SUMO1 modification of PKD2 channels regulates arterial contractility

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PKD2 (polycystin-2, TRPP1) channels are expressed in a wide variety of cell types and can regulate functions, including cell division and contraction. Whether posttranslational modification of PKD2 modifies channel properties is unclear. Similarly uncertain are signaling mechanisms that regulate PKD2 channels in arterial smooth muscle cells (myocytes). Here, by studying inducible, cell-specific *Pkd2* knockout mice, we discovered that PKD2 channels are modified by SUMO1 (small ubiquitin-like modifier 1) protein in myocytes of resistance-size arteries. At physiological intravascular pressures, PKD2 exists in approximately equal proportions as either nonsumoylated (PKD2) or triple SUMO1-modifed (SUMO-PKD2) proteins. SUMO-PKD2 recycles, whereas unmodified PKD2 is surface-resident. Intravascular pressure activates voltage-dependent Ca^{2+} influx that stimulates the return of internalized SUMO-PKD2 channels to the plasma membrane. In contrast, a reduction in intravascular pressure, membrane hyperpolarization, or inhibition of Ca^{2+} influx leads to lysosomal degradation of internalized SUMO-PKD2 protein, which reduces surface channel abundance. Through this sumoylation-dependent mechanism, intravascular pressure regulates the surface density of SUMO-PKD2–mediated Na^+ currents (I_{Na}) in myocytes to control arterial contractility. We also demonstrate that intravascular pressure activates SUMO-PKD2, not PKD2, channels, as desumoylation leads to loss of I_{Na} activation in myocytes and vasodilation. In summary, this study reveals that PKD2 channels undergo posttranslational modification by SUMO1, which enables physiological regulation of their surface abundance and pressure-mediated activation in myocytes and thus control of arterial contractility.

PKD2 channel | arterial smooth muscle | sumoylation | vasoconstriction | polycystin-2

Mammalian transient receptor potential (TRP) channels represent a family of ∼28 proteins that are subdivided into 6 classes, including polycystin (TRPP), canonical (TRPC), and vanilloid (TRPV) (1). TRP channels are expressed in almost every cell type, act as molecular sensors for a wide spectrum of stimuli, and can regulate multiple physiological functions, including contractility, sensory transduction, fertilization, cell survival, and development (1). Identifying novel mechanisms that regulate TRP proteins is important, as these processes may control physiological functions in a wide variety of different cell types.

PKD2, which is also referred to as polycystin-2 or transient receptor potential polycystin 1 (TRPP1), is a nonselective cation channel encoded by the *Pkd2* gene (2, 3). PKD2 is expressed in several cell types, including arterial myocytes, kidney epithelial cells, and cardiac myocytes (4). Mutations in PKD2 lead to Autosomal Dominant Polycystic Kidney Disease (ADPKD), the most common monogenic disorder identified in humans, which affects 1:400 to 1,000 individuals (5). ADPKD is characterized by growth of renal cysts, which impact kidney function (5). A significant proportion of patients with apparently normal renal function develop hypertension prior to the development of cysts, suggesting that PKD2 channels control blood pressure via an extrarenal mechanism (6–8).

PKD2 is expressed in arterial smooth muscle cells of several species (9–12). RNA interference-mediated knockdown of PKD2 inhibited pressure-induced vasoconstriction (myogenic tone) in cerebral arteries (11, 13). A recent study generated an inducible, smooth muscle-specific PKD2 channel knockout (*Pkd2* smKO) mouse to investigate vascular and in vivo blood pressure regulation by this protein (12). Data indicated that vasoconstrictor stimuli activate PKD2 channels in systemic artery myocytes, leading to a contraction that increases physiological systemic blood pressure (12). An increase in arterial myocyte PKD2 occurs during hypertension and contributes to the blood pressure elevation (12). Although PKD2 is recognized to control arterial contractility and blood pressure, mechanisms that regulate the function of this channel in myocytes are poorly understood. Here, we tested the unique hypothesis that posttranslational modification of PKD2 in myocytes is a physiological mechanism that controls channel function and arterial contractility.

Posttranslational modifications are diverse processes that can include phosphorylation, glycosylation, and ubiquitination (14–16). These alterations can modulate protein folding, expression, distribution, stability, and activity. Sumoylation is a reversible, posttranslational modification that occurs through the covalent attachment of a small ubiquitin-like modifier (SUMO) protein to a target protein (17). Sumoylation was initially considered to modify nuclear proteins, leading to the regulation of transcription, DNA repair, chromatin organization, and cell cycle regulation (17). Recent studies have identified several extranuclear targets of sumoylation, including potassium channels (18–22). Whether

Significance

PKD2 is an ion channel that is expressed in a wide variety of cell types, including arterial smooth muscle cells (myocytes). Mechanisms that regulate PKD2 function are poorly understood, particularly in arterial myocytes. Proteins can undergo posttranslational modification, which alters their properties, yet whether PKD2 channels are altered in this manner is unclear. Our study shows that a protein termed SUMO1 is attached to PKD2 channels in arterial myocytes. This posttranslational modification enables a physiological stimulus, intravascular pressure, to control the amount of SUMO-PKD2 that is located in the plasma membrane and to activate SUMO-PKD2 channels. We also demonstrate that the SUMO1 modification of PKD2 channels is necessary for intravascular pressure to regulate arterial contractility.

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TRP channels undergo sumoylation is unclear. Similarly uncertain is whether sumoylation modifies ion channels in arterial myocytes to control physiological functions, including contractility.

To determine whether PKD2 channels undergo posttranslational modification in myocytes, we compared their molecular identities in arteries of *Pkd2* smKO and control ($Pkd2^{fl/fl}$) mice. Our data show that PKD2 channels are modified by SUMO1 in arterial myocytes. We also demonstrate that intravascular pressure stimulates vasoconstriction by modulating the surface abundance of SUMO1-modified PKD2 channels in myocytes.

Results

PKD2 Channels Exist as 2 Different Molecular Weight Proteins in Arterial Myocytes. To profile the molecular identity of PKD2 channels in contractile arterial myocytes, we studied resistance-size (≤200 μm diameter) hindlimb arteries of inducible, smooth muscle cell-specific PKD2 knockout (*Pkd2* smKO) mice and their controls ($Pkd2^{fl/fl}$) (12). RT-PCR results confirmed that tamoxifen treatment abolished PKD2 messenger RNA in fresh-isolated arterial myocytes of *Pkd2* smKO mice, consistent with a previous report (*SI Appendix*, Fig. S1) (12). Western blotting revealed that myocyte-specific PKD2 ablation robustly reduced the intensity of 2 protein bands of ∼106 and 140 kDa in arteries (Fig. 1 *A* and *B*). The intensities of the ∼106- and 140-kDa bands in arterial lysate of *Pkd2* smKO mice were ∼44.0% and 2.1% of those in arteries of $Pkd2^{fl/fl}$ mice (Fig. 1 *A* and *B*). In $Pkd2^{fl/fl}$ arteries, the abundance of the large (L) and smaller (S) proteins were similar, with an L/S of ∼0.98 (Fig. 1 *A* and *C*). In contrast, in *Pkd2* smKO arteries, the L/S was ∼0.06 (Fig. 1 *A* and *C*). The calculated molecular weight of full-length unmodified PKD2 is ∼106 kDa, which corresponds to the smaller molecular weight protein (Fig. 1*A*). The larger protein detected by PKD2 antibodies that is essentially abolished in *Pkd2* smKO arteries may represent PKD2 that has undergone posttranslational modification.

To determine the molecular identity of the large and small proteins, immunoprecipitation was performed to enrich PKD2 from arterial lysate. Immunoprecipitated proteins were separated using electrophoresis and mass spectrometry (MS) performed to identify whether PKD2 was present in 2 different regions on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) gels between 90 to 110 kDa (low) and 115 to 160 (high) kDa. MS identified PKD2-specific peptide sequences in both low and high molecular weight regions (Table 1). These data raised the possibility that PKD2 exists as 2 different molecular weight proteins in arterial myocytes.

Arterial Myocyte PKD2 Channels Undergo Sumoylation. The relatively large difference in molecular weight between the 2 PKD2 proteins raised the possibility that PKD2 is sumoylated and/or ubiquitinated in arterial myocytes. To test these hypotheses, PKD2 was immunoprecipitated from arterial lysate and probed for SUMO1, SUMO2/3, or ubiquitin. A SUMO1 antibody detected a ∼140-kDa protein in PKD2 immunoprecipitate that corresponds to the large PKD2 protein identified in arterial lysate (Fig. 1*D*). In contrast, SUMO2/3 or ubiquitin antibodies did not detect any bands on PKD2 immunoblots (Fig. 1*D*). A reciprocal immunoprecipitation was performed using the SUMO1 antibody to pull down proteins from arterial lysate (Fig. 1*E*). PKD2 antibodies detected a ∼140-kDa protein in the SUMO1 immunoprecipitate (Fig. 1*E*). These results suggest that PKD2 is modified by SUMO1, but not by SUMO2/3 or ubiquitin, in arterial myocytes.

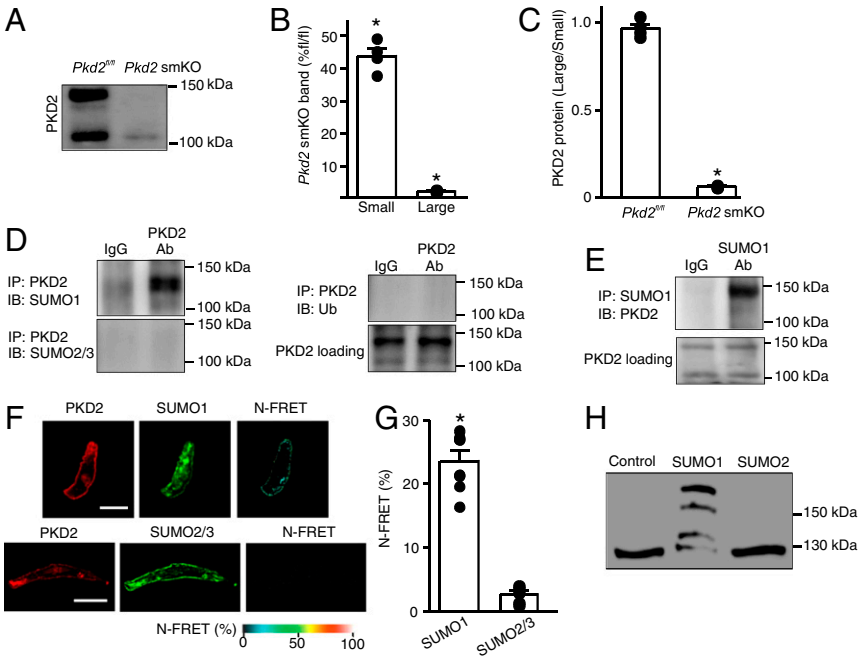

Fig. 1. PKD2 channels are sumoylated in arterial myocytes. (*A*) Representative Western blot illustrating that smooth muscle-specific knockout of PKD2 (*Pkd2* smKO) reduces the intensity of 2 protein bands detected in hindlimb arteries of control ($Pkd2^{fl/fl}$) mice. (*B*) Mean data comparing the relative amounts of large and small PKD2 proteins in arteries of *Pkd2* smKO arteries, when compared to $Pkd2^{fl/fl}$ arteries ($n = 4$ for each group). *$P < 0.05$ vs. $Pkd2^{fl/fl}$. (*C*) Mean data illustrating the ratio of large to small protein bands in arteries of $Pkd2^{fl/fl}$ and *Pkd2* smKO mice ($n = 4$ for each group). *$P < 0.05$ vs. $Pkd2^{fl/fl}$. (*D*) Representative Western blots illustrating that SUMO1, but not SUMO2/3 or ubiquitin, is detected (IB) in PKD2 immunoprecipitate (IP) and at the same molecular weight as the large PKD2 protein band present in arterial lysate (PKD2 loading control). Representative of 4 experiments. (*E*) Western blot illustrating that PKD2 is detected (IB) in SUMO1 immunoprecipitate (IP) at the same molecular weight as the large PKD2 protein band in arterial lysate (PKD2 loading). Representative of 4 experiments. (*F*) Immunofluorescence and immunoFRET images of PKD2 (Alex 546) and SUMO1 or SUMO2/3 (Alex 488). (Scale bars: 10 μm.) (*G*) Mean data for immunoFRET experiments ($n = 6$ for each). *$P < 0.05$ vs. SUMO2/3 FRET. (*H*) Representative Western blot of in vitro sumoylation reaction products probed with a GST antibody. Purified human GST-tagged PKD2 was used in the reaction. Blots were probed anti-GST antibody, $n = 4$.

Table 1. PKD2 peptide sequences that were identified using MS in both the low and high molecular weight regions that contained PKD2 protein bands

Identified peptide sequence	Amino acid positions in PKD2
[R].LEDQGAQCPSPAGGGDPLHR.[H]	[155–174]
[R].LLDGVAEDAR.[L]	[882–891]
[R].NPRPPSSQSAEGLEGGGGNGSANVHA.[-]	[941–966]

Förster resonance energy transfer (FRET) imaging was used to further examine PKD2 modification by SUMO proteins in isolated arterial myocytes. Alexa Fluor546- and Alexa Fluor488-tagged secondary antibodies bound to PKD2 and SUMO1 primary antibodies, respectively, generated mean normalized (N)-FRET of 24.3 ± 2.4% in isolated myocytes (Fig. 1 *F* and *G*). The vast majority of the FRET signal was located at the cell periphery (Fig. 1*F*). In contrast, N-FRET between PKD2 and SUMO2/3 bound secondary antibodies was only 2.7 ± 0.5% (Fig. 1 *F* and *G*). The Förster coefficient of the Alexa Fluor pair used for these experiments is ∼6.3 nm. We conclude from these experiments that PKD2 channels undergo posttranslational modification by SUMO1 in arterial myocytes.

SUMO1 Proteins Conjugate to PKD2 Channels. An in vitro assay was performed to measure sumoylation of PKD2 protein. Purified glutathione *S*-transferase (GST)-tagged PKD2 was incubated in the presence of components required to produce sumoylation, including E1 and E2 ligases and either SUMO1 or SUMO2. Western blotting of reaction products identified 4 different bands corresponding to unmodified PKD2 and PKD2 that was bound by either 1, 2, or 3 SUMO1 proteins (Fig. 1*H*). In contrast, purified PKD2 was not modified by SUMO2 (Fig. 1*H*). When taking into account that the GST tag increases the molecular weight of PKD2 by ∼28 kDa, the triple SUMO1-conjugated PKD2 (SUMO-PKD2) corresponds to that of the larger PKD2 protein identified in arteries. Thus, PKD2 is modified by 3 SUMO1 proteins in arterial myocytes.

Arterial Isolation Leads to Internalization and Lysosomal Degradation of SUMO-PKD2 Channels. Previous studies have shown that the application of either SUMO or sentrin-specific protease (SENP), a desumoylating enzyme, can modify the properties K2P1, K_V2.1, Na_V1.2, and I_{Ks} currents (18–21). We used a similar approach here to test the hypothesis that sumoylation can regulate PKD2 currents in arterial myocytes. Nonselective cation currents (I_{Cat}) were measured in fresh-isolated arterial myocytes using whole-cell patch-clamp electrophysiology and symmetrical Na^+ solutions, as done previously (12). SUMO1 or SENP were introduced into myocytes via the patch pipette at concentrations of 1 nM. This concentration is greater than, or equal to, those previously demonstrated to regulate K2P1, K_V2.1, Na_V1.2, and I_{Ks} currents (18–21). In isotonic bath solution, I_{Cat} densities were similar in patches obtained in control or with SUMO1- or SENP-containing pipette solution (*SI Appendix*, Fig. S2 *A* and *B*). Cell swelling activates PKD2 channels in hindlimb artery myocytes (11, 12). Reducing bath solution osmolarity from 300 to 250 mOsmol/L increased I_{Cat} density similarly in patches obtained with either control, SUMO1-, or SENP-containing pipette solution (*SI Appendix*, Fig. S2 *A* and *B*). In hypotonic bath solution, a reduction in bath [Na^+] from 115 to 40 mmol/L similarly reduced currents and left-shifted the reversal potential (E_{rev}) by ∼22.1, 20.3, and 22.4 mV (when corrected for liquid junction potential caused by the solution change), respectively, in control, SUMO1, or SENP (*SI Appendix*, Fig. S2 *A* and *C*). The E_{rev} shift caused by low [Na^+] bath solution is consistent with swelling-activated I_{Cat} being due primarily to Na^+ flux in arterial myocytes (*SI Appendix*, Fig. S2 *A–C*) (12). The addition of Gd^{3+}, a nonselective cation channel blocker, to the hypotonic/low Na^+ bath solution also similarly reduced I_{Cat} in control, SUMO, or SENP (*SI Appendix*, Fig. S2 *A* and *D*). These data indicate that the short-term application of SUMO or SENP does not regulate PKD2 currents over the ∼30-min time period of these experiments.

Cell current (*I*) is the product of the number of channels (*N*), their open probability (P_O), and single-channel current (*i*), such that $I = N \cdot Po \cdot i$. The lack of an immediate effect of SUMO or SENP suggests that sumoylation and desumoylation do not modify PKD2 channel P_O or *i*. Next, we examined whether sumoylation regulates the number (*N*) of PKD2 channels. As intravascular pressure activates PKD2 channels in myocytes, we examined the regulation of SUMO-PKD2 and PKD2 protein abundance by a loss of intravascular pressure caused by arterial isolation (12). The isolation and maintenance of arteries at 37 °C for 3 h lead to a large reduction in SUMO-PKD2 to ∼42.6% of that in fresh-isolated (0 h) control arteries (Fig. 2 *A* and *B*). In contrast, arterial isolation for 3 h did not change the amounts of PKD2, TRPM4, TRPC6, or Ca_V1.2 channels or PKD1, which can form a complex with PKD2 (Fig. 2 *A* and *B*) (4, 23, 24). Thus, arterial isolation leads to a selective reduction in SUMO-PKD2 protein in myocytes.

Biotinylation selectively labels surface proteins in intact arteries (25, 26). The use of this approach allows quantification of the amount of plasma membrane and intracellular (nonbiotinylated) proteins. Here, arterial biotinylation was used to measure the sumoylation state and abundance of plasma membrane and intracellular PKD2 channels in arteries. In fresh-isolated (0 h) arteries, ∼98.6% and 96.8% of SUMO-PKD2 and PKD2, respectively, were located in the plasma membrane (Fig. 2*C* and *SI Appendix*, Fig. S3). Arterial isolation caused a time-dependent loss of surface SUMO-PKD2 that was most prevalent between 1 h and 3 h (Fig. 2 *C* and *D*). Similarly to our data measuring total protein, 3 h after arterial isolation, ∼62.3% of surface SUMO-PKD2 was lost (Fig. 2 *C* and *D*). In contrast, surface PKD2 was unaltered over the same time course (Fig. 2 *C* and *D*). Notably, the decrease in surface SUMO-PKD2 was not associated with an increase in intracellular SUMO-PKD2, suggesting that internalized protein was degraded (Fig. 2*C* and *SI Appendix*, Fig. S3). These data indicate that arterial isolation leads to a substantial loss of surface SUMO-PKD2 channels in myocytes.

Proteins can be degraded by either proteasomes or lysosomes, or by a combination of both mechanisms. Con A, an internalization inhibitor, blocked the isolation-induced decrease in SUMO-PKD2 protein in arteries (Fig. 2 *E* and *F*). Bafilomycin, a blocker of lysosomal degradation, also reduced the isolation-induced decrease in SUMO-PKD2 protein (Fig. 2 *E* and *F*). In contrast, MG132, a proteasomal degradation inhibitor, did not alter SUMO-PKD2 protein loss (Fig. 2 *E* and *F*). Thus, arterial isolation leads to prominent internalization and lysosomal degradation of SUMO-PKD2 channels in arterial myocytes.

Intravascular Pressure Increases Surface SUMO-PKD2 Channel Abundance. Arterial isolation is associated with depressurization, which may underlie the loss of SUMO-PKD2 protein. To determine whether intravascular pressure regulates SUMO-PKD2 surface protein, isolated arteries were maintained at either low (10 mmHg) or physiological (80 mmHg) pressure for 3 h. Arterial pressurization at 10 mmHg led to a reduction in surface SUMO-PKD2 protein to ∼42.4% of that in fresh-isolated (0 h) arteries. In contrast, 80 mmHg prevented the loss of surface SUMO-PKD2 (Fig. 3 *A* and *B*). Low or physiological intravascular pressure did not alter the amount of PKD2 protein (Fig. 3 *A* and *B*). These data demonstrate that intravascular pressure stimulates an increase in surface SUMO-PKD2 protein in arterial myocytes.

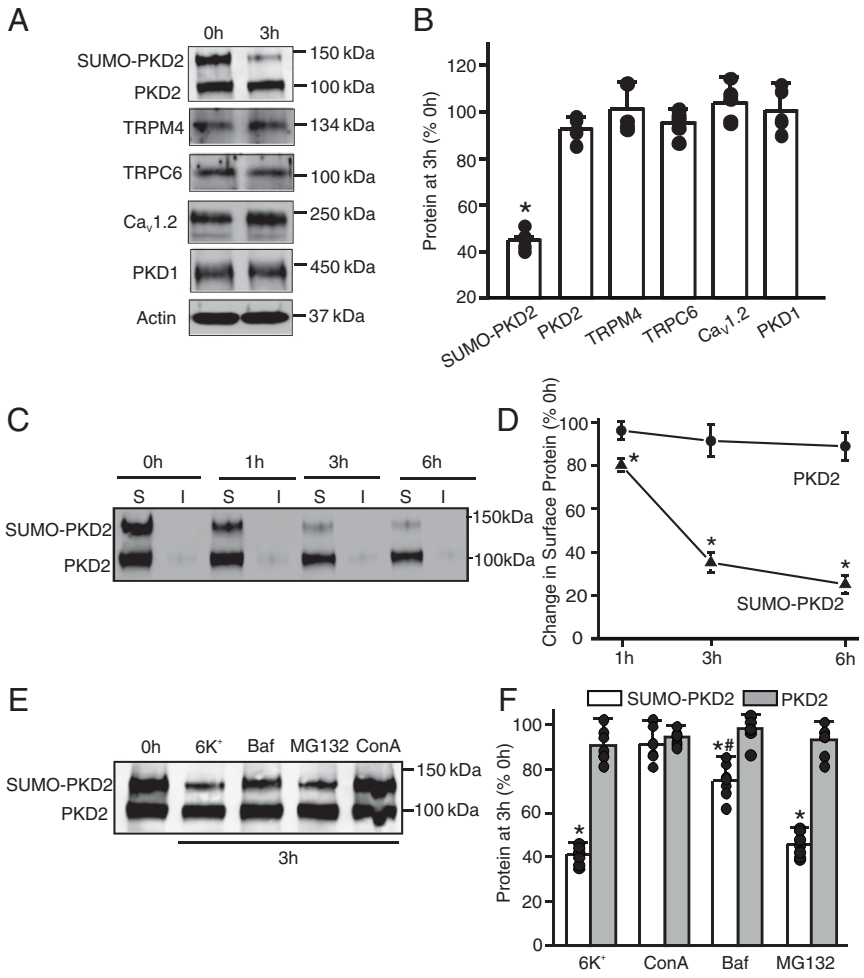

Fig. 2. Arterial isolation leads to desumoylation, internalization, and lysosomal degradation of PKD2 channels. (*A*) Representative Western blot showing proteins in fresh-isolated arteries (0 h) and 3 h after arterial isolation and depressurization. (*B*) Mean data illustrating that PKD2 loss is specific 3 h after arterial isolation ($n = 5$, for each). *$P < 0.05$ vs. 0 h. (*C*) Representative Western blot of arterial biotinylation illustrating surface (S) and intracellular (I) PKD2 protein at 1, 3, and 6 h after isolation in 6 K^+ PSS. (*D*) Time course of changes in SUMO-PKD2 and PKD2 ($n = 5$ for all data points). *$P < 0.05$ vs. 0 h. (*E*) Representative Western blot of PKD2 proteins in fresh-isolated arteries and 3 h after isolation in bafilomycin A (50 nM) and MG132 (15 μM). (*F*) Mean data ($n = 7$ for each). *$P < 0.05$ vs. 0 h; #$P < 0.05$ vs. 3 h/6 K^+.

An increase in intravascular pressure leads to depolarization of arterial myocytes, whereas a decrease in pressure results in hyperpolarization (27). We tested the hypothesis that pressure controls SUMO-PKD2 surface localization through the regulation of membrane potential. Arteries were processed either immediately (0 h) or 3 h after maintenance in physiological saline solution (PSS) containing either 6 or 30 mM K^+; 30 mM K^+ depolarizes arteries to ∼−40 mV, a potential that occurs at physiological pressure (27). Membrane depolarization (30 mM K^+) prevented the loss surface of SUMO-PKD2 that occurred in arteries placed in 6 mM K^+ PSS (Fig. 3 *C* and *D*). The removal of extracellular Ca^{2+} or nimodipine, a voltage-dependent $Ca_V1.2$ channel blocker, prevented membrane depolarization from stimulating an increase in surface SUMO-PKD2 protein (Fig. 3 *C* and *D*). In contrast, membrane depolarization, the removal of extracellular Ca^{2+}, or nimodipine did not alter PKD2 protein (Fig. 3 *C* and *D*). Collectively, these experiments demonstrate that membrane potential regulates the quantity of surface SUMO-PKD2 protein through a $Ca_V1.2$- and Ca^{2+}-dependent signaling mechanism.

Membrane Potential Regulates PKD2 Sumoylation, Which Determines Surface Protein Abundance. We investigated the hypothesis that membrane potential not only regulates the amount of surface SUMO-PKD2, but also modulates the sumoylation state of PKD2 channels in arterial myocytes. The synthetic flavone 2-D08, an inhibitor of Ubc9, the only known SUMO-conjugating enzyme, reduced the abundance of surface SUMO-PKD2 protein in depolarized arteries (Fig. 3 *C* and *D*). In contrast, 2-D08 did not alter the amount of surface PKD2 (Fig. 3 *C* and *D*). Coimmunoprecipitation was performed to test the hypothesis that membrane potential regulates the amount of SUMO1 that associates with PKD2 channels. Approximately 2.2-fold more SUMO1 immunoprecipitated with PKD2 channels in depolarized arteries (30 mM K^+) than in hyperpolarized arteries (6 mM K^+) (Fig. 4 *A* and *B*). Similarly, the amount of SUMO1 that associated with PKD2 channels was similar in fresh-isolated arteries and those maintained under depolarized conditions (30 mM K^+) for 3 h (Fig. 4 *A* and *B*). The 2-D08 reduced the amount of SUMO1 that associated with PKD2 channels in depolarized arteries, suggesting that Ubc9 actively maintains PKD2 channels in a sumoylated state (Fig. 4 *A* and *B*).

Next, we determined whether membrane potential regulates PKD2 sumoylation specifically in arterial myocytes. Arteries were exposed to different treatments, after which myocytes were isolated and immunoFRET was performed. Depolarization (30 mM K^+, 3 h) increased N-FRET generated between fluorescent

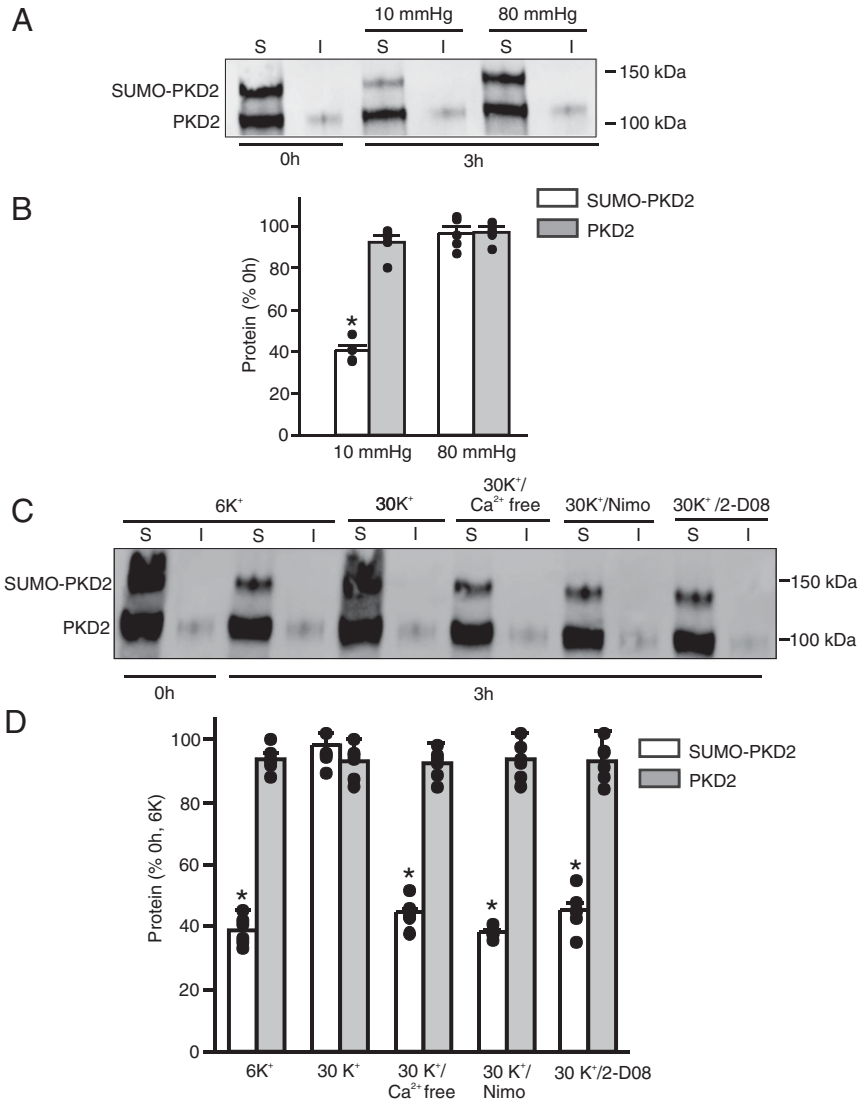

Fig. 3. Intravascular pressure and depolarization increase PKD2 sumoylation and surface abundance through voltage-dependent Ca^{2+} channel activation. (*A*) Representative Western blot of surface (S) and intracellular (I) PKD2 proteins in arteries that were fresh-isolated or pressurized for 3 h at 10 or 80 mmHg. (*B*) Mean data for surface SUMO-PKD2 and PKD2 protein in arteries that were pressurized to either 10 mmHg or 80 mmHg or in depressurized arteries maintained in 30 mmol/L K^+ when compared to PKD2 protein in fresh-isolated arteries (0 h); $n = 6$ for each group. *$P < 0.05$ vs. 0 h. (*C*) Representative Western blot of arterial biotinylation illustrating surface (S) and intracellular (I) PKD2 protein at 0 h in 6 K^+ PSS or after 3 h in 6 K^+ PSS, 30 K^+ PSS, Ca^{2+}-free 30 K^+ PSS or 30 K^+ PSS with nimodipine or 2-D08. (*D*) Mean data for surface SUMO-PKD2 and PKD2 protein in arteries at 3 h; $n = 6$ for each group. *$P < 0.05$ vs. 0 h/6 K^+.

FRET-pair antibodies bound to PKD2 and SUMO1, and this effect was prevented by 2-D08 (Fig. 4 *C* and *D*). Experiments were also performed using *Pkd2* smKO mice. Arterial isolation, membrane depolarization (30 mM K^+), or 2-D08 did not alter the amounts or distribution of PKD2 proteins in arteries of *Pkd2* smKO mice (*SI Appendix*, Fig. S4 *A* and *B*). These results, when combined with those in Fig. 1 *A*–*C*, indicate that myocytes contain the vast majority of SUMO-PKD2 present in cells of the arterial wall and that membrane depolarization stimulates an increase in surface SUMO-PKD2 channels in myocytes.

Membrane Depolarization Increases PKD2 Currents through Sumoylation in Arterial Myocytes. Whole-cell patch-clamp electrophysiology was used to investigate I_{Cat} regulation by processes that we have identified here to regulate PKD2 channel sumoylation. I_{Cat}s were measured in fresh-isolated myocytes and myocytes isolated from arteries maintained for 3 h in PSS containing either 6 mM K^+, 30 mM K^+, or 30 mM K^+ + 2-D08. In isotonic (300 mOsm) bath solution, voltage ramps generated outwardly rectifying currents that were similar in myocytes under all 4 conditions (Fig. 5*A* and *SI Appendix*, Fig. S5). Switching from isotonic to hypotonic bath solution (250 mOsm) increased I_{Cat} ~2.1- and 2.3-fold at −100 and +100 mV, respectively, in fresh-isolated myocytes (Fig. 5). The addition of Gd^{3+} inhibited swelling-activated I_{Cat} (Fig. 5). In myocytes of arteries that had been maintained in 6 mM K^+ for 3 h, the densities of Gd^{3+}-sensitive, swelling-activated I_{Cat}s were ~11.3% and 13.7% (at −100 and +100 mV, respectively) of those in fresh-isolated myocytes (Fig. 5). Arterial depolarization (30 mM K^+, 3 h) prevented this isolation-induced loss of swelling-activated I_{Cat} (Fig. 5). Furthermore, 2-D08 inhibited the ability of membrane depolarization to preserve swelling-activated I_{Cat} following arterial isolation (Fig. 5). Thus, depolarization-induced sumoylation maintains SUMO-PKD2 channels at the cell surface, enabling swelling to activate an I_{Cat} in arterial myocytes. Data also suggest that cell swelling activates SUMO-PKD2 channels.

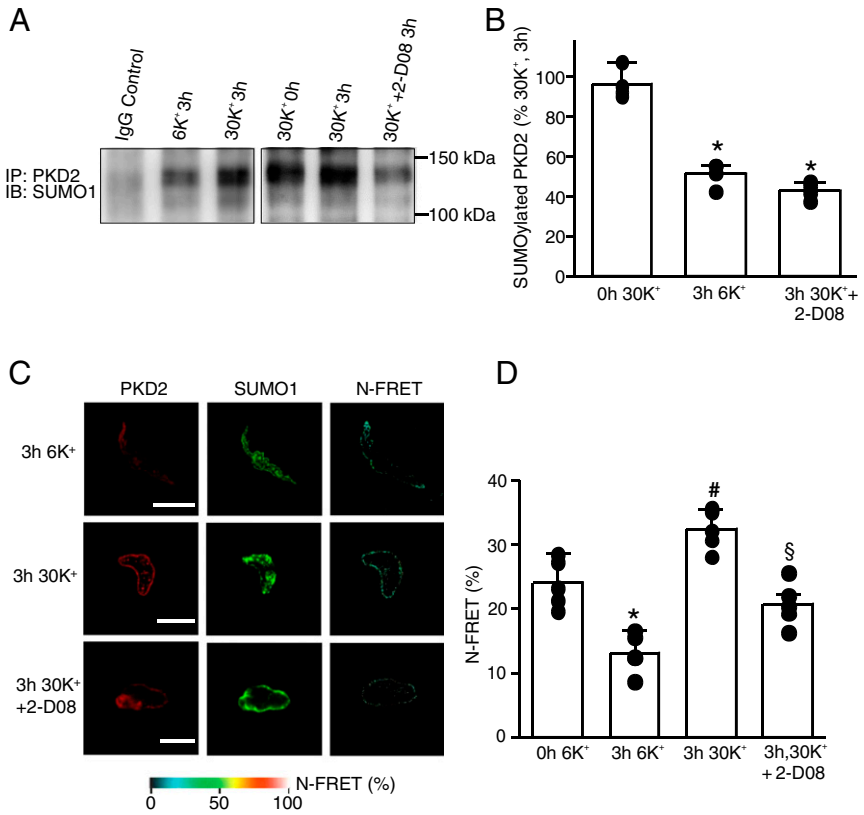

Fig. 4. Membrane depolarization stimulates PKD2 sumoylation. (*A*) Representative Western blots illustrating SUMO1 detection (IB) in PKD2 immunoprecipitate (IP) and at the same molecular weight as the large PKD2 protein band under different conditions. (*B*) Mean data of sumoylated PKD2, $n = 5$ for each group. *$P < 0.05$ vs. 0 h/30 K^+. (*C*) Immunofluorescence and immunoFRET images of PKD2 (Alex 546) and SUMO1 (Alex 488) after 3 h in 6 K^+ PSS, 30 K^+ PSS, or 30 K^+ PSS+2-D08. (Scale bars: 10 μm.) (*D*) Mean data for immunoFRET experiments ($n = 6$ for each). *$P < 0.05$ vs. 0 h/6 K^+, #$P < 0.05$ vs. 3 h/6 K^+, §$P < 0.05$ vs. 3 h/30 K^+.

Sumoylation of PKD2 Channels Is Required for Pressure-Induced Vasoconstriction. The significance of PKD2 channel sumoylation to pressure-induced vasoconstriction (myogenic tone) was measured in hindlimb arteries. First, we examined the contribution of myocyte PKD2 channels to myogenic tone. Hindlimb arteries from *Pkd2*$^{fl/fl}$ and *Pkd2* smKO mice were subjected to stepwise increases in intraluminal pressure (20 to 100 mmHg), and contractility was measured. Pressures greater than 40 mmHg stimulated a myogenic response in *Pkd2*$^{fl/fl}$ arteries, producing a constriction of ∼20.0% at 100 mmHg (Fig. 6*A*). This myogenic response was robustly attenuated in *Pkd2* smKO arteries, with 100 mmHg producing a vasoconstriction that was ∼21.2% of that in *Pkd2*$^{fl/fl}$ arteries (Fig. 6*A*). In contrast, depolarization (60 mM K^+) produced slightly larger vasoconstriction in *Pkd2* smKO than in *Pkd2*$^{fl/fl}$ arteries, consistent with up-regulation of $Ca_V1.2$ channels (*SI Appendix*, Fig. S6*A*) (12). These results suggest that PKD2 channels are a major contributor to the myogenic response in hindlimb arteries, supporting our previous data (12).

Next, we tested the hypothesis that PKD2 channel sumoylation controls myogenic vasoconstriction. Pressure response curves were measured in control, after which responses to 2-D08 were recorded at a steady pressure of 80 mmHg. The 2-D08 caused a slow but robust vasodilation in myogenic arteries that plateaued after ∼1.5 h, which is consistent with the time course of surface SUMO-PKD2 loss (Fig. 6*B*). The 2-D08 robustly attenuated myogenic tone at pressures greater than 40 mmHg (Fig. 6 *B* and *C*). For example, at 100 mmHg, myogenic tone in 2-D08 was only ∼19.4% of that obtained in control, which is similar to the reduction caused by *Pkd2* smKO (Fig. 6). In contrast, 2-D08 did not alter depolarization (60 mM K^+)-induced vasoconstriction, suggesting that this Ubc9 inhibitor does not inhibit voltage-dependent Ca^{2+} channels or Ca^{2+}-dependent vasoconstriction (*SI Appendix*, Fig. S6 *B* and *C*). Collectively, these results indicate that SUMO-PKD2 channels regulate arterial contractility.

Discussion

Here, we show that PKD2 channels undergo sumoylation, identify that posttranslational modification by SUMO1 regulates PKD2 surface abundance in myocytes, and demonstrate that PKD2 sumoylation is a functional mechanism by which intravascular pressure modulates arterial contractility. PKD2 can bind between 1 and 3 SUMO1 proteins and, in myocytes, exist in approximately equal proportions as either nonsumoylated or triple SUMO1-modified proteins. Physiological intravascular pressure stimulates voltage-dependent Ca^{2+} influx that inhibits the entry of internalized SUMO-PKD2 into a lysosomal degradation pathway, enabling recycling to the plasma membrane. In contrast, a reduction in intravascular pressure, membrane hyperpolarization, or a decrease in Ca^{2+} influx lead to SUMO-PKD2 degradation, which reduces surface channel abundance. Furthermore, our results suggest that pressure stimulates SUMO1-PKD2 channels, as the specific loss of these proteins inhibits swelling-activated I_{Cat} and myogenic tone. Thus, SUMO1 modification of PKD2 enables control over the surface abundance of these channels in myocytes to regulate arterial contractility.

SUMO-PKD2 was essentially absent in arteries of *Pkd2* smKO mice, suggesting that myocytes contain the majority of SUMO-PKD2 present in the vascular wall. In contrast, other vascular wall cell types appear to contain very little SUMO-PKD2. We show that PKD2 channels can be specifically modified by

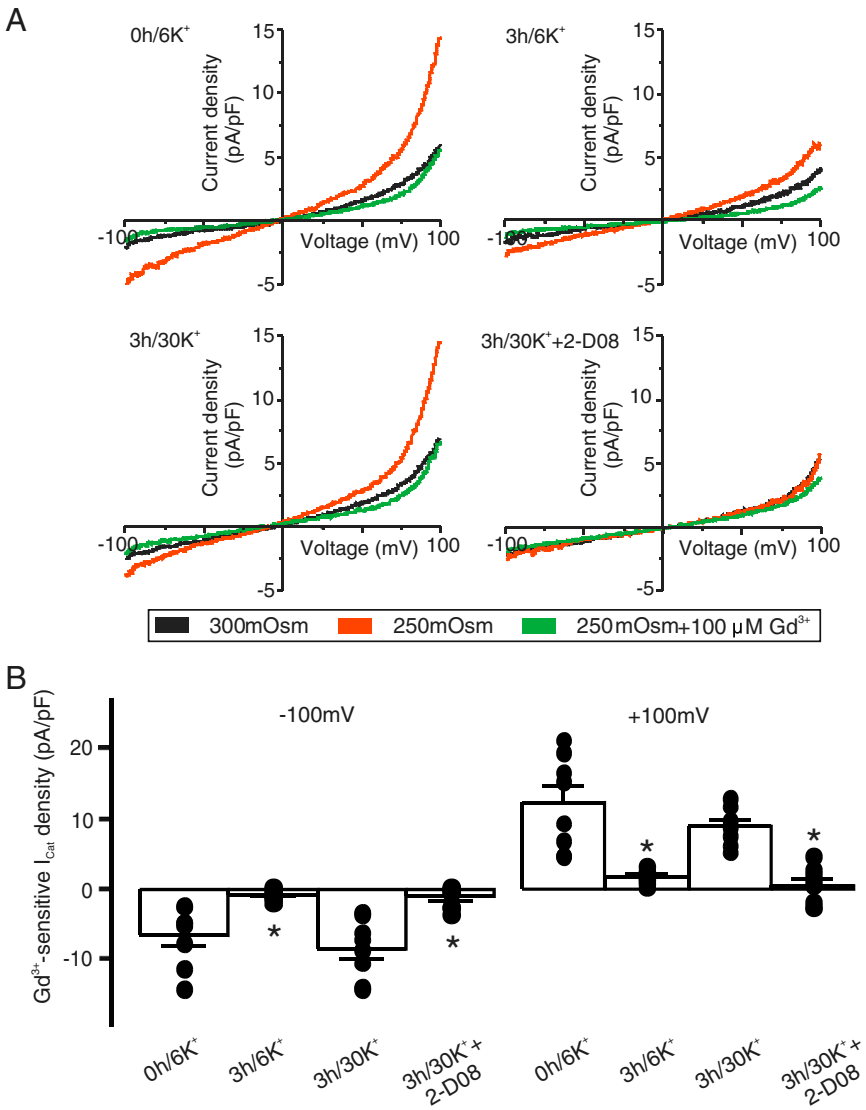

Fig. 5. Voltage-dependent regulation of PKD2 sumoylation modulates swelling-activated PKD2 current density in arterial myocytes. (*A*) Representative voltage ramps illustrating I_{Cat} recorded in isotonic (300 mOsm) and hypotonic (250 mOsm) bath solution with and without Gd^{3+} (100 μM) in the mouse hindlimb artery myocytes in 0 h/6 K^+, 3 h/6 K^+, 3 h/30 K^+, or 3 h/30 K^+/2-D08. (*B*) Mean data for Gd^{3+}-sensitive I_{Cat} recorded in hypotonic solution in myocytes under different conditions; $n = 6$ for all conditions. *$P < 0.05$ vs. 0 h control.

SUMO1, but not by SUMO2 or SUMO3. SUMO1 is an ∼11-kDa peptide, but, when run on an SDS/PAGE gel, size shifts caused by each SUMO can be larger than those expected purely from the calculated molecular weight (28). When proteins contain multiple sumoylation sites or SUMO chains form, large shifts in mobility can also occur (28). In our experiments, each SUMO1 modification increased the molecular weight of purified PKD2 by ∼11 kDa. Pure PKD2 and arterial myocyte PKD2 proteins were separated using identical SDS/PAGE conditions. Arterial myocyte PKD2 occurred as either a 106-kDa protein that did not contain SUMO1 or a ∼140-kDa protein that contained SUMO1. Therefore, we provide strong evidence that PKD2 channels are modified by 3 SUMO1 proteins in arterial myocytes.

Our data suggest that SUMO-PKD2 and nonsumoylated PKD2 channels are regulated by distinct processes in arterial myocytes. In depolarized and pressurized arteries, SUMO-PKD2 channels recycle, whereas PKD2 channels are surface-resident. Evidence supporting this conclusion includes that a reduction in intravascular pressure leads to the internalization and degradation of SUMO-PKD2 that is prevented by inhibitors of either internalization or lysosomal degradation. In depressurized arteries, lysosomal degradation does not lead to the appearance of SUMO-PKD2 in an intracellular compartment, but rather protein returns to the plasma membrane. Thus, SUMO-PKD2 constitutively recycles. In contrast, surface, intracellular, and total amounts of PKD2 channels are unaltered by a reduction in pressure, membrane hyperpolarization, or inhibition of $Ca_V1.2$ influx, suggesting that nonsumoylated channels do not recycle and are not subject to the same regulatory mechanisms of internalization and degradation as SUMO-PKD2. Con A blocked depressurization-induced desumoylation of surface SUMO-PKD2, suggesting that membrane potential and pressure do not alter the sumoylation state of surface-localized channels, but only of proteins that have been internalized.

Intravascular pressure stimulates arterial depolarization to between ∼−60 mV and −35 mV, which increases $[Ca^{2+}]_i$ from ∼100 to 250 nM in myocytes (29). A reduction in intravascular pressure led to a decrease in $[Ca^{2+}]_i$ that targeted internalized

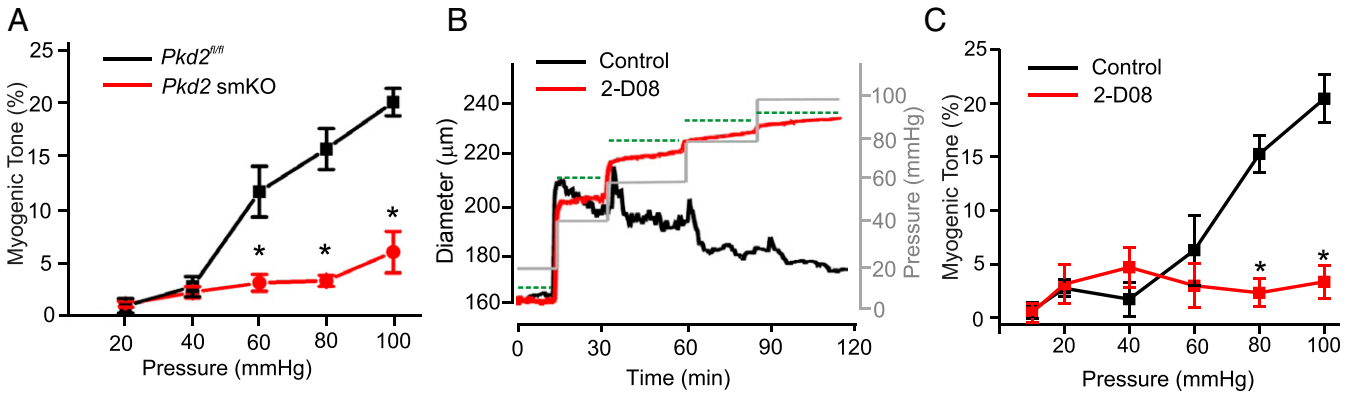

Fig. 6. Inhibition of sumoylation dilates pressurized arteries. (*A*) Mean data illustrating pressure-induced vasoconstriction in hindlimb arteries of *Pkd2*$^{fl/fl}$ ($n = 5$) and *Pkd2* smKO ($n = 5$) mice. *$P < 0.05$ vs. *Pkd2*$^{fl/fl}$. (*B*) Representative original traces illustrating diameter responses to a range of pressures between 20 mmHg and 100 mmHg in the same artery before (control, black trace) and after (10 μM, red trace) a 1.5-h exposure to 2-D08. Green dotted lines illustrate passive diameter recorded at each pressure in Ca^{2+}-free PSS. (*C*) Mean paired data ($n = 6$ for all data points). *$P < 0.05$ vs. control.

SUMO-PKD2 channels for lysosomal degradation. Thus, at physiological pressure, SUMO-PKD2 degradation is inhibited. Our data suggest that SUMO1 modification of PKD2 channels stimulates their return to the surface, because, if sumoylation is blocked with 2-D08, PKD2 channels are degraded, even in depolarized arteries. Similarly, 2-D08 reduced SUMO-PKD2 protein, but did not induce a concomitant increase in nonsumoylated PKD2, providing further support for this conclusion. In this light, it is possible that the sumoylation state of constitutively internalized SUMO-PKD2 channels is maintained by active processes. If this does not take place, SUMO-PKD2 is desumoylated and targeted for degradation. Conceivably, $[Ca^{2+}]_i$ may activate Ubc9, the only known SUMO-conjugating enzyme, and/or inhibit SENPs, which desumoylate proteins. Ca^{2+} may modulate the activity of these enzymes through an upstream Ca^{2+}-sensitive signaling pathway. Intracellular Ca^{2+} release increased PKD2 surface immunofluorescence in cultured rat proximal tubule cells, although, at that time, it was not known that PKD2 channels could be sumoylated. Similarly, supporting a link between $[Ca^{2+}]_i$ and sumoylation is evidence that calpain, a Ca^{2+}-activated protease, cleaves SAE2, a SUMO E1 enzyme, to inhibit protein sumoylation in cultured epithelial cells (30). In contrast to the observed Ca^{2+}-dependent regulation of SUMO-PKD2, $[Ca^{2+}]_i$ has no effect on the abundance or location of nonsumoylated PKD2, which is retained at the surface regardless of experimental conditions. To summarize, a pressure- or depolarization-induced increase in $[Ca^{2+}]_i$ inhibits constitutively recycling SUMO-PKD2 channels from entering into a lysosomal degradation pathway, thereby maintaining surface levels of these proteins. Conversely, a reduction in $[Ca^{2+}]_i$ stimulates lysosomal degradation of internalized SUMO-PKD2, leading to a decrease in surface channel abundance. In contrast to this dynamic regulation of SUMO-PKD2, over the same time course, TRPM4, TRPC6, and $Ca_V1.2$ channel proteins were unchanged, suggesting that SUMO-PKD2 loss is specific and not a result of widespread protein degradation. Many different vasoconstrictor and vasodilator stimuli, including receptor agonists, modulate $[Ca^{2+}]_i$ in arterial myocytes. Conceivably, these stimuli may also regulate PKD2 sumoylation and surface abundance to modulate arterial contractility. Future studies should investigate the possibility that PKD2 sumoylation is a vasoregulatory process for a variety of other stimuli in addition to intravascular pressure.

PKD2 has been proposed both to function independently and to interact with PKD1 through C-terminal coiled-coil domains and N-terminal loops and to form a receptor/channel complex containing 3 PKD2 and 1 PKD1 subunits (31–35). Whether PKD1 and PKD2 interact or form heterotetramers in arterial myocytes is unclear, as is the effect of PKD2 channel sumoylation on channel homomultimeric and heteromultimeric assembly. Our results demonstrate that the depressurization-induced reduction in PKD2 was not associated with a decrease in PKD1 protein. Either PKD2 does not form a heteromultimeric complex with PKD1 or PKD1 and PKD2 subunits first dissociate prior to subsequent degradation of PKD2 in arterial myocytes.

Patch-clamp electrophysiology and pressurized artery myography experiments support biochemical evidence that sumoylation-dependent modulation of PKD2 surface abundance regulates both myocyte I_{cat} and contractility. Arterial depressurization and sumoylation inhibition with 2-D08 decreased both surface SUMO-PKD2 protein and swelling-induced I_{Cat}. In contrast, arterial depolarization increased both surface SUMO-PKD2 abundance and swelling-induced I_{Cat}. Myography data show that 2-D08 application at physiological pressure reduced myogenic tone. In contrast, these procedures did not alter surface nonsumoylated PKD2 protein. Thus, cell swelling and pressure appear to activate SUMO-PKD2 channels, but not PKD2 channels. Put another way, SUMO-PKD2 appears to be the functional, surface channel in myocytes that contributes to pressure-induced vasoconstriction.

Sumoylation reduced the voltage dependence of activation of $K_V2.1$ and I_{Ks} currents, induced a hyperpolarizing shift in the inactivation of $K_v1.5$ channels, increased the voltage sensitivity of $Na_V1.2$ channels, and silenced K2P1 channels (18–22). In many of these previous studies, the application of either SUMO or SENP, a desumoylating enzyme, was demonstrated to modify the properties of currents or channel (18–21). In contrast, here the introduction of either SUMO or SENP into myocytes did not alter PKD2 currents over a period of ∼30 min. There are several explanations for this result, including that sumoylation does not modulate PKD2 channel open probability (P_O) or amplitude (i) in arterial myocytes. Other possibilities include that surface-localized PKD2 channels may not be capable of sumoylation or desumoylation. Surface SUMO-PKD2 channels are fully occupied by 3 SUMO1 proteins in arterial myocytes and thus cannot accept additional SUMO1. Similarly, surface nonsumoylated PKD2 channels may not be located near Ubc9, preventing active conjugation of SUMO1. We never observed more than half of total PKD2 to be sumoylated, suggesting that this is the maximum possible. Thus, the addition of SUMO1 through the patch pipette solution may not increase the sumoylation state of surface PKD2 proteins. Data also suggest that surface PKD2 channels do not undergo desumoylation. Evidence supporting this conclusion includes that concanavalin prevented the desumoylation of surface SUMO-PKD2 channels in depressurized arteries. Thus, the introduction of SENP via the pipette solution may not lead to the

desumoylation of surface SUMO-PKD2. Future studies should be designed to investigate further whether altering the sumoylation state of PKD2 channels alters gating under conditions where such modification does not lead to their internalization and degradation.

SUMO can bind to proteins through the canonical consensus motif Ψ-K-x-D/E, where Ψ is a hydrophobic residue, K is a lysine that conjugates to SUMO, x is any amino acid, and D or E are acidic residues (36). SUMO can also attach to proteins through both nonconsensus motifs and SUMO interaction motif (SIM) domains, the sequences of which are still being discovered (36). The majority of SUMO-binding sites in proteins are nonconsensus, making their identification more difficult (36). Prediction analysis using GPS-SUMO 2.0 (37) indicates that PKD2 contains 2 SUMO consensus motifs at K686 and K864, a nonconsensus motif at K759, and 3 SIM domains located between amino acids 229 and 233, 490 and 494, and 511 and 514. It is beyond the scope of this study to determine whether any of these predicted sumoylation sites in PKD2 bind SUMO1 or whether novel sequences that are not predicted by GPS-SUMO 2.0 are involved. The goal of this study is to determine whether posttranslational modification of PKD2 channels regulates physiological functions in arterial myocytes. Future studies should aim to identify specific sites that bind SUMO1 in PKD2 channels of contractile arterial myocytes.

Our data raise the possibility that SUMO proteins may conjugate to PKD2 channels in other cell types to regulate physiological functions. Similarly, altered PKD2 sumoylation may be associated with cardiovascular diseases and other pathologies. We have previously shown that hypertension is associated with an increase in surface PKD2 channels in arterial myocytes. In that study, we were unaware that PKD2 channels could undergo sumoylation, and we were not in a position to measure SUMO-PKD2 protein in arteries of hypertensive animals. Conceivably, dysregulation of PKD2 sumoylation may be involved in altered function in arterial myocytes. ADPKD is the most prevalent monogenic human disease worldwide and occurs due to mutations in either PKD1 or PKD2 proteins (6). ADPKD is characterized by the appearance of renal epithelial cysts, although a significant proportion of patients with apparently normal renal function develop hypertension prior to the development of cysts (6–8). More than 275 human variants in PKD2 have been identified (Autosomal Dominant Polycystic Kidney Disease Mutation Database, Mayo Clinic; https://pkdb.pkdcure.org). Whether PKD2 channels in kidney epithelial cells undergo sumoylation to regulate normal physiological functions is unclear. Similarly, the possibility that ADPKD mutations modify sumoylation to influence PKD2 channel surface trafficking, degradation, and function in kidney epithelial cells remains to be determined.

In summary, we show that PKD2 channels undergo sumoylation, identify that posttranslational modification by SUMO1 regulates PKD2 surface abundance in myocytes, and demonstrate that PKD2 sumoylation is a functional mechanism by which intravascular pressure modulates arterial contractility.

Materials and Methods

Animals. All procedures were approved by the Animal Care and Use Committee of the University of Tennessee. All experiments were performed using C57BL/6J mice (12 wk old) unless otherwise stated. C57BL/6J mice were purchased from Jackson Laboratories. *Pkd2*$^{fl/fl}$ and *Pkd2* smKO mice were bred and genotyped as previously described (12).

Tissue Preparation and Myocyte Isolation. Hindlimb (saphenous, popliteal, and gastrocnemius) arteries were harvested and placed into ice-cold PSS that contained 112 mM NaCl, 6 mM KCl, 24 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 10 mM glucose, gassed with 21% O_2, 5% CO_2, and 74% N_2 to pH 7.4. Arterial myocytes were dissociated using enzymes, as previously described (38).

RT-PCR. Fresh, dissociated hindlimb artery myocytes were visualized under a microscope and individually collected using an enlarged patch pipette. Total RNA was extracted from ~500 myocytes using the PureLink RNA Mini Kit (Thermo-Fisher Scientific). Complementary DNA was synthesized using SuperScript IV First-strand synthesis system (Thermo-Fisher Scientific) after which RT-PCR was performed. PCR products were separated on 2% agarose-tris-acetate ethylenediaminetetraacetic acid gels. Primers used to amplify transcripts are provided in *SI Appendix*, Table S1. The PKD2 forward primer spanned the junction of exons 9 and 10, and the reverse primer annealed to exon 13.

Western Blotting. Arteries were homogenized in radioimmunoprecipitation assay (RIPA) buffer, and protein concentration was normalized. Proteins were resolved on SDS/PAGE and blotted onto poly(vinylidene difluoride) membranes. Membranes were blocked and incubated with the following primary antibodies: $Ca_V1.2$ (Neuromab), PKD1 and PKD2 (Santa Cruz), TRPC6 and TRPM4 (Abcam), SUMO1, SUMO2/3, and ubiquitin (Santa Cruz), and actin (MilliporeSigma) overnight at 4 °C. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies. Membranes were developed and protein bands were imaged using an Amersham Imager 600 gel imaging system (GE Healthcare) and were quantified using ImageJ software.

MS. PKD2 protein was immunoprecipitated overnight, and both bands were resolved on SDS/PAGE. The gel regions corresponding to the higher and lower PKD2 bands were cut, washed twice in phosphate-buffered saline (PBS), and sent for MS to the Mass Spectrometry and Proteomics Resource Laboratory at Harvard University. Samples were enzymatically digested and analyzed by liquid chromatography MS/MS. MS spectra were correlated with the mouse proteome database for peptide identification.

Immunoprecipitation. Primary antibodies were cross-linked with magnetic beads. Proteins were pulled down overnight at 4 °C from hindlimb arterial lysate using immunoprecipitation kit (Pierce) per the manufacturer's instructions. Beads were washed, and bound proteins were eluted and analyzed using Western blotting and MS as discussed above.

Immunofluorescence and FRET Imaging. Isolated myocytes were plated onto poly-L-lysine–coated coverslips, fixed with paraformaldehyde, and permeabilized with Triton X-100. For cell surface immunofluorescence colocalization, cells were labeled with Alexa 488-conjugated wheat germ agglutinin (Invitrogen) prior to permeabilization. Myocytes were blocked and incubated with the following primary antibodies overnight at 4 °C: rabbit anti-PKD2 (Baltimore PKD Core) for immunofluorescence and both rabbit anti-PKD2 and mouse anti-SUMO1 or SUMO2/3 antibodies (Santa Cruz) for immunoFRET. Cells were washed and incubated with Alexa 546- or Alexa 488-conjugated secondary antibodies. Fluorescence images were acquired using a Zeiss LSM 710 laser-scanning confocal microscope. Pixel colocalization was measured using weighted colocalization coefficient values with Zeiss Pascal system embedded software. For immunoFRET analysis, images were background-subtracted, and N-FRET was calculated on a pixel-by-pixel basis using the Xia method and the Zeiss LSM FRET Macro tool (version 2.5).

In Vitro Sumoylation. Reactions were performed by incubating the following components at 37 °C for 4 h: E1-ligase (250 ng), E2-ligase (Ubc9, 1.2 μg), SUMO1 or SUMO2 protein (5 μg of either), and full length human GST-tagged PKD2 protein (5 μg) in buffer (55 mM Tris, pH 7.5, 5.5 mM $MgCl_2$, 2.2 mM ATP, 5.5 mM dithiothreitol). After reaction termination with SDS/PAGE sample buffer, products were separated on an SDS/PAGE gel, blotted onto nitrocellulose membranes, and probed with anti-GST antibody (Cell Signaling Technology).

Arterial Biotinylation. Arteries were biotinylated with EZ-Link Sulfo-NHS-LC-LC-Biotin and EZ-Link Maleimide-PEG2-Biotin for 1 h at 4 °C following procedures previously described (25, 39). Unbound biotin was quenched with glycine/PBS, washed with PBS, and then homogenized in RIPA buffer. Protein concentration was normalized, and biotinylated surface protein was captured by incubating cell lysates with avidin beads (Pierce) at 4 °C. Proteins present in biotinylated and nonbiotinylated samples were identified using Western blotting.

Pressurized Artery Diameter Measurements. Endothelium-denuded mouse first-order gastrocnemius artery segments (~1-mm length) were cannulated at each end in a temperature-controlled perfusion chamber (Living Systems

Instrumentation) and perfused with 37 °C PSS gassed with a mixture of 21% O_2, 5% CO_2, and 74% N_2. Arteries were subjected to stepwise increases in intravascular pressures (10 to 100 mmHg). Arterial diameter was recorded at 1 Hz using a charge-coupled device camera attached to a Nikon TS100-F microscope and the automatic edge detection function of IonWizard software (Ionoptix). Myogenic tone was calculated as $100 \times (1 - D_{active}/D_{passive})$, where D_{active} is active arterial diameter and $D_{passive}$ is the diameter determined in the presence of Ca^{2+}-free PSS supplemented with 5 mM ethylene glycol bis(2-aminoethyl)tetraacetic acid.

Patch-Clamp Electrophysiology. Isolated arterial myocytes were allowed to adhere to a glass coverslip in a recording chamber. The conventional whole-cell configuration was used to measure I_{cat} by applying voltage ramps between −100 mV and +100 mV from a holding potential of −40 mV. Currents were filtered at 1 kHz and digitized at 5 kHz using an Axopatch 200B amplifier and Clampex 10.4 (Molecular Devices). Offline analysis was performed using Clampfit 10.4.

Statistical Analysis. OriginLab and GraphPad InStat software were used for statistical analyses. Values are expressed as mean ± SEM. Data were analyzed using paired or unpaired Student's *t* test or ANOVA with Newman–Keuls post hoc test. All data are expressed as means ± SEM. Power analysis was performed to verify that the sample size gave a value of > 0.8 if P was > 0.05.

Expanded materials and methods are available as *SI Appendix*.

Data Availability Statement. All data discussed in the paper are available to readers at Figshare, https://doi.org/10.6084/m9.figshare.11239022.v1 (40).

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