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LABORATORY INVESTIGATION

Formation of reactive oxygen metabolites in glomeruli is suppressed by inhibition of cAMP phosphodiesterase isozyme type IV

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Formation of reactive oxygen metabolites in glomeruli is suppressed by inhibition of cAMP phosphodiesterase isozyme type IV. Several independent studies indicate that synthetic inhibitors of cyclic-3′,5′-nucleotide phosphodiesterase (PDE) isozymes, especially inhibitors of PDE-IV, are potent agents which suppress generation of reactive oxygen metabolites (ROM) by NADPH oxidase in leukocytes. Recent studies also show that NADPH oxidase is present in all cell types populating glomeruli. In view of this, we investigated PDE isozymes and their relation to ROM in isolated rat glomeruli. Glomeruli have the capacity to hydrolyze cAMP by isozymes PDE-II, PDE-III and PDE-IV, whereas cGMP is hydrolyzed by PDE-I and PDE-V. Inhibitor of PDE-IV rolipram inhibited significantly (cca 40 to 50%) ROM generation in response to stimulation by phorbol myristate acetate (PMA). Inhibitor of PDE-III cilostamide had only minor suppressive effects and inhibitors of other PDE isozymes did not influence ROM generation. Rolipram (3 μM) suppressed ROM generation without detectable increase in cAMP content. Incubation of glomeruli with forskolin, which increased cAMP content in glomeruli tenfold, inhibited ROM generation to a similar degree as rolipram. The suppression of ROM generation by rolipram was prevented by Rp-cAMPS, a specific inhibitor of protein kinase A (PKA) activity. Further, incubation of glomeruli with rolipram elicited marked *in situ* activation of PKA (+100%), as documented by increase in the (−cAMP/+cAMP) PKA activity ratio. We suggest that selective inhibitor of PDE-IV rolipram acted via the cAMP-signaling pathway and suppressed ROM generation possibly via phosphorylating *ras*-type GTP-binding protein component of NADPH oxidase and thereby blocking assembly of functional NADPH oxidase complex. Results also suggest that ROM burst in glomeruli is modulated by a distinct cAMP-signaling pathway which is controlled by PDE-IV. We surmise that inhibitors of PDE-IV might be effective agents for suppression of ROM generation in glomeruli *in vivo* and attenuation of glomerular injury.

Numerous experimental findings support the thesis that reactive oxygen metabolites (ROM) play an important role in the mechanism(s) of cell injury in a variety of pathophysiological situations [1] in various tissues, including kidney [1, 2]. With respects of glomerulopathies, ROM generated by infiltrating leukocytes, namely neutrophils and macrophages [1], or ROM generated within endogenous renal cells residing in glomeruli [3] could be a major and perhaps a determining noxious factor in the pathogenesis of various inflammatory or non-inflammatory glomerular diseases [1, 2, 4].

In view of cytotoxic effects of ROM generated in glomeruli [3], investigations of novel pharmacologic interventions which are aimed to decrease the content of ROM in glomeruli, either by blocking ROM generation and/or by increasing the rate of ROM inactivation in the glomerular cells, are highly desirable. One frequently employed experimental approach to lower ROM content in the kidney tissue relies on administration of enzymatic or nonenzymatic ROM scavengers or antioxidants sometimes applied in very high doses [1, 2, 4]. Another approach to prevent or diminish cell damage by ROM involves maneuver(s) aimed to decrease ROM generation *in situ*. Some studies of professional inflammatory-infiltrating cells, that is, neutrophils [5, 6], eosinophils [7] or macrophages [8] indicate that increased levels of intracellular cAMP can suppress the burst of ROM generation [5–10] via inhibition of NADPH oxidase, a major source of ROM in these cells [11, 12]. In this context, of particular interest and potential importance are recent observations which show that newly designed specific synthetic "2nd generation" inhibitors [13, 14] of some isozymes of cyclic-3′,5′-nucleotides phosphodiesterases (PDE),[1] namely inhibitors of PDE-IV, in a very low concentration can block the ROM

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[1] PDE denotes cyclic-3′,5′-nucleotide phosphodiesterase in general. cAMP-PDE denotes PDE activity using cAMP as substrate, cGMP-PDE denotes PDE activity using cGMP as substrate.

In this report, the PDE isozymes are classified as proprosed by Dr. J.A. Beavo [13, 14]. According to this nomenclature, PDE-I is the family (also called "type") of PDE isozymes dependent on ionized calcium (Ca^{2+}) and calmodulin (CaM); PDE-II is the family of isozymes cAMP-PDE activity of which is stimulated by low (μM range) concentration of cGMP; the PDE-III are isozymes with low K_m for cAMP and are sensitive to inhibition by cilostamide, "cardiotonic inhibitors" and cGMP but are not inhibited by rolipram or Ro-20-1724; PDE-IV are isozymes with very high affinity and selectivity for cAMP, are insensitive to compounds that inhibit PDE-III and are selectively inhibited by rolipram and Ro-20-1724; PDE-V are those isozymes which specifically hydrolyze cGMP and activity of which is independent of Ca^{2+}-CaM. PDE-V_A subtype is present in somatic cells, PDE-V_B and PDE-V_C are present in photoreceptor and interact with G-protein transducin.

formation in inflammatory infiltrating cells [5–7, 9, 10]. The NADPH oxidase complex is present not only in professional phagocytosing cells, that is, leukocytes and macrophages, but also in cells which populate glomerulus [15, 16]. In view of these considerations, we determined which major types of PDE isozymes are present in glomeruli, and we then tested the hypothesis that selective inhibitors of PDE isozyme(s) can suppress ROM formation in resident cells of glomeruli.

Methods

Male Sprague-Dawley rats (200 to 250 g body wt) had free access to tap water and food (Purina Laboratory Rat Chow, Ralston Purina Company, St. Louis, Missouri, USA) prior to experiments. Glomeruli were prepared from rat cortical tissue using a sequential sieving method as done in our previous studies [17–19].

Briefly, rats were anesthetized by i.p. injection of pentobarbital sodium (90 mg/kg body wt) prior to insertion of a polyethylene catheter (PE-90) into the abdominal aorta inferior to the renal arterial bifurcation. The vena cava was cut above the diaphragm and the kidneys were perfused (10 ml/min) *in situ* with 60 to 80 ml of pre-warmed (37°C) Hanks' balanced salt solution (HBSS) followed by ice-cold HBSS until both kidneys were completely blanched. The kidneys were then quickly removed and decapsulated and placed in ice-cold HBSS. All subsequent preparation was conducted at 0 to 4°C. Cortical tissue was then dissected free and finely minced before passing through a stainless steel sieve (250 μm pore size). The resulting suspension was passed several times through a 22 gauge needle to ensure complete dispersion and sequentially sieved through Nitex nylon mesh (Tetko Inc.) of 390, 250, and 211 μm pore openings. The cortical suspension was then centrifuged at 800 × g for five minutes. The pellet was resuspended in HBSS and passed over a 60 μM sieve to collect the glomeruli. The glomeruli were pelleted in a pre-weighed corex tube and the final suspension (approximately 30 mg/ml; wet/wt) was resuspended in HBSS and kept on ice until experimentally used. Yield and viability of glomeruli were similar as reported in our preceding studies [17–19].

For fractionation to "cytosol" and "membranes" [20, 21], glomeruli were homogenized in a medium of the following composition (final concentrations): 0.3 M sucrose, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 0.1 μM leupeptin, 0.1 μM pepstatin, and 0.1 μM phenylmethylsulfonyl fluoride and 20 mM Tris [tris-(hydroxymethyl) aminomethane] adjusted to pH 7.4, by 10 strokes in a Potter-type teflon-glass homogenizer. Homogenate was ultracentrifuged at 100,000 × g for 60 minutes and the supernatant was decanted and stored on ice. The pellet was rehomogenized and ultracentrifuged again as described above. The first and second supernatants were pooled to constitute the total soluble fraction or "cytosol." The washed pellet was resuspended in homogenizing buffer containing 0.1% Triton X-100 to release "latent" PDE activity [22]. This total membrane fraction is referred to as "membranes" [20, 21].

Phosphodiesterase (PDE) assay

The activity of PDE was measured by incubating the extracts or homogenate fractions (cytosol or membranes) in a reaction mixture (final volume 100 μl) of the following composition (final concentrations): 10 mM $MgSO_4$, 2 mM EGTA, 0.1% bovine serum albumin, 15 mM Tris-HCl adjusted to pH 7.4, and either 0.5 μM [^{3}H]-cAMP or [^{3}H]-cGMP as substrate [20, 21]. In experiments examining the activity of Ca^{2+}-calmodulin-dependent PDEs, the reaction mixture also contained 2.01 mM $CaCl_2$, to obtain 10 μM Ca^{2+}, and 10 μg/ml of calmodulin (CaM). The PDE activity in aliquot samples incubated without Ca^{2+} and CaM (but containing 2 mM EGTA, *vide supra*) was subtracted as basal activity [20, 21]. PDE-III and PDE-IV activities were determined as cAMP-PDE inhibitable by 3 μM cilostamide or rolipram, respectively [22]. At these concentrations (3 μM) both rolipram and cilostamide cause maximal inhibition of corresponding PDEs [20, 22]. In preliminary experiments we determined that by increasing the dose of rolipram from 3 μM to 10 μM has only a slightly higher inhibitory effect. Therefore, as in our preceding studies [22] we employed 3 μM of rolipram and cilostamide as maximum doses in the following experiments. The PDE-II activity was determined as the difference in cAMP-PDE without or with 5 μM cGMP [22]. In experiments examining the effect of PDE inhibitors, stock solutions of all inhibitors were made in 100% dimethylsulfoxide (DMSO) and the incubation media (including controls) contained, after final dilution, 0.1% DMSO. Hydrolysis of cyclic-3′,5′-nucleotides was less than 20% of the substrate and was linearly proportional to incubation time and enzyme protein concentration [20–22]. Protein content was determined by the Bradford's method [23].

Measurement of ROM by chemiluminescence

To monitor the production of ROM by glomeruli, light emmision from glomeruli was determined by a procedure described previously by Shah [3, 24] with minor modifications. The light emmision from glomeruli incubated in liquid scintillation vials in a medium containing luminol was measured with a liquid scintillation system (Model LS 6000SC, Beckman Instruments) set on out-of-coincidence mode. All PDE inhibitors, when used, were dissolved in DMSO to a final concentration of 0.1%. The reaction mixture in each vial consisted of 30 μM luminol, 0.1% DMSO alone (controls) or with added test agents in HBSS sufficient to achieve final volume of 2 ml. Then the background luminescence of each vial was determined. Glomeruli in suspension (100 μl of 30 mg/ml wet wt) preincubated at 37°C for 10 minutes were added to the vial, mixed, and a second count was taken. At the same time, an aliquot of glomeruli was removed for protein determination by a modified method of Lowry et al [25] as in our previous studies [21, 22]. After six minutes, phorbol myristate acetate (PMA), final concentration 10 μg/ml, was added and chemiluminescence measurements continued at one minute time intervals for up to 20 minutes after the addition of PMA, when the plateau was reached. After subtraction of the basal counts, data were expressed in cpm per milligram of protein. The time course of the chemiluminescence response is shown on Figure 1.

For measurements of cAMP, an additional aliquot of glomeruli was incubated under the same conditions as used for determination of chemiluminescence. At the end of the incubation period, the reaction was stopped by the addition of an equal volume of ice-cold 10% TCA. The glomeruli were homogenized and the mixture was stored frozen at −20°C until analysis. The TCA was extracted by water-saturated ether and cAMP content was determined in duplicate by radioimmunoassay (RIA) on

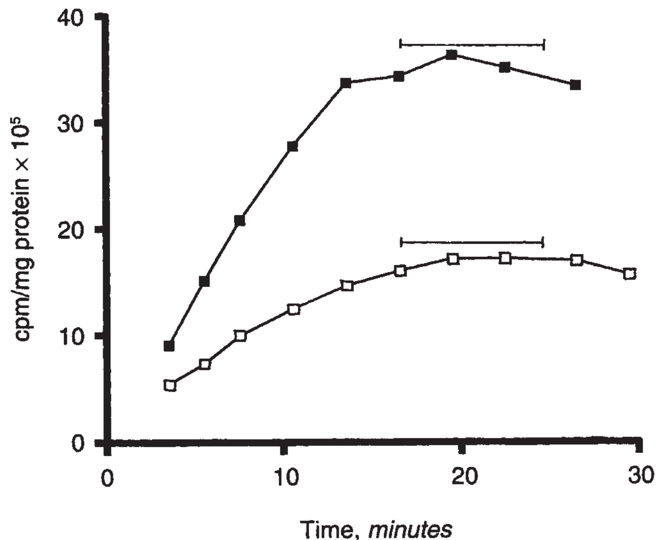

Fig. 1. *The time course of the chemiluminescence response of glomeruli to 10 μM PMA without (control -■-■-) or with 3 μM rolipram (-□-□-).* Bars above the lines show periods from which PMA-stimulated values were determined.

preacetylated samples as in our previous studies [17–21]. As a rule, the effect of the tested agents was assayed when glomeruli were exposed to stimulation by 10 μg/ml PMA. In a preliminary experiment we affirmed that 10 μg/ml PMA elicits maximum increase in chemiluminescence [3]. The effect of PDE inhibitors and other tested agents was expressed as a percent change computed as follows:

$$\frac{\text{cpm in control vial} - \text{cpm in presence of test agent}}{\text{cpm in control vial}} \times 100$$

In preliminary control experiments we determined the addition of superoxide dismutase (SOD) inhibited chemiluminescence by 62%. Further, we determined whether tested agents may interfere with chemiluminescence elicited by ROM. The chemiluminescence elicited by ROM generated by xanthine-xanthine oxidase system [3] was not influenced by the addition of rolipram, cilostamide, 8-MeoM-IBMX, zaprinast or forskolin.

To test whether rolipram may inhibit NADPH oxidase complex directly, isolated suspended glomeruli were disrupted by ultrasound treatment [26], centrifuged for 10 minutes at 800 × g at 4°C and the supernatant was used for NADPH oxidase assay [26]. The NADPH oxidase activity, O_2^- production, was determined in the supernatant by measuring the rate of superoxide dismutase-inhibitable ferricytochrome c reduction [26] without (control) or with addition of 3 μM rolipram.

Since the effects of PDE isozymes' inhibitors were measured in most experiments in glomeruli stimulated with PMA, that is in a state of maximally activated protein kinase C (PKC) [3], in control experiments we explored whether rolipram and cilostamide might display direct inhibitory effects upon PKC. The inhibitors were tested on PKC prepared from rat brain using a method employed previously by Walton et al [27]. We employed, in principle, the assay for PKC described by Abraham et al [28] with the following modifications. The mixture of protease inhibitors was not included, the incubation was carried out at 30°C instead of 37°C, the final reaction volume was 80 μl and the reaction was terminated by spotting 40 μl of incubation mixture onto Whatman P 81 paper, with subsequent immersing in 10 mM Na^+ pyrophosphate and 1% H_3PO_4 and three additional washes (15 min each) in 1% H_3PO_4. The PKC activity was assayed by incubating 80 μl mixture containing (final concentrations): 50 μM ^{32}P-γ-ATP (20 μCu/ml), 400 μg/ml histone III-S, 0.4 mM EDTA, 0.4 mM EGTA, 10 mM $MgCl_2$, 0.01% Triton X-100, 0.124 μg/ml PKC protein and 35 mM Tris-HCl (pH = 7.4). The PKC was assayed without (basal activity) or the stimulated activity with (final concentrations): 1 mM $CaCl_2$, 0.1 μM PMA and 100 μg/ml phosphatidyl-L-serine for 20 minutes at 30°C [28].

Measurement of cAMP-dependent protein kinase (PKA) activity in situ

To determine cAMP PKA activity we employed a method described by Corbin [29] that was also used in our previous studies [30, 31] with minor modifications. The glomeruli were incubated either without added agents (controls), or with 3 μM rolipram, or with 10 μM forskolin, similarly as described for chemiluminescence assay, except for the absence of luminol in the medium. After the addition of PMA, the incubation continued for 18 minutes and was terminated by chilling the mixture to 0 to 4°C and the glomeruli were pelleted by centrifugation at 2,500 × g for five minutes at 0 to 4°C. The medium was discarded and the packed glomeruli were homogenized in a medium containing (final concentrations): 0.25 M sucrose, 0.5 mM IBMX, 4 mM EDTA, and 20 mM MES-NaOH buffer, pH 6.8. The homogenate was centrifuged at 27,000 × g for 30 minutes at 0 to 4°C and the protein kinase activity was assayed in the supernatant. Before protein kinase assay, the protein concentrations in supernatants were determined by the modified method of Lowry et al as described above [25].

The cAMP-dependent protein kinase activity was measured by the incorporation of ^{32}P-γ-ATP into the synthetic oligopeptide substrate Kemptide [32]. The glomerular extract was incubated in a mixture containing (final concentrations): 20 mM MES-NaOH, 0.4 mM EGTA, 0.1 mM EDTA, 8 mM NaF, 0.6 mg/ml bovine serum albumin (BSA), 160 μM "Kemptide" (LRRASLG), pH 6.8, either with or without 1 μM added cAMP. The reaction was started by the addition of glomerular extract (2 μg of protein per tube); the incubation was carried out for six minutes at 30°C and stopped by placing the incubation mixture in 2 × 2 centimeter squares of phosphocellulose ion exchange paper (Whatman P 81) which were then immersed immediately into 10 mM $Na_4P_2O_7$ in 1% H_3PO_4. The papers were then washed (3 × 15 min) with 1% H_3PO_4 with constant agitation. The papers were dried and ^{32}P radioactivity was determined by liquid scintilation counting. Autophosphorylation of glomerular extract was negligible. The PKA activity was assayed in one aliquot without added cAMP, another aliquot with addition of 1 μM cAMP, (maximum stimulatory dose) and also in aliquot with 1 μM added cAMP plus maximum inhibitory dose (10 μM) of a specific PKA oligopeptide inhibitor WIPTIDE [33], a pseudosubstrate for PKA (TTYADFIASGRTGRRNAI-NH_2). The difference between activity without and with 10 μM WIPTIDE was taken as specific PKA activity; the residual protein kinase

activity in the presence of WIPTIDE was subtracted. *In situ* activity of PKA was determined as a ratio of PKA activity measured without added cAMP (−cAMP) to total PKA activity measured with 1 μM cAMP (+cAMP) and expressed as (cAMP/+cAMP) PKA activity ratio [29–31].

The results were evaluated statistically by *t*-test or paired *t*-test, as specified in the Results.

Materials

Rolipram, 4-(3-cyclopentyloxy-4-methylphenyl)-2-pyrolidone (ZK 62711) was a gift of Berlex Laboratories (Cedars Knolls, New Jersey, USA). Cilostamide, N-cyclohexyl-N-methyl-4(1,2-dihydro-2-oxo-6-quinohyloxy)butyramide(OPC-3639) was a gift of Otsuka Pharmaceutical Company (Osaka, Japan). Ro 20-1724 (4-3-butoxy-4-methoxybenzyl)-2-inidazolidinone was purchased from BIOMOL. Forskolin and bovine brain calmodulin were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). 8-Methoxymethyl,3-isobutyl-1-methyl-xanthine (8-MeoM-IBMX) was a gift of Dr. Jack N. Wells (Vanderbilt University, Nashville, Tennessee, USA). 8-MeoM-IBMX is a potent specific inhibitor of Ca^{2+}-calmodulin (CaM)-dependent PDE-I, but does not inhibit calmodulin [34]. The QAE-Sephadex A-25 resin was from Pharmacia, LKB, Inc. (Piscataway, New Jersey, USA); PKC was from Promega Corp. (Madison, Wisconsin, USA). The cAMP-RIA kits were from BIO MED TECH (BT-300); [8-^{3}H]-cGMP, ammonium salt, 10 to 30 Ci/mmol, [2,8-^{3}H]-cAMP, ammonium salt, NEN (NEX 275) 30 to 50 Ci/mmol, and ^{32}P-γ-ATP were puchased from Amersham Co. (Arlington Heights, Illinois, USA). The nylon nitex sieves were from Tetko Inc.; Kemptide and WIPTIDE were purchased from Peninsula Labs Inc. Rp-cAMPS and Sp-cAMPS were from Research Biochemicals International (Natick, Massachusetts, USA). All other reagents and chemicals, of the highest purity grade, were purchased from standard suppliers. [^{3}H]-cAMP and [^{3}H]-cGMP were, prior to use as substrates in PDE assays, purified according to a method of Kincaid and Manganiello [35].

Results

PDE activities in glomeruli

The activities of cAMP-PDE and cGMP-PDE were assayed in cytosol and total membrane fraction [20, 21] prepared from glomerular homogenate. Overall total cAMP-PDE activity, measured in the presence of activating agents, was higher ($P <$ 0.002, *t*-test) in cytosol (1048 ± 121 pmol cAMP/min/mg protein; $N = 10$) than total cGMP-PDE (553 ± 49 pmol cGMP/min/mg protein; $N = 11$). Both cAMP-PDE and cGMP-PDE activities in cytosol were more than three times higher than corresponding activities in membranes (Fig. 1).

The presence of five major types (families) of PDE isozymes was surveyed using sensitivity to specific modulators and selective synthetic inhibitors as criteria [13, 14]. Of all PDE isozymes which accept cAMP as a substrate, that is, cAMP-PDEs, both cytosol and membranes from glomeruli contained PDE-II, PDE-III and PDE-IV (Fig. 2), whereas Ca^{2+}/CaM-dependent PDE-I hydrolyzing cAMP was not detected. PDE-II activity was significantly higher than either PDE-IV or PDE-III (Fig. 2) and represented about 64% of all cAMP-PDE activity in the cytosol, as assayed under present conditions.

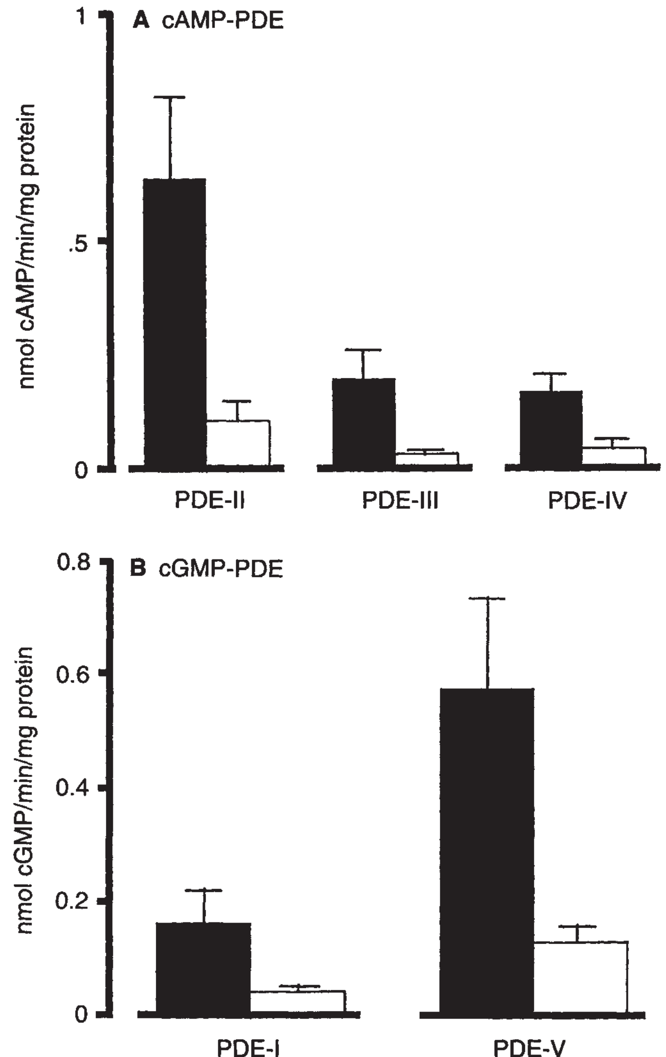

Fig. 2. *Activities of PDE isozymes in glomeruli hydrolyzing cAMP* (**A**, *cAMP-PDE) and hydrolyzing cGMP* (**B**, *cGMP-PDE).* Each bar denotes mean ± SEM of $N = 8$, from 3 independent experiments. Cytosolic (■) and membrane (□) fractions were prepared as described in **Methods**. Activities of cAMP-PDE and a cGMP-PDE were assayed with 0.5 μM cAMP or 0.5 μM cGMP, respectively, as substrates. Unless specified, PDE activities were assayed in the absence of Ca^{2+}. PDE-III is defined as cAMP-PDE supressible by 3 μM cilostamide; PDE-IV is cAMP-PDE activity supressible by 3 μM rolipram [20–22]. PDE-I is cGMP-PDE stimulated by 10 μM Ca^{2+} and 10 μg/ml CaM; PDE-V_A is cGMP-PDE activity assayed in the absence of Ca^{2+} and CaM. A (cAMP-PDE). PDE-II activity is significantly ($P < 0.005$, *t*-test) higher than PDE-III and PDE-IV, the latter two PDEs are not significantly different from each other. B (cGMP-PDE). PDE-I is significantly ($P <$ 0.01, *t*-test) lower than PDE-V. Activities of all PDE isozymes were significantly (by *t*-test) lower (4 times or more) in membranes than in cytosol.

Lack of PDE-I activity for sufficient hydrolysis of cAMP was unlikely due to employed assay conditions. To verify this we assayed simultaneously in the same experiment and using the same reagents, cytosol from glomeruli and cytosol from inner medullary collecting duct cells grown *in vitro* [21]. With the use of 0.5 μM cAMP as a substrate in cytosol from glomeruli no

Table 1. Effect of rolipram upon chemiluminescence, basal and stimulated by phorbol myristate acetate (PMA) in glomeruli incubated with rolipram

Additions	Chemiluminescence *cpm × 10⁴/mg protein* Basal	with 10 μg/ml PMA
Control (no additions)	120 ± 20	1,300 ± 229
3 μM rolipram	100 ± 21[a]	807 ± 181[a]
Δ%	−19 ± 6%[b]	−38 ± 7%[bc]

Each value denotes the mean ± SEM of $N = 6$ experiments.
[a] Value is significantly lower ($P < 0.05$; paired t-test)
[b] The percent inhibition is significant ($P < 0.05$; paired t-test)
[c] Value with PMA is significantly ($P < 0.05$, paired t-test) higher than basal value

Ca^{2+}/CaM-dependent activity was detected, whereas considerable PDE-I activity was readily found in cytosol of inner medullary collecting duct cells (basal activity of cAMP-PDE without Ca^{2+}/CaM was 316 ± 14 pmol cAMP/min/mg protein and after addition of Ca^{2+}/CaM the activity was significantly increased ($P < 0.05$, t-test, $N = 3$) to 538 ± 14 pmol cAMP/min/mg protein. PDE-II, measured with cAMP as substrate, was stimulated by added cGMP in a dose-dependent manner; the 1/2 max stimulatory concentration was found to be about 0.2 μM cGMP.

From PDEs hydrolyzing cGMP, the predominant activity was the Ca^{2+}-independent cGMP-PDE(PDE-V_A) and much lower was Ca^{2+}/CaM-dependent activity that is, PDE-I, which was about 22% of total cGMP-PDE activity (Fig. 2). The Ca^{2+}-independent PDE-V_A showed relatively low sensitivity to the inhibitory effect of 5 μM zaprinast (−18%) while 5 μM dipyridamole had higher inhibitory effect (−59%).

ROM generation in glomeruli

Glomeruli showed spontaneous basal ROM generation which increased about tenfold in response to incubation with 10 μg/ml PMA (Table 1, Fig. 1). All subsequent measurements of ROM generation were conducted in the presence of 10 μg/ml PMA (Fig. 1). In experiments examining the effect of PDE isozyme-specific inhibitors and other agents upon ROM generation, aliquots of the each preparation of glomeruli were incubated simultaneously under the same conditions in the presence of 10 μg/ml PMA. One aliquot was used to measure ROM generation by chemiluminescence (Fig. 3A) and the other to determine cAMP accumulation (Fig. 3B). Incubation of glomeruli with rolipram resulted in a pronounced inhibition (~ −40%) of ROM generation (Fig. 3, Table 1); rolipram also inhibited to a lesser degree (~ −20%) the basal ROM formation, that is, without stimulation by the added PMA (Table 1). Cilostamide, a selective inhibitor of PDE-III, caused significant but much less extensive (~ −14%) inhibition of ROM formation than rolipram (Fig. 3). On the other hand, neither 8-MeoM-IBMX (specific inhibitor of PDE-I) nor zaprinast (inhibitor of PDE-V) altered ROM formation (Fig. 3).

Notably, rolipram and cilostamide suppressed ROM without detectable increase of cAMP content in incubated glomeruli (Fig. 3B). Incubation of glomeruli with 10 μM forskolin inhibited ROM generation to similar degree as rolipram, but in contrast to rolipram, forskolin increased cAMP accumulation tenfold along with the inhibition of ROM generation (Fig. 3).

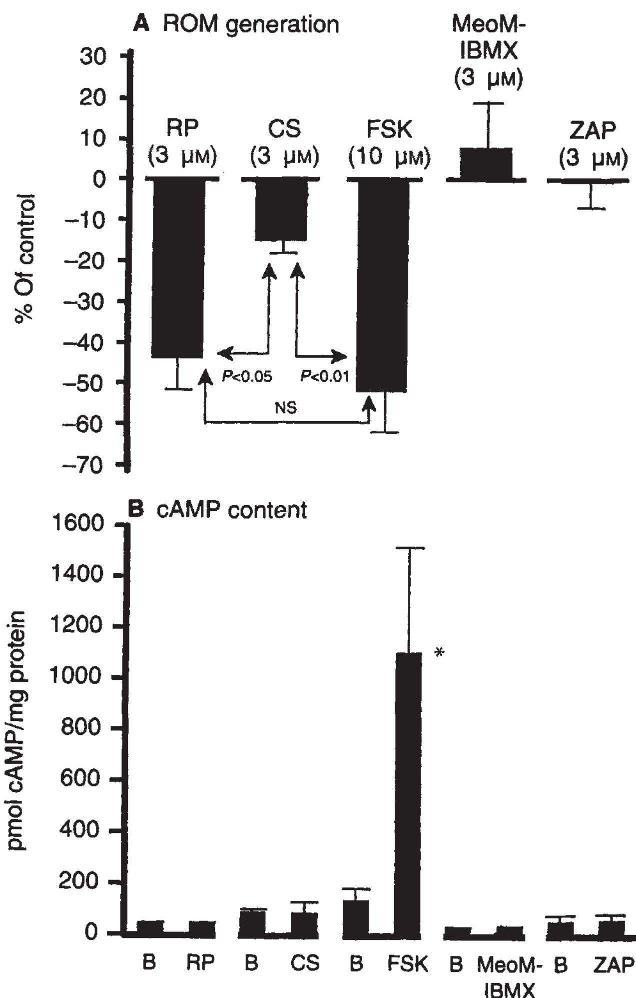

Fig. 3. *Effect of agents influencing cAMP and PDEs upon generation of ROM in glomeruli (A) and accumulation of cAMP (B).* RP = 3 μM rolipram; CS, 3 μM cilostamide; FSK, 10 μM forskolin, MeoM-IBMX, 3 μM 8-methoxymethyl-IBMX; ZAP, 3 μM zaprinast. A. "ROM formation" denotes effects of tested agents upon rate of ROM generation in the presence of 10 μg/ml PMA, expressed as Δ% change from the control, basal value (no additions). RP, CS and FSK caused significant (t-test) inhibition, 8-MeoM-IBMX and ZAP were without effect. B. Content of cAMP (glomeruli plus medium) accumulated in the incubation mixtures of aliquots of glomeruli in which ROM generation was determined, see corresponding bars in upper panel. Left side bar, B, denotes basal value without additions, right side bar denotes value with tested agents. Only FSK increased (*$P < 0.05$, t-test) the content of cAMP significantly. Each bar (ROM generation, A) or cAMP content (B) denotes mean ± SEM of 3 to 5 paired experiments.

Finally, addition of 100 μM Sp-cAMPS, an active stereoisomer of thio-derivative of cAMP [36], also decreased ROM generation (−24 ± 4%, mean ± SEM; $N = 3$, $P < 0.05$, paired t-test).

In view of these findings, we investigated whether rolipram might have inhibited the burst of ROM generation by direct action upon NADPH oxidase or rather via cAMP signalling pathway [9–12] due to its inhibition of PDE-IV. Very small changes in cellular cAMP content or redistribution of cAMP within cells can be detected by an increase of *in situ* activity [29, 37–39] of cAMP-dependent protein kinase (PKA). We observed that incubation of glomeruli with 3 μM rolipram, that is, under

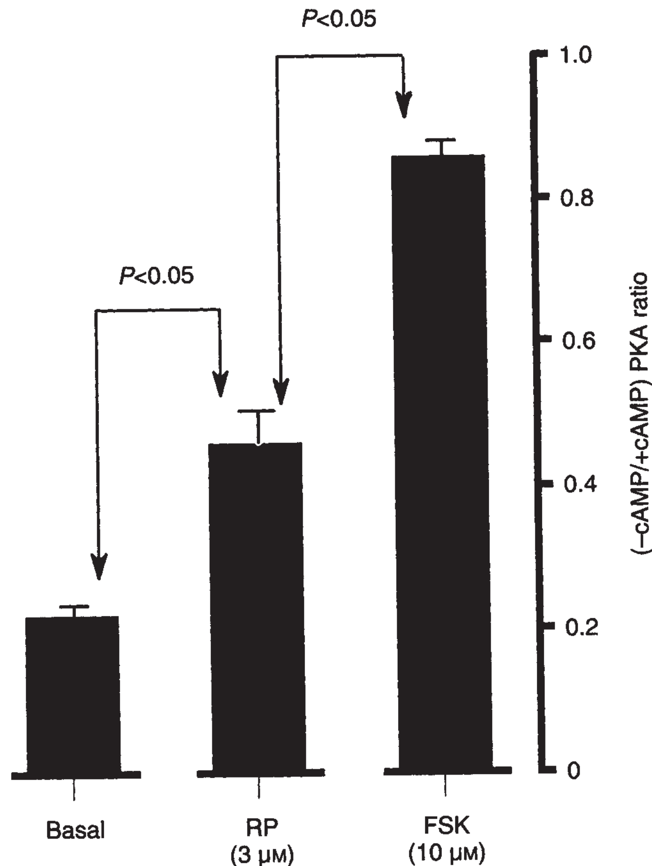

Fig. 4. *Effect of 3 μM rolipram (RP) and 10 μM forskolin (FSK) upon* in situ *activity of cAMP-dependent protein kinase in glomeruli.* Each bar denotes means ± SEM of 3 to 4 experiments. Ordinate: (−cAMP/+cAMP) protein kinase activity ratio.

conditions that rolipram caused inhibition of ROM formation (Fig. 3A and Table 1) without apparent change in total cAMP accumulation (Fig. 3), resulted in significantly (+111%) increased (Fig. 4) the *in situ* PKA activity, as determined by measurement of the (−cAMP/+cAMP) PKA activity ratio [29–31]. Incubation of glomeruli with forskolin resulted in almost complete *in situ* activation of PKA, which was about two times higher than PKA activation elicited by rolipram (Fig. 4). It is now well documented that Rp-cAMPS stereoisomer of thio-derivative of cAMP competes with cAMP for binding on regulatory subunits of PKA without activating the kinase [36]. In this respect, we have shown that inclusion of Rp-cAMPS to medium in which glomeruli were incubated with rolipram prevented the suppressory effect of rolipram upon ROM generation (Table 2). Finally, we explored whether rolipram might have inhibited NADPH oxidase activity and/or protein kinase C (PKC) activity by direct action. We found that 3 μM rolipram inhibited neither PKC (Table 3) nor influenced NADPH oxidase activity determined in cell-free extract (data not shown).

Table 2. Effect of cAMP antagonist R_p-cAMPS upon inhibition of ROM generation by rolipram

Conditions	Inhibition of ROM (Δ%) generation	*P* value[a]
3 μM rolipram	−42 ± 9%	< 0.05
3 μM rolipram + 100 μM R_p − cAMPS	−10 ± 14%	NS

The experimental design was the same as described for experiments portrayed on Fig. 3, see **Results**.

Data are mean ± SEM of $N = 3$ experiments. NS denotes not significant ($P > 0.5$).

[a] *P* value (paired *t*-test) for significance of inhibition

Table 3. Lack of interaction of rolipram and cilostamide with protein kinase C

Conditions	Protein kinase C activity *μmol $^{32}P_i$/20 min/mg protein*
Basal (no activators added)	4.6 ± 3.0
Stimulated with Ca^{2+}, PMA and PS	155.6 ± 5.0[a]
Stimulated with Ca^{2+}, PMA, PS and 3 μM rolipram	152.0 ± 5.2
Stimulated with Ca^{2+}, PMA, PS and 3 μM cilostamide	154.2 ± 6.2

For details of experimental design see **Methods**. Stimulatory agents of PKC were: 0.1 μM PMA, (phorbol myristate acetate); 1 mM Ca^{2+} (calcium chloride) and 100 μg/ml PS (phosphatidylserine).

Data are mean ± SEM; $N = 4$.

[a] Significantly higher than basal value ($P < 0.001$, *t*-test); not significantly different ($P > 0.5$; *t*-test) from values with added 3 μM rolipram or 3 μM cilostamide

Discussion

Our observations show that PDE isozymes which can hydrolyze cAMP in cells which populate glomeruli include PDE-II, PDE-III and PDE-IV (Fig. 2); PDE-I was detected only with use of cGMP but not cAMP as a substrate (Fig. 2). Localization of these PDE isozymes in the major cell types present in the (decapsulated) glomerulus, that is, podocytes, mesangial cells and endothelial cells, remains to be determined. According to a recent preliminary report [40], activities of PDE-IV and PDE-I hydrolyzing cGMP, as well as minor activity of PDE-II were observed in rat mesangial cells grown in primary culture. In our own preliminary experiments [41] on primary cultured rat mesangial cells we found PDE-IV, and to a lesser degree PDE-III, but no PDE-I hydrolyzing cAMP was detected. Although all nephron segments were not yet examined for PDE isozyme composition, the diversity is obvious by comparing glomeruli with inner medullary collecting ducts (IMCD). Unlike in glomeruli, IMCD lack PDE-II, and PDE-IV is a predominant isozyme hydrolyzing cAMP [42].

In view of our findings on PDE isozymes in glomeruli (Fig. 2) and cognizant that specific PDE isozymes can modulate ROM burst in inflammatory cells [5–10], we investigated the effects of selective PDE inhibitors upon ROM generation and upon cAMP signaling pathway in glomeruli. A chemiluminescence method has been employed primarily for its sensitivity in this study. It is realized that future analysis of the ROM system and dynamics of individual ROM species need to be determined by more specific methods.

Forskolin, a potent general activator of adenylate cyclase [43] which caused a huge (10×) increase in accumulation of cAMP (Fig. 3), decreased ROM formation in glomeruli, similarly as previously reported by Myanoshita, Takahashi and Endou [44]. In contrast, incubation of glomeruli with the PDE-IV inhibitor

rolipram (3 μM) caused no detectable increase in cAMP content but suppressed ROM formation to the same extent as forskolin (Fig. 3). Rolipram inhibited not only PMA-stimulated, but also basal, spontaneous ROM generation (Table 1). Another structurally distinct and selective PDE-IV inhibitor, Ro-20-1714 [13, 14], also suppressed ROM generation (data not shown). Cilostamide, a specific inhibitor of PDE-III, in the same concentration as rolipram (3 μM) also blunted ROM generation without change in cAMP content, albeit to a much lesser degree (Fig. 3) despite that PDE-III activity was as high as PDE-IV (Fig. 2).

Considering the pattern of responses to rolipram and to forskolin in terms of ROM generation and cAMP accumulation by glomeruli (Fig. 3), the mechanism by which rolipram inhibits ROM formation without noticeable increase of cAMP content called for clarification. Since rolipram did not act on ROM as scavenger (**Methods**), we explored whether rolipram might have acted via a cAMP-signaling pathway despite measurable increase in cAMP. Our finding that incubating glomeruli with 3 μM rolipram resulted in a clear-cut *in situ* activation of PKA (Fig. 4) strongly suggests that rolipram, by virtue of inhibiting PDE-IV, caused a discrete increase or redistribution of cAMP, perhaps only in some cell types and/or in some specific cellular compartment in glomeruli. Very small, analytically undetected increases or redistribution of cellular cAMP [29, 37, 38, 45] can cause increased cAMP binding onto the regulatory subunit of PKA [29, 37–39, 45–47], induce the dissociation of PKA heterotetramer into regulatory (R_2) and catalytic (C_2) subunits, and increase the rate of protein substrate phosphorylation by the catalytic subunit. Our interpretation suggesting that rolipram inhibited ROM generation via *in situ* PKA activation is supported by the finding that Rp-cAMPS, a specific inhibitor of PKA activation by cAMP [48], antagonized the inhibition of ROM generation caused by rolipram (Table 2), whereas its active stereoisomer Sp-cAMPS [36], when added to glomeruli significantly suppressed the ROM burst (**Results**). The notion that rolipram indeed supressed ROM burst via activation of cAMP-PKA signaling pathway is further supported by our observations that rolipram inhibited neither the activity of protein kinase C (Table 3) nor influenced NADPH oxidase when added directly into cell-free systems (**Results**).

It should be stressed that forskolin, which induced a several-fold (10×) increase in accumulation of cAMP (Fig. 3) and virtually complete *in situ* activation of PKA (Fig. 4), did not inhibit ROM burst to a higher degree than rolipram (Fig. 3). Such a comparison strongly suggests that rolipram activated the cAMP-signaling pathway either only in a specific population of glomerular cell types(s) and/or in a discrete cellular compartment. It follows that such a quantitatively limited effect upon glomerular cAMP by rolipram was sufficient to suppress the burst of ROM generation in the glomeruli to a maximal extent achievable under present experimental conditions (Fig. 3). We thus propose that ROM formation in glomeruli is modulated by a distinct cAMP-signaling pathway, a specific catabolic branch of which is rolipram-sensitive PDE isozyme type-IV. It remains to be determined whether inhibition of PDE-III by cilostamide accompanied by quantitatively minor ROM-suppressory effect, operates in the same sites of ROM generation and by similar mechanism as does rolipram. Lack of the effects of MeoM-IBMX or zaprinast (Fig. 3) underscores the specificity of the modulatory role of PDE-IV and also indicates that the inhibitory effect upon ROM generation is specific for cAMP compared to cGMP [13]. Thus, comparison of effects of forskolin and rolipram as well as other tested agents argues in favor of our proposition that the activation of the PDE-IV-controlled cAMP signaling mechanism alone elicites maximum suppressive effect upon ROM burst in glomeruli.

Interpretations of our present findings are limited by several features. The present experiments were conducted on whole glomeruli which consist of several types of cells: visceral epithelial, endothelial, and mesangial cells [49]. In the future studies it should be determined which sites are specific for ROS generation, that is, cell types and/or cell compartments, and whether these sites coincide with the cAMP-signaling system controlled by PDE-IV. Such studies will require extensive methodologic preparation. Another remaining problem challenging the interpretation of these studies is to determine the exact location of the abundant glomerulus PDE-II (Fig. 2), and also whether this PDE isozyme plays a regulatory role in cAMP metabolism pool that modulates ROM generation. It can be perhaps expected that PDE-II is localized in glomerular endothelial cells, in analogy with studies of PDEs in endothelial cells from other tissues [50]. Investigations into the role of PDE-II are, to a certain degree, more difficult than for other PDE isozymes, since no truly specific inhibitor of PDE-II is presently known [13, 14]. Finally, it remains to be discerned which ROM species are most relevant for the ROM burst response as well as for the pathogenic impact.

The mechanism by which rolipram-induced *in situ* activation of PKA suppresses ROM formation in glomerular cells should be briefly considered. It seems likely that most of ROMs in glomeruli, especially in response to inflammatory stimuli, are generated by the NADPH oxidase complex [15, 16, 51]; the activity of xanthine-xanthine oxidase system is rather low in glomeruli [52]. According to current knowledge the NADPH oxidase complex generates ROM only when assembled from three cytoplasmic and three membrane subunit components [51, 53].

Although the NADPH oxidase complex system is presently best described in leukocytes, it apparently exists in analogous variants in other cell types such as fibroblasts [54], in glomerular visceral epithelial cells [15], mesangial cells [15, 16], and glomerular endothelial cells [15, 16]. It is now known that the assembly of cytoplasmic components, the protein kinase C-phosphorylated $p47^{phox}$ and $p67^{phox}$ with membrane-bound flavocytochrome b_{558} into active NADPH oxidase complex requires the presence and co-assembly of two low molecular weight GTP-binding proteins (LMWG) of the Ras superfamily [55, 56]: Rac-1 in cytoplasm and membrane-bound Rap1a [51]. Recent studies indicate that cAMP-dependent phosphorylation of Rap1a catalyzed by PKA [57] blocks assembly of NADPH oxidase components into active complex [11] and consequently prevents ROM generation [58]. It would not be surprising if rolipram-induced cAMP-dependent activation of PKA (Fig. 4) suppresses the ROM generation in glomeruli by a similar mechanism [11, 57, 58], that is, by cAMP-dependent phosphorylation of Rap 1a, the LMWG component of NADPH oxidase complex in membranes of glomerular cells. However, at the present time this proposition remains only a working hypothesis posited by the authors.

In conclusion, this study provides evidence that selective

PDE-IV inhibitor(s) can, in micromolar concentrations, cause a marked suppression of ROM generation in glomerular cells. Our findings are consistent with the proposed working hypothesis that activation of a specific cAMP-signaling pathway which includes PDE-IV, can inhibit ROM formation via cAMP-dependent phosphorylation of a LMWG protein such as Rap1a [11, 57, 58], which in phosphorylated state blocks assembly of NADPH oxidase into active complex. Hence, selective PDE-IV isozyme inhibitors, relatively nontoxic and effective compounds such as rolipram [59], might possibly emerge as novel agents suited to attenuate, via a cAMP-mediated mechanism, the glomerular cell injury caused by ROM *in vivo*.

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