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

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Abstract

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 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 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 ERK1/2 and the release of arachidonic acid along with prostaglandins E2 and I2. Signaling was desensitized without receptor internalization but rapidly re-sensitized 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 the 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.

Introduction

G protein-coupled receptors (GPCRs) play 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 signalling pathways. The receptor GPR91[1], which is related to the family of P2Y purinoreceptors, was found to be activated by succinate[2] and was therefore renamed succinate receptor 1 (SUCNR1).

Among other organs, SUCNR1 is highly expressed in the kidney[2]. 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 compared to 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, we subsequently generated SUCNR1-expressing polarized Madin-Darby Canine Kidney (MDCK) cells and analyzed the regulation of SUCNR1 and its downstream signalling 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 (Fig.1A-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 (Fig.1C-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, labelling of mouse kidneys was performed. 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 (Fig.1E-H). SUCNR1-expressing cells in the TAL are often adjacent to these renin-expressing cells (Fig.1G-H), suggesting that SUCNR1 is localized to the macula densa (MD). To examine this possiblility at a subcellular level, immunogold

electron microscopy was performed. 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, 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 (Fig.lJ) 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 co-localize with the basolateral membrane marker anion exchanger (AE)2 (Fig.1K, inset bottom right and Suppl. Fig.S2A), which is especially clear in the later portions of the IMCD (Suppl Fig.S2B). Moreover, as shown in Fig.1L, IMCD cells expressing SUCNR1 also express renin. Finally, cells of the thin descending limb show weak labeling for SUCNR1 (Suppl. Fig.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 signalling properties compared to non-polarized, e.g. endothelial, cells[5]. Therefore, we employed Madin-Darby Canine Kidney (MDCK) cells as a model to study SUCNR1 localization, signalling 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 7 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 (Fig.3A). CLSM 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 co-localization with the basolateral plasma membrane marker E-Cadherin (Fig.3B). The localization of SUCNR1 in MDCK cells is thus similar to what is observed in TAL and CD principal cells *in vivo* (Fig.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 (Fig.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 (Fig.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 signalling in MDCK cells.

To determine the signalling 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 200μM 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 (Fig.4A). In contrast, stimulation of MDCK-SUCNR1 cells with succinate for 5 minutes resulted in increased phosphorylation of ERK1/2 (Fig.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[6;7]. As shown in fig.4B, SUCNR1 activation induced transient ERK1/2 phosphorylation, which was at its maximum at 2 minutes after addition of ligand and remained significantly increased compared to the control at t = 5 and 10 minutes ($P<0.05$; Student's T-test), suggesting that ERK1/2 activation by this receptors is G protein-mediated.

To further investigate receptor signalling, 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_{50}) of 23.1±12.8μM (Fig.4C). To assess which G proteins were involved in the SUCNR1-mediated calcium mobilization, we pre-incubated the cells with YM254890, an inhibitor of $G_{q/11}$, or pertussis toxin (PTX), an inhibitor of $G_{i/o}$. Inhibition of G_i resulted in a decrease in the maximum efficacy (E_{max}), but did not significantly affect the potency of the signal (EC_{50} = 22.1±14.1μM) (Fig.4C). Blockade of the $G_{q/11}$ pathway also resulted in a marked reduction of the maximal efficacy and also resulted in a shift of the EC_{50} to 67.4±14.7μM. Combined application of the blockers resulted in a complete absence of calcium mobilization in response to succinate (Fig.4C). These data suggest that the SUCNR1 utilizes both the $G_{q/11}$ and $G_{i/o}$ 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)[8], 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 200μM Suc, but released [^{3}H]AA could be measured after stimulation of endogenous purinoreceptors with 100μM ATP or induction of a calcium flux with 1μM 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 (Fig.5B). Supernatant collected from unstimulated SUCNR1 cells contained prostaglandin (PG)E2, 6-keto-PGF1α (a stable metabolite from PGI2) and PGF2α. Stimulation with 200μM 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 (Fig.5B). In these studies, PFG2α 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 signalling, 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 minutes markedly decreased the E_{max} value (88.6±5.3% reduced) and increased the EC_{50} value (from 26.3μM for control to 88.9μM;Fig.6A), which indicated that the SUCNR1 is indeed desensitized. Following desensitization, receptors may either be resensitized, or they can be down-regulated. After removal of the ligand, two washes and only a 15 minute resensitization period at 37ºC, the E_{max} and EC_{50} returned to control values

(Student's T-test; P>0.05; Fig.6A). In fact, even agonist pre-treatment for up to 4 hours 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 minutes followed by 5 minutes of stimulation with 200μM fresh succinate resulted in decreased levels of phosphorylated ERK1/2 to 24.6 ± 7.1% compared to the control cells that had not been subjected to succinate pre-treatment (Fig.6B). Pre-treatment as above followed by resensitization for 15 minutes at 37ºC and subsequent stimulation with succinate for 5 minutes reversed the desensitization effect to a large extent (72.6 ± 8.3% compared to control) (Fig.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[9-11]. 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 200μM succinate for 1 hr at 37ºC (Fig.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 to untreated control cells (Fig.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.

Signalling of SUCNR1 in the CD of diabetic mice

As SUCNR1-mediated pERK1/2 signalling may be involved in the onset of hypertension in diabetes, we assessed whether signalling events that occur in our polarized cell model can be extrapolated to the *in vivo* situation. For this, wild-type (SUCNR+/+) and SUCNR1 knock-out (SUCRN-/-) mice were made diabetic using streptozotocin, whereas control animals were left untreated. As shown in Fig.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 to non-diabetic animals. As no pERK1/2 was found in the CCD of diabetic SUCNR-/- mice (Fig.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[2]. Additionally, analysis of SUCNR1 mRNA expression in different nephron segments indicated SUCNR1 expression in the proximal tubule, distal nephron and JGA[2]. 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[4]. 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 expression in the luminal membrane of tubular cells of the cTAL, including macula densa cells (Figs.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 signalling in the MD, as confirmed by co-staining with the MD marker nNOS[12]. The possible implications of the localization and signalling 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 (Fig.1 and 2, Suppl. Fig.S1 and S2), epitope-tagged SUCNR1 was expressed in the apical membrane of MDCK cells (Fig.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 via the G_q and the G_i pathway and induced ERK1/2 phosphorylation[2]. Moreover, activation of the SUCNR1 induces the production and release of PGE2 as observed *in vivo*[4]. Our MDCK-SUCNR1 cells, however, also secrete PGI2, which is only released on the apical side (Fig.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±12.8μM) is of the same magnitude as described for non-polarized cells by He *et al.* (28-68μM)[2] and Toma *et al.* (69μM)[4]. Succinate excretion via the urine in healthy individuals

ranges between 2-12 mg/day[13], which, based on a daily urinary output of 1.5 liter, corresponds to a urinary succinate concentration between 11 and 67μM. 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 re-sensitized to control levels very quickly following removal of succinate (Fig.6). In line with this, the agonists-occupied SUCNR1 is not degraded (Fig.6C), and cell surface biotinylation and immunocytochemistry reveal a similar membrane localization of the SUCNR1 in untreated control and succinate-treated cells (Fig.6C-D). Assuming our MDCK cells mimic SUCNR1 regulation in the cTAL cells, the vesicles containing some SUCNR1 in MD cells (Fig.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[2]. One possibility is that the SUCNR1 is regulated differently in non-polarized (HEK293) cells as compared to 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 to 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[2]. 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[14]. As such, SUCNR1-expressing cells of the cTAL, MD and CCD will only sense succinate that fails to be reabsorbed in proximal tubules, 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[13]. 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 (Fig.1 and [12]), 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 rennin 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 (Fig.5B). As reported for tubular prostaglandins derived from cTAL/DCT and CD[15-17], 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[18], such as renin from the connecting tubule[19;20] or (pro)renin from the collecting duct in diabetes[21], 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 (Fig.1L), SUCNR1 may also directly regulate expression and release of renin and/or regulate water- and salt transport in this nehron 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[22], which, in the light of increased renal succinate levels found in diabetic animals[4], 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[12], 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[23]. The physiological impact of this discrepancy remains to be elucidated.

Besides the disease conditions indicated above, renal and/or tubular succinate levels may also be increased in other pathological states: at low oxygen levels, Krebs' cycle intermediates are converted to succinate[24;25] and, as described for the ischemic retina,

SUCNR1 signalling has a major role in the re-oxygenation and repair of the retina by stimulating angiogenesis[26]. Hypoxic and ischemic conditions in the kidney are also prevalent during organ transplantion, in acute and chronic renal failure, and coincide with increased urinary succinate levels[25;27;28], suggesting that tubular SUCNR1 activation may play 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[29]. Cells were transfected with 2.5μg plasmid DNA using Lipofectamine 2000 (Invitrogen, Paisley, UK) and individual clones selected and isolated as described[11]. Protein sample preparation and EndoH and PNGaseF digestion were performed as described[29]. Cell surface biotinylation experiments, ERK1/2 phosphorylation assays, calcium mobilization assays and [^{3}H] Arachidonic acid release assays were performed as described in the supplementary materials and methods. Samples for prostanoid analysis were prepared as described [30] 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, Poole, UK), 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).

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) 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 [12].

Immunohistochemistry

Kidney sections were prepared and immunolabeling was performed as previously described[4;12;31] 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), rabbit-anti-GPR91 (Millipore), rabbit anti-GPR91 (Novus Biologicals), chicken-anti-renin (kind gift of Hayo Castrup, University of Regensburg, Germany), chicken-anti-AQP2, and rabbit-anti-pERK1/2 antibodies (Cell Signaling).

Immunogold electron microscopy.

Ultrathin (70nm) 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 10nm colloidal gold particles (BioCell Research Laboratories, UK).

Disclosure

The authors state no competing financial interest

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Legends and titles

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). Abbreviations; G = glomerulus, T = thick ascending limb, P = proximal tubule, A = arteriole, MD = macula densa.

Figure 2. Immunoelectron microscopy of SUCNR1 in rat kidney.

A) Overview of the macula densa (MD) region of the thick ascending limb. B) In the MD cells immunogold labeling of SUCNR1 (H80) is observed intracellularly (arrowheads) and in the apical plasma membrane domains (inset, arrows). C) Using an alternative SUCNR1 antibody (Q15), a similar pattern of labeling is observed, with gold particles in direct contact with the plasma membrane (inset). D) In some MD cells, very few gold particles are observed in the plasma membrane. In these cells, labeling of distinct vesicles morphologically resembling endosomes (*) are apparent. Abbreviations; PT = proximal tubule, MD = macula densa, EM = extraglomerular mesangium, apm = apical plasma membrane, lm = lateral membrane.

Figure 3. Subcellular localization and glycosylation state of SUCNR1 in MDCK cells

A. Topology of the SUCNR1: The structure of GPCRs is composed of 7 transmembrane domains, indicated in roman numbers. Using bioinformatic tools on the amino acid sequences of SUCNR1, we predicted the topology of the receptor and the location of its potential N-glycosylation sites.

B. Subcellular localization of the SUCNR1: MDCK-SUCNR1-eYFP cells were seeded on filters, grown to confluence and labelled with the plasma membrane markers E-Cadherin (basolateral membrane) or wheat germ agglutinin (WGA; apical membrane) followed by CLSM analysis. Shown are representative XY scans and a corresponding cross-section.

C. Glycosylation state of the SUCNR1: MDCK-SUCNR1-eYFP cells were lysed, left untreated (control), or digested with endoH or PNGaseF (indicated). Samples were analyzed by SDS-PAGE and subjected to immunoblotting using a sheep-anti-GFP antiserum.

Figure 4. ERK1/2 and Calcium signalling by the SUCNR1

A. Ligand specificity of SUCNR1 signalling: Untransfected MDCK cells or SUCNR1-eYFP cells were grown to confluence and starved overnight and for a subsequent period of two hours. Next, cells were incubated with vehicle (control), 200μM succinate or αKG (indicated) for 5 minutes at 37ºC, followed by termination of the reaction on ice. Subsequently, cells were lysed and analyzed by SDS-PAGE followed by immunoblotting using anti-total ERK1/2 or anti-phospho-ERK1/2 antibodies. Signals were quantified using densitometry and are represented as fold increase over control (n=3).

B. Time-dependent ERK1/2 phosphorylation: MDCK-SUCNR1-eYFP cells were grown and starved as in A and subsequently incubated with 200μM succinate for the indicated times. Next, cells were cooled on ice and cell lysates analyzed and quantified as described under A (n=3).

C. Calcium signalling and G protein coupling of the SUCNR1: MDCK-SUCNR1-eYPF cells were seeded in 96 multiwell plates, left untreated (control), treated overnight with the $G_{i/o}$ inhibitor PTX, 2 hrs with the $G_{q/11}$ inhibitor YM254890, or a combination of both and subsequently loaded with FURA-2 AM. Cells were challenged with increasing concentrations of agonist and calcium mobilization was measured as changes in the λ340/ λ380 ratio using a FLEX-station. Pooled data of three individual experiments is shown.

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

A. Measurement of Arichadonic Acid release: Untransfected control cells, MDCK-SUCNR1-eYFP cells were seeded in 24 MW plates, starved and loaded with[^{3}H]Arachidonic acid (AA). Subsequently, cells were treated with 200μM Suc, 100μM ATP or 1μM ionomycin (indicated) for 15 minutes at 37ºC. Subsequently, released[^{3}H]AA was measured by counting the supernatant in a scintillation counter. Cells were lysed and total lysates were counted to determine total[^{3}H]AA incorporation into the cells. The graph shows the percentage[^{3}H]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 200μM succinate or 100μM ATP for 6 hrs at 37ºC. Subsequently, the apical (white bars) and basolateral (black bars) culture medium was removed from the cells and analysed for levels of prostaglandin E2, prostacyclin I2, and/or their metabolites. Asterisks indicate significantly ($P<0.05$; n=3) increased levels compared to their respective controls.

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 (■), pretreated with 200μM succinate for 15 minutes (▲), followed by ligand removal and resensitization for 15 minutes (▼). 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 3 independent experiments is shown.

B. ERK1/2 phosphorylation: MDCK-SUCNR1-eYFP cells were pre-treated with succinate as above, and subsequently challenged for 5 minutes with 200μM Suc. Cells were then placed on ice, washed twice with ice-cold PBS-CM, lysed in sample buffer and analysed for phosphorylated (P) or total ERK1/2 levels by immunoblotting as described in the legend to fig.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 200μM succinate for 1 hr at 37ºC. 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.

Figure 7. Immunolocalization of pERK1/2 in control and diabetic kidney

(A-D) Representative images of pERK1/2 immunofluorescence (red) in control non-diabetic SUCNR1+/+ (A), SUCNR1-/- (B), and diabetic SUCNR+/+ (C), GPR91 -/- (D) kidneys. (E) Summary of pERK1/2 in control non-diabetic (C) and diabetic (DM) kidney sections based on the number of pERK1/2-positive distol nephron cells per field. Error bars represent SEM. *: P<0.05 DM SUCNR1 GPR91 +/+ vs. DM SUCNR1-/-, #:P<0.05 vs DM SUCNR1+/+ (n=6). CNT: connecting segment, CCD: cortical collecting duct. Nuclei are stained blue. Scale bar is 20μm.

Supplemental Figure 1. Localization of SUCNR1 in mouse and rat kidney. A) Immunoperoxidase labeling using a SUCNR1 goat polyclonal antibody (Q-15) identified that SUCNR1 is localized to the TAL associated with the juxtaglomerular apparatus (JGA). B) Labeling using an alternative SUCNR1 rabbit polyclonal antibody (H-80) confirmed SUCNR1 expression in some TALs. C) SUCNR1 labeling is associated with the apical plasma membrane of the TAL. Abbreviations; G = glomerulus, T = thick ascending limb

Supplemental Figure 2: Localization of SUCNR1 in rat kidney IMCD. A) Immunoperoxidase labeling demonstrates that SUCNR1 is expressed in the IMCD. B) In the later portions of the IMCD (IMCD2/3), SUCNR1 labeling is associated with the apical plasma membrane. Abbreviations; IMCD = inner medullary collecting duct.

Supplementary 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). To generate C-terminal fusions of each receptor with enhanced Yellow Fluorescent Protein (eYFP) or the c-myc epitope tag, this cDNA was used in the polymerase chain reaction with the sense primer 91FWD (5'G**AAGCTTGCCACC**ATGGCATGGAATGCAACTTG3') to introduce a restriction site and the Kozak sequence (indicated in bold) upstream of the ATG start codon. To introduce a KpnI restriction site (indicated in bold) over the stop codon for fusion with eYFP, the antisense primer 91insKpnI (5'CAGGTACCCTTTTCTCTGAATGAAAGTAG3') was employed. For fusion of the C-terminal c-myc tag, a combination of 91FWD with 91myc (5'CACTGATGAGCTTCTGTTCCTTTTCTCTGAATG3') was used. Subsequently, the product was subjected to a second round of PCR using 91FWD in combination with 5'CT**GCGGCCGC**TACAGGTCCTCTTCACTGATGAGCTTCTG3' to fuse a NotI site to the c-myc tag. All PCR products were cloned into their corresponding restriction sites of the pEYFP-NI vector and their sequences verified.

Cell culture and transfection

MDCKII cells were maintained as described[1]. Cells were transfected with 2.5 μg plasmid DNA using Lipofectamine 2000 (Invitrogen, Paisley, UK) and individual clones

selected and isolated as described[2]. Protein sample preparation and EndoH and PNGaseF digestion were performed as described[1].

ERK1/2 assay

MDCK cells were seeded in 12 multiwell (MW) Costar plates at a density of 200,000 cells/cm^2 and grown to confluence. Subsequently, cells were starved overnight in serum-free culture medium, and the medium was replaced the next morning for an additional 2 hour starvation period. When the $G_{i/o}$ inhibitor Pertussis toxin (PTX, 25 ng/ml, Sigma, Poole, UK) was used, it was present throughout the starvation period, whereas the $G_{q/11}$ inhibitor YM-254890[3] was present during the final 2 hours of serum starvation. Next, cells were stimulated with the published Emax concentration[4] of 200 μM succinate (Sigma, Poole, UK) for the indicated time periods, followed by immediate cooling on ice and 2 washes with ice-cold PBS with 1mM $MgCl_2$ and 0.1mM $CaCl_2$ (PBS-CM). Cells were lysed in Laemmli buffer with 0.1 M dithiothreitol (DTT) and analyzed by immunoblotting followed by densitometry of the signals.

Cell surface biotinylation

Cells were grown on 6MW costar filters to confluence. Cells were left untreated or treated with succinate for 60 minutes followed by rapid cooling on ice and two washes with ice-cold PBS-CM. Cells were then subjected to cell surface biotinylation as described[2]

SDS-PAGE and immunoblotting

Protein samples were analyzed on 4-12% Novex gels (Invitrogen, Paisley, UK), and immublotted using an in-house generated sheep-anti GFP antiserum, or rabbit-anti c-myc antibodies (Cell Signalling, Hitchin, UK). Rabbit-anti ERK1/2 and mouse-anti phospho-ERK1/2 antibodies were from Cell Signalling. As secondary antibodies, HRP-conjugated donkey-anti goat (Sigma, Poole, UK), goat-anti rabbit or goat-anti mouse antibodies (GE Healthcare, Chalfont St. Giles, UK) were used. Detection was performed using Westpico Chemiluminescence substrate (Thermo Scientific).

Immunocytochemistry

Immunocytochemistry and confocal laser scanning microscopy (CLSM) of MDCKII cells was performed as described[1;5]. Primary antibodies used were rat-anti E-Cadherin, rabbit-anti calnexin (Sigma, Poole, UK), mouse-anti c-myc, rabbit-anti c-myc (Cell Signaling Technology, Hitchin, UK). Secondary antibodies used were Alexa^{633}-conjugated goat-anti rat, goat-anti rabbit, or Alexa^{488}-coupled goat-anti rabbit or goat-anti mouse (Molecular Probes, Eugene, OR).

Calcium mobilization assay

Cells were seeded in black-walled, clear-bottomed 96 MW plates at a density of 30,000 cells/well, grown overnight and subsequently loaded with 3 μg/ml FURA-2-AM (Sigma, Poole, UK) for 1 hour. Cells were washed three times in HEPES-glucose buffer (130 mM NaCl, 5 mM KCl, 20 mM HEPES, 10 mM D-glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$ (pH = 7.2) and allowed to equilibrate in 100 μl buffer for 15 min. at room temperature. Cells were transferred to a FLEXstation (Molecular Devices) and using the FLEXstation's

internal pipetting robot, 100 µl of two-times concentrated succinate was added to each well to stimulate the cells. Every three seconds λ=340/λ=380 ratios were measured for a total time period of 3 min. The difference between the maximum and minimum ratio measured over this period was used as readout for cytosolic calcium mobilization. Samples were measured in quadruplicate and experiments were performed at least in threefold, of which pooled data is presented. The data is shown as a relative increase compared to the negative control (no ligand).

Immunohistochemistry

Kidney sections were prepared and immunolabeling was performed as previously described[6-8]. For confocal laser scanning microscopy studies, the following secondary fluorescent antibodies were used at a 1:1,000 dilution: goat anti-rabbit IgG, Alexa Fluor 488; goat anti-chicken IgG, Alexa Fluor 546; donkey anti-goat IgG, Alexa Fluor 488 (Molecular Probes, Invitrogen). Laser confocal microscopy was carried out with a Leica TCS-SP2 laser confocal microscope. For immunoperoxidase labeling, staining was detected using a horseradish peroxidase-conjugated secondary antibodies (Dako) and visualized with 0.05% 3,3'-diaminobenzidine tetrachloride (DAB, Kemen Tek, Copenhagen, Denmark). Light microscopy was carried out with a Leica DMRE (Leica Microsystems). Antibodies used were rabbit anti-GPR91(H80, Santa Cruz) at 1:250 dilution, goat anti-GPR91 (Q15, Santa Cruz) at 1:200 dilution, rabbit anti-GPR91 (Millipore) used at 1:1000 dilution, rabbit anti-GPR91 (Novus Biologicals, Littleton, CO) chicken anti-renin antibody (a kind gift of Hayo Castrup, University of Regensburg,

Germany), a chicken anti-AQP2 antibody, and rabbit anti-pERK1/2 (Cell Signaling Technology).

Immunogold electron microscopy.

Ultrathin (70nm) cryosections from perfusion fixed rat kidney cortex were prepared on a Reichert Ultracut S cryo-ultramicrotome (Leica). Cryosections were blocked by incubation in PBS containing 0.05 M glycine and 0.1% skim milk powder and subsequently incubated overnight at 4°C with rabbit anti-GPR91(H80, Santa Cruz) at 1:150 dilution or goat anti-GPR91 (Q15, Santa Cruz) at 1:100 dilution in PBS containing 0.1% skim milk powder. The primary antibody was visualized using goat anti-rabbit IgG or rabbit anti-goat IgG conjugated to 10-nm colloidal gold particles (BioCell Research Laboratories, UK), diluted 1:50 in PBS with 0.1% skim milk powder and polyethyleneglycol (5 mg/ml). The cryosections were stained with 0.3% uranyl acetate in 1.8% methyl-cellulose for 6 min before examination in a FEI Morgagni electron microscope.

[^{3}H]Arachidonic acid release

Cells were seeded in 24 MW plates at a density of 100,000 cells/cm^2 and grown for 24 hours. Next, cells were loaded overnight with 0.25 μCi [^{3}H]arachidonic acid (AA, GE Healthcare, Chalfont St. Giles, UK) in starvation medium. Next, cells were washed 3X in HEPES-glucose buffer supplemented with 0.05% BSA followed by stimulation with 250 μl HEPES-glucose-BSA buffer with 200 μM agonist for 15 minutes at 37ºC. The reaction was stopped by placing the cells on ice. The buffer was removed and counted in a

scintillation counter. To determine total incorporation of [^{3}H]AA, the cells were lysed in 250 µl HEPES-glucose buffer with 0.5% Triton-X100 and the lysate counted in a scintillation counter. Data is represented as (released/total)*100%.

Prostanoid analysis

Samples were prepared as described[9] with minor modifications. MDCK cells were incubated with ligands for 6 hours and apical or basolateral supernatants collected. Subsequently, the supernatants were spiked with ~1 ng of deuterated internal standards, and the solvent was removed. The methoxime was obtained through reaction with an O-methylhydroxylamine hydrochloride-acetate buffer. After acidification to pH 3.5, prostanoid derivatives were extracted, and the pentafluorobenzylesters were formed. Samples were purified by thin layer chromatography, and a broad zone with R_F 0.03-0.4 was eluted. After withdrawal of the organic layer, trimethylsilyl ethers were prepared by reaction with bis(trimethylsilyl)-trifluoroacetamide and thereafter subjected to GC/MS/MS analysis on a Finnigan MAT TSQ700 GC/MS/MS (Thermo Electron Corp., Dreieich, Germany) equipped with a Varian 3400 gas chromatograph (Palo Alto, CA) and a CTC A200S autosampler (CTC Analytics, Zwingen, Switzerland).

Glycosylation motifs, transmembrane domain and topology predictions

The protein sequence for SUCNR1 (Acc. NP_149039) was analyzed using the transmembrane and N-glycosylation prediction tools available at the PredictProtein[10] website (www.predictprotein.org).

Statistical analysis

All quantified data were measured as triplicate samples, unless indicated otherwise, and experiments were performed at least in threefold. Statistical analysis was performed using the one-way Anova or the Student's T-test as indicated. P-values<0.05 were considered significant.

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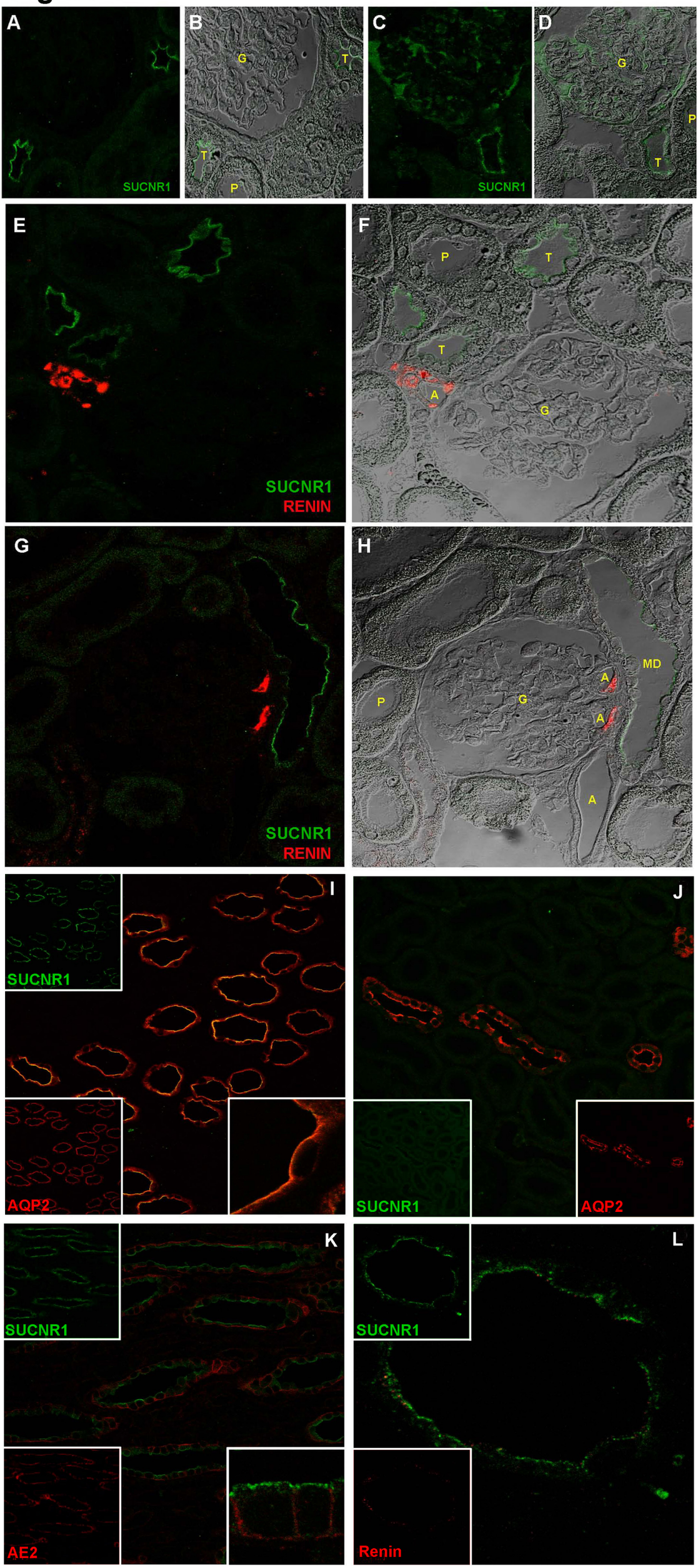
Figure 1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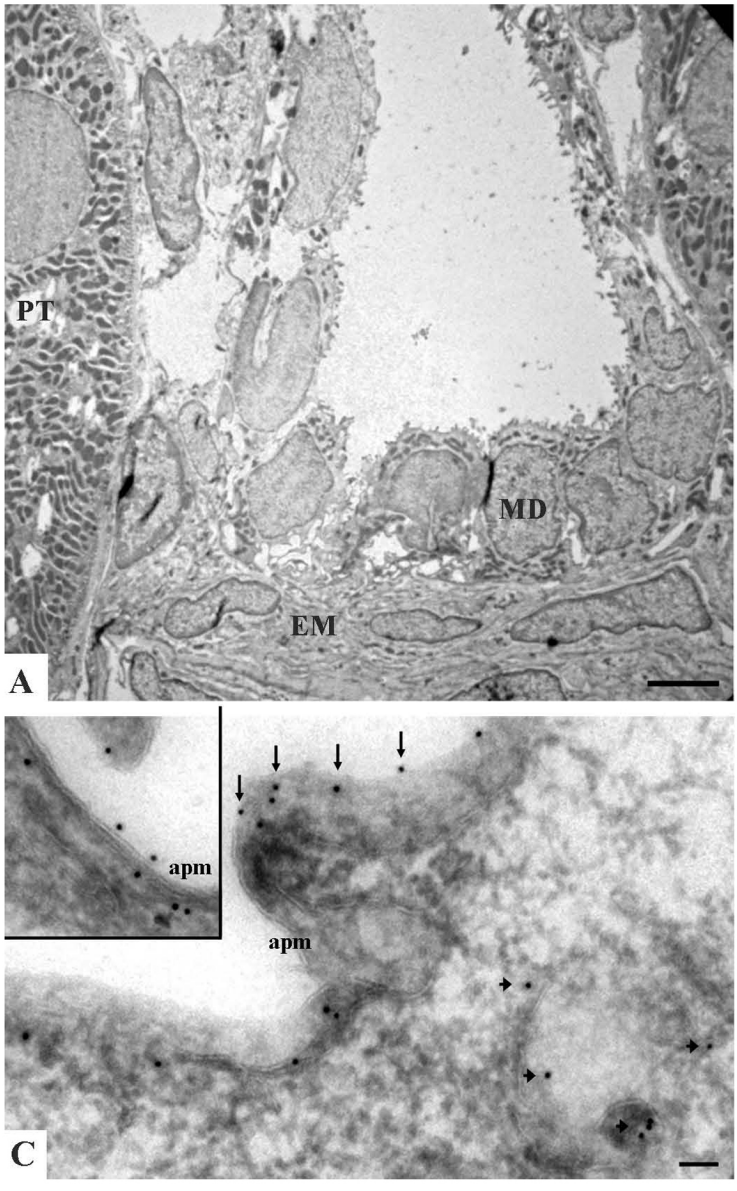

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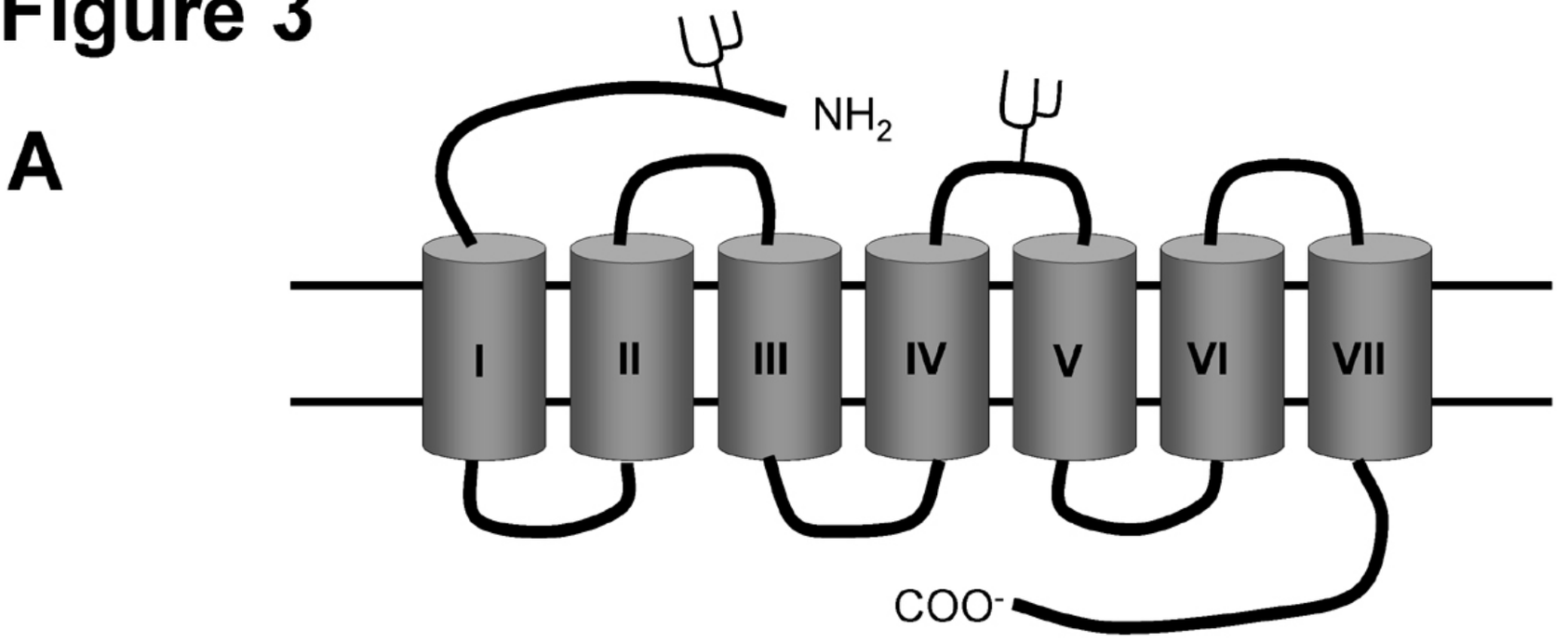
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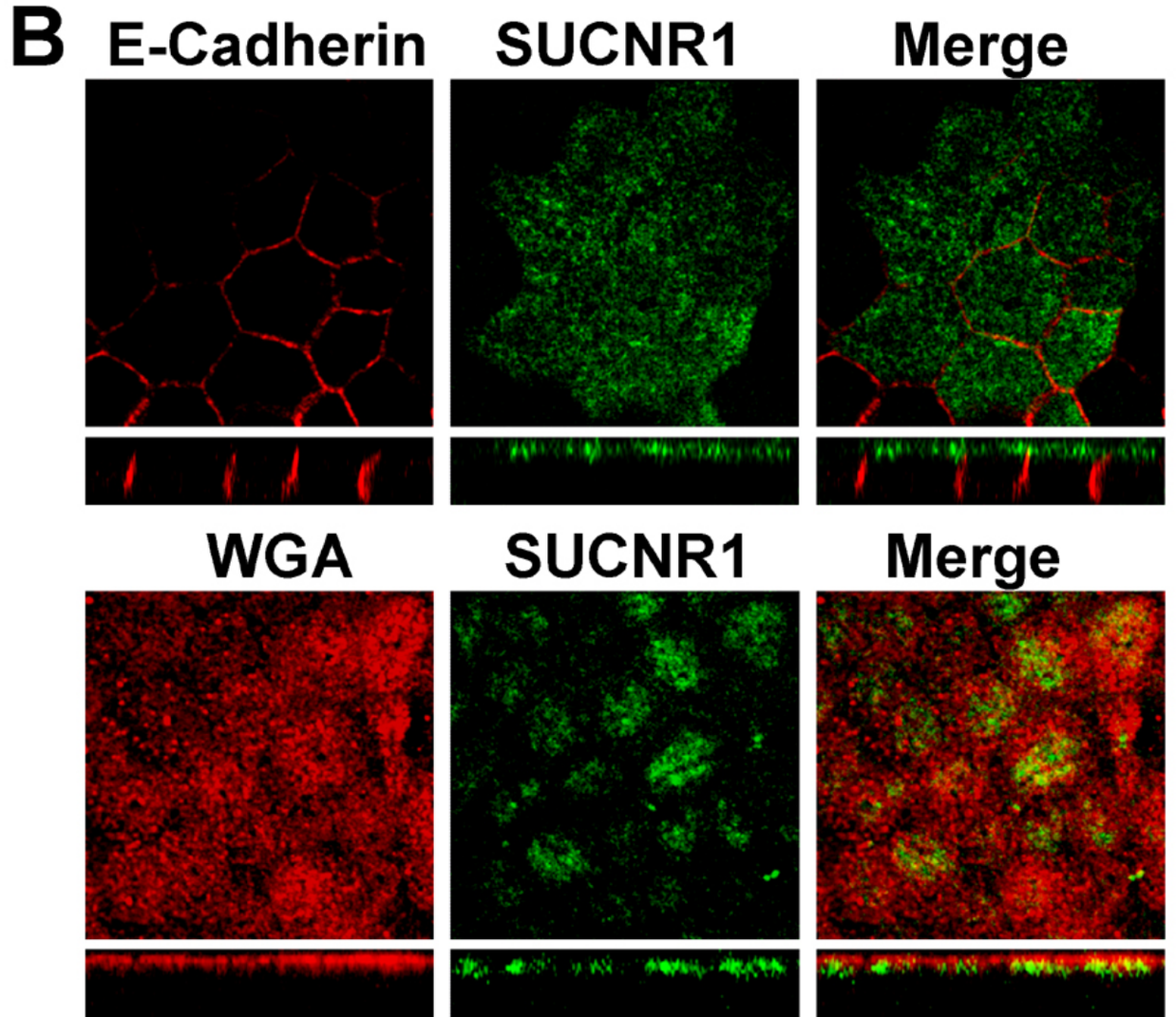
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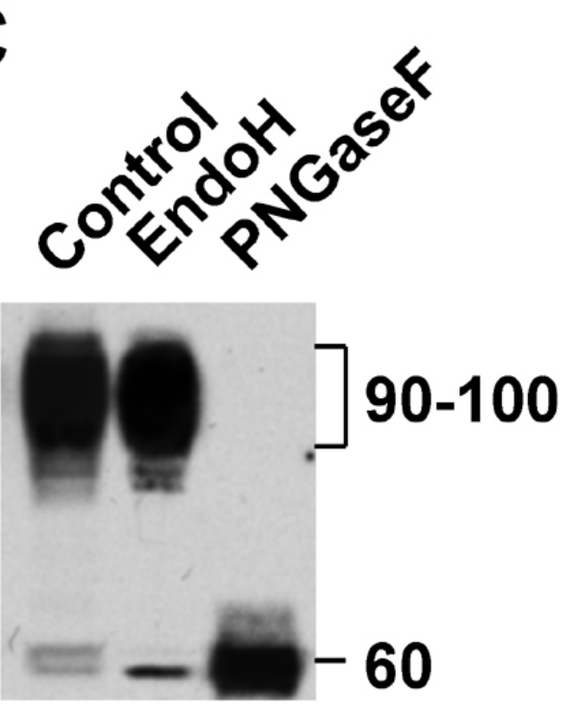
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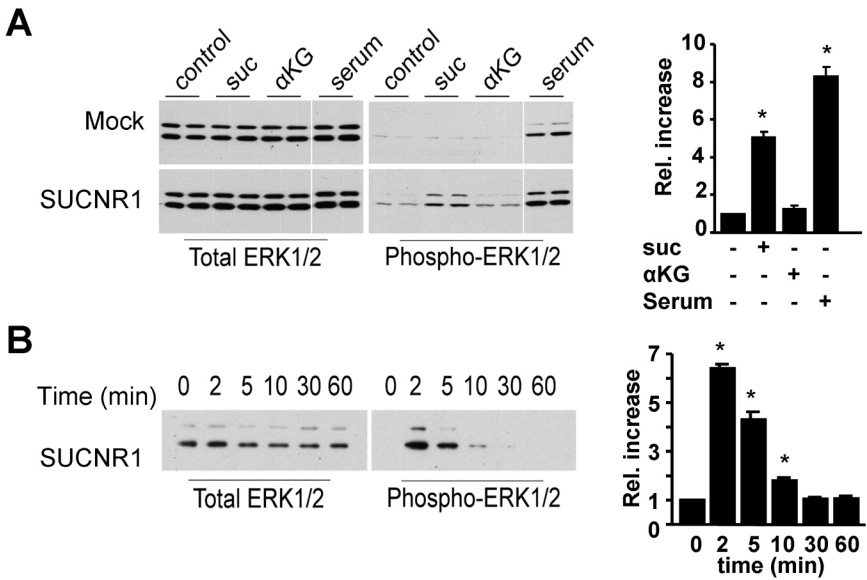
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YM254890 + PTX

Figure 5

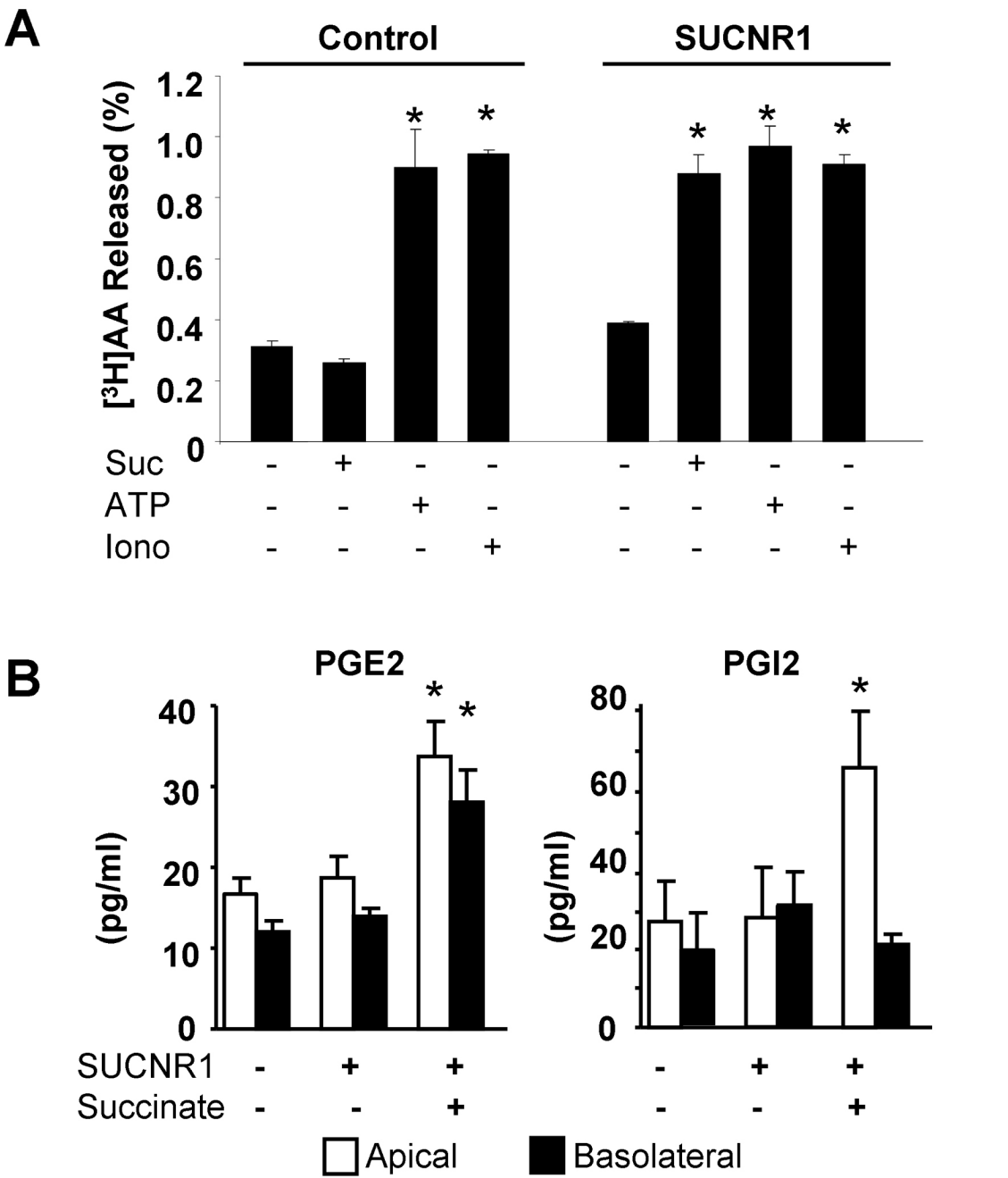
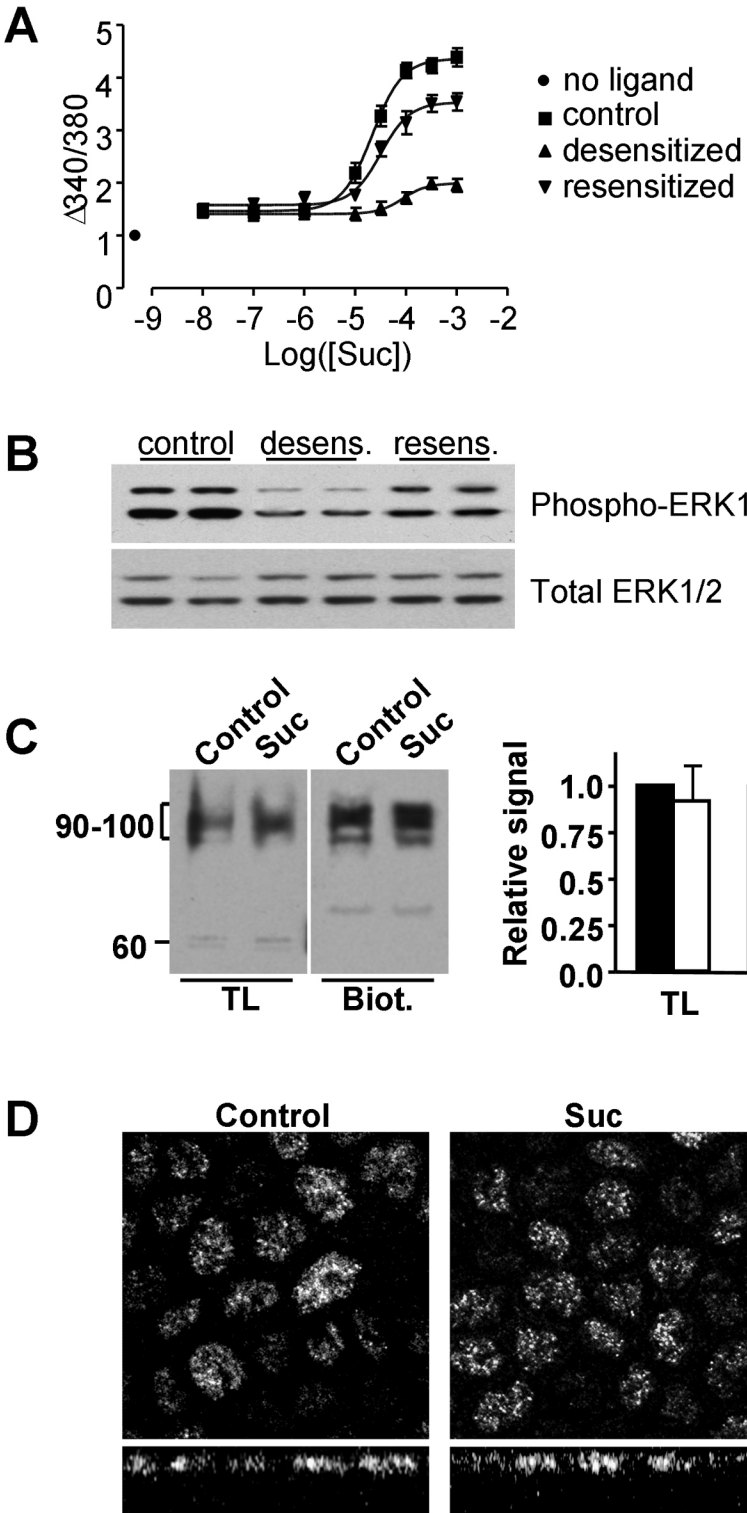
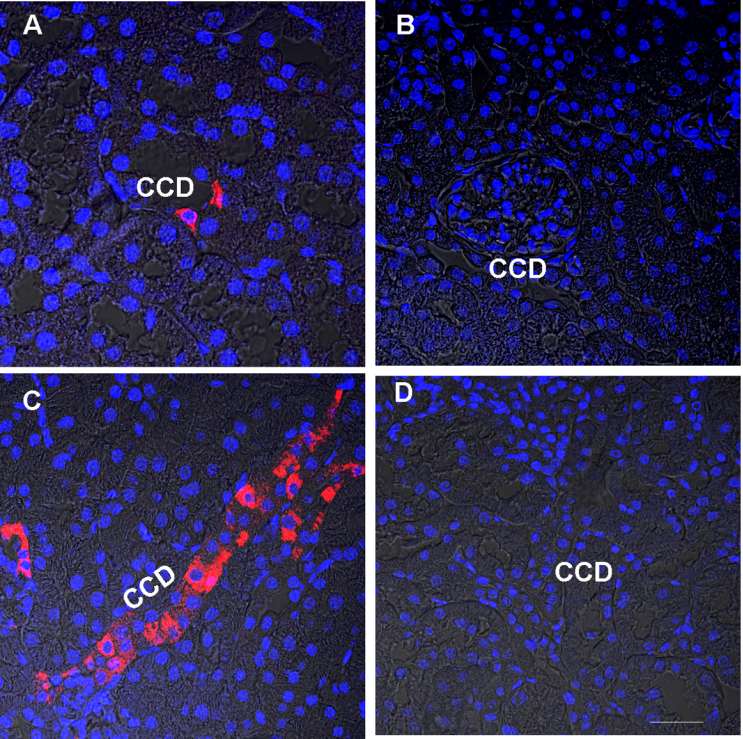
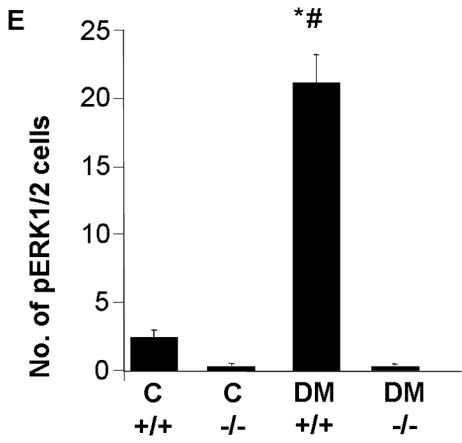

A
Control
SUCNR1
[3H]AA Released (%)
1.2
1.0
0.8
0.6
0.4
0.2
0
Suc
ATP
Iono
B
PGE2
PGI2
(pg/ml)
40
30
20
10
0
80
60
40
20
SUCNR1
Succinate
Apical
Basolateral

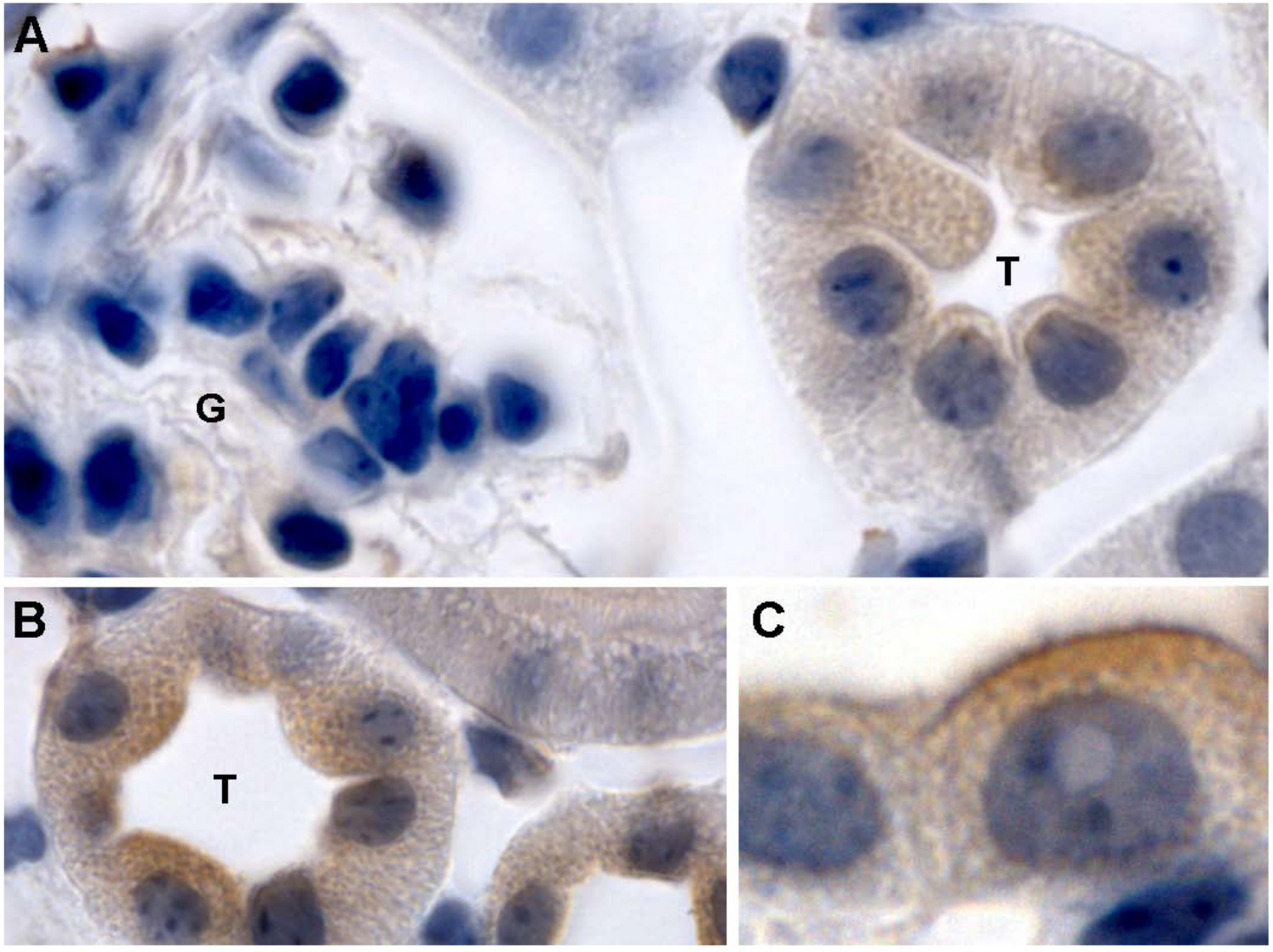
Figure 6


A
Δ340/380
5
4
3
2
1
0
-9 -8 -7 -6 -5 -4 -3 -2
Log([Suc])
no ligand
control
desensitized
resensitized
B
control
desens.
resens.
Phospho-ERK1/2
Total ERK1/2
C
Control
Suc
Control
Suc
90-100
60
TL
Biot.
Relative signal
1.0
0.75
0.5
0.25
0.0
TL
Biot.
D
Control
Suc


A
CCD
B
CCD
C
CCD
D
CCD


E
*#
25
20
15
10
5
0
No. of pERK1/2 cells
C
+/+
C
-/-
DM
+/+
DM
-/-

Suppl. Figure S1



Suppl. Figure S2