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Dopamine-induced recruitment of dopamine D1 receptors to the plasma membrane

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ABSTRACT **The recruitment of G protein-coupled receptors from the cytoplasm to the plasma membrane generally is believed to be a constitutive process. We show here by the use of both confocal microscopy and subcellular fractionation that, for at least one such receptor, this recruitment is regulated and not constitutive. Cells from a proximal tubular-like cell line, LLCPK1 cells, were incubated with either a D1 agonist, a dopamine precursor, or an inhibitor of dopamine metabolism to increase dopamine availability in the cell. Each of the three procedures led to a rapid translocation of dopamine D1 receptors from the cytosol to the plasma membrane.**

It is well established that the levels of receptors for hormones and neurotransmitters present on the plasma membrane alter in response to changes in the levels of the ligands for these receptors. There is a great deal of evidence that such ligands can alter the rate of sequestration or endocytosis of the receptors and thus play a major role in receptor desensitization. The present study provides evidence that ligand recruitment of receptors to the plasma membrane is also regulated by ligands.

The renal dopamine system is of utmost importance for the regulation of salt metabolism (1–3). Dopamine receptors belonging to the type one family (D1 receptors) have, with a variety of techniques, been demonstrated in renal tubular cells (4–9). The receptors can be activated by locally formed dopamine. In the proximal tubules, inhibition of dopamine degradation as well as treatment with a dopamine precursor leads to inhibition of tubular Na^+,K^+-ATPase activity and profound natriuresis (10). We describe here, with use of confocal laser scanning microscopy, the effect of dopamine accumulation on the localization of the D1 receptor.

METHODS

Cell Culture. LLCPK-1 cells taken from passages 3–13 were grown in DMEM, pH 7.6, supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Confluent cells were washed twice with warm PBS, harvested by trypsinization, replated on fibronectin-coated coverslips, and incubated for 1 hr at 37°C, 95%/5% CO_2.

Immunofluorescence. The cells were washed once with warm PBS and incubated with drug or vehicle (control) for the desired time, then fixed for 10 min in ice-cold 2% paraformaldehyde (D1 and Na^+,K^+-ATPase labeling) or for 15 min in ice-cold 3% glutaraldehyde, 0.5% paraformaldehyde (dopamine labeling). After washing with PBS, cells were incubated for 10 min in PBS containing sodium borohydride (2 mg/ml), permeabilized, and blocked with 7% normal goat serum (NGS), 5% nonfat dry milk (NFDM), and 0.1% Triton X-100 in PBS for 30 min. Unpermeabilized cells were treated by using the same protocol except that Triton X-100 was omitted from the buffers. The cells were then incubated overnight at 4°C with primary rabbit-anti-dopamine antibody (dopamine labeling) or rabbit-anti-D1 receptor (D1 labeling) in PBS containing 1.4% NGS, 1% NFDM, and 0.1% Triton X-100. After washing with PBS containing 0.1% Triton X-100, the cells were incubated for 1 hr at room temperature with secondary goat anti-rabbit-Texas Red X antibody (Molecular Probes) for D1 labeling or goat-anti-rabbit-Cy5 antibody (Jackson ImmunoResearch) for dopamine labeling in the same buffer as with the primary antibody. For double labeling with Na^+,K^+-ATPase, an additional incubation step with a mouse mAb against the α subunit of Na^+,K^+-ATPase (gift from M. Caplan, Yale University) was done following the same protocol as for the other primary antibodies and labeled with goat-anti-mouse-Oregon Green as secondary antibody (Molecular Probes). The cells were then washed again and mounted in Prolong antifade (Molecular Probes).

Confocal Microscopy. The immunolabeled cells were recorded with a Zeiss LSM410 invert confocal scanning laser microscope with excitation at 488 (Oregon Green), 543 (Texas Red X), and 633 nm (Cy-5) and detection at 510–525 nm for Oregon Green, 590–610 nm for Texas Red X, and long-pass 665 nm for Cy-5 using a $\times 63/1.4$ NA objective. Thin optical sections spaced 0.2 μm apart were recorded throughout the cells. Gain and brightness settings were controlled to use the full dynamic range of the detectors. The recorded images were postprocessed by using a Gaussian (3×3) filter for noise reduction. Optical sections taken through the center of the cells were used for the interpretation of the results.

Ouabain-Sensitive Rubidium-86 Uptake. Rubidium uptake was used as an index of Na^+,K^+-ATPase activity in LLCPK-1 cells. Briefly, confluent cells grown on six-well culture plates were incubated for 10 min with or without 1 mM ouabain. The cells were treated with 10 mM nitecapone in the presence or absence of 10 mM SCH23390 (D1 antagonist) for 20 min, and 0.05 ml of 50 mCi/ml 86Rb+ was added to each well; after 2 min the cells were lysed and the radioactivity in the cell lysate was measured. Na^+,K^+-ATPase activity was determined as the difference in 86Rb+ uptake in the absence and presence of ouabain.

cAMP Accumulation Assay. LLCPK-1 cells, preincubated with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) in DMEM for 10 min, were treated with either 5 μM glu-dopa or 5 μM nitecapone for 1 and 20 min, respectively, in the presence and absence of 5 μM SCH23390. The reaction was stopped by addition of 1 ml of ice-cold 0.1 M HCl. The cells were harvested, lysed, and centrifuged. cAMP accumulation in the supernatant was assayed by using a cAMP radioimmunoassay kit (DuPont/NEN).

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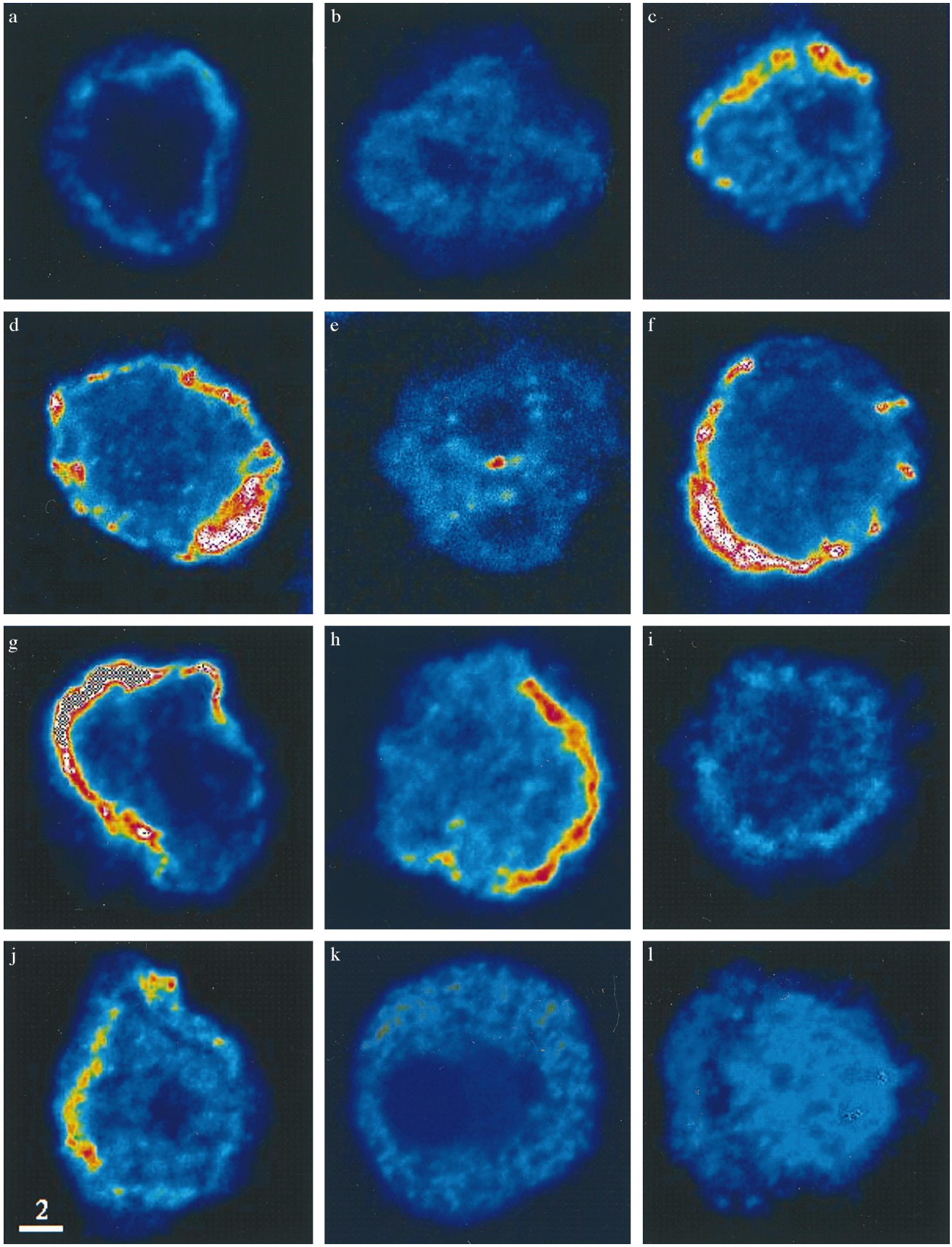

FIG. 1. Dopamine-induced recruitment of D1 receptors to the plasma membrane visualized by confocal microscopy. The images are represented by using a pseudo color scale, where blue is low intensity and yellow-red is high intensity. (Bar = 2 μm.) (*a*) Nonpermeabilized cell. (*b–l*) Cells treated as indicated, and then permeabilized with Triton X-100 after fixation and before incubation with the antibody. (*b*) Untreated. (*c*) Fenoldopam (1 μM), 1 min. (*d*) Fenoldopam (1 μM), 15 min. (*e*) Preincubation for 1 min with dopamine antagonist (SCH23390; 1 μM); incubation for 1 min with SCH23390 + 50 nM fenoldopam. (*f*) Preincubation for 1 min with 50 nM fenoldopam; incubation for 1 min with SCH23390 50 nM

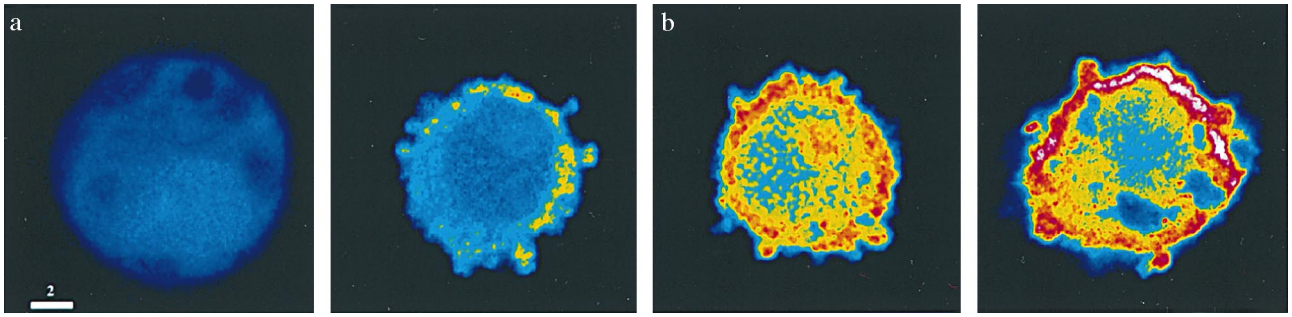

FIG. 2. The effect of glu-dopa and nitecapone on dopamine level and distribution in LLCPK-1 cells. Confocal micrographs showing dopamine in LLCPK-1 cells. The images are represented by using a pseudo color scale, where blue is low intensity and yellow-red is high intensity. From left to right are control, untreated, glu-dopa (5 μM, 1 min), and nitecapone (5 μM, 20 min). By using the fluorophore Cy5 (excitation maximum, 649 nm; emission maximum, 670 nm), the effect of autofluorescence from glutaraldehyde fixation was reduced.

Subcellular Fractionation. Outer cortical slices (250 μm) were dissected from rat kidney and kept in ice-cold buffer containing 124 mM NaCl, 26 mM $NaHCO_3$, 10 mM D-glucose, 1.5 mM $MgSO_4$, 1 mM *n*-butyric acid, 1.5 mM CaCl2, and 0.25 mM KH_2PO_4. The tissues were bubbled with 95% O_2 and 5% CO_2 in the same buffer for 10 min at 37°C. After treatment with drugs or vehicle for the indicated periods of time, the medium was aspirated and the tissue slices were frozen quickly. The slices were thawed and mixed with Harm's buffer containing 250 mM sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, pH 7.45, and protease inhibitors 5 μg/ml chymostatin, 5 μg/ml pepstatin, 10 μg/ml antipain, 10 μg/ml leupeptin, and 100 μM PMSF. The slices were homogenized and centrifuged at 2,000 $\times$ *g* for 10 min at 4°C. The postnuclear supernatant (PNS) was subjected to subcellular fractionation as described (26, 27). Briefly, PNS was layered on top of a prechilled 10–40% sucrose gradient with 1 ml of 65% cushion containing the protease inhibitors and centrifuged at 96,500 $\times$ *g* for 16 hr at 4°C. Fractions of 1 ml were collected from the bottom of the tubes. Each fraction (1–10) was electrophoresed and blotted on polyvinylene difluoride membrane. The unspecific sites were blocked with 5% nonfat dry milk in PBS/0.1% Tween. The catalytic α subunit of renal Na^+,K^+-ATPase in vehicle-treated tissue was probed with a mAb (gift from M. Caplan). D1 receptor in vehicle and drug-treated tissue was probed with affinity-purified rabbit-anti-D1 antibody. HRP-conjugated secondary antibodies were used to probe the primary antibodies. The detection of specific bands was visualized by using a chemiluminescence method (ECL Plus Kit, Amersham).

RESULTS

D1 receptors were localized by immunofluorescent staining with a well characterized and highly specific antibody (8), which recognizes the third extracellular loop of the receptor. In nonpermeabilized LLCPK-1 cells, D1 receptor immunoreactivity was detected on the cell surface (Fig. 1*a*). In permeabilized cells, the immunoreactive signal was present throughout the cell (Fig. 1*b*). Consistent with this observation, cytoplasmic localization of the D1 receptor has been reported previously in studies by using electron microscopy (8). Incubation of cells with fenoldopam, a D1 agonist, for 1 min (Fig. 1*c*) or 15 min (Fig. 1*d*) resulted in a translocation of the receptor to the region of the plasma membrane. When the cells were preincubated with a highly specific D1 antagonist, SCH23390, before the addition of fenoldopam, the effect was abolished (Fig. 1*e*). We next determined whether the accumulation of receptors in the plasma membrane was due to an increased translocation from the cytosol or to a decreased rate of internalization. For this purpose the antagonist was added 1 min after the agonist, at which time receptors already had accumulated in the plasma membrane. Under this condition, the antagonist had no effect on the amount of receptor present in the plasma membrane (Fig. 1*f*), indicating that the accumulation of D1 receptors in the plasma membrane was not due to a decreased rate of internalization. Because activated D1 receptors are coupled to adenylyl cyclase, we tested the effect of forskolin, a direct stimulator of adenylyl cyclase, on receptor distribution. Forskolin had a detectable effect at 10^{-9} M (not shown), and a prominent effect at 10^{-7} M (Fig. 1*g*) on the translocation of receptors to the plasma membrane.

LLCPK-1 cells possess aromatic amino acid decarboxylase (AADC), the enzyme that converts L-dopa to dopamine, and catechol-O-methyl transferase (COMT), the enzyme that degrades dopamine to its main urinary metabolite, homovanillic acid. We tested the effect of nitecapone (5 μM), an inhibitor of peripheral COMT, on dopamine content, D1 receptor localization, and cAMP level in LLCPK-1 cells. The cellular content of dopamine was examined by using a glutaraldehyde-conjugated dopamine antibody (14) (Fig. 2). In untreated but permeabilized cells dopamine immunofluorescence was detected mainly in the region of the plasma membrane. The immunoreactive signal became stronger after 20 min of nitecapone treatment. The dopamine signal in the treated cells was considerably more pronounced in the region of the plasma membrane than in the cytoplasm. Incubation of the cells with nitecapone for 20 min also caused accumulation of the D1 receptor in the plasma membrane region (Fig. 1*h*) and, through activation of the D1 receptor, an increase in cAMP levels (Fig. 3*A*). Nitecapone (5 μM) also caused a 15 $\pm$ 3% inhibition of Na^+,K^+-ATPase activity in LLCPK-1 cells, as determined by ouabain-sensitive rubidium uptake ($n = 9$, $P < 0.05$).

To determine more precisely the localization of the D1 receptor after its movement to the periphery of the cells, we carried out a study of the possible colocalization of the D1 receptor with Na^+,K^+-ATPase, which is well established to be an integral protein of the plasma membrane. In cells treated with 5 μM nitecapone, the translocated receptor colocalized with Na^+,K^+-ATPase, as demonstrated by double-labeling immunocytochemistry (Fig. 4). The localization of the D1 receptor after treatment with nitecapone or forskolin also was evaluated with another methodological approach. Slices from the outer cortex of rat kidney were incubated with nitecapone or forskolin and subjected to sucrose gradient centrifugation.

+ fenoldopam. (*g*) Forskolin (100 nM). (*h*) Nitecapone (5 μM), 20 min. (*i*) Glu-dopa (5 μM), 10 sec. (*j*) Glu-dopa (5 μM), 1 min. (*k*) Preincubation for 1 min with 5 μM SCH23390; incubation for 1 min with SCH23390 + 5 μM glu-dopa. (*l*) Preincubation for 10 min with 20 nM bafilomycin; incubation for 1 min with 5 μM glu-dopa.

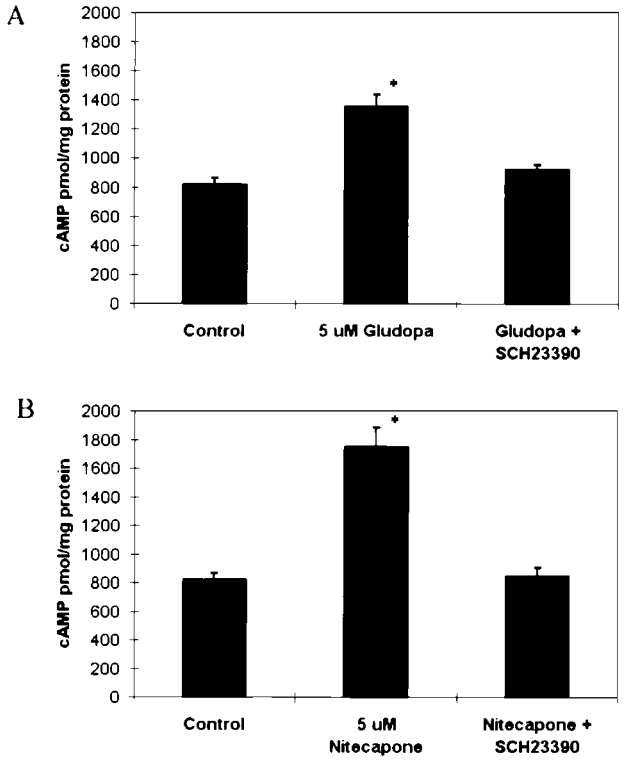

FIG. 3. The effect of nitecapone and glu-dopa on cAMP accumulation in LLCPK-1 cells. (*A*) Nitecapone treatment (5 μM; 20 min) caused an increase in cAMP accumulation (*, $P < 0.05$). This effect was abolished in the presence of a D1 receptor antagonist (SCH23390; 5 μM). (*B*) Glu-dopa treatment (5 μM; 1 min) caused an increase in cAMP accumulation (*, $P < 0.05$). This effect was abolished in the presence of 5 μM SCH23390. Results are means ± SEM for three experiments.

Both nitecapone and forskolin caused an increase of D1 abundance in the plasma membrane fraction (Fig. 5).

The plasma membranes of tubular cells have a high concentration of g-glutamyl transferase, which converts g-L-glutamyl-L-dopa (glu-dopa) to L-dopa (11). L-dopa enters the tubular cell via a specific transporter and is rapidly converted to dopamine via AADC (12, 13). Incubation of LLCPK-1 cells with glu-dopa for 1 min resulted in an increase in dopamine immunofluorescence (Fig. 2). Glu-dopa also caused an accumulation of the D1 receptor in the plasma membrane region. The effect of glu-dopa could be detected at 10 sec (Fig. 1*i*), was maximal at 1 min (Fig. 1*j*), was sustained for 15 min, and declined thereafter (data not shown). When the cells were preincubated with a D1 antagonist (SCH23390, 5 μM), glu-dopa-induced receptor translocation was abolished (Fig. 1*k*). Treatment of the cells with glu-dopa also resulted in receptor activation as judged from the effect on cAMP accumulation (Fig. 3*b*).

G protein-coupled receptors located in the cytoplasm are stored in vesicles and may require an acidic environment (15, 17). Renal proximal tubular cells have a high abundance of endocytic vesicles with an acidic interior, driven by H^+ATPase (18). We tested the possibility that the translocation effect depended on an acidic environment. For this purpose the effect of glu-dopa on D1 receptor localization was studied in cells pretreated with 20 nM of the endosomal H^+ATPase inhibitor bafilomycin. This dose of bafilomycin should selectively inhibit the endosome H^+ATPase (19, 20). Glu-dopa-induced translocation of the D1 receptor was abolished in those cells (Fig. 1*l*).

DISCUSSION

Several members of the G protein-coupled receptor family have been shown to move from the plasma membrane to the cytoplasm. This is a well regulated process that involves several steps of phosphorylation/dephosphorylation and interactions with other proteins. Much less is known about the reverse pathway, i.e., movement of receptors from the cytoplasm to the plasma membrane. The results from the present study show that recruitment of at least one G protein-coupled receptor, the D1 receptor, from the cytoplasm to the plasma membrane can be regulated by agonist availability. Similar findings were obtained with confocal imaging of pig kidney cells and subcellular fractionation of rat kidney slices, indicating that the results are applicable to renal epithelial cells of different species. Receptor translocation was prevented by bafilomycin, an inhibitor of vesicular H^+ATPase, indicating that the D1 receptors are stored/inserted in vesicles with an acidic content.

In the renal proximal tubule, dopamine acts as an autocrine hormone. The LLCPK1 cells mimic proximal tubular cells in that they possess D1 receptors and can form and metabolize dopamine. Incubation of LLCPK1 cells with a dopamine precursor, glu-dopa, or with an inhibitor of cathecol-O-methyltransferase, nitecapone, resulted in a rapid increase of dopamine signal, particularly in the region of the plasma membrane. Glu-dopa, nitecapone, and the D1 agonist, fenoldopam, caused translocation of the D1 receptor from the cytoplasm to the plasma membrane. Theoretically, the agonist effect could have been mediated by (*i*) dopamine binding to receptors

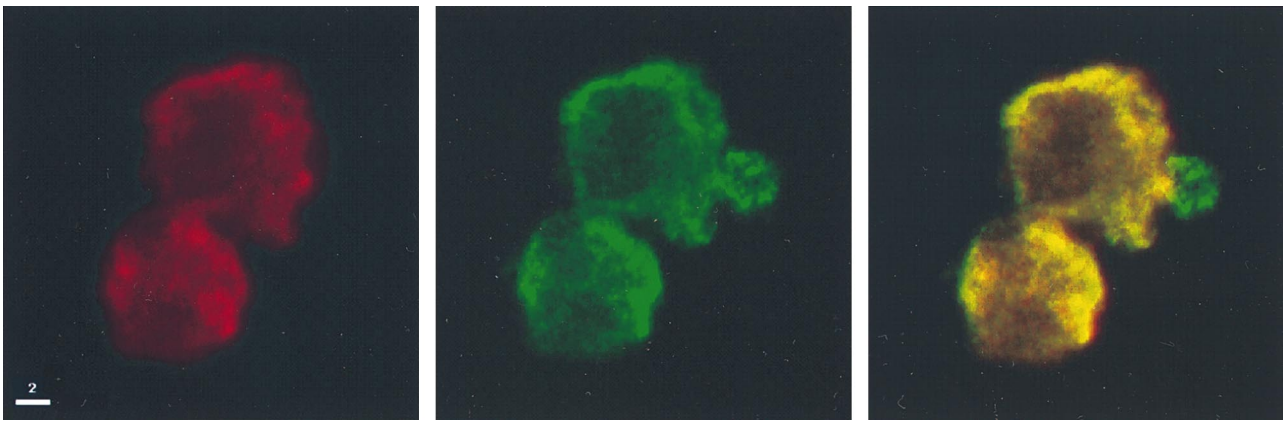

FIG. 4. Colocalization of D1 receptor and Na^+,K^+-ATPase in LLCPK-1 cells in response to nitecapone. Cells were treated with 5 μM nitecapone for 20 min and double-labeled for D1 receptor (red) and Na^+,K^+-ATPase (green) (16, 19, 20). Colocalization of D1 (red) and Na^+,K^+-ATPase (green) is seen as yellow. In the structure extending to the right of the upper cell only Na^+,K^+-ATPase is present, demonstrating that no significant crosstalk between the red and green channels was detected.

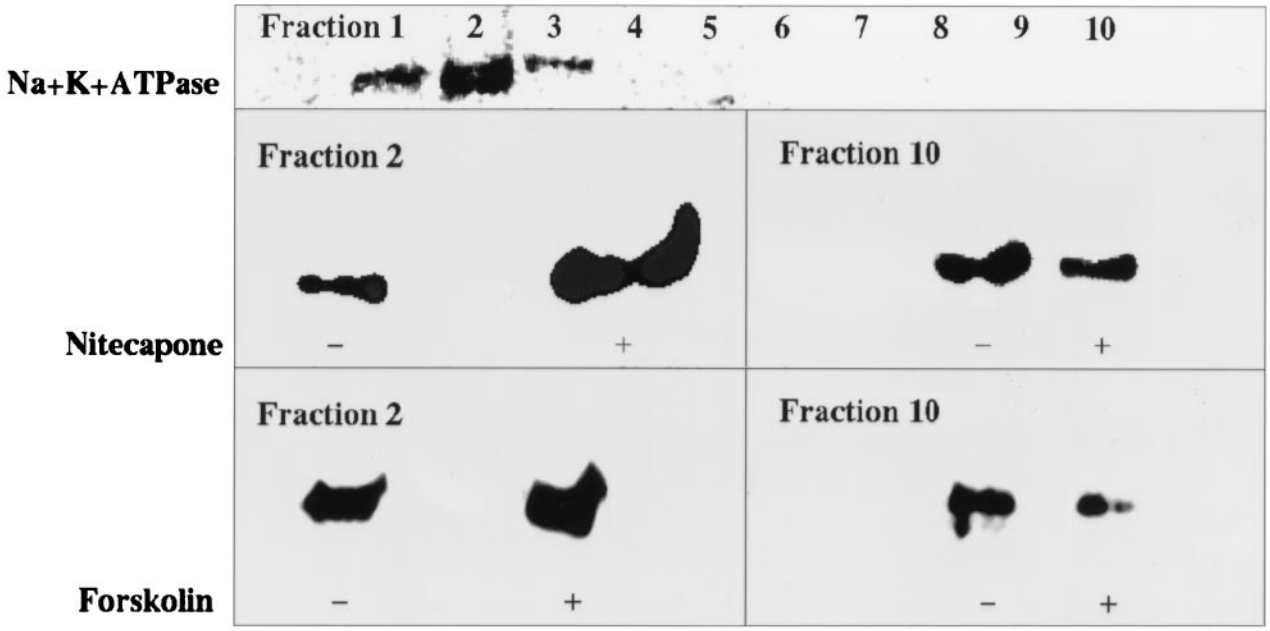

FIG. 5. Localization of D1 receptor and Na^+,K^+-ATPase in renal tissue slices in response to nitecapone and forskolin: subcellular fractionation studies. Slices (250 μm) from outer cortex, rich in proximal tubules, were dissected from rat kidney. The tissues were treated in the absence (−) or presence (+) of either nitecapone (5 μM; 20 min) or forskolin (5 μM; 1 min), homogenized, and subjected to subcellular fractionation by using a 10–40% sucrose gradient. Each fraction (1–10) from vehicle-treated tissue was electrophoresed, blotted on a polyvinylene difluoride membrane, and probed for Na^+,K^+-ATPase. The amount of Na^+,K^+-ATPase was highest in fraction 2 and absent from fractions 4–10. After treatment with nitecapone or forskolin, the fractions were probed for the D1 receptor. The amount of the D1 receptor increased in the Na^+,K^+-ATPase-containing fractions, illustrated by fraction 2, and decreased in the fractions devoid of Na^+,K^+-ATPase, illustrated by fraction 10. Both nitecapone and forskolin caused an increase of D1 abundance in the plasma membrane fraction (3.13 ± 0.38 and 2.27 ± 0.37 times, respectively, compared with control, $P < 0.01$). Results are representative of three experiments.

present in the plasma membrane, initiating an intracellular signal cascade that leads to translocation of the cytoplasmically located receptors, thereby amplifying and sustaining the effects of receptor activation; and (*ii*) binding of dopamine to a cytoplasmically located receptor, causing a conformational change of the receptor and subsequent translocation of the ligand receptor complex toward the plasma membrane.

The dopamine agonist fenoldopam cannot be used to differentiate between the two possibilities, because it would be expected to penetrate the cell membrane. The finding that the effects were induced by forskolin speaks in favor of the first alternative. Studies are in progress to distinguish between these possibilities.

Dopamine formed in proximal tubular cells acts as an intrarenal natriuretic hormone by inhibiting Na^+,K^+-ATPase and the Na^+/H^+ exchanger both in the proximal tubule and in more distal segments of the nephron. The natriuresis during high-salt diet depends on an increased renal dopamine tonus. Circumstantial evidence suggests that salt-sensitive hypertension may be associated with a defect in the renal dopamine system (21–25). The results from this study point to two important events in the activation of the renal dopamine system: the agonist-induced translocation of cytoplasmically stored dopamine 1 receptors and the escape of dopamine to be metabolized by COMT. Both of these events are of great importance for the understanding of the renal dopamine system and the regulation of salt metabolism.

It has been shown that insulin recruits functional γ-aminobutyric acid type A receptors to the plasma membrane in HEK 293 cells. It will be important to determine whether the ability of a ligand to regulate the localization of its own receptor, as shown here, is of broad physiological significance. For instance, there is indirect evidence (28) that autoreceptors and/or postsynaptic receptors in the nervous system may be regulated by their cognate ligand through a mechanism analogous to that reported here. Thus, the fascinating demonstration that postsynaptic fusion events contribute to long-term potentiation (28) might be accounted for by the type of phenomenon reported here.

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