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Phosphatidylinositol 3-Kinase-mediated Endocytosis of Renal Na^+,K^+-ATPase α Subunit in Response to Dopamine

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Dopamine (DA) inhibition of Na^+,K^+-ATPase in proximal tubule cells is associated with increased endocytosis of its α and β subunits into early and late endosomes via a clathrin vesicle-dependent pathway. In this report we evaluated intracellular signals that could trigger this mechanism, specifically the role of phosphatidylinositol 3-kinase (PI 3-K), the activation of which initiates vesicular trafficking and targeting of proteins to specific cell compartments. DA stimulated PI 3-K activity in a time- and dose-dependent manner, and this effect was markedly blunted by wortmannin and LY 294002. Endocytosis of the Na^+,K^+-ATPase α subunit in response to DA was also inhibited in dose-dependent manner by wortmannin and LY 294002. Activation of PI 3-K generally occurs by association with tyrosine kinase receptors. However, in this study immunoprecipitation with a phosphotyrosine antibody did not reveal PI 3-K activity. DA-stimulated endocytosis of Na^+,K^+-ATPase α subunits required protein kinase C, and the ability of DA to stimulate PI 3-K was blocked by specific protein kinase C inhibitors. Activation of PI 3-K is mediated via the D_1 receptor subtype and the sequential activation of phospholipase A_2, arachidonic acid, and protein kinase C. The results indicate a key role for activation of PI 3-K in the endocytic sequence that leads to internalization of Na^+,K^+-ATPase α subunits in response to DA, and suggest a mechanism for the participation of protein kinase C in this process.

INTRODUCTION

The unique distribution of different ion transport proteins to specific domains of the cell constitutes the basis for coordinated vectorial transport across epithelia (Rodriguez-Boulan and Nelson, 1989). The basolateral localization of Na^+,K^+-ATPase, for example, provides the gradient for sodium movement and thus for a number of sodium-coupled transport events across the apical domain of the cell (Katz, 1982; Kinne, 1988). Regulation of renal and intestinal Na^+,K^+-ATPase activity by catecholamines contributes to the ability of these organs to play an important role in the control of sodium and water homeostasis (Lee, 1982; Bertorello and Katz, 1993). Consequently, inhibition of renal Na^+,K^+-ATPase activity by dopamine (DA) during a high-salt diet leads to an increase in urinary sodium excretion (Bertorello *et al.*, 1988), and an impairment in this mechanism has been associated with the development of hypertension (Chen *et al.*, 1993; Nishi *et al.*, 1993).

The cellular mechanisms responsible for the regulation of Na^+,K^+-ATPase activity are not well understood, as underscored by the variety of signaling molecules implicated in such regulation (Bertorello and Katz, 1993). However, although the nature of the response (inhibition and stimulation) or its specificity varies in different tissues and with different

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agonists, it is clear that most intracellular signals converge in the activation of protein kinases (Bertorello and Aperia, 1989; Bertorello and Katz, 1993). Phosphorylation of Na^+,K^+-ATPase catalytic (α) subunit by protein kinases has been demonstrated in cell-free systems (Lowdnes *et al.*, 1990; Bertorello *et al.*, 1991; Chibalin *et al.*, 1992; Beguin *et al.*, 1994; Borghini *et al.*, 1994; Feschenko and Sweadner, 1995; Logvinenko *et al.*, 1997), as well as in intact cells (Middleton *et al.*, 1993; Fisone *et al.*, 1995; Carranza *et al.*, 1996a,b), although in the latter this effect is less well documented. The manner in which phosphorylation of Na^+,K^+-ATPase in intact cells may alter its catalytic activity and the role of additional, or alternative, mechanisms in this phenomenon remain to be elucidated.

We have recently demonstrated that inhibition of Na^+,K^+-ATPase activity by DA in renal proximal tubule (PCT) cells is associated with increased endocytosis of α and β subunits into early endosomes (EEs) and late endosomes (LEs) via a clathrin-coated vesicle (CCV)-dependent pathway (Chibalin *et al.*, 1997); this effect requires protein kinase C (PKC) activation and a dynamic actin microtubule cytoskeleton. The observation that inhibition of Na^+,K^+-ATPase activity is associated with its removal from the plasma membrane provides an important new insight into the cellular mechanisms responsible for Na^+,K^+-ATPase regulation.

Among several important functions (for review, see Toker and Cantley, 1997), activation of phosphatidylinositol 3-kinase (PI 3-K) has been linked to vesicular traffic and target of proteins to specific intracellular compartments (De Camilli *et al.*, 1996). This lipid kinase, described as a VSP34 gene product necessary for vacuolar transport in *Saccharomyces cerevisiae* (Schu *et al.*, 1993), is present in mammalian cells (Panayotou and Waterfield, 1992; Kapeller and Cantley, 1994), where its role in intracellular traffic has been postulated, interalia, on the basis of its ability to activate *rab*5, a GTP-binding protein responsible for regulating the endocytic traffic to early endosomes (Li *et al.*, 1995). Furthermore, the product of PI 3-K, phosphatidylinositol 3,4,5-phosphate, has been implicated in the regulation of AP-2, a protein responsible for recruiting clathrin to the target in the plasma membrane (Pearse and Robinson, 1990; Gaidarov *et al.*, 1996) and initiating the formation of the clathrin-coated pit that is followed by vesicle transport to early endosomes.

In this study we have examined the role of PI 3-K as a possible intracellular mediator triggering the endocytosis of the Na^+,K^+-ATPase α subunit in response to DA.

MATERIALS AND METHODS

Materials

Fenoldopam (SKF 82526) and *S*-sulpiride were kindly provided by Dr. Michael Murphy (University of Chicago). Quinpirole (LY 171555) and SCH 23390 were from Research Biochemicals (Natick, MA). Bisindolylmaleimide and haloenol lactone (*E*)-6-(bromomethylene)-3-(1-naphtalenyl)-2*H*-tetrahydropyran-2-one suicide substrate (HELSS) were purchased from Calbiochem (San Diego, CA). The cAMP analogue Rp-cAMPS was obtained from BioLog (Bremen, Germany), and 20-hydroxyeicosatetraenoic acid (20-HETE) was from Cayman Chemical Co. (Ann Arbor, MI). All other chemicals, including calphostin C, ethoxyresorufin, arachidonic acid (AA), and DA, were from Sigma (St. Louis, MO). HELSS, calphostin C, and bisindolylmaleimide were dissolved in DMSO (final concentration, $<0.01\%$). AA and 20-HETE were dissolved in ethanol (final concentration, $<0.1\%$) under N_2 flow, and the stock solution was stored at $-20°C$ protected from light. Fenoldopam and SCH 23390 were dissolved in distilled water, and quinpirole and *S*-sulpiride were dissolved in ethanol and methanol, respectively. LY 294009 was purchased from Calbiochem. The antibody against PI 3-kinase was a generous gift from Dr P. Shepherd (Cambridge University, Cambridge, United Kingdom) and was raised against a glutathione *S*-transferase fusion protein corresponding to the C-terminal region of the p85α subunit of human PI 3-kinase. The antibody against the Na^+,K^+-ATPase α subunit was kindly provided by Dr M. Caplan (Yale University, New Haven, CT). The identity of early endosomes was determined with a polyclonal antibody raised against a *rab*5 synthetic peptide corresponding to amino acids 193–211 within the carboxyl terminal of human *rab*5 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The LE fraction was identified with a mannose-6-phosphate receptor antibody (courtesy of Dr B. Hoflack, EMBL, Heidelberg, Germany). Identification of clathrin heavy chain was performed using a monoclonal antibody (Harlan Sera-Lab Ltd., Sussex, United Kingdom).

Preparation of Proximal Tubule Cells

PCT cells were prepared as described before (Seri *et al.*, 1990; Bertorello, 1992). Briefly, male Sprague Dawley rats (BK Universal, Sollentuna, Sweden) weighing between 150 and 200 g were used. After the kidneys were removed and the outer cortex was isolated, the tissue was moistened on ice to a paste-like consistency. The cortical minceate was incubated with 0.075 mg/100 ml collagenase (Type I, Sigma) in 50 ml of Hanks' medium (Life Technologies, Gaithersburg, MD). The incubation was performed at 37°C for 60 min, and the solution was continuously exposed to 95% O_2 and 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 pore size) to obtain a cell suspension. The PCT cells were washed three to four times by centrifugation at $100 \times g$ for 4 min to separate the remaining blood cells and traces of collagenase and were then kept on ice. Cells were resuspended to yield a protein concentration of ~3.5–5.0 mg/ml and were used immediately after preparation. It has been reported that phorbol esters regulate Na,K-ATPase differently depending on whether the tissue has been continuously oxygenated during its preparation and incubation with the PKC activator. Although this effect was not reported to be a modulating factor of the DA response, we have taken the precaution to incubate cells in oxygenated solutions in all of the steps until the tissue was disrupted to immunoprecipitate the p85 subunit or for preparation of clathrin vesicles, early and late endosomes.

Determination of Phosphatidylinositol 3-Kinase Activity

After preincubation with DA under different conditions, the cells were transferred in the cold, homogenized in 400 μl of lysis buffer

[140 mM NaCl, 10 mM HEPES, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM Na_3VO_4, 10% glycerol, 1% Nonidet P-40, 10 μg/ml aprotinin, 50 μM leupeptin, and 2 mM PMSF (pH 8.1)] and solubilized by continuous stirring for 1 h at 4°C (Heydrick *et al.*, 1993). After centrifugation, the supernatant was collected, and 1 mg of protein (in 500 μl) was incubated with an antiPI 3-K p85α antibody (unless otherwise stated) coupled to protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden). The immune complex was washed four times with buffer C [100 mM NaCl, 1 mM Na_3VO_4, and 20 mM HEPES (pH 7.5)] and resuspended in 40 μl of buffer D [180 mM NaCl and 20 mM HEPES (pH 7.5)]. The PI 3-K activity in the immunoprecipitate was assessed directly on the protein A-Sepharose beads. The reaction was initiated by addition of 20 μl of buffer E [50 mM NaCl, 0.015% Nonidet P-40, 12.5 mM $MgCl_2$, 250 μM [γ-^{32}P]ATP (30 μCi), 0.5 mg/ml phosphatidylinositol (Avanti Biochemicals, Birmingham, AL), and 20 mM HEPES (pH 7.5)]. The pellets were incubated for 10 min at room temperature, and the reaction was terminated by sequential addition of 80 μl of 1 M HCl and 160 μl of chloroform/methanol (1:1, vol/vol) and vigorous vortexing. After a brief centrifugation, 40 μl of the lower phase was spotted on aluminum-backed Silica Gel 60 TLC plates (EM Separations, Gibbstown, NJ). The lipids were resolved by chromatography in methanol/$CHCl_3$/pyridine/H_2O/formic acid (37.5: 30:22.5:8.67:1.33), 1 M boric acid, and 8.5 mM butylatedhydroxytoluene. The bands corresponding to phosphatidylinositol 3-phosphate were analyzed by autoradiography and quantitated using phosphoimaging. In three experiments performed independently (Figure 1), the PI 3-K activity was normalized to p85 in the immunoprecipitates. The amount of p85 did not vary significantly among the groups, and the increased PI 3-K activity (percent of control, 280 $\pm$ 70%; n = 3) was comparable to samples that were not normalized (percent of control, 202 $\pm$ 9.5%; n = 18). Thus, the PI 3-K activity under different experimental conditions was expressed as percent of control without any further normalization.

Preparation of Endosomes

Endosomes were fractionated on a flotation gradient, using essentially the technique described by Gorvel *et al.* (1991). Cells in suspension (1.5 mg protein/ml) pretreated for 30 min with wortmannin at room temperature were incubated with DA (1 μM) or vehicle. Incubation was terminated by transferring the samples to ice and addition of cold homogenization buffer containing 250 mM sucrose and 3 mM imidazole (pH 7.4). The cells were gently homogenized (15–20 strokes) to minimize damage of the endosomes using a Dounce homogenizer, and the samples were subjected to a brief (5 min) centrifugation (4°C, 3000 $\times$ *g*). The postnuclear supernatant was adjusted to 40.6% sucrose and loaded (1.5 ml) at the bottom of a 5.0-ml centrifuge tube, to which were added sequentially 16% sucrose (1.5 ml) in 3 mM imidazole and 0.5 mM EDTA in D_2O, 10% sucrose in the same buffer (1 ml), and finally homogenization buffer (1 ml). The samples were centrifuged (1 h, 110,000 $\times$ *g*) in a Beckman (Fullerton, CA) SW 50.1 rotor. EEs were collected at the homogenization buffer and 10% sucrose interface, and the LEs were collected at the 10 and 16% sucrose interface. The endosomal preparation was analyzed on SDS-PAGE and subjected to either silver staining or Western blot and autoradiography.

Preparation of Clathrin-coated Vesicles

Isolation of CCV was performed as described by Hammond and Verroust (1994). Briefly, after preincubation with or without DA, PCT cells were homogenized using a Potter homogenizer (three strokes; 30 s in 1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 M 2-(*N*-morpholino)-ethanesulfonic acid, and 0.2 mg/ml NaN_3, titrated to pH 6.5 with NaOH). The homogenate was centrifuged at 85,000 $\times$ *g* for 1 h, and 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 then centrifuged at 80,000 $\times$ *g* for 75 min and collected from the 10–40% interface; they were washed in homogenization buffer and pelleted at 85,000 $\times$ *g* for 1 h. Wheat germ agglutinin was added to a concentration of 1 mg/10 mg of protein and incubated overnight at 4°C. The agglutinated material was sedimented at 20,000 $\times$ *g* for 15 min. The resultant CCV preparation was analyzed by SDS-PAGE and subjected to either silver staining or Western blotting.

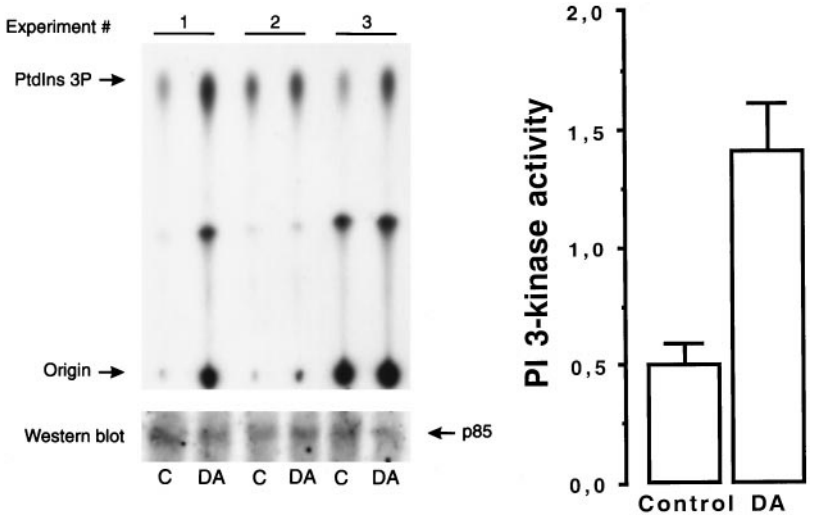

Figure 1. DA stimulates PI 3-kinase activity. PCT cells were incubated with 1 μM DA for 2.5 min at room temperature. The left panel shows three independent measurements of the formed PtdIns 3P with the corresponding Western blot of the immunoprecipitated p85. The right panel shows the quantitative data of the three experiments in which PI 3-K activity is expressed as the ratio of radiolabeled PtdIns 3P quantitated by phosphoimaging and normalized to the scanned p85 subunit (arbitrary units) in the immunoprecipitate.

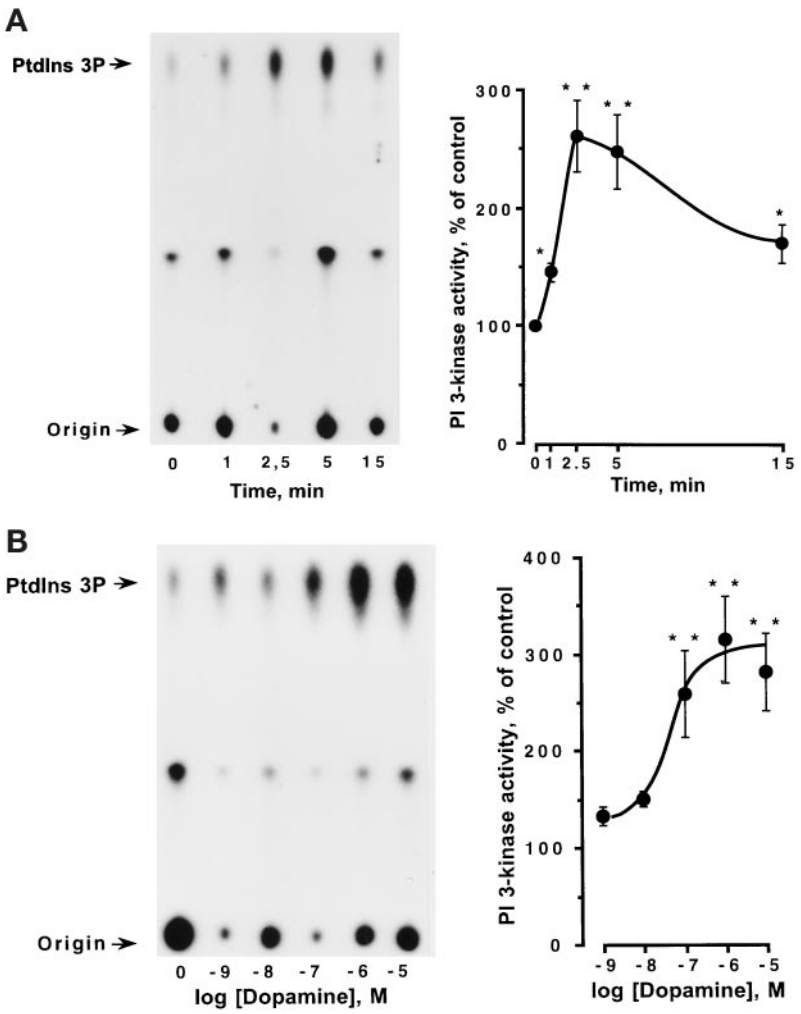

Figure 2. Time- and dose-dependent stimulation of PI 3-kinase activity by DA. PCT cells were exposed to 1 μM DA for different periods (A) or to different concentrations of DA for 2.5 min (B), both at room temperature. Left panels show a representative experiment in which the formed PtdIns 3P was analyzed by TLC. The right panels depict the corresponding phosphoimaging quantitations of radiolabeled PtdIns-3P. PI 3-K was expressed as the amount of Ptdins 3P formed compared with control (vehicle-treated) cells. Each data point represents the mean ± SEM of four (triplicate) experiments. *, $p < 0.05$; **, $p < 0.01$.

Miscellaneous

Proteins were analyzed by SDS-PAGE (7.5–15%) using the Laemmli (1970) buffer system. Protein content was determined according to the method of Bradford (1974). Western blots were developed with an enhanced chemiluminescence (Amersham, Buckinghamshire, United Kingdom) detection kit, used 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; the area of the peak minus the background (in arbitrary units) was quantitated; and the data were analyzed using the Desk Scan II software. Quantitation of the radiolabeled formed phosphatidylinositol 3-phosphate (PtdIns 3-P) was performed using a Fuji (Tokyo, Japan) Bas 1000 bio-imaging analyzer, and the data (arbitrary units) were analyzed using Tina 2.07 ray test software (Isotopenmessyeräte GmbH, Staulenhardt, Germany).

Statistics

Comparisons between two experimental groups were made with the unpaired Student's *t* test. For multiple comparisons one-way ANOVA with Sheffe's correction was used. $p < 0.05$ was considered significant.

RESULTS

Effect of Dopamine on Phosphatidylinositol 3-K Activity

In renal PCT cells DA stimulates PI 3-K twofold to threefold (Figure 1) and the increment occurred in a time-dependent (Figure 2A) and dose-dependent (Figure 2B) manner. Incubation with 1 μM DA induced a significant stimulation of PI 3-K activity at 1 min, which was maximal (~250% of control) after 2.5 min;

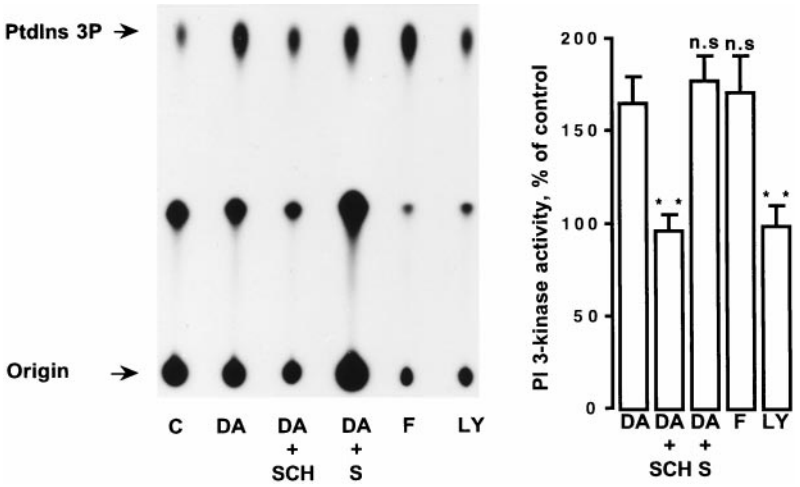

Figure 3. DA receptor type involved in the stimulation of PI 3-kinase activity. PCT cells were incubated with 1 μM DA (2.5 min at 23°C) in the presence or absence of D_1 and D_2 DA receptor antagonists [1 μM SCH 23390 (SCH) or 1 μM *S*-sulpiride (S), respectively] or D_1 and D_2 receptor agonists [1 μM fenoldopam (F) or 1 μM quinpirole (LY), respectively]. All of these ligands are highly selective or specific for these two DA receptor subtypes in renal tubules (Felder *et al.*, 1989c; Jose *et al.*, 1992). The left panel is a representative TLC separation of PtdIns-3P, and the right panel shows the corresponding quantitative data [mean ± SEM of three (triplicate) experiments] obtained by phosphoimaging quantitation of radiolabeled PtdIns 3P. PI 3-K was expressed as the amount of Ptdins 3P formed compared with control (vehicle-treated) cells. **, $p < 0.01$ (DA + SCH vs DA and LY vs F).

the dose necessary to obtain maximal stimulation was 1 μM.

Several isoforms of the DA receptor have been described in the kidney (Jose *et al.*, 1992) where the D_{1A} and D_2 subtypes are localized in the PCT cells. We therefore examined their involvement in the DA effect using selective receptor agonists and antagonists (Figure 3). Fenoldopam (D_1 agonist, 1 μM) stimulated PI 3-K activity, whereas quinpirole (D_2 agonist, 1 μM) had no effect. In agreement, the stimulatory effect of 1

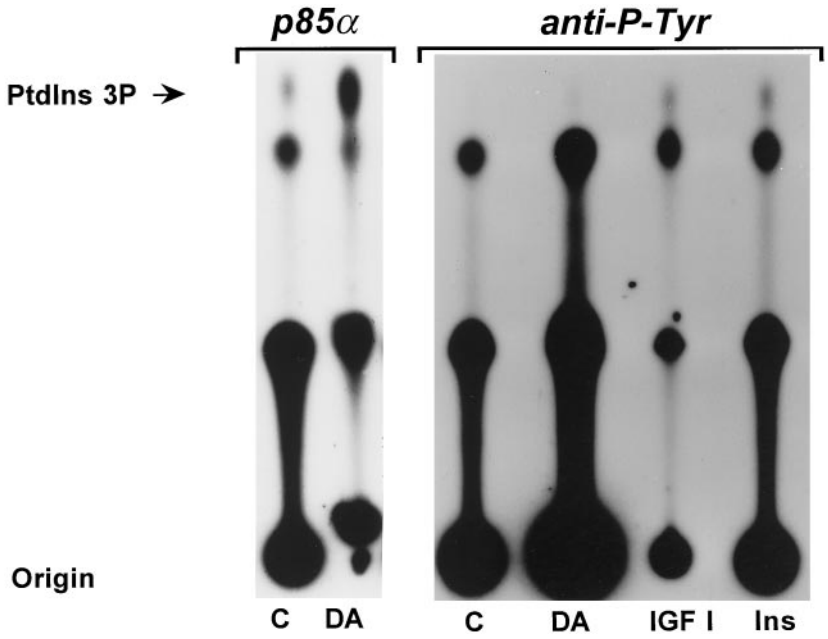

Figure 4. PI 3-kinase activity in vehicle- and DA-treated PCT cells was determined in immunoprecipitates with an anti-p85α and anti-phosphotyrosine antibody. Only the p85α revealed PI 3-K activity. Controls were performed in PCT cells treated with insulin-like growth factor I (100 ng/ml, 2.5 min) and insulin (10 nM, 2.5 min). Ins, insulin.

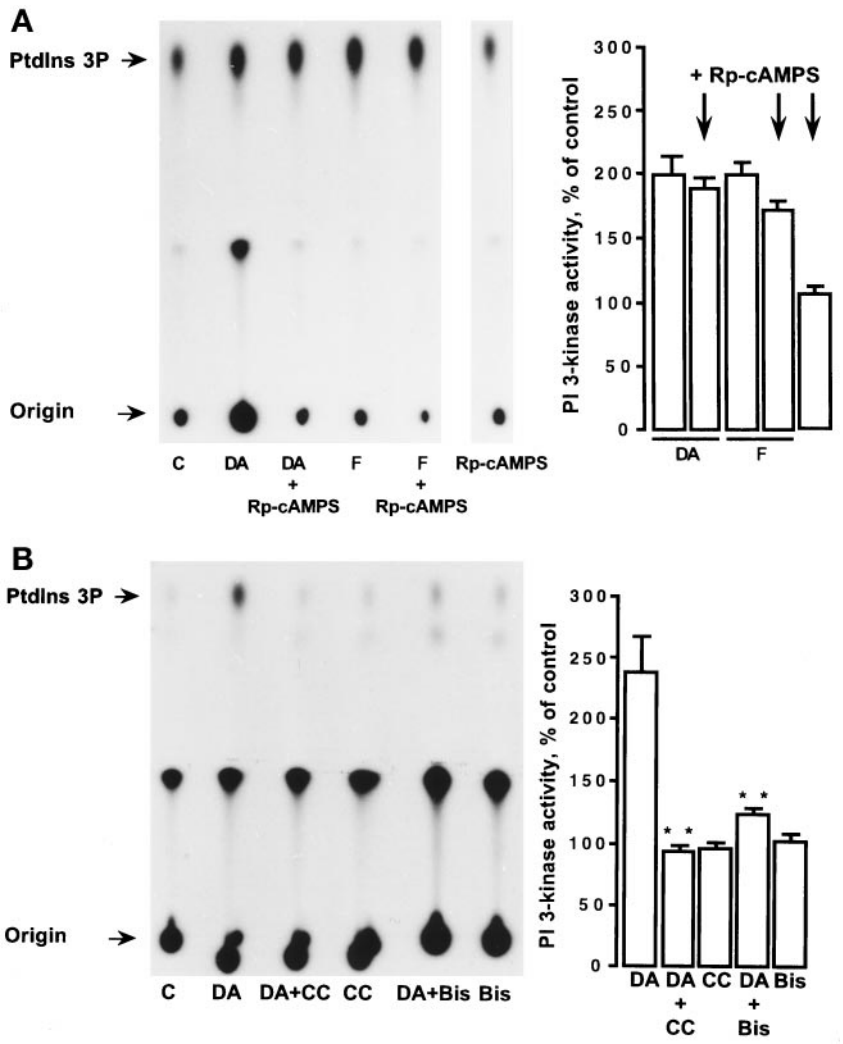

Figure 5. The stimulatory effect of DA on PI 3-kinase activity requires protein kinase C. PCT cells were incubated with 1 μM DA (2.5 min at 23°C) in the presence or absence of a cAMP-dependent protein kinase inhibitor Rp-cAMPS (500 μM; A) or PKC inhibitors calphostin C (1 μM) or bisindolylmaleimide (1 μM; B). Left panels show representative TLC separations of the formed Ptdins 3P during the PI 3-kinase assay, and the right panels show the quantitative data [mean ± SEM of three (triplicate) experiments] of formed radiolabeled Ptdins 3P obtained by phosphoimaging analysis. PI 3-K was expressed as the amount of Ptdins 3P formed compared with control (vehicle-treated) cells. F, fenoldopam.

μM DA on PI 3-K activity was abolished by 1 μM SCH 23390 (D_1 antagonist), whereas it was unaffected by coincubation with 1 μM *S*-sulpiride (D_2 antagonist).

In PCT cells incubation with either insulin-like growth factor I or insulin increased PI 3-K activity (Figure 4) in immunoprecipitates with a phosphotyrosine antibody (P-Tyr), whereas in DA-treated PCT cells the increased PI3-K was not evident by immunoprecipitation with a P-Tyr antibody (Figure 4), suggesting that Tyr phosphorylation is not involved in the DA effect. Regulation of PI 3-K has also been linked to Ser and Thr phosphorylation (Reif *et al.*, 1993) and directly by heterotrimeric G proteins (Stoyanov *et al.*, 1995). Binding to D_1 receptors can cause either an increase in the cellular levels of cAMP (Felder *et al.*, 1989c) or stimulation of protein kinase C activity via phospholipase C (Felder *et al.*, 1989a,b) and phospholipase A_2 (Satoh *et al.*, 1992). Therefore, we next examined the possibility that an increase in cAMP could have been responsible for the stimulation of PI 3-K activity. Incubation of renal PCT cells with forskolin (10 μM) did not cause a significant change in PI 3-K activity (our unpublished results). Furthermore, the cAMP analogue Rp-cAMPS, 500 μM, did not alter the ability of DA or fenoldopam to increase PI 3-K activity (Figure 5A).

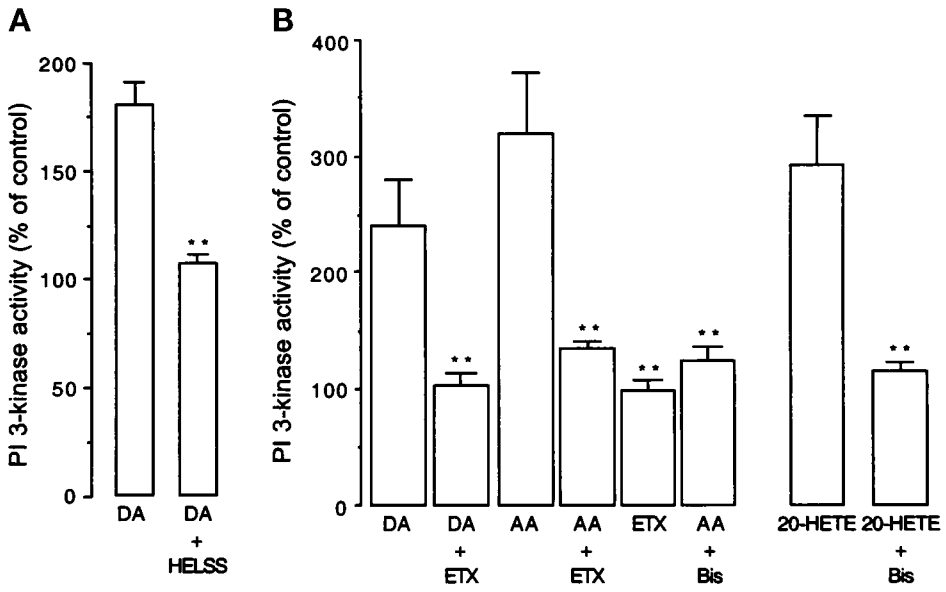

Figure 6. (A) Effect of DA on PI 3-K activity in the presence of the Ca^{2+}-independent phospholipase A_2 inhibitor HELSS. Each bar represents the mean ± SEM of three experiments performed independently and in triplicate. (B) DA and arachidonic acid (AA, 100 nM) stimulate PI 3-K activity, and their effect is blocked by ethoxyresorufin (ETX, 100 nM), a specific inhibitor of the cytochrome P450 monooxygenase pathway. The effect of AA or of its cytochrome P450 monooxygenase metabolite 20-HETE (10 nM) was also determined in the presence or absence of the PKC inhibitor bisindolylmaleimide (Bis, 1 μM). In all protocols the cells were incubated with the different agonists for 2.5 min at 23°C. Each bar represents the mean ± SEM of three separate (triplicate) experiments. **, $p < 0.01$ versus corresponding value without inhibitor.

Endocytosis of Na^+,K^+-ATPase α subunits in response to DA requires PKC activation (Chibalin *et al.*, 1998). It was thus of interest that the ability of DA to stimulate the PI 3-K was inhibited by calphostin C or bisindolylmaleimide, two specific PKC inhibitors (Figure 5B). These results suggest that activation of PKC is a necessary step required for PI 3-K stimulation.

The stimulatory effect of DA on PI 3-K activity was also abolished by pretreatment of the cells with HELSS, 25 μM (Figure 6A), suggesting that it requires activation of a Ca^{2+}-independent phospholipase A_2 (Lehman *et al.*, 1993; Portilla *et al.*, 1994), which may be followed by increased production of AA and of its cytochrome P450 monooxygenase metabolite 20-HETE. In renal PCT, AA and 20-HETE inhibit Na^+,K^+-ATPase activity (Satoh *et al.*, 1993), and this effect is linked to activation of PKC (Nowicki *et al.*, 1997). We therefore examined whether the DA and AA inhibition of Na^+,K^+-ATPase activity through stimulation of PI 3-K may occur via the cytochrome P450 pathway. When PCT cells were incubated with DA or AA, there was an increase in PI 3-K activity that was abolished by ethoxyresorufin, a selective cytochrome P450 monooxygenase inhibitor (Figure 6B). Additionally, 20-HETE, the principal metabolic product of this pathway in PCT cells, also stimulated PI 3-K activity. The effect of both AA and 20-HETE was blocked by the protein kinase C inhibitor bisindolylmaleimide (1 μM) (Figure 6B).

Activation of Phosphatidylinositol 3-kinase Is Necessary for Na^+,K^+-ATPase α Subunit Endocytosis

PI 3-kinase is inhibited by wortmannin (Arcaro *et al.*, 1993). Similarly, endocytosis of Na^+,K^+-ATPase α subunits in CCV and endosomes in response to 1 μM DA was blocked by wortmannin in a dose-dependent manner (Figure 7A), with a maximal inhibitory concentration of ≥100 nM. Because of the discrepancy between the dose of wortmannin necessary to prevent endocytosis and that reported to inhibit PI 3-K activity in vitro (1–10 nM), we examined the effect of different concentrations of wortmannin on DA-stimulated PI 3-K activity in vitro (wortmannin was added during the assay), and in intact cells (cells preincubated with wortmannin before the addition of DA; Figure 7B). Although the maximal inhibitory concentration of wortmannin during the in vitro assay was within the lower nanomolar range as reported by others, the maximal inhibitory concentration of wortmannin applied to intact cells was higher, corresponding to that needed to abolish endocytosis in CCV, EE, and LE.

DA-induced endocytosis of Na^+,K^+-ATPase α subunits was also evaluated in the presence of another selective PI 3-K inhibitor, LY-294002 (Vlahos *et al.*, 1994). Similar to the effect of wortmannin, the action of LY-294002 on PI 3-K differed when it was evaluated in

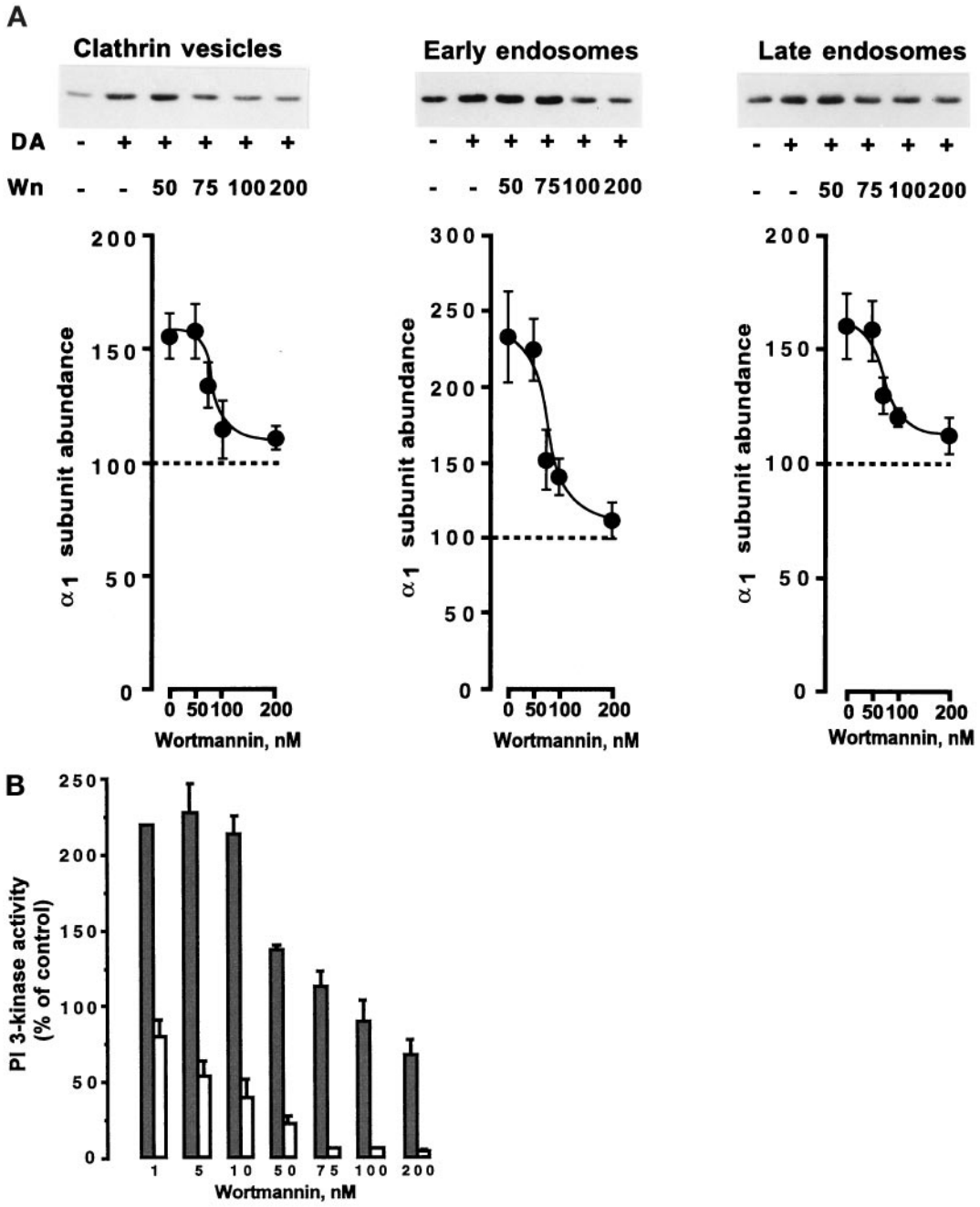

Figure 7. (A) DA-induced endocytosis of Na^+,K^+-ATPase α subunits in the presence of a PI 3-kinase inhibitor. PCT cells were preincubated with different concentrations of wortmannin at room temperature for 30 min, followed by incubation with DA, 1 μM, for an additional 15 min. DA-induced endocytosis of Na^+,K^+-ATPase α subunits was subsequently determined in CCV, EE, and LE and expressed as percent of untreated controls. Top panels depict Western blots, and bottom panels depict the quantitative data (mean ± SEM of five experiments). (B) Effect of wortmannin on PI 3-kinase activity from DA-treated PCT cells. PCT cells were incubated with DA (1 μM; 2.5 min at 23°C) alone and with DA in the presence of increasing concentrations of wortmannin (1–200 nM; hatched bars) 20 min (23°C) before incubation with DA. PI 3-kinase activity was determined in the immunoprecipitates with an anti-p85α antibody and expressed as percentage of control. Each bar represents the mean ± SEM of four experiments performed in triplicate. The effect of wortmannin on DA-stimulated PI 3-K activity was also determined in vitro (open bars). PCT cells were incubated with DA, 1 μM (2.5 min at 23°C). PI 3-kinase was immunoprecipitated with an anti-p85α antibody, and the activity (ability to phosphorylate PtdIns) was determined in vitro in the presence of different concentrations of wortmannin (1–200 μM). Each bar represents the mean ± SEM of three experiments performed in triplicate.

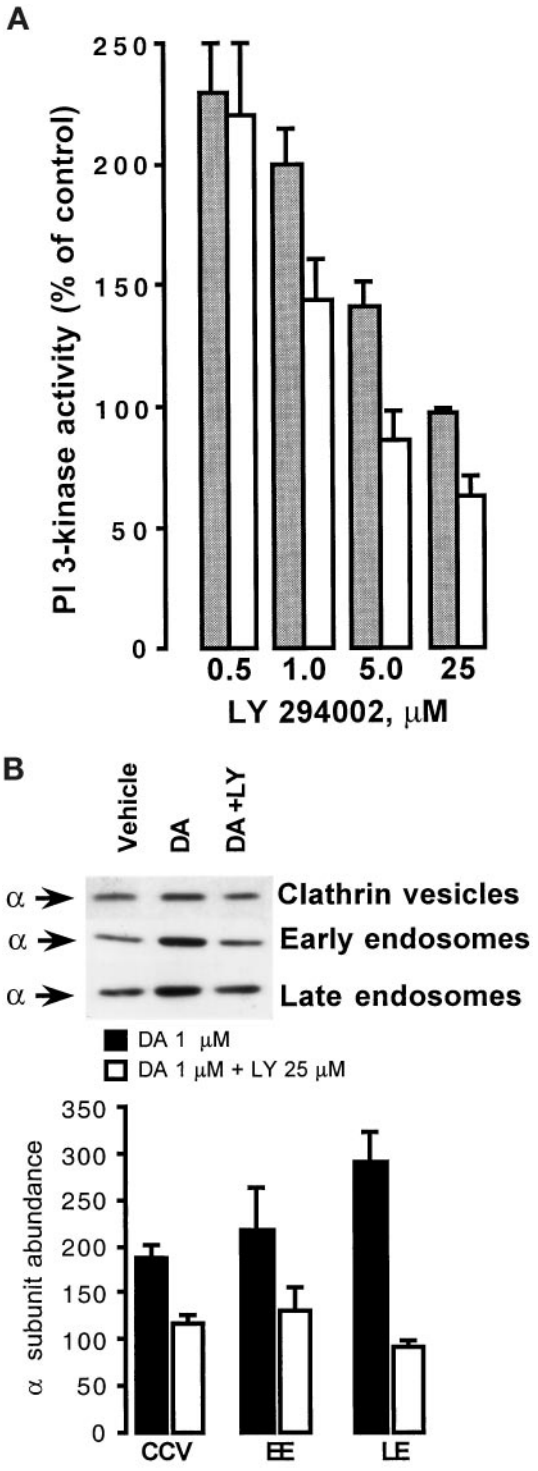

Figure 8. DA-stimulated PI 3-kinase activity (A) and increased α subunit endocytosis (B) in the presence of LY 294002. (A) Cells were either previously treated for 20 min at room temperature with different concentrations of LY294002 (hatched bars), and PI 3-K was determined, or PI 3-K was measured in vitro in the presence of different concentrations of LY 294002 after being precipitated from cells previously treated with 1 μM DA for 15 min at 23°C (open bars). PI 3-K activity was expressed as percent of control. Each bar represents the mean ± SEM of three experiments performed in duplicate. (B) Na^+,K^+-ATPase α subunit endocytosis in response to DA (1 μM; 15 min at 23°C) from cells that had been previously treated for 20 min at room temperature with 25 μM LY 294002. A representative Western blot is shown in the top panel, and the corresponding quantitative data from four experiments performed independently are presented in the bottom panel.

intact cells or in vitro (Figure 8A). This inhibitor also prevented the DA-induced endocytosis of Na^+,K^+-ATPase α subunits into CCV and EEs and LEs (Figure 8B).

DISCUSSION

Although activation of PI 3-K has been associated with internalization of membrane receptors, this study demonstrates that an integral membrane transport protein undergoes endocytosis in response to a membrane receptor signal, and that the link between the receptor (D_1) and the effector (Na^+,K^+-ATPase) requires activation of PI 3-K.

Our results indicate that DA activates PI 3-K activity in a time- and dose- dependent manner, and that this activation follows DA interaction with its D_1 receptor subtype.

Increased PI 3-K activity generally occurs by its association with tyrosine-kinase receptors, provided they can activate their intrinsic kinase activity and/or by binding of the p85 (regulatory) subunit of PI3-K to proteins that have been Tyr phosphorylated (Kappeller and Cantley, 1994). In these experiments, immunoprecipitation with a P-Tyr antibody did not reveal PI 3-K activity, suggesting that in PCT cells the effect of DA does not involve Tyr phosphorylation.

Activation of D_1 receptors in renal PCT increases cellular levels of cAMP (Felder *et al.*, 1989c), as well as enhancing PKC activity through stimulation of phospholipase C (Felder *et al.*, 1989a; Satoh *et al.*, 1992) and phospholipase A_2 (Satoh *et al.*, 1992). We therefore sought to determine which of these alternative pathways is involved in stimulation of PI 3-K activity by DA in PCT cells. Involvement of a cAMP-dependent protein kinase appears unlikely in view of the lack of effect of forskolin and the inability of an inhibitory cAMP analog to abolish the stimulation by either DA or the D_1 agonist fenoldopam. In contrast, two PKC inhibitors, calphostin C and bisindolylmaleimide, blocked PI 3-K stimulation by DA.

The intracellular signaling cascade initiated after DA binding to the D_1 receptor subtype that leads to the activation of PKC probably involves activation of a Ca^{2+}-independent phospholipase A_2 (endocytosis and kinase activity are blocked by HELSS), generation of AA, and increased production of its main PCT cyto-

chrome P450 metabolite, 20-HETE. It has been recently suggested that inhibition of Na^+,K^+-ATPase by 20-HETE in proximal tubules is mediated by PKC (Nowicki *et al*., 1997). In agreement, the stimulatory action of AA and 20-HETE on PI 3-K activity in our experiments was abolished by the PKC inhibitor bisindolylmaleimide (Figure 6).

Preliminary experiments performed in PCT cells metabolically labeled with [^{32}P]orthophosphate did not demonstrate any significant increase in the state of phosphorylation of the immunoprecipitated p85 in response to DA. Because Tyr phosphorylation appears not to be involved in this phenomenon, it is possible that DA activation of PI 3-K via PKC is accomplished by phosphorylation of an intermediate regulatory protein.

A role for phospholipase A_2 (PLA_2) in the signaling pathway of DA has been described in renal proximal tubules (Bertorello and Katz, 1993). In this study we found that the stimulation of PI 3-K is blocked by HELSS. The role of PLA_2 in endocytosis by activation PI 3-K activity is also supported by recent observations that endosomal fusion is blocked by inhibitors of PLA_2 and that this effect is prevented by arachidonic acid (Mayorga *et al*., 1993)

Inhibition of Na^+,K^+-ATPase by DA in renal PCT (Bertorello and Aperia, 1990; Takemoto *et al*., 1992) and in neostriatal neurons (Bertorello *et al*., 1990) requires the combined activation of both D_1 and D_2 receptors. Stimulation of cAMP production in renal PCT cells has been associated with phosphorylation of Na^+,K^+-ATPase α subunit and stimulation of its catalytic activity (Carranza *et al*., 1996b), whereas the inhibitory action of DA as well as endocytosis of the Na^+,K^+-ATPase α subunit is blocked by PKC inhibitors (Bertorello and Katz, 1993; Chibalin *et al*., 1997). Thus, it is likely that the inhibitory effect of DA on Na^+,K^+-ATPase activity is mediated by PKC, and cAMP may be an important cofactor in this regulatory mechanism.

We have used two inhibitors of PI 3-K to determine its role in Na^+,K^+-ATPase α subunit endocytosis in response to DA. Treatment of intact cells with wortmannin before incubation with DA abolished the incorporation of Na^+,K^+-ATPase α subunits in CCV, EE, and LE. Wortmannin prevents the activation of several phospholipases (PLC, PLD, and PLA_2) in response to mitogens (Cross *et al*., 1995). Although the effect of DA on Na^+,K^+-ATPase α subunit endocytosis is mediated by activation of a Ca^{2+}-independent PLA_2, it is unlikely that wortmannin elicited its effect by inhibition of PLA_2, because in intact cells maximal inhibition of PLA_2 occurs with 10 nM wortmannin (Cross *et al*., 1995), a concentration that in our experiments did not affect the ability of DA to increase incorporation of Na^+,K^+-ATPase α subunits in CCV, EE, and LE.

The mechanisms by which activation of PI 3-K could lead to endocytosis of the Na^+,K^+-ATPase α subunit are presently unknown. A likely posibility could be, as recently proposed by Li *et al.* (1995), that PI 3-K activates *rab*5, a necessary protein for transport of CCV to EE. Because in the present study the formation of CCV was blocked by wortmannin and activation of PI3-K activity occurred as early as after 1 min, these results suggest that PI 3-K is probably involved in the very early stages of endocytosis, such as clathrin-coated pit formation and CCV transport to early endosomes.

Taken together, our observations reveal a critical function of PI 3-K during endocytosis of an integral membrane protein, the Na^+,K^+-ATPase. Moreover, they demonstrate that stimulation of PI 3-K, which is usually associated with growth factor receptors, can also be accomplished by activation of catecholamine receptors, such as those for DA.

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