PKC-dependent superoxide production by the renal medullary thick ascending limb from diabetic rats

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Yang J, Lane PH, Pollock JS, Carmines PK. PKC-dependent superoxide production by the renal medullary thick ascending limb from diabetic rats. Am J Physiol Renal Physiol 297: F1220–F1228, 2009. First published September 9, 2009; doi:10.1152/ajprenal.00314.2009. —Type 1 diabetes (T1D) is a state of oxidative stress accompanied by PKC activation in many tissues. The primary site of O$_2^-$ production by the normal rat kidney is the medullary thick ascending limb (mTAL). We hypothesized that T1D increases O$_2^-$ production by the mTAL through a PKC-dependent mechanism involving increased expression and translocation of one or more PKC isoforms. mTAL suspensions were prepared from rats with streptozotocin-induced T1D (STZ mTALs) and from normal or sham rats (normal/sham mTALs). O$_2^-$ production by STZ mTALs was fivefold higher than normal/sham mTALs (P < 0.05). PMA (30 min) mimicked the effect of T1D on O$_2^-$ production. Exposure to calphostin C or chelerythrine (PKC inhibitors), Gö6976 (PKCa/β inhibitor), or rottlerin (PKCb inhibitor) decreased O$_2^-$ production to 20% of untreated baseline in both normal/sham and STZ mTALs. PKCb inhibitors had no effect. PKC activity was increased in STZ mTALs (P < 0.05 vs. normal/sham mTALs) and was unaltered by antioxidant exposure (tempol). PKCa protein levels were increased by 70% in STZ mTALs, with a ~30% increase in the fraction associated with the membrane (both P < 0.05 vs. sham). PKCb protein levels were elevated by 20% in STZ mTALs (P < 0.05 vs. sham) with no change in the membrane-bound fraction. Neither PKCb protein levels nor its membrane-bound fraction differed between groups. Thus STZ mTALs display PKC activation, upregulation of PKCa and PKCb protein levels, increased PKCa translocation to the membrane, and accelerated O$_2^-$ production that is eradicated by inhibition of PKCa or PKCb (but not PKCb). We conclude that increased PKCa expression and activity are primarily responsible for PKC-dependent O$_2^-$ production by the mTAL during T1D.

diabetes mellitus; rottlerin; calphostin C; chelerythrine; Gö6976

DIABETIC RENAL DISEASE is generally described as a glomerulopathy associated with diffuse or nodular glomerulosclerosis; however, in recent years it has become apparent that renal tubular structural and functional alterations arise early after the onset of type 1 diabetes (T1D). The earliest change involves epithelial cell hypertrophy, which is evident within days after onset of hyperglycemia, and the situation evolves progressively to include epithelial-mesenchymal transition and tubulointerstitial fibrosis (10, 55). Although reactive oxygen species generation is envisioned to represent a key step leading to tubulointerstitial fibrosis (42), little is known about the mechanisms underlying renal tubular production of oxygen radicals during T1D. In the normal kidney, the medullary thick ascending limb (mTAL) is the predominant site of renal superoxide anion (O$_2^-$) production (28, 61). Evidence suggests that disturbances in TAL function precede microalbuminuria and overt nephropathy in patients with T1D (41, 60); however, the effect of T1D on O$_2^-$ production by the mTAL is not established.

T1D increases de novo synthesis of diacylglycerol (DAG) in renal glomeruli and vascular cells, subsequently activating constitutive and novel PKC isoforms (3, 4, 18). The function of each individual PKC isoform is determined largely by the subcellular localization of isoform-specific anchoring proteins (receptors for activated C kinase; RACKs) that enable phosphorylation of the appropriate substrate(s) (34, 47). In rats with streptozotocin-induced T1D (STZ rats), different tissues have differential expressions of PKC isoforms (19). Glomeruli of diabetic rats generally display activation of PKCa, PKCb, PKCd, and/or PKCe (11, 25), while tubules from the renal cortex show activated PKCd and PKCe (11). The rat mTAL expresses Ca$^{2+}$-dependent (α, β1) and Ca$^{2+}$-independent (δ, ε, ζ) isoforms of PKC (1). In vascular cells and renal mesangial cells, hyperglycemia during T1D stimulates O$_2^-$ production via a PKC-dependent mechanism (16, 59). However, no information is available regarding T1D-induced changes in PKC activity in the mTAL and the influence of these changes on O$_2^-$ production by this nephron segment. We hypothesized that T1D stimulates O$_2^-$ production by the rat mTAL through a PKC-dependent mechanism involving increased translocation and/or expression of one or more PKC isoforms.

MATERIALS AND METHODS

Chemicals and reagents. The PKC activator PMA and the PKC inhibitors calphostin C, Gö6976, rottlerin, and 3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione (indolylmaleimide) were purchased from Calbiochem/EMD (Madison, WI). The PKCa inhibitor LY379196 (an analog of ruboxistaurin) was a generous gift from Lilly Research Laboratories (Indianapolis, IN). Monoclonal antibodies against PKCa, PKCb, and PKCd were purchased from BD/Transduction Laboratories (San Jose, CA). Odyssey Blocking Buffer and IRDye infrared-labeled secondary antibodies were purchased from LI-COR (Lincoln, NE). Polyclonal antibodies against the Na$^+$-$\text{K}^+$-2Cl$^-$ cotransporter (NKCC2) and heat shock protein 70 (HSP70) were purchased from Millipore/Chemicon/Upstate (Billerica, MA), and anti-β-actin was from Abcam (Cambridge, MA). Linplant sustained-release insulin pellets and microcrystalized palmitic acid vehicle pellets were purchased from Linshin Canada (Scarborough, ON). Anti-aromucoid (Tamm-Horsfall protein) was from ICN Biocentials (Costa Mesa, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Induction of type 1 diabetes. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Nebraska Medical Center and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats weighing ~300 g (Harlan, Indianapolis, IN) were anesthetized with methohexitonal sodium (50
mg/kg ip) to facilitate intravenous injection of 65 mg/kg streptozotocin (STZ rats) or vehicle (ice-cold PBS; pH 4; sham rats). The animals were allowed to recover from anesthesia and returned to the animal facility, where they had free access to food and water. The following day, the rats were anesthetized again for subcutaneous insertion of a 2.3 × 2.0-mm sustained-release insulin pellet (Linplant; STZ rats) or vehicle pellet (sham rats) via a 16-gauge needle to moderate the extent of the hyperglycemia. After recovery from anesthesia, the rats were provided ad libitum food and water for the ensuing 3–4 wk while housed in a temperature-controlled room with a 12:12-h light:dark cycle. During this period, STZ rats gained less weight (28 ± 2 g) than sham rats (77 ± 4 g). Blood glucose levels were measured with an Accu-Check III model 766 glucose meter (Boehringer Mannheim, Indianapolis, IN) before STZ or vehicle injection (averaging 92 ± 2 mg/dl) and twice weekly thereafter, during which values averaged 370 ± 9 mg/dl in STZ rats (n = 58) and 94 ± 1 mg/dl in sham rats (n = 34). Age-matched normal rats (n = 25) not subjected to STZ or vehicle treatment were used in some aspects of the study.

**Preparation of mTAL suspensions.** Fresh mTAL suspensions were prepared from the inner stripe of the outer medulla according to the method of Garvin and colleagues (48, 57), with slight modification. Briefly, under pentobarbital sodium anesthesia (50 mg/kg ip), kidneys were perfused via the abdominal aorta with 20 ml of HEPES-buffered Hanks’ balanced salt solution (HBSS) containing 233 U/ml collagenase. The HBSS consisted of (in mM) 138 NaCl, 5.3 KCl, 4.2 NaHCO3, 0.34 Na2HPO4, 0.4 MgSO4, 0.44 KH2PO4, 0.5 MgCl2·6H2O, 1.3 CaCl2·2H2O, 10 HEPES, and either 5.5 or 20 d-glucose (for normal/sham or STZ rats, respectively, to maintain the chronic in vivo condition of various pharmacological agents. At the end of this treatment period, until initiating a 30-min incubation at 37°C in the absence or presence of various pharmacological agents. At the end of this treatment period, the sample was placed in the chamber of a Berthold Sirius tubule suspension system together with 100 ml of perfusate. The composition of the perfusate was identical (20 mM MOPS, 50 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA, 1% NP-40, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and allowed to stand for 10 min on ice. After 15-min centrifugation, the supernatant was assayed for PKC activity according to the manufacturer’s instructions, with absorbance measured at 450 nm. Kinase activity (optical density/μg protein) was quantified by the change in absorbance over the background levels.

**Fractionation of mTALs into membrane- and cytosol-enriched components.** For assessing PKC isoform translocation, fresh mTALs were separated into cytosol-enriched and membrane-enriched fractions using a Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Pierce, Rockford, IL). This procedure differs from ultracentrifugation methods for separating soluble and particulate fractions (generally considered to represent cytosol and membrane), as it utilizes a mild detergent-based protocol and subsequent separation of the hydrophilic and hydrophobic proteins through phase partitioning. Cytosolic proteins are contained in the hydrophilic fraction, while the hydrophobic fraction contains integral and attached membrane proteins. Detergent was removed from the samples using a Pierce SDS-PAGE Sample Prep Kit before Western blot analysis was performed (see below). The manufacturer states that cross-contamination of cytosolic protein into the hydrophobic (membrane-enriched) fraction is typically <10%, due to the difficulty in obtaining complete sample separation at the interface between the two phases. We confirmed by Western blotting that NKCC2 was present primarily in the membrane-enriched fraction and HSP70 in the cytosol-enriched fraction prepared from mTALs (Fig. 2).

**Preparation of mTAL homogenates for western blotting.** Fresh mTALs from normal/sham and STZ rats were suspended in 200 μl lysis buffer (Celllytic MT Mammalian Tissue Lysis/Extraction Reagent) containing Protease Inhibitor Cocktail P8340 (1:10), sonicated on ice for two cycles of 20 s each at output 2 using a VC130...
Western blot analysis of PKC protein levels.

Fig. 2. Representative Western blots showing localization of Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter (NKCC2) and heat shock protein 70 (HSP70) in membrane-enriched (M) and cytosol-enriched (C) fractions, respectively, of mTAL suspensions prepared from normal rat kidneys.

Ultrasonic Processor (Sonics and Materials, Newtown, CT), and centrifuged at 13,000 g for 15 min. The soluble fraction was considered to be the mTAL homogenate, which was stored at -80°C until Western blot analysis of PKC protein levels.

Western blotting. The total protein level in each sample was measured using a Pierce BCA Protein Assay Kit before loading of mTAL homogenates (25–40 μg) or cytosol-enriched and membrane-enriched fractions (30–40 μg) into SDS-polyacrylamide gels (4–15%, Bio-Rad Laboratories, Hercules, CA). Proteins were separated by electrophoresis and transferred to a Millipore Immobilon-P polyvinylidene fluoride membrane at 300 mA for 90 min. The membrane was blocked for 60 min at room temperature, followed by overnight incubation at 4°C in blocking buffer containing PKCε-, PKCy-, or PKCδ-specific monoclonal antibodies (1:1,000) in the presence or absence of antibody against β-actin. The membrane was washed in PBS containing 0.1% Tween 20 and then incubated in a 1:5,000 dilution of secondary antibody against the appropriate IgG conjugated to infrared dyes. The reaction products and signals were detected with a LI-COR Odyssey Imager. For quantification of PKC isoform protein levels, PKC band intensities were normalized by the β-actin band intensities in each sample and expressed as the percentage of averaged sham values run on the same gel. In the PKC isoform translocation assay, PKC band intensities were normalized by protein amount loaded in each lane, and the proportion of membrane-bound PKC for each isoform was calculated relative to the total amount detected (membrane + cytosol).

Live/dead assay. The potential cytotoxic impact of the various PKC inhibitors used in this study was assessed using a Molecular Probes LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, Carlsbad, CA). Briefly, fresh mTAL suspensions prepared from normal rat kidney were incubated for 30 min in the absence/presence of PKC inhibitors or were exposed to 70% methanol (to kill the majority of cells). Aliquots (100 μl) of mTAL suspension were loaded into a 96-well plate in triplicate, and calcein-AM (2 μM) was added to each well. Calcein-A/M is a nonfluorescent fluorescein derivative that passively crosses the plasma membrane of viable cells and is converted by cytosolic esterases into green fluorescent calcein, which is retained by cells with intact membranes and inactive multidrug resistance protein. Hence, calcein fluorescence in this assay is an indicator of cell viability. After 30-min exposure to calcein-AM, fluorescence emission was determined using an automated plate reader at the manufacturer’s suggested settings. Results are expressed as the percentage of viable cells compared with untreated samples.

Statistical analysis. All data are reported as means ± SE, with n values representing the number of rats. Statistical comparisons utilized the unpaired t-test or ANOVA with post hoc comparisons made using the Student-Newman-Keuls method. If the data were not normally distributed, the Kruskal-Wallis ANOVA on ranks was employed, with post hoc comparisons utilizing the Holm-Sidak method. P values < 0.05 were considered significant.

RESULTS

Effects of T1D, PKC activation, and PKC inhibition on O2•\(^{-}\) production by mTAL suspensions. Lucigenin chemiluminescence was used to measure O2•\(^{-}\) production by freshly prepared mTAL suspensions from normal/sham and STZ rats, studied in the presence of 5.5 or 20 mM glucose, respectively. In preliminary experiments, 10 mM tempol reduced lucigenin chemiluminescence in mTAL suspensions from both sham rats (93 ± 2%; n = 4) and STZ rats (84 ± 4%; n = 4), indicating that lucigenin chemiluminescence values obtained under these conditions mainly reflect O2•\(^{-}\) production. Preliminary experiments revealed that acute (30 min) exposure of normal mTALs to 20 mM glucose HBSS caused a doubling of O2•\(^{-}\) production (from 388 ± 68 to 987 ± 196 RLU·s\(^{-}\)·mg protein\(^{-}\)1; n = 8; P < 0.05). As illustrated in Fig. 3, O2•\(^{-}\) production by untreated mTALs from normal/sham rats averaged 426 ± 33 RLU·s\(^{-}\)·mg protein\(^{-}\)1 (n = 18) and was accelerated fivefold in untreated suspensions from STZ rats (2,432 ± 388 RLU·s\(^{-}\)·mg protein\(^{-}\)1; n = 18; P < 0.05 vs. normal/sham mTALs). O2•\(^{-}\) production by STZ mTALs studied after 30-min exposure to a normoglycemic environment (5.5 mM glucose) averaged 2,945 ± 855 RLU·s\(^{-}\)·mg protein\(^{-}\)1 (n = 8), a value not significantly different from that evident in 20 mM glucose HBSS, thereby ruling out an osmotic effect of varying media glucose concentration in these experiments. Figure 3 also shows the impact of PKC activation and inhibition on O2•\(^{-}\) production by mTAL suspensions. Preincubation in 1 μM calphostin C, a broad-spectrum PKC inhibitor with an IC\(_{50}\) of 0.05 μM (20), decreased O2•\(^{-}\) production by 90% in both groups such that calphostin C-sensitive O2•\(^{-}\) production by 95% (1; H11002) of mTAL suspension were loaded into a 96-well plate in triplicate, and calcine-AM (2 μM) was added to each well. Calcein-AM is a nonfluorescent fluorescein derivative that passively crosses the plasma membrane of viable cells and is converted by cytosolic esterases into green fluorescent calcein, which is retained by cells with intact membranes and inactive multidrug resistance protein. Hence, calcein fluorescence in this assay is an indicator of cell viability. After 30-min exposure to calcein-AM, fluorescence emission was determined using an automated plate reader at the manufacturer’s suggested settings. Results are expressed as the percentage of viable cells compared with untreated samples.

Fig. 3. PKC-dependent O2•\(^{-}\) production by mTAL suspensions. mTAL suspensions from normal (or sham) and streptozotocin-diabetic (STZ) rats were studied in the presence of 5.5 and 20 mM glucose, respectively, to maintain in vivo conditions. Also shown are the effects of 30-min pretreatment with PKC inhibitors (1 μM calphostin C or 30 μM chelerythrine) and a PKC activator (1 μM PMA). *P < 0.05 vs. normal/sham. †P < 0.05 vs. untreated.
that activation of conventional/novel PKC isoforms is capable of accelerating O$_2^•$ production by mTAL suspensions. Overall, these data indicate that T1D stimulates mTAL O$_2^•$ production and that PKC activity is a key determinant of O$_2^•$ production by mTALs from both normal and STZ rats.

**Effects of T1D on PKC activity in rat mTAL.** As shown in Fig. 4, total PKC activity in mTALs from STZ rats was ~50% greater than that evident in mTALs from normal rats ($P < 0.05$; $n = 14$/group). Pretreatment with 1 μM calphostin C reduced PKC activity measured by this assay to virtually identical levels in both groups, with calphostin C-sensitive PKC activity in STZ mTALs significantly exceeding that evident in normal mTALs ($P < 0.05$; $n = 6$/group). Tempol exposure (10 mM; 30 min) did not significantly alter PKC activity in mTALs from sham or STZ rats ($n = 8$/group). These data reveal that PKC activity is increased in the rat mTAL during T1D and that this phenomenon is not dependent on the increased O$_2^•$ levels evident in mTALs from STZ rats.

**Effect of T1D on PKC isoform protein levels in rat mTAL.** Western blotting was used to assess total protein levels of PKC isoforms in mTALs from sham rats ($n = 7$–9) and STZ rats ($n = 7$–12). Figure 5 shows representative blots for PKCα, PKCβ, and PKCδ, as well as a summary of the densitometric data. In mTALs from STZ rats, PKCα and PKCβ protein levels were significantly increased to values averaging 170 ± 19 and 129 ± 9% of sham, respectively ($P < 0.05$); however, PKCδ protein levels did not differ between sham and STZ mTALs. Thus T1D triggers upregulation of PKCα and PKCβ protein expression in the rat mTAL without an effect on PKCδ expression.

**Effect of T1D on PKC isoform translocation in rat mTAL.** Western blot analysis was used to assess PKC isoform levels in membrane-enriched (hydrophobic) and cytosol-enriched (hydrophilic) fractions prepared from sham and STZ rats, while lesser proportions of PKCα (14 ± 1% of total) and PKCβ (15 ± 1% of total) were detected in the membrane fraction of these same rats, suggesting that PKCδ activity in the normal mTAL may be greater than that of the conventional isoforms. T1D had no effect on the proportion of PKCβ or PKCδ detected in the membrane fraction; however, there was a ~30% increase in the proportion of PKCα detected in the membrane-enriched fraction of mTALs from STZ rats compared with sham ($P < 0.05$). These data indicate that T1D stimulates PKCα translocation from cytosol to membrane, without effect on translocation of PKCβ or PKCδ.

**Effects of isoform-specific PKC inhibition on O$_2^•$ production by mTAL suspensions.** To investigate the functional role of PKC isoforms in the accelerated O$_2^•$ production evident in the mTAL during T1D, O$_2^•$ production was assessed by lucigenin chemiluminescence in the absence/presence of PKC inhibitors having relative isoform specificity (43, 45). At a concentration of 1 μM, Gö6976 abolishes enzymatic activities of recombinant PKCα and PKCβ (IC$_{50}$ = 1.3–6 nM), while having no effect on activities of the PKCδ, PKCε, or PKCζ (31). As shown in Fig. 7, 30-min incubation in 1 μM Gö6976 significantly decreased O$_2^•$ production by mTALs from normal and
STZ rats to 25 ± 8 and 14 ± 4% of untreated levels, respectively (both n = 6). At a concentration of 10 μM, rottlerin substantially reduces PKCβ activity (IC50 = 3.6 μM), while at least a 10-fold higher concentration is required to influence other PKC isozymes (7). In our experiments, 30-min incubation in 10 μM rottlerin decreased O2•− production to <5% of untreated levels in mTALs from normal and STZ rats (both n = 6; Fig. 7). Exposure to 30 nM LY379196 has been widely employed to reveal the role of PKCβ in signaling events (IC50 = 30–50 nM) (29, 58). However, in contrast to the effects of Gö6976 and rottlerin, LY379196 did not alter O2•− production by mTALs from either sham or STZ rats (both n = 4; Fig. 7). As 30 nM LY379196 had no effect in our studies, and because higher concentrations of this agent yield nonspecific effects (29), we performed follow-up experiments using a commercially available compound (50 nM indolylmaleimide1) that also selectively inhibits PKCβ (IC50 = 5–21 nM) (54). These experiments were performed more than 1 yr after the LY379196 investigation and utilized a new luminometer (same model) and freshly purchased lucigenin. These conditions yielded higher values for O2•− production than evident in the earlier experiments, averaging 2,336 ± 691 RLU·s−1·mg protein−1 in sham mTALs (n = 6); however, similar to the earlier experiments, a fivefold acceleration of O2•− production was evident in STZ mTALs (11,243 ± 2,915 RLU·s−1·mg protein−1; n = 6). The commercially available PKCβ inhibitor did not significantly alter mTAL O2•− production by either group (−38 ± 18% in sham, n = 6, P > 0.07 vs. untreated; −36 ± 16% in STZ, n = 7, P > 0.10 vs. untreated). Overall, these pharmacological data suggest that PKCα and PKCβ contribute to O2•− production by the mTAL in normal/sham and STZ rats, with PKCβ having no apparent involvement in this process.

Effects of PKC inhibition on mTAL viability. Because of the striking effects of some of the PKC inhibitors on O2•− production by mTAL suspensions, we determined the impact of these agents on cell viability. Treatment of mTALs from normal rats (n = 5) with 70% methanol reduced cell viability to 2.2 ± 0.4% of untreated samples; however, ANOVA did not indicate any significant impact of calphostin C, Gö6976, rottlerin, chelerythrine, or indolylmaleimide1 on cell viability. After 30-min exposure to these agents at the concentrations employed in the O2•− production experiments, mTAL viability exceeded 80% of untreated values (Table 1). Thus cytotoxic effects of the PKC inhibitors used in this study cannot explain the marked effects of most of these agents on O2•− production by mTAL suspensions.

**DISCUSSION**

Systemic oxidative stress is evident soon after onset of T1D in humans and is increased by early adulthood (5). The mTAL represents the primary source of O2•− in the normal kidney (28, 61), and acute exposure of rat mTALs to 25 mM glucose

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viability, % of untreated</th>
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<tr>
<td>Untreated</td>
<td>100.0 ± 4.6%</td>
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<tr>
<td>Calphostin C (1 μM)</td>
<td>94.8 ± 2.5%</td>
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<tr>
<td>Chelerythrine (30 μM)</td>
<td>83.9 ± 2.2%</td>
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<tr>
<td>Indolylmaleimide1 (50 nM)</td>
<td>91.2 ± 5.8%</td>
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<tr>
<td>Gö6976 (1 μM)</td>
<td>93.6 ± 3.7%</td>
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<tr>
<td>Rottlerin (10 μM)</td>
<td>100.2 ± 5.1%</td>
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<tr>
<td>Methanol (70%)</td>
<td>2.3 ± 0.4%*</td>
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Values are means ± SE; n = 5 experiments measured after 30-min exposure to the various treatments. mTAL, medullary thick ascending limb of the loop of Henle. Methanol treatment was employed to kill the majority of cells. *P < 0.05 vs. untreated.
has been reported to rapidly provoke a doubling of intracellular $O_2^{•−}$ concentration (35). Our results confirmed that $O_2^{•−}$ production by normal mTALs was more than doubled after 30-min exposure to 20 mM glucose, indicating that extracellular glucose levels in the range of those found in T1D increase $O_2^{•−}$ production by the mTAL. However, subsequent mechanisms likely also contribute to the fivefold acceleration in mTAL $O_2^{•−}$ production evident 3 wk after onset of T1D, as we found that this phenomenon was not reversed by acute exposure of mTALs from STZ rats to HBSS containing 5.5 mM glucose. Accordingly, rather than focusing on the acute glucose-induced response, our investigation targeted the mechanisms that are sustained 3 wk after onset of T1D.

Our data showed that calphostin C-sensitive PKC activity was increased in mTALs from STZ rats, consistent with reports that T1D stimulates PKC activity in multiple tissues (18, 39, 46). We also found that pretreatment with calphostin C or chelerythrine markedly reduced $O_2^{•−}$ production by mTALs from STZ rats, suggesting that PKC activity contributes to the accelerated $O_2^{•−}$ production by this nephron segment during early T1D. This observation extends to the mTAL the results of previous reports implicating PKC as a mediator of high glucose- and T1D-induced oxidative stress in multiple tissues (2, 13, 16, 17, 22, 30). Conversely, in several renal cell types, T1D- and high glucose-induced PKC activation is blocked by antioxidants (9, 11, 15, 26, 53), suggesting that reactive oxygen species can activate PKC. These reports raise the possibility of a positive-feedback system whereby hyperglycemia during T1D activates PKC, which triggers $O_2^{•−}$ production that, in turn, amplifies PKC signaling to exert changes in mTAL function (6, 27). However, we were unable to detect any impact of antioxidant exposure (tempol) on PKC activity in mTALs from sham or STZ rats, indicating that PKC-dependent $O_2^{•−}$ production by the mTAL during T1D is either of insufficient magnitude to provoke positive-feedback activation of PKC or occurs at a specific intracellular (or extracellular) site that does not engender PKC activation. This situation contrasts with recent evidence that $O_2^{•−}$ mediates PKC activation in the mTAL during angiotensin II-dependent hypertension (49) and underscores the complexity of mechanisms evident in the mTAL during different pathophysiological states associated with oxidative stress.

The inhibitory effects of calphostin C and chelerythrine on the normal mTAL implicate tonic PKC activation as a contributor to constitutive $O_2^{•−}$ production that likely plays a role in signal transduction, cell growth, and other processes in normal cells. In the normal rat kidney, NADH oxidase and mitochondrial respiratory chain enzymes represent the main sources of $O_2^{•−}$ in the outer medulla (61), with NAD(P)H oxidase representing the primary source in the cortical thick ascending limb (28). Presumably, the PKC-dependent $O_2^{•−}$ production evident in normal mTALs in the present study originated from these sources, and, indeed, evidence suggests that PKC is an important mediator of NAD(P)H oxidase activation in vascular smooth muscle (8, 44). The potential role of NAD(P)H oxidase in $O_2^{•−}$ production by the mTAL in normal and diabetic rats is an area of ongoing investigation in our laboratory.

In a variety of cell types, hyperglycemic conditions increase de novo DAG synthesis, subsequently activating conventional and/or novel isoforms of PKC (3, 15, 18, 46). We found that acute exposure to PMA (a DAG analog) provoked a marked increase in $O_2^{•−}$ production, thereby establishing that activation of one or more conventional or novel PKC isoform(s) is sufficient to increase $O_2^{•−}$ production by the mTAL. Accordingly, our attempts to identify the PKC isoforms involved in T1D-induced PKC activation focused on the conventional and novel PKC isoforms for which pharmacological inhibitors are readily available (PKCo, PKCβ, and PKCδ).

To detect activation of specific PKC isoforms in the mTAL, we relied on evidence of translocation from the cytosol to the membrane. Previous studies have assessed the distribution of PKC isoforms between soluble and particulate fractions of the rat mTAL (1, 49, 50, 56); however, we were unable to obtain by ultracentrifugation methods soluble and particulate mTAL fractions that reliably displayed an appropriate distribution of membrane and cytosolic proteins. Therefore, we utilized a phase-partitioning method that produced hydrophobic and hydrophilic fractions. Because of the small contamination of cytosolic protein in the membrane-enriched fraction (see MATERIALS AND METHODS), our data may underestimate the extent of PKC translocation to the membrane. Nevertheless, our detection of a significantly greater proportion of PKCo in the membrane-enriched fraction of mTALs from STZ rats, compared with sham rats, can be taken as evidence of translocation and probable activation of this isoform during diabetes. This observation extends to the mTAL the results of previous studies indicating that the diabetic milieu provokes PKCo activation/translocation in a variety of tissues and cells (i.e., glomeruli, aorta, mesangial cells) (59). Taken together with our observation of increased total PKCo protein levels in STZ mTALs, this constitutes strong evidence for increased PKCo activity in the mTAL during T1D.

We found no indication of a diabetes-induced change in the proportion of PKCβ or PKCδ present in the membrane-enriched fraction. This observation contrasts with reports that both PKCo and PKCδ are translocated to the membrane (including the nuclear membrane) of glomeruli during diabetes and in mesangial cells exposed to high glucose for at least 24 h (59). These translocation events depend on the localization of isoform-specific RACKs, which may vary between cell types (i.e., mesangial cells vs. mTAL). Alternatively, diabetes-induced activation of PKCβ and PKCδ in the mTAL may be associated with translocation to nonhydrophobic intracellular sites such as mitochondria, the nucleus, or the cytoskeleton (23, 33, 40), where they might phosphorylate substrates that contribute to $O_2^{•−}$ production. Our observations leave open the possibility of a T1D-induced increase in PKCβ activity in the absence of a change in the proportion of PKCβ evident in the membrane-enriched fraction, as the moderate increase in total PKCβ protein level detected in STZ mTALs should produce a modest increase in the absolute amount of PKCβ translocated to the membrane. In contrast, neither the proportion of PKCδ located in the membrane-enriched fraction nor the total amount of this isoform present in the cell differed between sham and STZ mTALs, thus making it unlikely that PKCδ activation contributes to the measured increase in PKC activity in the mTAL during T1D.

To gain further insight into the PKC isoform(s) that contributes to $O_2^{•−}$ production in mTALs from STZ rats, we exposed mTALs to PKC inhibitors with relative isoform specificity. These experiments revealed that PKCo/β and PKCδ inhibitors (Gö6976 and rottlerin, respectively) decreased $O_2^{•−}$ produc-
tion by mTALs from STZ rats, while neither PKCβ inhibitor (LY379196 or indolylmaleimide) had any effect. Because combined inhibition of PKCa and PKCβ prevented the diabetes-induced acceleration of $O_2^{-•}$ production by mTALs while inhibition of PKCβ alone had no effect, these observations suggest that increased PKCa activity contributes to accelerated $O_2^{-•}$ production by the mTAL during T1D. A role for PKCa in control of mTAL transport function has been implicated in several previous studies (49, 50, 56); however, to our knowledge, this is the first report that activation of PKCa is largely responsible for accelerated $O_2^{-•}$ production by this nephron segment during T1D. In contrast, even though PKCβ has been implicated in several renal complications of T1D (21, 24, 38), the functional impact of increased PKCβ levels/activity in the mTAL during T1D does not seem to include a role in increased $O_2^{-•}$ production.

The potential role of PKCδ in mTAL $O_2^{-•}$ production was investigated using rottlerin (7), the only commercially available PKCδ inhibitor. Although this agent has been reported to have toxic effects on rat mTALs in vitro (50), rottlerin did not alter mTAL viability in our experimental setting (Table 1). Our assay of PKCδ protein levels and translocation provided no evidence that T1D activates this isoform in the mTAL; however, a relatively high percentage of PKCδ protein was detected in the membrane fraction from both sham and STZ rats, suggesting significant constitutive activity. This constitutive activity may be critical for the mTAL to generate $O_2^{-•}$ under both normal and diabetic conditions, as evidenced by the marked inhibition of $O_2^{-•}$ production after rottlerin treatment. PKCδ translocation into the mitochondria promotes $O_2^{-•}$ generation (23), which could underlie the inhibitory effect of rottlerin on $O_2^{-•}$ production in our studies. However, there exists a growing list of PKCδ-independent actions of rottlerin (52), several of which could potentially contribute to the influence of rottlerin on $O_2^{-•}$ production. For example, rottlerin can act as a mitochondrial uncoupling agent (51), which represents a PKCδ-independent means through which this drug could reduce $O_2^{-•}$ production (akin to the ability of uncoupling protein 1 overexpression to abolish high glucose-induced ROS production by endothelial cells) (37). Rottlerin has also been reported to inhibit $O_2^{-•}$ production by NAD(P)H oxidase through a mechanism that does not involve direct blockade of PKCδ activity (52). Because of these uncertainties, as well as the lack of evidence for increased PKCδ activation in the mTAL during T1D, and despite the striking impact of rottlerin on $O_2^{-•}$ production by the mTAL from normal and diabetic rats, the available data fail to provide unequivocal support for a role of PKCδ in this phenomenon.

Despite our skepticism regarding a role for PKCδ in the accelerated $O_2^{-•}$ production by the mTAL during T1D, we cannot completely discard the possibility that both PKCa and PKCδ are involved in this phenomenon. It is possible that these isoforms are sequentially activated or function in alternative pathways to influence $O_2^{-•}$ production, similar to the involvement of multiple PKC isoforms in control of α1(T) collagen production by mesangial cells under high-glucose conditions (14). However, studies suggesting a functional interaction between PKCa and PKCδ generally indicate an inverse relationship between these isoforms (32, 36). Further study is required to determine how these PKC isoforms might interact in the mTAL during T1D.

In conclusion, we found that T1D accelerates $O_2^{-•}$ production and stimulates PKC activity in the rat mTAL. Antioxidant treatment does not alter PKC activity in the mTAL under these conditions, but broad-spectrum PKC inhibition markedly reduces $O_2^{-•}$ production by mTALs from both normal and diabetic rats. Upregulation of PKCa and PKCδ (but not PKCβ) protein levels is evident in T1D, together with translocation of PKCa to the membrane fraction. Inhibition of PKCa/β (but not PKCβ inhibition alone) revealed increased PKCα activity to be the primary contributor to accelerated $O_2^{-•}$ production by the mTAL during T1D. A moderate increase in PKCβ activity may arise in the mTAL during T1D but does not seem to contribute to accelerated $O_2^{-•}$ production under these conditions. A role for PKCδ in T1D-induced mTAL $O_2^{-•}$ production remains uncertain. These observations represent the first steps in appreciating the basic mechanisms underlying renal medullary oxidative stress during T1D, a situation which might have myriad consequences that include effects on Na+ balance, extracellular fluid volume homeostasis, blood pressure regulation, and the development of tubulointerstitial fibrosis as the renal complications of the disease progress.

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