Calcium (Ca$^{2+}$) Reduction Increases Cellular Proliferation and Apoptosis in Vascular Smooth Muscle Cells

Relevance to the ADPKD Phenotype

Sertac N. Kip, Larry W. Hunter, Qun Ren, Peter C. Harris, Stefan Somlo, Vicente E. Torres, Gary C. Siek, Qi Qian

Abstract—Cardiovascular complications are the leading cause of morbidity and mortality in autosomal dominant polycystic kidney disease. Pkd2$^{−/−}$ vascular smooth muscle cells (VSMCs) have an abnormal phenotype and defective intracellular Ca$^{2+}$ ([Ca$^{2+}$]) regulation. We examined cAMP content in vascular smooth muscles from Pkd2$^{−/−}$ mice because cAMP is elevated in cystic renal epithelial cells. We found cAMP concentration was significantly increased in Pkd2$^{−/−}$ vessels compared with wild-type vessels. Furthermore, reducing the wild-type VSMC [Ca$^{2+}$], by Verapamil or BAPTA-AM significantly increased cellular cAMP concentration (mainly by phosphodiesterase [PDE] inhibition), the rate of VSMC proliferation (determined by direct cell counting, $^{3}$H-incorporation, FACS analysis of cells entering S phase, and quantitative Western PCNA and ERK1/2 analyses), and the rate of apoptosis (by Hoechst staining, FACS analysis of the Annexin-V positive cells, and quantitative Western Bax, cytochrome c, and activated caspase 9 and 3 analyses). The low [Ca$^{2+}$], induced VSMC proliferation was independent of cAMP/B-Raf signaling, while that of apoptosis was promoted by cAMP. In summary, Pkd2$^{−/−}$ VSMCs have elevated cAMP levels. This elevation can also be induced by reducing [Ca$^{2+}$], in wild-type VSMCs. The [Ca$^{2+}$] reduction and cAMP accumulation can cause an increase in both cellular proliferation and apoptosis, resembling Pkd mutant phenotype. (Circ Res. 2005;96:873-880.)

Key Words: cAMP  phosphiodiesterase  proliferation  ERK  apoptosis

Autosomal-dominant polycystic kidney disease (ADPKD) is caused by mutations to either PKD1 or PKD2 genes encoding polycystin-1 (PC1) or polycystin-2 (PC2), respectively. PC1 is a plasma membrane receptor–like protein that has functions in cell-cell or cell-extracellular matrix interactions. PC2 is a nonselective cation channel protein with a large single-channel conductance and high permeability to calcium (Ca$^{2+}$). PC1 interacts with PC2. Their interaction can form a functional receptor-ion-channel-complex and regulate heterotrimeric G-protein–mediated signaling cascades. The mutations of either gene cause a nearly identical clinical phenotype (see review$^3$).

Cardiovascular complications are the leading cause of mortality and morbidity in ADPKD. The incidence of intracranial aneurysms and thoracic aortic dissections in ADPKD is approximately 10-fold higher than in the general population.$^2$ Even during the early stages of ADPKD, before the onset of hypertension or renal dysfunction, abnormal thickening of the intimal and media layers of vessels is evident.$^3$-$^4$ Although clinical and experimental evidence indicate a close relation between PKD/Pkd mutations and vascular complications, their pathogenesis is not understood. We have shown that when induced to develop hypertension, Pkd2$^{−/−}$ mice have an increased susceptibility to vascular injury, manifested as premature death or developing prominent irregular thickening in the tunica media layer of intracranial vessels.$^5$ The areas of irregular vessel wall thickening are correlated with abnormally increased or decreased number of VSMCs, indicating an imbalance between smooth muscle cell proliferation and apoptosis.

Elevated rates of proliferation and apoptosis are the major phenotypic features of ADPKD cells; these abnormalities have been detected in multiple organ systems including kidneys, lungs, liver, heart, brain, spleen, thymus, and testis.$^6$-$^7$ Recent studies suggest that reduced basal intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]), and elevated intracellular cyclic 3',5'-adenosine monophosphate (cAMP) play a role in this phenotype. Cystic renal tissues have elevated intracellular cAMP.$^8$-$^9$ In contrast to its growth inhibitory effect on wild-type renal epithelial cells, cAMP promotes proliferation in PKD/Pkd mutant renal epithelial cells.$^{10}$-$^{12}$ This proliferative response to cAMP can also be induced in wild-type renal epithelial cells by reducing their basal [Ca$^{2+}$].$^{13}$

Abstract—Cardiovascular complications are the leading cause of morbidity and mortality in autosomal dominant polycystic kidney disease. Pkd2$^{−/−}$ vascular smooth muscle cells (VSMCs) have an abnormal phenotype and defective intracellular Ca$^{2+}$ ([Ca$^{2+}$]) regulation. We examined cAMP content in vascular smooth muscles from Pkd2$^{−/−}$ mice because cAMP is elevated in cystic renal epithelial cells. We found cAMP concentration was significantly increased in Pkd2$^{−/−}$ vessels compared with wild-type vessels. Furthermore, reducing the wild-type VSMC [Ca$^{2+}$], by Verapamil or BAPTA-AM significantly increased cellular cAMP concentration (mainly by phosphodiesterase [PDE] inhibition), the rate of VSMC proliferation (determined by direct cell counting, $^{3}$H-incorporation, FACS analysis of cells entering S phase, and quantitative Western PCNA and ERK1/2 analyses), and the rate of apoptosis (by Hoechst staining, FACS analysis of the Annexin-V positive cells, and quantitative Western Bax, cytochrome c, and activated caspase 9 and 3 analyses). The low [Ca$^{2+}$], induced VSMC proliferation was independent of cAMP/B-Raf signaling, while that of apoptosis was promoted by cAMP. In summary, Pkd2$^{−/−}$ VSMCs have elevated cAMP levels. This elevation can also be induced by reducing [Ca$^{2+}$], in wild-type VSMCs. The [Ca$^{2+}$] reduction and cAMP accumulation can cause an increase in both cellular proliferation and apoptosis, resembling Pkd mutant phenotype. (Circ Res. 2005;96:873-880.)

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cAMP to the abnormal proliferation in PKD/Pkd mutant epithelial cells. The mechanism underlying an increased rate of apoptosis is less understood. Studies have shown that a vasopressin (V2) receptor blocker, by blocking adenyl cyclases (AC) and reducing renal cAMP content, inhibits cyst growth in animal models of PKD. This effect is accompanied by a marked reduction in apoptosis, suggesting that the elevated cellular cAMP contributes to the increased apoptosis in cystic renal epithelial cells.8,9

Information regarding cAMP content in Pkd mutant VSMCs and its effect on the VSMC phenotype is lacking. Previously, we have shown that Pkd2−/− VSMCs have a significant reduction in PC2 expression and basal [Ca2+]i, compared with those of wild-type VSMCs.5 However, the relationship between a reduced basal [Ca2+]i and their abnormal cellular phenotype is unknown. In this article, we tested the hypothesis that the [Ca2+]i and their abnormalities in both [Ca2+]i and cAMP lead to an abnormal VSMC phenotype. We show that a reduction in [Ca2+]i, in wild-type VSMCs causes an increase in intracellular cAMP, cellular proliferation, and apoptosis, mimicking PKD/Pkd mutant phenotype.

Materials and Methods

Genotyping, Isolation of the Thoracic Aortas from Adult Mice, and Generation of Cultured VSMCs

These methods were reported.5,14,15 All animal experiments were approved by the Institutional Animal Care and Use Committee. VSMCs were isolated using a commercial kit (Papain dissociation system, Worthington Biochemical) following the manufacturer protocol. Dissociated VSMCs were transferred into culture flasks containing DMEM (10% FCS), incubated at 37°C with 5% CO2, and the media changed every other day until the cells reached confluence. The purity of the VSMCs was confirmed both by morphological observation of the SMC-specific hill-and-valley growth pattern and homogeneous staining with smooth muscle α-actin mAB that distinguishes SMCs from fibroblasts as described previously.13

cAMP Concentration in Tissues and Cells

Vascular smooth muscles or primary cultured VSMCs were lysed in 10 volumes of 5% TCA, pelleted by centrifugation (600g × 10 minutes), and supernatants collected for cAMP determination using an enzyme immunoassay kit (Sigma). The results were expressed as pmol of cAMP/mg of protein.

Basal [Ca2+]i Measurements and Recording Techniques

VSMCs (treated or nontreated) grown on coverslides were loaded with fura-2 AM (Molecular Probes) for 30 minutes, rinsed with media, placed under an inverted Nikon Diaphot microscope, and excited at 340 and 380 nm. The emissions were collected by a 510-nm barrier filter, and images were acquired by a Photometric excitation at 340 and 380 nm. The emissions were collected by a Polytron homogenizer (4°C) in buffer containing (in mmol/L): 10 Tris (pH7.5); 20 KCl; 1 EGTA; 250 sucrose; 1 PMSE and protease inhibitor cocktail (Roche). Post nuclear supernatants (1000g × 15 minutes) were further centrifuged (15 000g × 20 minutes), the resulting pellets were enriched with mitochondrial proteins, and the resulting supernatants were further centrifuged (100 000g × 2 hours). The resulting supernatants represent cytosols. The lack of membrane proteins in cytosolic fraction was confirmed by immunoblotting with the membrane markers (Calreticulin and PC2). Protein content was determined by the Lowry assay (Bio-Rad). Proteins (20 to 40 μg) were denatured in 1X sample buffer with 5% 2-mercaptoethanol at 75° to 95°C for 10 minutes, fractionated by SDS-PAGE (Invitrogen), electrotransferred to PVDF membrane, detected with specific antibodies and visualized by Luminal reagent for enhanced chemiluminescence (Santa Cruz).

Statistical Analysis

Data are expressed as mean±SE. Student t test and two-way ANOVA were used for comparisons between different groups.

Antibodies and Reagents

Antibodies were obtained from Santa Cruz for PCNA (sc-56), ERK1/2 (sc-93), pERK1/2 (sc7383), and B-Raf (sc-166); from Sigma-Aldrich for SM-α-actin (A2547); from BD Pharmagen for cytochrome c (556433), cleaved Cy (552036), and cleaved C3 (551150); and from R&D systems for Bax (AF820). Unless otherwise specified, all reagents were obtained from Sigma.

Results

Pkd2+/− Vessels Have Increased Cellular cAMP Levels

Kidneys from Pkd2+/−, pcy mice and PCK rats have increased intracellular cAMP. To examine whether this is true in Pkd2+/− mutant arteries, we measured the cAMP content in the tunica media layer of individual aortas freshly isolated from sex-matched Pkd2+/− and wild-type littermates. As shown in Figure 1A, Pkd2+/− vascular smooth muscles have significantly higher levels of cAMP compared with the wild type (P<0.001), indicating an increased VSM cellular cAMP concentration.
Lowering the \([\text{Ca}^{2+}]\), Raised cAMP Levels in Wild-Type VSMCs

\(Pkd2^{−/−}\) VSMCs have a reduced basal \([\text{Ca}^{2+}]\); a low \([\text{Ca}^{2+}]\), could alter cAMP concentration by activating \(\text{Ca}^{2+}\)-inhibitable adenyl cyclases (especially AC5, 6) or by inhibiting \(\text{Ca}^{2+}\)-stimulatable phosphodiesterases (PDEs).\(^{16−18}\) To test whether a reduction of \([\text{Ca}^{2+}]\), as observed in \(Pkd2^{−/−}\) VSMCs, can lead to cAMP accumulation, we measured cAMP content in control (nontreated), Verapamil (20 \(\mu\text{mol/L}\)), or BAPTA-AM (a membrane permeable \(\text{Ca}^{2+}\) chelator, 20 \(\mu\text{mol/L}\)) treated wild-type cultured VSMCs (passage 4 to 6). The concentrations of Verapamil and BAPTA-AM were chosen because similar or higher variations in the concentrations of Verapamil or BAPTA-AM between 5 to 15 \(\mu\text{mol/L}\) did not change their effects on proliferation (Figure 3B) or on \([\text{Ca}^{2+}]\).

To dissect whether the AC activation and/or the PDE inhibition is responsible for this effect, cAMP was measured after adding forskolin (a prototype-AC activator,\(^{21}\) 10 \(\mu\text{mol/L}\)) or IBMX (a pan-PDE inhibitor, 300 \(\mu\text{mol/L}\)) to the VSMCs treated with Verapamil or BAPTA-AM. As shown in Figure 2, Forskolin further increased cAMP content (\(P<0.0001\)) up to 20-fold, whereas IBMX did not have a significant additive effect (\(P=0.98\)). These results suggest that cAMP accumulation is caused mainly by PDE inhibition.

To further determine whether PDE4, the major PDE in VSMCs, is responsible for this effect, cells were treated with a combination of Verapamil and Rolipram (a selective PDE4 inhibitor, 30 \(\mu\text{mol/L}\)) before cAMP content was measured. As shown in Figure 2 (black bars), the effect of Rolipram was similar to that of IBMX (Rolipram versus IBMX; \(P=0.67\)), suggesting that inhibition of PDE4 was mainly responsible for the low-\([\text{Ca}^{2+}]\)–induced cAMP elevation.

Reduction in Basal \([\text{Ca}^{2+}]\), Increased VSMC Proliferation

To study the relationship between the reduction of \([\text{Ca}^{2+}]\) and the rate of proliferation, wild-type VSMCs were treated with Verapamil or BAPTA-AM at the concentration that reduced \([\text{Ca}^{2+}]\), by \(\sim30\%\). The proliferative rates were measured by direct cell counting (data not shown); \(^{1}\)H-incorporation; FACS analysis for the number of S phase cells; quantitative Western analysis for PCNA, an indicator of active cellular proliferation; and phosphorylated ERK1/2. As shown in Figure 3A through 3D, under conditions of reduced \([\text{Ca}^{2+}]\), the rates of VSMC proliferation were significantly increased. Variations in the concentrations of Verapamil or BAPTA-AM between 5 to 15 \(\mu\text{mol/L}\) did not change their effects on proliferation (Figure 3B) or on \([\text{Ca}^{2+}]\).

cAMP Inhibited VSMC Proliferation at Normal or Reduced Basal \([\text{Ca}^{2+}]\), and Relation to the Expression Pattern of B-Raf Isoforms

cAMP is known to inhibit wild-type VSMC proliferation.\(^{22}\)

Others have shown that in renal epithelial cells, Verapamil or EGTA (an extracellular \(\text{Ca}^{2+}\) chelator), which reduce \([\text{Ca}^{2+}]\), switch the effect of cAMP from growth inhibition into growth
stimulation. To test whether this is true for VSMCs, we treated VSMCs with db-cAMP (membrane permeable active form of cAMP, 0.5 μmol/L) in the presence or absence of Verapamil or BAPTA-AM. We found that the addition of cAMP not only inhibited the proliferation of VSMCs with normal [Ca^{2+}], but also inhibited those with reduced [Ca^{2+}], (Figure 4A). To further determine whether this inhibitory effect is concentration-dependent, we repeated the experiments with combinations of db-cAMP and Verapamil or BAPTA-AM at various concentrations (0.002, 0.02, or 0.2 μmol/L of db-cAMP combined with 1 or 2 μmol/L of Verapamil or with 1 or 2 μmol/L of BAPTA-AM). The basal [Ca^{2+}], concentrations were reduced by 10% to 25% (1 μmol/L Verapamil or 1 or 2 μmol/L BAPTA-AM reduced [Ca^{2+}], by ≈10% to 25%). The number of S phase cells was determined by FACS analyses after 12 and 24 hours of treatments; no increase in proliferation was detected by comparing them to nontreated VSMCs in parallel experiments. We found that the apoptosis was significantly increased by DAPI-nuclear staining (see online Figure S2) as well as the FACS analysis staining with Annexin-V for apoptosis, we reexamined low-[Ca^{2+}]–induced apoptosis in the absence of cAMP inhibitor (Rp-cAMP, 100 μmol/L) or exogenous cAMP (db-cAMP, 0.5 μmol/L). As shown in Figure 5 (top left, white bars), Rp-cAMP largely rescued the low-[Ca^{2+}]–induced apoptosis, similar to that found in Pkd mutant epithelial cells.

In Pkd mutant or low [Ca^{2+}], wild-type renal epithelial cells, the cAMP-induced proliferation is accompanied by an elevated B-Raf expression. Of the multiple B-Raf isoforms (62 to 95 kDa), only the 95-kDa isoform is known to be associated with cAMP-induced proliferation. To determine whether the lack of proliferation to cAMP is due to a cell type-specific B-Raf expression, total cell lysate from various cell types; cystic epithelial cells are known to have elevated mitochondrial-mediated apoptosis. We hypothesized that the decreased [Ca^{2+}], in VSMCs leads to mitochondrial-mediated apoptosis. This hypothesis was tested by determining the apoptotic activities in wild-type VSMCs treated with Verapamil or BAPTA-AM (≈30% reduction in [Ca^{2+}],) and comparing them to nontreated VSMCs in parallel experiments. We found that the apoptosis was significantly increased by DAPI-nuclear staining (see online Figure S2) as well as the FACS analysis staining with Annexin-V for phosphotyrosinserine, an early cell-surface marker of apoptosis (Figure 5, top left, black bars). Three separate experiments, each in triplicate, with either Verapamil or BAPTA-AM, produced nearly identical results (P=0.0001 and P=0.0006, respectively). Quantitative Western analyses for the cellular apoptotic indicators were also performed. As shown in Figure 5, right panel, Bax, cytochrome c, and activated (cleaved) forms of caspase 9 and 3 were significantly elevated in VSMCs with reduced [Ca^{2+}], compared with those with normal [Ca^{2+}], which showed no increase in apoptosis. Collectively, these data indicate that a deficiency of [Ca^{2+}], in VSMCs causes an increase in apoptosis, similar to that found in Pkd mutant epithelial cells.

To determine whether the cAMP elevation contributes to apoptosis, we reexamined low-[Ca^{2+}]–induced apoptosis in the presence of cAMP inhibitor (Rp-cAMP, 100 μmol/L) or exogenous cAMP (db-cAMP, 0.5 μmol/L). As shown in Figure 5 (top left, white bars), Rp-cAMP largely rescued the low-[Ca^{2+}],...
induced apoptosis. Conversely, adding cAMP further heightened the rate of apoptosis (hatched bars). Similar results were obtained regardless of whether the reduction in [Ca\(^{2+}\)] was induced by Verapamil or BAPTA-AM. These observations indicate that cellular cAMP accumulation contributes to the increased apoptosis in low [Ca\(^{2+}\)], VSMCs.

Discussion

Heterozygous Pkd2\(^{+/−}\) VSMCs have an abnormal phenotype, manifested by an imbalance in proliferation and apoptosis, and defects in their intracellular Ca\(^{2+}\) regulation, manifested as reductions in basal [Ca\(^{2+}\)], and in the SR Ca\(^{2+}\) store. In this article, we have extended these findings by defining the relationship between basal [Ca\(^{2+}\)], and cellular cAMP concentration and by determining their roles in controlling the VSMC phenotype.

Our first set of experiments show that Pkd2\(^{+/−}\) mutant vascular smooth muscles contain a higher level of cAMP compared with that of wild-type ones from their littermates (Figure 1A). This finding is consistent with the observations in Pkd mutant renal epithelial cells, indicating a commonality in the underlying abnormality associated with Pkd mutations in both systems. Cellular cAMP is synthesized from ATP by ACs (AC1–9) and degraded to 5'-ATP by PDEs (PDE1–7). Because the activities of both can be directly or indirectly affected by diverse factors such as [Ca\(^{2+}\)], Gs/Gi signaling, and ERK signaling, the elevated cAMP content might be a downstream consequence of the [Ca\(^{2+}\)] dysregulation associated with Pkd mutations. This hypothesis was tested by determining the changes of cellular cAMP content in wild-type VSMCs after a reduction of [Ca\(^{2+}\)]. We found that a modest reduction in basal [Ca\(^{2+}\)], (to ≈70% of normal) inhibits the activity of PDEs, mainly PDE4 in this primary cultured mice VSMC system, and results in cAMP accumulation (Figure 1B and 1C).

The PDE4 family of PDEs is one of the predominant PDEs in VSMCs. They are cAMP-specific and encoded by four genes (PDE4A, PDE4B, PDE4C, and PDE4D); each gene has multiple isoforms resulting from either alternative mRNA splicing or different promoter utilization. Although their activities are not directly modulated by [Ca\(^{2+}\)], there is a complex circuitry of cross talks between ERK and cAMP that affects primarily the PDE4 activities. All four PDE4 (A through D) genes possess PQF and KIM motifs onto which ERK-2 can dock. Furthermore, many widely expressed members of PDE4s, specifically the long forms, possess a C-terminal SPS ERK-consensus motif; the Ser within this motif is specifically phosphorylated by ERK-2. This C-terminal Ser phosphorylation can profoundly inhibit PDE activities and lead to cAMP accumulation. We observed a marked increase in ERK-2 signaling in low [Ca\(^{2+}\)], VSMCs accompanying the accelerated cellular proliferation (Figure 3D). Therefore, it is conceivable that the PDE4 inhibition under this condition might be due to an ERK-2–mediated PDE4 phosphorylation. Further experiments to confirm this possible mechanism will be a focus of future study.

Cytosolic [Ca\(^{2+}\)] and cAMP are the two major intracellular second messengers and defects of either can cause abnormal cellular proliferation and/or apoptosis. Our next set of experiments was designed to address whether [Ca\(^{2+}\)] reduction leads to an appreciable change in VSMC proliferation. We show that lowering [Ca\(^{2+}\)], (≈70% of normal) by either Verapamil or BAPTA-AM induced a significant increase in VSMC proliferation demonstrated by multiple experimental methods (Figure 3). This effect was maintained even when...
the cells were treated with lower concentrations of Verapamil or BAPTA-AM (5 to 15 μmol/L, Figure 3B), suggesting that a mild to moderate disturbance in [Ca\(^{2+}\)] can have a strong effect on the VSMC phenotype. These observations are compatible with report by others showing that ketamine (an anesthetic agent that inhibits the SR Ca\(^{2+}\) release and lowers the [Ca\(^{2+}\)] causes human aortic VSMC proliferation accompanied by elevations of MAP kinase and pERK1/2.34 Contrary to these observations, others have reported that L-type Ca\(^{2+}\) channel blockers can inhibit VSMC proliferation induced by insulin or growth factors (IGF-1 or PDGF or FGF), while having no inhibitory effect when applied alone.35,36 We believe that the failure to detect a proliferative effect of Ca\(^{2+}\) channel blockers is likely due to the fact that these studies did not investigate or take into account the coexisting apoptosis. The net cell number would not be expected to change significantly if both the proliferative and apoptotic processes are simultaneously activated, as we have shown to be the case when VSMCs were treated with Verapamil. In fact, their results are similar to ours if only the net cell numbers are considered without considering the significantly different rates of apoptosis between the cells with normal and low [Ca\(^{2+}\)].

We next determined the effects of cAMP on VSMC proliferation. cAMP is known to inhibit VSMC proliferation and to promote cellular differentiation.22 Consistent with this observation, we found that cAMP inhibited proliferation in primary cultured wild-type VSMCs (Figure 4A). However, in contrast to a proliferative effect observed in low [Ca\(^{2+}\)], wild-type and Pkd mutant renal epithelial cells, exogenous cAMP inhibited proliferation of VSMCs with low [Ca\(^{2+}\)]. Furthermore, when the endogenous cAMP, induced by PDE inhibition, was blocked by Rp-cAMP, the rate of proliferation was further heightened (Figure 4B). These findings indicate that a higher level of cAMP, endogenous or exogenous, is growth inhibitory to VSMCs regardless of their [Ca\(^{2+}\)]. level. This opposite cAMP response might be explained by the different patterns of B-Raf expression in VSMCs compared with that of renal epithelial cells.

B-Raf is one of the three kinases (c-Raf-1, B-Raf, and A-Raf) that belong to a cytoplasmic serine/threonine kinase family. It has multiple isoforms, 95-kDa and 62 to 77 kDa (splice variants of 95-kDa form lacking the N-terminus).37 Only the 95-kDa isoform is known to mediate cAMP induced proliferation, in some cells through Rap-1/B-Raf signaling pathway.21 Although Rap-1 is ubiquitously expressed, B-Raf isoforms have a more cell type–restricted pattern of expression.37 Although renal epithelial cells express 95-kDa B-Raf,13,37 the B-Raf isoform expression in VSMCs has not been defined. We show that VSMCs express only the 68-kDa B-Raf; the 95-kDa isoform cannot be detected, nor be induced by decreasing [Ca\(^{2+}\)], or increasing cellular cAMP concentration (Figure 4C). This finding, compounded with the observation that the proliferative response to cAMP in the Pkd mutant or low [Ca\(^{2+}\)], wild-type renal epithelial cells is accompanied by a simultaneous rise in 95-kDa B-Raf isoform,11,13 indicates that the lack of 95-kDa B-raf in VSMCs likely accounts for the lack of proliferative response to cAMP.

We have shown that Pkd2\(^{-/-}\) VSMCs accumulate cAMP; in certain cell types, cAMP triggers apoptosis.22,38,39 Our last set of experiments examined whether the elevated apoptosis can be caused by cellular cAMP accumulation or a low [Ca\(^{2+}\)], in VSMCs. We found that cAMP triggers apoptosis in VSMCs with either normal or low [Ca\(^{2+}\)]; this apoptotic process appears mainly via a mitochondrial-mediated mechanism, demonstrated by a concordant increase in the mitochondrial-cytochrome c release and the recruitment of Bax into the mitochondrial fraction. This apoptotic effect was further confirmed by data showing that when endogenous cAMP was blocked, the rate of apoptosis was reverted to near normal range despite the fact that these VSMCs were still

**Figure 5.** Top left, FACS analyses after staining with Annexin-V to detect apoptotic cells. Lowering the basal [Ca\(^{2+}\)] increased the rate of apoptosis (black bars, n=3, \(P=0.0016\)). When endogenous cAMP was blocked by Rp-cAMP (100 μmol/L, white bars), the rate of apoptosis was decreased (n=3, \(P=0.001\)). Conversely, adding db-cAMP (0.5 μmol/L, lined bars) enhanced the apoptosis rate (n=4, \(P=0.043\)). Bottom left, Representative results from FACS analyses; the right top and bottom quadrants were Annexin-V positive cells. Nontreated VSMCs at 12 hours (A), Verapamil treated VSMCs at 12 hours (B), Verapamil treated VSMCs at 24 hours (C). Right, Quantitative Western analyses of the proteins isolated from VSMCs showed a time-dependent increase in Bax (mitochondrial fraction), cytochrome c (cytosolic protein), cleaved forms of caspase 3 and 9 (PNS) with Verapamil treatment. Nearly identical results were obtained with BAPTA-AM.
maintained in low [Ca\textsuperscript{2+}]i condition (Figure 5, top left). These results indicate that the elevated cellular cAMP, not the low [Ca\textsuperscript{2+}]i, is the main determinant of an accelerated apoptosis. Our findings are in accord with a recent study showing that the reduction in cellular cAMP by upregulating PDE4B in diffuse large B-cell lymphomas causes resistance to chemotherapy-induced apoptosis. This resistance is mediated by a low cAMP concentration, which leads to an increase in PI3 kinase activity and its downstream AKT signaling.\textsuperscript{40} Our chemotherapy-induced apoptosis. This resistance is mediated by the reduction in cellular cAMP by upregulating PDE4B in VSMCs. This study was supported by NIH DK63064 (Q.Q.), NIH GM56686 (G.C.S.), PKD Foundation 41A2R (Q.Q.), NIH DK44863 (V.E.T.), and Mayo Clinic School of Medicine (Q.Q.).

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Legends for Figure S1 and S2

Figure S1

(a) The global basal \([\text{Ca}^2+]_i\) in the primary cultured VSMCs was determined using a \(\text{Ca}^2+\) indicator Fura-2 AM. Verapamil (V, 1\(\mu\)M) and BAPTA-AM (B, 1 or 2\(\mu\)M) treatment for 12 or 24 hrs reduced basal \([\text{Ca}^2+]_i\) by \(\sim\)10-25\% (\(n \geq 60\), error bars indicate ± SE, \(p < 0.001\) for non-treated vs. V or B treated VSMCs).

(b) The combination of 1\(\mu\)M Verapamil (V) and db-cAMP (db) at concentration of 0.002, 0.02, or 0.20\(\mu\)M resulted in an inhibition in the % of S phase VSMCs after 12 or 24 hrs of treatment (\(n = 2\), \(p = 0.01\) for non-treated vs. V treated VSMCs). Similar results were obtained with 1\(\mu\)M BAPTA-AM, \(p < 0.01\) for non-treated vs. B treated VSMCs. No significant difference was detected in between 1\(\mu\)M V and 1\(\mu\)M B (\(n = 2\), \(p = 0.89\)) or 1 \(\mu\)M B and 2 \(\mu\)M B treated groups (\(n = 2\), \(p = 0.90\)).

Figure S2

(Upper panel): DAPI nuclear staining to detect nuclear condensations and fragmentations of apoptotic VSMCs. Reduction in basal \([\text{Ca}^2+]_i\) (to \(\sim\)70\% of normal) by either Verapamil or BAPTA-AM significantly increased the rate of VSMC apoptosis (\(n \geq 4\) separate parallel experiments, each in triplicate: 250 cells were counted/sample. \(p < 0.001\) for non-treated vs. Verapamil or BAPTA-AM treated VSMCs).

(Lower panel): Representative pictures of DAPI nuclear staining of the Verapamil treatment for (A) 6 hrs and (B) 12 hrs. Arrows show the condensed or fragmented nuclei. Bar = 10 \(\mu\)m.
Figure S1

A. [Ca^{2+}]_i, nM

- Treated/Non-Treated

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<th>24 hrs</th>
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<tr>
<td>V 1μM</td>
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B. % S Phase

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Figure S2
Legends for Figure S1 and S2

Figure S1

(a) The global basal [Ca^{2+}]_i in the primary cultured VSMCs was determined using a Ca^{2+} indicator Fura-2 AM. Verapamil (V, 1µM) and BAPTA-AM (B, 1 or 2µM) treatment for 12 or 24 hrs reduced basal [Ca^{2+}]_i by ∼10-25% (n ≥ 60, error bars indicate ± SE, p < 0.001 for non-treated vs. V or B treated VSMCs).

(b) The combination of 1µM Verapamil (V) and db-cAMP (db) at concentration of 0.002, 0.02, or 0.20µM resulted in an inhibition in the % of S phase VSMCs after 12 or 24 hrs of treatment (n = 2, p = 0.01 for non-treated vs. V treated VSMCs). Similar results were obtained with 1µM BAPTA-AM, p < 0.01 for non-treated vs. B treated VSMCs. No significant difference was detected in between 1µM V and 1µM B (n = 2, p = 0.89) or 1 µM B and 2 µM B treated groups (n = 2, p = 0.90).

Figure S2

(Upper panel): DAPI nuclear staining to detect nuclear condensations and fragmentations of apoptotic VSMCs. Reduction in basal [Ca^{2+}]_i (to ∼70% of normal) by either Verapamil or BAPTA-AM significantly increased the rate of VSMC apoptosis (n ≥ 4 separate parallel experiments, each in triplicate: 250 cells were counted/sample. p < 0.001 for non-treated vs. Verapamil or BAPTA-AM treated VSMCs).

(Lower panel): Representative pictures of DAPI nuclear staining of the Verapamil treatment for (A) 6 hrs and (B) 12 hrs. Arrows show the condensed or fragmented nuclei. Bar = 10 µm.
Figure S1

A) Bar graph showing [Ca^{2+}]_{i} in nM for treated versus non-treated conditions at 12 hours and 24 hours. Legend indicates treatments with B 1µM, B 2µM, and V 1µM.

B) Bar graph showing % S Phase at 12 hours and 24 hours. Treatments include V1 µM and V1 db 0.02, db 0.02, db 0.2.