Flow-Induced $[\text{Ca}^{2+}]_i$ Increase Depends on Nucleotide Release and Subsequent Purinergic Signaling in the Intact Nephron

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ABSTRACT

Flow induces cytosolic $\text{Ca}^{2+}$ increases ($[\text{Ca}^{2+}]_i$) in intact renal tubules, but the mechanism is elusive. Mechanical stimulation in general is known to promote release of nucleotides (ATP/UTP) and trigger auto- and paracrine activation of P2 receptors in renal epithelia. It was hypothesized that the flow-induced $[\text{Ca}^{2+}]_i$ response in the renal tubule involves mechanically stimulated nucleotide release. This study investigated (1) the expression of P2 receptors in mouse medullary thick ascending limb (mTAL) using P2Y2 receptor knockout (KO) mice, (2) whether flow increases induce $[\text{Ca}^{2+}]_i$ elevations in mTAL, and (3) whether this flow response is affected in mice that are deplete of the main purinergic receptor. $[\text{Ca}^{2+}]_i$ was imaged in perfused mTAL with fura-2 or fluo-4. It is shown that luminal and basolateral P2Y2 receptors are the main purinergic receptor in this segment. Moreover, the data suggest presence of basolateral P2X receptors. Increases of tubular flow were imposed by promptly rising the inflow pressure, which triggered a marked increase of $[\text{Ca}^{2+}]_i$. This $[\text{Ca}^{2+}]_i$ response was significantly reduced in P2Y2 receptor KO tubules (fura-2 ratio increase WT 0.44 ± 0.09 [n = 28] versus KO 0.16 ± 0.04 [n = 13]). Furthermore, the flow response was greatly inhibited with luminal and basolateral scavenging of extracellular ATP (apyrase 7.5 U/ml) or blockage of P2 receptors (suramin 300 $\mu$M). The flow response could still be elicited in the absence of extracellular $\text{Ca}^{2+}$. These results strongly suggest that increase of tubular flow elevates $[\text{Ca}^{2+}]_i$ in intact renal epithelia. This flow response is caused by release of bilateral nucleotides and subsequent activation of P2 receptors.


An increase of fluid flow over the apical side of cultured renal epithelia$^1$ or in intact renal tubules$^2$ elicits an increase of cytosolic $\text{Ca}^{2+}$ ($[\text{Ca}^{2+}]_i$). In cultured renal epithelium, this flow-induced $[\text{Ca}^{2+}]_i$ response seems to depend on the presence of the primary cilium.$^3$ The signal transduction pathways of this flow-stimulated $[\text{Ca}^{2+}]_i$ elevation, however, is poorly understood, and the available data are conflicting.$^1$–$^4$ One model for the flow-induced increase in $[\text{Ca}^{2+}]_i$ hypothesizes that application of mechanical stress/bending to the primary cilium results in conformational changes in polycystin-1 that further interacts with polycystin-2 to allow $\text{Ca}^{2+}$ influx at the base of the primary cilium.$^4$ This $\text{Ca}^{2+}$ influx then induces $\text{Ca}^{2+}$ release from intracellular stores. Noteworthy, mechanical stimulation of cells including epithelia in general promotes release of nucleotides (ATP, UTP).$^5$–$^9$ Nucleotide release occurs without apparent damage of the cells and is shown to underlie traveling waves of $[\text{Ca}^{2+}]_i$ in cellular networks.$^7$–$^10$ Also MDCK cells, the renal cell line used to establish the concept of the primary cilium as mech-
anoscensor, releases ATP after mechanical stimulation.\textsuperscript{5} The intact renal tubule and MDCK and other renal epithelial cells are known to express numerous luminal and basolateral P2 receptors.\textsuperscript{6,11} Their activation commonly triggers an increase of [Ca\textsuperscript{2+}].\textsuperscript{12} Therefore, we hypothesized that flow-induced [Ca\textsuperscript{2+}], signals in renal epithelia involve release of nucleotides and associated signaling events. Data supporting this hypothesis are presented in this article.

RESULTS

P2 Receptors in Mouse Medullary Thick Ascending Limb

We suggested that an increase of tubular flow triggers nucleotide release and subsequent stimulation of epithelial P2 receptors and increase of [Ca\textsuperscript{2+}]. To test our hypothesis, we first needed to establish whether P2 receptors are functionally expressed in medullary thick ascending limb (mTAL). Generally, transporting epithelia express luminal and basolateral P2 receptors.\textsuperscript{6} Studies indicate prominent luminal expression of P2Y\textsubscript{2} receptors in collecting duct, which inhibit electrogenic Na\textsuperscript{+} absorption.\textsuperscript{13,14} Therefore, we believed this receptor to be the prime candidate for extracellular nucleotide-mediated [Ca\textsuperscript{2+}], signaling in mTAL. If this is the case, then the P2Y\textsubscript{2} receptor knockout (KO) mouse would be a suitable tool to investigate the functional expression of P2 receptor in mTAL. Figure 1 shows that both 100 \(\mu\)M luminal UTP and ATP trigger brisk increases of [Ca\textsuperscript{2+}], of similar amplitude in wild-type (WT) but never in KO tubules (fura-2 ratio increase, luminal UTP, WT 0.71 ± 0.2 \([n=5]\), KO 0.02 ± 0.01 \([n=6]\); luminal ATP, WT 0.60 ± 0.13 \([n=5]\), KO 0.02 ± 0.01 \([n=6]\)). Other nucleotides such as UDP and ADP in the same concentration were without effect (data not shown). This strongly suggests that the P2Y\textsubscript{2} receptor is the only P2 receptor expressed on the luminal side of mTAL.

UTP also stimulated increases of [Ca\textsuperscript{2+}], from the basolateral side (Figure 2). Again, this response was present only in tubules from WT mice (fura-2 ratio increase, basolateral UTP, WT 0.25 ± 0.06 \([n=18]\), KO 0.01 ± 0.01 \([n=13]\)). Paired experiments demonstrated that luminal UTP was more potent than basolateral UTP at increasing [Ca\textsuperscript{2+}], in WT mTAL (luminal UTP, WT 0.71 ± 0.2 \([n=5]\) versus basolateral UTP, WT 0.24 ± 0.06 \([n=6]\); \(P=0.04\)). Basolateral ATP was also able to stimulate [Ca\textsuperscript{2+}], increases in WT and KO tubules. The pattern of the basolateral ATP-induced [Ca\textsuperscript{2+}], increase in WT tubules, however, was significantly different from that of basolateral UTP (Figure 3). Basolateral ATP increased [Ca\textsuperscript{2+}], with an initial peak followed by a sustained [Ca\textsuperscript{2+}], plateau in the continued presence of the agonist. The [Ca\textsuperscript{2+}], plateau value measured 100 s after the basolateral nucleotide addition was significantly higher in tubules that were stimulated with ATP compared with UTP (0.19 ± 0.02 \textit{versus} 0.01 ± 0.02, respectively; \(P=0.001\)). An ATP-induced [Ca\textsuperscript{2+}], plateau was also seen in KO tubules with an amplitude similar to WT (0.26 ± 0.08 \textit{versus} 0.19 ± 0.08; \(n=5\); \(P=0.462\)). In contrast, the initial basolateral ATP-induced [Ca\textsuperscript{2+}], peak was signifi-

![Figure 1. Effect of luminal UTP (100 \(\mu\)M; A) and ATP (100 \(\mu\)M; B) on [Ca\textsuperscript{2+}], in perfused mouse medullary thick ascending limb (mTAL). In wild-type (WT) tubules, both nucleotides elicited prompt and transient increases of [Ca\textsuperscript{2+}],. Mice that were deplete of the P2Y\textsubscript{2} receptor never responded to either luminal ATP or UTP.](www.jasn.org)

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ATP-inducible \([\mathrm{Ca}^{2+}]_i\) response in KO tubules unmasks other basolateral P2 receptors. The \([\mathrm{Ca}^{2+}]_i\) plateau was completely dependent on basolateral \([\mathrm{Ca}^{2+}]_i\). This supports that basolateral ATP stimulates basolateral \([\mathrm{Ca}^{2+}]_i\) influx and suggests this to occur via a \([\mathrm{Ca}^{2+}]_i\) permeable P2X receptor. In the entire series of experiments, the resting \([\mathrm{Ca}^{2+}]_i\) was slightly but statistically significantly lower in WT as compared with KO tubules (WT 0.95 ± 0.03 [n = 31]; KO 1.03 ± 0.03 [n = 34]). This indicates a small elevation of resting \([\mathrm{Ca}^{2+}]_i\) in KO mTAL. Subsequently, we investigated the source of the UTP-stimulated \([\mathrm{Ca}^{2+}]_i\), elevations. Luminal and basolateral UTP (100 \(\mu\)M) was tested in the bilateral absence of extracellular \([\mathrm{Ca}^{2+}]_i\) (Figure 4). Under these conditions, UTP still stimulated a transient \([\mathrm{Ca}^{2+}]_i\), increase amounting to 0.35 ± 0.04 (n = 3) ratio units from the luminal side and 0.31 ± 0.15 (n = 3) ratio units from the basolateral side. In summary, these data strongly suggest that (1) mouse mTAL express luminal and basolateral P2Y2 receptors, which trigger an increase of \([\mathrm{Ca}^{2+}]_i\) via release of \([\mathrm{Ca}^{2+}]_i\) from internal stores and likely also activation of store-operated \([\mathrm{Ca}^{2+}]_i\), entry; (2) luminal P2Y2 receptors are significantly more potent at triggering \([\mathrm{Ca}^{2+}]_i\), elevations than P2Y2 receptors that are localized on the basolateral side; and (3) mTAL express an additional basolateral ATP-sensitive P2 receptor, which triggers a sustained \([\mathrm{Ca}^{2+}]_i\), influx and plateau.

**Flow-Induced \([\mathrm{Ca}^{2+}]_i\), Response in Mouse mTAL**

Next we investigated whether an increase of luminal flow is able to trigger \([\mathrm{Ca}^{2+}]_i\), elevations in mTAL as seen in different cultured cells (MDCK) or intact rabbit or mouse cortical collecting duct. To this end, we followed the experience of the laboratory of Satlin.\(^2,15\) After establishment of tubule perfusion, the pressure on the inflow side was reduced to 10 cmH\(_2\)O for 10 min. This led to a significant reduction of the tubular lumen as visible online in...
the original fluorescence image of the tubule. Subsequently, the hydrostatic pressure of the inflow was abruptly elevated (within 1s) by 80 cmH₂O, leading to sudden distension of the tubule and concomitant elevation of tubular flow. Tubular distension was quantified in each tubule by measuring the area of tubular fura-2 fluorescence (pixels above threshold 345 nm). Figure 5 shows the effect of increasing inflow pressure on the tubular diameter and the corresponding [Ca²⁺]ᵢ signal. Below the traces 6 fura-2 ratio images show the time course of this effect. Obviously, this pressure increase instantaneously (within the first seconds) elevated mTAL [Ca²⁺]ᵢ in a biphasic manner with an initial peak followed by a decline of [Ca²⁺]ᵢ within 1 to 3 min. After 3 min, tubular inflow pressure was reduced to 30 cmH₂O, resulting in a slow decline of tubular lumen. Frequently, single local [Ca²⁺]ᵢ elevations were observed (image 5 and 6, Figure 5).

Flow Response Is Greatly Reduced in mTAL of P2Y₂ KO Mice

Then we studied whether this flow response was similar in P2Y₂ WT and KO tubules. Figure 6 summarizes the entire series of experiments and shows that the flow response in WT tubules is a robust phenomenon and could be stimulated in nearly all tested tubules. The mean [Ca²⁺]ᵢ peak in WT tubules amounted to 0.44 ± 0.09 ratio units (n = 28). In clear contrast, the flow-induced increase of [Ca²⁺]ᵢ was greatly reduced in KO tubules (0.16 ± 0.04 ratio units; n = 13; P < 0.05). The tubular distension as measured with pixel area above threshold approach was similar in both populations (18 ± 2.1% in WT, 22 ± 3.2% in KO). The actual diameters during the flow response changed in WT from 22.7 ± 0.6 to 25.6 ± 0.5 µm and in KO from 22.8 ± 0.7 to 26.6 ± 0.7 µm. The tubular distensions showed a significant degree of variation. In Figure 6B, we plotted the magnitude of the induced [Ca²⁺]ᵢ elevation as function of tubular distension for all WT and KO tubules. The data suggest that larger tubular distensions provoke bigger [Ca²⁺]ᵢ signals in WT but not in KO tubules. This strongly indicates that the flow response that is induced in mouse mTAL depends on the presence of P2Y₂ receptors and suggests that increased flow promotes tubular release of nucleotides.
extracellular Ca\(^{2+}\) (0.26 ± 0.08 ratio units; \(n = 8\)) was slightly but not significantly reduced as compared with WT controls (0.44 ± 0.09 ratio units; \(n = 28\)). These results indicate that a significant part of the flow response–mediated [Ca\(^{2+}\)]\(_{i}\) increase can be triggered in the absence of extracellular Ca\(^{2+}\) and therefore must result from release of Ca\(^{2+}\) from internal stores.

**Basolateral and Luminal Extracellular Scavenging of ATP and P2 Receptor Blockage Inhibits the Flow Response**

The results thus far suggest that an increase of tubular flow promotes release of nucleotides from the renal tubule. Next we examined the effect of apyrase on the flow response. Apyrase is known to scavenge extracellular ATP. These experiments were performed with the non-ratiometric Ca\(^{2+}\) dye fluo-4. Noteworthy, 7.5 U/ml apyrase reversibly reduced resting fluo-4 fluorescence by 12.3 ± 4.7% (\(n = 5\), \(P < 0.05\)). Importantly, in all experiments, basolateral apyrase led to a near-complete inhibition of the flow-induced [Ca\(^{2+}\)]\(_{i}\) increase as compared with the control response (\(F/F_0\) increase without apyrase 1.8 ± 0.16 [\(n = 18\)]; with basolateral apyrase 1.09 ± 0.01 [\(n = 8\)]; \(P < 0.05\); Figure 8). We also tested basolateral suramin, a nonspecific blocker of most P2 receptors. Addition of suramin (300 μM) basolaterally 3 min before stimulation also inhibited the flow response (\(F/F_0\) increase with basolateral suramin 1.13 ± 0.03; \(n = 7\); \(P < 0.05\); Figure 8). Apyrase (7.5 U/ml) and suramin (300 μM) were also tested from the luminal side and were included in the luminal perfusate before starting fluo-4 AM loading. Both antagonists were present for 30 min before the stimulus was applied. Luminal apyrase and suramin inhibited the flow response (\(F/F_0\) increase with luminal apyrase 1.34 ± 0.03 [\(n = 12\); \(P < 0.05\)]; with luminal suramin 1.22 ± 0.11 [\(n = 9\); \(P < 0.05\); Figure 8]). These data provide additional evidence that flow stimulation provokes bilateral ATP release from the mTAL.

**Length of Primary Cilium in mTAL of P2Y\(_2\) WT and KO Mice**

MDCK cells without primary cilium show no flow response, and intact renal tubules from orpk mice with shortened primary cilia display a reduced flow-stimulated increase of [Ca\(^{2+}\)].\(^{15}\) We therefore investigated whether the reduced flow response in mTAL of P2Y\(_2\) KO mice coincides with reduced cilia length. The data in Figure 9 indicate that this is not the case. The length of the primary cilium was 3.8 ± 0.01 μm in WT (\(n = 4\) mice and 22 cilia) and 3.9 ± 0.01 μm in KO tubules (\(n = 3\) mice and 16 cilia). Figure 9 shows immunolabelling...
luminal membrane to promote Ca\textsuperscript{2+}.4,16 There are several reasons to speculate that paracrine 1/2. This in turn triggers Ca\textsuperscript{2+} and subsequent spread of the Ca\textsuperscript{2+} Tubular flow triggers bending of the primary cilium and mech-

DISCUSSION

The intention of this study was to investigate whether purinergic signaling is involved in the flow-induced increase of [Ca\textsuperscript{2+}]\textsubscript{i}. In intact renal tubules. The following mechanistic model for the flow-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase was suggested: Tubular flow triggers bending of the primary cilium and me-

Figure 7. (A) Effect of increasing the inflow perfusion pressure on tubular [Ca\textsuperscript{2+}]\textsubscript{i} (top) and geometry (middle) in the absence of luminal and basolateral Ca\textsuperscript{2+}. Removal of luminal Ca\textsuperscript{2+} did not affect resting [Ca\textsuperscript{2+}]. On the contrary, removal of basolateral Ca\textsuperscript{2+} always promptly and reversibly lowered [Ca\textsuperscript{2+}]. This indicates that G-protein–coupled receptors may play a role in the flow response in the presence of basolateral or luminal apyrase, an enzyme established to scavenge extracellular ATP. Basolateral apyrase robustly inhibited the response. Also luminal apyrase effectively reduced the flow response but was less potent. These results strongly support that a flow stimulus triggers the release of nucleotides. Nu-

Flow and Pressure Increases in the Renal Tubule

The method to increase tubular flow involves the sudden elevation of the inflow pressure, which therefore must increase the tubular flow. We did not measure the tubular flow by collection of fluid. However, in each experiment, we quantified the degree of tubular distension, which inevitably occurs dur-
ing this maneuver and is in part the consequence of the mechanical compliance of the tubule. The surrounding tissue in situ is likely to limit tubular distension. We observed an average of approximately 10 to 20% distension in very close similarity to values that were obtained with the same protocol in rabbit and mouse collecting duct15,17 or in animals in which in vivo imaging of distal tubular dimensions was quantified after the infusion of, for example, loop diuretics.20 It needs to be borne in mind that a complex mechanical stimulus is applied in our experiments. Increasing the pressure on the inflow side led to the acute, moderate distension of the tubule and at the same time increased the tubular flow. Throughout this article, we have used the term “flow response,” being aware that an equally suitable term could be “pressure or stretch response.” Our results need to be seen in contrast to those that were obtained in cultured cells that were grown on glass in an open chamber. In this “classical” system, an increase of inflow pressure does not cause distension of the epithelial layer but will primarily lead to increases of flow over the apical cell surface. This flow response is strictly dependent on the presence of luminal Ca2+ and shows a distinct time delay of approximately 35 s between the start of flow increase and the peak [Ca2+]i elevation.1 The response that we observed has a short time delay of a few seconds and is clearly inducible in the absence of bilateral Ca2+. These differences may suggest that the flow response in cultured cells reflects a different phenomenon to that described in the intact renal tubule.

The role of the primary cilium as a flow-sensing organelle was mentioned at the beginning of this article. Our results do not address whether cilia are required for the observed flow response in mouse mTAL. Noteworthy, the flow response is reduced in perfused collecting ducts of a mouse model (orpk) with shortened primary cilia.15 In conjunction with our findings, this may suggest a link between the primary cilium and nucleotide release. It will need to be established whether bending of the primary cilium is a mechanical trigger to release ATP from renal epithelium.

**Purinergic Signaling in the Renal Tubule**

Nonlytic cellular release of nucleotides is an established phenomenon in a wide variety of cells, including renal epithelia.8,10,21 The mechanism of this release is not unraveled, and multiple modes have been suggested.21 Released nucleotides are likely perceived by the renal tubule because the entire tubular system expresses multiple luminal and basolateral P2 receptors.6,22,23 P2 receptors are either metabotropic P2Y or ionotropic P2X receptor, and most of the 15 subtypes link to an increase of [Ca2+]i. Frequently, multiple P2 receptors are expressed in the same cell type, which can cause major problems to pinpoint one relevant receptor for a given effect.6,22 In mouse mTAL, we show luminal functional expression of only the P2Y2 receptor, whereas the basolateral membrane contains the P2Y2.
and at least one other ATP-sensitive P2 receptor that is able to stimulate a sustained Ca\(^{2+}\) influx. Many tissues display cell-to-cell propagating [Ca\(^{2+}\)]\(_i\) waves. In addition to gap junction–mediated [Ca\(^{2+}\)]\(_i\) waves, traveling [Ca\(^{2+}\)]\(_i\) signals are shown to be caused by extracellular nucleotides. This phenomenon is termed ATP-induced ATP release and underlies, for example, a propagation of a [Ca\(^{2+}\)]\(_i\) wave in astrocytes or mammary epithelial cells.\(^{21,24}\)

**CONCLUSION**

Our results strongly suggest that an increase of tubular flow produces tubular distension and an increase of [Ca\(^{2+}\)]\(_i\) in the intact nephron, which involves bilateral release of nucleotides from the renal epithelium and subsequent activation of luminal and basolateral P2 receptors. We speculate that the cilia-dependent flow response in cultured renal epithelia also involves a purinergic signaling component.

**CONCISE METHODS**

**Mice**

Mouse handling complied with Danish animal welfare regulations. P2Y\(_2\) receptor KO mice were provided as KO breeder pairs (on a B6D2 genetic background) by Dr. B.H. Koller (University of North Carolina, Chapel Hill, Chapel Hill, NC). B6D2 P2Y\(_2\)\(^{-/-}\) mice were crossed with the SV129 mouse strain, generating B6D2/SV129 P2Y\(_2\)\(^{+/+}\) (WT) and B6D2/SV129 P2Y\(_2\)\(^{-/-}\) (KO) littermates. Genotyping was performed as described previously.\(^{25}\)

**Tubule Perfusion**

Experiments were performed using 4- to 7-wk-old P2Y\(_2\) receptor WT and KO mice of either gender. Mice had free access to standard rodent diet and tap water. Mice were killed by decapitation. Both kidneys were removed and placed in ice-cold dissection solution from the inner stripe of the outer medulla using fine forceps. Kidney tubules were transferred to a perfusion chamber on an inverted microscope. Isolated tubules were perfused to a perfusion chamber on an inverted microscope. Isolated tubules were perfused using a system of concentric glass pipettes as described previously.\(^{27}\) Care was taken to choose viable tubules by microscopic inspection and responsiveness to 5 mM basolateral Ca\(^{2+}\) at the end of each experiment. High extracellular Ca\(^{2+}\) stimulates the calcium-sensing receptor.\(^{28}\) Only tubules with a brisk [Ca\(^{2+}\)]\(_i\) elevation were included, and the response was similar in WT and KO tubules (data not shown).

**Video Imaging of Perfused Renal Tubules**

The set-up comprised an inverted microscope (Axiovert 100 TV; Zeiss, Jena, Germany) with the objective C-Apochromat, 63×/1.2NA (Zeiss), a monochromator (Polychrome IV; Till Photonics, Planegg, Germany), and a CCD camera (MicroMax, 5 MHz; Princeton Instruments, Princeton, NJ). Image acquisition and data analysis were performed with Metamorph/MetaFluor (Universal Imaging, West Chester, PA). Measurement of [Ca\(^{2+}\)]\(_i\) was performed with fura-2 or fluo-4. Tubules were incubated in 20 µM basolateral fura-2-AM or fluo-4-AM for 20 min at room temperature in control solution. In Ca\(^{2+}\)-free conditions, fura-2 loading was performed without Ca\(^{2+}\) in the luminal perfusate. As a measure of [Ca\(^{2+}\)]\(_i\), with fura-2, the fluorescence emission ratio at 345 nm/380 nm excitation was used (acquisition frequency 0.5 Hz). The fluo-4 measured [Ca\(^{2+}\)]\(_i\) changes (excitation 488 nm, emission >510 nm) were expressed relative to baseline values. Cellular excitation light–induced damage\(^{29}\) was controlled by neutral density filters in the excitation light path and a three-fold binning. Fluorescence was recorded from the entire tubule (approximately 150 µm). During dye loading, the tubule was continuously perfused. The experiment was started 5 to 10 min after washout of extracellular dye.

**Immunohistochemistry**

Freshly dissected mTAL were placed on cell-tak–coated (BD Biosciences, Erembodegem, Belgium) coverslips and washed in PBS. Tubules were fixed in 2.5% paraformaldehyde at room temperature. Tissue was washed and permeabilized with 0.3% Triton-X-100 PBS that contained BSA (15 g/L) for 15 min and incubated overnight with anti-acetylated tubulin (Sigma-Aldrich, Schnelldorf, Germany) at 4°C. After washing, sections were incubated with Alexa488-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR) in PBS supplemented with BSA and Triton X-100. The preparations were inspected with a c-Apochromat 63×/1.2NA objective on a confocal microscope (LSM 410; Zeiss).

**Solutions and Chemicals**

Experiments were performed at 37°C with the following control solution (in mM): 145 NaCl, 1 MgCl\(_2\), 1.3 Ca-gluconate, 5 d-glucose, 0.4 KH\(_2\)PO\(_4\), 1.6 K\(_2\)HPO\(_4\), and 5 HEPES. The Ca\(^{2+}\)-free solution contained (in mM) 145 NaCl, 1 MgCl\(_2\), 5 d-glucose, 0.4 KH\(_2\)PO\(_4\), 1.6 K\(_2\)HPO\(_4\), 5 EGTA, and 5 HEPES. The dissection solution contained (in mM) 118 NaCl, 24 NaHCO\(_3\), 1.2 MgCl\(_2\), 1.3 Ca-gluconate, 5 d-glucose, 0.4 KH\(_2\)PO\(_4\), and 1.6 K\(_2\)HPO\(_4\). Solutions were titrated with NaOH to pH 7.4 (37°C).

Fura-2-AM and fluo-4-AM were obtained from Invitrogen (Taastrup, Denmark). All other chemicals were obtained from Sigma-Aldrich Denmark (Vallensbæk, Denmark) and Merck (Darmstadt, Germany). Agonist and antagonist solutions were prepared directly before the experiment.

**Statistical Analyses**

Data are shown as means ± SEM. For experimental series, the \(n\) reflects the number of tubules used. On average, two tubules were used from each mouse. Paired and unpaired \(t\) test were used to compare mean values within one experimental series. \(P < 0.05\) was accepted to indicate statistical significance.

**DISCLOSURES**

None.
REFERENCES