Protein Kinase A Dependent Membrane Protein Phosphorylation and Chloride Conductance in Endosomal Vesicles from Kidney Cortex†

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ABSTRACT: Regulation of Cl conductance by protein kinase A may play a role in control of endosomal acidification [Bae, H.-R., & Verkman, A. S. (1990) *Nature*, *348*, 637–639]. To investigate the mechanism of kinase A action, cell-free measurements of Cl transport and membrane protein phosphorylation were carried out in apical endocytic vesicles from rabbit kidney proximal tubule. Cl transport was measured by a stopped-flow quenching assay in endosomes labeled in vivo with the fluorescent Cl indicator 6-methoxy-*N*-(3-sulfopropyl)quinolinium. Phosphorylation was studied in a purified endosomal preparation by SDS–PAGE and autoradiography of membrane proteins labeled by [γ-^{32}P]ATP. Endosomes had a permeability (P_{Cl}) for conductive Cl transport of 3.1×10^{-8} cm/s at 23 °C which was stilbene inhibitable. P_{Cl} was increased by 90 ± 20% by a 10-min preincubation with the catalytic subunit of kinase A (PKA, 10 units/mL) and MgATP (0.5 mM) with anion selectivity Cl > I > Br. The increase in P_{Cl} was blocked by 100 μM *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide (H-8) and was reversed by addition of alkaline phosphatase (AP, 40 units/mL) after incubation with PKA and MgATP; the increase in P_{Cl} was not blocked by pretreatment with AP. Incubation of endosomes with 1 μM [γ-^{32}P]ATP gave phosphoproteins at 35 and 48 kDa in the absence of PKA and an additional prominent phosphoprotein at 67 kDa in the presence of PKA; the 67-kDa band was nearly absent when excess unlabeled ATP or H-8 was present at the time of PKA addition or when AP was added after incubation; PKA-dependent phosphorylation of the 67-kDa band was enhanced (2.3 ± 0.2)-fold by pretreatment with AP. ^{32}P incorporation in excised bands showed about one 67-kDa phosphoprotein per endosome. These results suggest that, in a cell-free system, protein kinase A increases Cl conductance in endosomes from kidney proximal tubule by a phosphorylation mechanism. The labeled protein has a size similar to that of the 64-kDa putative kidney Cl channel reported by Landry et al. [(1990) *Science 244*, 1469–1472] but is much smaller than the ~170-kDa cystic fibrosis transmembrane conductance regulatory protein.

In epithelial cells, chloride channels exist in both plasma membranes and intracellular vesicles. Plasma membrane Cl channels are important for salt secretion and absorption in the airway, sweat duct, exocrine pancreas, intestine, cornea, and other tissues (Liedtke, 1989). Regulation of plasma membrane Cl channels in epithelia occurs primarily by a cAMP-dependent mechanism; however, in many cell types there is significant control by other messengers including calcium, protein kinase C, membrane-associated G-proteins, and cGMP (Liedtke, 1989). Abnormal regulation of apical membrane Cl channels by protein kinase A in some epithelia is a central defect in cystic fibrosis (Schoumacher et al., 1987). Recent complementation experiments suggested that CFTR (cystic fibrosis transmembrane conductance regulator), the cystic fibrosis gene product with predicted M_r 168 000, may itself be the cAMP-activated epithelial Cl channel (Drumm et al., 1990; Kartner et al., 1991; Anderson et al., 1991).

There is evidence that Cl channels are present on some intracellular membranes including endocytic vesicles, secretory vesicles, and trans-Golgi. The role of these Cl channels may be to permit intravesicular acidification by providing a Cl shunt to maintain electroneutrality for inward, ATP-dependent proton pumping (Mellman et al., 1986; Barasch et al., 1988). We reported that protein kinase A activated a Cl conductance in endosomal vesicles from proximal tubule by an assumed phosphorylation mechanism (Bae & Verkman, 1990). In those cell-free studies, ATP-dependent endosomal acidification was modulated by the magnitude of the Cl conductance. Recently, similar results were reported for regulation of the Cl channel in clathrin-coated vesicles (Mulberg et al., 1991). The relationship between the plasma membrane and intracellular Cl channels that are regulated by protein kinase A is unclear. It has been proposed that abnormal regulation of intracellular Cl channels is a central defect in cystic fibrosis which could, in principle, explain a number of diverse cellular abnormalities including decreased protein sialylation and increased protein sulfation and fucosylation (Barasch et al., 1991).

The molecular identity of intracellular Cl channels is unknown. Landry et al. (1990) used affinity chromatography to isolate a 64-kDa Cl channel from homogenates of kidney cortex and tracheal epithelium. This channel could be a plasma membrane or an intracellular Cl channel; in kidney cortex, the absence of plasma membrane Cl channels in proximal tubule cells (Berry & Rector, 1991) favors the possibility that the 64-kDa protein is an intracellular Cl channel. Xie et al. (1989) reported the partial purification and reconstitution of a stilbene-sensitive Cl transporter from clathrin-coated vesicles; recently Stone (1991) reported pre-

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liminary evidence that the Cl channel was a single polypeptide with M_r ~66 000. At this time the intracellular distribution of the CFTR protein is uncertain.

The purpose of this study was to evaluate the pattern of protein phosphorylation in endocytic vesicles from kidney proximal tubule and to compare protein phosphorylation with Cl conductance. It was found that the endosomal Cl conductance was stilbene-sensitive and was activated by protein kinase A dependent phosphorylation and decreased by phosphatase action. Under phosphorylating conditions which gave increased Cl conductance, there was a major phosphoprotein band at 67 kDa which was phosphorylated by PKA and whose appearance correlated with transmembrane Cl conductance.

METHODS

Vesicle Preparations. For anion transport measurements, endosomes were labeled with SPQ in vivo (Bae & Verkman, 1990). New Zealand White rabbits were infused intravenously with 200 mg of SPQ in 10 mL of phosphate-buffered saline (PBS) and killed 10 min later. The renal arteries were perfused with 50 mL of cold PBS, and the superficial renal cortex (0.5–1-mm depth) was dissected. Under these conditions it was shown that >95% of labeled endosomes originated from the apical membrane of proximal tubule cells (Lencer et al., 1990a). Renal cortex was homogenized in isethionate buffer (100 mM potassium isethionate, 100 mM mannitol, 1 mM magnesium acetate, 5 mM potassium phosphate, pH 7.4) by 10 strokes of the Potter–Elvehjem homogenizer. Large debris and mitochondria were removed by a low-speed centrifugation at 1000*g* for 10 min. A microsomal pellet containing SPQ-labeled endosomes was obtained by centrifugation of the supernatant at 100000*g* for 30 min. The microsomal pellet was washed once with isethionate buffer. The pellet was homogenized by 5–10 passes through a 25-gauge steel needle and used within 2 h. The microsome yield was 0.25–0.5 mg of protein per kidney. In some experiments, microsomes (1 mg of protein/mL) were incubated with the purified catalytic unit of protein kinase A (PKA), ATP, *N*-[2-(methylamino)-ethyl]-5-isoquinolinesulfonamide (H-8), and/or DNDS at 22 °C prior to stopped-flow experiments. In some experiments, microsomes were incubated with alkaline phosphatase (AP) and washed once prior to incubation with PKA and ATP.

For phosphorylation studies, purified endocytic vesicles from rabbit renal cortex were isolated by differential and Percoll density gradient centrifugation, a method described originally for isolation of rat renal cortical endosomes (Sabolic & Burckhardt, 1990). Kidney cortex from New Zealand White rabbits was homogenized in 35 mL of mannitol buffer (100 mM mannitol, 12 mM HEPES/Tris, pH 7.4) with 20 strokes in a loose-fitting, motor-driven glass/Teflon Potter–Elvehjem homogenizer (1000 rpm). The homogenate was diluted with 35 mL of mannitol buffer and centrifuged at 2500*g* for 15 min. The pellet was discarded and the supernatant was centrifuged at 20000*g* for 20 min. The resulting clear supernatant and the fluffy pellet were carefully separated, combined, and further centrifuged at 48000*g* for 30 min. The pellet was dispersed in mannitol buffer by 10 strokes with the Potter–Elvehjem homogenizer. A total of 6.1 g of concentrated Percoll was added to 32 g of vesicle suspension (16% w/w Percoll), and the mixture was centrifuged at 48000*g* for 30 min. The bottom 5 mL of the self-formed density gradient with the highest activity of the *N*-ethylmaleimide- (NEM-) sensitive H^+ pump was harvested, diluted with 30 mL of KCl buffer (300 mM mannitol, 100 mM KCl, 5 mM $MgSO_4$, 5 mM HEPES/Tris, pH 7.4), and centrifuged at 48000*g* for 30 min. The fluffy pellet was transferred to a 1.5-mL tube, diluted with KCl buffer, and centrifuged at 2500*g* for 15 min. The pellet contained membrane vesicles highly enriched in NEM-sensitive H^+ pump and strongly deenriched of marker enzyme activities for various (intra)cellular membranes (Sabolic et al., 1985; Sabolic & Burckhardt, 1990). For some studies, apical (brush border) membrane vesicles were isolated by the method of Biber et al. (1981). Enzyme enrichment factors for apical vesicles compared to cortical homogenate were 22 ± 2 (leucine arylamidase) and 1.5 ± 0.3 (ouabain-sensitive Na/K-ATPase).

Enzyme Assays and Electron Microscopy. Leucine arylamidase (LAP) (EC 3.4.11.2) was measured colorimetrically at ambient temperature using L-leucine-4-nitroanilide as a substrate as described in the commercial kit (Merck, Germany, Kit No. 5364). Ouabain-sensitive Na/K-ATPase activity (EC 3.6.1.3) was measured spectrophotometrically at ambient temperature using an ATP-regenerating system (Penefsky & Bruist, 1984).

H^+-pump activity in renal homogenate and isolated vesicles was measured by ATP-driven, Cl-stimulated quenching of acridine orange fluorescence (Sabolic et al., 1985). An aliquot of homogenate or vesicle suspension was added to 2 mL of KCl buffer containing 6 μM acridine orange and 5 μM valinomycin. Intravesicular H^+ uptake was initiated by adding 1.5 mM (final concentration) ATP. The fluorescence of acridine orange was monitored continuously at 37 °C in an SLM-Aminco 8000 fluorometer (Urbana, IL) interfaced to an IBM/PC computer (excitation wavelength 485 nm; emission wavelength 515 nm).

Electron microscopy was performed on ultrathin frozen sections of vesicle preparations that were stained with uranyl acetate according to the method of Tokuyasu (1980). Vesicles were fixed in 0.5% glutaraldehyde, washed with PBS, and embedded in 3% agar. The small solid agar blocks were infiltrated with 2.3 M sucrose overnight and frozen in liquid nitrogen. Then 60-nm sections were cut and mounted on carbon/Parlodion-coated grids. The specimen-containing grids were washed with PBS, fixed further with 1% glutaraldehyde, washed with water, and stained with 2% uranyl acetate for 5 min. The grids were then destained with and embedded in 2% methylcellulose, dried, and viewed in a Philips CM10 electron microscope.

Vesicle diameter was estimated by measuring with a calibrated eyepiece the greatest distance between two opposite points at the external surface of round vesicles. For oval vesicles, diameters along short and long axes of the vesicle were measured and averaged.

Anion Transport Measurements. Anion influx was measured at 22 °C by SPQ fluorescence quenching using a Hi-Tech SF51 stopped-flow apparatus (Wiltshire, England). A total of 0.08 mL of a microsomal suspension (0.1–0.2 mg of protein/mL) in the isethionate buffer containing 5 μM valinomycin was mixed with an equal volume of isosmotic buffer in which 50 or 100 mM isethionate was replaced by Cl, Br, or I. SPQ fluorescence was measured at 365-nm excitation wavelength (Hg–Xe arc lamp, 365 ± 5 nm six-cavity interference filter) and >420-nm emission wavelength (GG420 cut-on filter, Schott Glass, Duryea, PA). Instrument dead time was 1.7 ms. Each experiment consisted of 512 data points obtained at acquisition rates of 1–10 ms per point.

The initial rate of Cl influx (J_{Cl}) was calculated from the initial rate of fluorescence quenching (dF/dt) and the total entrapped SPQ fluorescence (F_o) from the equation $J_{Cl} = (1/K_x)\, d(F/F_o)/dt$, where K_x are Stern–Volmer constants (in M^{-1}: Cl 118, Br 156, I 272) (Verkman, 1990). F_o was de-

termined from the total amplitude of the time course of fluorescence quenching upon mixture of the microsomal suspension with 150 mM KSCN, a permeant anion which causes >99% SPQ quenching. F_0 was determined in every measurement to normalize for small differences in entrapped SPQ fluorescence in different samples. The initial rate, rather than the full time course, was analyzed because labeled vesicles are probably heterogeneous with respect to Cl transport properties and because Cl influx into vesicles containing a functional Cl channel would occur in a few seconds or faster. In addition, the full fluorescence vs time curve is very complex because of the changing electrochemical driving forces, endosome volume, and membrane potential.

Protein Phosphorylation. Membrane phosphorylations were carried out at 22 °C in 50 μL of KCl buffer containing 50 mM KCl, 100 mM mannitol, 1 mM $MgCl_2$, and 5 mM potassium phosphate, pH 7.45. Reactions were initiated by the addition of purified endosomes (50 μg of protein) to buffer containing 5 μCi of [γ-^{32}P]ATP. In some experiments, PKA (10 units), H-8 (100 μM), or EDTA (5 mM) was present. Dephosphorylation by alkaline phosphatase (AP) was studied by adding AP (40 units) and H-8 (100 μM) at 1 min after phosphorylation was initiated. Unless AP was present, phosphorylations were terminated by the addition of 500 μL of 6% trichloroacetic acid (TCA) at 4 °C. Proteins were precipitated at 12000*g* for 2 min, and the pellets were resuspended in buffer containing 75 mM Tris base and 2% SDS. When AP was present, phosphorylations were terminated by the addition of 1.0 mL of buffer at 4 °C. Membranes were precipitated by centrifugation at 25000*g* for 30 min and resuspended in SDS buffer. Proteins were separated by SDS–PAGE on polyacrylamide gels using a Bio-Rad minigel system. Gels were stained with Coomassie blue, dried, and exposed to X-ray film (Kodak X-Omat AR) at −80 °C.

The incorporation of ^{32}P into specific proteins was quantified by excising the bands from dried gels and measuring the radioactivity by liquid scintillation spectrophotometry (Beckman LS 3801). Background gel radioactivity, measured by excision of an equivalent mass of gel not containing a visible band, was subtracted. Background radioactivity was <5% of the radioactivity measured when PKA was present at the time of [γ-^{32}P]ATP incubation.

For experiments where membranes were treated with AP prior to phosphorylation, membranes (1.2 mg of protein/mL) were incubated at 20–22 °C for 10 min in KCl buffer, either with or without AP (80 units/mL). Endosomes were pelleted at 100000*g* for 30 min and resuspended in KCl buffer at 0.4 mg/mL. Phosphorylations were carried out as described above on 100-μL samples of treated and untreated membranes.

Results

Anion Transport Measurements. To provide a functional correlate to the membrane protein phosphorylation experiments, measurements of anion influx were performed. Figure 1 shows the time course of Cl influx into SPQ-loaded endocytic vesicles. Because unlabeled vesicles were nonfluorescent, the SPQ signal was specific for endocytic vesicles. Intravesicular Cl was zero at the start of the experiment. In response to a 50 mM inwardly directed Cl gradient, Cl influx caused quenching of entrapped SPQ fluorescence. Experiments were carried out in the presence of K and valinomycin to eliminate diffusion potentials so that conductive Cl influx was the rate-limiting process. It was shown previously that the primary route for Cl transport across these vesicles was conductive and that Na- and HCO_3-coupled Cl transporters were absent (Bae & Verkman, 1990).

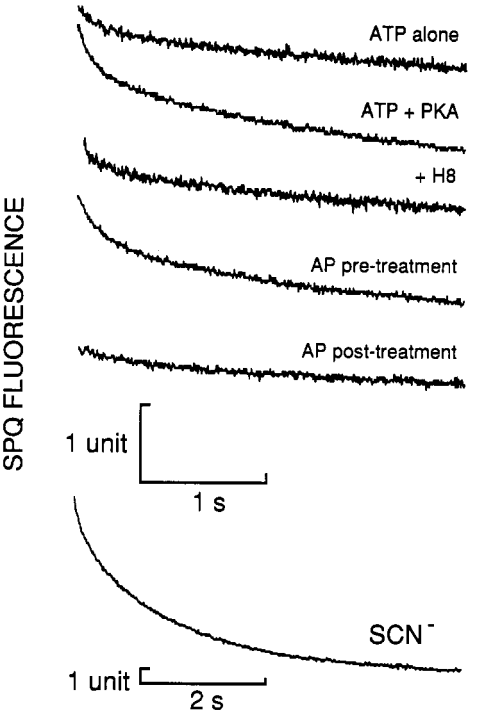

FIGURE 1: Time course of conductive Cl influx in SPQ-labeled endocytic vesicles. A total of 1 mL of vesicles (1 mg/mL) in 100 mM potassium isethionate, 100 mM mannitol, 1 mM magnesium acetate, and 5 mM potassium phosphate (pH 7.4) was incubated with PKA (10 units), ATP (0.5 mM), and/or AP (40 units) as described under Methods. Valinomycin (5 μM) was added to eliminate the development of diffusion potentials. Cl influx was measured by stopped-flow fluorometry by rapid (<1 ms) addition of 0.08 mL of the vesicle suspension with an equal volume of Cl-containing buffer to give a 50 mM inwardly directed Cl gradient. Each curve is the average of eight to ten experiments and has been displaced in the *y*-direction for visual clarity. Note the difference in scales for the Cl influx curves and the KSCN normalization experiment. Average relative initial Cl influx rates are given in the text.

Figure 1 shows that conductive Cl influx was enhanced by incubation of vesicles with PKA and ATP prior to the stopped-flow assay. The increase was not observed when the kinase A blocker H-8 was present throughout the experiment. Treatment of vesicles with AP prior to the PKA/ATP incubation had little influence on the activating effect of PKA and ATP, whereas treatment with AP after the PKA/ATP incubation caused a significant decrease in Cl influx. In four sets of paired measurements, the relative rates of initial Cl influx were 1.0 (ATP alone), 1.9 ± 0.2 (PKA + ATP), 1.1 ± 0.1 (PKA + ATP + H-8), 1.7 ± 0.3 (AP pretreatment), and 0.6 ± 0.2 (AP after PKA + ATP). The Cl permeability coefficient (P_{Cl}) for a relative Cl influx of 1.0 was 3.1×10^{-8} cm/s, calculated for a 50 mM Cl gradient, an endosome surface-to-volume ratio of 3.2×10^5 cm^{-1}, and a 0-mV membrane potential. We showed previously (Bae & Verkman, 1990) that Cl influx was not affected by a 10-min incubation with ATP or PKA alone, that Cl influx was inhibited by the stilbene DNDS, and that the activation of Cl conductance required Mg.

The anion selectivity of Cl conductance was examined in Figure 2. Studies were performed using 25 mM inwardly directed gradients of Cl, Br, and I. Analysis of initial anion influx rates (see Methods) for three sets of experiments gave an anion selectivity sequence Cl (1.0) > I (0.72) > Br (0.54). Note that the selectivity sequence is not obvious from inspection of initial curve slopes because the Stern–Volmer constants for quenching of SPQ by Cl, Br, and I differ. This sequence is different from the I > Cl selectivity reported for

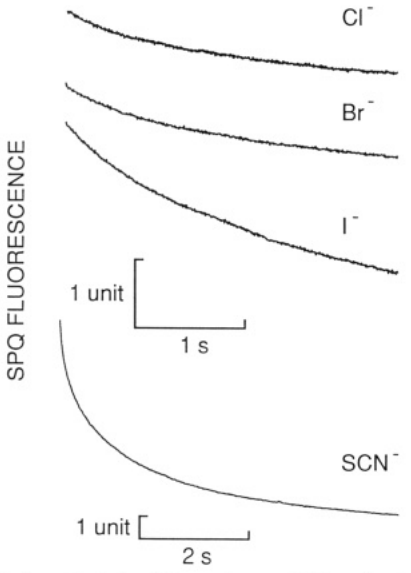

FIGURE 2: Anion selectivity of the endosomal Cl conductance. Vesicles as in Figure 1 were incubated with PKA and ATP and subjected to 25 mM inwardly directed gradients of Cl, Br, and I. Each curve is the average of eight to ten experiments. Relative initial anion influx rates from a series of measurements are summarized in the text.

the PKA-dependent rectifying Cl channel in the apical membrane of epithelial cells (Welsh, 1990).

Characterization of Endocytic Vesicles. To study the phosphoproteins produced by action of PKA, purified endosomes were isolated from rabbit renal cortex by differential and Percoll density gradient centrifugation. Figure 3A shows an electron micrograph of negatively stained endocytic vesicles. Vesicles, sharply delineated by the membrane bilayer, were fairly uniform in appearance. In 319 vesicles, the majority (82%) of measured diameters were between 100 and 250 nm (Figure 3B). The mean diameter was 190 ± 3.2 nm (SE).

The rates of NEM-sensitive intravesicular accumulation of protons in cortical homogenates and isolated vesicles were estimated from the rates of acridine orange fluorescence quenching in samples that had been preincubated without (–NEM) or with 1 mM NEM for 10 min prior to the addition of ATP. Representative curves are shown in Figure 3C. The rates of H^+ accumulation were linear for up to 0.8 and 0.15 mg/mL protein in homogenate and isolated vesicles, respectively (not shown). As given in Table I, compared to homogenate, isolated endocytic vesicles were enriched in NEM-sensitive H^+ pump by 22-fold. Furthermore, in accord with previous findings in endosomes isolated from the rat kidney (Sabolic & Burckhardt, 1990), preparations of rabbit renal endocytic vesicles exhibited a small enrichment in leucine arylamidase activity (Table I), indicating a slight contamination with proximal tubule brush-border membranes. As judged from the deenrichment in Na/K-ATPase activity, the

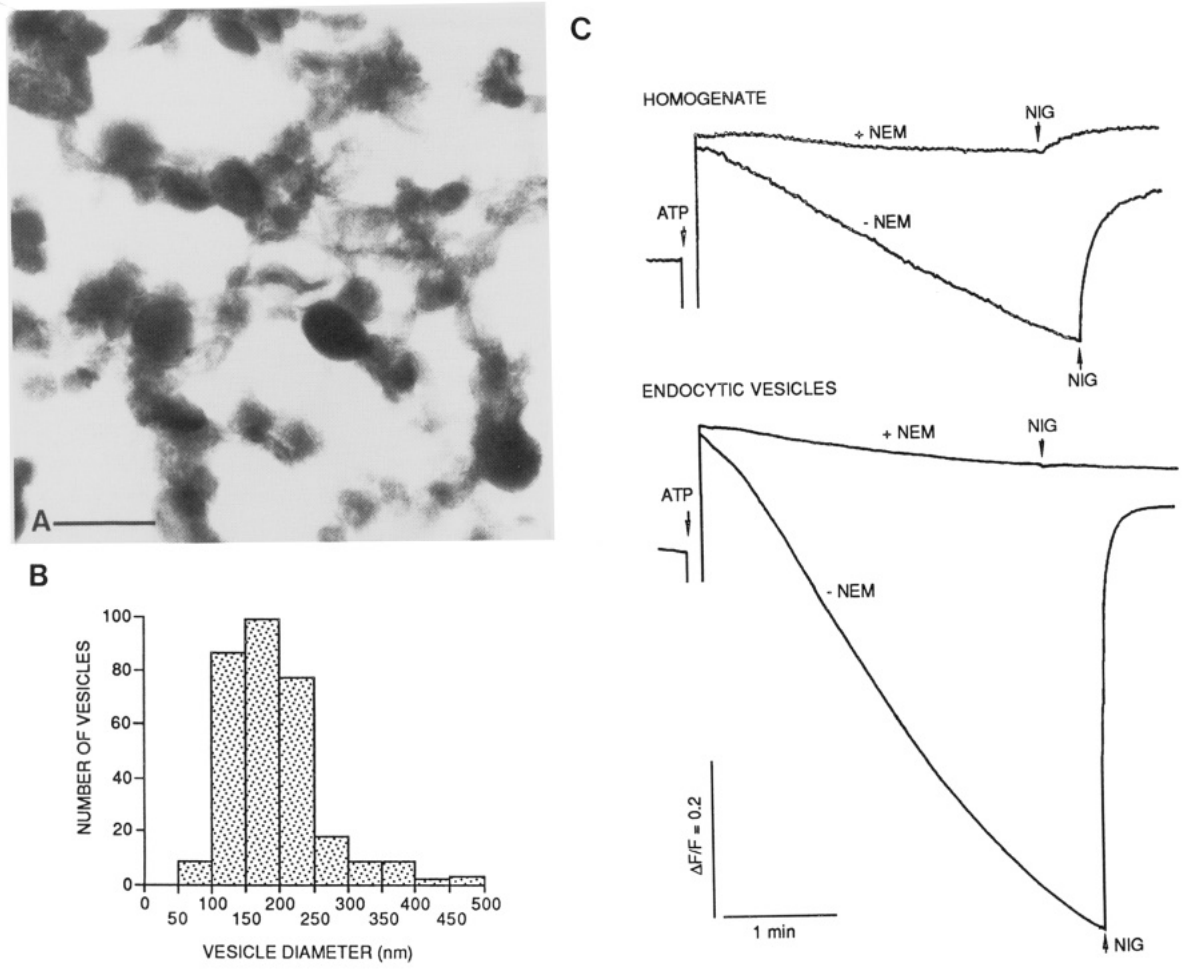

FIGURE 3: Morphological and transport properties of purified endocytic vesicles from rabbit renal cortex. (A) Electron micrograph of an ultrathin (60 nm) frozen section of isolated rabbit renal cortical endocytic vesicles negatively stained with uranyl acetate. The vesicles are sharply delineated by a membrane bilayer. Bar = 0.5 μm. (B) Histogram of diameters of endocytic vesicles calculated from analyses of electron micrographs. The average vesicle diameter was 190 ± 3.2 nm (n = 319). (C) ATP-driven, NEM-sensitive H^+ pump in renal cortical homogenate and isolated endocytic vesicles. H^+ pump was measured in KCl buffer containing acridine orange, valinomycin, and an aliquot of homogenate (0.63 mg of protein) or endocytic vesicles (0.054 mg of protein), in the absence or presence of 1 mM NEM. If present, NEM was added 10 min prior to ATP addition. Nigericin (NIG) (final, 5 μM) was added to dissipate the pH gradient.

Table I: Marker Enzyme Activities in Tissue Homogenate and Endocytic Vesicles Isolated from Rabbit Renal Cortex[a]

enzyme (n)	homogenate	endocytic vesicles	enrichment factor
LAP[b] (7)	63 ± 13	139 ± 31	2.3 ± 0.2
Na/K-ATPase[b] (7)	44 ± 9	16 ± 3	0.49 ± 0.11
NEM-sensitive H^+ pump[c] (13)	0.086 ± 0.01	1.7 ± 0.3	22 ± 2

[a] Shown are means ± SEM obtained in n membrane preparations. LAP = leucine arylamidase. NEM = N-ethylmaleimide. [b] nmol min^{-1} (mg of protein)$^{-1}$. [c] ΔF min^{-1} (mg of protein)$^{-1}$.

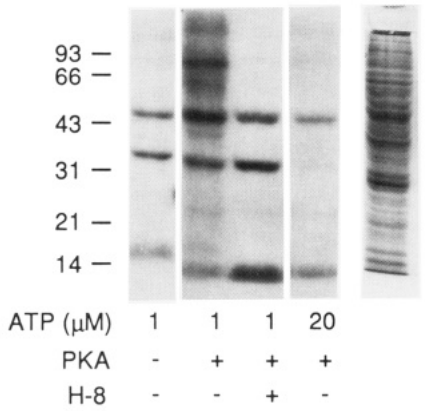

FIGURE 4: Autoradiogram showing protein kinase A dependent phosphoproteins in endocytic vesicles. Purified endosomes (1 mg of protein/mL) were incubated for 1 min with the indicated concentrations of ATP in 50 μL of buffer containing 100 mM mannitol, 50 mM KCl, 1 mM $MgCl_2$, and 5 mM potassium phosphate, pH 7.45. Where indicated, PKA (10 units) and H-8 (100 μM) were present. Reactions were terminated in 1 min with TCA, and precipitated proteins were dissolved in SDS buffer and separated on a 12% polyacrylamide gel as described under Methods. Lane 5 is a Coomassie blue stained gel. Molecular weight markers are indicated at the left. This experiment is representative of four similar studies on separate endosome preparations.

contamination by basolateral membranes was minimal. In seven vesicle preparations, the yield of protein was ~0.09% (homogenate, 1031 ± 59 mg; endocytic vesicles, 0.90 ± 0.07 mg), whereas the yield of H^+ pump was ~2% (homogenate, 86 ± 2 ΔF/min; endocytic vesicles, 1.70 ± 0.02 ΔF/min). For comparison, the protein and H^+ pump yields in rat renal endocytic vesicles were 0.13% and 5.6%, respectively (Sabolic & Burckhardt, 1990).

Phosphorylation Studies. To identify candidate proteins for the PKA-activated chloride channel, protein phosphorylation was examined in purified endosomes. As shown by the autoradiogram in Figure 4, incubation of purified endosomes with 1 μM [γ-^{32}P]ATP for 1 min gave two heavily phosphorylated bands (lane 1). When the catalytic subunit of PKA was included in the incubation (lane 2), additional bands were phosphorylated, including a prominent band at 67 kDa. The 67-kDa phosphoprotein was a consistent finding in six separate endosome preparations. Addition of the protein kinase A inhibitor H-8 blocked the phosphorylation of PKA-dependent proteins but not the PKA-independent bands. Phosphorylation was also inhibited by the chelation of free Mg with 3 mM EDTA (data not shown) or by a 20-fold dilution in the specific activity of the [γ-^{32}P]ATP (lane 4). A Coomassie blue stained gel is shown for comparison in lane 5. These results indicate that, under conditions used to study the activation of the chloride conductance, relatively few endosomal proteins are heavily phosphorylated by PKA and the phosphoproteins are not major Coomassie blue stained membrane proteins. In analogous phosphorylation studies of purified apical membrane

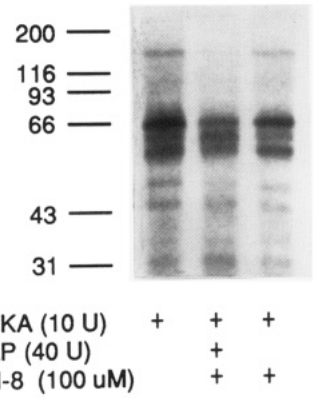

FIGURE 5: Autoradiogram showing dephosphorylation of endosomal membrane proteins by alkaline phosphatase. Purified endosomes were incubated with ATP and PKA as in Figure 4. After 1 min, H-8 with (lane 2) or without (lane 3) AP was added to the reaction mixture. Reactions were stopped after 10 min by a 50-fold dilution into KCl buffer and endosomes were separated from AP by centrifugation. Endosome proteins were separated on a 6% polyacrylamide gel as described under Methods. This experiment is representative of three similar studies on separate endosome preparations.

vesicles, there was diffuse protein phosphorylation in the range 25–60 kDa without the appearance of a 67-kDa phosphoprotein (not shown).

The incorporation of ^{32}P was quantified by measurement of radioactivity in individual excised bands. In three separate experiments, the total incorporated ^{32}P into the 67-kDa band represented 0.25 ± 0.04 pmol/mg of endosomal protein. Using a mean diameter for the vesicles of 190 nm and an internal endosome volume of 2 μL/mg protein, ~0.3 ^{32}P molecule was incorporated into each vesicle.[1] Incubation of vesicles with [γ-^{32}P]ATP and PKA for 10 min, instead of 1 min, increased ^{32}P incorporation into the 67-kDa band by ~20% (not shown), giving a maximal ^{32}P labeling stoichiometry of ~0.4 ^{32}P per vesicle.

The apparently low level of the 67-kDa phosphoprotein could be due to (a) a low level of expression on endosomes, (b) contamination of the preparation with nonendosomal membranes, (c) prephosphorylation of membrane proteins with nonradioactive phosphate which blocked ^{32}P incorporation, and/or (d) partial phosphorylation of membrane proteins under our experimental conditions. It is unlikely that partial phosphorylation is taking place because ^{32}P incorporation was increased by ~20% by prolonged incubation with [γ-^{32}P]ATP and PKA as described above. Significant membrane contamination is also unlikely. Direct counting of rhodamine–dextran-labeled endosomes as described by Lencer et al. (1990b) showed that >80% of the purified vesicles were endosomes (Sabolic and Lencer, unpublished data). Further studies were carried out to examine whether there was significant prephosphorylation of proteins in the endosomal preparation.

The effects of alkaline phosphatase on the phosphorylation of endosomal proteins were examined. Figure 5 is an auto-

[1] The amount of phosphorylation was calculated on the assumption that all protein applied to the gel effectively entered the gel. In a representative experiment, 660 cpm was measured in the excised band of a gel lane loaded with 16 μg of protein. Given a measured specific activity of [γ-^{32}P]ATP of 1.3 × 10^5 cpm/pmol of ^{32}P, 660 cpm in the 67-kDa band corresponds to 5.1 × 10^{-3} pmol of ^{32}P in the 67-kDa band, or 0.32 pmol of ^{32}P/mg of protein. Given a vesicle diameter of 190 nm and a volume of 2 μL/mg of protein, there are 5.6 × 10^{11} vesicles/mg of protein. Thus, there are (0.32 pmol of ^{32}P/mg)/(5.6 × 10^{11} vesicles/mg) or 5.7 × 10^{-13} pmol of ^{32}P/vesicle, which gives 0.34 ^{32}P in the 67-kDa band per vesicle.

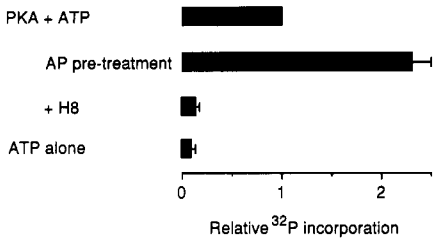

FIGURE 6: Quantitation of the 67-kDa phosphoprotein by band excision. Data are the mean and SE for three sets of paired measurements of ^{32}P incorporation into the 67-kDa band measured by band excision as described under Methods. The control condition (relative ^{32}P incorporation = 1.0) was a 1-min incubation of purified endosomes (50-μL reaction volume) with PKA (10 units) and ATP (5 μCi). Incubations were also carried out on endosomes pretreated with AP (second bar), in the presence of H-8 (100 μM) (third bar), and in the absence of PKA (fourth bar).

radiogram of an SDS gel of endosomal vesicles that have been incubated with [γ-^{32}P]ATP, PKA, and AP. The gel was run at a lower percentage of acrylamide to display phosphoproteins of higher molecular weight. In lane 1, endosomes were incubated for 1 min with [γ-^{32}P]ATP and PKA. There was strong phosphorylation of proteins at and near 67 kDa and weak phosphorylation of a higher molecular weight protein; these proteins were not phosphorylated in the absence of PKA (not shown). In lanes 2 and 3, vesicles were incubated with [γ-^{32}P]ATP and PKA for 1 min; then H-8 and AP (lane 2) or H-8 (lane 3) was added, and the incubation was continued for an additional 10 min. The H-8 was added to inhibit further phosphorylation by PKA after the addition of AP. Several phosphoproteins, including the prominent 67-kDa band and a higher molecular weight band, showed selective dephosphorylation by AP.

The possibility that the 67-kDa protein was prephosphorylated in the purified endosomes was examined by pretreating endosomes with alkaline phosphatase prior to phosphorylation by PKA. Since the treatment of endosomes with AP has been shown to inhibit chloride conductance (Bae & Verkman, 1990), we reasoned that some chloride channels may be prephosphorylated in isolated endosomal vesicles. If this were the case, then pretreatment with AP should enhance the subsequent phosphorylation by PKA. Phosphorylation of the 67-kDa band was quantified by band excision and expressed as the relative radioactivity in a "test" condition compared to a "standard" condition consisting of a 1-min incubation with PKA and [γ-^{32}P]ATP. In four sets of experiments as shown in Figure 6, pretreatment of endosomes with AP gave a (2.3 ± 0.2)-fold increase in phosphorylation over the level of phosphorylation achieved with untreated vesicles. In parallel measurements, there was little phosphorylation of the 67-kDa band when PKA was absent (ATP alone) or when H-8 was added with PKA.

Discussion

The purpose of this study was to determine the pattern of membrane protein phosphorylation in endocytic vesicles from kidney proximal tubule and to compare protein phosphorylation with membrane Cl conductance. Phosphorylations were performed in sealed endocytic vesicles so that only external (cytoplasm-facing) sites were exposed. It was found that activation of Cl conductance required the presence of ATP and PKA under phosphorylating conditions; Cl conductance was inactivated by phosphatase action. In [γ-^{32}P]ATP incorporation studies, phosphoproteins at 35 and 48 kDa were induced in the absence of PKA and were unaffected by a protein kinase A inhibitor. These bands may represent phosphorylation by other endogenous, membrane-bound endosomal protein kinases. In the presence of PKA, a prominent band centered at 67 kDa was observed consistently. Phosphorylation of the 67-kDa protein was reduced when the activity of protein kinase A was inhibited or when alkaline phosphatase was added after ^{32}P incorporation. In a variety of experimental conditions the appearance of the 67-kDa phosphoprotein correlated with activation of the endosomal Cl conductance.

The apical membrane of proximal tubule cells undergoes rapid constitutive endocytosis and exocytosis involving clathrin-coated pits (Rodman et al., 1986). Whereas the apical membrane of proximal tubule cells contains water channels, proton pumps, and Na-dependent proton and glucose transporters, endocytic vesicles derived from proximal tubule apical membrane do not contain Na-dependent transporters (Ye et al., 1989) but do contain proton pumps and Cl channels (Bae & Verkman, 1990) which are not present in the apical membrane (Berry & Rector, 1991). Therefore, the retrieval of apical membrane components is selective and probably associated with fusion of intracellular membranes containing Cl channels and proton pumps. For studies of water, proton, and Cl transport, endocytic vesicles from proximal tubule apical membrane were labeled selectively by fluid-phase fluorescent markers that were filtered by the kidney glomerulus. Because of (a) the high concentration of the fluorescent marker in the tubular fluid, (b) the more rapid rate of apical endocytosis compared with basolateral endocytosis and, (c) the prevalence of proximal tubules in renal cortex, the vast majority of endocytic vesicles labeled by the infusion protocol originated from apical endocytosis (Lencer et al., 1990a). The microsomal pellet containing the fluorescently labeled endocytic vesicles could be prepared rapidly with minimal membrane trauma; transport was studied selectively in the labeled vesicles without interference by nonfluorescent membranes present in the microsomal pellet.

Studies of ATP-dependent acidification in individual endocytic vesicles from proximal tubule showed considerable heterogeneity in pH (Shi et al., 1991). On the basis of a large body of data in fibroblasts and cultured epithelial cells, endocytic trafficking is associated with progressive acidification as internalized contents pass from early endosomes to sorting endosomes, late endosomes, and lysosomes (Mellman et al., 1986; Roederer et al., 1987). In principle, acidification could be regulated by the number/activity of proton pumps, ion pumps or channels, or by the passive proton conductance. There is evidence to implicate each of these mechanisms in specific cell types and endocytic vesicles (Xie et al., 1983; Cain et al., 1989; Fuchs et al., 1989; Van Dyke, 1988; Barasch et al., 1988). Recently, attention has been focused on intracellular Cl channels because of their potential role in the cellular pathophysiology of cystic fibrosis (Barasch et al., 1991). If the primary conductance in intracellular vesicles is to Cl, then regulation of Cl conductance would provide a simple means to control acidification driven by the electrogenic proton pump. Our finding of a phosphorylation-regulated Cl conductance in endocytic vesicles provides support for this hypothesis, but it must be cautioned that these results cannot be extrapolated to intracellular secretory vesicles or to endocytic vesicles in different cell types.

The 67-kDa phosphoprotein cannot be CFTR because of its small molecular size. The high molecular weight phosphoprotein seen best in Figure 5 was present in small quantities, corresponding to <1 phosphoprotein in 20 endosomes (<5% of the intensity of the 67-kDa band). Recently,

Crawford et al. (1991) reported that CFTR antibodies stained kidney proximal tubule strongly near the apical membrane; however, significant renal abnormalities are not associated with cystic fibrosis.

The 67-kDa phosphoprotein may be the endosomal Cl channel. Its molecular size is similar to that of the kidney Cl channel purified by Landry et al. (1990), and its phosphorylation correlated with endosomal Cl conductance. There were ~0.4 ^{32}P molecule incorporated in the 67-kDa band per endocytic vesicle. The majority of the total ^{32}P incorporation was shown to be protein kinase A dependent. The 0.4 ^{32}P incorporation value was increased by a factor of 2.3 when vesicles were pretreated with AP, suggesting that endosomal proteins are partially phosphorylated when isolated from kidney cortex. The stoichiometry of approximately one 67-kDa phosphoprotein per endocytic vesicle is consistent with a Cl channel whose prevalence would be predicted to be quite low. The 67-kDa phosphoprotein was not detected in contaminating apical vesicles as shown by phosphorylation studies on purified brush-border vesicles.

Data obtained in cell-free preparations must be interpreted cautiously because of heterogeneity in the vesicle purity and physical properties. It has been assumed that the phosphoproteins detected in the purified endocytic vesicle preparation corresponded to those present in the in vivo SPQ-labeled endocytic vesicles. Previous studies have confirmed that fluid-phase markers of endocytosis are highly enriched in the endosome purification procedure (Sabolic et al., 1985; Lencer et al., 1990a). It remains quite possible, however, that the purified endocytic vesicles are heterogeneous with respect to their phosphoprotein composition because of the presence of vesicles at different stages of endosomal maturation. In the stopped-flow assay of Cl transport, it is difficult to determine the fraction of SPQ-labeled endocytic vesicles which contain a protein kinase A dependent Cl conductance. Therefore, although our study identifies the endosomal proteins which are phosphorylated by protein kinase A, definitive assignment of phosphoprotein function will require protein purification and functional reconstitution.

Registry No. PK, 9026-43-1; Cl^-, 16887-00-6.

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