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Control of Na^+ and H^+ Transports by Exocytosis/Endocytosis Phenomena in a Tight Epithelium

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Abstract. The relationship linking Na^+ and H^+ transports and exocytosis/endocytosis located in the apical membranes of the frog skin epithelium was investigated under various conditions of ion transport stimulation. The exocytosis process, indicating insertion of intracellular vesicles, which were preloaded with fluorescent FITC-dextran (FD), was measured by following the FD efflux in the apical bathing solution.

Na^+ transport stimulators such as serosal hypotonic shock (replacement of serosal Ringer solution by half-Ringer or 4/5-Ringer), apical PCMPS (10^{-3} M) and amphotericin-B (20 μg/ml), were also found to stimulate the exocytotic rates of FD. Acidification of the epithelium by CO_2 or post NH_4 load, conditions which increase the proton secretion also stimulated the FD release in the apical bathing solution. On the other hand, alkalization of the epithelial cells increased the endocytosis rate. Hypotonic shock, acid load and PCMPS induced an increase in cell calcium which is probably the signal within the cell for exocytosis. In addition, quantitative spectrofluorimetric measurements of F-actin content after rhodamine-phalloidin staining, indicated a decrease in the F-actin content as a result of cell acidosis, hypotonic conditions and amphotericin additions. It is proposed that the insertion/retrieval of intracytoplasmic vesicles containing H^+ pumps plays a key role in the regulation of proton secretion in tight epithelia. In addition, it is suggested that cytoskeleton depolymerization of F-actin filaments facilitates H^+ pump insertion. A comparable working hypothesis for the control of Na^+ transport is proposed.

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Key words: Na^+ channel — H^+ pump — Hypotonic shock — Acidification — PCMPS — Exocytosis/endocytosis

Introduction

In ion transporting epithelia, the rates of absorption and secretion of ions and water can be controlled in various ways. Following changes in the hydromineral or acido-basic state of the animals, the transport capacity of channels, exchangers or pumps can be regulated by modifying the kinetic properties of the transport proteins or by "switching on" already-existing transporters present in the membrane. For instance, intracellular signals such as H^+, Ca^{2+}, Na^+ or regulatory proteins (G proteins) have been shown to be involved in the control of the permeability of amiloride-sensitive Na^+ channels in tight epithelia (for review, *see* Palmer, 1985; Garty & Benos, 1988; Cantiello & Aussiello, 1991). Hormones can act via one of these signals and/or any second messengers (AMPc, GMPc . . .) or can induce the synthesis of new transporters, in which case the action is delayed. A rapid regulation of cellular membrane transport can be achieved by exocytosis and endocytosis of transporters located in intracellular vesicles in proximity to the plasma membranes. This control mechanism has been found to occur for H^+ secretion in turtle urinary bladder (Gluck, Cannon & Al-Awqati, 1982), for histamine-stimulated acid secretion by H/K-ATPases (Forte, Machen & Forte, 1977), for vasopressin-activated water transport through the toad bladder and the kidney collecting tubule (Muller, Kachadorian & Di Scala, 1980; Wade, Stetson & Lewis, 1981) and for insulin stimu-

lation of glucose transport in fat and muscle cells (Suzuki & Kono, 1980).

The outermost living cell layer of the frog skin epithelium consists of two cell types with different transport functions. The granular cells are the main route of Na^+ transport while the mitochondria-rich cells (MRc) are responsible for proton excretion mediated by H^+ pumps located in their apical membranes (Rick et al., 1978; Ehrenfeld, Lacoste & Harvey 1989). Numerous experiments have been performed on the control of ion transport in this structure. We know of no study, however, in which the rate of exocytosis/endocytosis has been measured or in which the possibility of ion transport control (H^+ and Na^+) by the insertion of vesicles containing transporters or pumps has been investigated.

We therefore measured the rate of exocytosis/endocytosis located at the apical membrane of the frog skin epithelium using the fluid-phase marker FITC-dextran (FD), under steady-state conditions or on stimulation of the H^+ and Na^+ transports. Quantitative spectrofluorimetric measurements of the F-actin content of the epithelial cells by rhodamine-phalloidin binding were also undertaken to investigate the role of this major component of the cytoskeleton in the turnover rate of membrane transport proteins. Changes in intracellular calcium after various stimulation of Na^+ and H^+ transports (using Fura-2 as a calcium probe) were also followed.

Materials and Methods

Frogs, *Rana esculenta,* were kept at a constant temperature of 15 ± 1°C in running tap water. Studies were carried out on isolated abdominal skins removed from double-pithed animals. In most experiments, we cut the skin in two or three identical pieces, one serving as control while the other(s) were used in experiments. Each piece of skin was mounted flat between two Lucite chambers with an exposed surface area of 7 cm^2 and a 3 ml volume per hemichamber. This large surface/volume ratio was designed to increase variations in ion or fluorescein-dextran concentrations in the bathing solutions.

Two principal Ringer solutions were used. The air Ringer solution contained (in mM) 85 NaCl, 12 Na_2SO_4, 2,5 KCl, 2 $CaCl_2$, 2 $MgSO_4$, 2,5 Na_2HPO_4, 1,2 KH_2PO_4, 11 glucose and 5 N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4 and was bubbled with air. The HCO_3^-/CO_2-containing Ringer solution (pH 7.4) was identical in composition except that Na_2SO_4 was replaced by $NaHCO_3$ and the solution was gassed with a mixture of 5% CO_2 and 95% O_2. In some experiments, we used an acid Ringer solution (pH 6.2), identical in composition to the HCO_3^-/CO_2-containing Ringer solution (pH 7.4) but HCO_3^- free.

When using amphotericin as an ionophore, the apical bathing Ringer solution contained (in mM) 15 NaCl, 100 KCl, 2 $MgSO_4$, 2,5 Na_2HPO_4, 1,2 KH_2PO_4, 11 glucose and 5 N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4 and was bubbled with air.

Quantification of Exocytosis

All experiments were performed in open-circuit conditions in Ussing chambers. The technique is derived from that applied to the turtle urinary bladder by Arruda, Dytko and Taylor (1990). In most experiments, the serosal side was first bathed with the HCO_3^-/CO_2-containing Ringer solution for 30 min and then with the air Ringer solution for a further 30 min. During these 60 min, the apical air Ringer solution contained fluorescein dextran (FD; MW:70,000, Molecular Probes, Eugene, OR) at a concentration of 2 mg/ml. The stimulation of H^+ secretion by the CO_2/HCO_3^- Ringer solution (Ehrenfeld & Garcia-Romeu, 1977) was expected to increase the incorporation of intracytoplasmic vesicles containing the H^+ pump in the apical membrane, while withdrawal of CO_2 was expected to increase endocytosis of the apical membranes, leading to incorporation of FD into the cells. As will be seen below, these assumptions proved to be correct. In further experiments, the entire FD-loading period (60 min) was performed with an air Ringer solution on both sides of the skin.

At the end of the loading period, the mucosal and serosal faces of the skin were washed eight times and twice, respectively, with an air Ringer solution to eliminate the FD from the extracellular fluid. A further 15 min period, with an air Ringer solution on both faces of the skin completed the washing out of extracellular FD. The mucosal air Ringer solution was then renewed every minute for 10 min and every 10 min for a further 50 min. The removed samples were then bubbled with air for a period of 15 min to eliminate volatile CO_2, before measuring the fluorescence in a Perkin-Elmer spectrofluorimeter (model MPF 44B), using an excitation wavelength of 470 and an emission of 523 nm with excitation slit width set at 6 nm and emission slit width at 8 nm. The increase in fluorescence (sum of the successive samples) of the external bath plotted as a function of time was taken as an indication of the exocytosis through the apical cell membranes. A sample of the serosal solution was also measured at the end of the 60 min period.

Since large variations in the rate of appearance of FD in the mucosal solution were found from one skin to another, we used two or three pieces of skin when comparing different experimental conditions with this semi-quantitative technique.

In preliminary experiments, FD was dialyzed in dialysis tubing (model 12-14, Spectrum Medical Industries, Inc. Los Angeles, CA) for 18 hr at 4°C. Comparative experiments indicated no difference in FD effluxes between dialyzed and nondialyzed products.

We examined which cells were loaded with FITC-dextran once endocytosed; isolated epithelia were loaded apically with FITC-dextran, rinsed in a similar protocol described for FD washout experiments and examined under a fluorescence microscope (Model Polyvar, Reichert, Austria) using a B1 filter (excitation filter with a pass band of 450–495 nm, a dichroic mirror with DS 510 nm and a cutoff filter of 520 nm.

Measurement of F-actin in the Isolated Frog Skin Epithelium

A large surface area of frog skin epithelium was isolated from the chorion by the collagenase hydrostatic technique (Aceves & Erlij, 1971). Small pieces (4 mm diameter) of this were then cut

with a cork puncher (S = 0.13 cm^2) and incubated in the different experimental conditions described in Results. Care was taken to incubate all pieces (control and experimental) for the same time, since a decrease in the amount of cell F-actin was observed with time (*unpublished results*). Duplicates were performed for each experimental condition.

Epithelia were fixed for 30 min with 3% formaldehyde in piperazine-N,N'-bis (2 ethanesulfonic acid, PIPES) buffer (K_2HPO_4 20 mM, KH_2PO_4 7 mM, $MgSO_4$ 2 mM, ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) pH 7.4) and pieces stained with rhodamine-phalloidin (0.4 μM; Molecular Probes) for 18 hr at 28°C in saponin (0.2%) buffer solution in darkness on a rotator. Rhodamine-phalloidin concentration and staining time were sufficient to saturate epithelial cell F-actin. The epithelia were then washed for two, 10-min periods with a 0.2% saponin-containing buffer solution. The rhodamine-phalloidin was extracted for 90 min in methanol (Sigma spectrophotometry grade) in Wheaton tubes and the fluorescence of the methanol extracts was then measured in a Perkin-Elmer spectrofluorimeter set at 540 nm excitation and 575 mn emission. Endogenous fluorescence was measured in unlabeled epithelia, for correcting the experimental values.

Measurement of Sodium Absorption and Proton Secretion

Net sodium and proton fluxes were calculated from the ion concentration variations of the apical solution (in mM): 1 Na_2SO_4, 4 imidazole, pH 7.4) measured by atomic absorption and titration (for details of the techniques used, *see* Ehrenfeld & Garcia-Romeu, 1977). The serosal solution was a HCO_3^-/CO_2-containing Ringer solution, pH 7.4. Net fluxes are expressed in neq/hr/cm^2.

Intracellular Calcium Determinations

Experiments were performed with isolated cells of the frog skin epithelium which had been dissociated by trypsin incubation (final concentration: 0.012%), in a culture medium gently stirred with a magnetic bar. Acetoxy-methylester of Fura-2 (Fura-2/AM; Molecular Probes) was used as a probe of cell calcium. Cells were loaded with 10 μM of Fura-2 at 28°C, for 90 min, in an amphibian cell culture medium, in a CO_2 incubator. The cells (3–5 · 10^6 cells) were then washed and resuspended in the experimental media. The fluorescence was measured with a spectrophotometer system (PTI Deltascan, NJ). Excitation light was made to flicker between 340 and 380 nm (4 nm band pass) at a rate of 100–200 Hz, and the emission was monitored at 505 nm (6 nm band pass). The fluorescence intensity ratio (I_{340}/I_{380}) was measured every second and plotted graphically. Cellular autofluorescence was measured in an identical aliquot of cells not exposed to Fura-2 and did not exceed 5% of the fluorescence from dye-loaded cells. To calculate the calcium concentration, the Tsien equation was used (Grynkiewicz, Poenie & Tsien, 1985): $(Ca^{2+}) = K_d \times B(R - R_{min})/(R_{max} - R)$, where B is the ratio of fluorescence at 380 nm in the absence of calcium and in the presence of saturating calcium concentrations; K_d is the dissociation constant and was assumed to be 224 nM (Grynkiewicz et al., 1985); R_{max} was determined by the addition of digitonin (20 μM) to the Ringer solution containing calcium (2 mM) and R_{min} was determined by subsequent addition of ethylene glycol-bis (β-amino-ethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) at a final concentration of 20 mM.

In some experiments, *p*-chloromercuri-phenylsulfonic acid (PCMPS) was used at a final concentration of 0.5 or 1 mM.

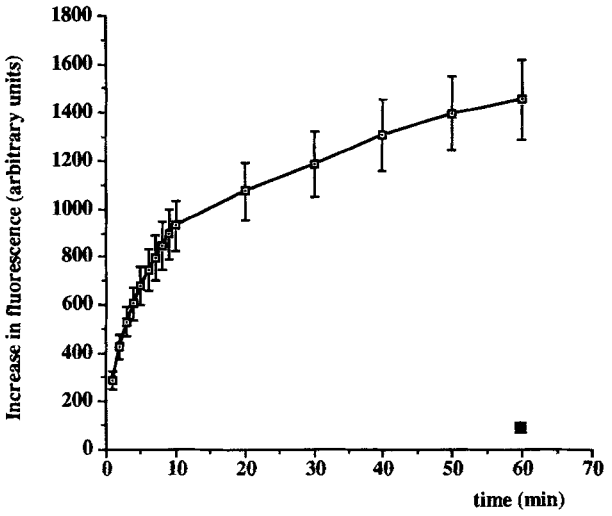

Fig. 1. Net increase in mucosal fluorescence (open squares) as a function of time in steady-state conditions. The net increase in serosal fluorescence at time 60 min is indicated by a filled square, (n = 97).

Results

Quantification of the Rate of Exocytosis by the Use of Fluorescein Dextran

The fluorescein dextran (FD) released into the apical solution from skins, loaded for one hour with FD, was followed as a function of time, both sides of the epithelium being bathed with an air Ringer solution. The accumulation of FD with time was a saturating function (Fig. 1); a rapid release of FD was observed during the first 10 min, followed by a slower outflow during the next 50 min of the experiment (after 10 min, the FD efflux was already two-thirds of that at 60 min). The quantity of FD appearance in the serosal solution at the end of the 60 min collecting period was 13 times less than that in the apical solution (serosal fluorescence in arbitrary units was 116 ± 15 as compared with the apical fluorescence of 1,461 ± 162, n = 97). A relatively high variability of the total FD released between the different skins and different batches of animals was recorded.

It was evident from this individual variability that paired experiments on the same skin were required for testing different experimental conditions.

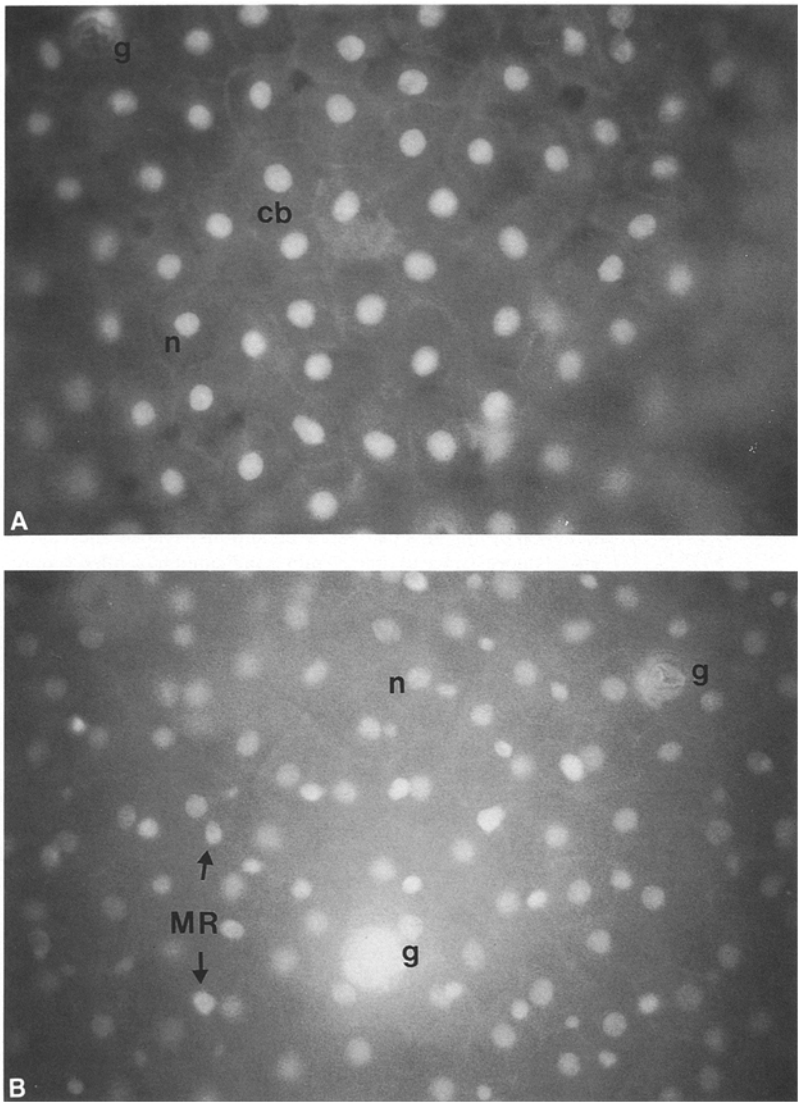

Fig. 2. Location of FITC-dextran (FD) in the isolated epithelium. (*A*) Photograph showing an isolated epithelium, not loaded with FITC dextran. The autofluorescence of the gland (*g*), the cell boundaries (*cb*) and the nuclei of the corneal cells (*n*) are clearly visible. (*B*) Photograph representing a FITC-dextran loaded epithelium. The difference between autofluorescence and the FITC-dextran fluorescence is clearly visible. FITC-dextran is located in the MR cells (MR), the neck of the glands (*g*), and disposed of in the body of the granular cells. The photograph is focused on the first-reactive cell layer and cell boundaries of corneal cells are at the limit of detection.

Location of the Dye in the Epithelium

The location of FD in the epithelium was examined using the same loading and washing procedures as those in the kinetic experiments. The FD was located in almost all cell types of the epithelium with the highest intensity of fluorescence in the mitochondria-rich cells (MR cells) and the gland cells bordering the gland duct (Fig. 2) and a lower intensity in the granular cells. Examination of the epithelium by confocal microscopy confirmed these observations and demonstrated the intracellular location of FD inside the cells. We observed that FD was also partly bound to the corneal cell layer despite our intensive washing procedure. We therefore considered that the presence of FD in the apical bathing solution (Fig. 1) was probably due to a combination of FD coming from constitutive exocytosis in the main cell types of the outermost living cell layer and of non-specific leakage.

Intracellular Acidosis Correlates with an Increased Rate of Exocytosis

Several procedures to acidify the epithelial cells have involved using CO_2 or NH_4 treatment of the epithelium (Harvey, Thomas & Ehrenfeld, 1988).

After FD loading (apical side), paired frog skins were bathed on their serosal side with an air Ringer solution or with a HCO_3-free Ringer solution (pH 6.2) gassed with a mixture of CO_2/O_2 (5%/95%, respectively) and the FD release into the apical solution was followed by the usual procedure. This treat-

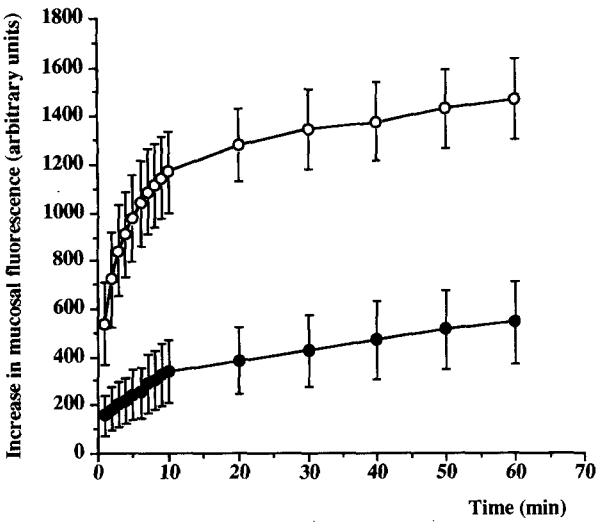

Fig. 3. Effect of an acid Ringer (pH 6.20, CO_2 5%) on the net increase in mucosal fluorescence in paired frog skins. Control skins, filled circles; acid-treated skins, open circles (n = 7).

ment was found to acidify the cells due to their high CO_2 permeability, presence of proton channels on the basolateral side of the membrane and reduced buffering power of the cells in the absence of HCO_3 in the medium (Harvey et al., 1988; Harvey, Lacoste & Ehrenfeld, 1991). As seen in Fig. 3, exocytosis of FD was rapidly stimulated by exposure of the serosal side of the frog skin to an acid Ringer (at 1 min, the FD released was 538 ± 170 arbitrary units of fluorescence as opposed to 156 ± 84 for control skins, n = 7). The stimulation induced by the acid load became maximal within the first 10 min and changed little in the following 50 min.

Proton secretion (JnH) was measured after such an acid load by titration analysis of the apical solution on a different experimental group of frog skins (measurements at 20 min intervals). Switching from an air Ringer solution to an acid Ringer was found to stimulate JnH rapidly (control period: −132 ± 15 neq/hr/cm^2 and acid period: −200 ± 29 neq/hr/cm^2, n = 6, $P < 0.01$).

Proton secretion is also stimulated with a constant pH (7.40) in the serosal solution, when the "air Ringer solution" is changed to a HCO_3/CO_2-containing Ringer solution (Ehrenfeld & Garcia-Romeu, 1977). This second protocol was used to follow the FD release into the apical solution of paired frog skins (Fig. 4). A transient acidification of the cell is expected since CO_2 is more permeable than bicarbonate ions. As with the first acidification procedure, a stimulation of the exocytosis of HCO_3/CO_2-treated skins was observed.

A third procedure for cell acidification was carried out and its effects on the FD release into the apical solution of FD-loaded epithelial cells followed. NH_4Cl (15 mM) addition to the serosal solution (air Ringer) was previously found to produce cell acidification, since NH_3 and NH_4 are both permeant through the basolateral membranes of the frog skin epithelium and their subsequent withdrawal results in a pronounced cell acidification (Harvey et al., 1988). A rapid stimulation of FD release (Fig. 5) was observed in the presence of NH_4 in the serosal Ringer solution. Return to an air Ringer solution, expected to induce a more acute cell acidification (arrow in Fig. 5), resulted in an additional stimulation of exocytosis.

Endo- and exocytosis processes have usually been found to show a high temperature sensitivity (Steinman, Silver & Cohn, 1974; Dixon, Clausen & Coachman, 1988). We tested the temperature dependence of the CO_2-induced increase of FD release into the apical solution (Fig. 6). At a temperature of 24°C, cell acidification increased the FD effuxes. At 7°C, the kinetics of FD release were completely different from those observed at 24°C; at the low temperature there was a considerably reduced FD release in the first minute, but although exocytosis was delayed and reduced in magnitude, it was not completely blocked over the subsequent period of measurements (between 10 to 60 min).

From these experiments. we can conclude that procedures which acidify the epithelial cells superimposed on a basic level of exocytosis ("constitutive exocytosis" plus nonspecific release), a stimulus-mediated exocytosis which parallels the stimulated proton secretion.

We investigated the effects of a cell alkalization on the rate of FD endocytosis. Paired skins were first incubated for 30 min in a CO_2/HCO_3 Ringer solution (serosal side) to stimulate exocytosis in the absence of the fluid phase marker. This period was followed by a FD loading period (30 min), the serosal side being bathed either with an air Ringer solution (one portion of the skin) or with a CO_2/HCO_3 Ringer solution (the other portion of the pair). Switching from a CO_2/HCO_3 Ringer to an air Ringer solution is known to alkalize the cells in view of the rapid CO_2 diffusion relative to HCO_3 permeability (Lacoste, Harvey & Ehrenfeld, 1991). After the usual Ringer washings of the apical and serosal sides of the skins, the FD release was followed in the apical solution with both portions of skin bathed on both sides with air Ringer solutions. As can be seen in Fig. 7, the FD release was less from skins maintained in CO_2/HCO_3 Ringer solution during the loading period (open circles), than that from those submitted to an alkalization by a transfer to air Ringer (filled circles). As the FD release is expected to be proportional to the amount of FD endocytosis during the loading

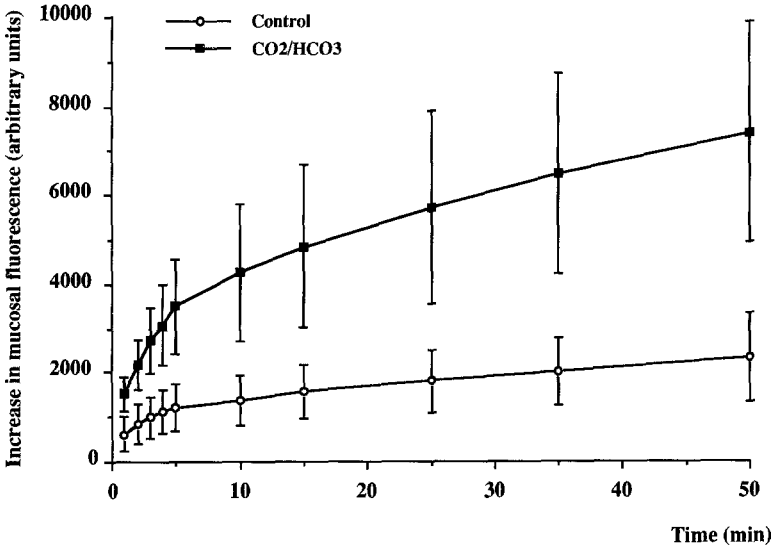

Fig. 4. Effect of a HCO_3^-/CO_2 containing Ringer solution (pH 7.40) on the net increase in mucosal fluorescence in paired frog skins. Control skins, open circles and experimental, filled squares (n = 4).

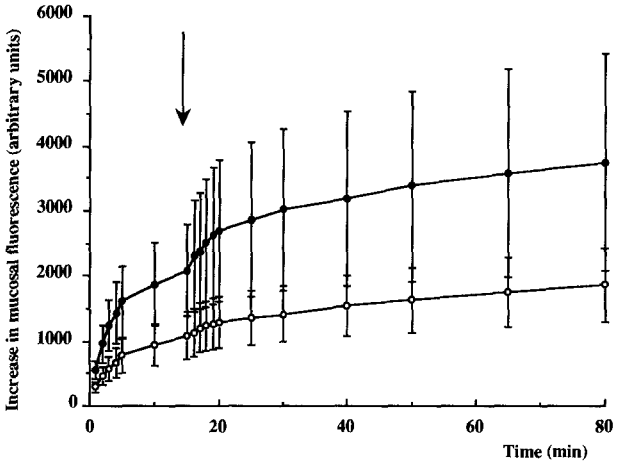

Fig. 5. Effect of an ammonium load (15 mM) on the net increase in mucosal fluorescence in paired frog skins. NH_4 (15 min) was added to the serosal solution of experimental skins at time zero. Arrow indicates NH_4 removal from the serosal bath (filled circles). Control skins, open circles (n = 5).

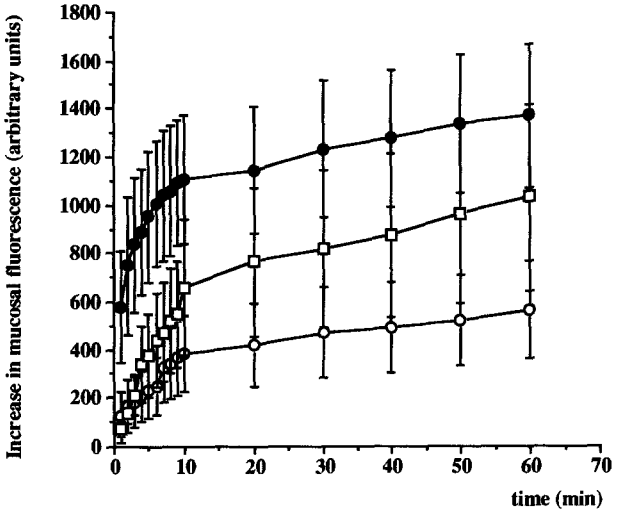

Fig. 6. Effect of low temperature on net increase in mucosal fluorescence in skins treated with 5% CO_2 (experiments at 24°C: filled circles, experiments at 7°C: open squares). Control skins (open circles) at 24°C, are bathed with an air Ringer solution, (n = 5).

period, this experiment suggests that, in that period, endocytosis was stimulated by a cell alkaline load.

Effects of Hypotonic Shock on Exocytosis

Hypotonic shocks have been found to stimulate Na^+ transport in the short-circuited frog skin bathed on both sides with a Ringer solution (Ussing, 1965). A similar result was found when the skin was mounted in open-circuit conditions and bathed on the apical side with a low Na^+-containing solution in which case, both Na^+ absorption and H^+ secretion were stimulated. This procedure was used to minimize individual differences between frog and to stimulate both ion transports (Garcia-Romeu & Ehrenfeld 1975). We investigated the effect of changing the osmolarity of the serosal solution on the FD release from FD-loaded epithelia (Fig. 8). The osmolarity was changed from an air Ringer solution (230 mOsm) to one diluted by half (115 mOsm) or by one-fifth (184 mOsm). In both series the hypotonic shocks caused an increase of FD release.

The effect of indomethacin, a blocker of prostaglandin synthesis, was investigated on the osmotically induced stimulation of FD release. We found that indomethacin at a concentration of 1 μM in one-third diluted Ringer completely blocked the osmoti-

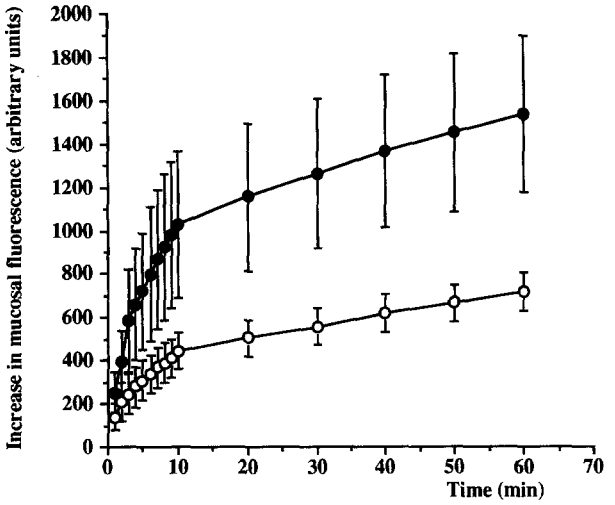

Fig. 7. Effect of loading conditions on the net increase in mucosal fluorescence. The skins were first incubated for 30 min with a HCO_3/CO_2 Ringer solution on the serosal side; then the loading period was carried out either without a change in the serosal solution (i.e., a HCO_3^-/CO_2 Ringer solution, open circles) or with an air Ringer solution (filled circles); $n = 5$. In both conditions, the FD release was with air Ringer on both sides of the skin.

cally induced stimulation of FD release in the apical solution (Fig. 9).

Effects of Amphotericin B and PCMPS on Exocytosis

Amphotericin is a polyene anti-fungal antibiotic which forms large pores in membranes. In the toad urinary bladder, this compound was found to cause an immediate rise in the short-circuit current and an increase in the passive permeability to chloride, potassium and water (Lichtenstein & Leaf, 1965).

The effect of apical treatment with amphotericin B (20 μg/ml) was tested on the FD release of skins bathed on both sides with an air Ringer solution. Figure 10*A* shows that the antibiotic induces a large increase in FD release with the maximum effect in the first 10 min. We have already shown (Fig. 8), that exocytosis is stimulated by serosal osmotic shocks. As amphotericin has been found to increase cationic, anionic and water permeabilities of membranes, the observed stimulation by the ionophore, may well be a consequence of cell swelling. We therefore repeated these experiments using an apical medium of a composition similar to that of the cell cytoplasm (high K^+, low Na^+, Ca^{2+} and Cl free); a cytoplasm-like solution has already been successfully used with ionophores to permeabilize apical cell membranes (Garty, 1984; Ehrenfeld, Lacoste & Harvey, 1992). Stimulation of the FD release was still observed with such a solution (Fig. 10*B*) in the

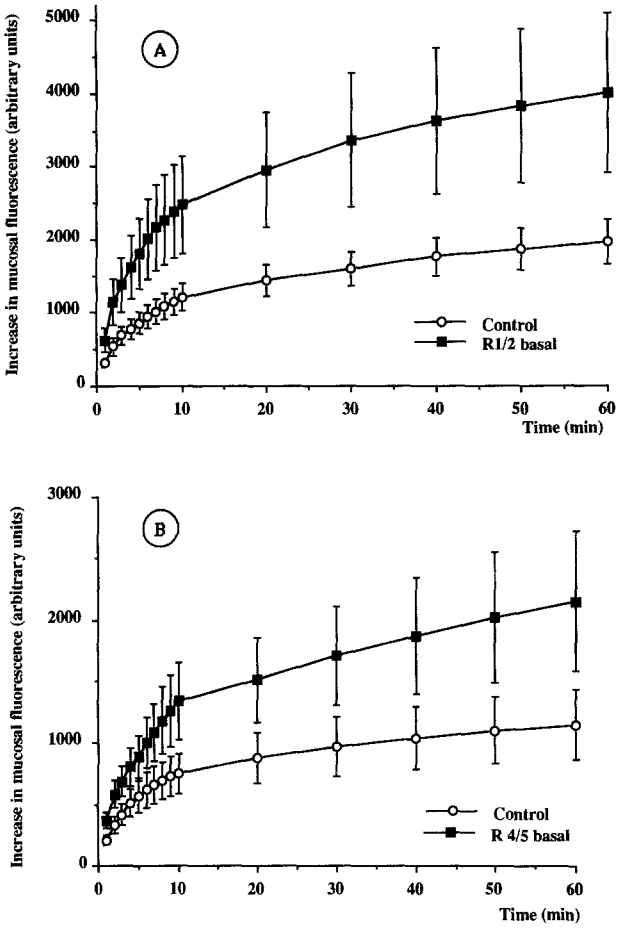

Fig. 8. Effect of serosal hypotonocity on the net increase in mucosal fluorescence. (*A*) Half-diluted Ringer solution ($n = 7$). (*B*) One-fifth diluted Ringer solution ($n = 4$). Control skins: open circles, experimental, filled squares.

presence of amphotericin (20 μg/ml), and was similar to that found with an air Ringer solution.

PCMPS has been reported to stimulate sodium transport in the short-circuited frog skin bathed on both sides with Ringer solutions (Dick & Lindeman, 1975); this agent is known to increase the number of conducting sodium channels and this is considered to be the consequence of releasing channels from a process of Na^+ self-inhibition (Li & Lindemann, 1983). PCMPS (1 mM) was tested on the rate of FD release into the apical solution (Fig. 11). A considerably augmented FD release was recorded (a threefold increase after 10 min of PCMPS treatment). It was therefore of interest to investigate the effects of PCMPS on the proton and sodium transports in conditions in which the transport of protons (secretion) and of sodium (absorption) are electrically linked, i.e., in open-circuit conditions with the apical side of the skin bathed with a low sodium-containing medium (1 mM Na_2SO_4). As previously

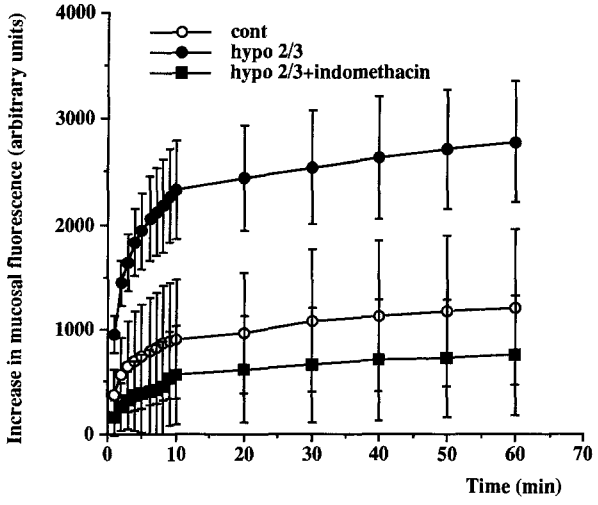

Fig. 9. Effect of indomethacin (1 μM) on the net increase in mucosal fluorescence of skins submitted to a hypotonic Ringer solution (dilution two-thirds of a normal Ringer). Control skins: open circles; hypotonically treated skins, filled circles and indomethacin plus hypotonically treated skins, filled squares, (n = 4).

found in this experimental condition (Fig. 12) in the absence of PCMPS, the magnitude of the proton transport was identical to that of the sodium transport but directed in the opposite direction; however, although experiments performed in short-circuit conditions with high sodium-containing solutions have been reported to have an effect on PCMPS (Dick & Lindemann, 1975; *unpublished results*), in our experiments PCMPS (1 mM) had no effect on the net transepithelial Na^+ and the H^+ transports; differences (not statistically significant) in JnNa and JnH were of 2 ± 18 neq/hr/cm^2 and 54 ± 58 neq/hr/cm^2, respectively. (n = 7).

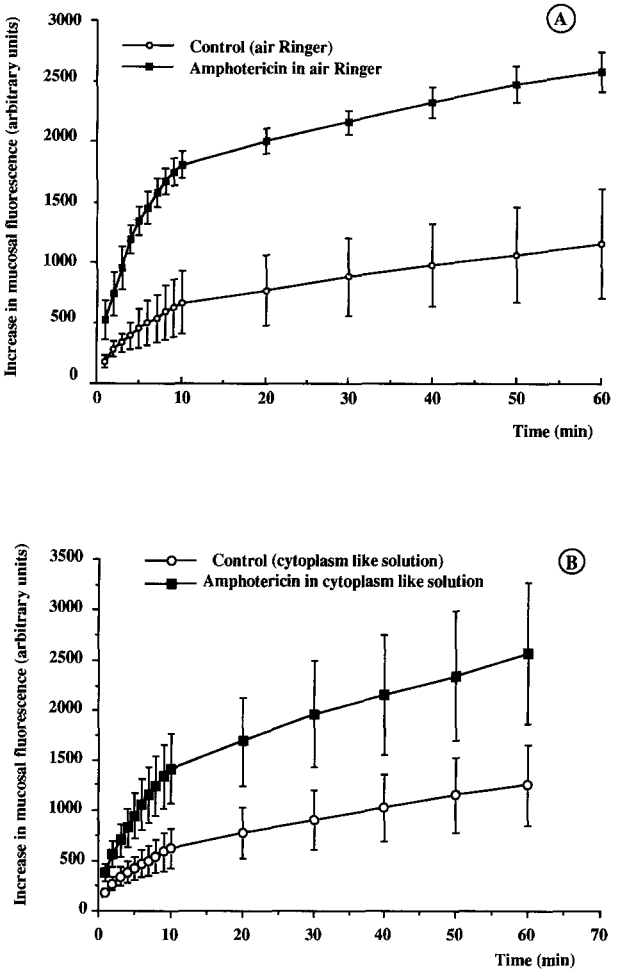

Fig. 10. Effect of amphotericin B (filled squares) on the net increase in mucosal fluorescence (*A*) Antibiotic in an air Ringer solution (n = 3). (*B*) Antibiotic in a "cytoplasm-like" solution, (n = 5).

F-Actin Content and the Effects of Exocytosis Stimulators

The cytoskeleton and in particular the state of polymerization and depolymerization of the F-actin filaments have been found to play an important role, for instance, in the hormone-induced water flow through urinary bladder (Ding et al., 1991) and in the discharge of catecholamine-containing granules in chromaffin cells (Burgoyne & Cheek, 1987). We therefore investigated the action of various stimuli found in this study to increase exocytosis, on the F-actin content of epithelial cells, measured with rhodamine-phalloidin.

The loading time and concentration of rhodamine-phalloidin necessary to label the isolated pieces of epithelia were first determined for selecting optimal experimental conditions. In Fig. 13 the F-actin content (as indicated by the rhodamine-phalloidin fluorescence) is plotted as a function of the concentration of the F-actin marker, with an incubation time of 3 hr, at 28°C. A saturating function links the rhodamine-phalloidin labeling and the concentration of the F-actin marker, the maximum being observed at concentrations between 1,000 and 2,000 nM (with an epithelial surface of 0.13 cm^2). To reduce the rhodamine-phalloidin concentration, we studied the relationship between incubation time and concentration of F-actin marker and found that 3 hr loading in the presence of 2,000 nM rhodamine-phalloidin was equivalent to 18 hr loading at a 400 nM concentration (222 ± 21 and 201 ± 23 arbitrary units of fluorescence for a 3 hr and an 18 hr loading period, respectively (n = 4). In addition, loading the epithelium with 400 or 2,000 nM rhodamine-phalloidin for a period of 18 hr revealed no difference

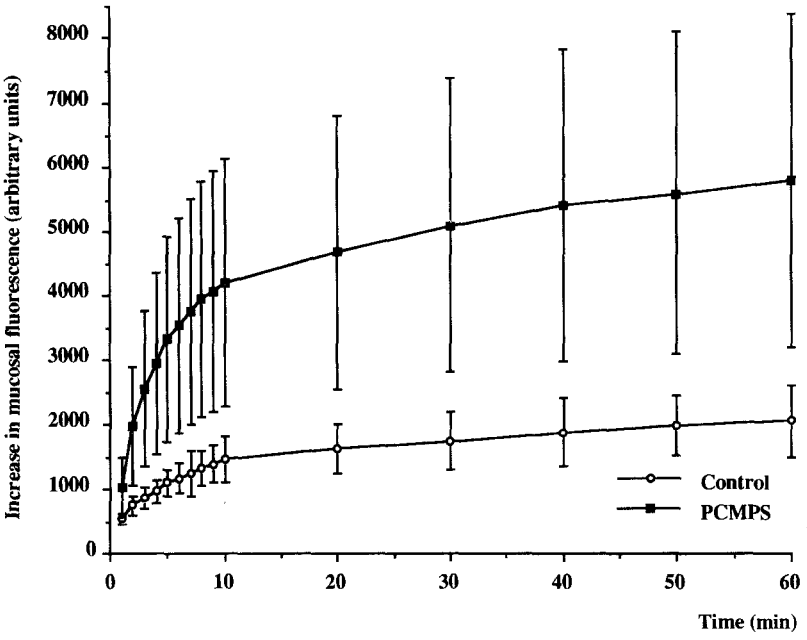

Fig. 11. Effect of PCMPS (1 mM) on the net increase in mucosal fluorescence. Control (open circles) and PCMPS-treated skins (filled squares), $n = 4$.

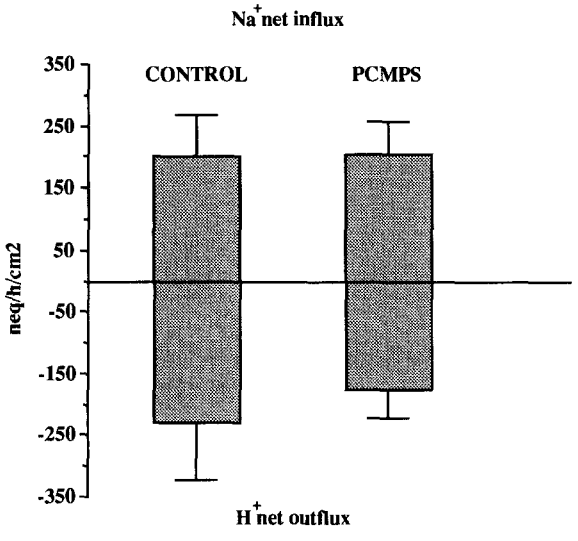

Fig. 12. Effect of PCMPS on Na^+ and H^+ transports in the frog skin in open-circuit conditions. Apical solution (mM): 1 Na_2SO_4, 4 imidazole, pH 7.4. Serosal solution: HCO_3^-/CO_2 Ringer solution pH. 7.4, ($n = 7$).

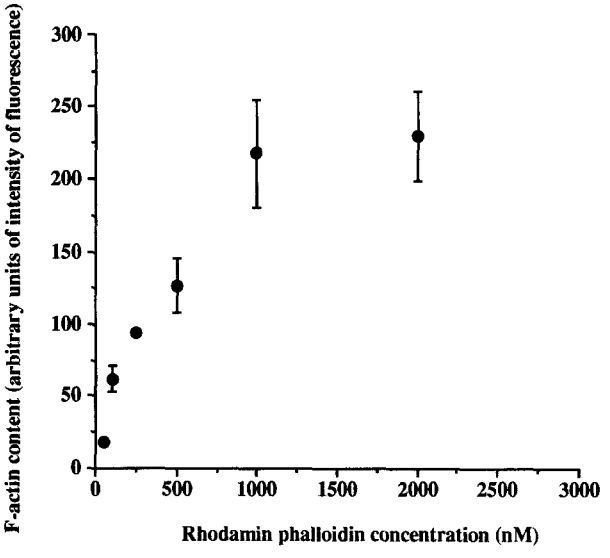

Fig. 13. F-actin content as a function of rhodamine-phalloidin concentration in the incubating medium with a loading time of 3 hr.

in fluorescence between the two batches (200 ± 20 and 195 ± 30 arbitrary units of fluorescence with 400 and 2,000 nM concentration, respectively). We therefore used an 18 hr loading period with rhodamine-phalloidin (400 nM) at 28°C to saturate the cell F-actin content in the following experiments. Addition of 40 μM of cold phalloidin reduced the fluorescence by 83% (17% of nonspecific binding).

In a first set of experiments, we tested the effect of cytochalasin D (5 μg/ml), a well-known disrupting agent (for review *see* Cooper, 1987) on the F-actin content of isolated epithelia (Fig. 14) after 2 hr drug treatment. Cytochalasin D reduced the F-actin content of epithelial cells by 26 ± 4% ($n = 8$, $P < 0.001$). Such a small inhibition by the well-known F-actin disrupter indicated that only small variations could be expected under the various experimental conditions tested.

As can be seen from Fig. 14, acidification by CO_2 or NH_4 treatment significantly reduced the F-actin

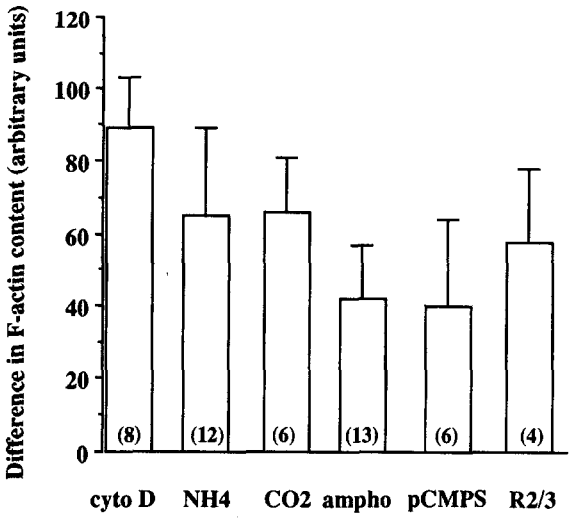

Fig. 14. Variations in the F-actin content of the frog skin epithelium under various experimental conditions. Cell acid load was produced either by incubating the epithelium with 15 mM NH_4Cl for 15 min followed by a 5 min reversal period or by incubation (15 min) in a CO_2 Ringer (pH 6.2). The incubation time for epithelia treated with amphotericin (20 μg/ml), PCMPS (1 mM) and two-thirds diluted Ringer solution (R2/3) was 15 min while that for cytochalasin D (5 μg/ml) was 120 min. *n* represents the number of epithelia used in each experimental condition.

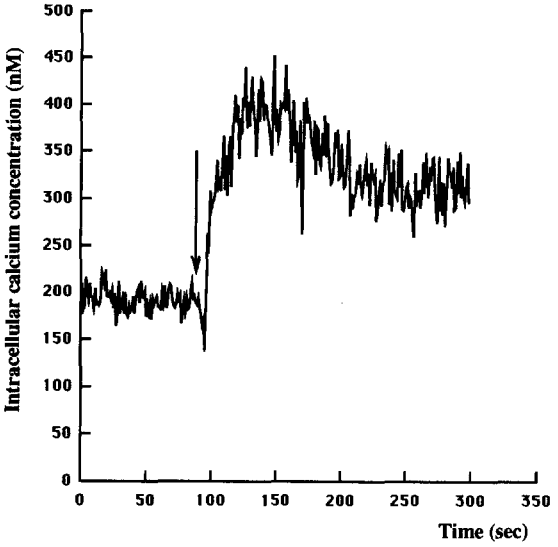

Fig. 15. Effect of hypotonic media (170 mOsm) indicated by the arrow, on $[Ca^{2+}]_i$ on isolated frog skin epithelial cells.

content of isolated epithelial cells (reductions of 19 ± 7% $P < 0.025$, $n = 12$ and of 19 ± 4% $P < 0.01$, $n = 6$ for NH_4 and CO_2 experiments, respectively). Hypotonic shock or amphotericin addition also reduced the F-actin content (reduction of 12 ± 4% $P < 0.05$, $n = 4$ and 14 ± 5% $P < 0.025$, $n = 13$, respectively), while no significant changes were observed with PCMPS (difference 11 ± 6 NS, $n = 6$).

Intracellular Calcium Measurements

Calcium has been found to be a signal for exocytosis in most of the cellular models so far studied for exocytosis and endocytosis phenomena (for recent review *see* Lindau & Gomperts, 1991). Using Fura-2 as a probe of intracellular calcium, we measured and followed the evolution of intracellular calcium of epithelial isolated cells changed from a Ringer solution (230 mOsm) to a hyposmotic one (170 mOsm).

The basic level of intracellular calcium in control experiments was 161 ± 10 nM, $n = 24$. When the cells were submitted to a hypotonic shock the cell calcium was considerably increased. An immediate Ca_i rise from 187 ± 20 to 320 ± 50 nM ($n = 10$, difference = 154 ± 19 nM, $P < 0.001$) was observed within the first 30 sec; it was followed by a slower regulatory phase (90 to 120 sec) to reach a Ca_i plateau at 302 ± 30 nM (difference between control and plateau was 116 ± 15 nM, $P < 0.001$). An illustration of a typical experiment showing the calcium changes produced by hypotonic shock is given in Fig. 15.

Acidification of isolated epithelial cells by HCl addition to the medium (final pH 5.5) was also found to produce an immediate but transient cell calcium increase (mean intracellular calcium in control: 169 ± 21 and 298 ± 28 nM, at the peak value of acid-treated cells (difference 129 ± 15, $P < 0.001$ $n = 8$). A typical experiment is given in Fig. 16*A*.

A progressive calcium rise was observed after PCMPS (0.5 mM) addition. However, the cell calcium increase was considerably smaller than after an osmotic shock or acid load. The mean difference in Ca_i between control and 10 min after PCMPS addition was 82 ± 11 nM, $P < 0.001$ ($n = 6$). Figure 16*B* represents a typical experiment.

Discussion

The insertion of vesicle-containing protein units by means of exocytosis/endocytosis phenomena has been found to play a role in the regulation of water and ion transport in a variety of epithelia (Masur, Hotzman & Waller, 1972; Wade et al., 1981). In this study, we found that various stimuli known to affect the sodium and proton transports can also modulate the rate of exocytosis at the apical membranes of the frog skin epithelium while at the same time, the state of polymerization of the F-actin filaments changes and Ca_i increases.

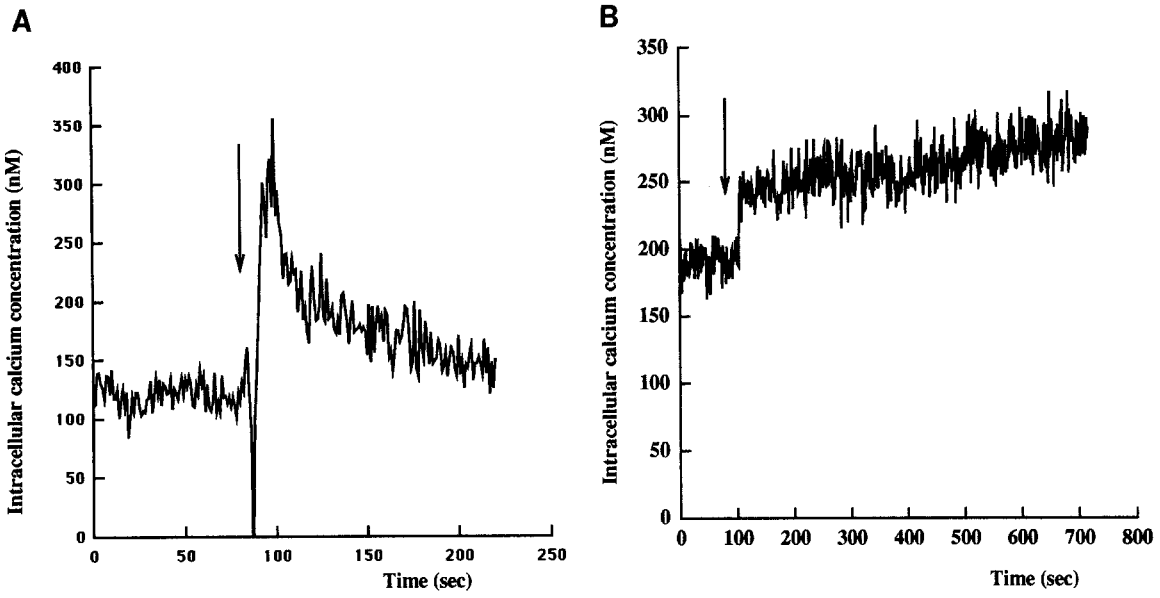

Fig. 16. Effect of an acid medium (*A*) and of PCMPS (*B*) on $[Ca^{2+}]_i$ in isolated epithelial cells.

Reliability of the technique of Exocytose Measurement

To quantify the rate of exocytosis, we chose a technique similar to that used on the turtle urinary bladder (Gluck et al., 1982; Arruda et al., 1990) with FITC-Dextran as a fluid phase marker. Turtle urinary bladder and frog skin have functional similarities (transport of Na^+ and H^+) and morphological ones (presence of principal cells responsible for sodium transport and mitochondria-rich cells involved mainly in proton secretion). However, the frog skin epithelium, with the presence of a cornified layer, several cell layers beneath the outermost living cell layer and glands, is a more complex structure making the validity of the technique questionable. Microscopic observation showed that FD loading from the apical side limited dye uptake to the outermost living cell layer (and cornea) with location of FD in the cellular body of MR cells, the granular cells and the exit duct of the glands. The dye could not penetrate the cellular junctions and its appearance in the serosal compartment was minimal. With our experimental approach it was not possible to determine the cellular origin of the FD appearing in the apical Ringer solution. We used intensive washings of the apical surface before measuring apical exocytosis to limit nonspecific FD-dextran release. Nevertheless, one must assume that the FD kinetics observed in steady-state conditions are a combination of constitutive exocytosis and nonspecific loading. However, the increased FD release into the apical Ringer solution in response to the various stimuli tested was attributed to higher rates of exocytosis for the following reasons: (i) all experiments were comparative on paired skins, (ii) acidification of the epithelial cells and hyposmotic shocks were carried out on the serosal side while the FD release was measured in the apical solution and therefore cannot be attributed to a direct contact effect, (iii) indomethacin (serosal addition) blocked the stimulated FD release of skins subjected to hyposmotic shocks. Thus, the method chosen for this investigation can be regarded as a semi-quantitative technique, revealing the exocytosis rate of the apical membranes of the first-reacting cell layer.

Exocytosis and Ion Transport

The increased rate of exocytosis due to cell acidosis is associated with an increased proton secretion (Ehrenfeld & Garcia-Romeu, 1977; present study). Intracellular vesicles located beneath the apical membranes of H^+-secreting cells (MR cells or intercatated cells) have been observed in a number of tight epithelia including the frog skin epithelium (Brown, 1989). It has been demonstrated, both in the mammalian collecting duct and the turtle urinary bladder, that when proton secretion is stimulated, a number of specialized vesicles containing H pumps move to and fuse at the apical membrane domain (Gluck et al., 1982; Stetson & Steinmetz, 1983; Schwartz & Al-Awqati, 1985; Madsen & Tisher 1986; Brown 1989). Previous studies have demonstrated that the MR cells represent approximately

50% of the first living cell layer of the frog skin epithelium and that their apical membrane infoldings may vary with the state of acidosis of the animals (Ehrenfeld et al., 1976; Ehrenfeld et al., 1989; Lacoste et al., 1992). Since the outermost living cell layer was the only layer to be loaded with the fluid phase marker (FD) and the MR cells were largely loaded with the fluorescent probe, it is likely that the increase in apical FD release was the result of vesicle (and therefore H-pump insertion) in the apical membranes of the MR cells resulting in increased H^+ transport.

Hypotonic shocks were also found to stimulate apical exocytosis as reflected by FD release into the apical bathing solution (this study). Changes in osmolarity have been found to affect the rate of exocytosis in a number of cell models: hyperosmotic media inhibited exocytosis in parathyroid cells (Brown et al., 1978), sea urchin eggs (Zimmerberg, 1983), chromaffin cells (Hampton & Holz, 1983), pheochomocytoma cells (Englert & Perlman, 1981), while the vesicle swelling induced by hyposmotic conditions was considered to be responsible for facilitating fusion of these vesicles with the plasma membrane (Finkelstein, Zimmerberg & Cohen, 1986). Recently, Okada et al. (1992) found that upon osmotic swelling epithelial cells exhibited significant increase in the membrane capacitance, suggesting a role of osmotic exocytosis in cell volume regulation.

In the open-circuited frog skin bathed on its apical side with a dilute saline solution, the hypotonic medium also stimulates, the transepithelial sodium and proton transports (Ehrenfeld & Garcia-Romeu, 1975). Under these conditions, the two ion transports are electrically linked and the proton secretion rate determines Na^+ entry (Ehrenfeld & Garcia-Romeu 1977; Ehrenfeld et al., 1985). Stimulation of Na^+ transport by hyposmotic conditions may therefore be indirectly related to the exocytosis of MR cell vesicles containing H-pumps. Since hyposmotic shocks were also found to produce an increase of cell calcium (Fig. 15), this signal could have triggered the exocytotic events leading to insertion of H-containing vesicles in the apical membranes of the MR cells and the resulting increase in FD release rate. However, stimulation of Na^+ transport is also observed in conditions in which H^+ secretion and Na^+ absorption are fully independent, i.e., with Ussing's technique (Ringer solution on both sides of the short-circuited frog skin). In these last conditions, the stimulation of Na^+ transport cannot be attributed to an increase in the H^+ secretion rate.

In addition, PCMPS or amphotericin application on the apical side of the frog skin considerably increased the rate of exocytosis. These agents are known to cause a greatly increased sodium transport in the short-circuited frog skin or urinary bladder bathed on both sides with Ringer solution (Lichtenstein & Leaf, 1965; Dick & Lindemann, 1975). However, in the present study, we found that PCMPS did not increase sodium absorption and proton secretion when the two ions were electrically linked (open-circuited skin bathed with a low sodium containing solution), while we confirmed (*unpublished results*) that PCMPS increased the SCC when both ions were independent (high apical sodium containing solution in SCC conditions). In the former case, the lack of a PCMPS effect can be explained by the high and strict dependence of the sodium transport on the proton pump activity partly providing the electrochemical gradient and the counter ion for sodium and thus determining Na^+ entry through the apical cell barrier (Ehrenfeld & Garcia-Romeu, 1977; Ehrenfeld et al., 1985). However, the higher rate of exocytosis induced by PCMPS cannot be attributed to the incorporation of vesicles containing H pumps in the MR cell membrane since in this case, Na^+ and H^+ transports would also have increased.

The possibility that exocytosis occurs in a cell type other than the MR cells, i.e., in the granular cells, has therefore to be considered. The granular cells (or principal cells) are the main route of both sodium and water transports after ADH stimulation of frog skin, turtle urinary bladder and kidney collecting duct (Carasso et al., 1966; Rick et al., 1980; Neil & Hayhurst, 1985; Durham & Nagel, 1986). In the kidney collecting duct and in the amphibian urinary bladder, ADH is thought to induce the cycling of cytoplasmic vesicles containing water channels into the apical membranes of the principal cells (for review *see* Brown, 1989). Exocytosis of vesicles present in the granular cells of the frog skin epithelium would explain the effects of hyposmotic shocks, PCMPS and amphotericin in the present study. These agents could have caused insertion of vesicles containing the Na^+ channels or regulatory subunits in the apical membrane of the granular cells, permitting a rapid stimulation of the sodium transport capacity of the frog skin epithelium. Another hormone, carbachol, was also found to increase the FD release without effects on proton secretion (*unpublished results*). It is interesting to note that acetylcholine (like carbachol) induces large variations of the short-circuit current (Na^+ transport) in the *Rana pipiens* skin, that indomethacin reduced the response to carbachol and that calcium was found to be an important determinant of this type of response (Cuthbert & Wilson, 1981). The inhibitory effect of indomethacin, found in that study (Fig. 9), on the increase in FD release could indicate that prostaglandin synthesis is implicated in the

process of exocytosis following a hyposmotic shock.

Calcium and Exocytosis in the Frog Skin Epithelium

We found a rapid (during the first minute) Ca_i increase of the frog skin epithelial cells in response to a hyposmotic shock, induced cell acid load and PCMPS application. This increased Ca_i could have been the result of calcium release from intracellular stores or of changes in cell membrane permeability to calcium. A similar Ca_i increase had already been observed in renal segments (McCarty & O'Neil, 1990; Montrose-Rafizadeh & Guggino, 1991) and in renal cell lines (Rothstein & Mack, 1990; Wong, Debell & Chase, 1990) under hyposmotic shock and in the turtle urinary bladder after a cell acid load induced by CO_2 (Cannon et al., 1985). Ca^{2+} has been found to be an intracellular signal involved in exocytosis/endocytosis phenomena in a number of cells (Llinas & Heuser, 1977; Blumenthal, 1987; Burgoyne, 1991; Lindau & Gomperts, 1991) and epithelia (Cannon et al., 1985; Arruda, Taylor & Dytko, 1988). It is probable that the calcium augmentation observed in the frog skin epithelium is associated with the increased rate of exocytosis observed after application of the various stimuli tested.

F-Actin and Exocytosis

In a number of cells actin polymerizes and depolymerizes in response to a variety of hormones, chemotactic agents and various agents (Cheek & Burgoyne, 1987; Ding et al., 1991). All epithelia possess a complex cytoskeleton including an F-actin network usually distributed in parallel filaments in the core of the microvilli, in a perijunctional belt and in a meshwork along the basolateral membranes (Pearl & Taylor, 1983; Grillone, Condeelis & Gennaro, 1984; Sasaki et al., 1984; Mooseker, 1985; Madara & Trier, 1987). The cytoskeleton is involved in many cellular functions such as phagocytosis, regulation of cell shape, locomotion and hormone action. In addition to these roles, the cytoskeleton and in particular F-actin filaments have recently been shown to be involved in transport functions in ion- and water-transporting epithelia. For instance, it is involved in the insertion by exocytosis of K-H pumps in the gastric cell membranes (Mercier et al., 1989), and in the exocytosis of vesicles containing water channels upon ADH stimulation (Pearl & Taylor, 1983; Parisi et al., 1985; Ding et al., 1991). Stabilization of F-actin was found to prevent cAMP-elicited Cl^- secretion in the T84 epithelial cell line (Shapiro et al., 1991). Furthermore, in a renal cell line (A6 cells), the actin-disrupter cytochalasin D and short actin filaments in the form of actin-gelsolin complexes were found to stimulate Na^+ channel activity demonstrating a role of actin filaments in this activity (Cantiello et al., 1991). We used the rhodamine-phalloidin binding technique to quantify the F-actin in our epithelial cells. This technique has been used with success in a variety of cells and epithelia (Shetterline, Rickard & Richards, 1984; Hall, Schlein & Condeelis, 1988; Ding et al., 1991). We found that acid load (after CO_2 and NH_4 treatment), amphotericin and osmotic shocks reduced the cell F-actin content (Fig. 14). The variations in F-actin concentration were small in all conditions tested even with the well-known F-actin disrupter, cytochalasin D, which also produced moderate effects. It has to be noted that a high concentration of cytochalasin D (5 μM) may induce polymerization along with depolymerization (Goddette et al., 1986) explaining the relatively low depolymerization found in that study. In a variety of cells including red blood cells and shark rectal gland it has been reported that hyposmotic conditions and PCMPS affected the F-actin network (Ralston & Crisp, 1981; Iida & Yahara, 1986; Cornet, Delpire & Gilles, 1987; Clark, O'Brien & Ralston, 1988; Mills et al., 1989). In the latter, PCMPS produced a massive cell swelling and cell swelling per se was also associated with a loss of the F-actin network (Kleinzeller & Ziyadeh, 1990). No effects on F-actin content were observed in this study with PCMPS, while a stimulation of exocytosis was evident. However, considering the complexity of the frog skin epithelium, we cannot exclude local changes in the outermost living cell layer after PCMPS addition. Recently, Hays et al. (1991) reported that AVP, forskolin and 8-bromoadenosine 3′5′-cyclic monophosphate reduced the total F-actin of the toad urinary bladder by 15% and suggested that depolymerization of the "actin barrier" is required for vesicle fusion leading in water transport increase. Our results are consistent with this view that F-actin participates in the control of ion and water transports.

We propose that exocytosis/endocytosis of vesicles containing H^+ pumps located in the MR cells would be an efficient and rapid regulatory control mechanism of proton transports through the apical membranes of the frog skin epithelium. The depolymerization of F-actin filaments observed with increased rates of exocytosis could facilitate vesicle fusion in the membranes. This view could be extended (Fig. 17) as a working hypothesis, to vesicles containing Na^+ channels (or regulatory subunits), whereas no direct evidence of Na^+ channel-contain-

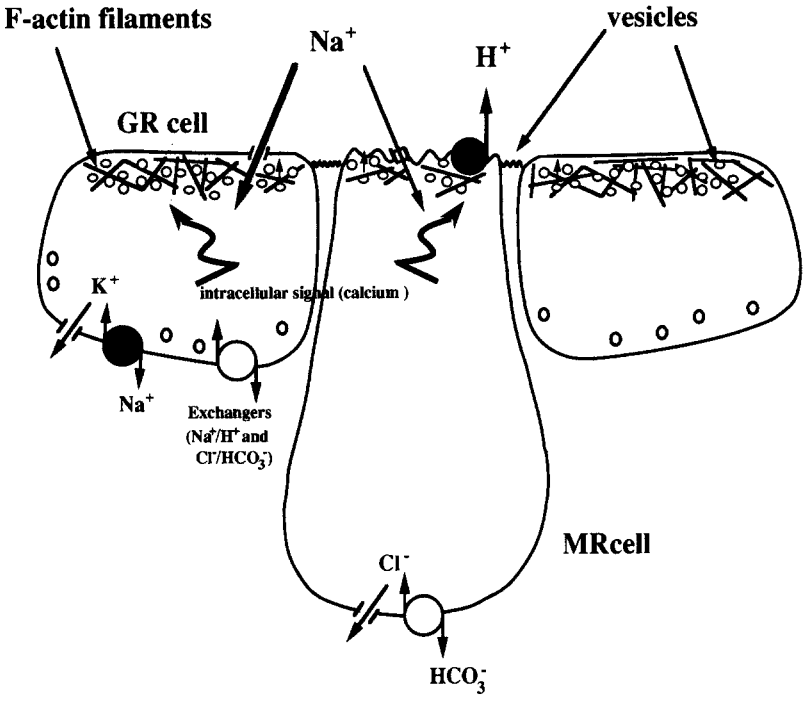

Fig. 17. Model of control of H^+ and Na^+ transports by exocytosis/endocytosis events. The two main cell types, granular cells (*GR*) and mitochondria-rich cells (*MR*) are represented. Vesicles close to apical membranes are held to contain transporters (channels, pump) or regulatory transporter proteins. In case of rapid stimulation of ion transport, these vesicles are inserted into the membranes. The F-actin filaments depolymerize and play a facilitating role in vesicle insertion.

ing vesicles has yet been demonstrated. Therefore, a study involving impedance analysis, as a measure of membrane surface area and simultaneous membrane conductance measurements, could be made to provide further evidence to support the present model.

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