THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 26, pp. 18489–18496, June 27, 2014

β1-Adrenergic Receptor Signaling Activates the Epithelial Calcium Channel, Transient Receptor Potential Vanilloid Type 5 (TRPV5), via the Protein Kinase A Pathway*

Received for publication, December 11, 2013, and in revised form, March 29, 2014 Published, JBC Papers in Press, May 14, 2014, DOI 10.1074/jbc.M113.491274

Eline A. E. van der Hagen[1], **Kukiat Tudpor**[1], **Sjoerd Verkaart, Marla Lavrijsen, Annemiete van der Kemp, Femke van Zeeland, René J. M. Bindels, and Joost G. J. Hoenderop**[2]

From the Department of Physiology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, 6500 HB Nijmegen, The Netherlands

Background: β1-Adrenergic receptors (β-ARs) are expressed in the distal part of the nephron where TRPV5-mediated active Ca^{2+} reabsorption takes place.

Results: The β1-AR agonist dobutamine, by inducing PKA-dependent phosphorylation, enhanced influx of Ca^{2+} through TRPV5.

Conclusion: β1-AR signaling potentially stimulates transcellular Ca^{2+} transport in the kidney.

Significance: Dobutamine, generally used as a positive inotrope, probably also has a calciotropic effect.

Epinephrine and norepinephrine are present in the pro-urine. β-Adrenergic receptor (β-AR) blockers administered to counteract sympathetic overstimulation in patients with congestive heart failure have a negative inotropic effect, resulting in reduced cardiac contractility. Positive inotropes, β1-AR agonists, are used to improve cardiac functions. Active Ca^{2+} reabsorption in the late distal convoluted and connecting tubules (DCT2/CNT) is initiated by Ca^{2+} influx through the transient receptor potential vanilloid type 5 (TRPV5) Ca^{2+} channel. Although it was reported that β-ARs are present in the DCT2/CNT region, their role in active Ca^{2+} reabsorption remains elusive. Here we revealed that β1-AR, but not β2-AR, is localized with TRPV5 in DCT2/CNT. Subsequently, treatment of TRPV5-expressing mouse DCT2/CNT primary cell cultures with the β1-AR agonist dobutamine showed enhanced apical-to-basolateral transepithelial Ca^{2+} transport. In human embryonic kidney (HEK293) cells, dobutamine was shown to stimulate cAMP production, signifying functional β1-AR expression. Fura-2 experiments demonstrated increased activity of TRPV5 in response to dobutamine, which could be prevented by the PKA inhibitor H89. Moreover, nonphosphorylable T709A-TRPV5 and phosphorylation-mimicking T709D-TRPV5 mutants were unresponsive to dobutamine. Surface biotinylation showed that dobutamine did not affect plasma membrane abundance of TRPV5. In conclusion, activation of β1-AR stimulates active Ca^{2+} reabsorption in DCT2/CNT; an increase in TRPV5 activity via PKA phosphorylation of residue Thr-709 possibly plays an important role. These data explicate a calciotropic role in addition to the inotropic property of β1-AR.

Ca^{2+} plays a pivotal role in bone skeletal development and acts as a second messenger in excitatory cells; thus maintenance of Ca^{2+} homeostasis is vital for the body (1). Ca^{2+} balance is tightly regulated by three primary organs: the gastrointestinal tract, bone, and the kidney (1). Ca^{2+} absorbed from the intestine is stored mostly in bone (99%) whereas the rest is either conjugated with other charged molecules or freely circulating in blood (2). The latter portion of Ca^{2+} is filtered in the glomerulus of the kidney and is reabsorbed to the circulation by the proximal tubule (PT,[3] 65%), thick ascending limb of Henle's loop (TAL, 20%), and DCT2/CNT (14%) (3). Mechanisms of Ca^{2+} reabsorption in these three segments are of different origin: passive Ca^{2+} reabsorption through the paracellular space in the PT and TAL, dependent on the electrochemical gradient, whereas active transcellular Ca^{2+} reabsorption in the DCT2/CNT is energetically driven by ATP hydrolysis (1). Ca^{2+} transport in the TAL and DCT2/CNT is subject to regulation by several factors including G protein-coupled receptors (GPCRs) (1). Agonists of two members of GPCRs, *i.e.* Ca^{2+}-sensing receptor and parathyroid hormone (PTH) receptor type 1, inhibit and stimulate Ca^{2+} reabsorption in TAL and in DCT2/CNT, respectively (4–6).

Active Ca^{2+} reabsorption in the DCT2/CNT is a crucial fine-tuning event determining final urinary Ca^{2+} excretion and consists of three consecutive steps: apical entry through the transient receptor potential vanilloid type 5 (TRPV5) Ca^{2+} channel, intracellular buffering and translocation to basolateral membrane by calbindin-D_{28K} and extrusion into the blood by the Na^{+}/Ca^{2+} exchanger 1 and plasma membrane Ca^{2+} ATPase type 1b (1, 7, 8). TRPV5-mediated Ca^{2+} influx is the rate-limiting step for the active renal Ca^{2+} reabsorption as shown by

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* This work was supported by The Netherlands Organization for Scientific Research (NWO-ALW) Grant 819.02.012.

[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: 286 Physiology, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Tel.: 31-24-3610580; Fax: 31-24-3616413; E-mail: Joost.Hoenderop@radboudumc.nl.

[3] The abbreviations used are: PT, proximal tubule; AR, adrenergic receptor; CHF, congestive heart failure; DAG, diacyl glycerol; DCT2/CNT, distal convoluted and connecting tubules; EGFP, enhanced green fluorescent protein; EPAC, exchange protein directly activated by cAMP; Epi, epinephrine; GPCR, G protein-coupled receptor; NE, norepinephrine; NKCC2, Na-K-Cl cotransporter 2; PTH, parathyroid hormone; TAL, thick ascending limb of Henle's loop; TRPV5, transient receptor potential vanilloid type 5.

hypercalciuria and osteopenia in TRPV5-deficient mice (3). Among six members of the vanilloid TRP family, TRPV5 is the most Ca^{2+}-selective channel possessing a constitutive inward rectifying property at low intracellular Ca^{2+} concentrations and physiological membrane potentials (9–11). Therefore, the amount of Ca^{2+} influx through the channel depends on channel activity and plasma membrane abundance (3). TRPV5 activity and plasma membrane abundance are regulated by various factors, including GPCRs. For example, activation of bradykinin receptor type 2 and PTH receptor type 1 initiate phosphorylation of TRPV5 through PLC/DAG/PKC and adenylyl cyclase/cAMP/PKA signaling cascades, respectively (6, 12).

Epinephrine (Epi) and norepinephrine (NE) have diverse hormonal and neurotransmitter functions in the body. Epi and NE were shown to be present in pro-urine filtered from blood but were also found to be synthesized by renal glomeruli/tubules and released from renal sympathetic nerves (13). Epi and NE can act through several members of GPCR adrenergic receptors: α1-AR, α2-AR, and three β-ARs. In kidney, α1-AR is expressed in arterioles whereas α2-AR is located predominantly in proximal tubules (14). α1-AR and α2-AR are responsible for stimulation of renal vasoconstriction and Na^{+} reabsorption, respectively (14). β-ARs can be divided into three subtypes: β1-AR, β2-AR, and β3-AR (15, 16). β1 and β2-ARs are reportedly expressed in rat and mouse DCT, whereas β3-AR is not detectable in the kidney (17–20). The roles of β1- and β2-ARs are well known, respectively, for myocardial contraction and vasodilation, whereas β3-AR is important for lipolysis (18, 21). β-AR blockers (β-blockers) are frequently administered to counteract sympathetic overstimulation in patients with congestive heart failure (CHF), resulting in reduced cardiac contractility (22). Positive inotropes, β1-AR agonists, are used to improve cardiac functions (22). Upon stimulation by Epi and NE, β-ARs activate Gα by the exchange of GDP for GTP, which can further enhance the activity of adenylyl cyclase and phospholipase C (PLC), mediators of cAMP/protein kinase A (cAMP/PKA)- and diacyl glycerol/protein kinase C (DAG/PKC)-dependent phosphorylation, respectively (20).

Even though Epi and NE are secreted in the pro-urine, to our knowledge, no effects of these hormones through signaling via β-ARs on renal active Ca^{2+} transport have been reported. We hypothesized that β-ARs regulate active Ca^{2+} reabsorption in DCT2/CNT. Thus, the present study aims to investigate (i) colocalization of β1- and β2-AR with TRPV5 in DCT2/CNT; (ii) the effect of β-AR activation by a β-AR agonist on active Ca^{2+} reabsorption; and (iii) the molecular mechanism of TRPV5 activation by β-AR.

EXPERIMENTAL PROCEDURES

Immunohistochemistry—Mouse kidney sections were incubated for 16 h at 4 °C with rabbit polyclonal antibody against β1-AR (1:100) or β2-AR (1:300) (NB100-92439 and NBP1-68227; Novus Biologicals). To visualize the receptors, an enhancer step was performed using a biotinylated goat anti-rabbit antibody. Biotin was then coupled to streptavidin-HRP and visualized with Thyramid (TSA Fluorescein System, NEL701A001KT; PerkinElmer Life Sciences). TRPV5 staining was described previously (4). Negative controls, *i.e.* conjugated antibodies solely, were devoid of any staining.

Isolation of DCT2/CNT Using COPAS Sorting and Primary Cell Culture—Transgenic mice expressing EGFP under the TRPV5 promoter were generated as described (23). Mice were maintained on a SSNIFF rodent complete diet (SSNIFF) with free access to water. The animal ethics board of Radboud University Nijmegen approved all of the experimental procedures. The process of renal tubular sorting by COPAS (Union Biometrica) has been described (24). Approximately 4,000–10,000 tubules from one mouse were pelleted (0.8 × g, 5 min, 4 °C) prior to culture or mRNA isolation as described below. For primary culture 2,000 fluorescent tubules, a mixture of tubules from two mice, were resuspended into warmed cell culture medium (Dulbecco's modified Eagle's medium (DMEM)/Ham's F12; Invitrogen) with supplements as described (24) and seeded onto 0.33-cm^2 polycarbonate Transwell® inserts (Corning Costar) previously coated with rat tail collagen (16 μl/insert of 0.75 mg/ml collagen in 95% v/v ethanol with 0.25% v/v acetic acid). Volumes used in the apical and basolateral compartments were 100 and 600 μl, respectively. Cells were cultured at 37 °C in 5% v/v CO_2, 95% v/v atmospheric air, and the medium was refreshed every day.

$^{45}Ca^{2+}$ Transport Measurement—For radioactive $^{45}Ca^{2+}$ transport experiments, primary cells cultured on Transwell inserts as described above were used. Cells were used 7–8 days after seeding; the day prior to the experiment transepithelial electrical resistance was measured using an epithelial volt-ohmmeter (World Precision Instruments). Cells were pretreated by adding 5 μM indomethacin to the culture medium for 30 min. Culture medium was removed, and cells were washed once with physiological salt solution (140 mM NaCl, 2 mM KCl, 1 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM D-glucose, 5 mM L-alanine, 5 μM indomethacin, 10 mM HEPES/Tris, pH 7.4). The apical compartment contained 100 μl, and the basolateral compartment contained 600 μl. Physiological salt solution was replaced with the same volumes of the prewarmed identical solution with and without 10 μM dobutamine hydrochloride (sc-203031; Santa Cruz Biotechnology); the apical medium contained 3 μCi/ml $^{45}Ca^{2+}$. Ten microliters of basolateral medium was collected at time points 0, 15, 30, 60, 120, and 180 min and analyzed for radioactivity in a PerkinElmer Life Sciences liquid scintillation counter. Unidirectional flux from the apical side to the basolateral side ($J_{A \rightarrow B}$) was calculated as described previously (25).

Semiquantitative Real-time PCR—To evaluate mRNA expression, RNA was extracted from pellets of 1,000 tubules isolated by COPAS using TRIzol® Reagent (Invitrogen) according to the manufacturer's protocol. The obtained RNA was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (Invitrogen). cDNA was used to determine mRNA expression levels by real-time PCR of *Adrb1* (β1-AR) and *Adrb2* (β2-AR). As controls *Trpv5* and *Slc12a1* (Na-K-Cl cotransporter 2, NKCC2) were included. The housekeeping gene *Gapdh* was used as an endogenous control. Primers targeting the genes of interest were designed using Primer3 and are listed in Table 1. Normal PCR using AmpliTaq Gold® (Invitrogen) was performed on HEK293 cDNA to check for

TABLE 1

***Mus musculus* oligonuceotide sequences used in real-Time PCR**

Adrb1, β1-adrenergic receptor gene; *Adrb2*, β2-adrenergic receptor gene; *Trpv5*, transient receptor potential vanilloid type 5 gene; *Slc12a1*, Na-K-Cl cotransporter 2 gene (NKCC2); *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase gene.

Gene	Forward primer	Reverse primer
Adrb1	GATCTGGTCATGGGATTGCT	AAGTCCAGAGCTCGCAGAAG
Adrb2	CCTTAACTGGTTGGGCTACG	GCTCTTGAAAGGCAATCCTG
Trpv5	CTGGAGCTTGTGGTTTCCTC	TCCACTTCAGGCTCACCAG
Slc12a1	GGCTTGATCTTTGCTTTTGC	CCATCATTGAATCGCTCTCC
Gapdh	TAACATCAAATGGGGTGAGG	GGTTCACACCCATCACAAAC

β1-AR and β2-AR expression (primers: *ADRB1* forward, CCCAGAAGCAGGTGAAGAAG and reverse, CCCAGCCAGTTGAAGAAGAC; *ADRB2* forward, GGCAGCTCCAGAAGATTGAC and reverse, TGGAAGGCAATCCTGAAATC); products were visualized on a 1.5% (w/v) agarose gel.

Cell Culture and Transfection—HEK293 cells were grown in DMEM (Bio Whittaker) containing 10% v/v fetal calf serum (PAA), 2 mM L-glutamine, 10 μl/ml nonessential amino acids at 37 °C in a humidity-controlled incubator with 5% CO$_2$. Cells were transiently transfected with the appropriate plasmids using polyethyleneimine (PolySciences) with a DNA:polyethyleneimine ratio of 1:6 for biochemical or live cell imaging experiments.

Cell Surface Biotinylation and Immunoblotting—HEK293 cells (9×10^4 cells/cm^2) were plated and transfected with 5 μg of TRPV5-HA pCINeo/IRES-GFP or pCINeo/IRES-GFP in 10-cm dishes. At 24 h after transfection cells were reseeded on poly-L-lysine-coated (Sigma) 6-well plates. At 48 h after transfection, cells were incubated for 1 h with 10 μM dobutamine or vehicle. Cells were homogenized in 1 ml of lysis buffer as described previously (6) using the NHS-LC-LC-biotin (Pierce). Finally, biotinylated proteins were precipitated using NeutrAvidin beads (Pierce). TRPV5 expression was analyzed by immunoblotting for the precipitates (plasma membrane fraction) and for the total cell lysates using the anti-HA antibody (6).

Intracellular Ca^{2+} and cAMP Measurements Using Fura-2/AM and Exchange Protein Directly Activated by cAMP (EPAC)-Enhanced GFP (EGFP)-mCherry—For combined $[\mathrm{Ca}^{2+}]_i$ and cAMP measurements, HEK293 cells were seeded onto coverslips (Ø 25 mm) and cotransfected with the cAMP sensor EPAC-EGFP-mCherry (26), kindly provided by Dr. K. Jalink) for cAMP measurements, and the appropriate TRPV5 pCINeo/IRES-EGFP construct from which the sequence encoding EGFP was deleted. After 24 h, cells were loaded with 3 μM Fura-2/AM (Molecular Probes) and 0.01% v/v Pluronic F-129 (Molecular Probes) in DMEM at 37 °C for 20 min. After loading, cells were washed with PBS and allowed to equilibrate at 37 °C for another 10 min in HEPES/Tris buffer (in mM: 132.0 NaCl, 4.2 KCl, 1.4 CaCl$_2$, 1.0 MgCl$_2$, 5.5 D-glucose, and 10.0 HEPES/Tris, pH 7.4). Changes in $[\mathrm{Ca}^{2+}]_i$ and cAMP were simultaneously monitored, using a modified Fura-2 protocol allowing simultaneous measurements of Ca^{2+} and cAMP (10). Briefly, the cAMP sensor EPAC-EGFP-mCherry was excited at 488 using a monochromator. Fluorescence emission light was directed by a 525DRLP dichroic mirror (Omega Optical) through a 535af26 emission filter (EGFP fluorescence) and a 565ALP emission filter (mCherry fluorescence) onto a CoolSNAP HQ monochrome CCD camera. The integration time of the CCD camera was set at 200 ms with a sampling interval of 5 s. All measurements were performed at room temperature. Quantitative image analysis was performed with Metamorph 6.0 (Molecular Devices). For each wavelength, the mean fluorescence intensity was monitored in an intracellular region and, for purpose of background correction, an extracellular region of identical size. After background correction, the fluorescence emission ratio of 340 nm and 380 nm excitation was calculated to determine the $[\mathrm{Ca}^{2+}]_i$ while the fluorescence emission ratio of the red and green fluorescence of the cAMP sensor was used to determine changes in cellular cAMP content.

Statistical Analysis—If not specified otherwise, the data are expressed as mean ± S.E. The significant differences between the means of two groups were analyzed by an unpaired Student's *t* test using the measurements per cell/sample ($n \geq 9$) of at least three independent experiments. The level of statistical significance is $p < 0.05$. All data were analyzed using GraphPad Prism.

RESULTS

β1-AR and β2-AR Are Expressed in DCT2/CNT—To investigate the role of β1-AR and β2-AR on active Ca^{2+} reabsorption in DCT2/CNT, immunohistochemical staining was performed on frozen mouse kidney sections. Stainings showed colocalization of β1-AR with TRPV5 in DCT2/CNT segments, but not β2-AR (Fig. 1, *a* and *c*). β1-AR and β2-AR antibody specificity was evaluated by negative controls of secondary antibody only (Fig. 1*b*, *neg ctrl panel*) and staining of liver tissue (Fig. 1*b*, *liver panel*) reported to be negative for both β1-AR and β2-AR (27, 28). mRNA expression of β1-AR (*Adrb1*) and β2-AR (*Adrb2*) in DCT2/CNT cells isolated from pTRPV5-EGFP mice using the COPAS Biosorter, was determined by real-time PCR. Results showed that both β1-AR and β2-AR are enriched in DCT2/CNT compared with total kidney cortex material, although the expression level of β1-AR is considerably higher (Fig. 2*a*), especially compared with the negative control NKCC2 (*Slc12a1*) normally expressed in TAL. TRPV5 expression was used as a positive control. Altogether, these results indicated that β1-AR is expressed at the DCT2/CNT part of the nephron together with TRPV5. At the cellular level, only part of the β1-AR signal did colocalize with TRPV5, suggesting expression in both apical and basolateral areas (Fig. 1*c*).

Dobutamine Stimulates Transcellular Ca^{2+} Transport in Mouse DCT2/CNT—To examine the role of β1-AR stimulation on Ca^{2+} reabsorption in the distal part of the nephron, primary DCT2/CNT tubules were cultured on Transwell inserts for 7/8 days allowing the formation of tight monolayers (transepithe-

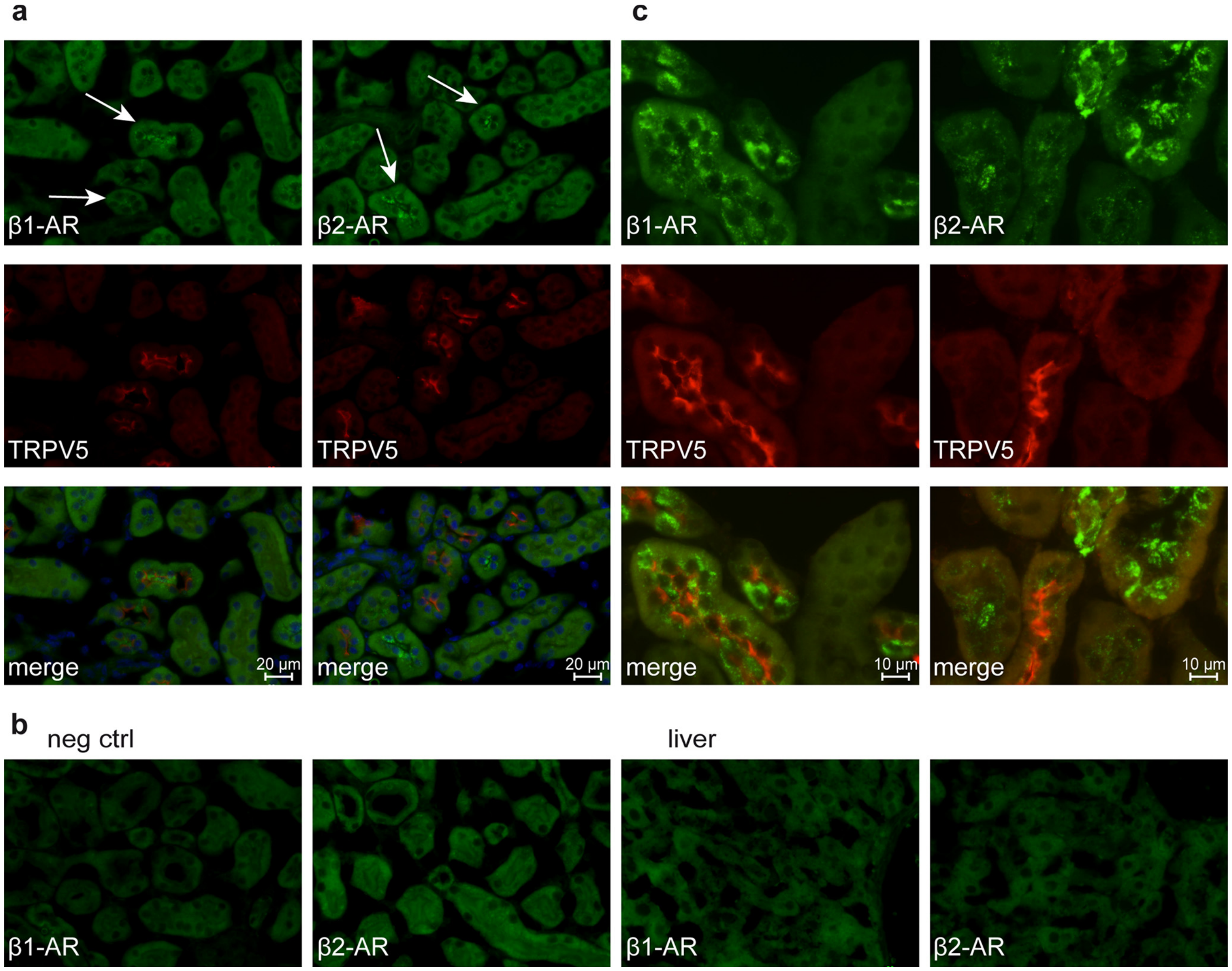

FIGURE 1. **β1-AR and β2-AR expression in mouse kidney.** *a*, localization of β1-AR, β2-AR, and TRPV5 is depicted by immunohistochemistry on mouse kidney tissue. *b*, negative controls of secondary antibody only and stainings of liver tissue are shown. *c*, higher magnification immunohistochemistry pictures of the localization of β1-AR, β2-AR, and TRPV5 are shown.

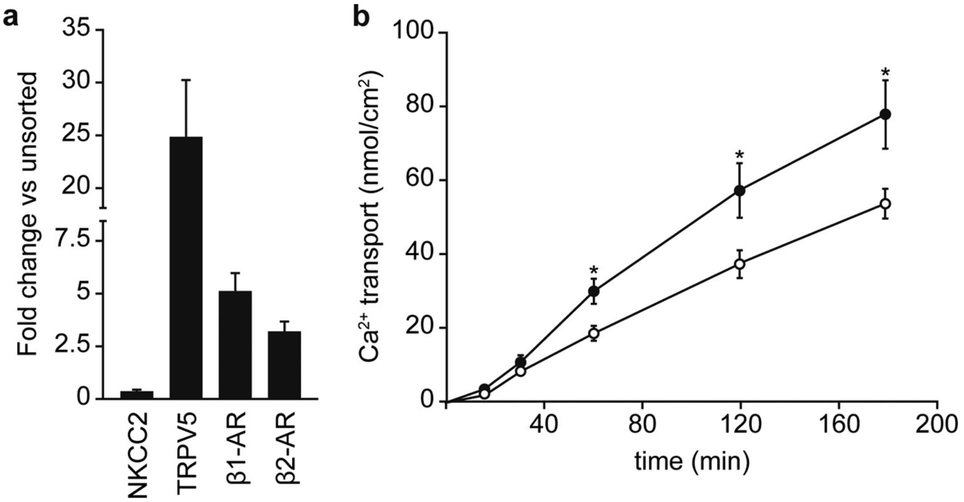

FIGURE 2. **Effect of β1-AR agonist (10 μM dobutamine) on transepithelial Ca^{2+} transport in mouse DCT2/CNT primary cultured monolayers.** *a*, mRNA enrichment of β1-AR (*Adrb1*) and β2-AR (*Adrb2*) in isolated mouse DCT2/CNT cells compared with total kidney cortex. TRPV5 (*Trpv5*) was used as positive control, NKCC2 (*Slc12a1*) as negative control ($n = 6$). *b*, Ca^{2+} transport under control (*open circles*) and dobutamine (*closed circles*) conditions, respectively. t_{15}, t_{30}, t_{60}, t_{120}, and t_{180} indicate time points of sample collections after 15-, 30-, 60-, 120-, and 180-min incubation. *, $p < 0.05$ compared with control at the same time point ($n = 7–8$).

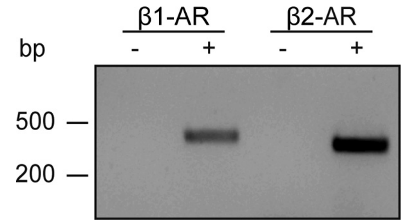

FIGURE 3. **Expression of β1- and β2-AR in HEK293 cells.** + and − signify PCR products of reverse-transcribed and non-reverse-transcribed mRNA, respectively. Product sizes were 346 bp for β1-AR and 328 bp for β2-AR.

lial electrical resistance of 603 ± 80 $\Omega\cdot\text{cm}^2$ 1 day prior to the experiment). Transepithelial transport of Ca^{2+} from the apical to basolateral compartment was measured by apical addition of $^{45}Ca^{2+}$ in the presence or absence of 10 μM dobutamine, a specific β1-AR agonist, in both the apical and basolateral solution. Transport of Ca^{2+} was significantly increased by 10 μM dobutamine (19 ± 2 and 30 ± 4 $\text{nmol}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ for control and dobutamine, respectively, $p < 0.05$) after 60 min (Fig. 2*b*).

β1-AR Agonist Stimulates cAMP Production in HEK293 Cells—To study the role of the Ca^{2+} channel TRPV5 in β1-AR-mediated activation of transcellular Ca^{2+} transport, we examined the effect of dobutamine in HEK293 cells, which were reported to express β1-AR (29). First, the expression of β1- and β2-AR in HEK293 cells was confirmed (Fig. 3). Identity of the observed bands was verified by sequencing of the respective PCR products. Because β1-AR activation leads to cAMP production (18) the β1-AR-mediated TRPV5 Ca^{2+} influx in HEK293 cells transfected with the cAMP sensor EPAC and TRPV5 was studied as described under "Experimental Procedures." The EPAC and Fura-2 ratios were measured before ($t = 0$ min, t_0) and after ($t = 3$ min, t_3) dobutamine treatment (10 μM) (Fig. 4, *a* and *d*). The EPAC ratio plotted at t_0 and t_3 indicated a significant increase in cAMP levels after a 2-min incubation with dobutamine as measured by a 5.1 ± 0.1% increase in the EGFP: mcherry ratio due to loss of the FRET signal upon binding of cAMP (Fig. 4*b*). Fura-2 ratios were increased by 19.5 ± 0.3% upon stimulation by dobutamine (Fig. 4*e*). The dobutamine-induced increase in EPAC and Fura-2 ratio was dose-dependent (Fig. 4, *c* and *f*), implying that β1-AR signaling can functionally stimulate TRPV5 activity in HEK293 cells.

Dobutamine Does Not Change TRPV5 Protein Abundance at the Plasma Membrane—Ca^{2+} influx through TRPV5 is regulated by plasma membrane abundance and activity of the channel (3). The effect of dobutamine on TRPV5 plasma membrane expression was assessed in HEK293 cells transiently transfected with TRPV5 using cell surface biotinylation. A 60-min incubation with 10 μM dobutamine did not affect plasma membrane abundance of TRPV5 (Fig. 5, *a* and *b*). Total expression of TRPV5 in the dobutamine-treated cells did not differ from control as well (Fig. 5*a*).

Dobutamine Stimulates TRPV5-mediated Ca^{2+} Uptake in HEK293 Cells via PKA Phosphorylation of Thr-709 Residue—TRPV5 channel activity is known to be stimulated by cAMP/PKA-mediated pathways (6). HEK293 cells were transfected with TRPV5 and loaded with the Fura-2 Ca^{2+} sensor to measure Ca^{2+} uptake. After a 1-min incubation with dobutamine, Ca^{2+} uptake reached maximal activation, ~2 times elevated compared with basal uptake levels (Fig. 6*a*). Interestingly, these stimulatory effects were prevented when the cells were preincubated (30 min) with the PKA inhibitor, H89 (Fig. 6, *a* and *b*), indicating that β1-AR activation is mediated by PKA phosphorylation of the TRPV5 channel. Previously, a threonine residue 709 on the C terminus of TRPV5 was identified as the sole TRPV5 PKA phosphorylation site (10). To study the effects of dobutamine on phosphorylation of TRPV5 at this residue, WT-TRPV5, nonphosphorylated mutant T709A-TRPV5, and the T709D-TRPV5 mutant mimicking phosphorylation at this residue were used. The stimulatory effect of dobutamine was absent in the nonphosphorylable T709A-TRPV5 mutant and the phosphorylation-mimicking T709D-TRPV5 mutant, the latter already showing activation, indicating that the Thr-709 residue is crucial for β1-AR-mediated PKA phosphorylation (Fig. 6, *c* and *d*).

DISCUSSION

The present study indicates that activation of β1-AR stimulates TRPV5-mediated Ca^{2+} transepithelial transport via PKA phosphorylation of the Ca^{2+} channel. Our conclusion relies on the following findings: (i) β1-AR colocalizes with TRPV5 in mouse DCT2/CNT; (ii) the β1-AR agonist dobutamine stimulates cAMP production and TRPV5-mediated Ca^{2+} uptake in HEK293 cells; (iii) dobutamine increases the TRPV5 channel activity which can be prevented by the PKA inhibitor H89 and mutation of the channel at the Thr-709 residue; (iv) dobutamine stimulates the apical-to-basolateral transepithelial Ca^{2+} flux in the mouse primary DCT2/CNT culture.

To our knowledge, the expression profile and the consequences of activation of β-ARs in mouse DCT2/CNT have not been characterized. Boivin and colleagues elaborately investigated β1- and β2-ARs in rat kidney by immunohistochemistry (17). In their study, β2-AR is faintly present in DCT and undetectable in CNT (17), whereas in our study β2-AR did not colocalize with TRPV5 in the mice DCT2/CNT. For β1-AR, Boivin *et al.* showed colocalization with calbindin-D_{28K} on the apical membrane (17) in line with our finding that β1-AR colocalizes with TRPV5, although we did not observe a clear apical localization.

Gesek and White demonstrated that the immortalized mouse DCT cell line expresses both β1- and β2-ARs (30). In the present study, both receptors were detected by PCR in material from DCT2/CNT as isolated by the COPAS biosorter. Considering the lower amounts of β2-AR detected in highly enriched DCT2/CNT material, combined with the absence of β2-AR in immunohistological stainings in DCT2/CNT, we conclude that β2-AR seems not present in the DCT2/CNT. Gesek and Friedman showed that isoproterenol stimulated both cAMP production and Na^+ uptake, but not Ca^{2+} influx into the cells (31). However, they selected thiazide-sensitive DCT1 cells, which contain no TRPV5, resulting in very low Ca^{2+} transport rates (20, 24, 31). In contrast, in the present study, DCT2/CNT tubules were selectively sorted from TRPV5-expressing cells. In the polarized mouse primary DCT2/CNT culture we demonstrated that dobutamine stimulates apical-to-basolateral transepithelial Ca^{2+} transport. Due to generally lower expression levels of β1-AR, in primary cultures compared with the *in vivo*

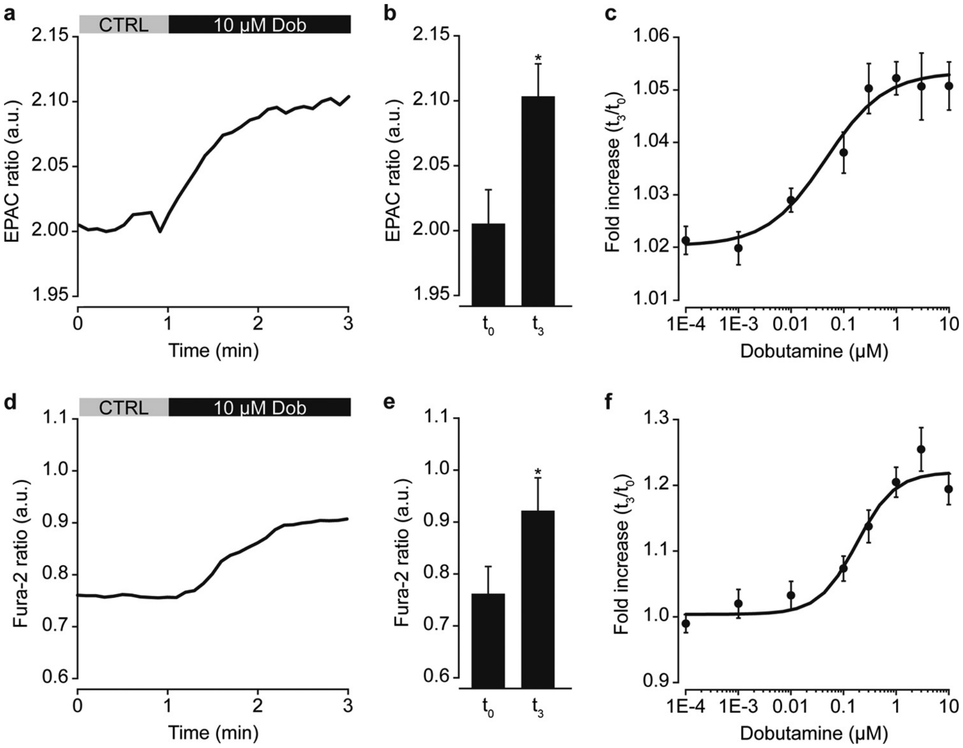

FIGURE 4. **Effect of β1-AR agonist dobutamine on cAMP production and Ca^{2+} uptake in HEK293 cells transiently transfected with TRPV5 and EPAC sensor.** *a*, averaged EPAC trace upon the addition of 10 μM dobutamine (*Dob*) ($n = 60$ cells). *b*, EPAC ratios before (t_0) and after (t_3) dobutamine stimulation. *, $p < 0.05$ compared with t_0 ($n = 60$ cells). *c*, dose-response curve of the effect of dobutamine treatment on cAMP production as measured by EPAC ratios (t_3 compared with t_0) ($n = 28$–76 cells/condition). *d*, averaged Fura-2 trace upon the addition of 10 μM dobutamine ($n = 59$ cells) *e*, Fura-2 ratios demonstrated before (t_0) and after (t_3) dobutamine stimulation. *, $p < 0.05$ compared with t_0 ($n = 59$ cells). *f*, dose-response curve of the effect of dobutamine treatment on Ca^{2+} uptake as measured by Fura-2 ratios (t_3 compared with t_0) (29–70 cells/condition).

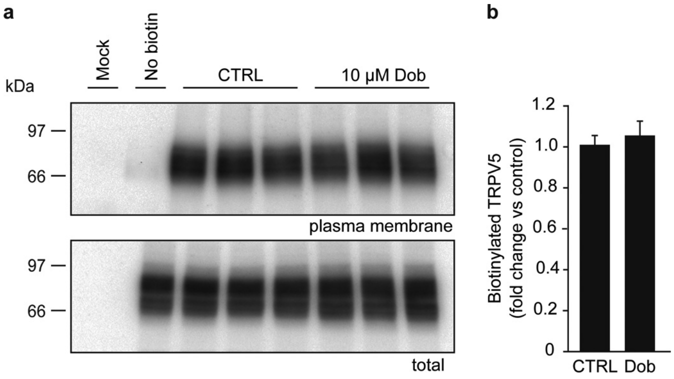

FIGURE 5. **Effect of β1-AR agonist on plasma membrane abundance of TRPV5.** *a*, TRPV5-transfected HEK293 cells were treated for 60 min with or without 10 μM dobutamine. NHS-LC-LC-biotin was added to medium for 30 min before the cells were lysed, and the biotinylated (plasma membrane) fraction was pulled down using neutravidin-agarose beads. Representative immunoblot is shown for TRPV5 analyzed for plasma membrane and total expression. *CTRL*, control; *Dob*, dobutamine-treated. *b*, calculated immunoblot densities ($n = 9$).

situation, relatively high concentrations of dobutamine were applied (10 μM) to stimulate transepithelial Ca^{2+} transport maximally.

HEK293 cells are generally used as a model for studying molecular mechanism of TRPV5-mediated Ca^{2+} uptake because they lack endogenous expression of this Ca^{2+} channel (32), allowing transfection of exogenous wild-type (WT) or residue-specific TRPV5 mutants. In addition, due to overexpression of TRPV5 faster and stronger effects are to be expected compared with primary cultures. Moreover, the cells were reported to express β1-AR, which could be stimulated by a nonspecific β-AR agonist isoproterenol, showing enhanced cAMP synthesis (29). Here, we show that the intracellular level of cAMP is increased within minutes after addition of dobutamine, indicating that β1-AR was functionally expressed in HEK293 cells and that the cells are suitable for studying β1-AR-initiated intracellular signaling. Accordingly, dobutamine was demonstrated to stimulate Ca^{2+} uptake in TRPV5-expressing HEK293 cells with maximal activation at 2 min. This rapid action of the agonist is likely nongenomic as exemplified by the hormonal action of PTH on TRPV5 activity that exerted similar maximal stimulation within 2 min. It is reported that TRPV5 activation is stimulated by the cAMP-dependent PTH1R signaling pathway leading to PKA-mediated phosphorylation of TRPV5 on the C terminus of the channel (6). Moreover, Topala and colleagues showed that the activation of the Ca^{2+}-sensing receptor induces TRPV5-mediated Ca^{2+} peak current in <1 s (4). They elaborated that the Ca^{2+}-sensing receptor agonist neomycin mediated PKC phosphorylation of the residues Ser-299 and Ser-654. Interestingly, Gkika and co-workers demonstrated that long term (1-h) stimulation of bradykinin receptor type 2 with tissue kallikrein resulted in increased plasma membrane accumulation of TRPV5 (12). In the present study, however, 1-h dobutamine incubation did not affect plasma membrane abundance or total expression of TRPV5. Altogether, activation of β1-AR enhanced TRPV5 activity via cAMP-dependent PKA phosphorylation of the TRPV5 channel at residue Thr-709 in the intracellular C terminus.

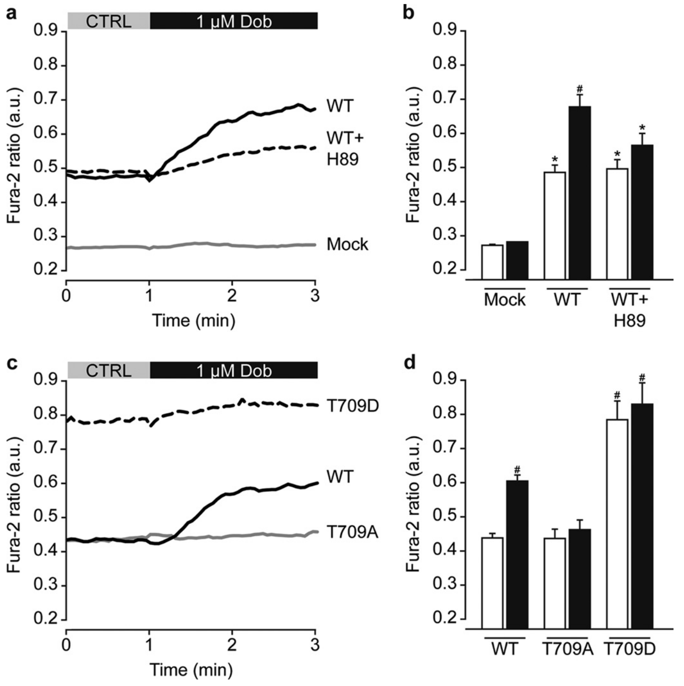

FIGURE 6. **Effect of β1-AR agonist on TRPV5-dependent Ca^{2+} uptake.** *a*, averaged Fura-2 traces of mock ($n = 10$ cells), WT-TRPV5-transfected preincubated with PKA inhibitor, H89, 10 μM ($n = 26$), and without ($n = 12$) upon the addition of 1 μM dobutamine. *b*, Fura-2 levels before (*open bars*) and after (*filled bars*) dobutamine stimulation of mock-transfected ($n = 10$ cells), WT-TRPV5 ($n = 12$), and H89-treated WT-TRPV5 ($n = 26$). *, $p < 0.05$ compared with untreated mock; #, $p < 0.05$ compared with the respective dobutamine-untreated (t_0). *c*, averaged Fura-2 traces of T709A-TRPV5 ($n = 21$) and T709D-TRPV5 ($n = 20$ cells) mutants upon the addition of dobutamine compared with WT-TRPV5 ($n = 12$). *d*, Fura-2 levels demonstrated before (*open bars*) and after (*filled bars*) dobutamine stimulation of WT-TRPV5 ($n = 12$ cells), T709A-TRPV5 ($n = 21$), and T709D-TRPV5 ($n = 20$). #, $p < 0.05$ compared with untreated WT.

Apart from the classic cAMP-dependent signaling mechanism of β-ARs, β1-AR has been reported to be PLC-dependent (20). In the study by Kang and colleagues, a nonspecific β-AR agonist isoproterenol stimulated Mg^{2+} uptake in isolated mouse DCT1 cell culture (20). Isoproterenol is known to bind preferentially to β1-AR and to a lesser extent to β2-AR. In the latter study, both PKA and PLC inhibitors inhibited the stimulatory effects of isoproterenol. Phorbol 12-myristate-13-acetate, a DAG analog, had no effect on Mg^{2+} reabsorption, but potentiated effects of isoproterenol (20). There is no report of dobutamine action on signaling mechanisms other than the cAMP-dependent pathway, and the PKA inhibitor H89 completely blocked the dobutamine action. Thus, the β1-AR signaling pathway in the present study seems to be mediated solely by the cAMP/PKA.

The present study indicates a novel calciotropic role of dobutamine in renal DCT2/CNT in addition to the positive inotropic effect in cardiac myocytes. In addition, β-blockers widely used in treatment of CHF might have adverse effects on maintaining body Ca^{2+} homeostasis. CHF initiates when the heart fails to supply sufficient blood to match the body demand due to reduced cardiac contractility (21, 33). Heart rate is accelerated (34), leading to a decreased effective circulating volume, which in turn triggers the renin-angiotensin-aldosterone system in the kidney (35). As a result of CHF, patients develop secondary aldosteronism leading to increased fecal and urinary Ca^{2+} excretion (36). In addition, patients are generally treated with loop diuretics and often suffer from vitamin D deficiency (36, 37). Moreover, hypercalciuria and osteopenia have been implicated in patients with advanced CHF awaiting cardiac transplantation (36, 38). Hence, potential calciotropic effects of β-blockers might further increase chances of developing hypocalcemia in these patients. In addition, β-blockers are used to resolve hypercalcemia among other symptoms in hyperthyroidism (39, 40). The mechanism of action of β-blockers in hyperthyroidism is unclear, but principally appears to antagonize overstimulated β-AR-mediated signaling. Interestingly, the Ca^{2+}-lowering effect of propranolol (nonselective β-blocker) was proposed to be caused by a direct effect on bone or renal Ca^{2+} handling (40).

In conclusion, the present study demonstrates that dobutamine, a β1-AR agonist, stimulates TRPV5 activity via a PKA-dependent pathway. PKA activation results in phosphorylation of the Thr-709 residue and consequently enhances TRPV5 activity. Epi and NE, by activating TRPV5 activity, could potentially be involved in normal physiological renal Ca^{2+} handling in the DCT2/CNT. Therefore, β-adrenergic receptor agonists and blockers, increasingly administered to patients, potentially exert adverse calciotropic effects.

Acknowledgments—We thank Judy Lin and Hans Meijer for excellent technical assistance and Dr. Praetorius for transgenic mice expressing EGFP under the TRPV5 promoter in DCT2 and CNT.

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