NOX2 interacts with podocyte TRPC6 channels and contributes to their activation by diacylglycerol: essential role of podocin in formation of this complex

Eun Young Kim,1* Marc Anderson,1* Cory Wilson,1 Henning Hagmann,2 Thomas Benzing,2 and Stuart E. Dryer1,3

1Department of Biology and Biochemistry, University of Houston, Houston, Texas; 2Department II of Internal Medicine and Center for Molecular Medicine Cologne, University of Cologne, Cologne, Germany; and 3Division of Nephrology, Baylor College of Medicine, Houston, Texas

Submitted 21 June 2013; accepted in final form 9 August 2013

Kim EY, Anderson M, Wilson C, Hagmann H, Benzing T, Dryer SE. NOX2 interacts with podocyte TRPC6 channels and contributes to their activation by diacylglycerol: essential role of podocin in formation of this complex. Am J Physiol Cell Physiol 305: C960–C971, 2013. First published August 15, 2013; doi:10.1152/ajpcell.00191.2013.—Canonical transient receptor potential-6 (TRPC6) channels have been implicated in the pathophysiology of glomerular diseases. TRPC6 channels are typically activated by diacylglycerol (DAG) during PLC-dependent transduction cascades. TRPC6 channels can also be activated by reactive oxygen species (ROS). We previously showed that podocin is required for DAG analogs to produce robust activation of TRPC6 channels in podocytes. Here we show that endogenous TRPC6 channels in immortalized podocytes reciprocally coimmunoprecipitate with the catalytic subunit of the NADPH oxidase NOX2 (gp91phox). The NOX2-TRPC6 interaction was not detected in cells stably expressing a short hairpin RNA targeting podocin, although NOX2 and TRPC6 were present at normal levels. Application of a membrane-permeable DAG analog [1-oleoyl-2-acetyl-sn-glycerol (OAG)] increased generation of ROS in podocytes, but this effect was not detected in podocin knockdown cells. OAG also increased steady-state surface expression of the NOX2 regulatory subunit p47phox. In whole cell recordings, TRPC6 activation by OAG was reduced in podocytes pretreated with the NOX2 inhibitor apocynin, by the pan-NOX inhibitor diphenylene iodonium, and by tempol, a ROS quencher. Cholesterol depletion and disruption of lipid rafts by methyl-β-cyclodextrin precipitated the NOX2-TRPC6 interaction as assessed by coimmunoprecipitation. These data suggest that active NOX2 assemblies with TRPC6 at podocin-organized sterol-rich raft domains and becomes catalytically active in response to DAG. The localized production of ROS contributes to TRPC6 activation by chemical stimuli such as DAG. Podocin appears to be necessary for assembly of the NOX2-TRPC6 complex in lipid rafts.

podocyte; glomerular filtration; TRPC6; slit diaphragm; podocin; NADPH oxidase

Podocytes are complex multipolar cells that form an essential component of the glomerular filtration apparatus (46). Major processes extend from the podocyte cell body, ramify extensively, and ultimately form foot processes that attach to the external surface of the glomerular capillary basement membrane. Specialized junctions between adjacent foot processes, known as slit diaphragms, form a porous matrix through which water and small solutes pass to enter the urinary space. The differentiated structure of podocyte foot processes and slit diaphragms is maintained by complex cytoskeletal elements and a host of associated proteins (12). In most glomerular diseases, it is possible to discern stereotypical ultrastructural changes in the shape of podocytes, especially in their foot processes, often at early stages of the disease process (33). Some foot processes become broader, while others retract all the way back into major processes, reflecting different stages of a pattern known as foot process effacement. Effacement is accompanied by marked changes in cytoskeletal organization within foot processes (57) and may be an adaptive mechanism to prevent the detachment of podocytes during stress (33). Several lines of evidence indicate that sustained Ca2+ overload into foot processes can drive their effacement (35).

Canonical transient receptor potential-6 (TRPC6) channels are Ca2+-permeable cationic channels that have been implicated in the pathophysiology of glomerular diseases. The initial reports described genetic analyses of familial forms of focal and segmental glomerulosclerosis (FSGS) (50, 68). In these families, FSGS was transmitted as an autosomal-dominant associated with mutations in TRPC6 channels. Most of the mutant TRPC6 channels identified in FSGS patients show a gain of function when they are heterologously expressed in human embryonic kidney (HEK-293) cells (19, 50, 68). In those studies, TRPC6 channels were usually activated in response to stimulation of G protein-coupled receptors coupled to PLC signaling cascades (50, 54, 68). In addition, there is evidence that TRPC6 expression in podocytes is increased in certain acquired (nongenetic) glomerular diseases (39). Moreover, podocyte-specific overexpression of wild-type and mutant TRPC6 channels in mice also results in foot process effacement, glomerulosclerosis, and albuminuria (32). It has also been suggested that acute glomerular toxicity evoked by puromycin aminonucleoside is driven in part by upregulation of TRPC6 secondary to generation of reactive oxygen species (ROS) (65).

Given this information, it is important to understand factors that normally lead to TRPC6 activation in podocytes. The canonical pathway for TRPC6 activation is through PLC, which leads to formation of diacylglycerol (DAG) from membrane phospholipids. Pioneering studies in heterologous expression systems showed that DAG is sufficient to cause a membrane-delimited activation of heterologously expressed TRPC6 channels (21). Application of membrane-permeable DAG analogs such as 1-oleoyl-2-acetyl-sn-glycerol (OAG) evokes robust activation of native TRPC6 channels in podocytes (1, 28). Moreover, PLC is required for activation of

* E. Y. Kim and M. Anderson contributed equally to this work.

Address for reprint requests and other correspondence: S. E. Dryer, Dept. of Biology and Biochemistry, Univ. of Houston, Houston, TX 77204-5001 (e-mail: s dryer@uh.edu).
endogenous TRPC6 channels in primary podocytes by ANG II (unpublished observations). Importantly, we also observed that ROS contribute significantly to activation of TRPC6 channels evoked by insulin (28) and also by N-methyl-D-aspartate receptor agonists, including metabolites of homocysteine (29), and by ANG II (data not shown). Other groups observed ROS-dependent activation of podocyte TRPC6 channels in response to elevated glucose (36) and puromycin aminonucleoside (65).

More recently, we showed that podocyte TRPC6 channels exhibit multimodal gating mechanisms, as they can be activated by mechanical stimuli, as well as by chemical stimuli, such as OAG (1). Importantly, the mechanical activation of TRPC6 channels in podocytes is not mediated by G protein signaling cascades or phospholipases, in contrast to the situation in vascular smooth muscle (24, 59). The dominant mode of TRPC6 activation in podocytes is determined in part by interactions with a cholesterol-binding hairpin loop membrane protein known as podocin (1). Podocin knockdown markedly enhances mechanical activation of endogenous TRPC6 channels but reduces, or even eliminates, activation evoked by OAG (1). Consistent with this finding, coexpression of podocin markedly enhanced OAG activation of TRPC6 channels in Xenopus oocytes (22). An important implication of these observations is that podocin deficiencies would be expected to cause a large gain of function in TRPC6 channels in foot processes, which are exposed to continuous mechanical stimulation derived from the cardiac cycle. This process is likely to contribute to the severe and early-onset foot process effacement observed in humans (6) and animal models (40) in which there is a podocin deficiency.

In the current study we address the mechanism whereby podocin is able to regulate activation of podocyte TRPC6 channels by chemical stimuli such as OAG. We report that endogenously expressed TRPC6 channels of immortalized podocyte cell lines occur in a complex with the NADPH oxidase NOX2. Moreover, OAG increases generation of ROS, which contribute significantly to activation of TRPC6. Impor-

---

Fig. 1. Interactions between NADPH oxidase 2 (NOX2), canonical transient receptor potential-6 (TRPC6) channels, and podocin. A: reciprocal coimmunoprecipitation of catalytic subunit of NOX2 (gp91phox) and TRPC6 in a wild-type mouse podocyte cell line. Initial precipitation was carried out with anti-NOX2 (subunit gp91phox), and immunoblot (IB) was probed with rabbit (r) anti-TRPC6 (left). Immunoprecipitation (IP) was carried out with goat (g) anti-TRPC6, and blot was probed with anti-NOX2 (right). Molecular weight markers are shown at left. B: NOX2 catalytic subunit also interacts with podocin, as shown by reciprocal coimmunoprecipitation carried out on podocyte lysates. C: in podocin knockdown (podocin KD) cells, it was no longer possible to detect coimmunoprecipitation of NOX2 and TRPC6 (2 blots on left), but anti-NOX2 could precipitate NOX2 and goat anti-TRPC6 could immunoprecipitate TRPC6 (2 blots on right). In all experiments, a sample of the original cell lysate was diluted and loaded on the gel and is labeled “input.” This confirmed expression of the proteins in the cells.
tantly, we observed that podocin is required for the NOX2-TRPC6 interaction, probably because of its ability to organize proteins within cholesterol-rich raft domains at the cell surface.

**MATERIALS AND METHODS**

**Cell culture.** Immortalized mouse podocyte cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin-streptomycin, and recombinant mouse γ-interferon and propagated at 33°C. Podocyte differentiation and expression of podocyte markers were induced by removal of γ-interferon and switch to 37°C for 14 days (31). The podocin knockdown (podocin KD) cell line is described in detail elsewhere (1). Briefly, a short hairpin RNA targeting the coding sequence of podocin was transfected into an immortalized mouse podocyte cell line by lentiviral transfection. Selection of stable cells was achieved by addition of 30 μg/ml blasticidin (Invivogen) to the cell culture medium (1). Chinese hamster ovary (CHO-K1) cells were purchased from American Type Culture Collection.

**Coimmunoprecipitation, cell surface biotinylation assays, and measurements of ROS generation.** Immunoblot and immunoprecipitation were carried out on podocyte lysates cleared by centrifugation, as described previously (28–31). For coimmunoprecipitation, rabbit anti-TRPC6 was obtained from Alomone Laboratories, goat anti-TRPC6 from Novus Biologicals, rabbit anti-NOX2 from Santa Cruz Biotechnology, and rabbit anti-podocin from Acris. Cell surface biotinylation assays were done using methods described previously (28–31). Briefly, podocytes were treated with sulfo-N-hydroxysuccinimidobiotin (Pierce Biotechnology, Rockford, IL) for 1 h to biotinylate surface proteins. The reaction was stopped, cells were lysed, and biotinylated proteins from the cell surface were recovered from lysates using streptavidin-agarose beads (Pierce Biotechnology). A sample of the initial podocyte lysate was retained for analysis of total protein. All samples were separated on SDS-PAGE, and proteins were quantified by immunoblot analysis using rabbit anti-p47phox (Santa Cruz Biotechnology). Immunoblots were analyzed by densitometry using ImageJ software (National Institutes of Health). Generation of ROS by podocytes was measured using the OxiSelect fluorometric assay (Cell Biolabs) according to the manufacturer’s instructions. In this assay, a cell-permeable probe, 2',7'-dichlorodihydrofluorescin diacetate, diffuses into cells and is deacetylated to a nonfluorescent product that is trapped in the cytosol. In the presence of cytosolic ROS, this probe is oxidized to a fluorescent product, 2',7'-dichlorodihydrofluorescein, which is measured using a fluorescence microplate reader. All biochemical experiments were repeated at least three times.

**Electrophysiology and drug treatments.** Whole cell recordings from podocytes were made as described previously (1, 28, 29). Bath salines contained (in mM) 150 NaCl, 5.4 CsCl, 0.8 MgCl₂, 5.4 CaCl₂, and 10 HEPES (pH 7.4). Recording pipette solutions in all experiments contained (in mM) 10 NaCl, 125 CsCl, 6.2 MgCl₂, 10 HEPES, and 10 EGTA (pH 7.2). The bath was perfused at a constant flow rate (0.3 ml/min), and outwardly rectifying currents were periodically evoked by ramp voltage commands (−80 to + 80 mV over 2.5 s) from a holding potential of −40 mV. In most experiments, this was

---

**Fig. 2.** Colocalization of TRPC6 and NOX2 in confocal images of cultured podocytes. A: localization of NOX2 (green) and TRPC6 (red). B: merged image. Nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI), and the signal from that probe is present in all images. Arrows in B show regions where colocalization of the signal is readily seen along outer margins of the cell.
done before and after application of 100 μM OAG delivered in external saline by bath superfusion. Currents were digitized and analyzed by pCLAMP software (version 10.1, Molecular Devices). Whole cell currents were quantified at +80 mV. In many of these experiments, podocytes were treated with tempol, apocynin, or diphenylene iodonium (DPI) (all from Sigma) in the cell culture incubators for 30 min prior to electrophysiological analyses. Podocytes were treated with 10 mM methyl-β-cyclodextrin (MBCD) for 18 h prior to electrophysiological analysis. All traces were filtered at 300 Hz using the digital Bessel filter implemented in pCLAMP.

**Cholesterol staining and confocal microscopy.** Podocytes or CHO-K1 cells were fixed in 4% paraformaldehyde for 30 min at 22°C, washed three times with PBS (pH 7.4), and treated with filipin (0.05 mg/ml in PBS containing 10% bovine serum albumin) for 1 h at 37°C to stain membrane cholesterol. The cells were then washed three times in PBS and treated for 10 min with a proprietary red fluorescent dye conjugated to phalloidin (Acti-stain 555, Cytoskeleton) at 22°C to label actin microfilaments. Cells were then washed three times in PBS and mounted using Vectashield medium. Images were collected on a Leica SP-8 confocal microscope using HCX PL APO CS 40 and 60 oil-immersion objectives. Filipin staining was detected using excitation at 355 nm while emission was monitored at 440 nm. Actin microfilaments were observed using excitation at 535 nm while emission was monitored at 585 nm. Note that these cells were not permeabilized prior to application of the fluorescent dyes. Three-dimensional stacks were constructed from 300 optical sections (370 nm) using Leica LAF-AS software. For NOX2-TRPC6 colocalization, differentiated podocytes were fixed in 4% paraformaldehyde at room temperature (22°C) for 30 min, washed three times with PBS, and then incubated for 1 h at 37°C with blocking solution (10% bovine serum albumin and 1 mg/ml glycine in PBS) with 0.1% Triton X-100. The cells were subsequently washed three times with PBS and then treated with rabbit anti-NOX2/gp91phox (Abcam) and goat anti-TRPC6 (Santa Cruz), both at a 1:500 dilution, in blocking solution overnight at 37°C. The podocytes were then washed and treated with conjugated Alexa 568-donkey anti-goat (Santa Cruz Biotechnology) and Alexa 488-mouse anti-rabbit (Santa Cruz Biotechnology) at a concentration of 1:1,000 in blocking solution for 1 h at 37°C. The cells were then washed three times with PBS and mounted in Vectashield HardSet medium containing 4’,6-diamidino-2-phenylindole, which allows for visualization of nuclei. The cells were imaged using a Leica SP8 confocal microscope and Leica LAF-AS software. The images represent single optical sections. 4’,6-Diamidino-2-phenylindole was used as a counterstain.

**RESULTS**

The NADPH oxidases comprise a class of enzymes that transfer electrons from NADPH to O2, thereby generating the superoxide anion (O2·−) and its downstream metabolites. NOX2, the prototype member of this family, is a heteromeric membrane protein that includes the catalytic subunit

---

**Fig. 3.** A diacylglycerol (DAG) analog increases generation of reactive oxygen species (ROS) in podocytes. A: fluorometric assay of ROS generation in wild-type (control) and podocin KD cells. Cells were treated for 12 h with 10 μM 1-oleoyl-2-acetyl-sn-glycerol (OAG) or vehicle. OAG increased ROS generation in wild-type, but not podocin KD, cells. Values are means ± SE. *P < 0.05 by 1-way ANOVA followed by Tukey’s post hoc test. B: cell surface biotinylation assay showing that OAG treatment increases steady-state surface expression of the NOX2 modulatory subunit p47phox. C: densitometric analyses of 3 repetitions of assay in B. Con, control. Values are means ± SE.
gp91phox along with at least five other smaller organizing and regulatory subunits (4). In the present study we observed that the endogenous NOX2 catalytic subunit gp91phox could reciprocally coimmunoprecipitate with TRPC6 when these analyses were carried out on lysates prepared from the differentiated cells of an immortalized mouse podocyte cell line (Fig. 1A). This complex could be detected when antibodies against TRPC6 or NOX2 were used for the initial precipitation.

---

**Fig. 4.** Analyses of whole cell currents in podocytes. Outwardly rectifying currents were evoked by ramp voltage commands (−80 mV to +80 mV over 2.5 s). Currents are from a single cell before and after exposure to 10 µM OAG. A: representative currents from a control podocyte. B: currents recorded from a podocyte pretreated with 10 µM apocynin for 30 min. C: currents from a podocyte pretreated for 30 min with 100 µM diphenylene iodonium (DPI). D: currents from a podocyte pretreated for 30 min with 10 mM tempol. Note reduction in OAG-evoked currents compared with control after treatment with apocynin, DPI, and tempol. E: summary of experiments in A–D. Values are means ± SE of currents measured at +80 mV before and after application of 10 µM OAG; each bar represents data from 7 cells. *P < 0.05 vs. control by Student’s paired t-test.
Neither NOX2 nor TRPC6 was detected in immunoprecipitates prepared using IgG, but both were readily detected in podocyte lysates (marked as input in Fig. 1). We and others previously showed that TRPC6 can coimmunoprecipitate with podocin (1, 22, 50) owing to direct interactions between the COOH termini of these proteins (1). Here we observed that NOX2 can also coimmunoprecipitate with podocin (Fig. 1B). Importantly, the TRPC6-NOX2 interaction could not be detected in a podocyte cell line in which podocin is stably knocked down using a short hairpin RNA delivered by lentiviral transfection (podocin KD cells). NOX2 and TRPC6 were expressed in this cell line at normal levels, and each antibody was clearly able to precipitate its own target antigen (Fig. 1C). Therefore, podocin is required for NOX2 and TRPC6 to occur in the same complex in podocytes. Consistent with immunoprecipitation, using confocal microscopy, we observed colocalization of TRPC6 and NOX2 in cultured podocytes (Fig. 2).

Previous studies in other cell types have shown that NOX2 can become active in response to cascades that lead to activation of various phospholipases (63) and production of DAG (16, 34). Therefore, we tested whether application of a membrane-permeable DAG analog, OAG, could increase generation of ROS in podocytes. Using a fluorometric assay, we observed that 12 h of exposure to 10 μM OAG evoked a statistically significant increase of cytosolic ROS accumulation in wild-type podocytes. However, this effect of OAG was not observed in podocin KD cells (Fig. 3A). The activity of NOX2 is regulated by translocation and binding of the regulatory subunit p47phox to the catalytic gp91phox subunit of NOX2. Consistent with this, we observed that treatment with OAG evoked an increase in the steady-state cell surface expression of p47phox (Fig. 3, B and C). Note that the ROS assay used in this study measures bulk cytosolic ROS generated from many different cellular sources, including mitochondrial respiration, and several different cellular oxidase enzymes, including NADPH oxidases. However, the cell surface biotinylation assay for p47phox is a more specific estimate of the steady-state levels of active complexes. These considerations can explain

Fig. 5. Nonhomogeneous distribution of cholesterol in podocytes based on filipin staining. A: merged images showing filipin (blue fluorescence) and phalloidin (red fluorescence) staining in a wild-type mouse podocyte. Note patchy distribution of filipin staining (i, ii, and iii). i: Elliptical distribution of filipin-stained patches in a single cell. ii: Higher magnification of area within white box in i. iii: Elliptical distribution of filipin staining in podocin KD cells. Elliptical distribution of staining was also present in many cells (ii and iii). D: wild-type podocytes treated for 18 h with 10 mM methyl-β-cyclodextrin (MBCD) do not exhibit filipin staining, although phalloidin staining is robust. Many podocytes appear somewhat smaller after MBCD treatment. E: 3-dimensional reconstruction of filipin and phalloidin staining from a wild-type podocyte.
why the increase in NOX2 assembly at the cell surface is substantially larger than the increase in bulk cytosolic ROS. Collectively, these data strongly suggest that DAG can increase the generation of ROS in the immediate vicinity of podocyte TRPC6, potentially leading to their activation.

To test this model, we made whole cell recordings from cultured podocytes under recording conditions that isolate cationic currents, as described previously (1, 28, 29). Currents were monitored during application of ramp voltage commands before and after application of 10 μM OAG. In previous studies we showed that small interfering RNA knockdown of TRPC6 or application of micromolar La3+ or SKF-96365 results in complete inhibition of OAG-evoked currents in this cell line (28). In the present study we again observed that OAG consistently evoked increases in outwardly rectifying cationic currents (Fig. 4A). However, 30 min of pretreatment with 10 μM apocynin (Fig. 3B) or 100 μM DPI (Fig. 4C) markedly reduced currents evoked by OAG. Apocynin binds to gp91phox and prevents its association with p47phox, thereby inhibiting NOX2 activation in cells and cell-free systems (27, 58), although quenching of ROS may also contribute to the actions of apocynin (20). DPI inhibits a variety of flavin-containing enzymes, including all forms of NADPH oxidase (25, 67). A similar reduction of responses to OAG was produced by 30 min of pretreatment with 10 mM tempol, a ROS quenching agent (66) (Fig. 4D). A summary of several repetitions of this experiment is shown in Fig. 4E. These data indicate that production of ROS produced by NADPH oxidases contributes to the increase in TRPC6 currents evoked by a DAG analog.

The data so far suggest that DAG and its analogs normally act on a TRPC6-NOX2 complex in podocytes that also requires podocin. Podocin is a hairpin loop membrane protein that normally associates with the inner leaflet of specialized plasma membrane domains in podocytes. Within glomeruli, podocin is almost exclusively localized at the slit diaphragm domain of podocytes.
foot processes (51, 55), an area of the podocyte plasma membrane with high cholesterol content (44). Podocin is a sterol-binding protein, and its ability to bind cholesterol is essential in order for it to modulate the gating of ionic channels (22). Immortalized podocytes do not form foot processes in vitro, but the plasma membrane is nevertheless far from homogeneous. This could be discerned using filipin staining. Filipin is a highly fluorescent polyene antibiotic that associates with nonesterified sterols in the plasma membrane and can, therefore, be used as a histochemical marker for plasma membrane domains rich in free sterols (37). We stained fixed but nonpermeabilized podocytes with filipin, and we used phalloidin to label underlying actin microfilaments. Filipin produced a striking patchlike staining pattern along the surface of podocytes, seen as blue fluorescence in two different cells in Fig. 5A. The filipin patches are much larger than single caveoli observed in electron microscopy, and these structures may instead be similar to the clusters of caveoli previously seen in smooth muscle cells (41). Very often these patches were arranged in an elliptical pattern. Notably, we have not seen this pattern in other cell types, such as CHO-K1 cells, where filipin staining is diffuse and relatively even across the entire cell surface (Fig. 5B). Because podocin is a cholesterol-binding protein, we hypothesized that it would be necessary to organize the patch pattern of filipin staining in podocytes. However, the same spatial pattern of filipin staining was observed even in podocin KD cells (Fig. 5C), indicating that the nonhomogeneous distribution of membrane sterols occurs even when podocin is not present to organize the protein content of raft domains (23, 55). The same characteristic elliptical arrangement of filipin patches was seen in many podocin KD cells. Importantly, filipin staining was abolished in podocytes treated for 18–24 h with 10 mM MBCD (Fig. 5D). MBCD is an agent that depletes plasma membrane cholesterol and, for this reason, can also be used to disrupt lipid rafts (71). When stacks of confocal sections were combined to reconstruct the three-dimensional arrangement of these patches in normal podocytes, it appeared that the filipin staining occurred within invaginations of the plasma membrane that were always on the periphery of the cell, above the actin microfilaments (Fig. 5E).

Does cholesterol play a role in regulating the NOX2-TRPC6 interaction? To test this, we treated podocytes with 10 mM MBCD and then determined if it was still possible to coimmunoprecipitate NOX2 and TRPC6 (Fig. 6). As was the case in the example shown in Fig. 1, in control cells we were able to readily detect an interaction between these proteins, regard-

![Graph](AJP-Cell Physiol • doi:10.1152/ajpcell.00191.2013 • www.ajpcell.org)

**Fig. 7.** Disruption of lipid rafts by cholesterol depletion reduces activation of TRPC6 by OAG. **A and B:** whole cell currents from single cells before and after superfusion of 10 µM OAG. **A:** robust response to OAG in control cell. **B:** much smaller OAG-evoked currents in cells treated for 18 h with 10 mM MBCD. **C:** summary of results in **A and B** showing basal and OAG-evoked currents in control and MBCD-treated cells (n = 7 per group). Two-way ANOVA indicates a significant effect of OAG and MBCD and a significant interaction effect (*P < 0.0001), indicating that MBCD treatment reduces responses to OAG. Basal currents are significantly higher after MBCD than control: +P < 0.05 by Tukey’s post hoc test.
less of which antibody was used for the initial immunoprecipitation (Fig. 6A). However, using the same procedures, we were no longer able to detect the NOX2-TRPC6 interaction in MBCD-treated cells (Fig. 6B). Both proteins were readily detected in lysates of MBCD-treated podocytes, but they were no longer concentrated within the same protein complexes. The same treatment with MBCD caused a significant reduction of TRPC6 activation evoked by 10 μM OAG (Fig. 7). In particular, two-way ANOVA indicated a significant ($P < 0.0001$) interaction effect between the effects of MBCD and the effects of OAG, indicating that MBCD reduced mean responses to OAG. This last result is consistent with models in which NOX2 contributes to chemical activation of TRPC6 channels in podocytes. Collectively, these data suggest that NOX2-TRPC6 interactions and the resulting modulation of TRPC6 activation require intact lipid rafts.

**DISCUSSION**

In previous studies of podocytes, we showed that generation of ROS contributes to regulation of TRPC6 channels by three different stimuli: insulin (28), activation of N-methyl-d-aspartate receptors (29), and ANG II (unpublished observations). Indeed, simply adding an exogenous source of ROS, such as $\text{H}_2\text{O}_2$, leads to robust TRPC6 activation in these cells (28, 36). This effect is due in part to increased surface expression of TRPC6 channels (28), but there is evidence that ROS also increase the probability of TRPC6 opening, strongly suggesting an effect on gating, as well as trafficking (17, 36). To modulate TRPC6 channels, ROS need to diffuse from the sites where they are generated to the immediate vicinity of TRPC6 channels on the cell surface. Diffusion is a relatively slow process over micrometer distances, especially if ROS have to pass through a gauntlet of scavenging enzymes before reaching a target. However, diffusion is very fast on nanometer scales (5). Therefore, if ROS were to contribute to a fast signaling cascade, such as the G protein-coupled pathway leading from ANG II to TRPC6 (1), we reasoned that a ROS-generating system would need to be colocalized with TRPC6.

In this study, we also observed that NOX2 interacts with TRPC6 and podocin and that podocin was necessary for coimmunoprecipitation of NOX2 and TRPC6. Previous studies showed that DAG can stimulate NADPH oxidase activity in neutrophil membranes by promoting assembly of active multimeric NOX2 complexes containing gp91$^{\text{phox}}$ and p47$^{\text{phox}}$ (8, 45, 49, 62). Biochemical studies have also shown that active NOX2 complexes preferentially assemble in lipid rafts (15, 18, 47, 56, 64). Collectively, these results are consistent with the model shown in Fig. 8. We propose that certain signals, such as ANG II, act through G proteins to activate PLC. The resulting liberation of DAG is likely to produce some sort of direct effect to promote TRPC6 activation (21), but these effects are strongly potentiated by ROS produced by assembly of active NOX2 in the same multiprotein complex. Podocin binds cholesterol and functions to tether TRPC6 and gp91$^{\text{phox}}$ to the raft domains. Consequently, in the absence of podocin, NOX2 and TRPC6 are no longer in sufficient proximity to allow ROS to function as efficient amplifiers of the DAG signal. This model is supported by several independent lines of evidence, including 1) the observed increase in current through podocyte TRPC6 channels evoked by exogenous ROS, such as $\text{H}_2\text{O}_2$ (28, 36), 2) the increase in ROS generation after treatment of podocytes with ANG II (69) or DAG analogs (present study), 3) inhibition of ANG II stimulation of TRPC6 channels by agents that quench ROS or by NOX2 inhibitors (unpublished observations), 4) suppression of DAG-evoked TRPC6 currents after podocin knockdown (1), 5) biochemical interaction between NOX2 and TRPC6 (present study), 6) loss of the NOX2-TRPC6 interaction after podocin knockdown (present study) or disruption of lipid rafts (present study), 7) translocation of p47$^{\text{phox}}$ to the podocyte surface in response to OAG (present study), 8) loss of DAG-evoked ROS production after podocin knockdown (present study), and 9) reduction of DAG activation of podocyte TRPC6 channels after disruption of lipid rafts (present study).

An important feature of this model is the central role of podocin in organizing the signaling complex that feeds into TRPC6. Our data from filipin staining in podocin KD cells indicate that podocin is not necessary to observe a nonhomogeneous distribution of cholesterol along the podocyte surface. Instead, it appears that podocin forms higher-order oligomers that allow it to play a role as a scaffold to recruit other proteins, such as TRPC6, nephrin, and CD2AP, into preexisting cholesterol-rich domains in podocytes (23, 55). Earlier freeze-fracture analyses using filipin suggest that cholesterol content of the slit diaphragm domain of the foot process plasma membrane is higher than that of other parts of the foot process surface in vivo (44). In the present study we have focused on TRPC6 channels, but it is possible that ROS produced by NOX2 can directly or indirectly affect other proteins at the slit diaphragm. For example, ROS can modulate the activity of RhoA/Rho kinase, MAP kinase, protein tyrosine phosphatases,
and tyrosine kinases (38), all of which could affect the activity and dynamics of proteins at the slit diaphragm.

In an earlier study (28), we observed that a different NADPH oxidase, NOX4, is required for mobilization of podocyte TRPC6 channels in response to insulin. Given this information, it is quite possible that some of the TRPC6-podocin complexes within podocytes also include NOX4. Nevertheless, the available evidence suggests that ROS generated by different cascades, for example, signals mediated through tyrosine kinase-type receptors and G protein-coupled receptors, can impinge on TRPC6 channels by distinctly different cascades that ultimately produce ROS by activation of different enzymes.

One question that emerges is whether the results obtained here can be extended to other cell types. Podocin is primarily expressed in the slit diaphragm domain of podocyte foot processes and is not thought to be expressed at significant levels in other cell types, or even in other areas on the podocyte plasma membrane (51). On the other hand, podocin is a member of a conserved family of hairpin loop membrane proteins that includes stomatins, prohibitins, flotillins, lower eukaryotic proteins such as Mec-2 and Unc-1 of Caenorhabditis elegans, and possibly even prokaryotic proteins (42). These proteins have a variety of different functions, but all appear to preferentially associate with sterol-rich raft domains. Stomatins are especially interesting in this regard, because it is nearly ubiquitously expressed in mammals (14). Moreover, stomatin regulates the gating and localization of several types of ion channels, including acid-sensing ion channels (7, 48), voltage-activated Ca\(^{2+}\) (Cav2.1) channels (11), pannexins (70), and possibly epithelial Na\(^{+}\) channels (13) and aquaporins (53). In this regard, there is evidence that acid-sensing ion channels can be regulated by their redox state and by NADPH oxidases (2, 9, 10). Therefore, it is possible that the organizational features of the model shown in Fig. 8 could apply to other signaling systems as well.

In a previous study, we demonstrated that podocin determines the dominant mode of TRPC6 gating in podocytes. More specifically, podocin knockdown suppressed chemical activation of TRPC6 by OAG but markedly enhanced TRPC6 activation evoked by membrane stretch or diacylglycerol. Am J Physiol Cell Physiol 305: C276–C289, 2013.

REFERENCES


