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Research Article

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Effect of Isoproterenol on Intracellular pH of the Intercalated Cells in the Rabbit Cortical Collecting Ducts

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Abstract

To examine the mechanisms which regulate the functions of the intercalated cells (ICs) in the cortical collecting duct (CCD), the effect of isoproterenol on intracellular pH (pHi) of ICs was studied with the in vitro microperfused rabbit CCD, using the single cell pHi determination technique with fluorescent dye, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein. The pHi of β -IC was significantly decreased with the addition of basolateral 10^{-6} M isoproterenol (7.21 ± 0.04 to 7.05 ± 0.04), whereas α -IC did not show any change. This response of β -IC to isoproterenol was dose-dependent and completely inhibited by the β -blockers, atenolol or propranolol. The addition of forskolin or 8-Br-cAMP mimicked the effects of isoproterenol, suggesting that the activation of adenylate cyclase induced the decrease in pHi. The rate of pHi changes after the Cl^- removal from the perfusate, which is considered to reflect the activity of luminal anion exchanger, was significantly higher with isoproterenol (0.032 ± 0.009 pH unit/s) than that in the control (0.023 ± 0.009 pH unit/s). The present studies provide direct evidence for the regulation of β -IC function by β -adrenergic receptor; and the luminal $\text{Cl}^-/\text{HCO}_3^-$ exchanger was considered to be stimulated by β -agonist, directly. (*J. Clin. Invest.* 1991; 87:1153–1157.) Key words: microperfusion • forskolin • cyclic AMP • anion exchanger • Na^+/H^+ exchanger

Introduction

The collecting ducts of the mammalian nephron are important in the modulation of acid-base balance. Recent studies have shown that the rabbit collecting ducts have principal cells and at least two types of intercalated cells, α and β (1–3). The α -intercalated cells are thought to secrete H^+ and reabsorb HCO_3^- , whereas the β -intercalated cells secrete HCO_3^- . To perform that function, the α -intercalated cells have an electrogenic proton translocating ATPase (H^+ -ATPase) on the luminal membrane (4) and $\text{Cl}^-/\text{HCO}_3^-$ exchanger on the basolateral membrane (5). On the other hand, the β -intercalated cells showed basolateral or diffuse staining by the monoclonal antibody to H^+ -ATPase (4). The recent report by Weiner and Hamm (6) has added the functional evidence of a basolateral Na^+/H^+ exchanger and luminal $\text{Cl}^-/\text{HCO}_3^-$ exchanger in β -intercalated cells. The mechanisms which regulate those transporters have not yet been fully understood, although several lines of evidence sug-

gested that cAMP and isoproterenol might regulate the function of the intercalated cells. Schuster has reported that cAMP and isoproterenol stimulates HCO_3^- secretion by the rabbit cortical collecting ducts (CCD) perfused in vitro (7). It has been also reported that the lumen to bath Cl^- flux was increased by cAMP and isoproterenol in rabbit CCD (8), suggesting that the luminal $\text{Cl}^-/\text{HCO}_3^-$ exchanger might be stimulated by those substances. However, these studies provide only indirect evidence of the regulating mechanisms of the intercalated cells, because the stimulation of H^+ -ATPase or Na^+/H^+ exchanger would result in the cell alkalization and therefore an increase in HCO_3^- secretion and the lumen to bath Cl^- flux.

The aim of the present studies was to examine the role of the β -adrenergic receptor on the regulation of the transport systems of the intercalated cells. To study the pHi of each cell type separately, we used single cell pH recording technique with the fluorescent, pH-sensitive dye 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF)¹ in the in vitro microperfused rabbit CCD.

Methods

Cortical collecting ducts (CCD) were dissected from female Japanese house rabbits weighing 1.5–2.0 kg and perfused as previously described (9, 10). All rabbits were given free access to standard food and water before death. A $\text{HCO}_3^-/\text{CO}_2$ buffered solution gassed with 95% O_2 and 5% CO_2 was used for dissection as well as for the perfusion and bath solutions. BSA (5% wt/vol) was added to the dissection medium. CCD was isolated freehand under a stereomicroscope and then transferred to a perfusion chamber. The two ends of the tubules were mounted in glass pipettes. The in vitro microperfusion was performed using standard technique with an $\text{HCO}_3^-/\text{CO}_2$ buffered solution containing (in millimolar) NaCl, 115; KCl, 5.0; NaHCO_3 , 25.0; Na_2HPO_4 , 1.6; NaH_2PO_4 , 0.4; CaCl_2 , 1.8; MgSO_4 , 1.0; D-glucose, 8.3; L-alanine, 5.0; and Na acetate, 10. When the bath or perfusate was changed to a Cl^- -free solution, gluconate was substituted for Cl^- and total Ca was increased to 10.4 for compensation of Ca^{2+} complexity by gluconate (10). After the start of microperfusion, we moved the microscope stage to set the tubule in a narrow groove portion ($\sim 100 \mu\text{l}$) of the perfusion chamber to achieve a quick solution change. The bath solution was preheated through a water-jacketed line, and bath temperature was maintained at 37°C. Bath flow rate was maintained at 3–3.5 ml/min by a perfusion pump (Minipuls 2, Gilson Medical Electronics, France) so that we could exchange the solution within 3 s. Bath solutions were exchanged by a four-way distribution valve before a bath entry. To change luminal fluid quickly, a tapered PE-10 tube was inserted into the tip of the perfusion pipette and placed during assembly of the perfusion equipment. Another end of the PE tube was connected to an injec-

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1. Abbreviations used in this paper: BCECF, 2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; BCECF-AM, acetomethyl ester of BCECF; CCD, cortical collecting ducts; IC, intercalated cell; OMCD, outer medullary collecting ducts; pHi, intracellular pH; H^+ -ATPase, proton translocating ATPase; PNA, peanut agglutinin.

tion needle and syringe via a three-way valve. An outlet of perfusion solution was placed at the back end of the perfusion pipette and connected to a reservoir, which was used for adjusting hydrostatic pressure to maintain luminal flow. By this system, it was possible to change luminal fluid within 5 s. The time required for the fluid change was confirmed by the appearance of a solution containing green dye before and after the experiments. The data was discarded, when there was a time variable of > 2 s.

Peanut agglutinin binding study. To identify β -intercalated cells, FITC-labeled peanut agglutinin (PNA, 10 $\mu\text{g}/\text{ml}$) was loaded from the lumen for 5 min at 37°C. After loading, the tubules were excited at 490 nm and observed under high magnification. A cell with a characteristic lectin cap on the edge of the tubule was identified as a β -intercalated cell and a red light spot, which indicates an area for pH measurement, was placed on that cell. Tubules were allowed to equilibrate for 2–3 h after PNA loading and the disappearance of fluorescence was confirmed before continuing the experiment. To study α -intercalated cells, CCD was taken from the deep cortex, and PNA-negative and BCECF-positive cells were used.

Measurement of intracellular pH. The acetomethyl ester of BCECF (BCECF-AM) was dissolved in DMSO as a 30-mM stock solution and was diluted to 15 μM with standard solution. BCECF was loaded from the lumen for 15–30 min. Using this loading method, BCECF-AM was selectively concentrated by intercalated cells (11). At 20 min after the end of loading, the experiment was started. This incubation period made the pH recordings stable. The measurement of fluorescence was performed by Olympus OSP System (Olympus Optical, Tokyo, Japan). The diameter of the beam of light focused on the tubule was usually 25 μm . The loaded tubules were excited alternately at 490 and 440 nm by spinning the sector mirror at 6,000 rpm. To avoid cell damage by the prolonged light exposure, the tubule was excited for 100 ms every 2–5 s. Fluorescence at 530 nm was measured by Olympus PMU unit. The area for fluorescence measurement was 5 μm diameter and positioned at the edge of the tubule to avoid the contamination of fluorescence from the other cells. The digital output of the photometer was recorded and processed on a personal computer. During 100 ms of excitation, 10 values of fluorescence ratio were obtained, and a mean of those values was taken as one measurement. Background fluorescence was < 5% of dye fluorescence at both exciting lengths and was subtracted before calculation of fluorescence ratio.

After a stable pH recording, basal pHi was determined as a mean of five or six measurements just before the bath solution was changed to $\text{HCO}_3^-/\text{CO}_2$ buffered solution containing isoproterenol, 8-Br-cAMP, water-soluble forskolin, or various substances (concentrations of these drugs were given in the figure legends and results). Within 2 min, pHi was stabilized and a mean of three or four measurements was taken as the change in pHi. After 3–5 min of recordings of pHi with those substances, the bath solution was changed to the standard solution. After these procedures, the cell types were confirmed by the Cl^- removal from the bath solution. In this way, β -intercalated cells used in the present studies showed cell acidification, and α -intercalated cells showed cell alkalization. All peanut lectin-positive cells were acidified by the bath Cl^- removal and identified as β -intercalated cells.

To examine the activity of $\text{Cl}^-/\text{HCO}_3^-$ exchanger on the luminal membrane of β -intercalated cells, the changes in pHi after Cl^- removal from the luminal perfusate were measured. After stable recordings of pHi, luminal perfusate was quickly changed to Cl^- -free solution. The rate of pHi change during the initial 20 s was calculated from the recordings.

Calibration. At the end of each experiment, calibration of intracellular BCECF was done by the addition of nigericin (14 μM) and HEPES-buffered solution, as previously described (12). The calibration solution contained 120 KCl, 1.2 CaCl_2 , 1.0 MgCl_2 , 2.0 NaH_2PO_4 , and 25 HEPES and was adjusted to pH 6.8, 7.0, 7.2, 7.4, 7.6, 7.8 using NaOH or HCl. Using the least square method, linear regression was performed and this calibration curve was used for the calculation of pHi from the measurement of fluorescent ratio.

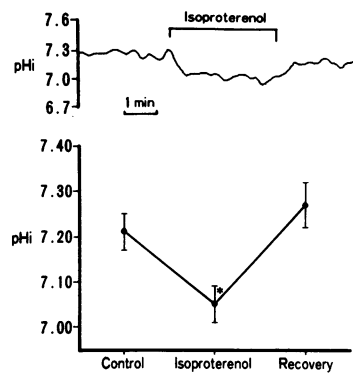


Figure 1. (Upper panel) A representative tracing of 10^{-6} M isoproterenol effect on pHi of β -intercalated cells. (Lower panel) Summary of isoproterenol effect on pHi of β -intercalated cells. * $P < 0.001$ compared with control period ($n = 12$).

Reagent and statistics. BCECF-AM was purchased from Molecular Probes Inc., Junction City, OR and water-soluble forskolin analogue (7 β -desacetyl-7 β -[γ (N-methylpiperazino)-butyryl]forskolin) was purchased from Calbiochem-Behring Corp., La Jolla, CA. All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO) or Wako Junyaku (Osaka, Japan).

Values are presented as means \pm SEM. All experiments were performed in a paired manner and analyzed by paired Student's t test.

Results

The upper panel of Fig. 1 shows the representative tracing of the effect of basolateral isoproterenol on pHi. Within 15 s after the exposure to isoproterenol, pHi started to decrease, gradually reaching a new steady state within 2 min. The summary of the isoproterenol studies is shown in the lower panel of Fig. 1. The resting pHi of β -intercalated cells was 7.21 ± 0.04 and decreased to 7.05 ± 0.04 after the addition of isoproterenol to the bath. The pHi returned to the basal pHi after the cessation of isoproterenol. To confirm that this pHi change was induced via β -adrenergic receptor, the effects of basolateral propranolol and atenolol (β_1 -selective antagonist) were studied. Fig. 2 shows the representative tracing and the summary of that study. No significant pHi change was seen after either the basolateral application of a β -blocker alone or the isoproterenol addition to the bath containing a β -blocker. The isoproterenol-induced cell acidification was dose dependent and the maximal response was obtained at 10^{-6} M (Fig. 3). On the other hand, isoproterenol did not induce significant pHi changes in α -intercalated cells in CCD (7.30 ± 0.16 to 7.32 ± 0.12 , $n = 4$).

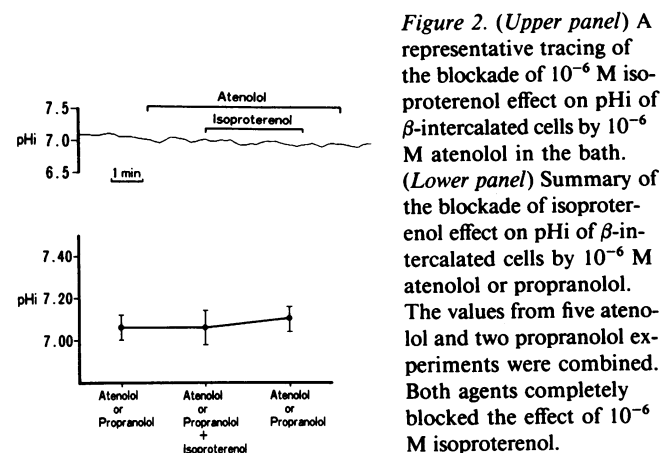


Figure 2. (Upper panel) A representative tracing of the blockade of 10^{-6} M isoproterenol effect on pHi of β -intercalated cells by 10^{-6} M atenolol in the bath. (Lower panel) Summary of the blockade of isoproterenol effect on pHi of β -intercalated cells by 10^{-6} M atenolol or propranolol. The values from five atenolol and two propranolol experiments were combined. Both agents completely blocked the effect of 10^{-6} M isoproterenol.

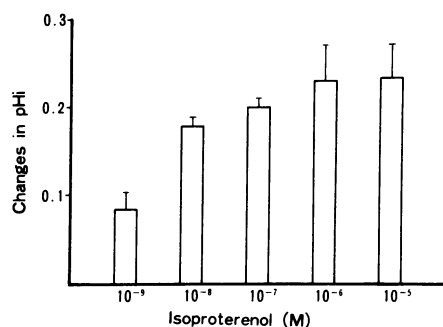


Figure 3. Relationship between pHi changes and the concentration of isoproterenol in β -intercalated cells. The values were determined from four experiments at each concentration.

To examine if this effect of isoproterenol on pHi was mediated by the activation of adenylate cyclase, the effect of water-soluble forskolin analogue and 8-Br-cAMP applied to the bath were studied. As shown in Figs. 4 and 5, both substances induced a significant decrease in pHi of β -intercalated cells and the pHi changes induced by both forskolin and 8-Br-cAMP were comparable to the isoproterenol-induced pHi changes.

To show which transporters are involved in the isoproterenol-induced cell acidification, we determined the effect of the inhibitor for Na^+/H^+ exchanger, amiloride, on the isoproterenol-induced pHi changes. Fig. 6 depicts the summary of that study. Basolateral application of amiloride itself induced cell acidification, suggesting that Na^+/H^+ exchanger was active at basal condition, although amiloride treatment did not abolish the effect of bath isoproterenol and cAMP. On the other hand, with the removal of Cl^- from the luminal perfusate, isoproterenol did not induce any pHi changes (Fig. 7), indicating that $\text{Cl}^-/\text{HCO}_3^-$ exchanger is required to show the isoproterenol action. To confirm the role of that exchanger, we determined the rate of pHi changes after luminal Cl^- removal with or without the presence of basolateral isoproterenol. This maneuver is considered to induce HCO_3^- influx to the cells. The rate of pHi changes after the exchanges of the perfusate (Fig. 8) was significantly faster with isoproterenol than that in basal condition.

Discussion

The present studies show that the β -adrenergic agonist, isoproterenol, decreased pHi of β -intercalated cells but not α -intercalated cells. This effect of isoproterenol was dose-dependent and completely inhibited by the β -blockers, propranolol and ateno-

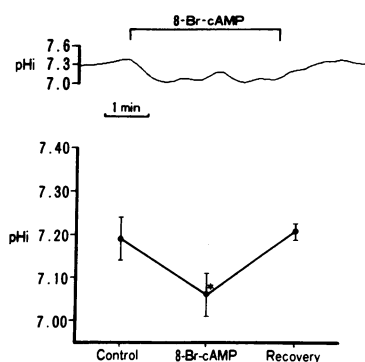


Figure 4. (Upper panel) A representative tracing of 10^{-4} M 8-Br-cAMP effect on pHi of β -intercalated cells. (Lower panel) Summary of 8-Br-cAMP effect on pHi of β -intercalated cells. * $P < 0.01$ compared with control period ($n = 9$).

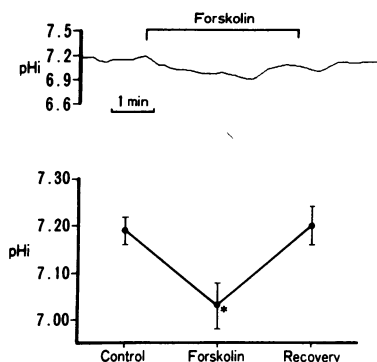


Figure 5. (Upper panel) A representative tracing of 10^{-5} M water-soluble forskolin analogue on pHi of β -intercalated cells. (Lower panel) Summary of forskolin effect on pHi of β -intercalated cells. * $P < 0.01$ compared with control period ($n = 8$).

lol. Furthermore, both 8-Br-cAMP and forskolin mimicked the effect of isoproterenol, suggesting that the activation of adenylate cyclase induces pHi changes. The rate of pHi changes by Cl^- -free perfusate indicated that $\text{Cl}^-/\text{HCO}_3^-$ exchanger on the luminal membrane was stimulated by isoproterenol via β -adrenergic receptor.

It has been reported that bicarbonate secretion was increased by cAMP in the in vitro microperfused rabbit CCD (7). The cAMP and isoproterenol also stimulated the lumen to bath Cl^- flux and the authors raised the possibility that β -intercalated cells are regulated by β -adrenergic receptor (8). This possibility was supported by the studies with isolated intercalated cells obtained by fluorescence-activated cell sorting (13). In that report, the isolated intercalated cells showed the activation of adenylate cyclase by isoproterenol. Furthermore, the autoradiographic studies revealed that β -adrenergic receptor was present in the collecting ducts (14, 15), and that the majority of receptors was predominately of the β_1 -subtype (16). The present studies are consistent with those reports and clearly showed that isoproterenol regulates the function of β -intercalated cells via its specific β_1 -adrenergic receptor. Both forskolin and 8-Br-cAMP acidified the β -intercalated cells, suggesting that the isoproterenol effect was mediated by the activation of adenylate cyclase. The adenylate cyclase activation by isoproterenol in the isolated intercalated cells (13) also supports the contention that the classical signal transduction system of β -adrenergic receptor is present in β -intercalated cells. However, our present studies do not exclude the possibility that adenylate cyclase independent mechanisms, which has been recently reported in multiple cell types (17), might also play a role in the effect of isoproterenol. On the contrary to our present studies, Emmons and Stokes have reported that cAMP induced cell alkalization

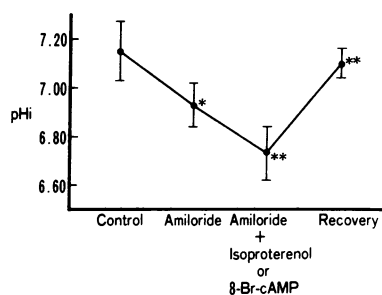


Figure 6. Summary of the effect of amiloride on isoproterenol-induced pHi changes in β -intercalated cells. * $P < 0.05$, ** $P < 0.01$ compared with the preceding period. The values from four isoproterenol and three 8-Br-cAMP experiments were comparable and combined.

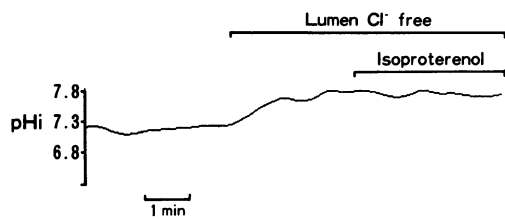


Figure 7. A representative tracing of the effect of Cl^- removal from the perfusate on isoproterenol effect on pHi of β -intercalated cells. In three experiments, no significant pHi changes were seen without Cl^- in the perfusate.

in β -intercalated cells (18). Differences in experimental procedures are not apparent, and we do not know the reason for these completely opposite results. However, their report appeared only in abstract form, and certain differences in experimental conditions might have resulted in different actions of cAMP.

Recently, several studies have immunohistochemically and functionally defined the acid-base transport systems in α - and β -intercalated cells. Using monoclonal antibody to H^+ -ATPase, Brown et al. (4) clearly showed that α -intercalated cell has that enzyme on the luminal membrane. In contrast, the β -intercalated cell has it on the basolateral membrane or shows diffuse distribution in the cells. Schuster et al. (5), using a monoclonal antibody to red blood cell anion exchanger, band 3 protein, have reported that the intercalated cells in CCD and outer medullary collecting ducts (OMCD) were divided to two cell types. The basolateral membrane of one type was stained by monoclonal antibody to band 3 protein and that localization of anion exchanger was consistent with the function of α -intercalated cells. Another cell type was stained by PNA but not by the monoclonal antibody. The newly developed pHi recording technique with fluorescent dye, BCECF, provided further evidence of the transport systems in two functionally different intercalated cells. Breyer and Jacobson have reported the existence of Na^+/H^+ exchanger and $\text{Cl}^-/\text{HCO}_3^-$ exchanger on the basolateral membrane of OMCD cells (19). The intracellular pH regulation of the β -intercalated cells were examined by Weiner and Hamm (11), and they have reported the Na^+/H^+ exchanger on the basolateral membrane and the $\text{Cl}^-/\text{HCO}_3^-$ exchanger on the luminal membrane. Those results are consistent with the previous reports of Cl^- and bicarbonate flux studies in the in vitro microperfused collecting ducts (10, 20, 21). From these reports, H^+ -ATPase, Na^+/H^+ exchanger, and $\text{Cl}^-/\text{HCO}_3^-$ exchanger were considered to play important roles in the regulation of pHi of the intercalated cells. Changes in any of those transporters could induce acidification of β -intercalated

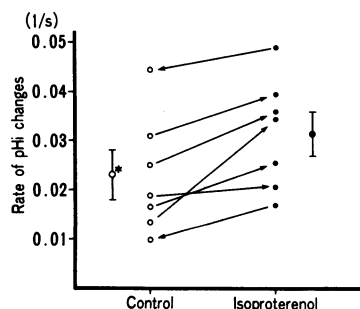


Figure 8. The rate of pHi changes induced by the Cl^- removal from the perfusate with or without 10^{-6} M isoproterenol in the bath. * $P < 0.05$ compared with control. An arrow indicates the order of the experiment.

cells by isoproterenol. In many cell types, indeed, the Na^+/H^+ exchanger is regulated by various hormones (22, 23). In the present studies, however, it was unlikely that the changes in Na^+/H^+ exchanger activity was the main component in the effect of isoproterenol, because it induced cell acidification even in the presence of amiloride. On the other hand, the results of luminal Cl^- removal studies clearly showed that luminal $\text{Cl}^-/\text{HCO}_3^-$ exchanger was stimulated by basolateral isoproterenol. As shown in the previous study (24), that maneuver induced cell alkalization by the influx of bicarbonate to the cells via reversed $\text{Cl}^-/\text{HCO}_3^-$ exchanger; and the rate of pHi changes is considered to reflect the activity of that exchanger (25). We have not examined the effect of *N*-ethylmaleimide to determine a role of H^+ -ATPase, because that inhibitor is non-specific and a specific inhibitor is not available now. The role of H^+ -ATPase on isoproterenol effect remains to be determined.

In summary, our present studies provide the first direct evidence of the regulation of β -intercalated cell function by β -adrenergic receptor. The $\text{Cl}^-/\text{HCO}_3^-$ exchanger on the luminal membrane is activated by β -agonist, whereas it is unlikely that Na^+/H^+ exchanger is involved in that action.

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