-rhodamine, was also excreted from the duct space by forskolin and IBMX (Fig. 2). The relative fluorescence intensity in the duct space was markedly decreased after 2 min treatment with forskolin. The relative fluorescence intensity was profoundly increased at 3 min after removal of forskolin. Forskolin enlarged the luminal space after 2 min treatment, but did not influence the outside diameter of the ducts (Fig. 3). The time course of changes in the fluorescence intensity is shown in Fig. 4. Forskolin induced the exclusion of the fluorescent dye from rat intralobular ducts, regardless of the nature of the fluorescent dye, suggesting that forskolin might activate fluid secretion in the ducts.

To clarify the involvement of bicarbonate in the forskolin-induced exclusion of the fluorescent dye, we checked the effects of methazolamide, an inhibitor for carbonic anhydrase. Methazolamide did not inhibit the forskolin-induced initial decrease in duct fluorescence, but inhibited forskolin-induced subsequent steady decrease in duct fluorescence (Fig. 5*a*). Pretreatment with methazolamide suppressed the intracellular acidification with forskolin and IBMX, and removal of methazolamide in the presence of fors-

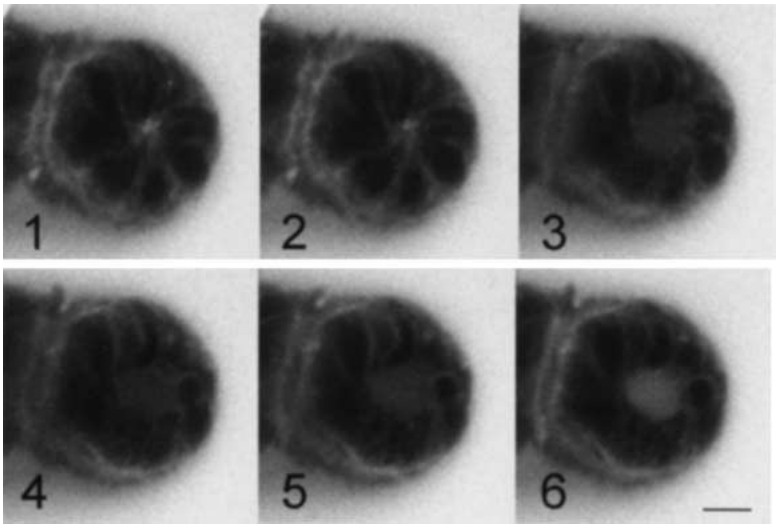

Fig. 3. Duct space enlargement by forskolin. Transverse cross-sectioning of isolated ducts was monitored under a confocal laser scanning microscope. The isolated duct was superfused with the solution containing sulforhodamine, and 10 μM forskolin and 100 μM isobutylmethylxanthine (IBMX) were added for 5 min. The fluorescence in the duct lumen was observed under a confocal laser microscope 1 min before (*1*), immediately before (*2*), 2 min (*3*), 5 min (*4*) after addition of forskolin and IBMX, and 3 min (*5*) and 7 min (*6*) after removal of forskolin. Bar indicates 10 μm.

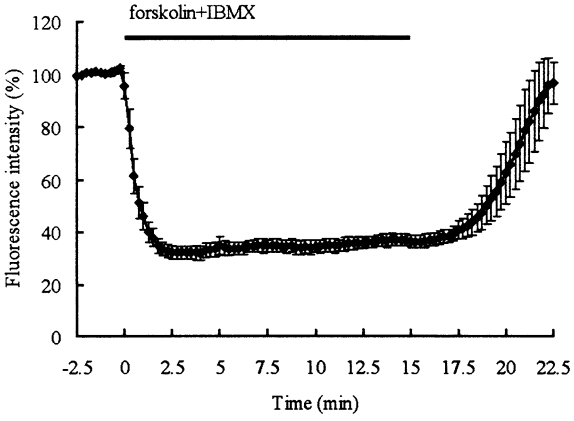

Fig. 4. Time course of forskolin-induced change in the fluorescence intensity of sulforhodamine in rat parotid ducts. The change in the fluorescence intensity in the duct space is expressed relative to that before addition of 10 μM forskolin and 100 μM isobutylmethylxanthine (IBMX). Application of forskolin and IBMX is indicated by the bar. The values are the mean +/− SE ($n = 5$).

kolin and IBMX induced acidification (Fig. 5*b*). Methazolamide suppressed the steady decrease in the duct fluorescence with forskolin and IBMX (Figs. 6 and 7*a*). Forskolin induced two phases of fluid secretion; the initial process was insensitive to methazolamide, and the subsequent one was sensitive to methazolamide.

Transporters for bicarbonate and Cl^- in the membranes of exocrine glands contribute to the fluid secretion [8–10, 14]. We checked the effect of removal of chloride ions and the effect of transporter inhibitors to clarify the involvement of uptake of bicarbonate and Cl^- in the forskolin-induced exclusion of the fluorescent dye. Removal of external Cl^- did not markedly influence the forskolin-induced decrease in the duct fluorescence (Fig. 7*b*). Methazolamide suppressed the steady decrease in the duct fluorescence with forskolin and IBMX, while DIDS and bumetanide (Fig. 8) did not show any profound influences. Bumetanide and removal of external Cl^- induced a slight inhibition of fluorescence recovery, suggesting that some minor portion of dye clearance is related to the Cl^- transport mechanism. However, bicarbonate production, but not uptake of Cl^-, is involved in the forskolin-induced major portion of the subsequent exclusion of fluorescent dye from the ducts.

The secretory process in the acinar and duct cells is triggered through the activation of luminal anion channels [16, 25, 27]. To clarify the pathway of bicarbonate secretion, we investigated the effects of channel inhibitors on forskolin-induced exclusion of the fluorescent dye. Glibenclamide, NPPB and DPC, but not amiloride, inhibited a forskolin-induced decrease in fluorescence in the duct space (Fig. 8). DMA, an inhibitor for Na^+/H^+ exchange, also inhibited the forskolin-induced decrease in fluorescence in the ducts (Fig. 8). Forskolin evokes a sustained fluid movement, possibly through CFTR and anion channels in rat parotid ducts, associated with the secretion of carbonic anhydrase-dependent bicarbonate linked with Na^+/H^+ exchange.

Discussion

Visualization of the duct secretion process using fluorescent dyes under a laser scanning microscope revealed that forskolin stimulated the exclusion of the fluorescent dyes, possibly through fluid secretion associated with the secretion of bicarbonate in rat parotid intralobular ducts. This process was mediated possibly through CFTR or anion channels activated with cyclic AMP. In addition to the secretory activation, forskolin enlarged the luminal space in the ducts in the absence of any change in the outside diameter, suggesting that forskolin induces the

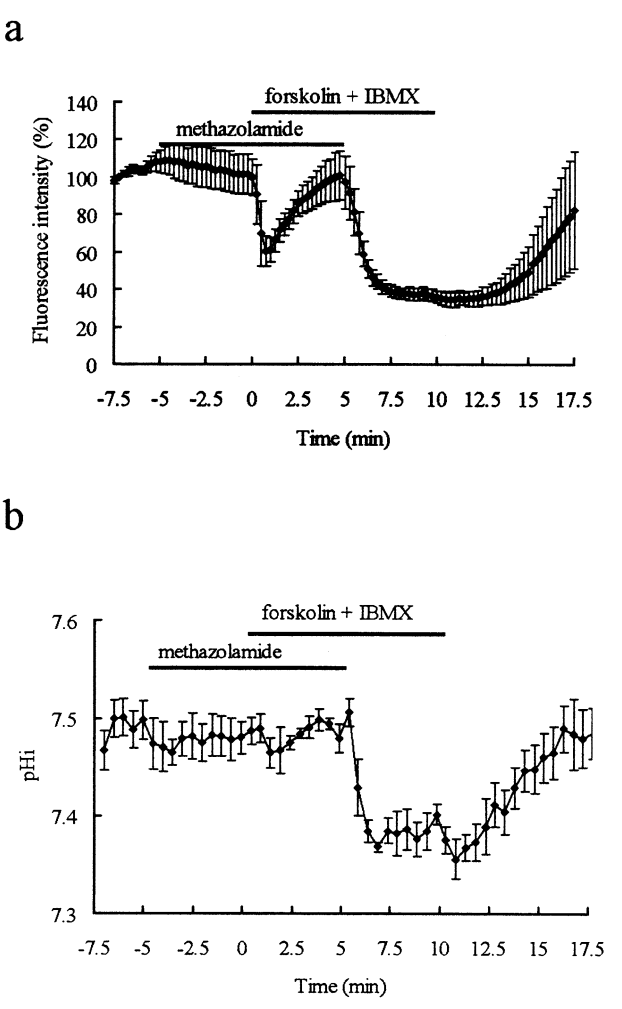

Fig. 5. Effect of methazolamide pretreatment on forskolin-induced change in the relative fluorescence intensity of 5 μM sulforhodamine and intracellular pH in the ducts. (*a*) Effect on the forskolin-induced change in the relative fluorescence intensity of 5 μM sulforhodamine. The isolated duct was superfused for 5 min with 1 mM methazolamide, for 5 min with 10 μM forskolin and 100 μM isobutylmethylxanthine (IBMX) in the presence of methazolamide, and then for 5 min with forskolin and IBMX. The calculation of the relative fluorescence intensity was described in Materials and Methods. The values are means +/− SE ($n = 3$). (*b*) Effect on forskolin-induced intracellular pH in rat parotid duct cells. The isolated duct was superfused for 5 min with 1 mM methazolamide, for 5 min with 10 μM forskolin and 100 μM IBMX in the presence of methazolamide, and then for 5 min with forskolin and IBMX. The values are means +/− SE ($n = 3$).

enlargement of duct space through cell shrinkage. Visualization of the duct secretory process, using the fluorescent dye, is a useful method for the clarification of the secretion mechanism in isolated ducts.

We demonstrated that forskolin activated the exclusion of neutral and anionic fluorescent dyes from the duct space and the enlargement of the inside diameter during the stimulation. Upon the removal of forskolin, fluorescence intensity in the duct space was markedly increased, but the restoration of the duct space was delayed. Taken together, the present findings suggest that fluorescent dyes are passively infiltrated into the duct space according to the concentration gradient and are persistently excluded by forskolin from the duct space, possibly through fluid secretion. It is unlikely that secretory function in the duct is due to the presence of acinar-like cells in the ducts, since the secretagogue-induced morphological changes in the acinar cells [30] were not observed in the parotid duct segments. If the acinar cells were mixed in the ducts, acinar-like cells might not be involved in the activation of the duct secretory function by forskolin, since forskolin does not activate the membrane current in the parotid acinar cells [12, 35], although ATP activates CFTR-like currents in the acinar cells [39, 40]. Forskolin-induced fluid secretion in the parotid ducts is consistent with that in the pancreatic ducts [6, 8, 14]. Exclusion of the fluorescent dye and vesicular substances from the parotid duct space is also induced by carbachol through an increase in intracellular calcium ions [23]. Dye exclusion from the salivary duct space is driven by the autonomic nervous activation and it is likely that the fluid secretory function might be common in the excretory ducts.

Pretreatment of the ducts with methazolamide did not suppress the initial transient exclusion of the fluorescent dye by forskolin, but inhibited the subsequent exclusion of the fluorescent dye. Forskolin-induced exclusion of fluorescent dyes is divided into two phases; one is an initial and transient exclusion, insensitive to methazolamide, and the other is the subsequent exclusion, sensitive to methazolamide. Initial secretion in the salivary acinar cells is due to the secretion of endogenous bicarbonate [21]. Low Cl^- solution and transporter inhibitors for $Na^+/K^+/2\ Cl^-$ and Cl^-/HCO_3^- did not markedly suppress the forskolin-induced later exclusion phase of the fluorescent dye. It is suggested that production of bicarbonate, but not via transporter, is responsible for a major portion of the forskolin-induced later exclusion of the fluorescent dyes. The source of bicarbonate is due to the production by carbonic anhydrase in the cytoplasm from CO_2 [3, 22, 37]. This conclusion is also supported by the observation that methazolamide suppressed forskolin-induced acidification. Luminal Cl^-/HCO_3^- exchange activity and other transporters of H^+/HCO_3^- function in submandibular gland ducts [17, 41], but Cl^-/HCO_3^- exchange activity does not work in the parotid ducts [28]. The parotid duct system produces HCO_3^- through carbonic anhydrase, but not through a HCO_3^- transporter.

Forskolin suppresses type III Na^+/H^+ exchangers [38], and this is consistent with our findings on forskolin-induced acidosis. However, the inhibitor for Na^+/H^+ exchange, DMA, inhibited the forskolin-induced decrease in the duct fluorescence, and this suggests that DMA might suppress other types of Na^+/H^+ exchangers activated by forskolin. Forskolin

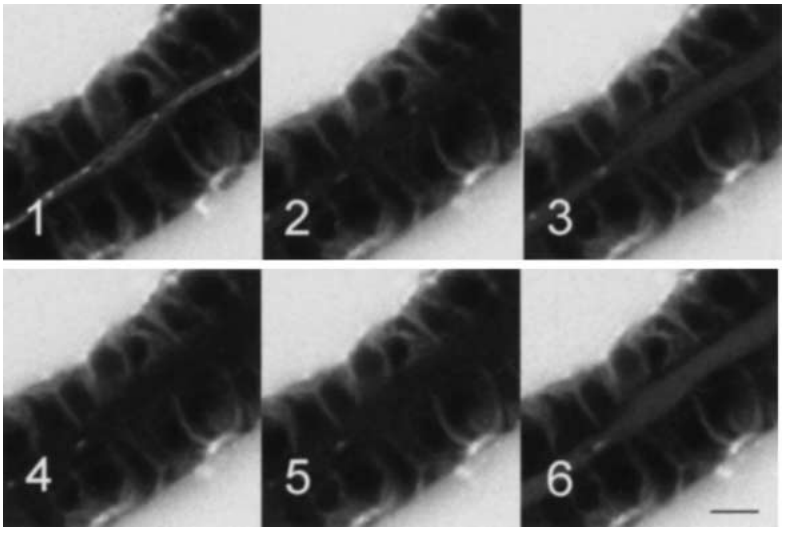

Fig. 6. Effect of methazolamide on the forskolin and isobutylmethylxanthine (IBMX)-induced decrease in the fluorescence of anionic dye, sulforhodamine, in rat parotid duct lumen, visualized under a confocal laser scanning microscope. The isolated duct was superfused with Krebs-Henseleit solution containing 5 μM sulforhodamine. The isolated duct was treated for 15 min with 10 μM forskolin and 100 μM IBMX, and methazolamide was added for 5 min in the presence of forskolin and IBMX. The fluorescence in the duct lumen was observed 1 min before (*1*), and 5 min (*2*) after the addition of forskolin and IBMX, 5 min (*3*) after the addition of methazolamide in the presence of forskolin and IBMX, 5 min (*4*) after removal of methazolamide in the presence of forskolin and IBMX, and 3 min (*5*) and 7 min (*6*) after removal of forskolin and IBMX. Bar indicates 10 μm.

induced cell shrinkage of duct cells, and cell shrinkage activates Na^+/H^+ exchange [2], Since many types of Na^+/H^+ exchangers are present in the parotids [26], it is likely that forskolin might suppress and/or activate Na^+/H^+ exchangers. Although Na^+/H^+ exchanger and Na^+/HCO_3^- cotransporter are involved in the regulation of the intracellular pH in mouse duct cells [4, 19], Na^+/H^+ exchangers contribute to pH regulation in parotid duct cells [28]. Forskolin might induce the fluid secretion, associated possibly with bicarbonate secretion, linked with H^+ outflux.

The exclusion of the fluorescent dyes in the parotid ducts by forskolin is mediated possibly through the activation of anion channels and CFTR, based on the present findings on the effects of anion channel inhibitors such as DPC, NPPB and glibenclamide on the exclusion of fluorescent dye. These channels are present in the duct [9, 10, 40]. The present conclusion is consistent with the observations that an increase in Cl^- permeability of pancreatic and submandibular duct cells by cAMP is suppressed by DPC [1, 7], and that CFTR and anion channels are permeable for Cl^- and bicarbonate [5, 7, 15, 16, 25, 31]. Glibenclamide

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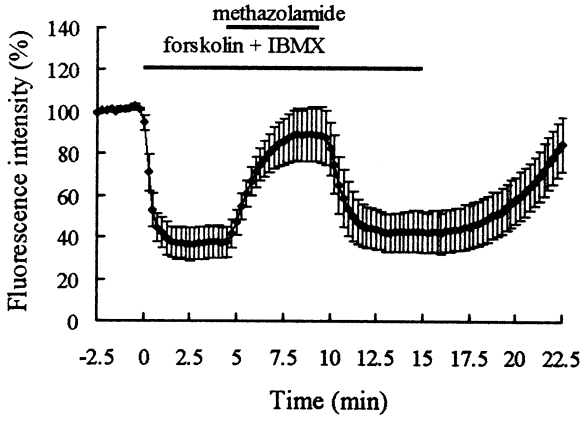

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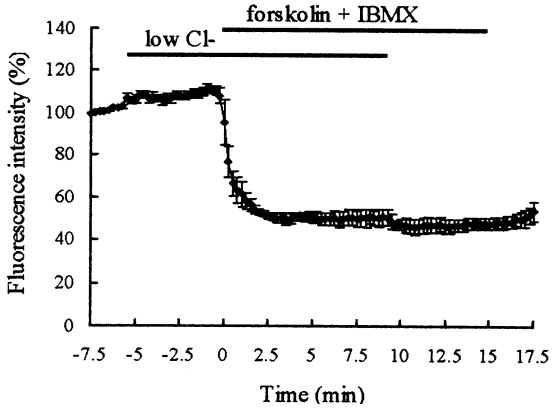

Fig. 7. Effects of methazolamide and removal of chloride ions on the forskolin-induced change in the relative fluorescence intensity of 5 μM sulforhodamine in the duct lumen. (*a*) Effect of methazolamide treatment. The isolated duct was superfused for 5 min with 10 μM forskolin and 100 μM isobutylmethylxanthine (IBMX), for 5 min with forskolin and IBMX in the presence of methazolamide, and then for 5 min with forskolin and IBMX. The calculation of the relative fluorescence intensity was described in Materials and Methods. The values are means +/− SE ($n = 5$). (*b*) The effect of low concentration of external Cl^-. The isolated duct was superfused for 5 min with low concentrations of external Cl^- solution, for 5 min with forskolin and IBMX in low concentrations of external Cl^- solution, and then for 5 min with forskolin and IBMX in normal concentrations of external Cl^- solution. The values are means +/− SE ($n = 4$).

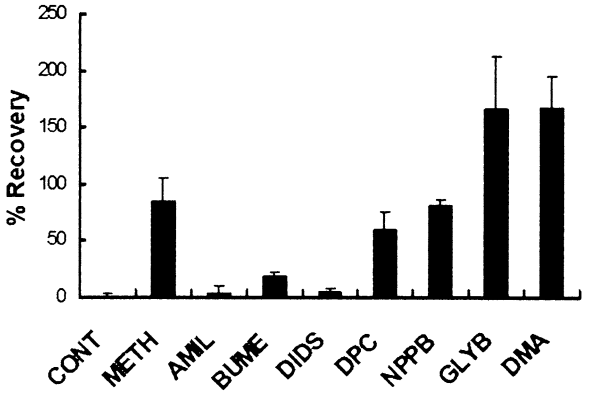

Fig. 8. The effect of inhibitors for transporters and ion channels on forskolin-induced fluorescence change of sulforhodamine in duct space. Methazolamide (1 mM), amiloride (10 μM), DPC (100 μM), NPPB (100 μM) glibenclamide (200 μM), dimethyl amiloride (DMA, 20 μM), bumetanide (500 μM), and DIDS (200 μM) were added for 5 min during the application of 10 μM forskolin and 100 μM isobutylmethylxanthine. The percentage of fluorescence recovery was calculated as the net fluorescence increase at 5 min after inhibitor addition relative to the net decrease at 5 min after forskolin and IBMX addition without inhibitors. The percentages of recovery are indicated as means +/− SE (n = 3–4).

inhibits the ATP-activated ROMK-type K_{ATP} channels in addition to CFTR [18], and it is not neglected that bicarbonate secretion through CFTR might be coupled with the K channel activation. However, gramicidine-perforated patch analysis also suggested the forskolin-induced bicarbonate secretion through anion channels including CFTR in rat parotid ducts [11]. It is plausible that activation of CFTR and anion channels with cyclic AMP is responsible for bicarbonate secretion from the ducts.

In the present study, visualization of dye-exclusion under a confocal laser scanning microscope suggested that the parotid intralobular ducts as exocrine glands might contribute to forskolin-induced secretion of bicarbonate, produced with carbonic anhydrase. The fluid secretion might lead to the exclusion of the fluorescent dyes. Forskolin also induces the enlargement of parotid ducts. Fluid secretion with bicarbonate secretion and duct enlargement by the secretagogues are the essential secretory function in the parotid ducts.

This study was supported, in part, by a Grant-in-Aid for Scientific Research (C12671808) from the Japan Society for the Promotion of Science (JSPS).

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