Identification and Quantification of 2',3'-cAMP Release by the Kidney

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List of Abbreviations: LC-MS/MS, tandem liquid chromatography-mass spectrometry; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat; 3',5'-cAMP, adenosine 3',5'-cyclic monophosphate; 2',3'-cAMP, adenosine 2',3'-cyclic monophosphate; MRP, multidrug resistance protein; m/z, mass-to-charge ratio; ANOVA, analysis of variance
ABSTRACT

We recently developed a sensitive assay for 3’,5’-cAMP using high performance liquid chromatography-tandem mass spectrometry. Using this assay, we investigated the release of 3’,5’-cAMP from isolated, perfused rat kidneys. To our surprise, we observed a dominant chromatographic peak that was due to an endogenous substance that had the same parent ion as 3’,5’-cAMP and that fragmented to the same daughter ion (adenine) as 3’,5’-cAMP. However, the retention time of this unknown was approximately 2.9 minutes compared to 6.3 minutes for authentic 3’,5’-cAMP. We hypothesized that the unknown substance was an isomer of 3’,5’-cAMP. Indeed, the unknown substance had the same retention time and mass spectral properties as authentic 2’,3’-cAMP. Renal venous secretion of 2’,3’-cAMP was greater in kidneys from 20-week-old genetically-hypertensive rats compared with age-matched normotensive rats (12.49 ± 2.14 versus 5.32 ± 1.97 ng/min per gram kidney weight, respectively; n=18). Isoproterenol (1 μmol/L; β-adrenoceptor agonist) increased renal venous 3’,5’-cAMP secretion (approximately 690% of control), but had no effect on 2’,3’-cAMP production. In contrast, rapamycin (0.2 μmol/L; activator of mRNA turnover) and iodoacetate + 2,4-dinitrophenol (50 μmol/L; metabolic inhibitors) increased the renal venous secretion of 2’,3’-cAMP (approximately 1000% and 4100% of control, respectively) while simultaneously decreasing the renal venous secretion of 3’,5’-cAMP. In conclusion, 2’,3’-cAMP is a naturally-occurring isomer of 3’,5’-cAMP that is: 1) not made by adenylyl cyclase; 2) released from kidneys into the extracellular compartment; 3) released more by kidneys from rats with long-standing hypertension; 4) derived from mRNA turnover; and 5) increased by energy depletion.
INTRODUCTION

Because 3’,5’-cAMP is an important second messenger in most cells comprising organ systems, it is desirable to be able to investigate, with a high level of precision, the production of 3’,5’-cAMP in intact organs. In this regard, it is fortunate that intracellular 3’,5’-cAMP is robustly transported to the extracellular compartment by various active transporters, including multidrug resistance protein (MRP) 4 and MRP5 (Kruh et al., 2001; Deeley et al., 2006). Consequently, increases in intracellular levels of 3’,5’-cAMP can be detected by measuring 3’,5’-cAMP in the venous effluent of organ systems, such as the kidney (Vyas et al., 1996).

With regard to the kidney, 3’,5’-cAMP is importantly involved in regulation of renal vascular resistance, glomerular filtration rate, renin release, tubular epithelial transport and the actions of hormones such as antidiuretic hormone (Cheng and Grande, 2007). Moreover, many of these physiological parameters are altered in kidneys from chronically-hypertensive animals, compared with normotensive animals, in part because of target organ damage induced by the chronic elevation of arterial blood pressure.

To study the role of 3’,5’-cAMP in the hypertensive kidney, we recently developed an assay to measure 3’,5’-cAMP in the renal venous effluent from isolated, perfused kidneys (Ren et al., 2008). Unlike the commercially available kits for 3’,5’-cAMP that rely upon the selectivity of antibody recognition, our assay was based on the platform technology of high-performance liquid chromatography-tandem mass spectrometry (Ren et al., 2008).

While investigating the production of 3’,5’-cAMP from isolated, perfused kidneys obtained from normotensive and hypertensive rats, we observed the release of a substance with
mass spectral characteristics similar to 3',5'-cAMP, but that was clearly not 3’,5’-cAMP. The purpose of this report is to describe the identification and quantification of this substance and to describe its origin and regulation.
METHODS

Animals. Studies utilized adult (20 weeks-of-age) male spontaneously hypertensive rats (SHR) and male normotensive Wistar-Kyoto (WKY) rats obtained from Taconic Farms (Germantown, NY). The Institutional Animal Care and Use Committee approved all procedures. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Isolated, Perfused Kidney Preparation. Rats were anesthetized with Inactin (90 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO), and the left kidney was isolated and perfused with Tyrode’s solution containing 3-isobutyl-1-methylxanthine (10 μmol/L; an inhibitor of phosphodiesterases; Sigma-Aldrich, St. Louis, MO) using a Hugo Sachs Elektronik-Harvard Apparatus GmbH (March-Hugstetten, Germany) kidney perfusion system as previously described (Gao et al., 2003). Briefly, all branches of the left renal artery and vein were ligated. A polyethylene-50 cannula was placed into the left renal artery, and a polyethylene-90 cannula was placed into the left renal vein. The left kidney was removed, attached to the perfusion system and kidneys were perfused (single pass mode) at a constant flow (5 ml/min), and perfusion pressure was monitored with a pressure transducer.

Protocols. Kidneys were isolated from adult SHR and WKY and perfused in vitro as described above. After a 30 to 60-minute stabilization period, perfusate exiting the renal vein was collected for 1 minute and immediately placed on ice. Next, in some experiments, isoproterenol (a β-adrenoceptor agonist; 1 μmol/L; Sigma-Aldrich) was added to the arterial perfusate, and five minutes later another 1-minute renal venous sample was collected. In other experiments, after the basal sample of venous perfusate was collected, the kidney was treated
with either rapamycin (0.2 μmol/L; Sigma-Aldrich) or iodoacetate + 2,4-dinitrophenol (50 μmol/L each; Sigma-Aldrich) and venous sampling was repeated at the indicated times.

**High Performance Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS).**

3',5'-cAMP and 2',3'-cAMP were purchased from Sigma-Aldrich. The internal standard (^{13}C_{10}-adenosine) was from Medical Isotopes Inc. (Pelham, NH). All standards were stored at -20°C, and the internal standard was stored at -80°C. Solutions of standards (50 ng/μL) and internal standard (1 ng/μL) were prepared in ultrapure water and stored at -20°C. The mixed solution of cAMPs (1 ng/μL) was prepared each day by dilution in ultrapure water and kept at 4°C. Additional dilutions of standards were prepared from this solution by serial dilution. Methanol (for LC-MS/MS) was from Riedel-Dehae (Seelze, Germany), and analytical grade formic acid was from Fluka (Buchs, Switzerland).

3',5'-cAMP and 2',3'-cAMP were resolved by reversed-phase LC (Agilent Zorbax eclipse XDB-C-18 column, 3.5 μm beads; 2.1x100 mm) and quantified using a triple quadrupole MS (TSQ Quantum-Ultra, ThermoFisher Scientific, San Jose, CA) operating in the selective reaction monitoring (SRM) mode with a heated electrospray ionization source. The mobile phases were delivered by an ultra pressure LC system (Accela, ThermoFisher Scientific, San Jose, CA) and consisted of linear gradient changes involving two buffers: Buffer A was 0.1% formic acid in water; Buffer B was 0.1% formic acid in methanol. The mobile phase flow rate was 300 μL/min. The gradient (A/B) was: 0 to 2 min, 98.5%/1.5%; 2 to 4 min, to 98%/2%; 5 to 6 min, to 92%/8%; 7 to 8 min, to 85%/15%; 9 to 11.5 min, to 98.5%/1.5%. Sample tray temperature was set at 4°C and the column temperature was kept at 20°C.

For maximum sensitivities, instrument parameters were optimized as follows: ion spray voltage, 3.8 kV; ion transfer capillary temperature, 270°C; source vaporization temperature,
220°C; Q2 CID gas, argon at 1.5 mTorr; sheath gas, nitrogen at 50 arbitrary units; auxiliary gas, nitrogen at 40 arbitrary units; Q1 and Q3 resolution, 0.70 u full-width half-maximum; source CID, off; scan width, 0.2 u; scan time, 0.05 seconds; and tube lens offset, 123 V. Two SRM transitions were monitored: 278 m/z → 141 m/z for $^{13}$C$_{10}$-adenosine as internal standard with a collision energy of 19 V and 330 m/z → 136 m/z for 3’,5’-cAMP and 2’,3’-cAMP with a collision energy of 28 V. Calibration standard curves were constructed at concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 and 10 pg/μL in ultrapure water.

**Statistics.** Data were analyzed by 2-factor analysis of variance (ANOVA). The criterion of significance was p<0.05. All values in text and figures are means ± SEM.
RESULTS

As shown in Figure 1, with selective reaction monitoring (SRM; 330 m/z → 136 m/z), injection of a sample of renal venous perfusate into the LC-MS/MS system gave rise to two prominent peaks in the chromatogram, one with a retention time similar to that of authentic 3',5'-cAMP (approximately 6.3 minutes) and the other with a retention time of approximately 2.9 minutes. When a renal venous perfusate sample was obtained from the same kidney as shown in Figure 1 but during administration of isoproterenol, an agonist that stimulates adenylyl cyclase via β-adrenoceptors, the 3',5'-cAMP peak was greatly increased, whereas the unknown peak was not (Figure 2). Thus, it was unlikely that the unknown peak was endogenous 3',5'-cAMP. For the unknown substance giving rise to the peak at 2.9 minutes to be observed, the substance would have to have a parent ion of 330 m/z (molecular weight of 3',5'-cAMP plus 1) with a daughter fragment of 136 m/z (molecular weight of adenine fragment plus 1). We hypothesized, therefore, that the unknown substance was a positional isomer of 3',5'-cAMP, possibly 2',3'-cAMP. Because, at least to our knowledge, 2',3'-cAMP has not been reported as an endogenous product released from intact cells, tissues or organs, we thought it necessary to secure the identity of the putative 2',3'-cAMP peak. Therefore, we obtained authentic 2',3'-cAMP and determined its retention time relative to the unknown substance using our LC-MS/MS system, again with selective reaction monitoring (SRM; 330 m/z → 136 m/z). As shown in Figure 3, authentic 2',3'-cAMP and the unknown substance had the same retention time (approximately 2.9 minutes). To further confirm the identity of the putative 2',3'-cAMP peak, we obtained another renal venous perfusate sample, split the sample, added authentic 2',3'-cAMP to one part and injected both samples. The area under the putative 2',3'-cAMP peak
increased from 545,047 arbitrary units to 1,763,662 arbitrary units with the addition of authentic 2',3'-cAMP, and the ratio of the area under the putative 2',3'-cAMP peak to the area under the 3',5'-cAMP peak increased from 1.69 to 3.24. Thus, addition of authentic 2',3'-cAMP increased the signal of the putative 2',3'-cAMP peak without introducing any new peaks in the chromatogram. This indicated that the substance giving rise to the putative 2',3'-cAMP peak had the precise retention time, parent ion and daughter ion of authentic 2',3'-cAMP.

To further secure the identity of the substance giving rise to the putative 2',3'-cAMP peak, we injected authentic 2',3'-cAMP and authentic 3',5'-cAMP into the LC-MS/MS system, and then acquired mass spectral scans from 100 m/z to 400 m/z at different collision energies to fragment the parent ion. As shown in Figure 4A, at a collision energy of 10 V, both authentic 2',3'-cAMP and authentic 3',5'-cAMP gave rise to a mass spectrum consisting of mainly the parent ion (330 m/z). Thus, 10 V was insufficient to fragment the parent ion of either form of cAMP. However, when the collision energy was increased to 18 V (Figure 4B), there was substantial fragmentation of authentic 2',3'-cAMP to the 136 m/z fragment; in fact the 136 m/z fragment became the most abundant ion. In contrast, although authentic 3',5'-cAMP showed some fragmentation at 18 V of collision energy, the 330 m/z remained the most abundant ion. For both forms of cAMP, at a collision energy of 23 V, mainly the 136 m/z daughter ion was observed (Figure 4C). These data indicate that 2',3'-cAMP can be further identified and differentiated from 3',5'-cAMP by using the collision energy necessary to fragment the parent ion.

As shown in Figure 5A, when renal venous perfusate was injected into the LC-MS/MS and a mass spectral scan was obtained at the center of the putative 2',3'-cAMP peak and the 3',5'-cAMP peak, at 10 V of collision energy, only a single mass was observed at 330 m/z for
both peaks. Importantly, when the collision energy was increased to 18 V (Figure 5B), for the putative 2',3'-cAMP peak, the major fragment was 136 m/z; whereas for the 3',5'-cAMP peak, the major fragment was still 330 m/z. At 23 V of collision energy (Figure 5C), both the putative 2',3'-cAMP and 3',5'-cAMP peaks gave rise mainly to a daughter ion of 136 m/z. Thus the susceptibility of the putative 2',3'-cAMP peak to fragmentation by collision energy was very similar to that observed for authentic 2',3'-cAMP and was different from that observed for 3',5'-cAMP.

Taken together, the above results indicate that the putative 2',3'-cAMP peak was indeed, and without any reasonable doubt, 2',3'-cAMP. Therefore, we turned our attention to quantifying the amounts of 2',3'-cAMP versus 3',5'-cAMP release from isolated, perfused kidneys obtained from 20 week-old SHR and WKY rats. As shown in Figure 6, the basal renal venous secretion of 3',5'-cAMP was similar in SHR versus WKY kidneys and was significantly (p<0.0001) and similarly increased by isoproterenol (7.7-fold and 6.2-fold in SHR and WKY kidneys, respectively). In contrast, the renal venous 2',3'-cAMP secretion was 2.4-fold greater (p=0.0052) in SHR compared with WKY kidneys and isoproterenol had no effect on the renal venous secretion of 2',3'-cAMP (Figure 7).

Biochemical studies suggest that 2',3'-cAMP can be produced by RNases (Thompson et al., 1994), and recent evidence supports enhanced mRNA turnover in SHR tissues (Klöss et al., 2005). Therefore, we hypothesized that 2',3'-cAMP may derive from degradation of mRNA. To test this hypothesis we used two approaches to accelerate mRNA turnover. One approach was infusion of rapamycin, a drug that activates mRNA turnover via the mTOR pathway (Banholzer et al., 1997; Hashemolhosseini et al., 1998; Albig and Decker, 2001), into the kidney. In this experimental series, 4 kidneys were from SHR and 4 were from WKY; however, the
results were similar so the data were combined. As shown in Figure 8, rapamycin caused a time-related and large (approximately 1000% of basal; p=0.0381) increase in renal venous 2’,3’-cAMP secretion while inhibiting the renal secretion of 3’,5’-cAMP (approximately 50% of basal; p=0.0007).

Because previously published studies show that tissue ischemia triggers mRNA degradation (Akahane et al., 2001a; Akahane et al., 2001b; Almeida et al., 2004), in a second approach to stimulate mRNA degradation, we treated kidneys with a combination of metabolic inhibitors (iodoacetate, inhibitor of glycolysis; 2,4-dinitrophenol, inhibitor of oxidative phosphorylation). In this experimental series, 4 kidneys were from SHR and 2 were from WKY; however, as with the rapamycin study the results were similar so the data were combined. As shown in Figure 9, the combination of iodoacetate + 2,4-dinitrophenol caused a time-related and massive (approximately 4100% of basal; p<0.0001) increase in renal venous 2’,3’-cAMP secretion while inhibiting the renal secretion of 3’,5’-cAMP (approximately 40% of basal; p=0.0002).
DISCUSSION

The present study confirms that 2’,3’-cAMP is released from the kidney. What is the source of 2’,3’-cAMP? It is well-known that mRNA is degraded by the action of multiple ribonucleases (RNases), and that RNases catalyze the hydrolysis of the P-O^5' bond of mRNA (Wilusz et al., 2001). What is not widely appreciated is that this hydrolysis reaction, at least with isolated RNases, proceeds via two steps: transphosphorylation to form 2’,3’-cyclic nucleotides (such as 2’,3’-cAMP (right panel of Figure 10) which differs from 3’,5’-cAMP (left panel of Figure 10)) and hydrolysis of these cyclic intermediates to form 3’-nucleotides (Markham and Smith, 1952). Studies by Thompson et al. (Thompson et al., 1994) using ^31P NMR spectroscopy to monitor the accumulation of the 2’,3’-cyclic nucleotides during the transphosphorylation and hydrolysis reactions catalyzed by various RNases and by small molecules confirm this conclusion. In this regard, the experiments by Thompson et al. show that 2’,3’-cyclic nucleotides accumulate during catalysis by monomeric RNase A, a dimer and a trimer of RNase A, bovine seminal RNase, RNase TI, barnase and RNase I. These enzymes, which are of widely disparate phylogenetic origin, release rather than hydrolyze, most of the intermediate 2’,3’-cyclic nucleotides formed by transphosphorylation of mRNA. In contrast, 2’,3’-cyclic nucleotide intermediates do not accumulate during catalysis of mRNA by hydroxide ion or imidazole buffer (Thompson et al., 1994). Moreover, trapping experiments to assess the throughput of the reaction catalyzed by RNase A show that only 0.1% of the mRNA substrate is found to be both transphosphorylated and hydrolyzed without dissociating from the enzyme (Thompson et al., 1994).
The biochemical findings in isolated systems described above suggest, but do not prove, that mRNA degradation is the source of 2',3'-cAMP from intact tissues. Importantly, the present study demonstrates that both rapamycin and metabolic inhibitors cause a massive increase in the renal venous secretion of 2',3'-cAMP while simultaneously decreasing the renal production of 3',5'-cAMP. These findings add support to the conclusion that mRNA is the source of 2',3'-cAMP released from the isolated, perfused kidney because both rapamycin (Banholzer et al., 1997; Hashemolhosseini et al., 1998; Albig and Decker, 2001) via the mTOR pathway and energy depletion via tissue ischemia (Akahane et al., 2001a; Akahane et al., 2001b; Almeida et al., 2004) are general activators of mRNA degradation.

Based on the aforementioned considerations, we conclude that the most likely source of 2',3'-cAMP in the kidney is mRNA. Because most eukaryotic mRNAs contain a polyadenine tail, eukaryotic mRNA contains on average greater than 25% adenine nucleotides and therefore mRNA turnover would be an extremely efficient mechanism for generating intracellular 2',3'-cAMP. Moreover, because mRNA turnover is initiated by hydrolysis of the polyadenine tail (Wilusz et al., 2001), release of 2',3'-cAMP would be a leading event in the degradation of mRNA.

Would 2',3'-cAMP be expected to egress to the extracellular compartment? Research by us (Jackson and Mi, 2000; Dubey et al., 2001; Jackson and Dubey, 2001; Jackson et al., 2003; Jackson et al., 2006; Jackson et al., 2007), as well as by others (Davoren et al., 1963; Broadus et al., 1970; Kuster et al., 1973; Cramer and Lindl, 1974; King and Mayer, 1974; Doore et al., 1975; O'Brien and Strange, 1975; Brunton and Mayer, 1979; Barber and Butcher, 1983; Fehr et al., 1990; Kather, 1990; Li et al., 2007; Chiavegatti et al., 2008; Giron et al., 2008), demonstrate that 3',5'-cAMP (the product of adenylyl cyclase) is robustly and actively transported to the
extracellular compartment. In this regard, our unpublished studies show that the active transporter, multidrug resistance protein 4 (MRP4), is responsible for most, if not all, of the transport (egress or efflux) of 3’,5’-cAMP from the cytoplasm to the extracellular space in preglomerular vascular smooth muscle cells (manuscript in preparation). MRP4 and the related MRP5 are widely expressed and relatively indiscriminant organic anion transporters (Kruh et al., 2001; Deeley et al., 2006; Borst et al., 2007) that differ from other MRP-like proteins in their ability to transport cyclic nucleotides (Kruh et al., 2001; Deeley et al., 2006; Borst et al., 2007). Thus, MRP4 and MRP5 are likely involved in determining extracellular levels of cyclic nucleotides. MRP4, in particular, is expressed at relatively high levels in kidney cells, where it contributes to urinary excretion of 3’,5’-cAMP and 3’,5’-cGMP (van Aubel et al., 2002). It is very likely therefore that 2’,3’-cAMP produced by mRNA turnover would egress to the extracellular space via active transport by MRP4, MRP5 or both, depending on the cell type.

The results of the present study clearly support the concept that endogenous 2’,3’-cAMP formed from the metabolism of mRNA actually appears in the extracellular compartment.

Could extracellular levels of 2’,3’-cAMP be used as a convenient and easily accessible biomarker for mRNA turnover/degradation? Because 2’,3’-cAMP is known to be made by RNases and because cyclic nucleotides can be transported out of cells by MRPs, measurement of 2’,3’-cAMP in the extracellular compartment of tissues and organs most likely is a biomarker for mRNA turnover/degradation. The elevated production of 2’,3’-cAMP in SHR kidneys supports this conclusion. Klöss et al. report a greater than 50% reduction in the expression of human-antigen R in the aortas from SHR with long-standing hypertension, but not in aortas from prehypertensive SHR (Klöss et al., 2005). The elav-like (embryonic-lethal abnormal vision) mRNA-binding protein human-antigen R stabilizes many mRNAs by binding to highly
conserved AU-rich elements (AREs; AUUUA) in the 3’-untranslated region (3’-UTR) (Fan and Steitz, 1998). AREs are targeted for rapid mRNA decay, and thus the presence of AREs within the 3’-UTR of numerous mRNAs plays a critical role in regulating mRNA stability and degradation (Fan and Steitz, 1998). Therefore, a reduction in human-antigen R in organs from animals with long-standing hypertension would be predicted to result in decreased mRNA stability, increased mRNA degradation and therefore increased extracellular levels of 2′,3′-cAMP.

In the present study we were able to differentiate 2′,3′-cAMP from 3′,5′-cAMP using LC-MS/MS. Do commercially available assay kits for 3′,5′-cAMP discern between these positional isomers of cAMP? Although we cannot generalize to all commercially available kits, we have examined the popular Amersham cAMP Biotrak Enzymeimmunoassay System (GE Healthcare, Piscataway, NJ) and find that this kit does not detect 2′,3′-cAMP.

In summary, this study shows for the first time that 2′,3′-cAMP is produced by an intact organ and is secreted into the extracellular compartment. It is noteworthy that polyadenine tails of mRNA would provide a large cellular reservoir of potential 2′,3′-cAMP, that hydrolysis of the polyadenine tail of eukaryotic mRNA is an early event in mRNA turnover/breakdown and that 2′,3′-cAMP is likely transported to the extracellular compartment. Thus it is conceivable that an important role of the polyadenine tail of mRNA is to provide, in response to tissue injury, extracellular 2′,3′-cAMP which might then mediate biological functions, for example tissue protection, and this possibility is under investigation.
REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1: LC-MS/MS SRM chromatogram of renal venous perfusate obtained from untreated, isolated, and perfused SHR kidney. Two transitions were monitored: 278 m/z → 141 m/z for the internal standard (top panel) which was $^{13}$C$_{10}$-adenosine; 330 m/z → 136 m/z for endogenous 3',5'-cAMP (bottom panel). Note the prominent peak with a retention time of approximately 2.9 minutes (bottom panel), which was much too short to be 3',5'-cAMP which has a retention time of approximately 6.3 minutes.

Figure 2: Figure illustrates a chromatogram of renal venous perfusate obtained from the same kidney as in Figure 1, but during the administration of isoproterenol (1 μmol/L). Two transitions were monitored: 278 m/z → 141 m/z for the internal standard (top panel) which was $^{13}$C$_{10}$-adenosine; 330 m/z → 136 m/z for endogenous 3',5'-cAMP (bottom panel). Comparing to Figure 1, note the marked increase in the area of the peak corresponding to 3',5'-cAMP (6.3 minutes), whereas the area of the unknown peak (2.9 minutes) was little changed.

Figure 3: Figure illustrates a chromatogram of a renal venous sample (top panel) versus authentic 2',3'-cAMP (bottom panel). The same transition was monitored in each panel: 330 m/z → 136 m/z for endogenous 3',5'-cAMP. Note that authentic 2',3'-cAMP had a retention time precisely that of the unknown substance.

Figure 4: Panels A, B and C illustrate the mass spectrum of authentic 2',3'-cAMP and 3',5'-cAMP at different levels of collision energy (10, 18 and 23 V, respectively).
Figure 5: Panels A, B and C illustrate the mass spectrum of the putative 2',3'-cAMP peak and 3',5'-cAMP peak at different levels of collision energy (10, 18 and 23 V, respectively).

Figure 6: Bar graph depicts the renal venous secretion rate of 3',5'-cAMP in kidneys obtained from SHR and WKY rats both under basal conditions and with the addition of isoproterenol (1 μmol/L) to stimulate adenylyl cyclase via β-adrenoceptors. Statistical analysis is from 2-factor analysis of variance.

Figure 7: Bar graph depicts the renal venous secretion rate of 2',3'-cAMP in kidneys obtained from SHR and WKY rats both under basal conditions and with the addition of isoproterenol (1 μmol/L) to stimulate adenylyl cyclase via β-adrenoceptors. Statistical analysis is from 2-factor analysis of variance.

Figure 8: Line graph depicts the time-related effects of rapamycin (0.2 μmol/L) on renal venous secretion rate of 2',3'-cAMP and 3',5'-cAMP in kidneys obtained from 4 SHR and 4 WKY rats. The effects of rapamycin were independent of strain so the results from all 8 kidneys were combined. Data are shown as % of time 0 (basal) levels of 2',3'-cAMP and 3',5'-cAMP at the indicated time after administration of rapamycin. Statistical analysis is from 2-factor analysis of variance.

Figure 9: Line graph depicts the time-related effects of iodoacetate + 2,4-dinitrophenol (50 μmol/L) on renal venous secretion rate of 2',3'-cAMP and 3',5'-cAMP in kidneys obtained from
4 SHR and 2 WKY rats. The effects of iodoacetate + 2,4-dinitrophenol were independent of strain so the results from all 6 kidneys were combined. Data are shown as % of time 0 (basal) levels of 2’,3’-cAMP and 3’,5’-cAMP at the indicated time after administration of iodoacetate + 2,4-dinitrophenol. Statistical analysis is from 2-factor analysis of variance.

Figure 10: Comparison of chemical structures for 3’,5’-cAMP (left panel) versus 2’,3’-cAMP (right panel).
Renal Venous Perfusate Sample: Basal

Internal Standard

SRM: 278 m/z → 141 m/z
Collision Energy: 19 volts

Unknown

SRM: 330 m/z → 136 m/z
Collision Energy: 28 volts

3',5'-cAMP

Retention Time (minutes)
Renal Venous Perfusate Sample: During Isoproterenol

Figure 2

SRM: 278 m/z → 141 m/z
Collision Energy: 19 volts

SRM: 330 m/z → 136 m/z
Collision Energy: 28 volts

Internal Standard

Unknown

3',5'-cAMP
Figure 3

Renal Venous Perfusate Sample: During Isoproterenol

Relative Abundance (Full Scale: 1.3 x 10^4)

- Unknown
- 3',5'-cAMP

SRM: 330 m/z → 136 m/z
Collision Energy: 28 volts

Relative Abundance (Full Scale: 1.0 x 10^4)

- 2',3'-cAMP
- Authentic 2',3'-cAMP

SRM: 330 m/z → 136 m/z
Collision Energy: 28 volts
Figure 4A

Collision Energy: 10 volts

Scan of Authentic 2',3'-cAMP

Scan of Authentic 3',5'-cAMP
Figure 4B

Collision Energy: 18 volts

Scan of Authentic
2',3'-cAMP

Scan of Authentic
3',5'-cAMP

Relative Abundance

m/z
Collision Energy: 23 volts

Scan of Authentic 2',3'-cAMP

Scan of Authentic 3',5'-cAMP

Figure 4C
Collision Energy: 10 volts

Scan of “2’,3’-cAMP” Peak

Scan of “3’,5’-cAMP” Peak

Figure 5A
Collision Energy: 18 volts

Scan of “2’,3’-cAMP” Peak

Scan of “3’,5’-cAMP” Peak

Figure 5B
Collision Energy: 23 volts

Scan of “2’,3’-cAMP” Peak

Scan of “3’,5’-cAMP” Peak

Figure 5C
Figure 6

3',5'-cAMP

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ng/min/gram kidney

2-Factor ANOVA

- Strain: p=0.8427
- Isoproterenol: p<0.0001
Figure 7

2',3'-cAMP

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2-Factor ANOVA

Strain: p=0.0052
Isoproterenol: p=0.4088
Figure 8
Figure 9
Figure 10

3',5'-cAMP

2',3'-cAMP