

G-protein-coupled Receptor-mediated Traffic of Na,K-ATPase to the Plasma Membrane Requires the Binding of Adaptor Protein 1 to a Tyr-255-based Sequence in the α -Subunit*

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Motion of integral membrane proteins to the plasma membrane in response to G-protein-coupled receptor signals requires selective cargo recognition motifs that bind adaptor protein 1 and clathrin. Angiotensin II, through the activation of AT1 receptors, promotes the recruitment to the plasma membrane of Na,K-ATPase molecules from intracellular compartments. We present evidence to demonstrate that a tyrosine-based sequence (IVVY-255) present within the Na,K-ATPase α 1-subunit is involved in the binding of adaptor protein 1. Mutation of Tyr-255 to a phenylalanine residue in the Na,K-ATPase α 1-subunit greatly reduces the angiotensin II-dependent activation of Na,K-ATPase, recruitment of Na,K-ATPase molecules to the plasma membrane, and association of adaptor protein 1 with Na,K-ATPase α 1-subunit molecules. To determine protein-protein interaction, we used fluorescence resonance energy transfer between fluorophores attached to the Na,K-ATPase α 1-subunit and adaptor protein 1. Although angiotensin II activation of AT1 receptors induces a significant increase in the level of fluorescence resonance energy transfer between the two molecules, this effect was blunted in cells expressing the Tyr-255 mutant. Thus, results from different methods and techniques suggest that the Tyr-255-based sequence within the NKA α 1-subunit is the site of adaptor protein 1 binding in response to the G-protein-coupled receptor signals produced by angiotensin II binding to AT1 receptors.

The dopaminergic and renin-angiotensin systems act coordinately to regulate blood pressure, in part, by affecting sodium transport in renal proximal tubules (1–3). The apical Na/H-exchanger and basolateral Na,K-ATPase (NKA)² are responsible for ~75% of the renal-filtered sodium reabsorption (4, 5). By

maintaining a low intracellular sodium concentration, Na,K-ATPase provides the driving force for renal proximal tubule sodium reabsorption (6). Both the Na/H-exchanger and NKA are regulated by the hormones dopamine and angiotensin II (Ang II). Dopamine activation of type 1 dopamine receptors (D1Rs) induces natriuresis and inhibits renal proximal tubule NKA activity (1, 6–10). The antinatriuretic Ang II, through activation of type 1 angiotensin II receptors (AT1Rs), stimulates sodium reabsorption in renal proximal tubule cells (11–14) and activates NKA (15, 16). We have demonstrated that NKA regulation by dopamine and Ang II is due to a reduced/increased number of NKA molecules at the plasma membrane and not to modulation of the intrinsic NKA activity (16). At the cellular level, Ang II-dependent activation of NKA is mediated by recruitment of NKA molecules from intracellular compartments to the plasma membrane via a clathrin-coated vesicle-dependent mechanism (16, 17). Protein kinase C β -dependent phosphorylation of the NKA α 1-subunit constitutes the triggering signal for NKA plasma membrane recruitment. Phosphorylation of Ser-11 and Ser-18 in NKA α 1 may induce a conformational change in the protein to facilitate the binding of adaptor protein 1 (AP-1). Although we are beginning to better understand the organization of signals initiated by stimulation of AT1Rs and the traffic of NKA molecules from intracellular compartments to the plasma membrane, the mechanism involved in cargo selection remains unclear. The present study was performed to identify the NKA α 1 sequence that interacts with AP-1.

EXPERIMENTAL PROCEDURES

Materials—Ouabain, angiotensin II, chelerythrine chloride, and mouse anti-AP-1 antibody were obtained from Sigma. Alexa Fluor 488-tagged anti-mouse and Cy3-tagged anti-rabbit antibodies were purchased from Molecular Probes (Eugene, OR). *N*-hydroxysulfosuccinimide-biotin was obtained from Pierce. ExactaCruz E antigen detection system was purchased from Santa Cruz Biotechnology. Anti-Na,K-ATPase α 1 monoclonal mouse antibody was a generous gift of Dr. Robert W. Mercer (Washington University, St. Louis, MO). Anti-Na,K-ATPase α 1 polyclonal rabbit antibody was a generous gift of Dr. Jack H. Kaplan (University of Illinois, Chicago, IL). Other reagents were of the highest quality available.

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² The abbreviations used are: NKA, Na,K-ATPase; Ang II, angiotensin II; AP-1, adaptor protein 1; AT1R, type 1 angiotensin II receptor; D1R, type 1 dopamine receptor; IP, immunoprecipitation; FRET, fluorescence resonance

energy transfer; FRET^N, normalized FRET; FRET^C, corrected FRET; WT, wild-type; PMA, phorbol 12-myristate 13-acetate.

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Cell Culture and Transfection—OK cells stably expressing the wild-type rat Na,K-ATPase $\alpha 1$ or mutant forms of this protein were cultured in Dulbecco's modified Eagle's medium containing 10% calf serum and antibiotics. To prevent the expression of the endogenous NKA $\alpha 1$, cells were maintained all the time in 3 μM ouabain medium (18, 19). Plasmid preparation and site-directed mutagenesis were performed as described previously (20). Stable expression of Na,K-ATPase WT $\alpha 1$ and mutants was performed as described by Pedemonte *et al.* (18, 19).

Cell Treatment—The optimal concentrations of the reagents and the extension of the treatments used in these experiments were as determined previously (21–23). In some experiments, before and during the treatment with hormones, the cells were incubated with 2 μM chelerythrine chloride for 30 min to inhibit protein kinase C activity.

Determination of Rb⁺ Uptake—Measurements of NKA-mediated transport by Rb⁺ uptake were performed with attached cells as described previously (18, 19). Cells were transferred to serum-free Dulbecco's modified Eagle's medium containing 50 mM HEPES, pH 7.4, and either 3 μM or 5 mM ouabain (incubation medium). All treatments and determinations were performed at 23 °C. Then, a trace amount of [⁸⁶Rb]RbCl was added to the cell medium. After 20 min, cells were washed three times with ice-cold saline and dissolved with SDS, and accumulated radioactivity was determined. NKA-mediated Rb⁺ uptake was calculated from the difference in tracer uptake between samples incubated in 3 μM or 5 mM ouabain. The ouabain-insensitive Rb⁺ uptake (measured in the presence of 5 mM ouabain) was 25–30% of the total Rb⁺ uptake measured. In some experiments, cells were treated with hormones before the Rb⁺ uptake determination. The concentrations used and the time of treatment are described in the respective figures.

Determination of Plasma Membrane Pool of NKA by Biotinylation—For biotinylation experiments, the cells were treated with 1 μM PMA or 1 μM Ang II for 10 min at room temperature, and then the medium was changed to ice-cold 10 mM Tris-HCl, pH 7.5, 2 mM CaCl₂, 150 mM NaCl, and 1.5 mg/ml *N*-hydroxysulfosuccinimide-biotin. After incubation for 1 h at 4 °C, the cells were scraped in immunoprecipitation (IP) buffer (20 mM Tris, 2 mM EDTA, 2 mM EGTA, and 30 mM sodium pyrophosphate, pH 7.3) containing a protease inhibitor mixture, frozen in liquid nitrogen, thawed rapidly, probe-sonicated twice in an ice-water bath, and frozen/thawed again. The cell suspension was centrifuged at 14,000 $\times g$ at 4 °C for 5 min. The supernatant was separated, and protein concentration was determined. Aliquots containing equal amounts of protein were transferred to clean tubes, and 0.2% Triton X-100 and 0.2% SDS were added. The suspension was incubated at 4 °C for 1 h with anti- $\alpha 1$ antibody and for 2 h with protein A/G-agarose, which had been prewashed three times with phosphate-buffered saline and once with IP buffer containing 0.2% Triton X-100. After separation, the agarose beads were washed four times with IP buffer containing 0.2% Triton X-100 and 0.2% SDS and once with 50 mM Tris-HCl, pH 7.4, and finally resuspended in Laemmli sample buffer. Electrophoresis, Western blot analysis with ExtrAvidin peroxidase conjugate, and densi-

tometric analysis were performed as described previously (16, 21, 24).

Determination of AP-1 Coprecipitation with NKA $\alpha 1$ —OK cells stably expressing either the WT or Y255F mutant form of NKA $\alpha 1$ were treated with 1 μM Ang II (or vehicle) for 10 min at room temperature. The cells were then scraped in ice-cold radioimmune precipitation assay buffer followed by the ExactaCruz IP protocol (performed as described by the manufacturer). Cell lysates (containing an equal amount of protein for each treatment) were precleared with Preclearing Matrix E (Santa Cruz Biotechnology) for 30 min at 4 °C. Preimmune normal mouse serum and control antibody anti-actin C-2 IgG1 (both reagents are from Santa Cruz Biotechnology; catalog nos. sc-45051 and sc-8432, respectively) and NKA $\alpha 1$ IgG1 antibody were incubated with the ExactaCruz IP matrix to form IP complexes. These were washed twice with phosphate-buffered saline and then incubated with cell lysates for 1 h at 4 °C. The IP matrixes were pelleted, washed three times with radioimmune precipitation assay buffer, and finally resuspended in Laemmli sample buffer. After separation by SDS-PAGE, the proteins were transferred to a piece of polyvinylidene difluoride membrane, which was assayed by Western blot analysis with an AP-1 antibody. The protein bands were analyzed by densitometry. The polyvinylidene difluoride membrane was stripped and tested with an anti-NKA $\alpha 1$ antibody to determine precipitated NKA as a control of PAGE loading. The ratio of integral densities of protein bands identified by AP-1 and NKA $\alpha 1$ antibodies in the same membrane was calculated for each sample. Then, the membrane was stripped again and tested with an anti-phosphoserine antibody to determine the level of NKA $\alpha 1$ phosphorylation.

Preparation of Cells for Microscopic Studies—Cells attached to glass coverslips and grown to 90% confluence were incubated for 10 min at room temperature with 1 μM Ang II in serum-free Dulbecco's modified Eagle's medium containing 50 mM HEPES, pH 7.4. The cells were washed twice with phosphate-buffered saline containing 1.2% sucrose (PBSS), fixed with freshly prepared 4% paraformaldehyde in PBSS for 10 min, washed twice with PBSS, incubated with L-lysine/sodium *m*-periodate for 20 min, washed with PBSS, permeabilized with 0.2% bovine serum albumin and 0.2% Triton X-100 in PBSS for 10 min, and washed again twice with PBSS. The cells were blocked with 5% bovine serum albumin, 1% normal goat serum, and 0.2% Tween 20 for 1 h. Cells were incubated with mouse anti-AP-1 and rabbit anti-NKA $\alpha 1$ antibodies and washed with PBSS. In the dark, cells were incubated with Alexa Fluor 488-tagged anti-mouse and Cy3-tagged anti-rabbit antibodies for 1 h. Then, the coverslips were washed, air-dried, and mounted on glass slides using Gel-Mount containing anti-fading agents (Biomedica, Foster City, CA).

Optical Setup of Fluorescence Microscopy—To obtain high resolution three-dimensional images of the cells, the fluorescence imaging work station consisted of an Olympus IX-81 inverted fluorescence microscope (Olympus Corp., Tokyo, Japan) equipped with a 60X/100X oil immersion objective lens, cooled Hamamatsu ORCA-ER CCD camera (Hamamatsu Photonics, Hamamatsu-city, Japan), a halogen 100-watt light source, a motorized filter and shutter, and a scan wizard for

collection of x/y and z image sequences and wavelength positions over time, all controlled by SimplePCI software (Compix, Cranberry Township, PA). To obtain deconvolved images of cells, at least seven z-stacks of the field with a step of 0.5–1.0 μm were acquired, and deconvolution was performed using AutoDeblur software (AutoQuant Imaging, Troy, NY). For all intensity measurements, the Dynamic Intensity Analysis module of SimplePCI was used.

Fluorescence Resonance Energy Transfer—For FRET determinations, images were acquired sequentially through three channels using the following filter sets (Chroma Corp., Rockingham, VT): Alexa Fluor 488 (excitation, 470/40 nm; emission, 500/40 nm), Cy3 (excitation, 540/25 nm; emission, 605/55 nm), and Alexa Fluor 488/Cy3 (excitation, 470/40 nm; emission, 605/55 nm). Corrected FRET (FRET^C) was calculated on a pixel-by-pixel basis for the entire image using the equation $\text{FRET}^{\text{C}} = \text{FRET} - (0.45 \times \text{Alexa Fluor 488}) - (0.20 \times \text{Cy3})$ (25), where FRET, Alexa Fluor 488, and Cy3 correspond to background-subtracted images of cells stained with Alexa Fluor 488 and Cy3 acquired through the FRET, Alexa Fluor 488, and Cy3 channels, respectively. Values of 0.45 and 0.20 are the fractions that were calculated for the bleed-through of Alexa Fluor 488 and Cy3 fluorescence, respectively, through the FRET filter channel. Calibrations of bleed-through were performed in cells treated with only one primary/secondary set of the antibodies, either anti-AP-1/Alexa Fluor 488 or anti-NKA $\alpha 1$ /Cy3. In the cells, treated simultaneously with both sets of antibodies, normalized FRET (FRET^N) values were calculated according to the equation $\text{FRET}^{\text{N}} = \text{FRET}^{\text{C}} / (\text{Alexa Fluor 488} \times \text{Cy3})^{1/2}$, where FRET^C, Alexa Fluor 488, and Cy3 are the mean intensities of FRET^C, Alexa Fluor 488, and Cy3 fluorescence, respectively (26).

Pseudocolor FRET^N images were obtained using the ISee imaging program from Digital Analysis Technology (Raleigh, NC). Pseudocolor FRET^N images are displayed with *deep blue* (cold) indicating low values and *bright red* (hot) indicating high values.

Statistical Procedures—Each experiment was repeated at least three times. Comparison between the two experimental groups was determined with the non-paired Student's *t* test. $p < 0.05$ was indicated with an *asterisk* in the figures. For the microscopic images, at least six randomly chosen sets of cells were used in each experimental condition. The figures show representative images for each experiment.

RESULTS

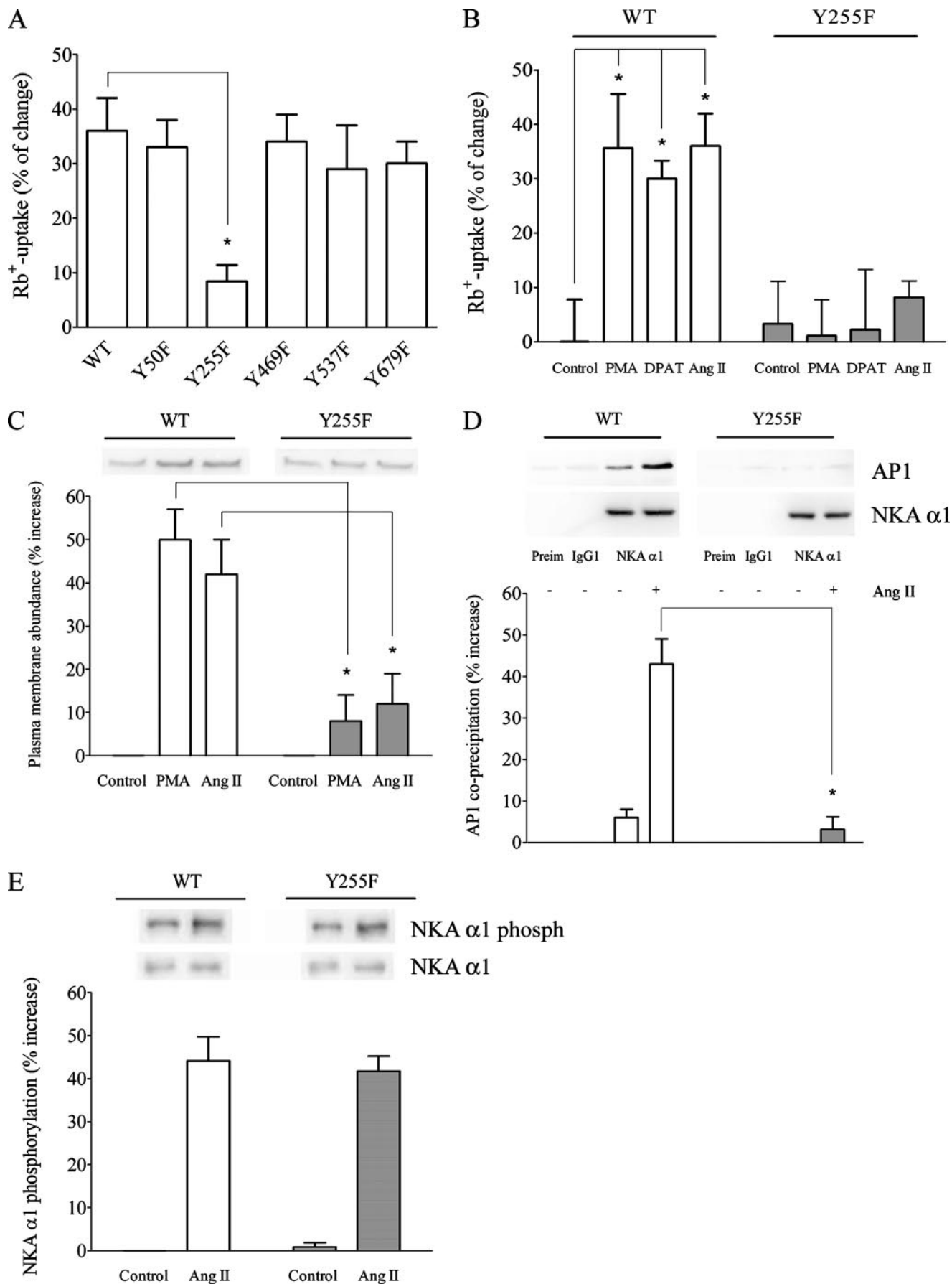
Adaptor proteins such as AP-1 interact with target proteins through binding to defined protein sequences carrying the NPXY motifs, where N, P, and Y are asparagine, proline, and tyrosine, and X can be any amino acid, or the more broadly used Ypp ϕ motifs, where Y is tyrosine, p residues are highly variable but tend to be hydrophilic, and ϕ is a residue with a bulky hydrophobic chain (27). The sequence of residues may be in the reverse order. Analysis of the primary sequence of the NKA subunits revealed the presence of five of these consensus sequences within the cytosolic loops of $\alpha 1$ at Tyr-50, Tyr-255, Tyr-469, Tyr-537, and Tyr-679. The small cytosolic loop of the β -subunit has none of the consensus sequences. One by one,

the tyrosine residues in the $\alpha 1$ consensus sequences were mutated to phenylalanine. The mutation introduced the minimum possible change by eliminating the tyrosine hydroxyl group and keeping the aromatic characteristic of the residue side chain. Plasmids containing the rat wild-type NKA $\alpha 1$ (WT $\alpha 1$) and its mutated forms were stably expressed in OK cells, a cell culture line that is widely used as a model of renal proximal tubule epithelia (28–30). Cells expressing the WT and mutant forms of NKA $\alpha 1$ were selected and maintained all the time in the presence of 3 μM ouabain medium to prevent the expression of the endogenous $\alpha 1$ -subunit (19, 31). Transport activity mediated by the NKA was determined in these cells. The basal ouabain-sensitive Rb^+ uptake in WT $\alpha 1$ cells was 9.0 ± 0.7 nmol/min/mg protein, and similar values were determined in cells expressing the different NKA $\alpha 1$ mutants, which indicates that the NKA was expressed at the same level in the different cell lines. Ang II-dependent stimulation of AT1Rs (16) increased the ouabain-sensitive Rb^+ uptake to 12.4 ± 0.5 nmol/min/mg protein (a 38% increase) in cells expressing the wild-type NKA $\alpha 1$ (Fig. 1). Whereas similar transport levels were observed in most of the NKA $\alpha 1$ mutants, the hormonal stimulation was significantly reduced in cells expressing the NKA $\alpha 1$ Y255F mutant (Fig. 1A). We have previously demonstrated that 8-OH-DPAT, a serotonin 1A receptor agonist, and the phorbol ester PMA, an activator of protein kinase C, stimulate the same signaling pathway as Ang II to induce the plasma membrane recruitment of NKA molecules (32). Consistent with this observation, the substitution of Tyr-255 for phenylalanine in NKA $\alpha 1$ impairs the stimulation of NKA activity by either 8-OH-DPAT or PMA (Fig. 1B).

To determine the effect of the $\alpha 1$ Y255F mutation on the Ang II-induced plasma membrane recruitment of NKA molecules, we measured the plasma membrane abundance of NKA molecules in cells that have been treated or not treated with Ang II. For this, we used the technique of plasma membrane protein biotinylation, which has been validated in several of our previous publications (16, 21, 24). After treatment of the cells with either PMA or Ang II, the cells were rapidly transferred to a 4 °C bath, the temperature at which the plasma membrane proteins were labeled with *N*-hydroxysulfosuccinimide-biotin. The low temperature stops the hormonal reaction and prevents the intracellular trafficking of NKA molecules. Changes in NKA activity in response to AT1Rs activation are paralleled by increases in abundance of newly recruited NKA units at the plasma membrane (Fig. 1C). On the contrary, in cells expressing the $\alpha 1$ Y255F mutant, stimulation of AT1Rs failed to increase NKA abundance at the plasma membrane. Basal amounts of NKA molecules at the plasma membrane (controls, Fig. 1C) determined in cells expressing the NKA WT $\alpha 1$ and the $\alpha 1$ Y255F mutant were not significantly different, indicating that NKA was expressed at the same level in both cell lines.

We further evaluated whether the IVVY-255 sequence represents the AP-1 binding site by looking at its interaction with NKA using co-IP and FRET techniques. As determined by co-IP assays, stimulation of AT1Rs increases the association of NKA and AP-1 molecules in cells expressing the wild-type $\alpha 1$, whereas this effect was greatly reduced in cells expressing the $\alpha 1$ Y255F mutant (Fig. 1D). Besides being used for IP, aliquots

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from the cell lysates were separated by PAGE and analyzed by Western blotting with an anti-NKA $\alpha 1$ antibody (data not shown). The same amount of NKA $\alpha 1$ was determined in the cell lysates corresponding to both cell types and all treatment conditions. Thus, the differences presented in the figure are not due to different amounts of NKA available for IP. Furthermore, as shown in Fig. 1D, similar amounts of NKA $\alpha 1$ were immunoprecipitated from cells expressing WT $\alpha 1$ and the Y255F mutant. In cells expressing the NKA $\alpha 1$ Y255F mutant, the antibody recognition for AP-1 is not significantly different from the background level observed for the IP controls performed with preimmune serum or anti-actin control antibody (the same IgG type as the anti-NKA $\alpha 1$ antibody). Results presented in Fig. 1D suggest that treatment of the cells with Ang II specifically increases the association of AP-1 with NKA molecules and that this association is prevented in cells expressing the NKA $\alpha 1$ Y255F mutant. These results are consistent with those presented in Figs. 1 and 2.

We have previously demonstrated that stimulation of AT1Rs induces the phosphorylation of Ser-11 and Ser-18 of NKA $\alpha 1$ and that mutation of these amino acids prevents the Ang II-dependent regulation of NKA activity (16, 17). Consistent with this, Fig. 1E shows that the Ang II-dependent increased coprecipitation of NKA and AP-1 is accompanied by an increased phosphorylation of NKA $\alpha 1$. The absence of difference in phosphorylation level between cells expressing WT and mutant Y255F $\alpha 1$ shows that the process of AP-1-mediated recruitment of NKA $\alpha 1$ lies downstream of its phosphorylation.

Further support for the interaction between NKA and AP-1 molecules was obtained using FRET between fluorophores attached to the two proteins. Because it can only occur at a very close distance, FRET is the best technique to measure protein-protein interaction. We have previously optimized the use of this technique (33). After treatment with Ang II and/or other reagents, the cells were exposed to polyclonal rabbit and monoclonal mouse antibodies against NKA $\alpha 1$ and AP-1, respectively. Subsequently, the cells were treated with Cy3-tagged anti-rabbit and Alexa Fluor 488-tagged anti-mouse secondary antibodies. Cy3 and Alexa Fluor 488 are an excellent donor-acceptor pair for FRET determinations (34–36). Results depicted in Fig. 2A, vertical panels, demonstrate bright-field

images (first panel), as well as images of the cells obtained in the Cy3 channel (second panel), Alexa Fluor 488 channel (third panel), and FRET channel (fourth panel). For determinations of normalized FRET, images of cells labeled with only one of the fluorophores were also acquired (data not shown). FRET^N, which is independent of the fluorophore concentrations (37), was determined through pixel-by-pixel intensity recalculation, and it is presented in Fig. 2 as pseudocolor images. In non-treated cells, NKA $\alpha 1$ is mostly localized in the plasma membrane region, and AP-1 is in cytosolic regions surrounding the nucleus. We have previously demonstrated that the intracellular compartments containing NKA molecules are close to the plasma membrane and that it is difficult to distinguish between NKA molecules that are at the plasma membrane and in intracellular compartments (38). In cells treated with Ang II, it can be observed that AP-1 molecules have been recruited to the plasma membrane region where the NKA-containing intracellular compartments are located, and consequently the level of FRET^N between the fluorophores attached to NKA and AP-1 has been significantly increased (arrows on Fig. 2A). In cells in which the Ang II-dependent phosphorylation of NKA $\alpha 1$ has been impaired by pretreatment with the protein kinase C inhibitor chelerythrine chloride, the increased level of FRET^N was greatly reduced. As a control, some of the cells were treated with the D1R agonist fenoldopam, which, contrary to Ang II, induces the endocytosis of NKA molecules (39, 40), and no increased level of FRET^N was observed (Fig. 2B).

Determinations of FRET^N were also performed in cells expressing the Y255F and Y537F mutants of $\alpha 1$. Ang II stimulation of AT1Rs induced an increased level of FRET^N between the fluorophores attached to NKA $\alpha 1$ and AP-1 in cells expressing the Y537F mutant, but not in cells expressing the Y255F mutant (Fig. 2B).

DISCUSSION

Interaction of a clathrin-dependent translocated protein with AP-1 is a key event in the recruitment of target proteins to the plasma membrane (41). In this report, we present evidence that the sequence IVVY-255 within the NKA $\alpha 1$ is the site of binding of AP-1 in response to AT1R stimulation. In support of this conclusion, we present results obtained by different and

FIGURE 1. Importance of Tyr-255 for the Ang II-dependent stimulation of NKA activity. A, the effect of Tyr-255 mutation on the Ang II stimulation of NKA-mediated Rb⁺ uptake. OK cells expressing WT or mutant forms of NKA $\alpha 1$ were treated with 1 μ M Ang II for 10 min before the Rb⁺ uptake assay. The percentage of Rb⁺ uptake increase for each experimental condition was calculated with respect to a control that was not treated with Ang II. *, $p < 0.05$ with respect to OK WT $\alpha 1$ cells. B, the effect of the Y255F mutation on the stimulation of NKA-mediated Rb⁺ uptake by different activator/agonists. OK cells expressing the WT or Y255F mutant form of NKA $\alpha 1$ were treated for 10 min with 1 μ M PMA, 3 μ M 8-OH-DPAT, or 1 μ M Ang II as described under "Experimental Procedures" before the Rb⁺ uptake assay. The percentage of increase for each experimental condition was calculated with respect to a non-treated control. *, $p < 0.05$ with respect to the control. C, the effect of the Y255F mutation on the Ang II-induced plasma membrane recruitment of NKA molecules. Cells were treated with either PMA or Ang II, and then the abundance of NKA molecules at the plasma membrane was determined by biotinylation as described under "Experimental Procedures." Representative Western blots are shown in the upper panel. Quantitation of the Western blots is presented in the lower panel as the percentage of increase of NKA abundance at the plasma membrane. *, $p < 0.05$ with respect to the same treatment of OK cells expressing WT $\alpha 1$. D, the effect of the Y255F mutation on NKA-AP-1 coprecipitation. OK cells expressing either the WT or Y255F mutant form of NKA $\alpha 1$ were treated with 1 μ M Ang II for 10 min and then dissolved with radioimmune precipitation assay buffer. Cell lysates were precleared with Preclearing Matrix E and incubated with ExactaCruz IP matrix conjugated to preimmune normal mouse serum (Preim), anti-actin C-2 (IgG1), and NKA $\alpha 1$ antibodies for 1 h. The matrix was separated by centrifugation and treated with Laemmli buffer. The precipitated proteins were separated by PAGE and transferred to polyvinylidene difluoride membrane, and Western blot analysis was performed with an anti-AP-1 antibody. Representative Western blots for AP-1 (AP1) and precipitated NKA $\alpha 1$ are shown in the upper panel. Quantitation data of precipitated AP-1 to NKA ratios are presented in the bar graph (lower panel) as a percentage of change of the Ang II-induced coprecipitation AP-1/NKA ratio with respect to a non-treated control. *, $p < 0.05$ with respect to the same treatment of OK cells expressing WT $\alpha 1$. E, Ang II-mediated phosphorylation of NKA $\alpha 1$. OK cells were treated with 1 μ M Ang II for 10 min, NKA $\alpha 1$ was immunoprecipitated, and Western blot analysis was performed with an anti-phosphoserine antibody. Representative Western blots for NKA $\alpha 1$ phosphorylation (phosph) and precipitated NKA $\alpha 1$ are shown in the upper panel. Quantitation data of phosphorylated NKA $\alpha 1$ to precipitated NKA ratios are presented in the bar graph (lower panel) as a percentage of change of the Ang II-induced NKA $\alpha 1$ phosphorylation/NKA ratio with respect to a non-treated control.

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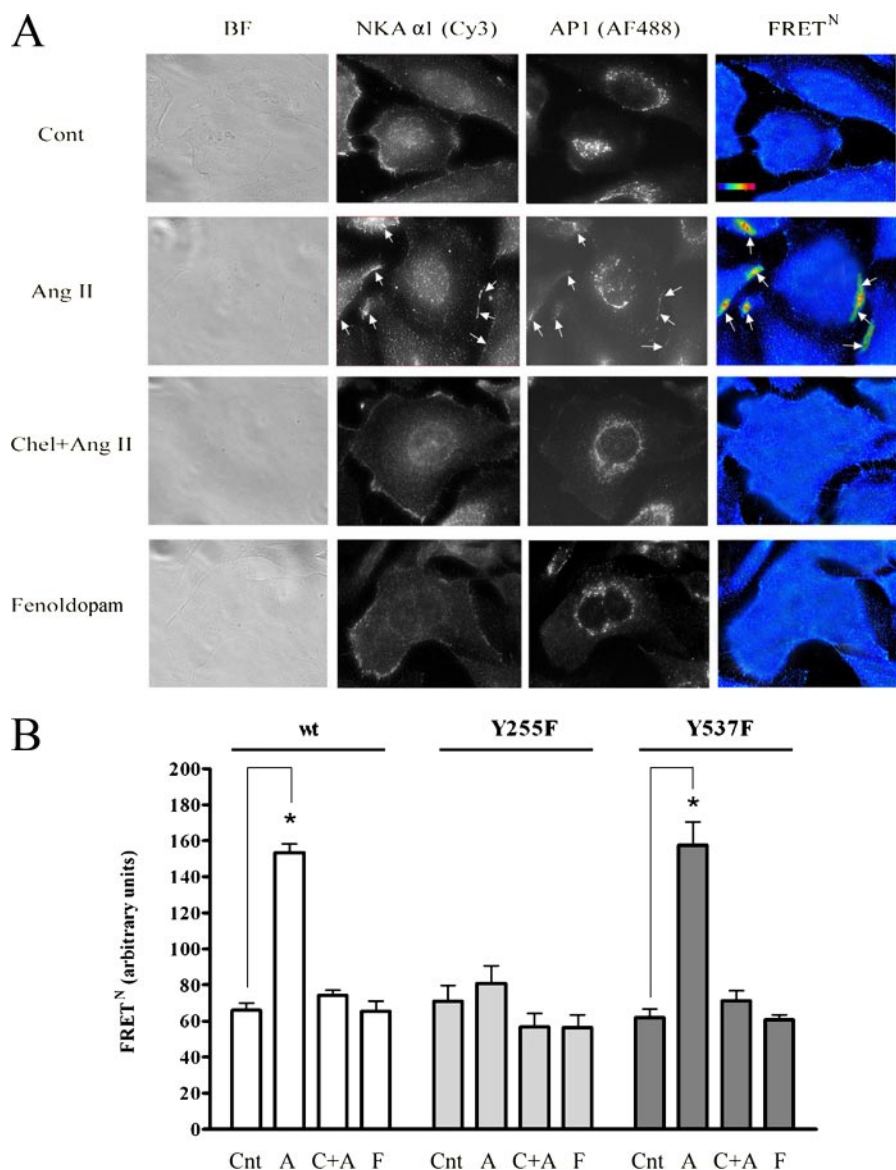


FIGURE 2. Interaction between NKA $\alpha 1$ and AP-1 determined by FRET. *A*, images of OK cells expressing NKA WT $\alpha 1$ and treated with Ang II (1 pM, 10 min), the protein kinase C inhibitor chelerythrine chloride (2 μ M, 30 min), or the D1R agonist fenoldopam (1 μ M, 10 min). *Left panels* show bright-field images of representative cells for each treatment. Fluorescence images (*right panels*) were acquired through the Cy3 channel, the Alexa Fluor 488 channel, and the FRET channel. Cells were treated and images were acquired as described under "Experimental Procedures." *Cont*, control; *BF*, brightfield. *B*, levels of normalized FRET. FRET^N was calculated as described under "Experimental Procedures." *, $p < 0.05$ with respect to non-treated cells. *Cnt*, control; *A*, angiotensin II; *C+A*, chelerythrine + angiotensin II; *F*, fenoldopam.

independent methods and techniques. The reduced Ang II-dependent stimulation of NKA activity was not due to a reduced expression of the $\alpha 1$ Y255F mutant. That the level of expression of NKA $\alpha 1$ is the same in cells expressing the wild-type $\alpha 1$ and the $\alpha 1$ Y255F mutant is illustrated by the display, under basal conditions, of the same level of NKA activity, NKA molecule abundance at the plasma membrane, and fluorescence.

Besides binding to Tyr-based sequences, AP-1 may also interact with dileucine motifs with the sequence $z_{2-4}xLL$, where z is a negatively charged residue and x a polar residue (42). The NKA $\alpha 1$ cytosolic loops have two of these consensus sequences, EPKHL-499 and LLLPDE-559. We have previously demonstrated that PMA, which activates the NKA activity through the same signaling pathway as Ang II (16), stimulates

the NKA containing the WT $\alpha 1$ at the same level as NKA molecules containing the mutants L499A and L554A (20). Therefore, so far, the NKA $\alpha 1$ IVVY-255 sequence is the only site of AP-1 binding that is relevant to the Ang II-dependent regulation of NKA activity.

Cells expressing the WT and mutant forms of NKA $\alpha 1$ were selected and maintained all the time in 3 μ M ouabain medium, which prevents the expression of the endogenous $\alpha 1$ -subunit (19, 31). Thus, endogenous non-mutated $\alpha 1$ should not have interfered with determinations performed in cells expressing the various mutants of $\alpha 1$. We have previously demonstrated that, as part of the mechanism of endocytosis of NKA molecules induced by stimulation of D1Rs, AP-2 interacts with the 537-YLEL sequence within NKA $\alpha 1$ (20). We determined that the mutation Y537F has no effect on Ang II-dependent stimulation of NKA activity and in the NKA $\alpha 1$ /AP-1 interaction (as determined by FRET). Furthermore, contrary to the effect of Ang II, stimulation of D1Rs (which produced inhibition of NKA activity) did not increase the interaction of NKA $\alpha 1$ with AP-1. These results, as well as the observation that preventing the Ang II-induced phosphorylation of NKA $\alpha 1$ impairs the NKA $\alpha 1$ /AP-1 interaction, support the conclusion that the NKA $\alpha 1$ /AP-1 interaction is a very specific response to the activation of AT1Rs. Ang II-dependent plasma membrane recruitment of NKA molecules requires the protein

kinase $C\beta$ -mediated phosphorylation of NKA $\alpha 1$ (16). The fact that the Ang II-dependent increment of FRET^N was impaired when the cells were treated with the protein kinase C inhibitor chelerythrine chloride is evidence of the causal link between the specific phosphorylation of $\alpha 1$ and the NKA/AP-1 interaction. The results of these experiments strongly suggest that the Ang II-dependent increment on FRET^N is not due to some unknown artifact, such as membrane ruffling or constitutive protein delivery to the plasma membrane. It should be noted that the effect of Ang II is an acute effect produced in a few minutes. Thus, the effects observed are not due to the delivery of newly synthesized proteins to the plasma membrane.

AP-1, through its μ -subunit, interacts with the cargo Tyr-based sequences (43, 44). Although AP-1 containing the μA

isoform has been implicated in the bidirectional traffic between the *trans*-Golgi network and endosomes, AP-1 containing the μ B isoform seems to be selectively expressed in polarized epithelial cells and to be responsible for delivery of proteins to the basolateral membrane domain (43, 44). Although we have not studied it, it is likely that the μ B isoform is expressed in OK, which is derived from opossum kidney proximal tubule epithelial cells. Because the regulation of NKA by Ang II occurs in a short term fashion and does not require protein synthesis (and thereby no traffic through the *trans*-Golgi network), it seems likely that AP-1 containing the μ B isoform would be responsible for the results described in this report.

Clathrin vesicle-dependent traffic of membrane proteins requires the specific interaction of the protein cargo with clathrin adaptors (42). Such adaptors are organ-specific and have a particular distribution within the cell cytoplasm. Whereas, induced by D1Rs activation, AP-2 binds to the NKA α 1 Tyr-537-based sequence during plasma membrane endocytosis (20), in this report we present evidence that Ang II-induced clathrin-dependent traffic of NKA molecules requires the binding of AP-1 to another sequence located at Tyr-255 within the NKA α 1. These observations unequivocally demonstrate the existence of two specific clathrin adaptor-binding sequences that provide selectivity regarding whether the NKA molecules would traffic from or to the plasma membrane in response to G-protein-coupled receptor signals.

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