The Isolation and Characterization of a Novel Corticostatin/Defensin-like Peptide from the Kidney*

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We report the isolation and characterization of RK-1, a novel peptide found in the kidney. RK-1 is related to the corticostatin/defensins and has the sequence MPC-SCKYCDPWEIDGSCGLFNKCYICREK but differs from the very cationic corticostatins/defensins in having only one arginine and a calculated charge at pH 7 of +1. Like some myeloid corticostatin/defensins RK-1 inhibits the growth of Escherichia coli. Since corticostatin/defensins effect ion flux in responsive epithelia we used volume changes in villus enterocytes as a model system to study the effects of RK-1 on ion channels in epithelial cells. At concentrations 10^{-9} M RK-1 decreased enterocyte volume in a dose-dependent manner through a pathway that requires extracellular calcium and is inhibited by nigerulpine, a dihydrorpyridine-sensitive "L"-type Ca^{2+}-channel blocker. In other assay systems for corticostatin/defensins, such as the inhibition of adrenocorticotropic hormone-stimulated steroidogenesis, or cell lysis, RK-1 was inactive or only weakly active. These results demonstrate the existence of a novel system of biologically active peptides in the kidney represented by RK-1 which is antimicrobial and can activate epithelial ion channels in vitro.

Corticostatin/defensins (CS/def)s\(^1\) are low molecular weight arginine-rich peptides with between 29 and 34 amino acids (1\textendash11) and three disulfide bridges (12). Many CS/defs are antimicrobial at micromolar concentrations (1) probably as a result of their ability to create voltage-dependent pores by insertion into the lipid phase of biological membranes (13). Some CS/defs, the corticostatins, are specific antagonists of the hormone adrenocorticotropic (3, 5, 6, 9, 11, 14, 15), some are chemotactic for monocytes at 10^{-10} M (16), some are cytotatic for HL-60 promyelocyte-like cells at 10^{-9} and 10^{-8} M (17), and some inhibit natural killer-cell activity in vitro at 10^{-9} M (18).

Many CS/defs stimulate a calcium-dependent volume reduction in villus enterocytes at concentrations of 10^{-10} to 10^{-8} M (19) in response to changes in the ion flux across the cell membrane. The maintenance of cell volume in villus epithelial cells in isotonic medium reflects a balance between the rates of salt influx and efflux (20). The addition of calcium ionophore to these cells under isotonic conditions activates charybdotoxin-sensitive K^{+} channels (21) and calcium-activated Cl^{-} channels (21). The consequent loss of these osmolytes together with water results in a reduction in cellular volume. The volume response to CS/defs requires extracellular calcium and is dihydrorpyridine-sensitive implying a pivotal role for L-type calcium channels in this process.

The expression of genes for CS/def-like polypeptides is not confined to myeloid cells. The intestine contains antimicrobial CS/def-like peptides called cryptidins found in Paneth's cells of the crypt but not in cells of the inflammatory system (22\textendash25). The ability of some CS/defs to elicit water movement in enterocytes (19) may contribute to the onset of diarrhea, as has been suggested for guanylin, another peptide found in Paneth's cells (26). Interestingly a further system of cationic cystine-rich peptides, the f-defensins, that are structurally distinct from CS/defs occurs in bovine tracheal epithelia (27) and in bovine neutrophils (28), suggesting that specialized peptidergic defensive systems may occur in several tissues.

The existence of two specialized families of CS/def-like peptides, the defensins and the cryptidins, raises the possibility of other tissue-specific CS/def-related systems. Urinary tract infections may lead to kidney failure and are a significant source of Gram-negative septicemia (29). Neither myeloid nor enteric CS/defs occur in the kidney, but other kidney-specific CS/def-like peptides might contribute to the defense mechanisms of the kidney.

We used a homology screening system based on the unusual physicochemical properties of the CS/defs to detect renal-specific peptides. Chemical assays have been successful in the isolation of biologically active gastroendocrine peptides (30) but have rarely been attempted in other fields. Here we used three selection criteria: the elution position on reverse phase HPLC, an apparent size of approximately 30 residues assessed by size-exclusion HPLC and amino acid analysis, and a cystine content of about 20%. Using these criteria we isolated a peptide, RK-1, that is abundant in the kidney and is a distant relative of the myeloid/enteric CS/defs. Having proven the existence of a renal CS/def-like peptide, RK-1 was then subjected to a series of assays characteristic of the myeloid CS/defs to compare its activities to those of the classic CS/def system. These experiments show that RK-1 possesses the Ca^{2+} channel activating effects associated with the myeloid CS/defs.

MATERIALS AND METHODS

Tissue Extracts—Freshly dissected whole rabbit kidneys, or frozen kidneys or kidney cortex (Pel-Freez, Rogers, AK), were extracted by homogenization between 1 and 5 min (depending on amount of tissue being homogenized) using a Polytron homogenizer in a high salt low pH...
buffer (1 M HCl, 5% formic acid, 1% NaCl, and 1% trifluoroacetic acid, 4 °C). A volume to weight ratio of approximately 10:1 extraction medi-

tum-ultracentrifugation was used. Fresh tissue was diced before homogenization, and 

frozen tissue was homogenized whole while still frozen. The high acidity and 

high ionic strength of this buffer solubilizes peptides but precipi-

tates most higher molecular weight proteins and inhibits most proteo-

lytic enzymes. In previous studies we showed that the structure of 

human myeloid defensins extracted from lung tissues was identical to 

that obtained from peripheral neutrophils when using this extraction 

protocol (17). After centrifugation at 2000 × g for 20 min at 4 °C, 

the pellet was re-extracted as above, and the pooled supernatants filtered 

over glass wool in a cold room to remove low density solids. The filtered 

extract was passed through ODS cartridges (Sep-Pak C18 Bondapak, 

Waters, Milford, MA) connected in series and sequentially eluted first 

using 15% acetonitrile in 0.1% trifluoroacetic acid, then 40% aceton-

itrile in 0.1% trifluoroacetic acid, and finally 80% acetonitrile in 0.1% 

trifluoroacetic acid. The sequential elution allows us to take advantage 

of the differential retention of some peptides between C18 µBondapak 

cartridges and C18 µBondapak columns. This greatly simplifies the 

purification. The eluates were lyophilized and stored at −40 °C until 

use.

HPLC Purification Procedures—The Sep-Pak eluates were resus-

pended in 0.1% trifluoroacetic acid and fractionated by reversed-phase 

HPLC using a Waters C18 µBondapak column (7.8 mm × 30 cm) eluted 

over a 3-h period using a gradient of 0–80% acetonitrile in 0.1% tri-

fluoroacetic acid and an elution rate of 1.5 ml min⁻¹ with a 10-min 
equilibration period in 0.1% trifluoroacetic acid before the gradient. 

Fractions eluting close to the elution positions of rabbit, rat, and human 

CS/defs were subjected to amino acid analysis to determine the cystine 

content. Fractions with a high cystine content were subsequently puri-

fied by size-exclusion HPLC using two Waters 1-125 Protein-Pak col-

umns connected in series and eluted in 40% acetonitrile with 0.1% 

 trifluoroacetic acid at a flow rate of 1 ml min⁻¹. UV absorbance fractions 

were again subjected to amino acid analysis. Fractions with a high 
cystine content, and which eluted on size exclusion chromatography 
in the position of unreduced CS/defs, were further purified using a second 
reversed-phase gradient of 12–24% acetonitrile in 0.1% trifluoroacetic 
acid on a 10×250 mm column (3.9 mm × 30 cm). At this stage the peptide was 

pure as judged by amino acid analysis, electrospray mass spectrometry, and 
gas-phase microsequencing.

Amino Acid Analysis and Microsequencing—Amino acid analysis, 

and S-pyridylethylated peptides were performed as described previously (6). 

Purified S-pyridylethylated peptides were then subjected to gas-phase 

Edman microsequencing using an Applied Biosystems (Foster City, CA) 

Model 477A Gas-Liquid protein sequencer with a microscale reaction 

cartridge and Applied Biosystems' MIFCST program cycles (31). 

Villus Cell Assay—Villus cells were isolated as described elsewhere 

(20). Briefly, segments of adult guinea pig jejunum were placed 

over metal spiral rods and vibrated in Ca²⁺-free phosphate-buffered phys-

iologic (pH approximately 6.0). After 12 h aliquots of the cell suspension were 
diluted in a 10 × serial fashion, streaked onto agar plates using a 

flamed loop, and then incubated overnight at 37 °C.

Cell Growth Assays—All cell lines were obtained from the American 

Type Culture Collection (ATCC, Rockville, MD). [H]Thymidine incorpo-

ration was performed as follows (33). 0.8 × 10⁵ cells/well of a 24-

multiwell plate were incubated for 24 h in RPMI 1640 medium (Flow 

Laboratories, McLean, VA) supplemented with 10% fetal bovine serum. 

The medium was removed and washed twice with serum-free medium, 

and the cells incubated in serum-free medium with various concentra-

tions of the human defensin HP-1 or the purified rabbit kidney peptide 

for 24 h. [H]Thymidine incorporation was then determined as de-

scribed (see Ref. 33). Cell counts were not performed because we have 

previously shown that the decrease in thymidine incorporation in re-

sponse to HP-1 is a function of cell death (33). Cells used were A549, 

SK-MES-1, and CHO-K1, which are derived from a lung carcinoma, 

a pleural effusion from a patient with a squamous carcinoma of the lung, 

and Chinese hamster ovariess, respectively. These cells were chosen 

because we had previously shown they are among the most responsive 

cell lines to the cytotoxic effects of myeloid defensins (5, 17).

RESULTS

Purification of RK-1—Rabbit kidney extracts were fractionated 

by RP-HPLC and analyzed for cystine content across the region of the chromatogram corresponding to the elution positions of the 

myeloid CS/defs (Fig. 1A). A cystine-rich compound was found eluting at fractions 70–73 (about 24–26% acetonitrile) in the 15–40% acetonitrile eluate of the C18 Sep-Pak (Fig. 1A). No cystinyl compounds were found in the 0–15% acetonitrile
trile eluate of the C₁₈ Sep-Pak although when chromato-
graphed this material overlaps the cysteine-rich region in Fig.
1A. Clearly therefore the sequential elution of the Sep-Paks
greatly simplifies the purification. The major cysteine-rich
component from Fig. 1A (fraction 72–75, indicated by an arrow)
was fractionated by size-exclusion HPLC (not shown), and
eluted in the position of the known CS/defs. The less abundant
cystine containing component in Fig. 1A (fraction 67, 68, and
69) eluted by size-exclusion chromatography in the approxi-
mate position of insulin (6 kDa, not shown). The CS/def-sized
material, which we called RK-1, was then purified to homoge-
neity by a second step of RP-HPLC fractionation (Fig. 1B) and
its purity assessed by amino acid analysis. The final yield of
RK-1 is 6.04 nmol/kidney or 0.81 nmol/g wet weight (n = 4, 40
kidneys per determination). We were unable to detect RK-1 in
other tissues (bone marrow, lung, and liver) using the chro-
matographic and analytical techniques used to identify RK-1 in
the kidney.

Sequence Analysis of RK-1—Approximately 80 pmol of the
intact pyridylethylated derivative of RK-1 was subjected to
gas-phase microsequence analysis which identified all residues
except residue 29. The average repetitive yield was 89%. To
identify residue 29 a total of 200 pmol of the pyridylethylated
peptide was digested with Asp-N-protease, which digests on
the N-terminal side of aspartic acid, and the digest was then
fractionated by RP-HPLC. The C-terminal digestion fragment
was identified by amino acid analysis. Approximately 50 pmol
were sequenced with an average repetitive yield of 86.3% giv-
ing a sequence corresponding to residues 16–32 in the se-
dence given below. The deduced amino acid composition of the
intact peptide corresponds closely with the composition deter-
mined by amino acid analysis (Table I). RK-1 and pyridyleth-
ylated RK-1 were also analyzed by electrospray mass spectrom-
ya. The amino acid analysis and ratio of amino acids obtained by amino
acid analysis of RK-1 and the theoretical ratio of amino acids deduced
from sequence analysis and electrospray mass spectrometry

| Amino acid | Pmoles | AA ratio | Theoretical AA ratio |
|------------|--------|----------|----------------------|
| Asp        | 364.76 | 3.29     | 3.00                 |
| Glu        | 313.43 | 2.83     | 2.00                 |
| Ser        | 308.76 | 2.78     | 3.00                 |
| Gly        | 250.60 | 2.26     | 2.00                 |
| His        | 0.00   |          |                      |
| Arg        | 131.38 | 1.18     | 1.00                 |
| Thr        | 0.00   |          |                      |
| Pro        | 251.24 | 2.27     | 2.00                 |
| Tyr        | 197.90 | 1.78     | 2.00                 |
| Val        | 105.63 | 0.95     | 1.00                 |
| Met        | 56.99  | 0.51     | 1.00                 |
| Cys        | 553.82 | 4.99     | 6.00                 |
| Ile        | 210.89 | 1.90     | 2.00                 |
| Leu        | 116.23 | 1.05     | 1.00                 |
| Phe        | 123.08 | 1.11     | 1.00                 |
| Trp        | 0.00   |          | 1.00                 |
| Lys        | 452.60 | 4.08     | 4.00                 |
| Other      | 0.00   |          |                      |

The sequences are aligned at the cysteines with dashes introduced to maximize the alignment.

- RK-1: Ser-Lys-Tyr-Cys*-Asp-Pro-Trp-Glu*'-Val-Ile-Asp-Gly*-Ser-Cys*-Gly-Leu-Phe-Asn-Ser-Lys-Tyr-Ile-Cys*-Cys*-Arg-Glu-Lys (where * indicates a residue conserved with the myeloid/enteric CS/defs)

This sequence is compared with representatives of the myeloid and enteric CS/defs in Table II and as a dendrogram in Fig. 2. The distance between peptides along the branches represents their relatedness expressed in arbitrary units. For example, HP-1 and HP-3 which differ by one amino acid are close, whereas HP-1 and HP-4 which have only 11 amino acids in common are placed far apart on the dendrogram. In this analysis there is no more difference between defensins and cryptdins than between defensins of different species. In contrast RK-1 maps on a separate limb of the den-

**Effect of RK-1 on the Growth of E. coli in Culture**—As shown in Fig. 3 RK-1 is antimicrobial at concentrations between 15 and 150 μg/ml. This is compared with the bone marrow corti-
ocostatin/defensin NP-2 in Fig. 3 which is approximately 10-fold
more active in this assay.

**Effect of RK-1 on Villus Cell Volume in the Presence of Na⁺ or in a Na⁺-free Medium**—The addition of RK-1 (10⁻⁸ M) to guinea pig villus enterocytes suspended in isotonic Na⁺ containing medium caused a volume reduction within 2 min, and by 5 min the enterocytes shrank to a relative volume (0.94 ± 0.01) that did not change for the rest of the experiment (Fig. 4A). The effect of RK-1 on enterocyte volume was dose responsive: 10⁻¹⁰ M and 10⁻¹⁰ M had no effect on cell volume, while 10⁻⁹ M stimulated volume reduction which was slower (1.1 ± 0.2% cell⁻¹ min⁻¹ versus 2.0 ± 0.4% cell⁻¹ min⁻¹, p < 0.05) and reduced (final relative volume, 0.96 ± .01 versus 0.93 ± .01, p < .05) in comparison with 10⁻¹⁰ M RK-1. Volume reduction was also observed at 10⁻⁸ M which was the highest concentration tested. In Ca²⁺-free medium containing 100 μΜ EGTA the effect of 10⁻⁸ M RK-1 on enterocyte volume was prevented (final relative volume, 0.98 ± .01 versus 0.93 ± .01, p < .001). In Ca²⁺-
(1 mM) containing medium, in the presence of the dihydropyrini-
dine Ca²⁺ channel blocker nigrulidine (0.2 μM), 10⁻⁸ M RK-1 did not cause a volume reduction (final relative volume 1.00 ± .01, p < .001, Fig. 4A). Previously we have shown that sus-
pending the villus enterocytes in isotonic Na⁺-free medium, which hyperpolarizes the membrane, provides an increased
driving force for conductive Cl⁻ efflux, and results in larger
volume changes (20, 21). We therefore examined the effect of 10⁻⁸ M RK-1 on isotonic enterocyte volume in medium where Na⁺ was replaced isotonically with NMDG⁺ (Fig. 4B). When suspended in NMDG⁺ medium, without agonist, the villus cells
loose ~8% of their volume, which is consistent with Na⁺ influx
having been prevented while Na⁺ efflux together with anion
efflux continues through a leak pathway. The addition of RK-1
caused an increase in the initial rate of cell shrinkage com-
pared with NMDG⁺ medium alone (4.1 ± 0.4% cell⁻¹ min⁻¹
versus 1.9 ± 0.4% cell⁻¹ min⁻¹, p < 0.001) as well as a greater
volume reduction (final relative volume 0.84 ± 0.01 versus 0.92
± .01, p < 0.001, Fig. 4B). The volume reduction caused by
RK-1 in NMDG⁺ was prevented by Ca²⁺-free (EGTA, 100 μM)
NMDG⁺ medium (final relative volume 0.93 ± 0.02 versus 0.84

**Table I**
The amino acid analysis and ratio of amino acids obtained by amino acid analysis of RK-1 and the theoretical ratio of amino acids deduced from sequence analysis and electrospray mass spectrometry

| Amino acid | Pmoles | AA ratio | Theoretical AA ratio |
|------------|--------|----------|----------------------|
| Asp        | 364.76 | 3.29     | 3.00                 |
| Glu        | 313.43 | 2.83     | 2.00                 |
| Ser        | 308.76 | 2.78     | 3.00                 |
| Gly        | 250.60 | 2.26     | 2.00                 |
| His        | 0.00   |          |                      |
| Arg        | 131.38 | 1.18     | 1.00                 |
| Thr        | 0.00   |          |                      |
| Pro        | 251.24 | 2.27     | 2.00                 |
| Tyr        | 197.90 | 1.78     | 2.00                 |
| Val        | 105.63 | 0.95     | 1.00                 |
| Met        | 56.99  | 0.51     | 1.00                 |
| Cys        | 553.82 | 4.99     | 6.00                 |
| Ile        | 210.89 | 1.90     | 2.00                 |
| Leu        | 116.23 | 1.05     | 1.00                 |
| Phe        | 123.08 | 1.11     | 1.00                 |
| Trp        | 0.00   |          |                      |
| Lys        | 452.60 | 4.08     | 4.00                 |
| Other      | 0.00   |          |                      |

**Table II**
A comparison of RK-1 with a representative myeloid CS/def, rabbit CS-1 (3), and the enteric CS/def murine cryptdin 1 (22)

The sequences are aligned at the cysteines with dashes introduced to maximize the alignment.

| Peptide    | MPCSCKKY-CDPWEVIDGSCGFNSKYI-CCKREK | MPDVMFCSKY-CDPWEVIDGSCGFNSKYI-CCKREK |
|------------|------------------------------------|--------------------------------------|
| RK-1       | 1+                                 | 1+                                  |
| Murine cryptdin | LRDLVLCYCRSGKGRMNGTCKRHLLYTLCCR | LRDLVLCYCRSGKGRMNGTCKRHLLYTLCCR |
| Myeloid CS1 | GICACRRRFCPNERFSGYCVRNGARYVRCSSR | GICACRRRFCPNERFSGYCVRNGARYVRCSSR |

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Neither 0.2 mM niguldipine nor 1 nM GVIA ω-conotoxin alone had any effect on enterocyte volume. Niguldipine added together with RK-1 prevented volume reduction (final volume 0.91 ± 0.01 versus 0.83 ± 0.01, p < 0.001, Fig. 4B). GVIA ω-conotoxin (10 nM) added with RK-1, partially attenuated the volume reduction (final relative volume 0.88 ± 0.1 versus 0.84 ± 0.01, p < 0.05). Together these results suggest that the action of RK-1 is mediated through dihydropyridine-sensitive Ca\(^{2+}\) channels in villus epithelial cells.

Effect of RK-1 on ACTH-stimulated Adrenal Steroidogenesis—A total of 12 μM RK-1 reduced the corticosterone output of rat adrenal cell suspensions in response to 150 pg of ACTH by 40% (not shown). Because of the high levels of peptide required we were unable to reach the I.D. 50. In comparison CS-1 has an I.D. 50 of 25 nM (3). RK-1 is therefore not corticostatic.

Effect of RK-1 on Mammalian Cell Survival—RK-1 had no effect on the growth of the mammalian epithelial cell lines SK-MES-1, CHO-K1, and A549, although the related peptide HP-1 was cytotoxic at the concentrations assayed (Fig. 5) as previously reported (33).

**DISCUSSION**

Here we demonstrate the existence of a novel peptide found in the kidney which we call RK-1 and which has antimicrobial activity against *E. coli*, and activates ion channel activity. RK-1 possesses the cystine motif of the CS/def's and has potent biological activities. It is, however, structurally distinct from both the myeloid and enteric peptides indicating the evolution of at least three families of CS/def-like peptides from a common ancestor with each family restricted in its distribution to either

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**FIG. 2.** A dendrogram of the degree of sequence relatedness among the CS/def's and RK-1 was obtained using the program CLUSTAL in PC/GENE. CS-1 to CS-5 are rabbit myeloid CS/def's, HP-1 to HP-4 are human defensins, R-1 to R-5 are rat CS/def's, Cry 1 to Cry 5 are murine cryptdins (2–11, 21, 22). For simplicity only some of the enteric cryptdins are shown (44).

**FIG. 3.** A comparison of the effects of RK-1 (open circles, n = 14) and CS-4/NP-2 (closed circles, n = 5), a rabbit myeloid defensin on the growth of *E. coli* (strain Y1090), expressed as percent of control incubations (no peptide). Error bars represent S.E.

**FIG. 4.** The effect of 10^{-8} M RK-1 on cell volume of villus enterocytes in a sodium containing (A) and sodium free (B) medium. In A, open circles represent the effect of RK-1 on volume (n = 4), closed circles show the volume of untreated cells in isotonic medium over the same time course (n = 4), and closed squares show the effect of RK-1 on villus cell volume in the presence of 0.2 mM niguldipine (n = 4). In B, open circles show the effect on cell volume of RK-1 alone (n = 7), open diamonds show the effect of RK-1 in a calcium-free medium (n = 7), closed diamonds show the effect of RK-1 in the presence of 1 mM GVIA ω-conotoxin (n = 7), and closed squares the effect of RK-1 in the presence of 0.2 mM niguldipine (n = 7). Vertical bars represent the S.E.
neutrophils and alveolar macrophages, the intestinal crypts, or the kidney. RK-1 is the least cationic of the CS/def-like peptides and unlike the myeloid and enteric CS/defs, the positive charge is carried mainly on lysine residues. To distinguish RK-1 and related peptides, peptides from the myeloid or enteric CS/defs, and to avoid the potential problems associated with naming a peptide from a tissue source or a bioassay, we propose the generic term lysyl-CS/def with the individual peptides given initials according to their species.

Having proven the existence of a CS/def-related peptide in the kidney we performed several assays to compare its biological characteristics with those of myeloid and enteric CS/defs. Among these we examined the antibacterial activity of RK-1 against E. coli, and its ability to kill mammalian epithelial cells. E. coli was selected because it is a common bacterial pathogen in urinary tract infections (29) and is a standard test organism for CS/defs and cryptdins. RK-1 is antimicrobial in this assay, although less so than the bone marrow-derived peptide CS-4/DP-2. Although the role of CS/defs in host defense is well established (1) some CS/defs are only weakly antimicrobial (32). The conditions used here were based on those described by Lehrer et al. (32) and may not reflect the conditions in the kidney. Further experiments using other conditions and organisms will be required to show the optimal conditions for RK-1 antimicrobial activity.

The villus enterocyte volume assay is a useful model to study the effects of biological mediators on ion channels in epithelial cells (19–21). RK-1, like the corticostatins (19), stimulates the effects of biological mediators on ion channels in epithelial cells. The dihydropyridine-sensitive voltage-dependent Ca\(^{2+}\) channels in epithelial cells are physiologically relevant but indicates that the molecular and cellular machinery exists for RK-1-like peptides to act through Ca\(^{2+}\) channels in the kidney.

In summary, the major finding of this paper is the existence of a novel and potently biologically active peptide in the kidney. The similarity of this molecule with the CS/defs reveals a network of CS/def-like peptides with each branch of the family restricted to a very narrow range of tissues. The evidence presented suggests two hypotheses for the function of RK-1. First, based on its homology with the defensins and cryptdins and its antimicrobial activity, RK-1 may be involved in disease resistance in the kidney. Second, the biological effects of RK-1 on calcium dependent volume regulation may indicate a role for RK-1 as a local regulator of calcium channels. Our demonstration of a novel peptide in the kidney exemplified by RK-1 should open important new avenues of investigation into renal physiology and pathology.

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