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Inhibition of gastric H^+,K^+-ATPase by 4-(2-butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl)oxybutyric acid (DCPIB), an inhibitor of volume-regulated anion channel

Takuto Fujii [a], Yuji Takahashi [a], Hiroshi Takeshima [b], Chisato Saitoh [a], Takahiro Shimizu [a], Noriaki Takeguchi [a], Hideki Sakai [a,*]

[a] Department of Pharmaceutical Physiology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan
[b] Department of Biological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan

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ABSTRACT

4-(2-Butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl)oxybutyric acid (DCPIB) has been used as an inhibitor of volume-regulated anion channel (VRAC), which is expressed in almost all cells (IC_{50} is around 4 μM). Here, we found that DCPIB significantly inhibited the activities of gastric proton pump (H^+,K^+-ATPase) in isolated gastric tubulovesicles and the membrane sample of the H^+,K^+-ATPase-expressing cells, and their IC_{50} values were around 9 μM. In the tubulovesicles, no significant expression of leucine rich repeat containing 8 family member A (LRRC8A), an essential component of VRAC, was observed. The inhibitory effect of DCPIB was also found in the membrane sample obtained from the cells in which LRRC8A had been knocked down. On the other hand, DCPIB had no significant effect on the activity of Na^+,K^+-ATPase or Ca^{2+}-ATPase. In the H^+,K^+-ATPase-expressing cells, DCPIB inhibited the $^{86}Rb^+$ transport activity of H^+,K^+-ATPase but not that of Na^+,K^+-ATPase. DCPIB had no effect on the activity of Cl^- channels other than VRAC in the cells. These results suggest that DCPIB directly inhibits H^+,K^+-ATPase activity. DCPIB may be a beneficial tool for studying the H^+,K^+-ATPase function in vitro.

1. Introduction

Volume-regulated anion channel (VRAC) is ubiquitously expressed and has a pivotal role in the cell volume regulation: the anion channel is activated by osmotic cell swelling to maintain the original cell size via Cl^- efflux (Nilius et al., 1996; Okada, 1997). VRAC activation is also involved in apoptotic and necrotic cell death. Recently, leucine rich repeat containing 8 family, member A (LRRC8A), also named SWELL1, was found to be an essential component of VRAC (Qiu et al., 2014; Voss et al., 2014). 4-(2-Butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl) oxybutyric acid (DCPIB) is a potent inhibitor of VRAC (Decher et al., 2001). DCPIB has been widely used to evaluate the physiological and pathophysiological function of VRAC.

Gastric H^+ secretion is mediated by proton pump (H^+,K^+-ATPase) which belongs to a family of P_2-type ATPases including Na^+,K^+-ATPase and Ca^{2+}-ATPase (Munson et al., 2005; Shin et al., 2009). Gastric H^+,K^+-ATPase is expressed in both the apical canalicular membrane and intracellular tubulovesicles of gastric parietal cells (Fujii et al., 2009), and it actively transports H^+ and K^+ in opposite direction. Upon stimulation, tubulovesicles fuse each other and connect with the apical canalicular membrane, resulting in massive acid secretion. So far, Cl^- channels such as CLIC-6 (Nishizawa et al., 2000; Sachs et al., 2007), CFTR (Sidani et al., 2007) and SLC26A9 (Xu et al., 2008), Cl^-/H^+ antiporter ClC-5 (Takahashi et al., 2014), and K^+,Cl^- cotransporter KCC4 (Fujii et al., 2009) have been suggested as Cl^--transporting proteins involved in gastric acid (HCl) secretion.

H^+,K^+-ATPase is a key molecule in the final step of gastric acid secretion and is a therapeutic target for various acid-related diseases such as gastric ulcers, duodenal ulcers, gastroesophageal reflux diseases, and Zollinger Ellison syndrome (Sachs et al., 2007). Proton pump inhibitors (PPIs) such as omeprazole, rabeprazole, lansoprazole, and pantoprazole are potent inhibitors of gastric H^+,K^+-ATPase and are generally used in the treatment of the acid-related diseases (Shin and Sachs, 2008).

So far, the target of DCPIB other than VRAC has not been well elucidated. In the present study, we examined effects of DCPIB on H^+,K^+-ATPase, Na^+,K^+-ATPase, and Ca^{2+}-ATPase, and found that

* Corresponding author. Fax: +81 76 434 5051.
E-mail address: sakaih@pha.u-toyama.ac.jp (H. Sakai).

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DCPIB potently inhibits the activity of gastric H^+,K^+-ATPase, while it had no effect on Na^+,K^+-ATPase or Ca^{2+}-ATPase.

2. Materials and methods

2.1. Materials

DCPIB was obtained from Tocris bioscience (Ellisville, MO, USA). Niflumic acid, 2-methyl-3-cyanomethyl-8-(phenylmethoxy)imidazo[1,2-α]pyridine (SCH28080, an inhibitor of gastric H^+,K^+-ATPase), ouabain (an inhibitor of Na^+,K^+-ATPase), thapsigargin (an inhibitor of SERCA Ca^{2+}-ATPase), and anti-β-actin antibody were from Sigma-Aldrich (St. Louis, MO, USA). 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) was from Research Biochemical International (Natick, MA, USA). Diphenylamine-2-carboxylic acid (DPC) and A23187 were from Wako Pure Chemical Industries (Osaka, Japan). A monoclonal anti-H^+,K^+-ATPase α-subunit antibody (1H9) was from Medical and Biological Laboratories (Nagoya, Japan). Alexa Fluor 488-conjugated anti-mouse IgG antibody was from Invitrogen (Carlsbad, CA, USA). All other reagents were of the molecular biology grade or the highest grade of purity available.

2.2. Preparation of the gastric mucosal sample and tubulovesicles from hog stomach

Isolated hog stomach was obtained from Toyama meat center (Toyama, Japan). For preparing the gastric mucosal sample, fundic region of the mucosa was scraped and minced in the homogenize solution containing 250 mM sucrose, 1 mM EDTA, 10 mM Tris–HCl (pH 7.4), and protease inhibitor cocktail (10 μg/ml aprotinin, 10 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A). The tissue suspension was homogenized and centrifuged at 500*g* for 10 min at 4 °C. The supernatant was centrifuged at 100,000*g* for 90 min at 4 °C, and the mucosal sample was obtained by resuspending the pellet in the homogenize solution. For preparing the tubulovesicles (TV), fundic region of the mucosa was scraped and homogenized in 250 mM sucrose, 1 mM EGTA, and 5 mM Tris–HCl (pH 7.4). The suspension was centrifuged at 1,000*g* for 10 min and the supernatant was further centrifuged at 13,500*g* for 30 min. The supernatant was centrifuged at 100,000*g* for 30 min. The pellet was applied to 250 mM sucrose/7% ficoll step gradient and centrifuged at 132,000*g* for 1 h. TV were collected from the interface between 250 mM sucrose and 7% ficoll layers, and the TV sample was freeze-dried for increasing leakiness of the vesicular membranes. All procedures were carried out at 4 °C.

2.3. Cell culture and transfection of siRNA

Hog renal proximal tubular LLC-PK1 cells and human embryonic kidney 293 (HEK293) cells stably expressing rabbit gastric H^+,K^+-ATPase α- and β-subunits (LLC-PK1-αβ and HEK293-αβ cells, respectively) were each maintained in the Dulbecco's Modified Eagle's Medium (DMEM) (Wako) supplemented with 10% fetal bovine serum (Nichirei biosciences, Tokyo, Japan), 100 unit/ml penicillin (Wako), 100 μg/ml streptomycin (Wako), 0.5 mg/ml G418 (Enzo Life Sciences, NY, USA), and 0.2 mg/ml zeocin (Invitrogen) (Fujii et al., 2007, 2013). HEK293-αβ cells were transfected with 100 pmol siRNA using JetPRIME (Polyplus-transfection, NY, USA). The transfected cells were cultured in a growth medium for 48 h. The siRNA for LRRC8A (GGUACAACCACAUCGCCUA) (Voss et al., 2014) and negative control siRNA were purchased from Nippon Gene.

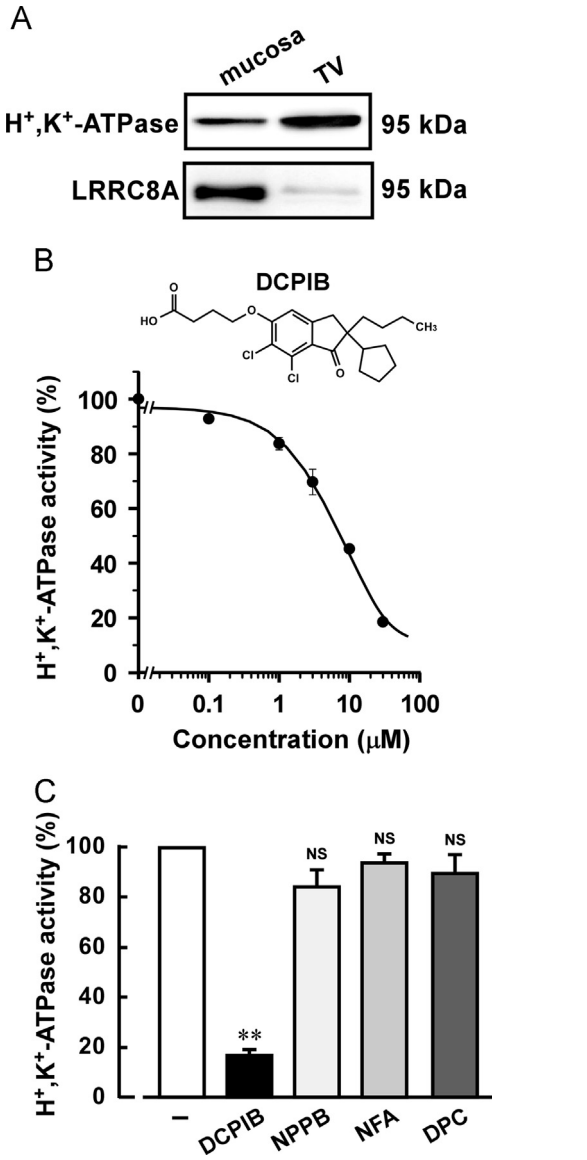

Fig. 1. Effects of anion channel blockers on the activity of hog gastric H^+,K^+-ATPase in TV. (A) Western blotting using antibodies for H^+,K^+-ATPase α-subunit (95 kDa) and LRRC8A (95 kDa) was performed in TV and the membrane sample of gastric mucosa (5 μg of protein). (B) Concentration dependent inhibition of H^+,K^+-ATPase by DCPIB. The SCH28080-sensitive K^+-ATPase (H^+,K^+-ATPase) activity in TV was measured at various concentrations of DCPIB ($n=4$–6). The activity in the absence of DCPIB was taken as 100%. In the inset, the chemical structure of DCPIB is shown. (C) Effects of 30 μM of DCPIB, NPPB, niflumic acid (NFA), and DPC on the H^+,K^+-ATPase activity in TV ($n=4$–5). The control activity in the absence of the inhibitors (−) was 95.8 ± 7.5 μmol Pi/mg protein/h and was taken as 100%. NS, not significantly different from control ($P>0.05$). ** Significantly different from control ($P<0.01$).

2.4. Preparation of membrane sample from cultured cells

LLC-PK1-αβ and HEK293-αβ cells were incubated in a low ionic salt buffer (0.5 mM $MgCl_2$ and 10 mM Tris–HCl, pH 7.4) supplemented with the protease inhibitor cocktail for 10 min at 0 °C. Subsequently, they were homogenized with 40 strokes in a dounce homogenizer, and the homogenate was diluted with an equal volume of a solution containing 500 mM sucrose and 10 mM Tris–HCl (pH 7.4). The cell suspension was homogenized with 40 more strokes and was centrifuged at 800*g* for 10 min. The supernatant was centrifuged at 100,000*g* for 90 min, and the pellet was suspended in a solution containing 250 mM sucrose and 5 mM Tris–HCl (pH 7.4).