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Effects of $[Ca^{2+}]_i$ and pH on epithelial Na^+ channel activity of cultured mouse cortical collecting ducts

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SUMMARY

$[Ca^{2+}]_i$ and pH have been demonstrated to affect Na^+ transport in epithelium mediated *via* the apical epithelial Na^+ channel (ENaC). However, it still remains unclear whether the effects of $[Ca^{2+}]_i$ and intracellular pH (pH_i) on ENaC activity are direct. In this study, inside-out recording was employed to clarify the effects of pH_i and $[Ca^{2+}]_i$ on ENaC activity. We found that elevation of $[Ca^{2+}]_i$ induced a significant inhibition of ENaC open probability without altering channel conductance. The inhibitory effect was due to a direct interaction between Ca^{2+} and ENaC, and is dependent on $[Ca^{2+}]_i$. pH_i also directly regulated ENaC open probability. Lower pH_i (<7.0) reduced the ENaC open probability as shown in shorter opening time, and higher pH_i (>7.0) enhanced the ENaC open probability as shown in augmented opening time. pH_i did not cause any alteration in channel conductance. The effects of pH_i on ENaC open probability could be summarized as an S-shaped curve around pH 7.2.

Key words: calcium, epithelial sodium channel, pH.

INTRODUCTION

Transport of Na^+ across epithelial cells in kidney collecting ducts occurs *via* apical amiloride-sensitive epithelial Na^+ channels (ENaCs) and coupled basolateral Na^+,K^+-ATPase. ENaC activity is regulated by a variety of hormones, e.g. aldosterone and antidiuretic hormone (ADH), as well as by the luminal contents (Blokkebak-Poulsen et al., 1991; Palmer and Frindt, 1987). A millimolar concentration of Ca^{2+} intravesicularly was found to block the amiloride-sensitive $^{22}Na^+$ flux in rabbit kidney tubule. This inhibitory effect of Ca^{2+} was dependent on pH (Blokkebak-Poulsen et al., 1991) and probably due to increase of $[Ca^{2+}]_i$ (Chase and Al-Awqati, 1981). However, other cations, Cd^{2+}, Ba^{2+} and Mg^{2+}, in millimolar concentrations only slightly inhibited $^{22}Na^+$ influx. $[Ca^{2+}]_i$ elevation in tubule cells is mediated by either Ca^{2+} release from internal store or Ca^{2+} uptake across the apical membrane *via* different calcium channels (Brunette et al., 2004; Leclerc et al., 2004) (Brunette and Leclerc, 2002), and is proportional to the outward Na^+ gradient (Chase and Al-Awqati, 1981). Ca^{2+} as an important second messenger, has been implicated to inhibit ENaCs (Wang and Chan, 2000). In particular, increase in cytoplasmic Ca^{2+} results in decrease of apical Na^+ permeability in the frog skin (Grinstein and Erlij, 1978), toad urinary bladder (Brem et al., 1985; Palmer, 1985; Taylor et al., 1987) and the rabbit cortical collecting tubule (Frindt et al., 1993; Frindt and Windhager, 1990; Windhager et al., 1986). However, it is still unclear whether this inhibition is due to a direct effect. Studies on planar bilayers concluded that cytoplasmic Ca^{2+} can inhibit ENaCs by direct interaction (Ismailov et al., 1997) whereas investigations using A6 cells (Ling et al., 1997) and rat CCD cells (Palmer and Frindt, 1987) suggest cytoplasmic Ca^{2+} inhibits ENaCs by indirect interaction and through other second messengers. So far, there has been little work carried out to address this issue in a native cell line, which endogenously expresses the ENaC.

Protons have long been recognized to affect epithelial Na^+ absorption. Trans epithelial Na^+ transport rate is increased by intracellular alkalization but decreased by intracellular acidosis (Blokkebak-Poulsen et al., 1991; Harvey et al., 1988; Lyall et al., 1995). Controversially, in toad bladder, acidification of the cytoplasm can stimulate Na^+ transport (Garty et al., 1987; Leaf et al., 1964). Electrophysiology experiments based on transfected oocytes and A6 cells demonstrated that the α subunit of ENaC is directly regulated by pH_i (Chalfant et al., 1999) and a reduction of pH_i decreased ENaC activity. However, amiloride-sensitive Na^+ fluxes in toad urinary bladder are not affected by changes in pH_i over the range of pH 7–8, suggesting a indirect regulation mechanism by pH_i (Garty and Asher, 1985). It is postulated that pH exerts its effect by altering the inhibitory effect of Ca^{2+} on ENaC (Blokkebak-Poulsen et al., 1991). Therefore, in this study, inside-out recording were employed to elucidate the direct effects of $[Ca^{2+}]_i$ and pH_i on ENaC activity of cultured mouse cortical collecting ducts.

MATERIALS AND METHODS

Cell culture

M1 cells (mouse kidney cortical collecting duct cells) at the 21st passage were purchased from the European Collection of Cell Cultures (Salisbury, UK). Cells were grown on the medium containing DMEM:Ham's F12 medium (1:1; Sigma, Ayrshire, UK), 2 mmol l^{-1} glutamine (Gibco, Paisley, UK), 5 μmol l^{-1} dexamethasone (Sigma) and 5% FBS (Sigma) in an incubator at 37°C and 5% CO_2. Aldosterone (1.5 μmol l^{-1}; Sigma) was added to the culture medium 24 h before experimentation to enhance ENaC expression. When cells in the culture flasks reached 70% confluence, they were seeded at low density onto either coverslips or culture inserts (BD, Franklin Lakes, NJ, USA).

Single channel patch clamp recording

Single channel recordings were performed as previously described (Gorelik et al., 2005). Briefly, M1 cells on a coverslip or insert were placed into a recording chamber mounted on a Nikon inverted

microscope (Nikon TE 2000U). Patch pipettes with a resistance of 7 MΩ were fabricated from borosilicate glass capillaries (1.5 o.d., 0.86 i.d.; Warner, Reading, UK) on a Sutter Puller (P97; Novato, CA, USA). Bath solution A contained (in mmol l^{-1}): 110 NaCl, 4.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 Hepes, 5 Na-Hepes, pH 7.2. Bath solution B used in the pH_i experiments contained (in mmol l^{-1}): 110 NaCl, 4.5 KCl, 1 $MgCl_2$, 5 Hepes, 5 Na-Hepes, EGTA 0.1. The pH was adjusted by addition of NaOH or HCl. In solution B, free $[Ca^{2+}]_i$ was maintained at a concentration of 2 μmol l^{-1}. Total $CaCl_2$ required in solution B, corresponding to different pHs, was calculated using a standard equation (WEBMAXC STANDARD, version 21/05/2007, Stanford, CA, USA). The pipette solution contained (in mmol l^{-1}): 110 NaCl, 4.5 KCl, 0.1 EGTA, 5 Hepes, 5 Na-Hepes, pH 7.2. Different concentrations of Ca^{2+} for the bath media were calculated using the WEBMAXC equation (version 21/05/2007, Stanford). EGTA (200 nmol l^{-1}) were used as the chelator. All media were made on the day of experiments. Currents were recorded with an Axon (Sunnyvale, CA, USA) 1D amplifier and Axon Clampex 9.0. The data were acquired at 20 KHz and filtered with 5 kHz of low pass filter. The channel events were analyzed using pClampfit 9.0 (Axon; single channel search in analyze function). Data was further filtered at 200 Hz before analysis. The 50% threshold cross method was utilized to determine valid channel openings. When multiple channel events were observed in each patch, the total number of functional channels (N) in the patch was determined by observing the number of peaks detected on all point amplitude histograms. NP_o, the product of the number of channels and the open probability, or the open probability (P_o), itself was used to measure the channel activity within a patch. The NP_o was calculated according to the method of Yue et al. (Yue et al., 2002). Because the recording membrane patch usually contained multiple channels, in most cases the changes in NP_o (but not P_o) were directly observed and compared. Owing to the variance of channel open probability, the first 2–3 min single channel recording (inside-out recording) in normal bath medium was usually used as the control. In experiments in which Ca^{2+} or pH were altered, the NP_o under the different conditions was directly compared with the NP_o of the control. The above ratio was employed to determine the effects of Ca^{2+} or pH on ENaC activity. In some cases the NP_o of the ENaCs during modifications of Ca^{2+} and pH was compared to that of ENaCs in normal medium when modified medium was washed off. The data were utilized to confirm observations. The data are presented as means ± s.e.m., and the statistical differences were compared using Student's paired t-test, taking $P<0.05$ as significant.

RESULTS

Characterization of ENaC currents in M1 cells

Single channel recording was utilized to characterize ENaCs in M1 cells. In a cell-attached recording, an inward current was detected when the pipette voltage was held at +20 mV and +40 mV (Fig. 1A). These inward currents had a slope conductance of 5.1±0.25 pS (N=30) between the command voltages (20 and 40 mV, hyperpolarization). The cell membrane under a patch pipette was excised from the cell to obtain either inside-out or outside-out recordings. In an inside-out recording, current with the slope conductance of 5.07±0.11 pS (N=80) was obtained (Fig. 1B). The same conductance current was also observed in the outside-out recordings (Fig. 1C). The current was almost abolished by bath application of 5 μmol l^{-1} amiloride (Fig. 1C) and was reversed when amiloride was washed off. The data suggest that the small conductance currents obtained in M1 cells were from ENaCs.

$[Ca^{2+}]_i$ directly inhibited ENaC activity

In a cell-attached recording, $[Ca^{2+}]_i$ elevation caused by application of 1 μmol l^{-1} tharpsigargin (TG), induced an inhibitory effect on ENaC activity (Fig. 2), shown as a short opening time for ENaC currents (N=6). In inside-out recordings, changes in the cytoplasmic

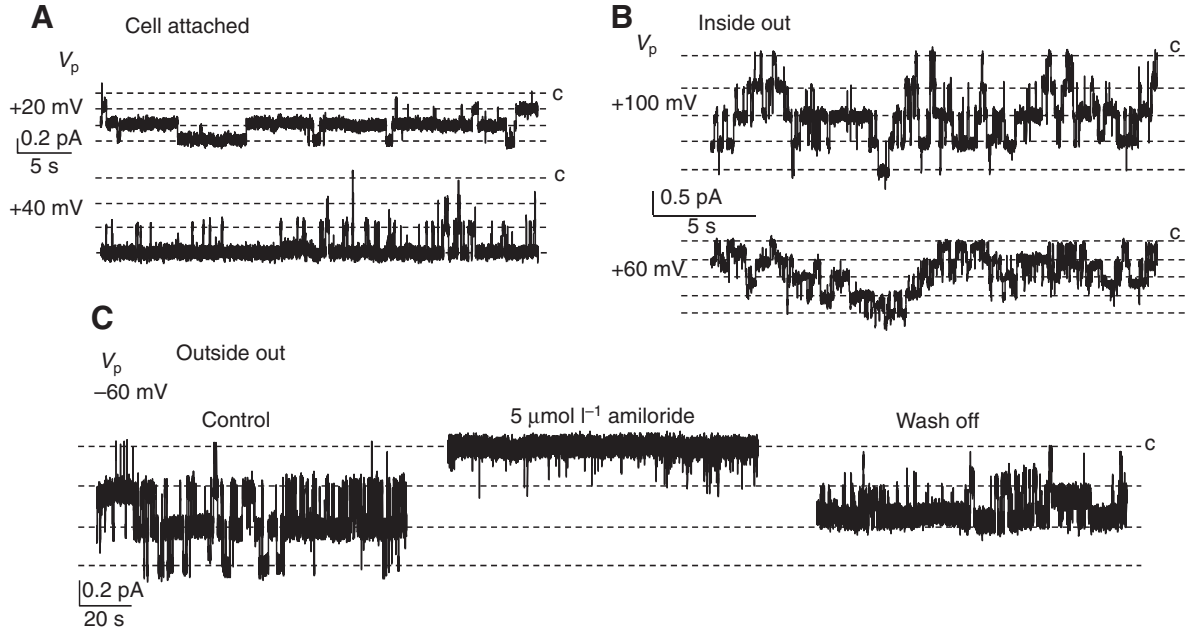

Fig. 1. Epithelial Na^+ channel (ENaC) currents revealed by single channel recording from cultured M1 cells. (A) In a cell-attached recording, inward currents were recorded when the pipette voltage (V_p) was held at +20 mV and +40 mV. (B) In an inside-out recording, the single channel currents were detected when the pipette voltage was held at +100 mV and +60 mV. (C) In an outside-out recording, the currents were monitored when the pipette voltage was held at –60 mV. Bath application of 5 μmol l^{-1} amiloride almost abolished the ENaC currents. The currents were reversed when amiloride was washed off. c indicates the closing level.

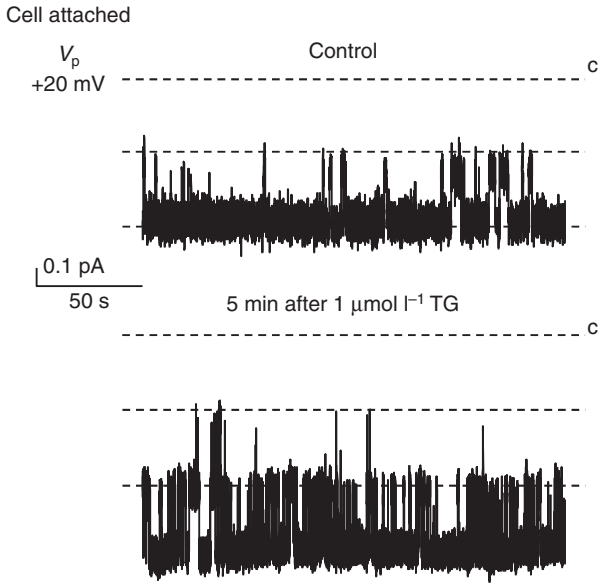

Fig. 2. Effect of elevation of $[Ca^{2+}]_i$ on ENaC activity. The top panel shows single channel currents were detected when the pipette voltage was held at +20 mV, in a cell-attached recording. After the cell was incubated with 1 μmol l^{-1} thapsigargin (TG) for 5 min, ENaC activity were reduced as shown in the lower trace.

Ca^{2+} concentration significantly altered ENaC activity (Fig. 3). The maximum open probability of ENaCs was seen when $[Ca^{2+}]_i$ was below 500 nmol l^{-1} (N=11). The ENaC P_o was reduced following the $[Ca^{2+}]_i$ elevation. The inhibitory effect of $[Ca^{2+}]_i$ on ENaC was saturated at 100 μmol l^{-1} Ca^{2+} (N=11).

High intracellular pH enhanced ENaC open probability

When cytoplasmic pH rose from 7.2 to 7.6 or 8.0, ENaC open probability significantly increased (Fig. 4) to 195.7±19.8% (N=6) or 231.1±25.3% (N=7) of the control P_o in a reverse manner. This enhancing effect occurred rapidly when the pH of the medium was increased. A change in single channel conductance with increasing pH_i was not been observed in our experimental conditions.

Low intracellular pH reduced ENaC open probability

When cytoplasmic pH was decreased from 7.2 to 6.8 or 6.2, ENaC open probability significantly decreased (Fig. 5) to 57.7±15.5% (N=6) or 29.5±11.1% (N=5) of the control P_o in a reverse manner. This inhibition occurred rapidly when the pH of the medium was decreased. A change in single channel conductance was not observed when the pH_i was decreased. However, channel opening status became flickering in pH_i 6.2, τ_{open}=3.1±0.92 mS (N=6) compared with τ_{open}=22.5±5.6 mS (N=11) in the control (data fitted with exponential standard), when single channel recordings of 5 min duration were analyzed at the control and different pH_is.

A long single channel recording containing multiple repeated alternations in pH_i is shown in Fig. 6. It demonstrates the robust effects of pHi on ENaC activity. A summary of the effects of pH_i is shown in Fig. 7. An S-shaped curve around pH 7.2 was obtained from the experimental data (values are mean ± s.e.m., N=5 or 6 cells).

DISCUSSION

This study presents the evidence that $[Ca^{2+}]_i$ directly interacts with ENaCs and negatively regulates ENaC open probability without alteration in channel conductance. In micromolar concentrations of $[Ca^{2+}]_i$, pH_i can regulate ENaC activity. Low pH_i significantly reduces the ENaC open probability whereas high pH_i enhances the ENaC open probability. The conductance of ENaCs is not affected

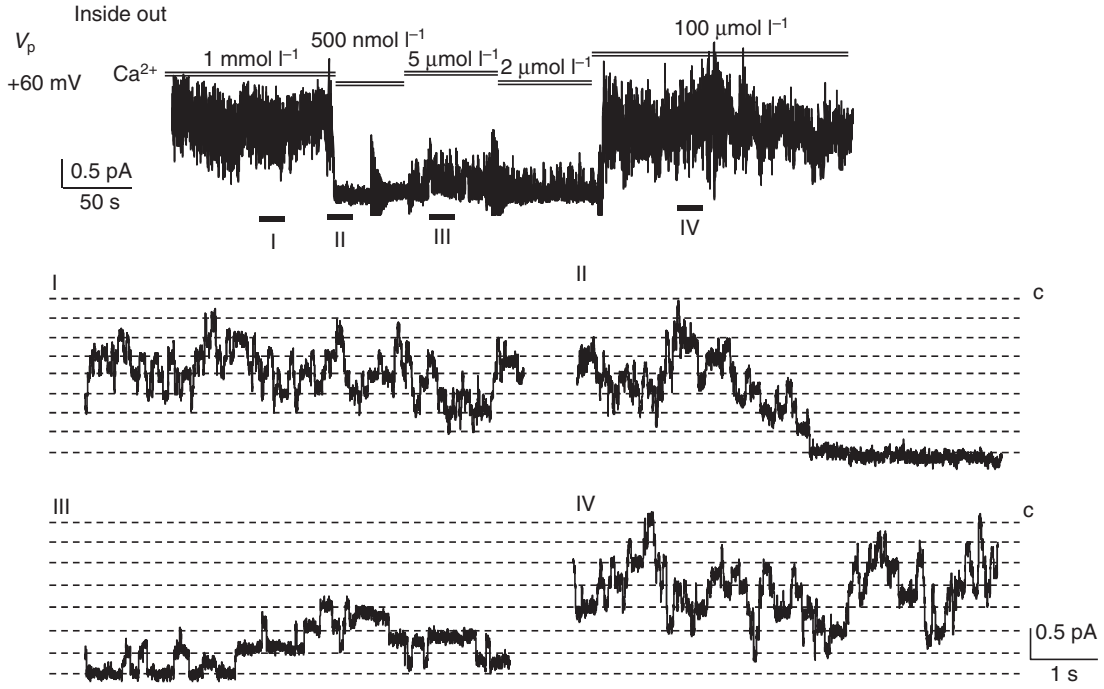

Fig. 3. Direct effects of $[Ca^{2+}]_i$ on ENaC activity. In an inside-out recording, 500 nmol l^{-1} Ca^{2+} led to maximum ENaC open probability (P_o) and even 5 μmol l^{-1} Ca^{2+} exerted significant inhibition on ENaC P_o. The inhibitory effect of Ca^{2+} on ENaC activity depended on the Ca^{2+} concentration. In the lower panels selected currents are shown on an expanded time scale.

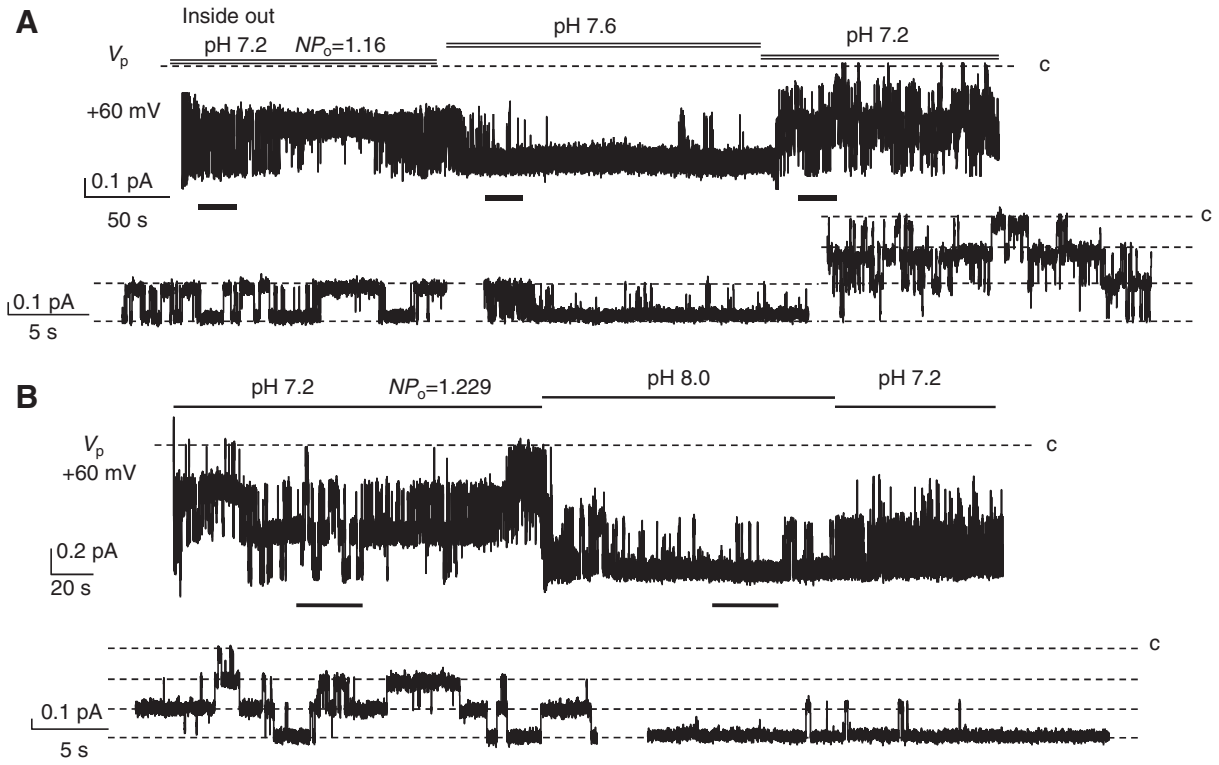

Fig. 4. Effect of high pH_i on ENaC activity. In inside-out recordings, with pH_i at 7.6 (A) and 8 (B), channel activity was significantly enhanced. This effect was reversed when pH_i was returned to 7.2. The lower traces in each panel show the currents on an expanded time scale, as indicated by the bars.

by pH_i. The effect of pH_i on ENaC activity can be seen in the S-shaped curve around pH_i 7.2.

In order to simplify the experimental conditions, media providing 115 mmol l^{-1} Na^+ (Canessa et al., 1994) across the membrane were used in most of our excised membrane recording, e.g. inside-out and outside-out recordings. This made it easy to determine the electrical potential across the membrane (V_m) and channel conductance. The disadvantage of this protocol is that it does not mimic the physiological condition of the cytoplasm. Nevertheless, it is possible to study the ion channel kinetics of a patch of excised membrane in an isolated artificial environment. Because Ca^{2+} was included in the cytoplasmic medium in inside-out recordings, short opening and closing times (τ) of ENaCs were observed. By contrast, long opening and closing times of these channels in the range of seconds were observed in a cell-attached recording (Benos et al., 1995; Ismailov et al., 1995; Ismailov et al., 1997). In this study another protocol was also employed, using 100 nmol l^{-1} Ca^{2+} in the cytoplasmic medium. In this case, it was difficult to determine the presence of ENaCs in the patch of membrane because of invisible current transition, which could be interpreted as channel full opening, channel intermittent silence or channel absence. We therefore included 2 μmol l^{-1} Ca^{2+} in the cytoplasmic medium to determine the ENaC currents by current transition. Such concentration of $[Ca^{2+}]_i$ might be found in nature in cells under activation. In order to clarify the effects of a variety of pH_i on ENaC, we fixed the cytoplasmic Ca^{2+} at a certain level. Although inside-out recordings have minimal cytoplasmic effects, there are still some factors associated with ENaCs that could exert unexpected effects on ENaC activities. The conclusion in this study is primarily based on current knowledge of macro-structures of ENaCs and signalling pathways.

Our results are inconsistent with previous studies on rat collecting duct (Palmer and Frindt, 1987), which found free Ca^{2+} in the cytoplasm has no effect on the ENaC activity. Their results suggested that Ca^{2+} does not interact with ENaCs directly, and $[Ca^{2+}]_i$ elevation causes the inhibition of ENaC activity through an indirect process, for example alterations in pH_i, activation of calmodulin, PKC and prostaglandin (Palmer and Frindt, 1987). In their experiments, Ca^{2+} ionophore ionomycin was used to elevate $[Ca^{2+}]_i$. Ionomycin can cause damage to the membrane, alter pH_i as a result of ionophore-mediated $2H^+/Ca^{2+}$ exchange (Erdahl et al., 1994) and induce an apoptotic cascade. The profound effects of ionomycin could be reflected in their inconsistent observations: ionomycin failed to enhance ENaC in first minute in half of their experiments. In this study, TG was therefore employed to enhance $[Ca^{2+}]_i$ without causing damage to cell membrane structure and cell activity. Our results are consistent when cytoplasmic Ca^{2+} was increased by either TG in intact cells or bath perfusion in inside-out recordings, suggesting intracellular Ca^{2+} does affect ENaC activity. The obvious explanation for the discrepancy with the previous study could be a difference in biological samples, since the previous study was performed in CCD ducts freshly isolated from rat whereas cultured mouse CCD cells was used in this study. Our results agreed with observations in frog skin (Ussing and Zerahn, 1951), membrane vesicles derived from toad bladder (Garty and Asher, 1985), mammalian ENaC in bilayers (Ismailov et al., 1995; Ismailov et al., 1997) and MDCK cells expressing rat ENaCs (Ishikawa et al., 1998).

The inhibitory effect of intracellular Ca^{2+} on ENaCs could be either a direct interaction between Ca^{2+} (Ling and Eaton, 1989) and ENaCs or an indirect mechanism, e.g. *via* protein kinase C (PKC) (Awayda et al., 1996; Ling and Eaton, 1989). Inhibition of ENaC activity by PKC is due to direct phosphorylation of ENaC subunits. Activation of PKC generally requires Ca^{2+},

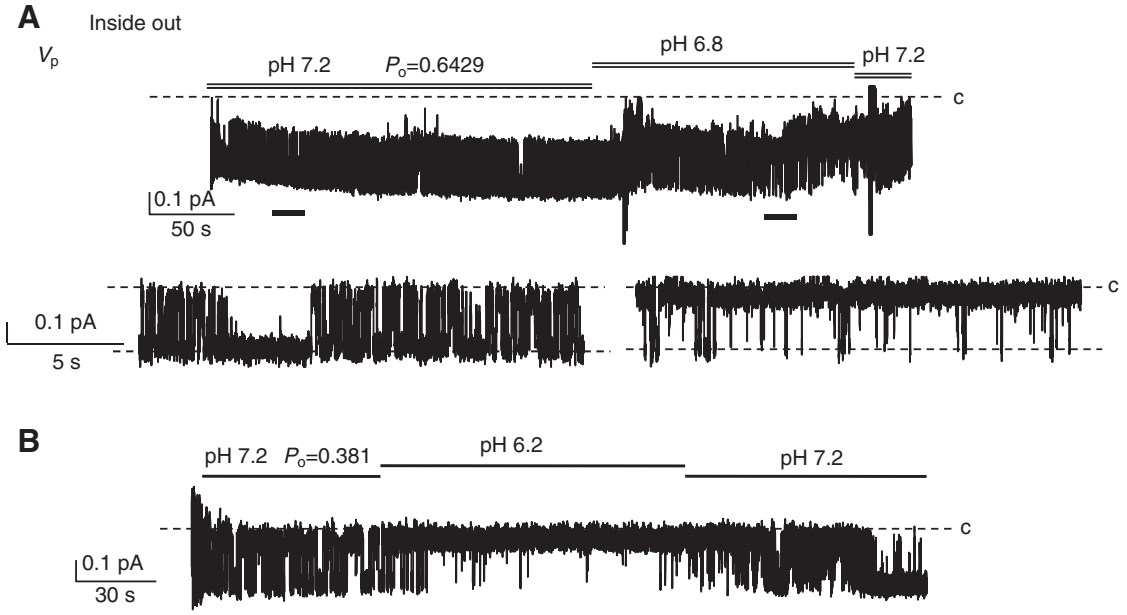

Fig. 5. Effect of low pH_i on ENaC activity. In inside-out recordings, with pH_i at 6.8 (A) and 6.2 (B), channel activity was significantly decreased. This effect was reversed when pH_i was returned to 7.2. The lower traces in each panel show the currents on an expanded time scale, as indicated by the bars.

diacylglycerol and phospholipid. However, inhibition of ENaCs is still observed when other components except Ca^{2+} are free in the inside-out recordings, implying that cytoplasmic Ca^{2+}, in addition to PKC, can directly inhibit ENaCs. Additionally, the inhibitory effect of Ca^{2+} on ENaC is almost instant when cytoplasmic Ca^{2+} is altered. This observation also supports the direct interaction between Ca^{2+} and ENaCs. Our observation is consistent to other conclusions obtained from planar bilayers (Ismailov et al., 1995) and in mouse endometrial epithelium (Wang and Chan, 2000).

Many pathways or mechanisms (Zeiske et al., 1999) could lead to alternations in pH_i, which consequently regulate ENaC activity. For example, natriferic hormones mediate the activities of the Na^+/H^+ exchanger, H^+-ATPase, H^+/K^+-ATPase and H^+ conductive pathways in epithelial cells, resulting in the change in pH_i (Johanson and Murphy, 1990; Lyall et al., 1994; Lyall and Biber, 1995; Lyall

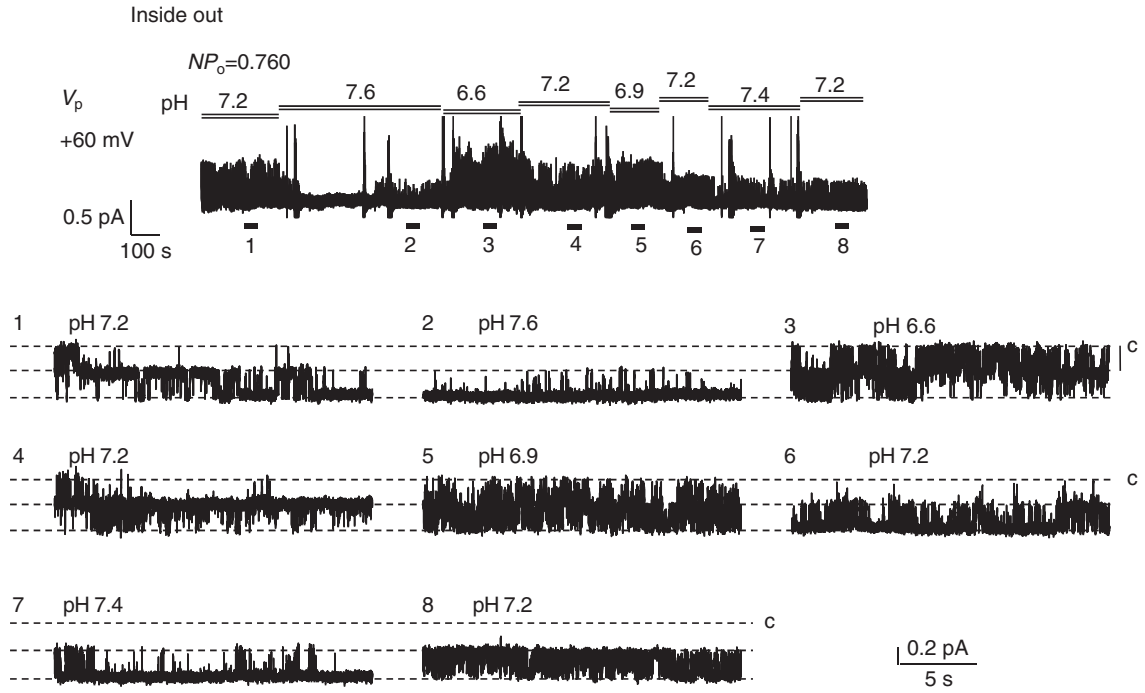

Fig. 6. Effect of pH_i on ENaC activity. In an inside-out recording, low pH_i significantly and reversibly reduced ENaC activity whereas high pH_i reversibly enhanced ENaC activity. Lower traces show selected parts of the current recordings as indicated (1–8).

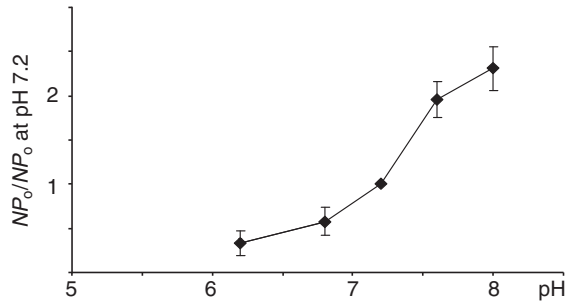

Fig. 7. A summary of pH_i effect on ENaC activity. The open probability (NP_o) values at each pH are normalized to that at pH 7.2.

et al., 1995). The changes in pH_i act as an intermediate in the second messenger cascade initiated by the hormones to regulate Na^+ uptake (Lyall et al., 1994; Lyall and Biber, 1994; Lyall et al., 1997; Stewart et al., 1998). Although alternations in pH_o do not directly regulate ENaC activity in the short term (<10 min), high or low pH_o (Lyall et al., 1997) might affect the activity of Na^+/H^+-ATPase (Korbmacher et al., 1988; Shimada and Hoshi, 1987; Wolosin et al., 1988) and the H^+ conductive pathways (Lyall and Biber, 1994; Prigent et al., 1985), resulting in the changes in pH_i. Consistent with previous reports (Palmer and Frindt, 1987; Zeiske et al., 1999), decrease of pH_i inhibits ENaC activity. In addition, pH_o has also been shown to affect ENaC activity but in a long-term manner (Awayda et al., 2000). The possible explanations are focused upon the elevation in $[Ca^{2+}]_i$ in the medium or alternation in interaction of Ca^{2+} and ENaCs due to changes in pH_o. The ability of Ca^{2+} to inhibit Na^+ uptake in toad bladder was greatly reduced by decreasing pH_i from 7.4 to 7.0 (Garty et al., 1987). In this case, it would be expected that a decrease of pH_i may relieve the inhibition of the channel by Ca^{2+}. However, our finding is opposite to that in toad bladder. The inhibitory effects of Ca^{2+} and H^+ on ENaCs are probably superimposed on each other.

In our experiments, pH 7.2 was used as the normal pH_i. According to the literature, standard cellular pH_i varies from cell to cell. In rabbit collecting duct, normal pH_i is 7.28 (Satlin, 1994) and pH_i decreases to 6.5 with 'acid loading' (Chaillet et al., 1985). Measurements of pH_i in a variety of mammalian skeletal muscle preparations indicate pH_i is mostly in the range of 6.8 to 7.1 and the normal pH_i in liver cells is about 7.00 (Park et al., 1979). In medullar collecting duct of hamster, normal pH_i is 6.97 and amiloride was found to completely inhibit Na^+-dependent pH_i recovery (Matsushima et al., 1990). Nevertheless, our results show increasing pH_i enhances ENaC activity whereas decreasing pH_i reduces ENaC activity.

In summary, pH_i and $[Ca^{2+}]_i$ could directly interact with the ENaC to regulate its activity by altering the open probability without changing conductance. Elevation of $[Ca^{2+}]_i$ directly reduces the ENaC P_o. In the cytoplasmic membrane, acidification can reduce ENaC P_o whereas high pH_i enhances ENaC P_o.

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