Receptor-mediated inhibition of renal Na\(^{+}\)-K\(^{+}\)-ATPase is associated with endocytosis of its \(\alpha\)- and \(\beta\)-subunits

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Chibalín, Alexander V., Adrian I. Katz, Per-Olof Berggren, and Alejandro M. Bertorello. Receptor-mediated inhibition of renal Na\(^{+}\)-K\(^{+}\)-ATPase is associated with endocytosis of its \(\alpha\)- and \(\beta\)-subunits. Am. J. Physiol. 273 (Cell Physiol. 42): C1458–C1465, 1997.—The mechanisms involved in receptor-mediated inhibition of Na\(^{+}\)-K\(^{+}\)-ATPase remain poorly understood. In this study, we evaluate whether inhibition of proximal tubule Na\(^{+}\)-K\(^{+}\)-ATPase activity by dopamine is linked to its removal from the plasma membrane and internalization into defined intracellular compartments. Clathrin-coated vesicles were isolated by sucrose gradient centrifugation and negative lectin selection, and early and late endosomes were separated on a flotation gradient. Inhibition of Na\(^{+}\)-K\(^{+}\)-ATPase activity by dopamine, in contrast to its inhibition by ouabain, was accompanied by a sequential increase in the abundance of the \(\alpha\)-subunit in clathrin-coated vesicles (1 min), early endosomes (2.5 min), and late endosomes (5 min), suggesting its stepwise translocation between these organelles. A similar pattern was found for the \(\beta\)-subunit. The increased incorporation of both subunits in all compartments was blocked by calphostin C. The results demonstrate that the dopamine-induced decrease in Na\(^{+}\)-K\(^{+}\)-ATPase activity in proximal tubules is associated with internalization of its \(\alpha\)- and \(\beta\)-subunits into early and late endosomes via a clathrin-dependent pathway and that this process is protein kinase C dependent. The presence of Na\(^{+}\)-K\(^{+}\)-ATPase subunits in endosomes suggests that these compartments may constitute normal traffic reservoirs during pump degradation and/or synthesis.

Experimental Procedures

Preparation of proximal convoluted tubule cells. Proximal convoluted tubule (PCT) cells were prepared as described previously (2, 31). Briefly, male Sprague-Dawley rats (BK Universal, Sollentuna, Sweden) weighing between 150 and 200 g were used. After the kidneys were removed and the cortex was isolated, the tissue was minced on ice to a paste-like consistency. The cortical mince was incubated with 0.075 g/100 ml collagenase (type I, Sigma) in 50 ml Hanks' medium (Life Technologies, Gaithersburg, MD). The incubation was carried out at 37°C for 60 min. The solution was continuously exposed to 95% O\(_2\)-5% CO\(_2\). The incubation was terminated by placing the tissue on ice and pouring it through graded sieves (180, 75, 53, and 38 µm in pore size) to obtain a cell suspension. The PCT cells were washed three to four times by centrifugation at 100 g for 4 min to separate the remaining blood cells and traces of collagenase. The cells were kept on ice until studied and were used immediately after preparation. Cells were resuspended in Hanks' medium to yield a protein concentration of ~1–3 mg/ml.

Cell fractionation using sucrose density gradients. Renal proximal tubules (~0.7–0.9 mg protein) were suspended in 400 µl homogenization buffer containing (in mM) 20 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 MgCl\(_2\), 2 EDTA, 250 sucrose, and 1 phenylmethylsulfonyl fluoride (PMSF) as well as 0.5 µg/ml each of leupeptin, pepstatin, aprotinin, and antipain. The tissue was homogenized (15–20 strokes) in a Dounce homogenizer. The homogenate was centrifuged (1,000 × g for 4 min), the supernatant was removed, and the remaining blood cells and traces of collagenase were removed by centrifugation (10,000 × g for 10 min). The pellet was resuspended in Hanks' medium to yield a protein concentration of ~1–3 mg/ml.

Cell fractionation using sucrose density gradients. Renal proximal tubules (~0.7–0.9 mg protein) were suspended in 400 µl homogenization buffer containing (in mM) 20 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 MgCl\(_2\), 2 EDTA, 250 sucrose, and 1 phenylmethylsulfonyl fluoride (PMSF) as well as 0.5 µg/ml each of leupeptin, pepstatin, aprotinin, and antipain. The tissue was homogenized (15–20 strokes) in a Dounce homogenizer. The homogenate was centrifuged (1,000 × g for 5 min), and the pellet was resuspended in 200 µl homogenization buffer and centrifuged again, as above. Supernatants from the two centrifugations were pooled, and this postnuclear supernatant (PNS) was layered on a 4-ml linear sucrose gradient (1.074–1.242 g/ml in 20 mM HEPES, 2 mM EDTA, and 1 mM PMSF) and centrifuged in a SW50.1 Beckman rotor at 110,000 g and 4°C for 18 h. Fractions (290 µl each) were collected from the top of the gradient, and their density was determined by refractometry.

Preparation of endosomes. Endosomes were fractionated on a flotation gradient, using essentially the technique described by Gorvel et al. (14). Cells in suspension (1.5 mg protein/ml) were incubated with phorbol (1 µM) or vehicle. Incubation...
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Fig. 1. Cellular distribution of Na⁺-K⁺-ATPase catalytic α-subunit in renal proximal convoluted tubule (PCT) cells and effect of incubation with dopamine (1 µM) during 15 min at room temperature (top panel). After incubation with dopamine or vehicle, homogenates of PCT suspensions were layered on a 4-ml linear sucrose gradient and processed as described in EXPERIMENTAL PROCEDURES. After fractionation, samples were pooled as follows: A, fractions 1.083 to 1.129 g/ml; B, fractions 1.139 to 1.183 g/ml; and C, fractions 1.194 to 1.240 g/ml. Samples [3 µg Na⁺-K⁺-ATPase, 7.5 µg mannose 6-phosphate receptor (MPR) and rab5, and 50 µg GLUT-2] of protein were separated by SDS-PAGE (7.5–15%) and transferred to nitrocellulose membranes. Qualitative determination of Na⁺-K⁺-ATPase α-subunit immunoreactivity, GLUT-2, rab5, and MPR, as indicators of early and late endosomes, respectively, was performed by Western blot analysis. Data are representative of 3 experiments performed independently.

Preparation of clathrin-coated vesicles. Isolation of clathrin-coated vesicles (CCV) was performed as described by Hammond and Verroust (17). Briefly, after incubation with dopamine, PCT cells were homogenized using a Potter homogenizer (3 strokes, 30 s) in 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.5 mM MgCl₂, 0.1 M 2-(N-morpholino)ethanesulfonic acid, and 0.2 mg/ml NaN₃, titrated to pH 6.5 with NaOH. The homogenate was centrifuged at 15,000 g for 15 min, and the supernatant was further centrifuged at 85,000 g for 1 h. The pellet was resuspended in the same buffer and applied to a discontinuous sucrose gradient (wt/vol): 60, 50, 40, 10, and 5%. Samples were centrifuged at 80,000 g for 75 min, collected from the 10–40% interface, and then washed in homogenization buffer and pelleted at 85,000 g for 1 h. Wheat germ agglutinin was added to a concentration of 1 mg/10 mg protein and incubated overnight at 4°C. The agglutinated material was sedimented at 20,000 g for 15 min. The resultant CCV preparation was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and subjected to either silver staining or Western blotting. It is important to note that although this method will isolate all CCV, it may also leave inside-out vesicles in the supernatant.

Determination of Na⁺-K⁺-ATPase activity. Na⁺-K⁺-ATPase activity was determined as described before (5). Briefly, aliquots (~3 µg protein) were transferred to the Na⁺-K⁺-ATPase assay medium (final volume 100 µl) containing (in mM) 50 NaCl, 5 KCl, 10 MgCl₂, 1 EGTA, 50 tris-(hydroxymethyl)aminomethane-HCl, 7 Na₂ATP (Calbiochem, La Jolla, CA), and [γ-32P]ATP (Amersham; sp act 3,000 Ci/mmol) in tracer amounts (3.3 nCi/µl). Cells were transiently exposed to a thermic shock (10 min at ~20°C) to render membranes permeable to ATP. The samples were then incubated at 37°C for 15 min, and the reaction was terminated by rapid cooling to 4°C and addition of 700 µl trichloroacetic acid-charcoal (5%/10% wt/vol) suspension. After separation of the charcoal phase (12,000 g for 5 min) containing the unhydrolyzed nucleotide, the liberated 32P was counted in an aliquot (200 µl) from the supernatant. Na⁺-K⁺-ATPase activity was calculated as the difference between test samples (total ATPase activity) and samples assayed in the same medium but devoid of Na⁺ and K⁺ and in the presence of 1 mM ouabain (ouabain-insensitive ATPase activity).

Miscellaneous. Identification of clathrin heavy chain was performed using a monoclonal antibody (Harlan Sera-Lab, Sussex, UK). The presence of glucose transporter GLUT-2 was evaluated with a polyclonal antibody (Biogenesis, Stinsford Road Poole, UK). The identity of early endosomes was determined with a polyclonal antibody raised against a rab5 synthetic peptide corresponding to amino acids 193–211 within the carboxy terminal of human rab5 (Santa Cruz Biotechnology, Santa Cruz, CA). The late endosome fraction was identified with a mannose 6-phosphate receptor (MPR) antibody (courtesy of Dr. B. Hoflack, EMBL, Heidelberg, Germany). Identification of the Na⁺-K⁺-ATPase α- and β-subunits was performed using monoclonal antibodies raised against the α₁- and β₁-subunits (25) (courtesy of Dr. M. Caplan, Yale University, New Haven, CT). Proteins were analyzed by SDS-PAGE (7.5–15%) using the Laemmli buffer system (21). Protein content was determined according to Bradford (9). Western blots were developed with an ECL detection kit (Amersham, UK), as recommended by the manufacturer. Measurements were performed using multiple exposures of autoradiograms to ensure that signals were within the linear range of the film. Scans were performed using a Scan Jet IIc scanner (Hewlett-Packard, Palo Alto, CA). Each band was scanned twice in different regions, the scans were averaged, and the area of the peak minus the background (in arbitrary units) was quantitated.

Results.

In this study, we used rat renal PCT cells to assess the mechanisms involved in the rapid inhibition of
Na\textsuperscript{+,K\textsuperscript{+}}-ATPase activity by dopamine. Cell fractionation applying linear sucrose gradients has been used successfully to determine the subcellular localization of Na\textsuperscript{+,K\textsuperscript{+}}-ATPase \(\alpha\)-subunit during \textit{Xenopus} oocyte maturation (27, 30). To identify the distribution pattern of Na\textsuperscript{+,K\textsuperscript{+}}-ATPase, homogenates were therefore fractionated on a continuous sucrose gradient (1.074–1.242 g/ml). Fractions were grouped into three samples (A–C, see Fig. 1). Na\textsuperscript{+,K\textsuperscript{+}}-ATPase \(\alpha\)-subunit immunoreactivity was higher in fraction B (enriched in plasma membrane fragments) than in fraction C. Incubation of PCT cells with 1 \(\mu\)M dopamine for 15 min caused a shift in the \(\alpha\)-subunit immunoreactivity away from fraction B, while increasing markedly its abundance in fraction C. MPR immunoreactivity, denoting the presence of late endosomes and to a lesser extent plasma membrane fragments (15), was present in fractions B and C (Fig. 1, 2nd panel), whereas rab5 immunoreactivity, which indicates the presence of early endosomes (14, 16), was found predominantly in fraction C (Fig. 1, 3rd panel). These results suggested that the \(\alpha\)-subunit may have been incorporated into endosomal compartments. Indeed, Western blot analysis revealed increased incorporation of the \(\alpha\)-subunit in early and late endosomes prepared from PCT cells exposed to dopamine (1 \(\mu\)M; 15 min) as compared with PCT cells incubated with vehicle (Fig. 2A). The increased incorporation of Na\textsuperscript{+,K\textsuperscript{+}}-ATPase \(\alpha\)-subunits into early endosomes was time
dependent and became clearly apparent at 5 min (Fig. 2B, top panel), whereas increased incorporation into late endosomes occurred after a lag of 15 min (Fig. 2B, bottom panel). Even earlier times (2.5 and 5 min, respectively) were revealed by scanning densitometry (Fig. 2E).

The early and late endosomes were separated on a flotation gradient (14), and their identities were verified by their immunoreactivity to rab5 and MPR antibody, respectively (Fig. 2, C and D). Late endosomes were rich in MPR, whereas early endosomes were rich in rab5 but showed no MPR immunoreactivity, indicating that they were not contaminated with late endosomes or plasma membrane fragments. Unlike the translocation of the α- and β-subunits (see also Fig. 5), the cell distribution of MPR did not change in response to dopamine (Figs. 1, middle panels, and 2D). In separate experiments, a specific marker of basolateral membranes, GLUT-2, also did not change its distribution in early and late endosomes in response to 1 µM dopamine (Fig. 3), in contrast to the α-subunit abundance.

Receptor-mediated endocytosis generally occurs by formation of clathrin-coated pits and CCV, followed by vesicle transport (22). In this study, CCV were isolated from PCT cells, utilizing sucrose density gradients and negative lectin selection (17). Silver staining of CCV revealed both the heavy chains (160 kDa) and light chains (30 kDa) of clathrin, as well as an 100-kDa protein corresponding in size to the α-subunit (Fig. 4A). The identity of CCV was further documented by Western blot analysis, using a specific antibody against the clathrin heavy chain in the CCV preparation, which was compared with brain and kidney homogenates (Fig. 4B). Contamination of CCV with early endosomal vesicles in this preparation was excluded by the lack of immunoreactivity with rab5 (data not shown). The α-subunit abundance increased...
in CCV treated with dopamine (1 µM) in a time-dependent manner: it became evident at 1 min, peaked at 2.5 min, and lasted up to 30 min of incubation (Fig. 4, C and D). This time course coincides with the early presence of the α-subunit in CCV and its subsequent appearance (at 5 min) in early endosomes (see Fig. 2B). Despite the increase in Na\(^{+}\)-K\(^{+}\)-ATPase α-subunit abundance in CCV and early endosomes from dopamine-treated cells, the enzymatic activity remained unchanged (Table 1), indicating that the endocytosed subunits became inactive.

In PCT cells, dopamine inhibits Na\(^{+}\)-K\(^{+}\)-ATPase activity by activation of PKC (3, 29), which may also be involved in endocytosis. Consistent with this hypothesis, the ability of dopamine to increase incorporation of α-subunits in CCV was abolished by calphostin C, a specific PKC inhibitor found by us to block dopamine inhibition of the pump in intact tubules (24, 26) (Fig. 5, A and D, left panel). Similar to CCV, calphostin C also prevented the increased α-subunit incorporation induced by dopamine in early and late endosomes (Fig. 5, A and D, left panel), whereas calphostin C alone had no effect on α-subunit distribution (data not shown). In addition to the α-subunit, dopamine also increased the incorporation of Na\(^{+}\)-K\(^{+}\)-ATPase β-subunit into early and late endosomes, and this effect as well was blocked by calphostin C (Fig. 5, B and D, right panel). Similar inhibition of α-subunit endocytosis (data not shown) was obtained with another specific PKC inhibitor, bisindolylmaleimide.

Table 1. Na\(^{+}\)-K\(^{+}\)-ATPase activity in basolateral membranes, clathrin-coated vesicles, and early endosomes prepared from vehicle- and dopamine-treated PCT cells

<table>
<thead>
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<th>Na(^{+})-K(^{+})-ATPase Activity, nmol Pi · mg protein (^{-1}) · min (^{-1})</th>
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<tr>
<td>PCT cells</td>
<td></td>
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<tr>
<td>Control</td>
<td>112.0 ± 8.0 (4)</td>
</tr>
<tr>
<td>Dopamine</td>
<td>60.0 ± 2.0* (4)</td>
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<tr>
<td>Basolateral membranes</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>183.0 ± 20 (5)</td>
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<tr>
<td>Dopamine</td>
<td>102.0 ± 12* (5)</td>
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<tr>
<td>Clathrin-coated vesicles</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>19.8 ± 4.0 (3)</td>
</tr>
<tr>
<td>Dopamine</td>
<td>19.6 ± 3.0 (3)</td>
</tr>
<tr>
<td>Early endosomes</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>14.0 ± 2.9 (3)</td>
</tr>
<tr>
<td>Dopamine</td>
<td>15.4 ± 1.5 (3)</td>
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Values are means ± SE of number of experiments given in parentheses. *P < 0.05. PCT, proximal convoluted tubule.

Fig. 5. Internalization of Na\(^{+}\)-K\(^{+}\)-ATPase α- and β-subunits in PCT cells incubated with dopamine is protein kinase C dependent.

A: PCT cells were incubated with dopamine at room temperature for 2.5 min (CCV) and 15 min (EE and LE). Dopamine (1 µM)-induced incorporation of α- (A) and β-subunits (B) in CCV, EE, and LE is blocked by coincubation with calphostin C (1 µM). C: in contrast, inhibition of Na\(^{+}\)-K\(^{+}\)-ATPase activity with ouabain (Ouab; 1 mM) is not associated with increased endocytosis of its α-subunit in endosomes. Amount of protein loaded in each lane was 1 µg (Na\(^{+}\)-K\(^{+}\)-ATPase α-subunit) and 5 µg (Na\(^{+}\)-K\(^{+}\)-ATPase β-subunit). Experiments were independently performed 4 times. Densitometric scans (D) were expressed as α-subunit enrichment (percentage of control), and mean ± SE values are shown. Asterisks represent values statistically (P < 0.05) different from dopamine-treated cells without calphostin C (open bars).
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Fig. 6. Endocytosis of Na⁺-K⁺-ATPase α-subunits from PCT cells that have been previously incubated with norepinephrine (5 µM) for 30 min at room temperature or phallacidin (1 µM). To ensure incorporation of phallacidin, cells were subjected to a thermic shock (10 min at −20°C), and, after a recovery period of 10 min at room temperature, dopamine was added for an additional 15 min. Thermic shock did not affect endocytosis of Na⁺-K⁺-ATPase subunits induced by dopamine as seen in PCT cells that have not received phallacidin treatment but were subjected for 10 min to −20°C. Neither phallacidin (% of control of α-subunit abundance: CCV, 95.2 ± 7.3, n = 4; EE, 98.8 ± 9.3, n = 5; LE, 106.6 ± 6.3, n = 5) nor norepinephrine (% of control of α-subunit abundance: CCV, 95.0 ± 1.0, n = 4; EE, 98.5 ± 5.2, n = 4; LE, 105.8 ± 2.3, n = 4) affects sedimentation properties of CCV, EE, and LE. Total amount of protein subjected to SDS-PAGE separation was 1 µg. Western blots (A) are representative of 4 experiments, and statistical comparison of densitometry scans is shown in B.

Is the activation of PKC a requisite for endocytosis of pump subunits? To address this question, we next examined their distribution after exposure to ouabain, a specific Na⁺-K⁺ pump inhibitor that lacks the PKC effect. Although ouabain (1 mM, 10 min) inhibited pump activity, it had no effect on the internalization of the α-subunit into endosomes (Fig. 5C) (β-subunit was not examined). This observation further supports the concept that receptor-mediated inhibition/endocytosis of the pump in epithelial cells requires activation of a specific intracellular signaling system.

The trafficking of proteins between the plasma membrane and several subcellular compartments is dependent on the integrity of the cortical actin cytoskeleton and the microtubule system. Therefore, we also examined the role of the actin/microtubule system in this process by using microtubule depolymerizing agents, norepinephrine (Noc) and phallacidin (Phall), which stabilize the cortical actin cytoskeleton but do not affect the α-subunit distribution [Noc: α-subunit abundance (% of control): CCV, 107 ± 3, n = 4; early endosomes, 98.5 ± 5.2, n = 4; late endosomes, 105.8 ± 2.3, n = 4; Phall: α-subunit abundance (% of control): CCV, 95.2 ± 7.3, n = 4; early endosomes, 98.8 ± 9.3, n = 5; late endosomes, 106.6 ± 6.3, n = 5]. In the presence of Noc, 1 µM dopamine failed to increase Na⁺-K⁺-ATPase α-subunit endocytosis into early and late endosomes (similar results were obtained with colchicine, another microtubule depolymerizing agent), but not into CCV, whereas Phall treatment prevented their incorporation into CCV as well (Fig. 6). The ability of dopamine to modulate Na⁺-K⁺-ATPase activity was also affected by Phall and Noc treatment. Although Phall prevented the inhibitory action of dopamine (97 ± 5% of control, n = 4), Noc only attenuated the inhibition (72.6 ± 5.3% of control, n = 11; P < 0.01). In parallel experiments without Noc, dopamine inhibited Na⁺-K⁺-ATPase activity maximally by 65 ± 5% of control (n = 4).

DISCUSSION

In contrast to the wealth of information about its structure and kinetic properties, the regulation of Na⁺-K⁺ pump activity and abundance is less well understood. Recent progress in this area led to definition of two types of regulation: short and long term, or rapid and sustained, which are mediated by different mechanisms (reviewed in Refs. 7 and 11). It is generally accepted that rapid modulation of Na⁺-K⁺-ATPase activity involves stimulation of serine/threonine protein kinases, primarily PKA and PKC (1, 3, 5, 8, 12, 23, 28, 29), leading to activation of other intracellular mediators (e.g., eicosanoids) and/or the reversible phosphorylation of its catalytic (α) subunit. However, the molecular mechanisms that translate the phosphorylation effect into modulation of pump activity remain obscure. In this paper, we propose the concept (summarized in schematic fashion in Fig. 7) that receptor-mediated inhibition of Na⁺-K⁺-ATPase activity by dopamine in PCT cells is associated with a stepwise endocytosis of its α- and β-subunits. The proposed mechanism appears to be analogous to that of other systems such as vasopressin-dependent regulation of aquaporin-2 water channels (20) in collecting duct cells, or insulin-dependent regulation of the glucose trans-

Fig. 7. Proposed schema of intracellular traffic sequence of Na⁺-K⁺-ATPase subunits after inhibitory action of dopamine. For explanation, see text.
porter GLUT-4 in skeletal muscle (18) and, in the opposite direction, of Na\(^{+}-\)K\(^{-}\)-ATPase in lacrimal glands (33) and skeletal muscle (19). Further evidence for the proposed sequence of events is provided by the lack of endocytosis in the presence of a microtubule depolymerizing agent. Failure of nocodazole (Noc to inhibit the incorporation of \(\alpha\)-subunits in CCV is consistent with the fact that this process is microtubule independent (16). In contrast, stabilizing the cortical actin cytoskeleton with Phall inhibited the incorporation of \(\alpha\)-subunits into CCV. Although inhibition of Na\(^{+}\)-K\(^{-}\)-ATPase activity of endocytosis in the presence of a microtubule depolymerizing agent. Failure of nocodazole (Noc to inhibit the incorporation of \(\alpha\)-subunits in CCV is consistent with the fact that this process is microtubule independent (16). In contrast, stabilizing the cortical actin cytoskeleton with Phall inhibited the incorporation of \(\alpha\)-subunits into CCV. Although inhibition of Na\(^{+}\)-K\(^{-}\)-ATPase activity is strongly associated with formation of CCV (Phall treatment blocked the effect of dopamine), disrupting microtubules affect transport to endosomes but only partially the inhibition of enzyme activity.

The endocytosis of Na\(^{+}\)-K\(^{-}\)-ATPase subunits observed in response to dopamine is not likely to be due to increased fluid-phase endocytosis, because other membrane proteins such as GLUT-2 and MPR did not change their cellular distribution in the presence of dopamine. The results described above constitute in our view persuasive biochemical evidence for the increased abundance of enzyme subunits in defined intracellular organelles during Na\(^{+}\)-K\(^{-}\)-ATPase inhibition. Localization of Na\(^{+}\)-K\(^{-}\)-ATPase subunits in early endosomes has previously been suggested in Chinese hamster ovary cells, where they could contribute to the interior membrane potential and thereby affect H\(^{+}\) transport and intravesicular pH (13). Because enzyme subunits capable of ATP hydrolysis are present in endosomes under basal conditions, the present study also raises the possibility that these compartments might constitute traffic reservoirs during pump synthesis as well as during endocytosis, serving, for example, as intracellular storage sites from which the enzyme’s subunits may shuttle rapidly to the plasma membrane. As demonstrated in this paper, receptor-mediated endocytosis likely increases the number of Na\(^{+}\)-K\(^{-}\)-ATPase subunits in this preexisting pool of organelles, a process dependent on PKC activation. It is unclear at present whether the endocytosed units are eventually recycled to the plasma membrane or follow a degradative pathway. That Na\(^{+}\)-K\(^{-}\)-ATPase activity in early and late endosomes could be modulated by okadaic acid (unpublished observations) suggests, however, that this compartment is endowed with protein phosphatase activity, and that dephosphorylation may be a necessary step if the subunits are to be returned to the plasma membrane. It should be emphasized that although our results underscore the importance of PKC, they do not allow an assessment of whether activation of PKC is needed to phosphorylate the \(\alpha\)-subunit itself or at other stages during endocytosis, such as regulation of CCV formation, or for actin or microtubule function.

Finally, preliminary evidence from our laboratory that endocytosis of membrane pumps also occurs in Madin-Darby canine kidney and opossum kidney cells, where enzyme activity can apparently be inhibited by either PKA or PKC, and in pancreatic \(\beta\)-cells, where the signal is not receptor mediated but rather dependent on glucose metabolism, suggests that the mechanism described here in PCT cells may constitute a general mode of Na\(^{+}\)-K\(^{-}\)-ATPase regulation.

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