Localization of the succinate receptor in the distal nephron and its signaling in polarized MDCK cells

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When the succinate receptor (SUCNR1) is activated in the afferent arterioles of the glomerulus it increases renin release and induces hypertension. To study its location in other nephron segments and its role in kidney function, we performed immunohistochemical analysis and found that SUCNR1 is located in the luminal membrane of macula densa cells of the juxtaglomerular apparatus in close proximity to renin-producing granular cells, the cortical thick ascending limb, and cortical and inner medullary collecting duct cells. In order to study its signaling, SUCNR1 was stably expressed in Madin-Darby Canine Kidney (MDCK) cells, where it localized to the apical membrane. Activation of the cells by succinate caused Gq and Gi-mediated intracellular calcium mobilization, transient phosphorylation of extracellular regulated kinase (ERK)1/2 and the release of arachidonic acid along with prostaglandins E2 and I2. Signaling was desensitized without receptor internalization but rapidly resensitized upon succinate removal. Immunohistochemical evidence of phosphorylated ERK1/2 was found in cortical collecting duct cells of wild type but not SUCNR1 knockout streptozotocin-induced diabetic mice, indicating in vivo relevance. Since urinary succinate concentrations in health and disease are in the activation range of SUCNR1, this receptor can sense succinate in the luminal fluid. Our study suggests that changes in the luminal succinate concentration may regulate several aspects of renal function.


KEYWORDS: GPCR; hypertension; MDCK; signaling; SUCNR1

G-protein-coupled receptors (GPCRs) have a major role in the regulation of many (patho-) physiological processes in the human body. Their role is to transfer signals from the extracellular environment to the inside of the cell via effector proteins and multiple cellular signaling pathways. The receptor GPR91,1 which is related to the family of P2Y purinoreceptors, was found to be activated by succinate2 and was therefore renamed succinate receptor 1 (SUCNR1).

Among other organs, SUCNR1 is highly expressed in the kidney. Importantly, there are strong indications that the SUCNR1 is involved in hyperglycemia and diabetes-induced hypertension, because rat and mouse models of hypertension and metabolic syndrome have increased succinate levels when compared with healthy control animals,3 and injection of succinate into normal, but not SUCNR1 knockout mice induces the production and release of renin and hypertension.2 Also, SUCNR1-mediated renin release has been linked to hyperglycemia and diabetes.3,4 Therefore, the SUCNR1 may be an important protein in the kidney-derived onset of hypertension.

Consistent with its role in renal regulation of volume balance, immunohistochemistry identified expression of SUCNR1 near the juxtaglomerular apparatus (JGA), in particular the vascular endothelial cells of the afferent arteriole and in the glomerulus. Moreover, stimulation of the SUCNR1 in vascular endothelial cells leads to the mobilization of intracellular calcium and production of nitric oxide and prostaglandin (PG) E2 release, which contribute to the release of renin from granular cells and vasodilation of the afferent arteriole.4

Interestingly, however, SUCNR1 mRNA has also been detected in proximal and distal tubules,2 suggesting an additional role for SUCNR1 in the renal tubule. To increase our understanding of the function of SUCNR1 in renal physiology, we analyzed the expression of SUCNR1 along the nephron, and found that SUCNR1 is also expressed in the polarized cells of the thick ascending limb of Henle’s loop (TAL) and the cortical and inner medullary collecting duct (CCD and IMCD, respectively). As a model for these cells,

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we subsequently generated SUCNR1-expressing polarized Madin–Darby Canine Kidney (MDCK) cells and analyzed the regulation of SUCNR1 and its downstream signaling pathways on the (sub)cellular level and in vivo.

RESULTS

Localization of SUCNR1 in the kidney
To assess whether SUCNR1 may have a role in renal tubular physiology, we set out to determine the cellular and subcellular localization of SUCNR1 in the kidney. Staining of rat kidney sections for SUCNR1 with antibody Q-15 followed by confocal laser scanning microscopy (CLSM) clearly shows that the receptor localizes to the luminal membrane of cells morphologically resembling cells of the thick ascending limb (TAL) of Henle’s loop (Figure 1a and b). No staining was observed after preabsorption of the antibody with the immunizing peptide (not shown). The specificity of this localization was confirmed by staining with a second SUCNR1-specific antibody, H-80 (not shown). In addition, staining with this antibody reveals expression of SUCNR1 in glomerular cells (Figure 1c and d), which, as reported,4 likely represent endothelial cells of the glomerular capillaries and the afferent arteriole. To assess whether the localization of SUCNR1 is conserved between species, labeling of mouse kidneys was carried out. Also here, SUCNR1 was detected in the luminal membrane of the cortical TAL (Supplementary Figure S1).

As SUCNR1 activity is involved in the release of renin from the kidney and considering the complex architecture of the JGA, we further assessed whether the cells expressing SUCNR1 also express renin, or are in close proximity to renin-expressing cells. Co-staining of rat kidney sections revealed that in the cortex, the tubular cells expressing SUCNR1 do not express renin themselves and that renin is only expressed in granular arteriolar cells (Figure 1e–h). SUCNR1-expressing cells in the TAL are often adjacent to these renin-expressing cells (Figure 1g and h), suggesting that SUCNR1 is localized to the macula densa (MD). To examine this possibility at a subcellular level, immunogold electron microscopy was carried out. As shown in Figure 2, SUCNR1 was detected in MD cells, both in the apical membrane and in distinct intracellular vesicles morphologically resembling endosomes.

As shown in Figure 1i, double immunofluorescence labeling of rat kidney inner medulla for SUCNR1 and aquaporin-2 (AQP2), a marker for collecting duct principal cells, revealed that SUCNR1 localizes to the IMCD. At high magnification (inset, bottom right), it is clear that the majority of SUCNR1 labeling is associated with the apical plasma membrane domains.

Figure 1 | Renal localization of the SUCNR1. (a) Immunofluorescence labeling using a SUCNR1 antibody (Q-15) identified that SUCNR1 is localized to distinct tubule segments. (b) Overlay with differential interference contrast (DIC) image identifies that SUCNR1 is localized to the cortical thick ascending limb of Henle’s loop (TAL). (c) Immunofluorescence labeling using an alternative SUCNR1 antibody (H-80) localized SUCNR1 to both cells within the glomerulus and a region of the TAL associated with the juxtaglomerular apparatus (JGA). (d) Overlay with DIC image. (e) Double immunofluorescence labeling of SUCNR1 (Q15) (green) and renin (red) determined that SUCNR1 does not colocalize with renin in juxtaglomerular cells. (f) Overlay with DIC image. (g) Double immunofluorescence labeling of SUCNR1 (Q15) (green) and renin (red). (h) Overlay with DIC image clearly shows that SUCNR1 is detected in tubules morphologically resembling the macula densa. (i) Double immunofluorescence labeling of renal medulla for SUCNR1 (green) and AQP2 (red) determined that SUCNR1 localizes to the IMCD. At high magnification (inset, bottom right), it is clear that the majority of SUCNR1 labeling is associated with the apical plasma membrane domains. (j) Double immunofluorescence labeling of SUCNR1 (green) and AQP2 (red) in the cortex, where SUCNR1 is very weakly expressed in AQP2-containing tubules. (k) In the IMCD, SUCNR1 (green) does not colocalize with the basolateral membrane marker AE2 (red). (l) In the IMCD, SUCNR1 (green) can be observed in renin-expressing cells (red). A, arteriole; AQP-2, aquaporin-2; G, glomerulus; IMCD, inner medullary collecting duct; MD, macula densa; P, proximal tubule; T, thick ascending limb; SUCNR1, succinate receptor.
magnification, it is clear that the majority of SUCNR1 labeling is associated with the apical plasma membrane domains (inset, bottom right). The same double labeling performed on the renal cortex (Figure 1j) shows that SUCNR1 is weakly expressed in AQP2-containing cells, indicating that the receptor is also expressed in principal cells of the cortical collecting duct (CCD). No SUCNR1 staining was found in CCD-intercalated cells. In the IMCD, SUCNR1 also localizes to the apical membrane, as it does not colocalize with the basolateral membrane marker anion exchanger (AE)2 (Figure 1k, inset, bottom right and Figure S2A), which is especially clear in the later portions of the IMCD (Figure S2B). Moreover, as shown in Figure 1l, IMCD cells expressing SUCNR1 also express renin. Finally, cells of the thin descending limb show weak labeling for SUCNR1 (Figure S2B).

Taken together, these data indicate that the SUCNR1 is, besides endothelial cells of the glomerular vasculature, also expressed in the luminal membrane of tubular cells from different renal segments where it may be a sensor for succinate in the pro-urine.

Localization of SUCNR1 in MDCK cells
Since TAL cells and principal cells of the CCD and IMCD are polarized, they may have alternative SUCNR1 localization, trafficking, and signaling properties compared with non-polarized, for example, endothelial cells. Therefore, we employed Madin–Darby Canine Kidney (MDCK) cells as a model to study SUCNR1 localization, signaling and regulation. MDCK cells were stably transfected with expression constructs encoding C-terminally eYFP or c-myc-tagged SUCNR1 and individual clones were isolated. N-terminal epitope tags could not be used, because these affected the localization of SUCNR1 (not shown).

Based on bioinformatical analysis, the SUCNR1 consists of seven transmembrane domains, which is characteristic for GPCRs, and has two putative N-glycosylation sites: one at Asn4 in the N terminus and one at Asn164 in the second extracellular loop (Figure 3a). Western blot analysis of MDCK–SUCNR1–eYFP cells revealed a clear expression in the apical domain of the cells, as it showed overlap with staining of apically applied wheat-germ agglutinin, and no colocalization with the basolateral plasma membrane marker E-Cadherin (Figure 3b). The localization of SUCNR1 in MDCK cells is thus similar to what is observed in TAL and CD principal cells in vivo (Figure 1), indicating that the MDCK cell is a proper model for MD and principal cells, and that the C-terminal tag does not interfere with trafficking of the receptor.

Western blot analysis of SUCNR1–eYFP cells with GFP antibodies showed a strong signal of approximately 90–100 kDa and a weaker doublet of bands of approx. 60 kDa (Figure 3c). The upper band of this doublet was absent following digestion with both PNGaseF and EndoH, suggesting that the lower and upper band represent the unglycosylated and the high-mannose glycosylated immature forms of the receptor, respectively. Upon digestion of the protein mix with PNGaseF, but not endoH, the 90–100 kDa band completely disappeared into the 60 kDa signal (Figure 3c). This indicates that SUCNR1 is only glycosylated at Asn (no O-glycosylation) and that the 90–100 kDa band is the N-linked complex-glycosylated form of the receptor. Together, these observations reveal that SUCNR1 is expressed as a mature and properly folded receptor when expressed in MDCK cells.

SUCNR1 signaling in MDCK cells
To determine the signaling characteristics of the SUCNR1 in polarized cells, we first assessed whether this receptor could be activated selectively by succinate. As shown in Figure 4a, administration of the E_max concentration of 200 μmol/l Suc_2 to untransfected MDCK cells did not induce phosphorylation of extracellular regulated kinase (ERK1/2), whereas these cells showed a robust signal to addition of serum (Figure 4a). In contrast, stimulation of MDCK–SUCNR1 cells with succinate for 5 min resulted in increased phosphorylation of ERK1/2 (Figure 4a), whereas they remained unresponsive to administration of a related compound from the Krebs’ cycle, α-ketoglutarate (αKG).

Activation of the ERK1/2 pathway by GPCRs can be G-protein-mediated, which is a rapid and transient process (< 10 min), or it can be mediated via β-arrestins, resulting in a slower and more sustained phosphorylation of ERK1/2.
As shown in Figure 4b, SUCNR1 activation induced transient ERK1/2 phosphorylation, which was at its maximum at 2 min after addition of ligand and remained significantly increased compared with the control at $t=5$ and 10 min ($P<0.05$; Student’s t-test), suggesting that ERK1/2 activation by these receptors is G-protein-mediated.

To further investigate receptor signaling, we employed FURA-2 measurements to assess receptor-induced intracellular calcium mobilization. SUCNR1-specific activation by succinate dose-dependently increased calcium mobilization with a half-maximum potency (EC<sub>50</sub>) of 23.1 ± 12.8 μmol/l (Figure 4c). To assess which G proteins were involved in the SUCNR1-mediated calcium mobilization, we pre-incubated the cells with YM254890, an inhibitor of G<sub>q/11</sub>, or pertussis toxin (PTX), an inhibitor of G<sub>i/o</sub>. Inhibition of G<sub>i</sub> resulted in a decrease in the maximum efficacy ($E_{\text{max}}$), but did not significantly affect the potency of the signal (EC<sub>50</sub> = 22.1 ± 14.1 μmol/l) (Figure 4c). Blockade of the G<sub>q/11</sub> pathway also resulted in a marked reduction of the maximal efficacy and also resulted in a shift of the EC<sub>50</sub> to 67.4 ± 14.7 μmol/l. Combined application of the blockers resulted in complete absence of calcium mobilization in response to succinate (Figure 4c). These data suggest that the SUCNR1 uses both the G<sub>q/11</sub> and G<sub>i/o</sub> pathways to increase intracellular calcium and induce ERK1/2 phosphorylation.

**SUCNR1 activation triggers the release of arachidonic acid and the production of prostaglandins**

As intracellular calcium mobilization and ERK1/2 phosphorylation can induce the release of arachidonic acid (AA),<sup>4</sup> a precursor of prostaglandins, we tested whether activation of SUCNR1 was able to induce the release of AA from MDCK cells. As shown in Figure 5a, untransfected control cells did not respond to 200 μmol/l Suc, but released [³H]AA could be measured after stimulation of endogenous purinoreceptors with 100 μmol/l ATP or induction of a calcium flux with 1 μmol/l of the calcium ionophore ionomycin. In SUCNR1 cells, however, stimulation with succinate triggered the
release of $[^3]$H]AA 2.61 ± 0.09 fold over basal levels. This release was not significantly different ($P > 0.05$; one-way ANOVA) from the $[^3]$H]AA release measured upon ATP or ionomycin treatment of the SUCNR1 cells.

Next, we determined the identity of the prostaglandins released by the SUCNR1 cells (Figure 5b). Supernatant collected from unstimulated SUCNR1 cells contained prostaglandin (PG)E2, 6-keto-PGF1α (a stable metabolite from PGI2) and PGF2α. Stimulation with 200 μmol/l succinate significantly ($P < 0.05$) increased PGE2 in both the apical and basolateral medium, whereas 6-keto-PGF1α/PGI2 was only increased in the apical medium (Figure 5b). In these studies, PGE2 levels remained unaltered (not shown), whereas thromboxane (Tx)B2 (a stable metabolite from TxA2) was not detected in the supernatant under any of the conditions above. These data indicate that stimulation of SUCNR1 in polarized cells increases the release of PGE2 to both sides and of PGI2 to only the luminal side.

Desensitization and internalization of the SUCNR1

To prevent continuous signaling, many GPCRs undergo desensitization and/or internalization shortly after agonist stimulation. Calcium mobilization measurements showed that pre-treatment of MDCK–SUCNR1–eYFP cells with succinate for 15 min markedly decreased the $E_{max}$ value (88.6 ± 5.3% reduced) and increased the $EC_{50}$ value (from 26.3 μmol/l for control to 88.9 μmol/l; Figure 6a), which indicated that the SUCNR1 is indeed desensitized. Following desensitization, receptors may either be resensitized, or they can be downregulated. After removal of the ligand, two washes and only a 15 min resensitization period at 37 °C, the $E_{max}$ and $EC_{50}$ returned to control values (Student’s t-test;
by resensitization for 15 min at 37°C and subsequent stimulation with succinate for 5 min reversed the desensitization effect to a large extent (72.6 ± 8.3% compared with control) (Figure 6b), indicating that also SUCNR1-induced ERK1/2 phosphorylation is subject to desensitization and resensitization.

To further explore the potential internalization of SUCNR1-eYFP in response to succinate, we performed cell-surface biotinylation experiments. Immunoblot analysis revealed that the cell-surface (biotinylated) fraction of the mature 90–100 kDa form of SUCNR1-eYFP was not reduced by treatment with 200 μmol/l succinate for 1 h at 37°C (Figure 6c). This demonstrates that, despite desensitization, SUCNR1 is resistant to agonist-induced internalization. In line with this, CLSM analysis of SUCNR1-eYFP cells treated as above showed no different subcellular localization compared with untreated control cells (Figure 6d). These observations indicate that SUCNR1 undergoes rapid desensitization and resensitization, and that the receptor is not subject to agonist-mediated internalization in polarized MDCK cells.

**Signaling of SUCNR1 in the CD of diabetic mice**

As SUCNR1-mediated pERK1/2 signaling may be involved in the onset of hypertension in diabetes, we assessed whether signaling events that occur in our polarized cell model can be extrapolated to the *in vivo* situation. For this, wild-type (SUCNR1+/+) and SUCNR1 knock-out (SUCNR1+/–) mice were made diabetic using streptozotocin, whereas control animals were left untreated. As shown in Figure 7a and b, only very few cells in the CCD stained positive for pERK1/2 of wild-type and SUCNR1+/– non-diabetic control mice. In contrast, the amount of pERK1/2-positive CCD cells in diabetic animals is significantly (*P < 0.05*) increased compared with non-diabetic animals. As no pERK1/2 was found in the CCD of diabetic SUCNR1+/– mice (Figure 7d), the observed increase in pERK1/2 levels in the CCD of diabetic mice is likely the consequence of SUCNR1 activation.

**DISCUSSION**

**SUCNR1 is expressed in the luminal membrane of the cortical thick ascending limb and principal cells of the collecting duct**

Northern blot analysis revealed that the kidney is a major site of expression of the succinate receptor SUCNR1. In addition, analysis of SUCNR1 mRNA expression in different nephron segments indicated SUCNR1 expression in the proximal tubule, distal nephron, and JGA. Indeed, using SUCNR1-specific antibodies, Toma et al. showed SUCNR1 expression in endothelial cells of the afferent arteriole and in the glomerulus, where it appeared to regulate renin release following detection of blood succinate levels. Our data confirm SUCNR1 expression in the glomerular vasculature with antibody H-80, likely representing endothelial cells of the glomerular capillaries and the afferent arterioles. Using two different antibodies, however, we also observed SUCNR1

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**Figure 5: Activation of SUCNR1 triggers the release of arachidonic acid and the production of prostaglandins.**

(a) Measurement of arachidonic acid release: Untransfected control cells, MDCK-SUCNR1-eYFP cells were seeded in 24 MW plates, starved and loaded with [3H]Arachidonic acid (AA). Subsequently, cells were treated with 200 μmol/l succinate (Suc), 100 μmol/l ATP or 1 μmol/l ionomycin (Iono) for 15 min at 37°C. Subsequently, released [3H]AA was measured by counting the supernatant in a scintillation counter. Cells were lysed and total lysates were counted to determine total [3H]AA incorporation into the cells. The graph shows the percentage of [3H]AA that was released. Bars indicated with an asterisk are significantly different from the untreated controls (*P < 0.05; n = 3*). (b) Production and release of prostaglandins: MDCK-SUCNR1-eYFP cells were seeded on filters and grown to confluence. Subsequently, cells were left untreated (control) or treated with 200 μmol/l sucinate or 100 μmol/l ATP for 6 h at 37°C. Subsequently, the apical (white bars) and basolateral (black bars) culture medium was removed from the cells and analyzed for levels of prostaglandin E2, prostacyclin I2, and/or their metabolites. Asterisks indicate significantly (*P < 0.05; n = 3*) increased levels compared with their respective controls. MDCK, Madin–Darby Canine Kidney; eYFP, enhanced yellow fluorescent protein; SUCNR1, succinate receptor.

P > 0.05; Figure 6a). In fact, even agonist pre-treatment for up to 4 h did not affect the rate at which receptor resensitization occurred (data not shown). Similar to results of the Fura-2 measurements, pre-treatment of SUCNR1 cells with succinate for 15 min followed by 5 min of stimulation with 200 μmol/l fresh succinate resulted in decreased levels of phosphorylated ERK1/2 to 24.6 ± 7.1% compared with the control cells that had not been subjected to succinate pre-treatment (Figure 6b). Pre-treatment as above followed
expression in the luminal membrane of tubular cells of the cTAL, including macula densa cells (Figures 1 and 2), of which the latter were located in close proximity to the renin-producing JGA cells, and in CCD and IMCD principal cells. In the course of our study, Vargas et al. also reported SUCNR1 expression and signaling in the MD, as confirmed by co-staining with the MD marker nNOS. The possible implications of the localization and signaling of the SUCNR1 in cTAL, MD and CD cells is given below.

**SUCNR1 signals in response to physiological levels of succinate**

Similar to its localization in renal MD, cTAL and CD principal cells (Figures 1 and 2, Figures S1 and S2), epitope-tagged SUCNR1 was expressed in the apical membrane of MDCK cells (Figure 3b), suggesting that these cells represent a good model for SUCNR1 localization and regulation in renal tubular cells. Our data reveal that stimulation of SUCNR1 in polarized cells induces the same signaling cascade as found in non-polarized cells, as succinate increased intracellular calcium levels through the Gq and the Gs pathway and induced ERK1/2 phosphorylation. Moreover, activation of the SUCNR1 induces the production and release of PGE2 as observed in vivo. Our MDCK-SUCNR1 cells, however, also secrete PG12, which is only released on the apical side (Figure 5). Whether the latter also occurs in vivo and what physiological effect this may lead to remains to be established.

The EC50 value for succinate-induced Ca2+ mobilization in MDCK cells (23.1 ± 12.8 μmol/l) is of the same magnitude as described for non-polarized cells by He et al. (28–68 μmol/l) and Tomo et al. (69 μmol/l). Succinate excretion via urine in healthy individuals ranges between 2–12 mg/day, which, based on a daily urinary output of 1.5 l, corresponds to a urinary succinate concentration between 11 and 67 μmol/l. This indicates that the urinary succinate levels in healthy individuals are in the physiological range to activate the tubular SUCNR1.

**SUCNR1 undergoes rapid de- and resensitization at the apical plasma membrane**

Interestingly, in our MDCK cells, the SUCNR1 undergoes desensitization following succinate binding, but is resensitized to control levels very quickly following removal of succinate (Figure 6). In line with this, the agonists-occupied SUCNR1 is not degraded (Figure 6c), and cell-surface biotinylation and immunocytochemistry reveal a similar

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**Figure 6 Desensitization and resensitization of the SUCNR1.**

(a) Calcium measurements: MDCK-SUCNR1-eYFP cells were seeded in 96 MW plates and were left untreated ( ), pre-treated with 200 μmol/l succinate (Suc) for 15 min ( ), followed by ligand removal and resensitization for 15 min ( ). Cells were then challenged with increasing concentrations of agonist and calcium mobilization was measured and analyzed as described in the legend to Figure 4. Pooled data of three independent experiments are shown. (b) ERK1/2 phosphorylation: MDCK-SUCNR1-eYFP cells were pre-treated with succinate as above, and subsequently challenged for 5 min with 200 μmol/l Suc. Cells were then placed on ice, washed twice with ice-cold PBS-CM, lysed in sample buffer, and analyzed for phosphorylated (P) or total ERK1/2 levels by immunoblotting as described in the legend to Figure 4. (n = 3). (c) Plasma membrane localization of the SUCNR1: MDCK-SUCNR1-eYFP cells were seeded on filters and grown to confluence. Subsequently, cells were left untreated (control) or treated with 200 μmol/l succinate for 1 h at 37°C. Cells were rapidly cooled on ice, subjected to apical cell-surface biotinylation and samples were analyzed by SDS-PAGE followed by immunoblotting using anti-GFP antibodies. A representative blot is shown. Immunoblot signals for the cell-surface fraction of succinate-treated and untreated control cells were quantified using densitometry and were found to be not significantly different. (P > 0.05; n = 3) TL; Total lysates, Biot.; biotinylated cell-surface fraction. (d) Subcellular localization of the SUCNR1: MDCK-SUCNR1-eYFP cells were grown and pre-treated as described under c. Subsequently, cells were fixed and analyzed by CLSM. Shown are representative XY scans (n = 4) and a corresponding cross-section. MDCK, Madin-Darby Canine Kidney; eYFP, enhanced yellow fluorescent protein; SUCNR1, succinate receptor.
membrane localization of the SUCNR1 in untreated control and succinate-treated cells (Figure 6c and d). Assuming our MDCK cells mimic SUCNR1 regulation in the cTAL cells, the vesicles containing some SUCNR1 in MD cells (Figure 2) may represent recycling vesicles, continuously recycling their endocytosed proteins to the plasma membrane.

Our data are in contrast to those of He et al. who reported succinate-induced internalization of the SUCNR1 in HEK293 cells. One possibility is that the SUCNR1 is regulated differently in non-polarized (HEK293) cells as compared with polarized cells. On the other hand, however, the study of He et al. lacks important controls to substantiate their conclusion: Fluorescence microscopy is less appropriate to determine plasma membrane expression levels compared with cell-surface biotinylation assays. Moreover, the intracellular expression of the SUCNR1 in cells without succinate stimulation and plasma membrane SUCNR1 localization after succinate stimulation were not determined. It remains to be established whether the SUCNR1 is internalized in endothelial cells and whether the regulation of the SUCNR1 in MDCK cells mimics that in the polarized cTAL/MD/CD cells in vivo.

**Putative role of the SUCNR1 in the renal tubule**

Succinate is freely filtered in the glomerulus but is normally reabsorbed in the proximal tubules, mainly via sodium-dicarboxylate co-transporters. As such, SUCNR1-expressing cells of the cTAL, MD, and CCD will only sense succinate that fails to be reabsorbed in proximal tubule cells, or that is excreted by or beyond proximal tubule cells. As indicated above, however, succinate is found in the urine of healthy individuals at levels that will stimulate the SUCNR1. As such, the newly-identified localization of SUCNR1 in the luminal membrane of MD, cTAL, and CD principal cells may suggest several physiological functions for tubular SUCNR1.

As shown here and as recently described (Figure 1 and12), the succinate-induced release of PGE2 likely signals to granular cells, which are in close proximity to the basolateral side of the MD, to produce and release renin. Considering the known role of the glomerular endothelial SUCNR1 in inducing renin expression and release, and the proximal tubular reabsorption of succinate, the tubular SUCNR1 may further increase renin production and release only in conditions of hyperglycemia and diabetes when filtrated succinate is in excess of reabsorbed levels.

Besides SUCNR1-mediated release of PGE2 to the basolateral side, tubular activation of the SUCNR1 in the cTAL and CD may induce apical release of PGE2/PGI2, as observed in our MDCK cells (Figure 5b). As reported for tubular prostaglandins derived from cTAL/DCT and CD, such luminal prostaglandins may reduce blood pressure and hypertension by reducing NaCl reabsorption in the cTAL and diminishing water and/or NaCl reabsorption in the DCT and collecting duct. Alternatively, these luminal prostaglandins may trigger the release of RAS components from downstream tubular cells, such as renin from the connecting tubule or (pro)renin from the collecting duct in diabetes, which, via angiotensin II may stimulate sodium retention in the collecting duct via the epithelial sodium channel ENaC. However, as the SUCNR1 and renin are co-expressed in
IMCD cells (Figure 1l), SUCNR1 may also directly regulate expression and release of renin and/or regulate water- and salt transport in this nephron segment. Moreover, SUCNR1-mediated stimulation of ERK1/2 phosphorylation in the renal tubules of diabetic mice, as shown in Figure 7, is also observed in diabetic nephropathy, which, in the light of increased renal succinate levels found in diabetic animals, strongly suggests the involvement of SUCNR1 in this pathological condition.

Interestingly, previous studies showed that in MD cells, ERK1/2 phosphorylation was increasing in time following SUCNR1 stimulation, which usually points to the presence of pERK1/2 in the cytosol, where it can activate numerous cellular pathways. In contrast, in our MDCK cells, which are derived from more distal tubular cells, we found a transient rise in ERK1/2 phosphorylation, which is usually associated with migration of pERK1/2 to the nucleus and cell proliferation. The physiological impact of this discrepancy remains to be elucidated.

Besides the disease conditions indicated above, renal and/or tubular succinate levels may also increase in other pathological states: at low oxygen levels, Krebs’ cycle intermediates are converted to succinate and, as described for the ischemic retina, SUCNR1 signaling has a major role in the re-oxygenation and repair of the retina by stimulating angiogenesis. Hypoxic and ischemic conditions in the kidney are also prevalent during organ transplantation, in acute and chronic renal failure, and coincide with increased urinary succinate levels, suggesting that tubular SUCNR1 activation may have a similar role in the kidney. The exact role of the tubular SUCNR1 in renal physiology and disease, however, remains to be established.

MATERIALS AND METHODS

Expression constructs
cDNA encoding the human SUCNR1 was a kind gift of Dr Hampe and Dr Schaller (University of Hamburg, Hamburg, Germany). C-terminal fusions of SUCNR1 with enhanced yellow fluorescent protein (eYFP) or the c-myc epitope tag were generated as described in the Supplementary materials and methods.

Cell culture and cell assays
MDCKII cells were maintained as described. Cells were transfected with 2.5 μg plasmid DNA using Lipofectamine 2000 (Invitrogen, Paisley, UK) and individual clones selected and isolated as described. Protein sample preparation and EndoH and PNGaseF digestion were performed as described. Cell-surface biotinylation experiments, ERK1/2 phosphorylation assays, calcium mobilization assays, and [3H] Arachidonic acid release assays were carried out as described in the Supplementary materials and methods. Samples for prostanoid analysis were prepared as described and analyzed as described in the Supplementary materials and methods.

Immunoblotting and immunocytochemistry
Immunoblotting and immunohistochemistry were performed as described in the Supplementary materials and methods. Primary antibodies used were Rabbit-anti-ERK1/2, mouse-anti-phospho-ERK1/2 (Cell Signalling), rat-anti-E-Cadherin, and rabbit-anti-calnexin (Sigma, Poole, UK). Secondary antibodies used were HRP-conjugated donkey-anti-goat (Sigma), goat-anti-rabbit or goat-anti-mouse antibodies (GE Healthcare, Chalfont St Giles, UK) and Alexa633-conjugated goat-anti-rat, goat-anti-rabbit, or Alexa488-coupled goat-anti-rabbit or goat-anti-mouse antibodies (Molecular Probes, Eugene, OR, USA).

SUCNR1 –/+ mice and induction of diabetes
All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee at University of Southern California. Breeding pairs of SUCNR1 –/+ mice (C57BL6 background) were provided by Amgen (Thousand Oaks, CA, USA) and bred at University of Southern California. In male SUCNR1 –/+ mice, or their wild-type littermates, diabetes was induced by daily streptozotocin injection for 4 days as described.

Immunohistochemistry
Kidney sections were prepared and immunolabeling was performed as previously described and analyzed as described in the Supplementary materials and methods. Secondary antibodies used were goat-anti-rabbit, Alexa Fluor488; goat-anti-chicken, Alexa Fluor546; donkey-anti-goat, Alexa Fluor488 (Molecular Probes, Invitrogen). Primary antibodies used were rabbit-anti-GPR91(H80), goat-anti-GPR91(Q15, Santa Cruz, Heidelberg, Germany), rabbit-anti-GPR91 (Millipore, Watford, UK), rabbit anti-GPR91 (Novus Biologicals, Littleton, CO, USA), chicken-anti-renin (kind gift of Hayo Castrup, University of Regensburg, Germany), chicken-anti-AQP2, and rabbit-anti-pERK1/2 antibodies (Cell Signalling, Hitchin, UK).

Immunogold electron microscopy
Ultra thin (70 nmol/l) cryosections from perfusion fixed rat kidney cortex were prepared and analyzed as described in the Supplementary materials and methods. Primary antibodies were rabbit-anti-GPR91(H80) or goat-anti-GPR91(Q15). Primary antibodies were visualized using goat-anti-rabbit or rabbit-anti-goat antibodies conjugated to 10 nmol/l colloidal gold particles (BioCell Research Laboratories, UK).

DISCLOSURE
The authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1.
Figure S2.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES


