Molecular and Functional Identification of Cyclic AMP–Sensitive \( \text{BK}_{\text{Ca}} \) Potassium Channels (ZERO Variant) and L-Type Voltage-Dependent Calcium Channels in Single Rat Juxtaglomerular Cells

Ulla G. Friis, Finn Jørgensen, Ditte Andreasen, Boye L. Jensen, Ole Skøtt

Abstract—This study aimed at identifying the type and functional significance of potassium channels and voltage-dependent calcium channels (\( \text{Ca}^2+ \)) in single rat JG cells using whole-cell patch clamp. Single JG cells displayed outward rectification at positive membrane potentials and limited net currents between −60 and −10 mV. Blockade of \( \text{K}^+ \) channels with TEA inhibited 83% of the current at +105 mV. Inhibition of \( \text{K}_V \) channels with 4-AP inhibited 21% of the current. Blockade of calcium-sensitive voltage-gated \( \text{K}^+ \) channels (\( \text{BK}_{\text{Ca}} \)) with charybdotoxin or iberiotoxin inhibited 89% and 82% of the current, respectively. Double immunofluorescence confirmed the presence of \( \text{BK}_{\text{Ca}} \) and renin in the same cell. cAMP increased the outward current by 1.6-fold, and this was inhibited by 74% with iberiotoxin. Expression of the cAMP-sensitive splice variant (ZERO) of \( \text{BK}_{\text{Ca}} \) was confirmed in single-sampled JG cells by RT-PCR. The resting membrane potential of JG cells was −212 mV and activation of \( \text{BK}_{\text{Ca}} \) with cAMP hyperpolarized cells on average 16 mV, and inhibition with TEA depolarized cells by 17 mV. The cells displayed typical high-voltage activated calcium currents sensitive to the L-type \( \text{Ca}^2+ \) blocker calciseptine. RT-PCR analysis and double-immunofluorescence labeling showed coexpression of renin and L-type \( \text{Ca}^2+ \) 1.2. The cAMP-mediated increase in exocytosis (measured as membrane capacitance) was inhibited by depolarization to +10 mV, and this inhibitory effect was blocked with calciseptine, whereas \( \text{K}^+ \)-blockers had no effect. We conclude that JG cells express functional cAMP-sensitive \( \text{BK}_{\text{Ca}} \) channels (the ZERO splice variant) and voltage-dependent L-type \( \text{Ca}^2+ \) channels. (\textit{Circ Res.} 2003;93:213-220.)

Key Words: \( \text{BK}_{\text{Ca}} \) ■ \( \text{Ca}^2+ \) ■ juxtaglomerular cells

Renin release from juxtaglomerular (JG) cells in the renal afferent arterioles is controlled by several intracellular messenger systems. Vasodilators generally stimulate renin release by increasing the intracellular concentration of cAMP and vasoconstrictors generally inhibit renin release by increasing the intracellular calcium concentration.

The membrane potential often acts as an integrator in cellular signaling and it has been suggested to be involved in the control of renin release. Based on electrophysiological measurements, Fishman\(^1\) suggested that hyperpolarization was associated with stimulation and depolarization with inhibition of renin secretion. Cell membrane depolarization, followed by activation of \( \text{Ca}^2+ \), and calcium influx, has been suggested to inhibit renin release.\(^2,3\) However, the inability of depolarization to change calcium concentration in JG cells and the lack of effect of calcium channel blockers on renin release have questioned the relevance of \( \text{Ca}^2+ \) in the control of renin secretion.\(^4,6\)

The ion channels responsible for setting the membrane potential of JG cells are not known with certainty. When measured in the whole-cell patch-clamp mode, there are limited net currents in JG cells at membrane potentials between −60 and −10 mV, whereas at more positive potentials, an outward current is activated.\(^4,7\) This outward current is activated by cAMP.\(^8\)

We have used the patch-clamp technique in combination with molecular techniques to define the ion channels involved in the outward current and to study the presence of \( \text{Ca}^2+ \) in JG cells and their possible interaction with the secretory process.

Materials and Methods

Isolation of Rat JG Cells

Male Sprague-Dawley rat (60 to 80 g; Animal Facility, University of Southern Denmark, Denmark) JG cells were isolated from renal cortex.\(^8\) All animal procedures conformed to the Danish law on experiments on animals and with the guidelines for the care and handling of animals established by the US Department of Health and Public Services and published by the National Institutes of Health. For patch clamp, the cells were transferred to cover slips, and for the renin secretion studies the cells were separated on a Percoll density gradient (25% Percoll at 27 000 \( \times \) g for 30 minutes), resuspended in RPMI-1640 medium, and seeded in 96-multiwell plates.

Original received March 7, 2003; revision received June 25, 2003; accepted June 25, 2003.

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\textit{Circulation Research} is available at http://www.circresaha.org DOI: 10.1161/01.RES.0000085041.70276.3D

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Renin Secretion From JG Cell Cultures
Cultured cells were incubated for 20 hours, washed, and experiment-
al agents added. After 20 hours, the medium was removed, and the
cells were harvested. Renin concentration was determined by RIA
for ANG 1. Renin secretion rates were calculated as fractional
release of total renin content.

Immunofluorescence
JG cells attached to glass slides were rinsed in TBS, fixed in
99.9% EtOH, and air-dried. Kidney cryosections were blocked
with TTBS containing 5% goat serum + 1% BSA and JG cells
with 5% skim milk. The specimens were incubated with primary
antibodies (mouse monoclonal anti-renin [SWANT, Bellinzona,
Switzerland], polyclonal rabbit anti-rat α1c [Alomone Labs,
Jerusalem, Israel], polyclonal rabbit anti-rat BKCa [Chemicon,
Temecula, Calif]) for 2 hours and were washed in TTBS. Next,
the specimens incubated with secondary antibody for 30 minutes.
Secondary antibodies were as follows: goat-anti rabbit IgG
conjugated to Alexa Fluor 488 combined with goat-anti mouse
IgG conjugated to Alexa 568 for sections, and goat anti-rabbit
IgG conjugated to Alexa Fluor 586 combined with goat anti-
mouse IgG conjugated to Alexa Fluor 488 for JG cells (Molecular
Probes). The specimens were mounted with fluorescence medium
(DAKO). Negative controls with omission of primary antibody
were always run in parallel, and where peptides were available,
we included preabsorption controls (α1c peptide).

Reverse Transcription–Polymerase Chain Reaction
RT-PCRs were performed as previously described. Templates for
RT-PCRs were total RNA isolated from rat organs, from microdis-
sected rat renal preglomerular vessels, and from single JG cells
sampled with patch pipettes. RNA was isolated by acid guanidium-
thiocyanate phenol-chloroform method using yeast tRNA as carrier.
Reverse Transcription–Polymerase Chain Reaction

Electrophysiological Identification of K+ Channels in JG Cells
The whole-cell recording configuration was obtained in 149
single cells isolated from 80 preparations. The average Cm
was 2.42±0.10 pF, and the cells displayed outward current
rectification at positive membrane potentials (Figure 1A).
Inward current rectification was never observed, not even
when the internal solution was changed to a high-chloride
solution, and the pulse protocol was changed to cover a
voltage range from −200 mV to +70 mV (Figure 1B).

Addition of the potassium channel blocker tetraethylam-
monium (TEA, 5 mmol/L) resulted in a large inhibition of
the outward current (Figures 1C and 1D; P<0.05, n=4),
indicating that most of the outward current in JG cells
is carried by K+.

The voltage-gated Kv channel blocker 4-aminopyridine
(4-AP) inhibited 20.6±9.5% of the outward current at +105
mV (Figure 1D; P<0.05, n=12), indicating that only a minor
part of the outward current is carried by K+ through 4-AP-
sensitive Kv channels. The effects of TEA and 4-AP were
 additive: at +105 mV, TEA (5 mmol/L) together with 4-AP
(1 mmol/L) inhibited 91.5±2.9% of the current (Figure 1D;
P<0.05, n=4).

The identity of the K+ channels was investigated by superfusion with charybdotoxin or ibetoxin, which inhibit
calcium-sensitive voltage–gated BKCa channels (Figures 2A
and 2B). At +105 mV, charybdotoxin (1×10−7 mol/L) inhibited
88.7±1.2% of the outward current (P<0.05, n=4). Similarly,
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Statistics
All values are given as mean±SEM. Paired Student’s t test was used
to calculate statistical difference. The change in Cm was calculated
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shown) were reversible. Thus, most of the outward current is carried through BK<sub>Ca</sub> channels.

The gating of BK<sub>Ca</sub> channels is sensitive to calcium. The Ca<sup>2+</sup> dependence of the outward current was, therefore, tested with EGTA (2 mmol/L) in the pipette solution, which almost completely abolished the outward current (Figure 2D; n=7).

Outward current in JG cells is enhanced by cAMP, and the ZERO splice variant of the SLO-BK<sub>Ca</sub> channel gene is sensitive to cAMP. We therefore examined whether BK<sub>Ca</sub> channels carry cAMP-activated currents. Figure 2E shows the I-V relationships recorded immediately after the whole-cell configuration was obtained (circles), after the cells have been dialyzed with cAMP (1 μmol/L) for around 3 minutes (squares), and after the cells have been superfused with iberiotoxin (10<sup>-7</sup> mol/L) (triangles). Dialysis with cAMP for 3 minutes resulted in an 1.6-fold increase in the outward current at +105 mV (P<0.05, n=5), and iberiotoxin inhibited 74.1% ± 5.7% of this current (P<0.05, n=5), indicating that the cAMP-mediated increase in outward current is carried through BK<sub>Ca</sub> channels.

**Molecular Identification of BK<sub>Ca</sub> Channels in JG Cells**

The sensitivity of BK<sub>Ca</sub> channels to cAMP is determined by alternative splicing of the SLO gene at splice junction 2, where the presence of a 59 amino acid insert (STREX-variant) abolishes cAMP sensitivity. RT-PCR analysis using primers adjacent to splice junction 2 of the SLO gene was applied to discriminate between STREX and ZERO BK<sub>Ca</sub> channel splice variants. First, rat organs with reported differ-
Significance of cAMP-Mediated BKCa Activation for Cell Capacitance Changes and Renin Secretion

To address the functional significance of BKCa activation, the patch-clamp technique was used to record cell capacitance ($C_m$) changes in response to cAMP. An increase in $C_m$ indicates addition of membrane to the cell surface area and is a measure of exocytosis.6 An original trace from a cell dialyzed with 1 μmol/L cAMP is shown in Figure 4A. Iberiotoxin (1×10⁻⁷ mol/L) was added via a pipette from t=200 seconds (arrow). $C_m$ increased by 13.5±4.9% ($P<0.05$; n=5; Figure 4B), which is similar to the increase obtained in the absence of iberiotoxin.6 This indicates that in spite of the effect on whole-cell currents, iberiotoxin does not influence the cAMP-mediated increase in membrane capacitance.

Renin release was studied over prolonged time in primary cultures of JG cells. Under control conditions, these cells released 20.4±3.1% of total content (n=10) (Figure 4C). The adenylyl cyclase activator, forskolin (10 μmol/L), increased renin release to 31.2±1.8% of total renin content. 4-AP (10⁻⁴ to 10⁻² mol/L; n=10, with four wells assigned per condition in one experiment) did not affect forskolin-mediated renin release (Figure 4C). Similar (negative) results were obtained with TEA (10⁻⁴ to 10⁻² mol/L) (n=12) (Figure 4D), indicating that K⁺ current activation is not necessary for cAMP-mediated renin release.

Role of BKCa Channels for Regulation of JG Cell Membrane Potential

Next, we addressed the contribution of BKCa to the resting membrane potential under current clamp. Under control (resting) conditions, the membrane potential in JG cells was $-32±2.7$ mV (n=5). With cAMP (1 μmol/L) in the patch pipette, the membrane potential gradually hyperpolarized (Figure 5A, original trace). On average, the membrane potentials were hyperpolarized by 16.0±3.8 mV (Figure 5C; $P<0.05$; n=9). Addition of TEA to the bath (5 mmol/L) depolarized the JG cells from $-35.7±3.4$ to $-18.7±2.4$ mV (Figure 5B, original trace (first arrow), and Figure 5C; $P<0.05$; n=3). The effect was reversible (Figure 5B, second arrow), and on average, the membrane repolarized from $-18.7±4.7$ to $-37.8±1.8$ mV (n=3) after washout of TEA. Similar results were obtained, when the zero current potential was read from the $I$-$V$ curves obtained in the voltage-clamp mode and acquired in the beginning and after a 10-minute recording period (cAMP data) or before and after change of extracellular solution (TEA, charybotoxin, iberiotoxin, and

**Figure 3.** Demonstration of BKCa channels (the ZERO variant). A, PCR amplification (32 cycles) of cDNA from rat organs for BKCa (rSLO gene) (top) and β-actin (bottom). Primers spanned the splice site 2 junction and discriminate between the STREX (478 bp) and ZERO (301 bp) variants, which determine cAMP sensitivity of the channel. Both variants were expressed in cerebellum, pituitary gland, and skeletal muscle, whereas in kidney, only the ZERO variant was detected (top). β-Actin was amplified in all cDNA samples (bottom). A negative control without cDNA was run in parallel (cDNA). Size marker is ﾂX174DNA/HaeIII fragments. B, PCR amplification (32 cycles) of cDNA from microdissected rat renal preglomerular (PG) vessels (~1 mm vessel/reaction) for BKCa-rSLO and renin. Predominant variant was ZERO. No amplification was seen in the absence of reverse transcriptase (–RT). Renin (194 bp) was used as positive control. Size marker is ﾂX174DNA/HaeIII fragments. D through G, Immunolabelings of freshly isolated JG cells. D and E, JG cells were double-labeled with mouse anti-renin (D) and rabbit anti-BKCa (E). F, Overlay of D and E. G, Bright-field image of the labeled cell shown in D through F. Bar=10 μm.
4-AP data) (Figure 5C). Thus, cAMP (1 μmol/L) lead to membrane hyperpolarization (ΔmV, 14.1 mV ± 2.8 mV; P<0.05, n=14), whereas TEA (5 mmol/L) lead to membrane depolarization (ΔmV, 21 mV ± 4 mV; P<0.05, n=3). A similar depolarizing effect was observed after addition of charybdotoxin (1×10^{-7} mol/L) (ΔmV, 19.3 mV ± 3.3 mV; P<0.05; n=4) and iberiotoxin (3×10^{-7} mol/L) (ΔmV, 18.6 mV ± 8 mV; P<0.05, n=5), whereas 4-aminopyridine (1 mmol/L) was without any affect on the zero current potential (ΔmV, 0.7±5.2 mV, n=6). These data suggest that BK_{Ca} channels are predominant in setting the resting membrane potential in JG cells and are responsible for cAMP-mediated hyperpolarization.

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evokes an inward Ba\textsuperscript{2+} current of around 4 pA (Figure 6B, control), which is blocked by the specific L-type voltage-dependent calcium channel (VDCC) blocker calciseptine (2×10\textsuperscript{-8} mol/L) (Figure 6B), indicating that this inward current passes through L-type Ca\textsuperscript{2+} channels. On average, calciseptine inhibited the current by 91.5\% (P<0.05; n=4; Figure 6C).

Double-immunofluorescence labeling of rat kidney cryosections and of isolated JG cells with antibodies directed against renin and the smooth muscle/cardiac α\textsubscript{1c} subunit of an L-type voltage-gated calcium channel showed that immunoreactive proteins were colocalized in terminal afferent glo-

merular arterioles (Figures 7A through 7C) and in single JG cells (Figures 7D through 7G). Preabsorption of the α\textsubscript{1c} antibody with the peptide used to raise the antibody prevented labeling. Omission of primary antibodies prevented fluorescence signals from the cells. RT-PCR analysis of pipettesampled single JG cells showed expression of mRNA encoding the α\textsubscript{1c} subunit (Figure 7H).

**Effect of Calcium Channel Activation on JG Cell Membrane Capacitance Changes**

We tested the effect of depolarization-evoked calcium channel activation on cAMP-induced cell membrane capacitance changes. With control solutions, average C\textsubscript{m} did not change significantly during recording times up to 10 minutes (at holding potentials of −30 mV or +10 mV) (Figure 6D). At negative holding potentials of −55 and −30 mV, with minimal Ca\textsuperscript{2+}-channel activation, cAMP elicited a large increase in membrane capacitance (Figure 6D). With more positive holding potentials, the C\textsubscript{m} increase was inhibited and at a holding potential of +10 mV, the cAMP-induced increase in C\textsubscript{m} was abolished (Figures 6D and 6E, lower trace). To establish a causal link between diminished CAMP sensitivity of C\textsubscript{m} and calcium channel activation, the experiments were repeated in the presence of calciseptine. At a holding potential of +10 mV, calciseptine re-established the effect of cAMP on C\textsubscript{m} (+10.8% ± 1.8%) (P<0.05 versus control, n=5) (Figures 6D and 6E, upper trace). These data indicate that the lack of effect of cAMP at +10 mV is due to inhibition of exocytosis caused by calcium entering the JG cell through VDCCs.

**Discussion**

JG cells are characterized by voltage-dependent outward currents, which are enhanced by cAMP. Over 50% of mouse JG cells display inward currents at negative membrane potentials.\textsuperscript{4,7} Such currents were not observed in 326 rat JG cells,\textsuperscript{8} suggesting a species difference.

Inhibition of calcium-sensitive voltage-gated (BK\textsubscript{Ca}) channels with charybdotoxin or iberiotoxin blocked most of the current, as well as the increase in cAMP-induced current. Inhibition of voltage-gated potassium channels (K\textsubscript{v}) by 4-aminopyridine (4-AP) inhibited a minor part of the current. The SLO gene encodes BK\textsubscript{Ca} channels. Five alternative splice sites exist in the COOH terminal part of the molecule in mammals. At splice site 2, there can either be an inserted exon (STREX [Stress axis regulated exons]) or no insert (ZERO).\textsuperscript{10,14,15} BK\textsubscript{Ca} channels with the STREX insert are inhibited by protein kinase A (PKA), whereas the ZERO variant is activated by PKA.\textsuperscript{12} Our demonstration of cAMP-stimulated ZERO variant expression in JG cells is consistent with our electrophysiological finding of cAMP-stimulated BK\textsubscript{Ca} in these cells.

In the current clamp configuration, the membrane potential was −32 mV, which is close to the potential of −38 mV in afferent arterioles in the perfused hydropneophrotic kidney.\textsuperscript{16} cAMP hyperpolarized the membrane potential, and blockade of K\textsuperscript{+} channels with TEA depolarized the JG cells. Similar results were observed by analysis of zero current potentials in the voltage clamp configuration where cAMP led to hyper-
polarization, whereas closure of the BK$_{\text{Ca}}$ channels with inhibitors depolarized the JG cells. Based on these findings, we conclude that the BK$_{\text{Ca}}$ channels participate in the setting of the resting membrane potential in JG cells.

The membrane potential of the JG cells will be important for the ability of cAMP to activate the BK$_{\text{Ca}}$ channels. The JG cells are electrically coupled to the vascular smooth muscle cells of the afferent arteriole. In nonperfused vessels, the membrane potential is relatively hyperpolarized. Thus, in nonperfused hydronephrotic kidneys, the JG cell membrane potential is in the order of $-60$ to $-75 \text{ mV}$. and at this negative potential, cAMP-mobilizing agents never caused hyperpolarization. When JG cells are uncoupled from the arteriole and the membrane potential is more depolarized, cAMP will open the channels and cause hyperpolarization. This is consistent with the results of Fishman who measured a membrane potential of $-45 \text{ mV}$ in JG cells adhering to enzyme-treated isolated glomeruli, and the cells hyperpolarized on exposure to epinephrine.

It is unlikely that the cAMP-induced hyperpolarization per se stimulates renin secretion, because blockade of BK$_{\text{Ca}}$ did not inhibit the cAMP-induced increase in cell membrane capacitance and because forskolin-induced renin release from isolated JG cells was unaffected by TEA. In isolated perfused rat kidneys, TEA was also without effect on isoproterenol-stimulated renin release.

The presence of mRNA and protein for L-type Ca$^{2+}$ (Ca$_{\text{L}}$) and the demonstration of currents through Ca$^{2+}$ in cell membrane capacitance and because forskolin-induced renin release from isolated JG cells was unaffected by TEA. in isolated perfused rat kidneys, TEA was also without effect on isoproterenol-stimulated renin release.

The presence of mRNA and protein for L-type Ca$_{\text{L}}$ (Ca$_{\text{L}}$, 1.2) and the demonstration of currents through Ca$_{\text{L}}$, which are blocked by an L-type specific inhibitor, demonstrate that the JG cells possess L-type Ca$_{\text{L}}$. The finding that strong depolarization of the JG cells inhibits cAMP-stimulated increases in cell membrane capacitance is consistent with the view that activation of the L-type Ca$_{\text{L}}$ is associated with inhibition of renin secretion. The ability of calciseptine to reverse the inhibition supports this interpretation. The whole-cell currents through the Ca$_{\text{L}}$ were small, but correction for cell surface area (C$_{\text{m}}$) showed that the channel density was similar to that of the smooth-muscle cell line, A7r5, and rat tail artery myocytes. A reason why it has been difficult to measure Ca$^{2+}$-currents in JG cells may be that the cells are about 10 times smaller than vascular myocytes. The expression of Ca$_{\text{L}}$ channels in JG cells and their inhibitory effect on cAMP-induced renin secretion is consistent with the finding that the calcium channel opener Bay K 8644 inhibits isoproterenol-induced renin release in anesthetized rats, and that depolarization with a high K$^+$ concentration reversed forskolin-induced renin secretion in the isolated rat by a mechanism that was dependent on extracellular calcium.

Previous attempts to address this question by whole-cell patch clamp have been made on isolated glomeruli with attached afferent arterioles and JG cells. It is unlikely that the cell-to-cell coupling in this preparation, capacitative currents cannot be fully compensated, and it is therefore difficult to clamp the voltage reliably at voltages deviating from the zero current potential. This may explain why it was not possible to observe an increase in Ca$^{2+}$ using a depolarizing voltage step protocol. When depolarizing a similar preparation with K$^+$, Russ and coworkers reported a small dose-dependent increase in Ca$^{2+}$, that was abolished by a dihydropyridine Ca$^{2+}$ antagonist. In many smooth muscle cells, a global increase in intracellular calcium concentration after calcium influx depends on calcium-induced calcium-release from intracellular stores via ryanodine or IP$_3$ receptors. Ryanodine has been shown not to modify calcium release from intracellular stores in the renin-secreting cell line As4.1. A less efficient coupling of calcium influx to calcium release in JG cells could contribute to the difficulties in using Ca$^{2+}$, as a measure of the function of Ca$_{\text{L}}$.

Renin release is sensitive to changes in cell volume, and the volume-sensitive step is located later in the secretory pathway than the calcium-sensitive process. The change in effective osmolality, which is associated with K$^+$ depolarization (exchange of less permeable Na$^+$ with more permeable K$^+$), may therefore lead to cell swelling and
obscure the effect of activation of Ca, in various preparations. Anisosmotic addition of K⁺ leads to transient shrinkage and inhibition of renin release.²⁶

During stimulation of renin release by maneuvers that increase the cellular cAMP concentration (sympathetic nervous activity, prostaglandin E₂, dopamine, etc), the concomitant activation of the BKCa channels may protect the cells against depolarization and activation of VDCCs. When acute depolarization occurs, the calcium influx may inhibit renin release, but activation of the BKCa channels may repolarize the cells and terminate the inhibitory signal. A 40 mV depolarization (to +10 mV) inhibited cAMP-induced renin secretion, whereas the 20-mV depolarization caused by inhibition of K⁺ channels was not sufficient. In vivo, the ability of the BKCa channels to stabilize membrane potential may prevent sufficiently strong depolarization and thereby limit the functional significance of Ca, in the control of renin secretion.

In conclusion, we show expression and function of cAMP-sensitive, BKCa channels of the ZERO splice variant and of L-type voltage-dependent calcium channels (CaV 1.2) in juxtaglomerular renin-secreting cells. The BKCa channels are involved in the control of membrane potential, whereas they do not play a direct role in the control of renin secretion. Activation of the L-type Ca, inhibits cAMP-induced renin release, thereby providing the first evidence at the cellular level for a functional role of these channels.

Acknowledgments

The present work was supported by grants from Carlbergfondet, the Danish Medical Research Council (U.G.F., 52001085; B.L.J., 22010159; O.S., 99022742), the Danish Heart Foundation (U.G.F., 00-2-1-3-22831; B.L.J., 01-1-2-36-33896; O.S., 99-2-2-36-22743), the NOVO Nordic Foundation, the AJ Andersen Fond, the Medical Association Research Foundation, and the Foundation of 17.12.1981. The authors wish to thank Annette K. Rasmussen, Gitte Dybnose, Mette Fredenslund, and Inge Andersen for excellent technical assistance.

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Molecular and Functional Identification of Cyclic AMP-Sensitive BK$_{Ca}$ Potassium Channels (ZERO Variant) and L-Type Voltage-Dependent Calcium Channels in Single Rat Juxtaglomerular Cells

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Circ Res. 2003;93:213-220; originally published online July 3, 2003; doi: 10.1161/01.RES.0000085041.70276.3D

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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