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ENERGIZATION OF SODIUM ABSORPTION BY THE H^+-ATPase PUMP IN MITOCHONDRIA-RICH CELLS OF FROG SKIN

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Summary

The frog skin *in vivo* is capable of active transepithelial H^+ secretion (J_H) which is matched by Na^+ absorption (J_{Na}). Studies *in vitro* demonstrate that J_H is generated by an H^+-ATPase pump localized in apical membranes of mitochondria-rich (MR) cells, whereas J_{Na} occurs through an amiloride-sensitive pathway in principal (P) cells. The H^+ pump is sensitive to inhibitors of carbonic anhydrase (e.g. acetazolamide) and to specific inhibitors of mitochondrial F_1F_o H^+-ATPase (oligomycin) and vacuolar (V)-type H^+-ATPase (*N*-ethylmaleimide) and to inhibitors of both these types of H^+-ATPases (dicyclohexylcarbodiimide, DCCD). J_H is independent of external K^+, which differentiates it from gastric H^+/K^+-ATPase and is strictly dependent on aerobic metabolism. The proton pump is primarily implicated in whole-body acid–base regulation. Acute stimulation of J_H in response (seconds–minutes) to an acid load involves insertion of H^+ pumps (exocytosis) from a cytosolic pool into the apical membrane. The chronic response (days) to metabolic acid load involves morphological changes (increased apical membrane surface area and number of MR cells). Whole-cell patch-clamp recordings of membrane capacitance and current fluctuations from MR cells demonstrate that a respiratory acid load and aldosterone produce rapid exocytotic insertion of DCCD-sensitive conductive membrane.

A secondary role of the H^+ pump is to energize sodium absorption (J_{Na}) *via* principal cells from dilute solutions in the absence of a permeant anion under open-circuit conditions. The apparent 1:1 stoichiometry between J_H and J_{Na} is a result of transepithelial electrical coupling between these electrogenic fluxes. The H^+ pump in MR cells generates a transepithelial current (serosa to apical) which acts as a physiological voltage-clamp to hyperpolarize the apical membrane of P cells. This hyperpolarization can facilitate passive Na^+ entry across the apical membrane against a threefold chemical gradient. Since both J_H and J_{Na} are sensitive to membrane potential, inhibition or activation of one will produce similar effects on the transport of the other ion. For example, inhibition of J_H by ethoxzolamide will reduce J_{Na}. Conversely, blocking J_{Na} with amiloride also inhibits J_H. These effects can be avoided or reversed if variations in membrane potential are prevented by voltage-clamping the epithelium. A paradoxical activation of J_{Na} is observed when J_H is stimulated by an acid load (CO_2), despite inhibition of Na^+ channel activity by H^+ in P cells. Patch-clamp studies reveal the presence of Na^+ channels in MR cells which may provide a route for Na^+ absorption energized by the H^+ pump when Na^+ channels in P cells are closed by an acid load.

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Key words: mitochondria-rich cells, principal cells, H^+ secretion, Na^+ absorption, Na^+ channels, H^+-ATPase, intracellular pH, patch-clamp, exocytosis, membrane capacitance, aldosterone.

Introduction

The frog skin *in vivo* absorbs sodium and secretes hydrogen ions (Krogh, 1939) in an apparent 1:1 stoichiometery (Garcia-Romeu *et al.* 1969). The *in vitro* amphibian skin and urinary bladder mounted in an Ussing chamber can also actively secrete hydrogen ions under appropriate electrochemical gradients (Frazier and Vanatta, 1971; Ludens and Fanestil, 1972; Machen and Erlij, 1975; Ehrenfeld and Garcia-Romeu, 1977; Ramsay, 1982). The outermost living cell layer of frog skin epithelium (the first reactive cell layer) is composed of two cell types, cuboidal granular cells and mitochondria-rich cells (MR). The granular cells along with the deeper spinosum and germinativum cells form a functional sodium transport syncytium (Rick *et al.* 1978). The Na^+ absorption function of the epithelium is carried out by these principal (P) cells (Koefoed-Johnsen and Ussing, 1958; Kristensen and Ussing, 1985), whereas active H^+ secretion is restricted to the mitochondria-rich cells (Harvey and Ehrenfeld, 1988*a*; Ehrenfeld *et al.* 1989).

The specialized transport functions of MR cells and principal cells are found in a wide range of high-resistance epithelia. Na^+ absorption and H^+ secretion mechanisms are similar in amphibian skin and urinary bladder, gill of freshwater fish and crustaceans and in mammalian colon and renal collecting tubule (Kelly *et al.*, 1980; Schwartz *et al.* 1982; Madsen and Tisher; 1985; Durham and Nagel; 1986; Koeppen, 1987; Steinmetz, 1988; Harvey and Ehrenfeld, 1988*a*; Sauer *et al.* 1990; Larsen, 1991; Kirschner, 1982, 1988; Krippeit-Drews *et al.* 1989; Avella and Bornancin, 1990; Perrone *et al.* 1990; Frömter, 1988). The proton secretion function and high carbonic anhydrase content of MR cells in amphibian skin (Ehrenfeld *et al.* 1985; Rosen and Friedley, 1973; Katz and Gabbay, 1988) indicate a close similarity with the alpha-type intercalated cell of collecting tubule and turtle urinary bladder (Steinmetz, 1974, 1986; Schwartz and Steinmetz, 1977; Dixon and Al-Awqati, 1979; Husted *et al.* 1981; Steinmetz and Andersen, 1982; Brown *et al.* 1988*a*). Proton secretion in frog skin is affected by agents which inhibit different types of H^+-ATPases (for reviews on pharmacology of H^+ pumps, see Senior and Wise, 1983; Solioz, 1984; Stone and Xie, 1988; Forgac, 1989; Al-Awqati, 1986). Inhibitors of V-ATPase, such as dicyclohexylcarbodiimide (DCCD) and *N*-ethylmaleimide (NEM), are effective blockers of J_H, as are diethylstilboestrol (DES) and oligomycin (Ehrenfeld *et al.* 1985, 1990; Sabolic and Burckhardt, 1986; Harvey and Ehrenfeld, 1988*a*), which interact with F_1F_o-class ATPases (Fanestil and Park, 1981; Strid *et al.* 1988). Inhibition of J_H by vanadate (which is ineffective against F-ATPases and V-ATPases) may be direct (Arruda *et al.* 1981) or indirect *via* effects on metabolism or insertion of H^+ pumps into the plasma membrane. In this paper I will focus mainly on the role of the vacuolar H^+-ATPase (V-ATPase) pump in energizing Na^+ absorption across frog skin. I will describe novel uses of ion-sensitive microelectrodes and patch-clamp techniques to study the cellular pathways involved in J_H and J_{Na} and the rapid regulation of H^+ secretion in single MR cells by the exocytotic insertion of membrane containing active proton pumps.

Energization of Na^+ uptake by the proton pump

The transport pathways for transepithelial H^+ secretion and Na^+ absorption through mitochondria-rich and principal cells are summarized in Fig. 1. Most *in vitro* studies of

Na^+ absorption in frog skin use high-NaCl Ringer's solution bathing both sides of the tissue whereas *in vivo* Na^+ uptake occurs from more dilute external solutions. For example, frogs can absorb Na^+ in the absence of a permeant anion from fresh water containing less than 2 mmol l^{-1} Na^+. Under these conditions, J_{Na} and J_H are coupled in an apparent 1:1 stoichiometry and electroneutrality is conserved by equal movements of Na^+ and H^+ in opposite directions, but through different cell types (Harvey and Ehrenfeld, 1988*a*). The action of the V-ATPase pump in energizing Na^+ uptake across apical membranes of P cells is illustrated by the experiment in Fig. 2. A double-barrelled Na^+-sensitive microelectrode was used to measure intracellular Na^+ activity (A^i_{Na}) in P cells during electrical and pharmacological modulation of J_{Na} and J_H.

When J_H is inhibited by acetazolamide, the loss of transepithelial proton current produces a depolarized transepithelial potential (serosal side becomes more positively charged) and the apical membrane of principal cells also depolarizes. This results in a decrease in the driving force for Na^+ uptake and a fall in intracellular Na^+ activity A^i_{Na} (Fig. 2). Short-circuiting the tissue renders J_{Na} independent of J_H and restores Na^+ uptake (Fig. 2). The dependence of J_{Na} on electrical gradients is critical under physiological ion gradients. In P cells exposed to dilute apical saline (2 mmol l^{-1} Na^+) under open-circuit conditions, intracellular Na^+ activity is three times that in the external solution. A favourable electrical gradient exists, however, for passive uptake of Na^+ even down to an external Na^+ concentration of 0.1 mmol l^{-1} (Harvey and Kernan, 1984). Under these conditions, Na^+ uptake in P cells is energized by a transepithelial electrical gradient generated by rheogenic H^+ secretion through MR cells. Sodium uptake through P cells can be calculated from the instantaneous rate of change in intracellular Na^+ activity (ΔA^i_{Na}) after closure of apical Na^+ channels by amiloride (Harvey and Kernan, 1984). The equivalence between ΔA^i_{Na} and J_{Na} (Fig. 2) supports the hypothesis that principal cells constitute the main pathway for Na^+ absorption when J_{Na} is either coupled or uncoupled to J_H.

In the absence of an impermeant anion, J_{Na} is rate-limited by charge balance from rheogenic proton secretion. Hormones such as oxytocin or vasopressin, which increase apical Na^+ permeability in P cells but which do not directly stimulate J_H, fail to activate Na^+ absorption when J_{Na} and J_H are coupled (Ehrenfeld *et al.* 1990). Thus, the rate of acid secretion, or more precisely the acid–base status of the animal, will set an upper limit to Na^+ absorption.

Effects of electrical potential and acid load on Na^+ and H^+ transport

A transepithelial voltage-clamp produces opposite effects on J_{Na} and J_H (Fig. 3A). At negative serosal potentials, J_{Na} is stimulated whereas J_H decreases. Transepithelial potential gradients have been shown to act, *via* changes in apical membrane potential, on separate electrogenic pathways for Na^+ uptake in principal cells and H^+ secretion in mitochondria-rich cells (Ehrenfeld *et al.* 1985; Harvey and Ehrenfeld, 1986). The effects on Na^+ uptake of acid-loading the epithelium are dependent on the relationship existing between J_{Na} and H^+ secretion (Harvey and Ehrenfeld, 1988*b*). Under conditions where J_{Na} and J_H are coupled (open-circuited skins exposed to dilute external saline or

impermeant anions), an acid load produces a reversible increase in net Na^+ absorption (Fig. 3B,C). The basal and acid-stimulated J_{Na} are completely blocked by micromolar concentrations of amiloride and not affected by ethylisopropyl amiloride, an inhibitor of electroneutral Na^+/H^+ exchange. These findings implicate amiloride-blockable Na^+ channels as the pathway for the acid-stimulated Na^+ uptake process. Under short-circuit conditions, when J_{Na} is independent of J_H, net Na^+ absorption is inhibited by metabolic or respiratory acid loads (Fig. 3D).

Proton pumps are localized to mitochondria-rich cells

The 1:1 electrical coupling between J_{Na} and J_H in the absence of a permeant anion would seem to indicate that the underlying transport mechanisms are localized to the same cell. Measurements of intracellular Na^+ activity with double-barrelled ion-sensitive microelectrodes demonstrate that Na^+ uptake occurs essentially through principal cells even under conditions of tight coupling between J_{Na} and J_H. Indirect evidence places the proton pump in MR cells. Correlation between J_H and MR cell number and apical surface area (Ehrenfeld *et al.* 1990), the high carbonic anhydrase activity of these cells (Rosen and Friedley, 1973; Katz, 1986) and the inhibition of J_H by acetazolamide (Ehrenfeld and Garcia-Romeu, 1977) constitute the strongest proof. Adaptation to different environmental salinities and acid–base or hormonal challenges can change the number and morphology of MR cells, which correlate with alterations in J_H (Frazier, 1978; Ilic and Brown, 1980; Katz, 1986; Page and Frazier, 1987; Ehrenfeld *et al.* 1989), as found for intercalated cells of turtle urinary bladder (Husted *et al.* 1981; Schwartz *et al.* 1985; Steinmetz and Stetson, 1987) and in renal collecting duct (Madsen and Tisher, 1984).

I have sought direct evidence for the presence of V-ATPase pumps in MR cells by scanning pH gradients above MR cells. Double-barrelled pH-sensitive microelectrodes

Fig. 1. Schematic representation of ion transport through mitochondria-rich (MR) cells and principal (P) cells of isolated frog skin. (A) Conditions which resemble the *in vivo* state. Net Na^+ uptake in P cells is indirectly electrically coupled to proton secretion in MR cells with 1:1 stoichiometry under open-circuit conditions in the absence of transepithelial anion fluxes. Under control conditions (apical side bathed in Na_2SO_4 or 2 mmol l^{-1} NaCl solution buffered to pH 7.4 with 4 mmol l^{-1} imidazole, serosal side bathed in NaCl Ringer's solution buffered with 5 % CO_2, 24 mmol l^{-1} HCO_3^-), proton secretion is driven by a V-ATPase pump in the apical membranes of MR cells and the bulk of Na^+ absorption occurs through amiloride-sensitive channels in P cells (Na^+ chemical gradients are shown in mmol l^{-1}). The MR cell and the P cell generate equal and opposite H^+ and Na^+ currents, respectively. An acid load stimulates both carbonic-anhydrase-dependent H^+ secretion and amiloride-sensitive Na^+ absorption, despite inhibition of Na^+ and K^+ channels by H^+ in P cells. Proton-coupled Na^+ absorption in an acid load may occur *via* MR cells which can buffer changes in intracellular pH by activation of H^+ pumps and the dynamic buffering power of carbonic anhydrase. (B) Under classical experimental conditions in which the tissue is short-circuited (with or without a permeant anion) or open-circuited in NaCl Ringer's solution bathing both sides, Na^+ absorption occurs exclusively at a high rate through P cells and is uncoupled from H^+ secretion. Electroneutrality is conserved by equivalent movement of Cl^- (through the shunt or *via* MR cells) or by electrons from the external electron-motive force. An acid load under these conditions inhibits Na^+ uptake through P cells, resulting in a reduction of transepithelial Na^+ absorption. *I*, current; *E*, electrochemical potential.

placed 0.2 μm above the apical membrane of MR cells detected a fall in extracellular pH (pHe) under conditions when active H^+ secretion was abruptly stimulated (voltage-clamping V_t from −100 mV to +100 mV) (Fig. 4). The voltage-activated extracellular acidification (ΔpHe) was observed solely above the pit of MR cells and never when the pH electrode was above principal cells. ΔpHe was blocked by the H^+-ATPase probe (Solioz, 1984) dicyclohexylcarbodiimide (10 μmol l^{-1}) or by the carbonic anhydrase inhibitor ethoxzolamide (100 μmol l^{-1}) added to the apical solution (Fig. 4). The instantaneous rate of change in pHe was used to calculate H^+ flux in single HR cells; it

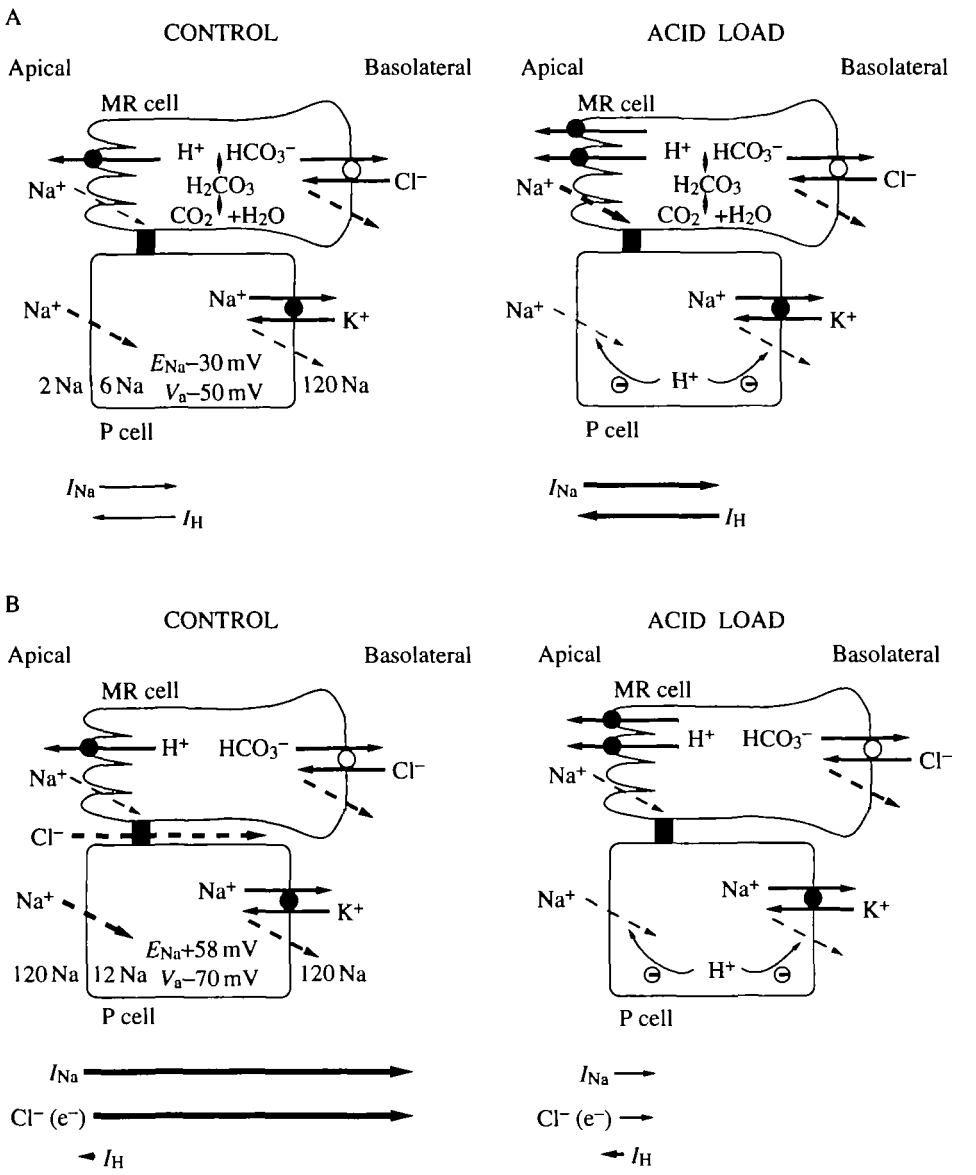

Fig. 1

was 8×10^{-18} equiv s^{-1}. At an imposed V_t of 100 mV, the DCCD-sensitive transepithelial current was 10 μA cm^{-2}, equivalent to a transepithelial H^+ secretion rate of 373 equiv h^{-1} cm^{-2}, which could be generated by 1.3×10^5 fully activated MR cells per square centimetre of tissue (normally 1 cm^2 of frog skin contains approximately 10^5 MR cells). If the pump number correlates with the density of studs (200 000 per cell) associated with rod-shaped particles in MR cells of amphibian skin and urinary bladder (Brown *et al.* 1987*b*; Steinmetz and Stetson, 1987), then single-pump current is 3.9×10^{-17} A.

Direct evidence for Na^+ channels in mitochondria-rich cells

A Na^+ transport role for MR cells seems necessary to explain the stimulated J_{Na} during an acid load (in low external salinity, OCC) when Na^+ uptake *via* principal cells is reduced by H^+-induced closure of Na^+ channels. Investigations into sodium absorption by MR cells have led to conflicting conclusions, although there is a consensus that MR cells and P cells have specialized functions. An amiloride- and ouabain-sensitive swelling of MR cells has been demonstrated by video-image analysis in toad skin (Spring and Ussing, 1986; Larsen *et al.* 1987), which is consistent with the presence of apical Na^+ channels and basolateral Na^+/K^+-ATPase. Electron microprobe elemental analysis of MR cells in frog skin (Rick *et al.* 1978; Dörge *et al.* 1989) failed to show significant changes in cellular Na^+, K^+ or Rb^+ levels in response to diuretics, although recent microprobe data revealed that MR cells, which showed changes in Na^+ content following ouabain and amiloride treatment, accounted for 50 % and 25 %, respectively, of the total MR cell

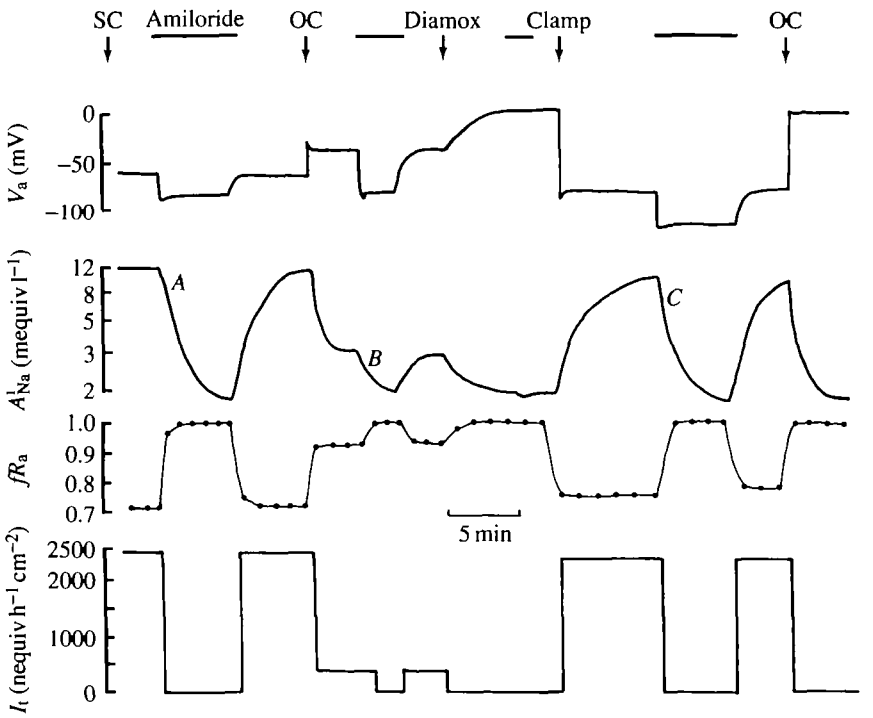

Fig. 2.

population (Rick, 1992). Microelectrode measurements of membrane potential in turtle urinary bladder and renal nephron intercalated cells do not support the hypothesis that these cells could constitute a major pathway for transepithelial Na^+ uptake (Durham and Nagel, 1986; Koeppen, 1987; Bello-Reuss, 1991), but recent patch-clamp studies provide direct evidence for highly selective Na^+ channels in apical membranes of frog skin MR cells (Harvey and Larsen, 1992).

Single MR cells maintain their shape and polarity when isolated from amphibian skin by collagenase treatment and membrane currents can be studied using the patch-clamp technique (Larsen and Harvey, 1992). Recordings of single-channel Na^+-selective currents (I_{Na}) in an excised inside-out MR cell apical membrane of frog skin are shown in Fig. 5. The single-channel current–voltage (I–V) relationship was described by the Goldman–Hodgkin–Katz (GHK) equation for Na^+ flux. The channel is highly selective for Na^+ relative to K^+ and has a single-channel chord conductance of 9 pS with 120 mmol l^{-1} Na^+ in the patch pipette and 12 mmol l^{-1} Na^+ in the bath perfusate. In symmetrical Na^+ solutions (120 mmol l^{-1}) the I–V relationship was linear and single-channel slope conductance was 3.6 pS. The GHK equation fitted to the I–V data yielded a value for the permeability selectivity coefficient, P_{Na}/P_K, of 35. Using this value, it is possible to determine the Na^+ concentration of MR cells when the membrane potential is

Fig. 2. Pen recording of measurements of apical membrane potential (V_a) and intracellular Na^+ activity (A^i_{Na}) in a frog skin principal cell obtained with a double-barrelled Na^+-sensitive microelectrode (for details see Harvey and Kernan, 1984) under conditions when Na^+ absorption was uncoupled from proton secretion in short-circuit (SC) current, when J_{Na} (I_t) and J_H were electrically coupled in open-circuit (OC), when J_H was inhibited by acetazolamide (Diamox) and when J_{Na} was activated by voltage-clamp during inhibition of J_H (Clamp). Apical membrane fractional resistance (fR_a) and transepithelial Na^+ current (I_t) were calculated from changes in V_a and V_t produced by the amiloride-sensitive component of bipolar transepithelial current pulses required to clamp V_t at ±20 mV for 200 ms duration (not shown). Apical solution, 55 mmol l^{-1} Na_2SO_4 buffered to pH 7.4 with 4 mmol l^{-1} imidazole; serosal solution, CO_2/HCO_3^--buffered Ringer, pH 7.4. Amiloride (10^{-5} mol l^{-1}) was added to the apical superfusate during periods marked by solid bars and produced a hyperpolarization of V_a, a fall in A^i_{Na} and an increase in fR_a to unity due to block of apical Na^+ channels. In SC conditions, J_{Na} is high and occurs essentially through P cells. This can be verified by comparing I_t with the instantaneous rate of decline in A^i_{Na} (ΔA^i_{Na}) after addition of amiloride. At A, I_t was 2490 nequiv h^{-1} cm^{-2} and ΔA^i_{Na} was 13 mmol l^{-1} min^{-1}, which is equivalent to Na^+ influx of 2260 nequiv h^{-1} cm^{-2} (epithelial volume/surface=2.9 μl cm^{-2}). When the tissue was abruptly changed from SC conditions to OC conditions, J_{Na} was instantaneously reduced, since it becomes rate-limited by the requirement for charge balance provided by H^+ secretion. A^i_{Na} decreases and fR_a is increased as less Na^+ enters the P cell. Under these conditions, when J_{Na} and J_H are coupled, Na^+ uptake still occurs essentially through principal cells as verified by ΔA^i_{Na} measured at *B*: 3 mmol min^{-1} or 520 nequiv h^{-1} cm^{-2} (compared with I_t=500 nequiv h^{-1} cm^{-2}). Addition of the carbonic anhydrase inhibitor diamox (10^{-4} mol l^{-1}) to the apical superfusate under OC conditions produced membrane depolarization, a fall in A^i_{Na}, an increase in fR_a and a reduction in transepithelial Na^+ absorption. Amiloride had no additional effect on these transport parameters indicating that net Na^+ entry through the P cell was completely abolished. Voltage-clamping the epithelium to a V_t of 100 mV produced a recovery in A^i_{Na} and fR_a. In this situation when J_H is inhibited, the voltage-driven J_{Na} passes through the P cell and at *C* the ΔA^i_{Na} was 14 mmol min^{-1} (2440 nequiv h^{-1} cm^{-2} equivalent to I_t).

clamped to 0 mV by high external K^+ (120 mmol l^{-1}). Under these conditions, the I_{Na} reversal potential is equal to the Nernst potential for Na^+ and the GHK fit to single-channel Na^+ currents in intact cells gives E_{Na} of 50 mV and $[Na^+]_i$ of 16 mmol l^{-1}.

Another approach for detecting Na^+ channels in MR cells uses the whole-cell patch-clamp configuration. In this case, MR cells are patch-clamped *in situ* from the basolateral side of an intact epithelium mounted in an Ussing chamber. This experimental design has the advantage of maintaining functional polarity of the cells and allowing asymmetrical

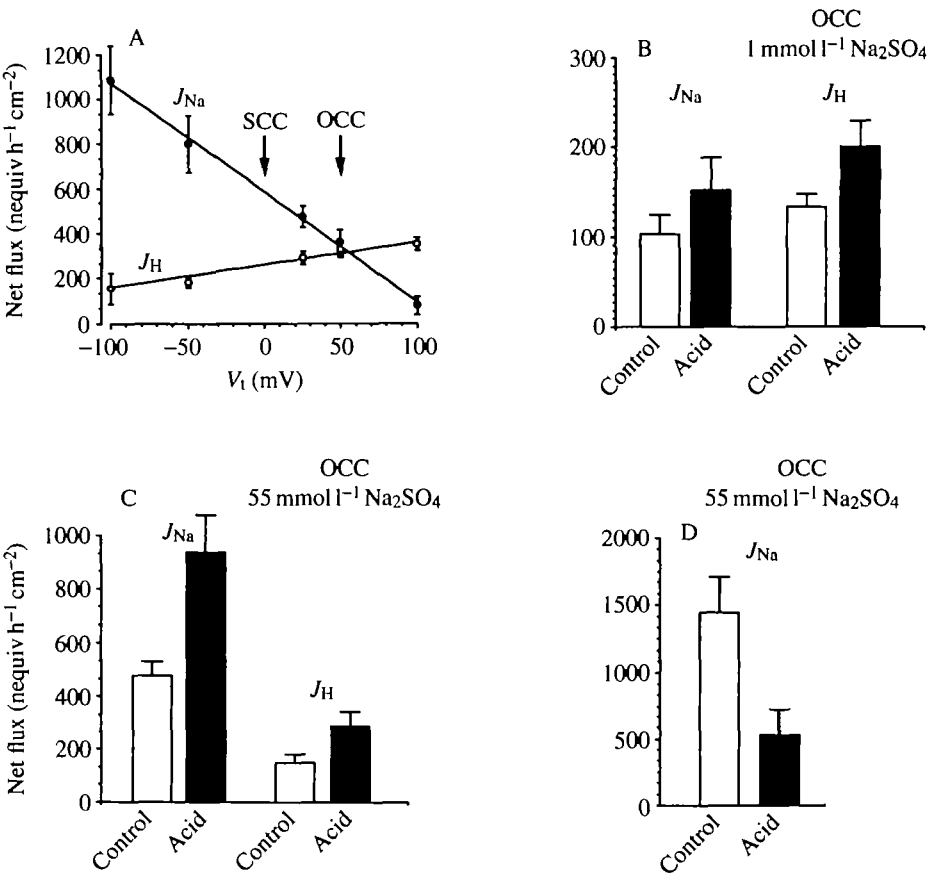

Fig. 3. (A) Relationship between net active H^+ secretion (J_H) and Na^+ absorption (J_{Na}) in isolated frog skin (bathed in 1 mmol l^{-1} Na_2SO_4 solution on the apical side and CO_2/HCO_3^--buffered Ringer's solution on the serosal side) at different imposed transepithelial electrical potentials (V_t). J_{Na} and J_H show opposite dependence on V_t. Hyperpolarization of the epithelium (serosa negative) reduces J_H and increases J_{Na}. Note that J_{Na} and J_H are equal only in open-circuit conditions (OCC). (B) Stimulation of J_{Na} and J_H by an acid load under open-circuit conditions from a 1 mmol l^{-1} Na_2SO_4 solution. (C) Stimulatory effect of an acid load on J_{Na} and J_H under open-circuit conditions in an apical 55 mmol l^{-1} Na_2SO_4 solution. (D) The same ionic gradients as in C but under short-circuit conditions (SCC), where an acid load inhibits Na^+ absorption. Acid loads were created by application of 5 % CO_2 to the apical side in C and D or by loading with 15 mmol l^{-1} NH_4Cl from the serosal side in B. (Data from Harvey and Ehrenfeld, 1988*a*.) Bars show mean+S.E.

application of transport inhibitors or activators. Whole-cell voltage-clamp of MR cells *in situ* is possible since these cells are chemically and electrically uncoupled from neighbouring principal cells (Farquhar and Palade, 1964; Rosen and Friedley, 1973; Rick *et al.* 1978; Ilic and Brown, 1980; Kristensen and Ussing, 1985; Spring and Ussing,

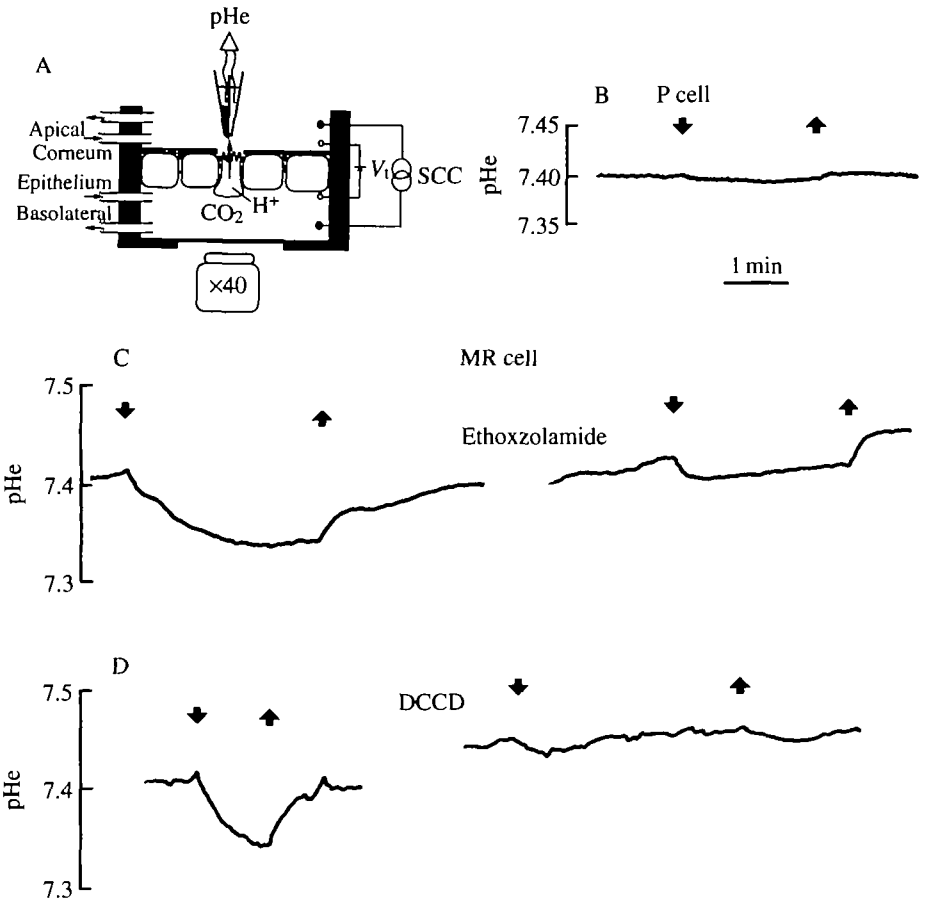

Fig. 4. Direct evidence for voltage-dependent H^+-ATPase pumps located in apical membranes of mitochondria-rich cells in isolated epithelia. (A) Schematic representaion of experimental set-up to measure external pH (pHe) changes with double-barrelled pH-sensitive microelectrodes positioned 0.2 μm above an MR cell in response to a transepithelial voltage-clamp. Apical solution, 1 mmol l^{-1} Na_2SO_4 buffered to pH 7.4 with 4 mmol l^{-1} imidazole; serosal solution, 5 % CO_2/24 mmol l^{-1} HCO_3^--buffered Ringer, pH 7.4. The epithelium was voltage-clamped at −100 mV to reduce (or reverse) electrogenic J_H and stepped to +100 mV (during periods marked by arrows in the accompanying pen recordings of pHe) to stimulate J_H. In B pHe was unchanged by voltage-clamp when the pH microelectrode was positioned 0.2 μm above the stratum corneum over a principal cell. In C and D the electrode was positioned over an MR cell. Voltage-clamping the tissue to +100 mV produced a reversible external acidification which was reduced in C by the carbonic anhydrase inhibitor ethoxzolamide (10^{-4} mol l^{-1}). In D, application of 10^{-5} mol l^{-1} dicyclohexylcarbodiimide (DCCD) to the apical bath prevented the voltage-induced external acidification above an MR cell.

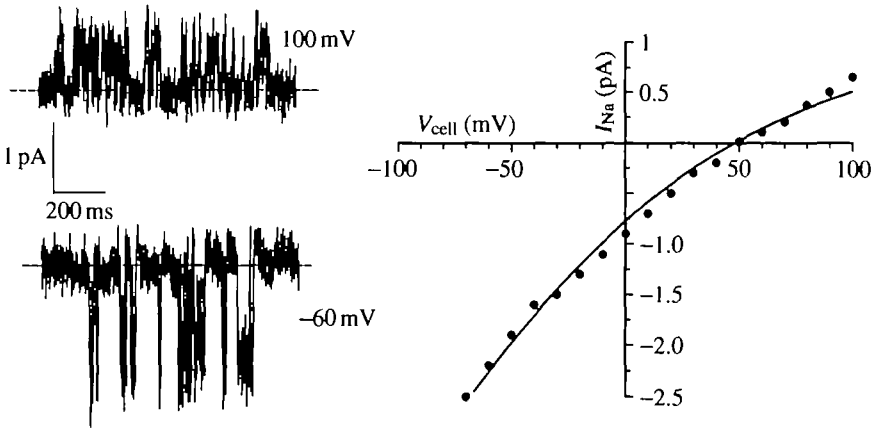

Fig. 5. Patch-clamp recording of single Na^+ channel activity in a patch excised from the apical membrane of an isolated MR cell. The patch pipette was filled with (in mmol l^{-1}) NaCl, 120; $BaCl_2$, 5; KCl, 2; $MgCl_2$, 1; $CaCl_2$, 2; Hepes, 10; pH 7.4 and the bath (cytosolic side) contained NaCl, 12; KCl, 104; CsCl, 4; $MgCl_2$, 1.17; $CaCl_2$, 2.96; EGTA, 5 (100 nmol l^{-1} free Ca^{2+}); Hepes, 10; pH 7.4. Single-channel current records are shown in A at two membrane potentials, +100 mV and −60 mV. The closed state is shown by the dashed line. Upward current deflections signify Na^+ moving across the membrane from the cytosolic side into the patch pipette. The single-channel current–voltage relationship is given in B. Fitting the Goldman–Hodgkin–Katz equation to the I–V data (solid line through data points) gave values for single-channel chord conductance, g, of 9 pS and a selectiviy ratio, P_{Na}/P_K, of 35:1.

1986). Amiloride-sensitive apical membrane current–voltage relationships ($I_a - V_a$) can be recorded from MR cells (in which K^+ and Cl^- currents were eliminated by Ba^{2+}, Cs^+ and ionic substitution) by subtraction of whole-cell I−V relationships recorded in the absence and the presence of 5 μmol l^{-1} apical amiloride (B. J. Harvey and E. H. Larsen, unpublished results). The almost ideal fit of the GHK equation for Na^+ flux to $I_a - V_a$ relationships provides firm evidence that the MR cell apical membrane possesses Na^+ channels. Fluctuation analysis of amiloride-sensitive whole-cell current yields estimates of single-channel conductance and the number of Na^+ channels in the MR cell apical membrane (Fig. 6). Power density spectra were fitted by a single Lorentzian function which describes whole-cell noise generated by a homogeneous population of fluctuating ion channels. The voltage-dependence of amiloride-sensitive noise shows minima at E_{Na}, as expected for current fluctuations generated by openings and closures of Na^+ channels. The low conductance and high Na^+/K^+ selectivity of Na^+ channels in MR cells resembles quite closely the biophysical characteristics of patch-clamped Na^+ channels in principal cells (Palmer and Frindt, 1986). The Na^+ channel density of 450 channels per MR cell or 45×10^6 channels per cm^2 tissue (for 10^5 MR cells per cm^2) is in the range 17×10^6–77×10^6 channels per cm^2 estimated by noise analysis in amphibian skin (van Driessche and Zeiske, 1985). It is theoretically possible for MR cells to account for all of J_{Na}. However, MR whole-cell Na^+ currents and channel density are five times smaller than in granular cells (Harvey and Larsen, 1992), which constitute a high-capacity and syncytial route for Na^+ absorption. The sensitivity of both MR and P cell Na^+ channels to cytosolic H^+

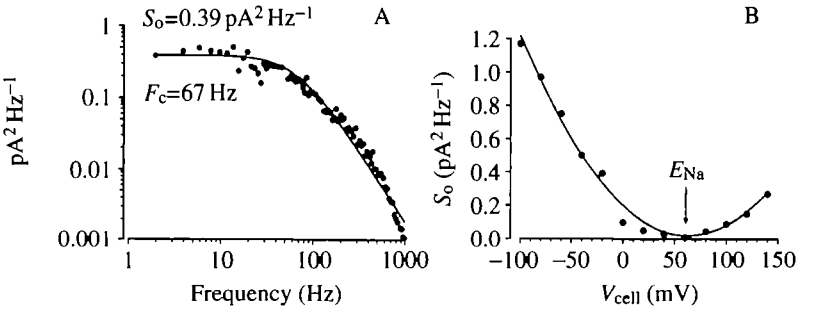

Fig. 6. Stationary fluctuation analysis of MR cell Na^+ currents recorded from an isolated MR cell in whole-cell patch-clamp configuration. The bath contained (in mmol l^{-1}) sodium acetate, 120; $BaCl_2$, 5; KCl, 2; $MgCl_2$, 1; $CaCl_2$, 2; Hepes, 10; (pH 7.4); DCCD, 10^{-4} and the patch pipette was filled with NaCl, 5.7; sodium gluconate, 6.3; potassium gluconate, 104; CsCl, 4; $MgCl_2$, 1.17; $CaCl_2$, 2.96; EGTA, 5 (100 nmol l^{-1} free Ca^{2+}); Hepes, 10 (pH 7.4); and GTPγS 10 μmol l^{-1}. (A) Power density spectrum (PDS) of whole-cell noise recorded at a pipette (cell) voltage V_p of −25mV. The PDS was fitted by a single Lorentzian function, which is indicative of the presence of spontaneously fluctuating channels. A single-channel chord conductance, g_s, of 8.45 pS was calculated from the Lorentzian plateau of 0.39 pA² s^{-1} MR cell, the corner frequency, f_c of 67 Hz and the single Na^+ channel open probability, P_o, of 0.25 (measured in excised patches under similar electrochemical gradients as used in the whole-cell recordings). These data and the measured noise variance of 41 pA², Nernst potential E_{Na} of 58 mV and membrane current I_m of 78 pA at V_p of 25 mV were used to calculate a single-channel Na^+ current of 0.7 pA and a density of 4.46 Na^+ channels per MR cell. The ion-selectivity of the channel that generates the spontaneous whole-cell noise was evaluated by examining the voltage-dependence of the plateau Lorentzian function. The relationship between S_o (see below) and V_{cell} is shown in B and displays a minimum at a V_{cell} of 60 mV. Since Na^+ is the only ion with a reversal potential close to this value, it can reasonably be concluded that the whole-cell noise is generated by spontaneously fluctuating Na^+ channels. *Whole-cell current noise analysis.* The whole-cell configuration was obtained from the cell-attached mode after breaking the membrane patch by applying a brief negative pressure pulse (-2×10^3 N m^{-2}) in the pipette. Patch pipettes were coated with Sylgard to reduce stray capacitance through the glass wall. Whole-cell current fluctuations were amplified (RK300 Biologic, Claix, France), digitized (A/D converter CED 1401, Cambridge Electronic Design, UK) and sampled in real time on a computer hard disk at an acquisition rate of 2 kHz. The noise signal was split into low- and high-pass filtered components with cut-off frequencies at 1 kHz and 0.1 Hz, respectively. The filtered signals were analysed by a fast Fourier transform using the SPAN program (J. Dempster, Strathclyde Electrophysiology Software, Scotland). The variance of the fluctuations in current noise is presented as a function of the a.c. frequencies contained in the current noise (power density spectra). An average power spectrum was compiled from 200 record segments (each of 250 ms duration). Channel noise was recorded at pipette holding potentials between +150 mV and −100 mV. Background or channel-free noise was recorded in the presence of a saturating concentration of the inhibitor amiloride. A Lorentzian curve was fitted to the power spectrum using a Levenberg–Marquadt iterative fitting algorithm. For a channel that fluctuates between distinct open and closed states, the power spectrum will contain a Lorentzian defined by the equation: $L(f)=S_o/[1+[f/f_c)^2]$, where S_o is the low-frequency asymptote or plateau value and f_c is the corner or cut-off frequency at which the spectral power is $S_o/2$. The variance in channel current noise (σ^2) was calculated by integrating the power spectrum $\sigma^2=(\Pi>f_c S_o)/2$ and the single-channel conductance (g_s) was then determined from $g_s=\sigma^2/[I_m(V_P-E_r)\times(1-P_o)]$, where I_m is the mean d.c. current recorded in the power spectrum, V_p is the pipette holding voltage, E_r is the reversal potential and P_o the open-channel probability. Single-channel current (I_s) can then be obtained from $I_s=g_s(V_p-E_r)$. The channel density (N) was estimated from: $N=\Pi f_c S_o/2I_s^2P_o(1-P_o)$.

(Harvey *et al.* 1988; B. J. Harvey and E. H. Larsen, unpublished results) and the CO_2-activation of J_H-coupled J_{Na} implies that MR cell Na^+ channels are protected against an acid load. This protection could be achieved by the dynamic buffering power of carbonic anhydrase-catalysed hydration of CO_2. The high rate of H^+ secretion through the H^+ pump and the CO_2-induced exocytotic insertion of pumps may also contribute to a rapid regulation of intracellular (pHi) (Cohen and Steinmetz, 1980; van Adelsberg and Al-Awqati, 1986).

Counting proton pumps in single MR cells

The H^+-ATPase pump of epithelia is thought to be composed of a cytoplasmic domain catalytic subunit and an intramembranous proton channel linked by an antechamber of high buffering power (Andersen *et al.* 1985; Steinmetz, 1988). This structure is analogous to that envisaged for V-ATPases and F-ATPases (Sebald *et al.* 1982; Senior and Wise, 1983; Gluck and Caldwell, 1988; Forgac, 1989; Futai *et al.* 1989). The rheogenic nature of proton secretion and its passage through a proton channel in the apical membrane opens the possibility of applying whole-cell patch-clamp recording to detect H^+ pump current fluctuations. Using DCCD as a probe for the proton channel (Solioz, 1984), power density spectra were constructed from CO_2-induced noise in single MR cells *in situ*, in which conductive movements of Na^+, K^+ and Cl^- were eliminated (Fig. 7). Electrical access to the whole-cell membrane was achieved by perforating the membrane patch in cell-attached mode with the ionophore amphotericin B in the patch pipette. This technique (Horn and Marty, 1988) provides a low electrical resistance access to the cell while, presumably, maintaining metabolism and the cytoskeleton intact. (Horn and Marty used nystatin to perforate rat lacrymal gland cells; lower access resistance is achieved with amphotericin B in MR cells). Current fluctuations induced in MR cells by increasing P_{CO_2} in the serosal bath could be fitted by a single Lorentzian function, indicative of the presence of a homogeneous population of spontaneously fluctuating channels. The concomitant disappearance of the Lorentzian noise component and transepithelial H^+ current when DCCD was added to the apical bath strongly suggest that the whole-cell current fluctuations originate from spontaneously fluctuating proton channels in the apical membrane. Changing from CO_2-free to 5 % CO_2 medium produced a threefold increase in the calculated number of H^+ pumps in the membrane accompanied by a 27 % increase in single-pump current. The voltage-dependence of the Lorentzian plateau showed minima at membrane potentials of approximately -170 mV, which is in reasonable agreement with measured transepithelial voltages (130–210 mV) required to halt or reverse J_H (Dixon and Al-Awqati, 1979, 1980; Andersen *et al.* 1985; Ehrenfeld *et al.* 1985).

Aldosterone stimulates H^+ secretion in amphibian skin and urinary bladder and in rat colon (Ludens and Fanestil, 1974; Al-Awqati *et al.* 1976; Perrone *et al.* 1990), activates H^+-ATPase activity in renal collecting tubule (Mujais, 1987; Khadouri *et al.* 1989), selectively binds to MR cells (Sapirstein and Scott, 1975) and changes their morphology (Voûte *et al.* 1972). In the presence of 5 % CO_2/24 mmol l^{-1} HCO_3^-, aldosterone produced an additional stimulation of DCCD-sensitive apical membrane current

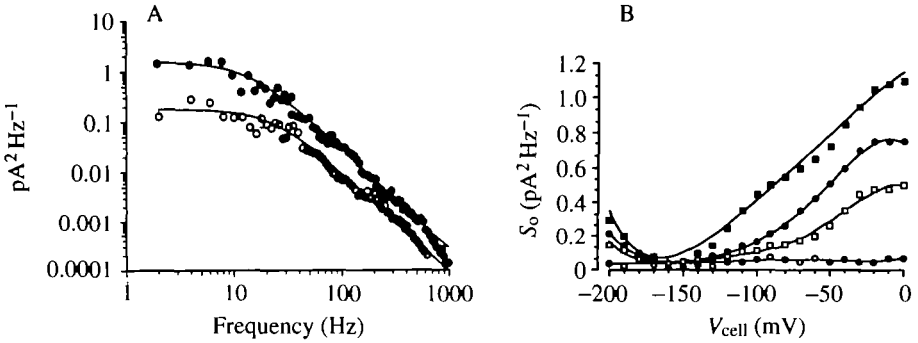

Fig. 7. Noise analysis of CO_2- and aldosterone-treated single MR cells. Membrane current fluctuations were recorded using the perforated whole-cell patch-clamp configuration. The experimental arrangement and solutions are described in detail in Fig. 8. (A) Power density spectra recorded in CO_2-free (○) and in 5 % CO_2/24 mmol l^{-1} HCO_3^- (●) Ringer's solution are shown for the same MR cell voltage-clamped at 100 mV. In CO_2-free solutions (N=9), S_o=0.18±0.011 pA^2 Hz^{-1} and f_c=22.4±2.4 Hz. Single-pump current (I_p) was 0.166 pA and the number of pumps, N was 602 pumps per MR cell. In 5 % CO_2 solutions (N=9), S_o=1.057±0.099 pA^2 Hz^{-1}, f_c=13.5±1.19 Hz, I_p=0.21 pA, N=7695 pumps per MR cell (assuming P_o=0.9). In aldosterone-treated cells (N=9), S_o=1.3±0.4 pA^2 Hz^{-1}, f_c=44.3±2.5 Hz, I_p=0.299 pA and N=11 129 pumps per MR cell. (B) Voltage-dependence of PDS plateau Lorentzian functions in the presence of apical (10^{-5} mol l^{-1}) DCCD (□), CO_2-free Ringer's (○), 5 % CO_2 (●) and aldosterone in presence of 5 % CO_2 (■). The minimum Lorentzian plateau S_o for the latter three conditions was recorded at a V_{cell} of −170 mV.

fluctuations in single MR cells (Fig. 7B) and a 150 % increase in the calculated number of H^+ pumps, without significantly affecting single-pump current. From electron micrographs, the MR cell apical area is approximately 12.5 μm^2 and the density of proton pumps in CO_2 is approximately 600 μm^{-2}. Since MR cells account for approximately 10 % of tissue area, this calculation yields a proton current of 12 μA cm^{-2}, which is equivalent to measured rates of transepithelial H^+ secretion (350 nequiv h^{-1} cm^{-2}).

The single-pump current calculated from noise analysis is about 500 times greater than that estimated from titration and morphological data. This could be explained if H^+ pumps functioned in clusters, and their distribution over the membrane was non-uniform.

CO_2- and aldosterone-stimulated exocytosis in single MR cells

Exocytosis of cytosolic vesicles into plasma membranes can be a rapid means of increasing the transport capacity of a cell (Almers, 1990; Schaerer *et al.* 1991). CO_2 stimulates H^+ secretion in turtle urinary bladder (Schwartz and Steinmetz, 1971) by rapid insertion of vesicles containing preformed V-ATPase holoenzymes into the luminal membrane (Gluck *et al.* 1982; Stetson and Steinmetz, 1983, 1986; Brown *et al.* 1987*a*; Steinmetz, 1988). This phenomenon has also been described as a mechanism controlling acid secretion in renal tubule (Schwartz and Al-Awqati, 1985). The exocytotic event is dependent on changes in cell pH and calcium concentration (Cannon *et al.* 1985; van Adelsberg and Al-Awqati, 1986; Arruda *et al.* 1988, 1990), although the role of

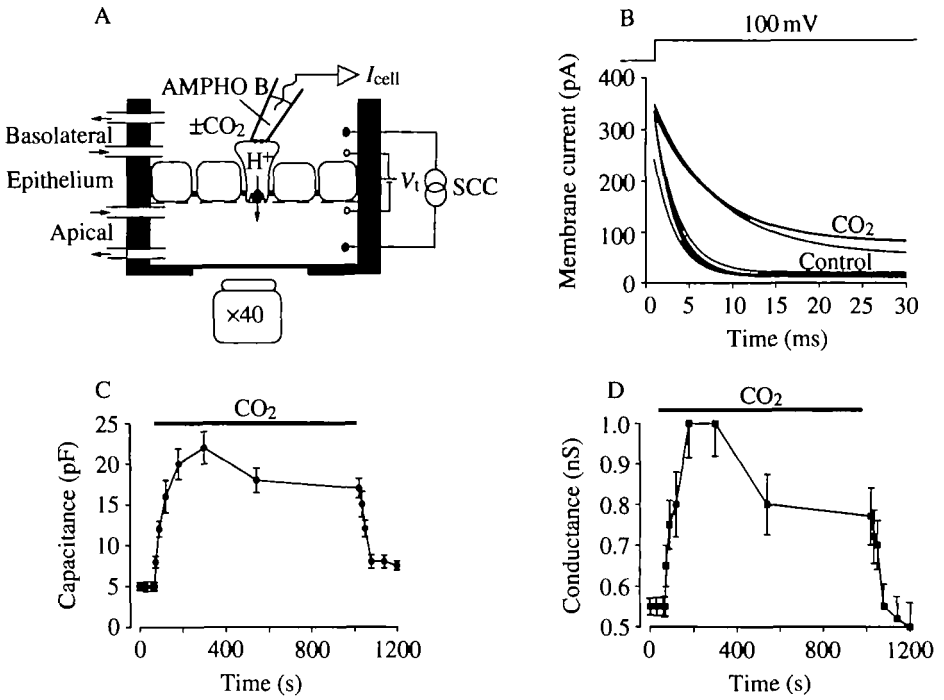

Fig. 8. Exocytosis induced by carbon dioxide and quantified from measurements of membrane capacitance in single MR cells *in situ*. A diagram of the experimental arrangement is shown in A. The isolated epithelium was mounted in a miniature Ussing chamber on the stage of an inverted Leitz microscope and viewed at ×40 magnification. The basolateral membranes of MR cells *in situ* were exposed by collagenase treatment and the slow whole-cell configuration of the patch-clamp technique was used to record MR cell membrane capacitative current transients (Lindau and Neher, 1988). The patch membrane was perforated by adding the ionophore amphotericin B oxycholate (final concentration 40 μg ml^{-1}) to the patch pipette solution which contained (in mmol l^{-1}) potassium gluconate, 104; acetate, 3; pyruvate, 5; L-aspartic acid, 5; KCl, 20; $MgCl_2$, 1; $CaCl_2$, 1; EGTA, 10; ATP, 3; Hepes, 10 (pH 7.2) and 100 μmol l^{-1} ADP; 10 μmol l^{-1}, GTPγS. The serosal bath was NaCl Ringer containing 5 mmol l^{-1} $BaCl_2$ and equilibrated in CO_2-free air (pH 7.4) or gassed in 5 % CO_2/24 mmol l^{-1} HCO_3^- (pH 7.4). The apical side was superfused with 1 mmol l^{-1} $MgSO_4$, 4 mmol l^{-1} imidazole (pH 7.4). Membrane capacitance was calculated from the time constant (τ) of decay in capacitative membrane currents immediately following a step in pipette voltage (V_p) from 0 to +100 mV. The change in whole-cell clamp current in the time domain (I_c) was fitted by $I_c=(I_{max}-I_{min})\exp(-\tau/t)+I_{min}$. Since the membrane pipette seal resistance (>50 GΩ) is much larger than the access resistance ($R_a \approx 300$ MΩ) of the patch membrane, R_a can be calculated from V_p/I_{max} and membrane conductance $G_m=I_{min}/(V_p-R_aI_{min})$ and membrane capacitance from $C_m=\tau>(1/R_a+G_m)$. Twenty consecutive I_c transients were recorded and averaged.for a single determination of C_m. Pulse duration was $\tau\times 10$ ms at 100 ms intervals and an entire run took on average 4 s. The I_c signals were recorded using a patch-clamp amplifier (Biologic RK300, Claix, France) with cut-off Bessel filter set at 5 kHz and digitized at 10 kHz (Cambridge Electronic Design CED1401 A/D converter) and stored by computer for analysis. (B) Membrane current transients recorded from a single perforated whole MR cell following voltage-clamp from 0 to +100 mV pipette (cell) positive. Four consecutive membrane capacitance transients (each an average response to 20 voltage pulses) are shown for CO_2-free and 5 % CO_2 conditions. The rate of decay in whole-cell clamp current is slower in CO_2 (signifying increased capacitance). (C) MR cell membrane capacitance and (D) conductance before, during and after exposure to 5 % CO_2 (N=10 cells). An intracellular acid load reversibly stimulated both exocytosis and conductance.

intracellular acidification *per se* in CO_2-induced J_H has been convincingly challenged (Adrogué *et al.* 1987). A dynamic control of acid secretion may be achieved by exocytotic insertion and endocytotic retrieval of V-ATPase pumps (Al-Awqati *et al.* 1983; Reeves *et al.* 1983; Schwartz and Al-Awqati, 1986; Dixon *et al.* 1986, 1988; Stetson, 1989). Exocytosis increases cell membrane area, which can be recorded as an increase in cell membrane electrical capacitance (Clausen and Dixon, 1986; Rich *et al.* 1990). Measurements of single-cell membrane capacitance (C_m) and exocytotic rates can be reliably made in the whole-cell patch-clamp recording configuration (Horn and Marty, 1988; Lindau and Neher, 1988). The application of this technique to single MR cells *in situ* is shown in Fig. 8. Superfusion of an MR cell in 5 % CO_2-buffered Ringer's solution induces a rapid and reversible increase in membrane capacitance (C_m) from 4.6±10.7 pF to 22±11.5 pF with a half-time for maximum change in C_m of 37±16 s (N=10). The changes in MR cell membrane area and apical membrane DCCD-sensitive conductance occurred simultaneously, demonstrating that the newly inserted membrane contained functional H^+ pumps. This result correlates well with an enhanced acid-secreting capacity of the epithelium with DCCD-sensitive transepithelial current increasing from 4.6 μA cm^{-2} (172 nequiv h^{-1} cm^{-2}) in CO_2-free medium to 15.7 μA cm^{-2} (584 nequiv h^{-1} cm^{-2}) in 5 % CO_2.

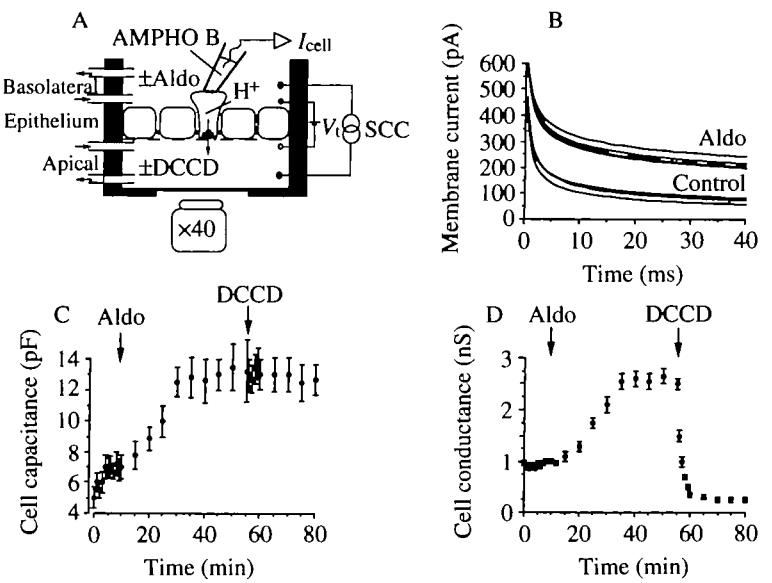

Fig. 9. Effects of aldosterone (10 nmol l^{-1}) on membrane capacitance and conductance of amphotericin-perforated MR cells which had been exposed to 5 % CO_2/24 mmol l^{-1} HCO_3^- Ringer's solution for 2 h. The experimental set-up is shown in A and bath/pipette solutions are similar to those used for the CO_2-induced exocytosis experiments. (B) Recording of 80 averaged membrane current transients in control and after aldosterone (30 min). Both the time constant and d.c. whole-cell current were increased by hormone treatment. (C) The response of cell capacitance and (D) conductance to aldosterone (N=6 cells). The hormone triggers insertion of membrane with conductive properties. Addition of DCCD (10^{-5} mol l^{-1}) to the apical bath reduced membrane conductance without affecting membrane capacitance.

A similar approach was used to investigate the mechanism of aldosterone-stimulated H^+ secretion in frog skin (Fig. 9). Aldosterone produces a slow increase in membrane capacitance and conductance of single MR cells (half-time 12 min). The conductance, but not the capacitance, was reduced by DCCD applied to the apical side, which indicates that aldosterone induces the insertion of new membrane containing functional H^+ pumps into the apical membrane. DCCD acts as a blocker of proton current without affecting exocytosis/endocytosis. Aldosterone and CO_2-induced exocytosis are additive but mutually independent. How the hormone increases the activity of the membrane shuttle is at present unknown.

Energization of Cl^- transport by the proton pump

Besides their proton secretion function, there is evidence that MR cells are the site of passive apical Cl^-/HCO_3^- exchange (Ehrenfeld and Garcia-Romeu, 1978). Cl^- absorption/base secretion may not involve the alpha-type proton-secreting MR cell but rather a base-secreting MR cell similar to the beta-type intercalated cell in turtle urinary bladder and renal collecting tubule (Stetson and Steinmetz, 1985; Schwartz *et al.* 1985; Brown *et al.* 1988*b*). A concentration- and voltage-dependent Cl^- conductive uptake pathway has also been attributed to amphibian MR cells (Larsen and Rasmussen, 1982; Willumsen and Larsen, 1986; Katz *et al.* 1985; Katz and Scheffey, 1986; Nagel and van Driessche, 1991). Although this claim is currently a matter of contention (Dörge *et al.* 1988), recent whole MR cell patch-clamp studies provide direct evidence for voltage-gated Cl^- currents (Larsen and Harvey, 1992), which may involve a novel gamma-type MR cell (Larsen, 1991). If Cl^- channels are inactivated in gamma-type MR cells exposed to low external Cl^- concentration, the parallel operation of H^+-ATPase pumps and Cl^-/HCO_3^- exchange will produce an apparent rheogenic transepithelial Cl^- absorption (Larsen *et al.* 1992). The possibility that proton pumps in MR cells energize Na^+ and Cl^- absorption from dilute solutions *in vivo* is given added support from the positive correlation between Na^+ and Cl^- absorption rates and MR cell number (Ehrenfeld *et al.* 1990; Devuyst *et al.* 1991).

Conclusions and perspectives

The V-ATPase in mitochondria-rich cells appears to be a major determinant of sodium absorption through principal cells of amphibian skin *in vivo* or *in vitro* in the absence of transepithelial anion flux. The apparent 1:1 coupling between J_{Na} and J_H results from the equilibrium of circular current flow across the epithelium and is a manifestation of Kirchhoff's current law. An acid load produces closure of apical Na^+ channels in principal cells and the MR cells provide an alternative cellular pathway for acid-stimulated amiloride-sensitive Na^+ uptake.

Patch-clamp studies, as described here, of membrane conductance and capacitance of MR cells *in situ* in a polarized and functional epithelium offer unique opportunities to discover the mechanisms of ion transport regulation modulated by acid–base and hormonal challenges. This tool could also be used to probe further the analogy between

proton channels and antidiuretic hormone water channels (Harris *et al.* 1991; Harvey *et al.* 1991).

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