Nierenfunktion Kinase-defizienter Mäuse

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Declaration

I hereby declare that this work has been carried out by me under the supervision of **Prof. Dr. Med. Florian Lang** at the Institute of Physiology, Eberhard-Karls-University Tübingen, Germany. Only the sources indicated have been used. The work part thereof has not been submitted for the award of any degree elsewhere.

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To my parents, my grandmother and my brother,

To my boyfriend,

For all the moral support they gave me every time

And just for being there for me

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Content

Abbreviations

ASDN	Aldosterone sensitive distal nephron
bw	Body weight
Ca^{2+}	Calcium
CCD	Cortical collecting duct
cd	Control diet
Dexa	Dexamethasone
DOCA	Deoxycorticosterone acetate
ddH_2O	Double distilled water
EDTA	Ethylene diamine tetra acetate
ECaC	Epithelial calcium channel
ENaC	Epithelial sodium channel
GFR	Glomerular filtration rate
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
hm	Hypomorphic
ht	Hematocrit
i.p.	Intraperitoneal
kg	Kilogram
ko	Knockout
MCD	Medullary collecting duct
mg	Milligram
$MgCl_2$	Magnesium chloride
MR	Mineralocorticoid receptor
NaCl	Sodium Chloride
NHERF2	Sodium-hydrogen exchanger regulatory factor 2
NKCC2	Natrium-Potasium-Clor contransporter

PDK1	Phosphoinositide dependent kinase 1
PI-3K	Phosphatidylinositide-3-kinase
PKB	Protein kinase B
PPARγ	Peroxisome proliferator activated receptor γ
PTH	Parathyroid hormone
ROMK	Renal outer medullary potassium channel
rt	Room temperature
SDS	Sodium dodecyl sulphate
SGK 1	Serum and glucocorticoid inducible kinase 1
SGK 1- SGK 3	Serum and glucocorticoid inducible kinases 1-3
SGLT1	Sodium glucose transporter 1
TGF	Tissue growth factor
TNFα	Tumor necrosis factor alpha
TRPV5	Transient receptor potential cation channel-5
wt	Wildtype
- /-	Knock-out
+/+	Wildtype

Summary

The serum- and glucocorticoid-inducible kinase (SGK1) is ubiquitously expressed and under genomic control by cell stress (including cell shrinkage) and hormones (including gluco- and mineralocorticoids). Two novel isoforms of SGK where identified and termed SGK2 and SGK3, whose catalytic domains share 80% amino acid sequence identity with each other and with SGK1 (Kobayashi et al., (1999) *Biochem J*).

The present studies examined some aspects of the renal function of mice deficient of kinases SGK1 and SGK1-SGK3 (knock-out, ko) compared with their wildtype littermates (wildtype, wt).

SGK1 stimulate the renal epithelial Ca^{2+} channel TRPV5 in *Xenopus* oocytes cells. Therefore one of these studies was based on *in vivo* experiments performed to explore the role of SGK1 in the regulation of renal Ca^{2+} handling.

The effects of mineralocorticoids, leading to development of renal fibrosis, on renal tubular Na^{+} reabsorption and salt appetite involve the SGK1. The kinase is highly expressed in fibrosing tissue. The question was raised - what is the involvement of SGK1 in renal fibrosis?

Whereas SGK1 is under genomic control of mineralocorticoids and glucocorticoids, SGK3 is constitutively expressed. The SKG1-knockout ($sgk1^{-/-}$) mouse has an impaired ability to retain NaCl when it is fed a salt-deficient diet. In the SGK3-knockout ($sgk3^{-/-}$ mouse fed standard and salt-deficient diets, NaCl excretion is normal. The possibility was considered that SGK1 and SGK3 could mutually replace each other, thus preventing severe NaCl loss in $sgk1^{-/-}$ and $sgk3^{-/-}$ mice.

For *in vivo* experiments regarding the regulation of Ca^{2+} channel the $sgk1^{-/-}$ mice under control diet (cd), immunohistochemistry revealed lower abundance of TRPV5 and calbindin D-28K protein than in $sgk1^{+/+}$ mice (colocalized in distal convoluted tubules and connecting tubules). Renal Ca^{2+} excretion under control diet was significantly lower in $sgk1^{-/-}$ than in $sgk1^{+/+}$ mice. No significant differences were observed between genotypes under the control diet in the plasma concentrations of intact parathyroid hormone and $1.25(OH)_2D_3$. Under control diet absolute and fractional urinary excretion of Ca^{2+} was significantly lower in $sgk1^{-/-}$ than in $sgk1^{+/+}$ mice.

Under a Ca^{2+}-deficient diet TRPV5 protein abundance markedly increased in both genotypes, clearly pointing to SGK1-independent regulation of TRPV5.

In Western blot analysis, the membrane protein level of TRPV5 and cytosolic calbindin D-28K were lower in $sgk1^{-/-}$ than in $sgk1^{+/+}$ mice. Quantitative real-time PCR measurements did not reveal statistically significant differences between the genotypes in the levels of mRNA encoding TRPV5 or calbindin D-28K.

Under control diet in both genotypes renal excretion was less than 2% of the calculated dietary Ca^{2+} intake and fecal Ca^{2+} excretion. Under a Ca^{2+}- deficient diet, renal elimination of Ca^{2+} decreased significantly in both $sgk1^{-/-}$ and $sgk1^{+/+}$ mice. The ability of the $sgk1^{-/-}$ mice to decrease urinary Ca^{2+} output again points to SGK1- independent regulation of renal tubular Ca^{2+} reabsorption. However, the decrease of absolute and fractional excretion upon exposure to the Ca^{2+}-deficient diet was blunted in the $sgk1^{-/-}$ mice. Thus, the Ca^{2+}-deficient diet dissipated the differences of absolute and fractional Ca^{2+} excretions between $sgk1^{-/-}$ and $sgk1^{+/+}$ mice. Replacement of tap water in the drinking bottle

with 1% saline led to the expected increase in urinary Na^+ output, paralleled by a moderate increase in urinary Ca^{2+} output, but the urinary excretion of Ca^{2+} remained slightly lower in *sgk1*$^{-/-}$ than in *sgk1*$^{+/+}$ mice. To explore the sensitivity of renal Ca^{2+} excretion to inhibition of Na^+ reabsorption, the NKCC2 inhibitor furosemide (20 µg/g bw) was applied with or without the carboanhydrase inhibitor acetazolamide (50 µg/g bw). Furosemide alone increased renal Ca^{2+} excretion in *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice and abolished the difference of Ca^{2+} excretion between genotypes. The additional administration of acetazolamide did not further increase natriuresis or calciuria.

In conclusion, despite decrease in TRPV5 abundance in the connecting tubule, SGK1 knockout mice are anticalciuric presumably due to a decrease in extracellular volume and enhancement of Na^+ and Ca^{2+} reabsorption upstream of the aldosterone-sensitive distal nephron including the loop of Henle.

To study the role of SGK1 in renal fibrosis, *sgk1*$^{-/-}$ and their wild-type littermates *sgk1*$^{+/+}$ were implanted with desoxycorticosterone acetate (DOCA)-release pellets and offered 1% saline as drinking water for 12 weeks.

Treatment with DOCA/high salt led to a marked increase of fluid and Na^+ intake in both genotypes. Three weeks after the initiation of DOCA/high salt treatment, the fluid intake increased by 3.0 ± 0.2-fold in *sgk1*$^{-/-}$ mice, and by 11.1 ± 1.9-fold in *sgk1*$^{+/+}$ mice increasing even more during the whole study. After 12 weeks of treatment, body weight increased significantly ($p<0.05$) more in *sgk1*$^{+/+}$ mice than in *sgk1*$^{-/-}$ mice and the urinary volume augmented significantly by 24.8 ± 5.0-fold in *sgk1*$^{+/+}$ mice and 3.9 ± 0.4-fold in *sgk1*$^{-/-}$ mice with further increase until 12 weeks. DOCA/high salt treatment tended to enhance creatinine clearance in *sgk1*$^{+/+}$, but decreased it in *sgk1*$^{-/-}$ mice.

The DOCA/high salt treatment tended to increase the fractional excretion of K^+ (not significant) in both genotypes. Within 3 weeks of DOCA/high salt treatment, the fractional Na^+ excretion reached 42.7 ± 8.8-fold in *sgk1*$^{+/+}$ mice and 14.5 ± 3.6-fold (n=9) in *sgk1*$^{-/-}$ mice and the fractional Ca^{2+} excretion increased 11.5 ± 3.7-fold in *sgk1*$^{+/+}$ mice and 6.0 ± 1.3-fold (n=9) in *sgk1*$^{-/-}$ mice. After 12 weeks of DOCA/high salt treatment blood pressure was significantly higher in *sgk1*$^{+/+}$ mice than in *sgk1*$^{-/-}$ mice. Urinary albumin excretion was markedly increased following treatment of *sgk1*$^{+/+}$ mice with DOCA/high salt, the increase was almost absent in *sgk1*$^{-/-}$ mice, even though the increase of systemic blood pressure was similar up to 7 weeks of treatment of *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice.

Histological analysis revealed a marked influence of DOCA/high salt treatment on the morphology of kidneys from *sgk1*$^{+/+}$ mice. The treatment was followed by marked glomerular enlargement with segmental glomerulosclerosis and tubulointerstitial fibrosis in *sgk1*$^{+/+}$ mice, effects clearly blunted in *sgk1*$^{-/-}$ mice.

In conclusion, SGK1 is required for the development of severe albuminuria following DOCA/high salt treatment whereas lack of SGK1 protects against DOCA/high-salt-induced albuminuria and renal fibrosis.

To study the possibility that SGK1 and SGK3 could mutually replace each other, thus preventing severe NaCl loss in *sgk1*$^{-/-}$ and *sgk3*$^{-/-}$ mice, double knock-out mutants (*sgk1*$^{-/-}$ / *sgk3*$^{-/-}$) were compared with their wildtype littermates (*sgk1*$^{+/+}$/*sgk3*$^{+/+}$). The *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice share the delayed hair growth with *sgk3*$^{-/-}$ mice and the modestly impaired renal salt retention with *sgk1*$^{-/-}$ mice. Blood pressure was slightly, but significantly ($p<0.03$), lower in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ than in *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ mice, a difference that was maintained in mice fed low- and high-salt diets. Plasma aldosterone concentrations were significantly ($p<0.01$) higher in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ than in *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ mice fed control and low-salt diets. During salt

depletion, absolute and fractional excretions of Na^+ were significantly ($p< 0.01$) higher in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ than in *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ mice. The fractional Na^+ excretion was significantly decreased by low salt diet in both *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ mice ($p<0.0001$) and *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice ($p<0.0001$).

After birth, the *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice gained body weight slightly slower than *sgk1*$^{+/+}$/*sgk3*$^{+/+}$, mice at 12 weeks being almost identical in body weight. Exposure to low salt diet did not significantly alter body weight. Food intake per body weight was slightly but significantly larger in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ than in *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ mice both at control diet ($p<0.0084$) and at low salt diet ($p<0.0013$). Fluid intake in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice similarly tended to increase following salt deficient diet and tended to be larger in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ than in *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ mice. Fecal dry weight was significantly higher in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice on control diet, whereas this difference disappeared under low salt diet. Fecal Na^+ excretion significantly decreased under low salt diet in both *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ ($p<0.0002$) and *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ ($p<0.0001$) mice but no significant differences were observed between *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ and *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice on either diet. Fecal K^+ excretion, plasma concentrations of Na^+ and K^+ and the hematocrit were similar in *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ and *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice on either diet as well as at control and low salt diet in both genotypes. Plasma K^+ concentration tended to augment upon treatment with low salt diet and tended to be higher in *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ than in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice. No significant differences were observed in creatinine clearance and in urinary flow rate between *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ and *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice on either diet or between control diet and low salt diet in either genotype. Under low salt diet urinary sodium excretion decreased significantly in both *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ mice ($p<0.0002$) and *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice ($p<0.0001$). The absolute and fractional urinary K^+ excretion tended to be higher in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ than in *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ mice, a difference reaching statistical significance under low salt diet ($p<0.03$). The low salt diet did not significantly influence the absolute or fractional K^+ excretion in the *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ mice whereas it tended to increase K^+ excretion in the *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice. Under control diet blood pressure was significantly ($p<0.05$) lower in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ than in *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ mice. Under both low ($p<0.05$) and high ($p<0.05$) salt diet blood pressure remained significantly lower in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ than in *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ mice.

Zusammenfassung

Die Serum- und Glucocorticoid-induzierbare Kinase (SGK1) wird ubiquitär exprimiert, wobei die Expression durch zellulären Stress (einschließlich Zellschrumpfung) sowie Hormone (einschließlich Gluco- und Mineralocorticoide) reguliert wird. Zwei weitere Isoformen der SGK wurden identifiziert und mit SGK2 und SGK3 bezeichnet. Die Aminosäuresequenzen der katalytischen Domänen von SGK2 und SGK3 stimmen zu 80% miteinander und mit SGK1 überein (Kobayashi et al., (1999) *Biochem J*).

Die vorliegenden Studien untersuchen einige Aspekte der Nierenfunktion von Mäusen, die keine SGK1 bzw. keine SGK1 und SGK3 exprimieren (knock-out, KO), verglichen mit der Wildtyp-Maus (wildtype, WT).

SGK1 stimuliert den renalen epithelialen Ca^{2+}- Kanal TRPV5 in den Zellen. Aus diesem Grund basiert eine der vorliegenden Studien auf *in vivo*- Experimenten zur Unteruschung der Funktion von SGK1 in der Regulation des renalen Ca^{2+}-Transports.

SGK1 ist an verschiedenen Effekten von Mineralocorticoiden beteiligt, welche zur Entwicklung renaler Fibrose führen sowie die tubuläre Na^{+}- Rückresorption und das Verlangen nach Salz beeinflussen. Gewebe, die von Fibrose betroffen sind, zeigen eine hohe Expression der Kinase. Dies führte zu der Frage, welche Rolle SGK1 bei renaler Fibrose spielt.

Während SGK1 auf Expressionsebene durch Gluco- und Mineralocorticoide reguliert ist, wird SGK3 grundsätzlich exprimiert. Die Fähigkeit der SGK1-defizienten Maus ($sgk1^{-/-}$), unter salzarmer Ernährung NaCl zurück zu halten, ist vermindert. Dagegen ist die NaCl-Exkretion der SGK3-defizienten Maus ($sgk3^{-/-}$) sowohl bei einer Standard-Diät als auch bei salzarmer Ernährung unverändert. Daraufhin wurde in Erwägung gezogen, dass SGK1 und SGK3 sich gegenseitig in ihrer Funktion ersetzen und so einen schweren NaCl-Verlust in der $sgk1^{-/-}$- und $sgk3^{-/-}$-KO Maus verhindern können.

Immunohistochemische Untersuchungen der *in vivo*-Experimente bezüglich der Regulation des Ca^{2+}-Kanals zeigten unter Kontrolldiät (KD) eine geringere Häufigkeit von TRPV5 und des Calbindin D-28K Proteins in $sgk1^{-/-}$-Mäusen verglichen mit den $sgk1^{+/+}$-Mäusen (beide Proteine kommen im distalen und verbindenden Tubulus nebeneinander vor). Die renale Ca^{2+}-Exkretion unter KD war bei den $sgk1^{-/-}$-Mäusen im Vergleich zur $sgk1^{-/-}$-Maus signifikant verringert. Bezüglich der Plasmakonzentrationen an intaktem Parathyroidhormon $1.25(OH)_2D_3$ bestand zwischen beiden Genotypen kein signifikanter Unterschied. Sowohl die absolute als auch die fraktionelle Exkretion von Ca^{2+} mit dem Urin unter KD war bei den $sgk1^{-/-}$ KO-Mäusen signifikant geringer als bei den $sgk1^{+/+}$- Mäusen.

Unter Ca^{2+}-armer Ernährung nahm die Häufigkeit des TRPV5-Proteins in beiden Genotypen deutlich zu, was auf eine SGK1-unabhängige Regulation von TPRV5 hinweist.

Western blot-Analysen zeigten ein geringeres Vorkommen von TRPV5 und des cytosolischen Calbindin D-28K Proteins in $sgk1^{-/-}$-KO Mäusen verglichen mit der $sgk1^{+/+}$-WT Maus. Jedoch zeigte die quantitative *real-time* PCR keine statistisch signifikanten Unterschiede zwischen beiden Genotypen in Hinblick auf die mRNA-Spiegel von TRPV5 oder Calbindin D-28K.

Unter KD betrug die renale Exkretion bei beiden Genotypen weniger als 2% der kalkulierten Ca^{2+}-Aufnahme aus dem Gehalt in der Diät und der fäkalen Ca^{2+}-Ausscheidung. Unter Ca^{2+}-armer Ernährung nahm die renale Eliminierung von Ca^{2+} sowohl bei der *sgk1*$^{-/-}$-KO Maus als auch bei der *sgk1*$^{+/+}$-Maus signifikant ab. Die Fähigkeit der *sgk1*$^{-/-}$- Maus, den renalen Ca^{2+}-Verlust noch weiter zu reduzieren, deutet erneut auf eine SGK1-unabhängige Regulation der tubulären Ca^{2+}-Rückresorption in der Niere hin. Allerdings war die beobachtete Abnahme sowohl der absoluten als auch der fraktionellen Ausscheidung unter Ca^{2+}-armer Diät bei der *sgk1*$^{-/-}$-KO Maus abgeschwächt. Folglich verringerte die Ca^{2+}-arme Diät die Unterschiede zwischen WT und KO bezüglich der absoluten und fraktionellen Ca^{2+}-Exktretion. Wurde das Wasser in der Trinkflasche durch 1% Kochsalzlösung ersetzt, nahm die renale Na^{+}-Ausscheidung wie erwartet zu. Dieser Effekt wurde von einem moderaten Anstieg der renalen Ca^{2+}- Ausscheidung begleitet. Dabei war die Ca^{2+}- Ausscheidung bei den *sgk1*$^{-/-}$- Mäusen etwas geringer im Vergleich zu den *sgk1*$^{+/+}$-Mäusen. Zur Untersuchung der Abhängigkeit der renalen Ca^{2+}-Ausscheidung von einer Hemmung der Na^{+}-Rückresorption wurde der NKCC2 - Inhibitor Furosemid (20 µg/g KG) allein bzw. in Kombination mit dem Carboanhydrase-Inhibitor Acetazoamid (50 µg/g KG) appliziert. Furosemid allein führte sowohl bei den *sgk1*$^{-/-}$- Mäusen als auch bei den *sgk1*$^{+/+}$-Mäusen zu einem Anstieg der renalen Ca^{2+}- Ausscheidung und zur Aufhebung der Unterschiede in der Ca^{2+}-Exkretion zwischen beiden Genotypen. Die zusätzliche Gabe von Acetazoamid hatte keinen weiteren Anstieg der Natriurese oder Calciurie zufolge.

Zusammenfassend kann gesagt werden, dass SGK1-KO Mäuse trotz einer geringeren Häufigkeit von TRPV5 im verbindenden Tubulus keine Calciurie aufweisen. Dies beruht auf einem verringerten extrazellulären Volumen sowie einer verstärkten Rückresorption von Na^{+} und Ca^{2+} stromaufwärts des Aldosteron-sensitiven distalen Nephrons einschließlich der Henle-Schleife.

Zur Untersuchung der Rolle von SGK1 in der renalen Fibrose wurden *sgk1*$^{-/-}$-Mäuse und deren *sgk1*$^{+/+}$- Wurfgeschwister 12 Wochen lang mit die Desoxycorticosteronacetat (DOCA)-Freisetzung anregenden Pellets gefüttert und das Trinkwasser mit 1% Kochsalz versetzt.

Die DOCA/Hochsalz-Diät führte bei beiden Genotypen zu einem deutlichen Anstieg der Flüssigkeits- und Na^{+}-Aufnahme 3 Wochen nach Beginn der beschriebenen Diät konnte ein Anstieg der Flüssigkeitsaufnahme um das 3.0 ± 0.2-fache bei den KO-Mäusen bzw. um das 11.1 ± 1.9-fache bei den WT-Mäusen beobachtet werden, wobei es im restlichen Verlauf der Studie zu einer weiteren Steigerung in der Flüssigkeitsaufnahme kam.

Nach 12 Wochen Diätgabe wiesen die *sgk1*$^{+/+}$ - Mäuse eine signifikant ($p<0.05$) höhere Zunahme an Körpergewicht auf als ihre *sgk1*$^{-/-}$-Wurfgeschwister. Weiterhin nahm das Urinvolumen bis zum Ende der 12 Versuchswoche signifikant um das 24.8 ± 5.0-fache bei den *sgk1*$^{+/+}$- WT Mäusen bzw. um das 3.9 ± 0.4-fache bei den *sgk1*$^{-/-}$-KO Mäusen zu. Die DOCA/Hochsalz-Diät führte tendenziell zu einer Steigerung der Kreatinin-Clearance bei den WT-Mäusen, bei den KO-Mäusen dagegen jedoch zu einem Rückgang der Kreatinin-Clearance.

Die Gabe der DOCA/Hochsalz - Diät hatte tendenziell eine Zunahme der fraktionellen K^{+}-Ausscheidung (nicht signifikant) bei beiden Genotypen zufolge. Bereits nach 3 Wochen der DOCA/Hochsalz - Diät wies die Na^{+}-Exkretion einen 42.7 ± 8.8-fachen Anstieg bei den *sgk1*$^{+/+}$- Mäusen bzw. einen Anstieg um das 14.5 ± 3.6-fache (n=9) bei den *sgk1*$^{-/-}$- Mäusen auf. Die fraktionelle Ca^{2+}-Ausscheidung stieg um das 11.5 ± 3.7-fache bei den *sgk1*$^{+/+}$-Mäusen und um das 6.0 ± 1.3-fache (n=9) bei den *sgk1*$^{-/-}$- Mäusen an. Nach 12 Wochen der

DOCA/Hochsalz - Diät wiesen die *sgk1*$^{+/+}$-Mäuse einen signifikant höheren Blutdruck auf als ihre *sgk1*$^{-/-}$ - Wurfgeschwister. Die Albuminausscheidung über den Urin war bei den *sgk1*$^{+/+}$- Mäusen nach Gabe der DOCA/Hochsalz - Diät deutlich gesteigert. Dieser Effekt blieb bei den *sgk1*$^{-/-}$ - Mäusen dagegen nahezu aus, obwohl der systemische Blutdruck bei beiden Genotypen während der ersten 7 Wochen des Experimentes gleich war.

Histologische Untersuchungen zeigten einen deutlichen Einfluss der DOCA/Hochsalz-Diät auf die Morphologie der Niere der *sgk1*$^{+/+}$- Mäuse. Die Diät führte zu einer merklichen Vergrößerung der Glomerula mit segmentaler Glomerulosklerose und tubulointerstitieller Fibrose bei den *sgk1*$^{+/+}$ - WT Mäusen, während diese Effekte bei den *sgk1*$^{-/-}$- KO Mäusen nur in abgeschwächter Form auftraten.

Zusammengefasst zeigt sich, dass SGK1 an der Entwicklung einer schweren Albuminurie als Folge einer DOCA/Hochsalz-Diät beteiligt ist, während das Fehlen von SGK1 vor einer DOCA/Hochsalz-Diät induzierten Albuminurie und renaler Fibrose schützt.

Zur Untersuchung der Hypothese, dass SGK1 und SGK3 sich gegenseitig ersetzen können, um so einem schweren NaCl-Verlust bei *sgk1*$^{-/-}$- und *sgk3*$^{-/-}$ -KO Mäusen entgegen zu wirken, wurden Doppel-KO-Mutanten (*sgk1*$^{-/-}$ / *sgk3*$^{-/-}$) mit ihren WT-Wurfgeschwistern (*sgk1*$^{+/+}$/*sgk3*$^{+/+}$) verglichen. Die *sgk1*$^{-/-}$/*sgk3*$^{-/-}$- Mäuse wiesen den gleichen verminderten Haarwuchs wie die *sgk3*$^{-/-}$- Mäuse sowie die mäßig beeinträchtigte renale Salzretention wie die *sgk1*$^{-/-}$- Mäuse auf. Der Blutdruck der Doppel-KO Mutanten war leicht, aber signifikant ($p<0.03$) verringert gegenüber der *sgk1*$^{+/+}$/*sgk3*$^{+/+}$- WT Maus. Dieser Unterschied konnte sowohl bei salzarmer als auch bei salzreicher Diät beobachtet werden. Die Plasma-Aldosteronkonzentrationen waren unter Gabe von KD und salzarmer Diät bei den *sgk1*$^{-/-}$/*sgk3*$^{-/-}$- Mäusen signifikant ($p<0.01$) höher als bei den *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ - Mäusen. Unter Salzdepletion waren die absolute und fraktionelle Na^+-Exkretion bei den *sgk1*$^{-/-}$/*sgk3*$^{-/-}$- Mäusen signifikant ($p< 0.01$) höher als bei den *sgk1*$^{+/+}$/*sgk3*$^{+/+}$-Mäusen. Die fraktionelle Na^+-Ausscheidung war unter salzarmer Ernährung sowohl bei den *sgk1*$^{-/-}$/*sgk3*$^{-/-}$-Mäusen als auch bei den *sgk1*$^{+/+}$/*sgk3*$^{+/+}$-Mäusen signifikant verringert ($p<0.0001$).

Nach der Geburt nahmen die *sgk1*$^{-/-}$/*sgk3*$^{-/-}$- Mäuse etwas langsamer an Gewicht zu als die *sgk1*$^{+/+}$/*sgk3*$^{+/+}$-Mäuse, nach 12 Wochen wiesen beide Genotypen jedoch das selbe Körpergewicht auf. Die Fütterung der salzarmen Diät hatte keinen signifikanten Einfluss auf das Körpergewicht der Tiere. Die Nahrungsaufnahme pro Körpergewicht war bei den *sgk1*$^{-/-}$/*sgk3*$^{-/-}$-Mäusen nur wenig, aber signifikant höher als bei den *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ -Mäusen sowohl unter Kontrolldiät ($p<0.0084$) als auch unter salzarmer Ernährung ($p<0.0013$). Die Flüssigkeitsaufnahme der *sgk1*$^{-/-}$/*sgk3*$^{-/-}$-Mäuse schien infolge salzarmer Ernährung zuzunehmen und war tendentiell höher als bei den *sgk1*$^{+/+}$/*sgk3*$^{+/+}$-Mäusen. Das Trockengewicht der Fäzes war bei den *sgk1*$^{-/-}$/*sgk3*$^{-/-}$-Mäusen unter Kontrolldiät signifikant höher. Dieser Unterschied konnte unter salzarmer Ernährung nicht festgestellt werden. Die fäkale Na^+-Exkretion nahm bei salzarmer Diät signifikant sowohl bei den *sgk1*$^{+/+}$/*sgk3*$^{+/+}$-Mäusen ($p<0.0002$) als auch bei den *sgk1*$^{-/-}$/*sgk3*$^{-/-}$-Mäusen ($p<0.0001$) ab, während unter KD keine signifikanten Unterschiede bezüglich der fäkalen Na^+-Ausscheidung zwischen beiden Genotypen festgestellt werden konnten. Die fäkale K^+-Ausscheidung, die Plasmakonzentrationen von Na^+ und K^+ sowie der Hämatokrit waren bei den *sgk1*$^{+/+}$/*sgk3*$^{+/+}$- und *sgk1*$^{-/-}$/*sgk3*$^{-/-}$-Mäusen sowohl unter KD als auch bei salzarmer Ernährung gleich. Die Plasmakonzentration an K^+ schien unter salzarmer Diät zuzunehmen und war tendentiell höher bei den *sgk1*$^{+/+}$/*sgk3*$^{+/+}$-Mäusen im Vergleich zu den Doppel-KO Mäusen. Bezüglich der Kreatinin-Clearance und des Urinvolumens bestanden keine signifikanten Unterschiede

zwischen beiden Genotypen bei beiden Diäten sowie zwischen den beiden Ernährungsformen bei dem jeweiligen Genotyp. Unter salzarmen Ernährungsbedingungen nahm die Na^+-Ausscheidung sowohl bei den *sgk1*$^{+/+}$/*sgk3*$^{+/+}$-Mäusen ($p<0.0002$) als auch bei den *sgk1*$^{-/-}$/*sgk3*$^{-/-}$- Mäusen ($p<0.0001$) signifikant ab. Die absolute und fraktionelle K^+-Ausscheidung schien bei den *sgk1*$^{-/-}$/*sgk3*$^{-/-}$-Mäusen höher zu sein als bei den *sgk1*$^{+/+}$/*sgk3*$^{+/+}$-Mäusen. Dieser Unterschied erreichte unter salzarmer Diät statistische Signifikanz ($p<0.03$). Die salzarme Ernährung hatte bei den *sgk1*$^{+/+}$/*sgk3*$^{+/+}$-Mäusen keinen signifikanten Einfluss auf die absolute oder fraktionelle K^+-Exkretion, wogegen es bei den *sgk1*$^{-/-}$/*sgk3*$^{-/-}$- Mäusen zu einer tendenziell gesteigerten K^+-Ausscheidung kam. Unter Kontrolldiät war der Blutdruck der *sgk1*$^{-/-}$/*sgk3*$^{-/-}$-Mäuse signifikant niedriger ($p<0.05$) im Vergleich zu ihren *sgk1*$^{+/+}$/*sgk3*$^{+/+}$-Wurfgeschwistern. Sowohl unter salzarmer Ernährung ($p<0.05$) als auch bei salzreicher Diät ($p<0.05$) war der Blutdruck der *sgk1*$^{-/-}$/*sgk3*$^{-/-}$- Mäuse signifikant niedriger als bei den *sgk1*$^{+/+}$/*sgk3*$^{+/+}$- Mäusen.

1. Introduction

1.1. Kidney anatomy

The kidneys are organs that filter wastes (such as urea) from the blood and excrete them, along with water, as urine. The medical field that studies the kidneys and diseases of the kidney is called nephrology (nephro- meaning kidney is from the Ancient Greek word nephros; the adjective renal meaning related to the kidney is from Latin rēnēs, meaning kidneys). In humans, the kidneys are located in the posterior part of the abdomen. There is one on each side of the spine; the right kidney sits just below the liver, the left below the diaphragm and adjacent to the spleen. Above each kidney is an adrenal gland (also called the suprarenal gland). The asymmetry within the abdominal cavity caused by the liver results in the right kidney being slightly lower than the left one while the left kidney is located slightly more medial. The kidneys are retroperitoneal, which means they lie behind the peritoneum, the lining of the abdominal cavity. They are approximately at the vertebral level T12 to L3. The upper parts of the kidneys are partially protected by the eleventh and twelfth ribs, and each whole kidney is surrounded by two layers of fat (the perirenal and pararenal fat) which help to cushion it. Congenital absence of one or both kidneys, known as unilateral or bilateral renal agenesis can occur. In a normal human adult, each kidney is about 10 cm long, 5.5 cm in width and about 3 cm thick, weighing 150 grams. Together, kidneys weigh about 0.5% of a person's total body weight. The kidneys are "bean-shaped" organs, and have a concave side facing inwards (medially). On this medial aspect of each kidney is an opening, called the hilum, which admits the renal artery, the renal vein, nerves, and the ureter. The outer portion of the kidney is called the renal cortex, which sits directly beneath the kidney's loose connective tissue/fibrous capsule. Deep to the cortex lies the renal medulla, which is divided into 10-20 renal pyramids in humans. Each pyramid together with the associated overlying cortex forms a renal lobe. The tip of each pyramid (called a papilla) empties into a calyx, and the calices empty into the renal pelvis. The pelvis transmits urine to the urinary bladder via the ureter.

Blood supply

Each kidney receives its blood supply from the renal artery, two of which branch from the abdominal aorta. Upon entering the hilum of the kidney, the renal artery divides into smaller interlobar arteries situated between the renal papillae. At the outer medulla, the interlobar arteries branch into arcuate arteries, which course along the border between the renal medulla and cortex, giving off still smaller branches, the cortical radial arteries (sometimes called interlobular arteries). Branching off these cortical arteries are the afferent arterioles supplying the glomerular capillaries, which drain into efferent arterioles. Efferent arterioles divide into peritubular capillaries that provide an extensive blood supply to the cortex. Blood from these capillaries collects in renal venules and leaves the kidney via the renal vein. Efferent arterioles of glomeruli closest to the medulla (those that belong to juxtamedullary nephrons) send branches into the medulla, forming the vasa recta. Blood supply is intimately linked to blood pressure.

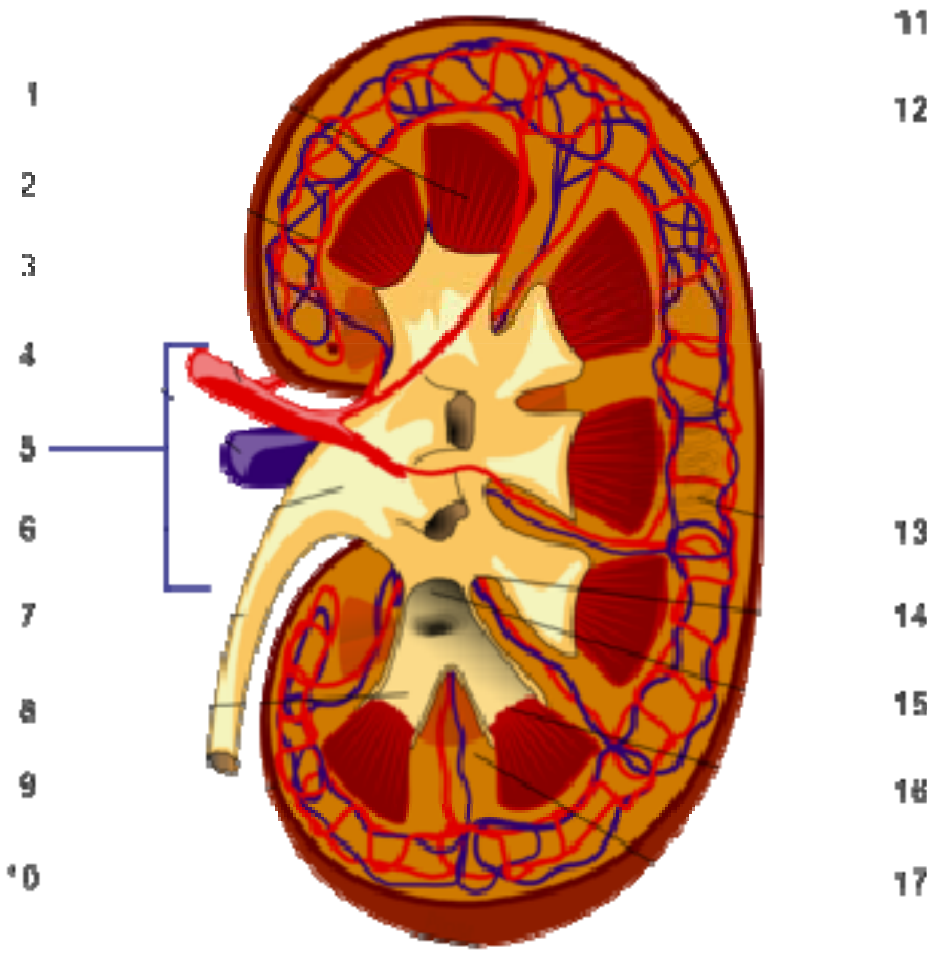

Figure nr. 1 - Kidney structure.
Parts of the kidney: 1. Renal pyramid; 2. Efferent vessel; 3. Renal artery; 4. Renal vein; 5. Renal hilum; 6. Renal pelvis; 7. Ureter; 8. Minor calyx; 9. Renal capsule; 10. Inferior renal capsule; 11. Superior renal capsule; 12. Afferent vessel; 13. Nephron; 14. Minor calyx; 15. Major calyx; 16. Renal papilla; 17. Renal column.

The basic functional unit of the kidney is the nephron, of which there are more than a million within the cortex and medulla of each normal adult human kidney. Nephrons regulate water and solute within the cortex and medulla of each normal adult human kidney. Nephrons regulate water and soluble matter (especially electrolytes) in the body by first filtering the blood under pressure, and then reabsorbing some necessary fluid and molecules back into the blood while secreting other, unneeded molecules. Reabsorption and secretion are accomplished with both cotransport and countertransport mechanisms established in the nephrons and associated collecting ducts.

Collecting duct system

The fluid flows from the nephron into the collecting duct system. This segment of the nephron is crucial to the process of water conservation by the organism. In the presence of antidiuretic hormone (ADH; also called vasopressin), these ducts become permeable to water and facilitate its reabsorption, thus concentrating the urine and reducing its volume.

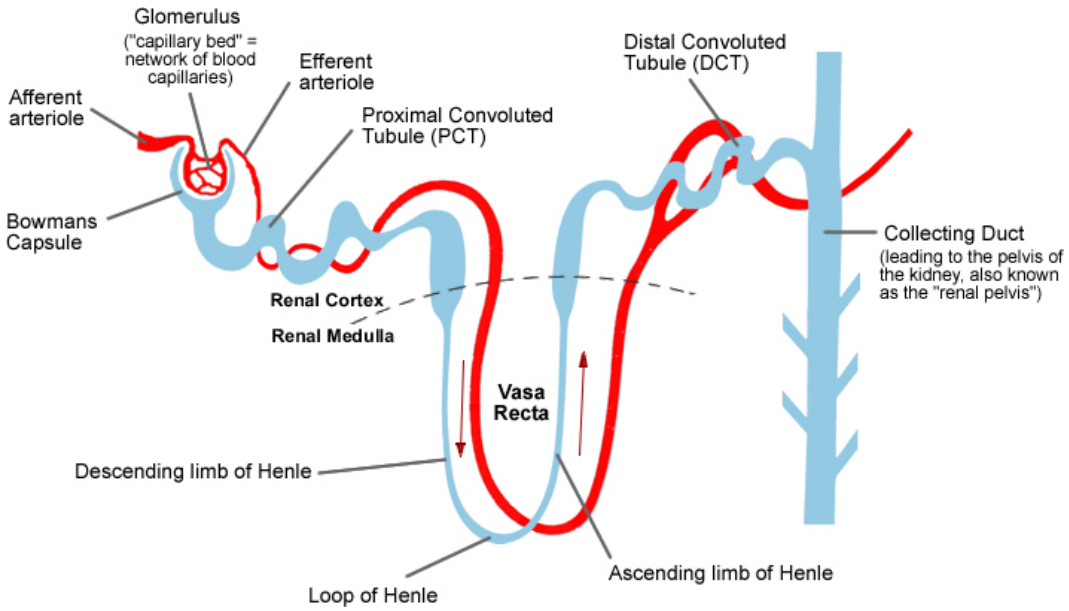

Figure nr. 2 - Nephron structure.

Conversely, when the organism must eliminate excess water, such as after excess fluid drinking, the production of ADH is decreased and the collecting tubule becomes less permeable to water, rendering urine dilute and abundant. Failure of the organism to decrease ADH production appropriately, a condition known as syndrome of inappropriate ADH (SIADH), may lead to water retention and dangerous dilution of body fluids, which in turn may cause severe neurological damage. Failure to produce ADH (or inability of the collecting ducts to respond to it) may cause excessive urination, called diabetes insipidus (DI). A second major function of the collecting duct system is the maintenance of acid-base homeostasis. After being processed along the collecting tubules and ducts, the fluid, now called urine, is drained into the bladder via the ureter, to be finally excluded from the organism.

Functions

The kidneys have four major responsibilities:

- Balancing body fluids
  Healthy kidneys know how much fluid to get rid of and how much to save for the body to function properly.
- Balancing body chemicals
  Healthy kidneys are responsible for maintaining a proper balance of various chemicals your body needs to function. One of the most important of these is potassium, which is needed for nerve and muscle control. Too much or too little potassium can cause weak muscles and heart problems. Healthy kidneys are also active in balancing the levels of calcium, phosphorus and magnesium in your blood.
- Removal of waste products
  A waste product called urea is formed from the breakdown of protein from the foods you eat. Another waste product called creatinine is formed by normal muscle activity.
- Release of essential hormones
  Normal, healthy kidneys release several vital hormones into the bloodstream, including:
  1. renin, which helps regulate blood pressure,
  2. erythropoietin (EPO), which helps bone marrow make red blood cells,
  3. a activated form of vitamin D, which regulates calcium absorption from food and helps maintain healthy bones,
  4. urodilatin.

Every 24 hours, the kidneys filter and clean about 200 quarts of fluid from the blood. Of this, about 198 quarts are absorbed back and retained in the body, and about two quarts are sent to the bladder in the form of urine.

Excretion of waste products

The kidneys excrete a variety of waste products produced by metabolism, including the nitrogenous wastes: urea (from protein catabolism) and uric acid (from nucleic acid metabolism) and water.

Homeostasis

The kidney is one of the major organs involved in whole-body homeostasis. Among its homeostatic functions are acid-base balance, regulation of electrolyte concentrations, control of blood volume, and regulation of blood pressure. The kidneys accomplish these homeostatic functions independently and through coordination with other organs, particularly those of the endocrine system. The kidney communicates with these organs through hormones secreted into the bloodstream.

Acid-base balance

The kidneys regulate the pH, by eliminating H ions concentration called augmentation mineral ion concentration, and water composition of the blood. By exchanging hydronium ions and hydroxyl ions, the blood plasma is maintained by the kidney at a slightly alkaline pH of 7.4. Urine, on the other hand, is acidic at pH 5 or alkaline at pH 8. The pH is maintained through four main protein transporters: NHE3 (a sodium-hydrogen exchanger), V-type H-ATPase (an isoform of the hydrogen ATPase), NBC1 (a sodium-bicarbonate cotransporter) and AE1 (an anion exchanger which exchanges chloride for bicarbonate). Due to the polar alignment of cells in the renal epithelia NHE3 and the H-ATPase are exposed to the lumen (which is essentially outside the body), on the apical side of the cells, and are responsible for excreting hydrogen ions (or protons). Conversely, NBC1 and AE1 are on the basolateral side of the cells, and allow bicarbonate ions to move back into the extracellular fluid and thus are returned to the blood plasma.

Blood pressure

Sodium ions are controlled in a homeostatic process involving aldosterone which increases sodium ion reabsorption in the distal convoluted tubules.When blood pressure becomes low, a proteolytic enzyme called Renin is secreted by cells of the juxtaglomerular apparatus (part of the distal convoluted tubule) which are sensitive to pressure. Renin acts on a blood protein, angiotensinogen, converting it to angiotensin I (10 amino acids). Angiotensin I is then converted by the Angiotensin-converting enzyme (ACE) in the lung capillaries to Angiotensin II (8 amino acids), which stimulates the secretion of Aldosterone by the adrenal cortex, which then affects the kidney tubules.

Aldosterone stimulates an increase in the reabsorption of sodium ions from the kidney tubules which causes an increase in the volume of water that is reabsorbed from the tubule. This increase in water reabsorption increases the volume of blood which ultimately raises the blood pressure.

Plasma volume

Any significant rise or drop in plasma osmolality is detected by the hypothalamus, which communicates directly with the posterior pituitary gland. A rise in osmolality causes the gland to secrete antidiuretic hormone, resulting in water reabsorption by the kidney and an increase in urine concentration. The two factors work together to return the plasma osmolality to its normal levels.

1.2. Protein kinase

A protein kinase is a kinase enzyme that modifies other proteins by chemically adding phosphate groups to them (phosphorylation). This usually results in a functional change of the target protein (substrate) by changing enzyme activity, cellular location, or association with other proteins. Up to 30% of all proteins may be modified by kinase activity, and kinases are known to regulate the majority of cellular pathways, especially those involved in signal transduction, the transmission of signals within the cell. The human genome contains about 500 protein kinase genes; they constitute about 2% of all eukaryotic genes. The chemical activity of a kinase involves removing a phosphate group from ATP and covalently attaching it to one of three amino acids that have a free hydroxyl group. Because protein kinases have profound effects on a cell, their activity is highly regulated. Kinases are turned on or off by phosphorylation (sometimes by the kinase itself - *cis*-phosphorylation/autophosphorylation), by binding of activator proteins or inhibitor proteins, or small molecules, or by controlling their location in the cell relative to their substrates.

Disregulated kinase activity is a frequent cause of disease, particularly cancer, where kinases regulate many aspects that control cell growth, movement and death. Approximately fifty of the hundred or so known genes that have been directly linked to induction of cancer (i.e. oncogenes) encode protein kinases. The remainder of the oncogenes specify proteins that either activate kinases or are phosphorylated by kinases. Although the findings are less direct, aberrant cell signalling through protein kinases has also been associated with cardiovascular disease, diabetes, inflammation, arthritis and other immune disorders, and neurological disorders such as Alzheimer's disease. Over 400 human diseases have been connected to protein kinases. Drugs which inhibit specific kinases are being developed to treat several diseases, and some are currently in clinical use.

With the sequencing of the complete human genome, it is now possible to identify all the related genes within distinct families. It is most efficient if a company can develop expertise around a single family of highly similar proteins. Lessons learned from one family member are most rapidly transferable to related proteins. Protein kinases are the largest family of related genes identified so far that encode enzymes with measurable catalytic activities that are suitable to screen for inhibitory drugs.

Protein kinases are amongst the most meticulously investigated enzymes by researchers. There is a wealth of data about these enzymes that already serves as a solid foundation from which to build. The primary structures of over 500 human protein kinases are already known and the three-dimensional structures of many different protein kinases have been elucidated. Extensive artificial mutagenesis of several protein kinases has been performed to establish detailed structure-function relationships. After the proteases, protein kinases represent the most attractive candidates for molecular modelling studies to design new drugs.

It is estimated that over 25% of the drug discovery efforts in pharmaceutical and biotech companies are focused on protein kinase inhibitors. These drugs have demonstrated applications for treatment of a wide range of diseases including cancer, inflammation, diabetes, congestive heart failure, and neurological damage. Over 60 protein kinase inhibitors are currently in advanced clinical trials, and three are now available in the market place (Herceptin, Gleevec, and Ireesa). The pharmaceutical industry has clearly come to fully recognize the therapeutic potential of protein kinase inhibitors.

In the genome of the yeast *Saccharomyces cerevisiae*, protein kinases represent the largest family of related genes (121 out of 6144 yeast genes encode protein kinases). Many of these kinase genes have mammalian counterparts that will substitute for them in genetically re-engineered yeast. In the fly *Drosophila melanogaster*, 319 of its 13338 genes encode protein kinases. In the worm *C. elegans*, 437 of its 18266 genes specify protein kinases. For all of these organisms, this translates to approximately 2% of the total genes corresponding to protein kinases. Recently, the complete genome of the mustard plant *Arabidopsis thaliana* was reported and it features 1049 putative protein kinases out of 25706 genes. This represented about 4% of that plant's genome. The human genome appears to encode 500 protein kinases in addition to many pseudo-protein kinase genes, and these have been sub classified into over 57 families. There may well be additional protein kinases that remain to be identified. Protein kinases are readily recognized, because they feature characteristic amino acid sequences that distinguish these enzymes from other proteins.

There are 5 types of protein kinase (according to the type of action):

- *Serine/threonine-specific protein kinases*
- *Tyrosine-specific protein kinases*
- *Histidine-specific protein kinases*
- *Aspartic acid/glutamic acid-specific protein kinases*
- *Mixed kinases*

1) Serine/threonine-specific protein kinases

Serine/threonine protein kinases phosphorylate the -OH group of serine or threonine (which has similar sidechains). Activity of these protein kinases can be regulated by specific events (e.g. DNA damage), as well as numerous chemical signals, including cAMP/cGMP, diacylglycerol, and Ca^{2+}/calmodulin. These kinases are not specific to a similar consensus sequence - that is there is no common "target sequence" to be phosphorylated. Since the substrate to be phosphorylated aligns with the kinase by several key amino acids (usually through hydrophopic forces and ionic bonds), a kinase is usually specific, not to a single substrate, but to a whole "substrate family" having common properties. The kinases are usually inactivated by a pseudosubstrate that binds to the kinase like a real substrate but lacks the amino acid to be phosphorylated. Its removal activates the kinase. The catalytic domain of these kinases is highly conserved.

- *Phosphorylase kinase*: phosphorylase kinase was the first Ser/Thr protein kinase to be discovered (Krebs EG et al., (1959) *J Biol Chem*).

- *Protein kinase A:* protein kinase A consists of two domains, a small domain with several β sheet structures and a larger domain containing several α helices. The binding sites for substrate and ATP are located in the catalytic cleft between the domains (or lobes). When ATP and substrate bind, the two lobes rotate so that the terminal phosphate group of the ATP and the target amino acid of the substrate move into the correct positions for the catalytic reaction to take place.

Regulation

Protein kinase A has several functions in the cell, including regulation of glycogen, sugar, and lipid metabolism. It is controlled by cAMP: in the absence of cAMP, the kinase is a tetramer of two regulatory and two catalytic subunits (R_2C_2), with the regulatory subunits blocking the catalytic center of the catalytic subunits. Binding of cAMP to the regulatory subunit leads to dissociation of active RC dimers. Also, the catalytic subunit itself can be regulated by phosphorylation. Downregulation of protein kinase A occurs by a feedback mechanism: one of the substrates that are activated by the kinase is a phosphodiesterase, which converts cAMP to AMP, thus reducing the amount of cAMP that can activate protein kinase A.

- *Protein kinase C:* protein kinase C is actually a family of protein kinases that require Ca^{2+}, diacylglycerol, and a phospholipid such as phosphatidylcholine for activation. Thus, protein kinase C is activated through the same signal transduction pathway as phospholipase C. At least twelve members of the protein kinase C family have been identified in mammals, due to their high sequence homology. The protein kinase C usually means the protein kinase Cα enzyme.

Structure and regulation

Protein kinase C enzymes consist of an N-terminal regulatory domain and a C-terminal catalytic domain. The kinases are inactive in the absence of activating agents, due to autoinhibition of the regulatory domain. They can be activated tumor promoters such as tetradecanoyl-phorbol-acetate (TPA) or by the cofactors Ca^{2+}, diacylglycerol and a phospholipid. The common linear structure of protein kinase C enzymes is: N-pseudosubstrate - TPA-binding - (Ca^{2+}-binding) - ATP-binding - substrate-binding- C.

Upon activation, protein kinase C enzymes are translocated to the plasma membrane by RACK proteins (membrane-bound receptor for activated protein kinase C proteins). The protein kinase C enzymes are known for their long-term activation: they remain activated after the original activation signal or the Ca^{2+}-wave is gone. This is presumably achieved by the production of diacylglycerol from phosphatidylcholine by a phospholipase; fatty acids may also play a role in long-term activation.

Function

The consensus sequence of protein kinase C enzymes is similar to that of protein kinase A, since it contains basic amino acids close to the Ser/Thr to be phosphorylated. Their substrates are MARCKS proteins, MAP kinase, transcription factor inhibitor IκB, the vitamin D_3 receptor VDR, Raf kinase, calpain, and the EGF receptor.

Ca^{2+}/calmodulin-dependent protein kinases:

Also called *CaM kinases*, these kinases are primarily regulated by the Ca^{2+}/calmodulin complex. These kinases show a memory effect on activation. Two types of CaM kinases are:

- *Specialized CaM kinases*. An example is the myosin light chain kinase (MLCK) that phosphorylates myosin, causing muscles to contract.
- *Multifunctional CaM kinases*. Also collectively called *CaM kinase II*, which play a role in many processes, such as neurotransmitter secretion, transcription factor regulation, and glycogen metabolism.

Structure and autoregulation

The CaM kinases consist of an N-terminal catalytic domain, a regulatory domain and an associative domain. In the absence of Ca^{2+}/calmodulin, the catalytic domain is auto inhibited by the regulatory domain, which contains a pseudo substrate sequence. Several CaM kinases aggregate into a homo-oligomer or hetero-oligomer. Upon activation by Ca^{2+}/calmodulin, the activated CaM kinases autophosphorylate each other, in an intermolecular reaction.

This has two effects:

- An increase in affinity for the calmodulin complex, prolonging the time the kinase is active.
- Continued activation of the phosphorylated kinase complex even after the calmodulin complex has dissociated from the kinase complex, which prolongs the active state even more.

2) Tyrosine-specific protein kinases

Tyrosine-specific protein kinases phosphorylate tyrosine amino acid residues, and are, like serine/threonine-specific kinases, used in signal transduction. They act primarily as growth factor receptors and in downstream signaling from growth factors; some examples:

- Platelet-derived growth factor (PDGF) receptor;
- Epidermal growth factor (EGF) receptor;
- Insulin receptor and insulin-like growth factor (IGF1) receptor;
- Stem cell factor (*scf*) receptor (also called *c-kit*).

- *Receptor tyrosine kinases*:

These kinases consist of a transmembrane receptor with a tyrosine kinase domain protruding into the cytoplasm. They play an important role in regulating cell division, cellular differentiation, and morphogenesis. More than 50 receptor tyrosine kinases are known in mammals.

Structure

The extracellular domain serves as the ligand-binding part of the molecule. It can be a separate unit that is attached to the rest of the receptor by a disulfide bond. The same mechanism can be used to bind two receptors together to form a homo- or heterodimer. The transmembrane element is a single α helix. The intracellular or cytoplasmic domain is responsible for the (highly conserved) kinase activity, as well as several regulatory functions.

Regulation

Ligand binding causes two reactions:

- dimerization of two monomeric receptor kinases or
- stabilization of a loose dimer.

Many ligands of receptor tyrosine kinases are multivalent. Some tyrosine receptor kinases (e.g., the platelet-derived growth factor receptor) can form heterodimers with other similar but not identical kinases of the same subfamily, allowing a highly varied response to the extracellular signal.

Trans-autophosphorylation (phosphorylation by the other kinase in the dimer) of the kinase.

The autophosphorylation causes the two subdomains of the intrinsic kinase to shift, opening the kinase domain for ATP binding. In the inactive form, the kinase subdomains are aligned so that ATP cannot reach the catalytic center of the kinase. When several amino acids suitable for phosphorylation are present in the kinase domain (e.g., the insulin-like growth factor receptor), the activity of the kinase can increase with the number of phosphorylated amino acids; in this case, the first phosphorylation is said to be a *cis*-autophosphorylation, switching the kinase from "off" to "standby".

Signal transduction

The active tyrosine kinase phosphorylates specific target proteins, which are often enzymes themselves. An important target is the ras protein signal-transduction chain.

- *Receptor-associated tyrosine kinases:*

Tyrosine kinases recruited to a receptor following hormone binding are receptor-associated tyrosine kinases and are involved in a number of signaling cascades, principally those involved in cytokine signaling (but also others, including growth hormone). One such receptor-associated tyrosine kinase is Janus kinase (JAK), many of whose effects are mediated by STAT proteins.

3) Histidine-specific protein kinases

Histidine kinases are structurally distinct from most other protein kinases and are found mostly in prokaryotes as part of two-component signal transduction mechanisms. A phosphate group from ATP is first added to a histidine residue within the kinase, and later transferred to an aspartate residue on a 'receiver domain' on a different protein, or sometimes on the kinase itself. The aspartyl phosphate residue is then active in signaling.

Histidine kinases are found widely in prokaryotes, as well as in plants and fungi. The pyruvate dehydrogenase family of kinases in animals is structurally related to histidine kinases, but instead phosphorylate serine residues, and probably do not use a phospho-histidine intermediate.

4) Aspartic acid/glutamic acid-specific protein kinases

5) Mixed kinases

Some kinases have mixed kinase activities. For example, MEK (MAPKK), which is involved in the MAP kinase cascade, is a mixed serine/threonine and tyrosine kinase.

1.3. The SGK kinases

The serum- and glucocorticoid-regulated kinase (SGK) was originally cloned from rat mammary tumor cells as a glucocorticoid responsive gene. The human isoform was subsequently cloned as a cell volume–sensitive gene upregulated by both hypertonic and isotonic cell shrinkage (Waldegger S. et al., (1997) *Proc Natl Acad Sci*; Waldegger S. et al., (2000) *Cell Physiol Biochem*).

Because of the discovery of the 2 isoforms SGK2 and SGK3 (Kobayashi T. et al., (1999) *Biochem J*) the originally cloned kinase is labeled SGK1. SGK1 is expressed in renal tubular epithelial cells (Naray-Fejes-Toth A. et al., (1999) *J Biol Chem*; Loffing J. et al., (2001) *Am J Physiol Renal Physiol*) and its transcription is strongly stimulated by mineralocorticoids (Chen SY. et al., (1999) *Proc Natl Acad Sci USA*) suggesting a role in renal Na^+ regulation. Indeed, coexpression of SGK1 with the renal epithelial Na^+ channel (ENaC), in *Xenopus* oocytes markedly upregulates Na^+ channel activity by enhancing channel protein abundance in the cell membrane.

SGK kinases are expressed in a wide variety of species including shark and *Caenorhabditis elegans*. Yeast expresses two orthologs, Ypk1 and Ypk2, which are involved in endocytosis and required for survival. Yeast lacking Ypk1 and Ypk2 can be rescued by mammalian SGK1. SGKs participate in the regulation of transport, hormone release, neuroexcitability, cell proliferation, and apoptosis.

Little is known about genomic regulation of SGK2 and SGK3, which appear to be less sensitive to hormonal regulation than SGK1.The serum- and glucocorticoid-inducible kinase-1, SGK1, is a known downstream effector of the PI3K cascade. SGK1 belongs to the "AGC" family of serine-threonine kinases and shares approximately 45% to 55% homology with Akt in its catalytic domain.

In contrast to Akt, SGK1 is also regulated at the transcriptional level in response to various hormones, growth factors, and extracellular stresses in a cell type – dependent manner, allowing *sgk1* to be available for its targets only when needed. SGK1 was originally cloned from murine mammary tumor cells as a glucocorticoid-responsive gene. Human SGK1 was subsequently cloned as a cell volume-sensitive gene upregulated by hypertonic cell shrinkage. Increasing evidence suggests that expression, enzymatic activity, and cellular localization of SGK1 are regulated in response to various stimuli controlling not only cell volume and epithelial transport, but also cardiac action potential and cell proliferation, survival, and apoptosis. Excessive transcription of SGK1 has been shown to parallel diabetic nephropathy, glomerulonephritis, hepatic cirrhosis, pulmonary fibrosis, and polymorphisms of the SGK1 gene correlated with hypertension. Despite the wide tissue distribution of *sgk1* and its sensitivity to various stimuli, the role of SGK-1 in the cardiovascular and pulmonary system remained ill defined. Because heparin, an inhibitor of thrombin formation, has been shown to decrease SGK1 mRNA in aortic smooth muscle cells, we hypothesized that SGK1 may play a role in thrombin signaling in human pulmonary artery smooth muscle cells (PASMC). We found that SGK1 is activated and induced by thrombin, that it regulates TF expression and activity in PASMC, and that it is present in remodeled pulmonary vessels with media hypertrophy associated with ph (Rachida S. et al., (2006) *Circ Res*).

1.3.1. The SGK1

SGK1 was originally cloned as an immediate early gene transcriptionally stimulated by serum and glucocorticoids in rat mammary tumor cells (Firestone G. et al., (2003) *Cell Physiol Biochem*). The human isoform has been discovered as a cell volume-regulated gene upregulated by cell shrinkage.

Transcription of SGK1 is upregulated by both serum and glucocorticoids. Several other hormones and mediators stimulate SGK1 transcription, including mineralocorticoids, gonadotropins (Lang F. et al., (2006) *Physiol Rev*), 1.25-dihydroxyvitamin D3 ($1.25(OH)_2D_3$), transforming growth factor-α (TGF-α) (Kumar JM. et al., (1999) *J Am Soc Nephrol*; Lang F. et al., (2000) *Proc Natl Acad Sci USA*), interleukin-6 (Mc Ewen BS. et al., (1995) *Vitam Horm*), fibroblast and platelet-derived growth factor (Mizuno H. et al., (2001) *Genes Cell*), thrombin (Belaiba R. et al., (2006) *Circ Res*), endothelin (Wolf Sc. et al., (2006) *Biochem Pharmacol*), as well as other cytokines (Verenivov A. et al., (2001) *Physiol Biochem*). Moreover, activation of peroxisome proliferator-activated receptor γ (PPAR γ) stimulates SGK1 gene transcription (Hong G. et al., (2003) *Faseb J*). The human isoform has been identified as a cell volume-regulated gene that is transcriptionally upregulated by cell shrinkage.

In renal epithelial (A6) cells, SGK1 expression is stimulated by cell swelling rather than cell shrinkage (Rozansky DJ. et al., (2002) *Am J Physiol Renal Physiol*). SGK1 transcription is further stimulated by excessive glucose concentrations (Lang F. et al., (2000) *Proc Natl Acad Sci USA*; Saad S. et al., (2005) *Kidney Int*), heat shock, ultraviolet (UV) radiation, and oxidative stress. SGK1 transcription is inhibited by heparin (Delmolino LM. et al., (1997) *J Cell Physiol*) and by mutations in the gene MECP2, which underlies Rett syndrome (RTT), a disorder with severe mental retardation (Nuber UA. et al., (2005) *Hum Mol Genet*).

Intestinal SGK1 transcription is further regulated by dietary iron (Marzullo L. et al., (2004) *Gene*). Signaling molecules involved in the transcriptional regulation of SGK1 include protein kinase C (Lang F. et al., (2000) *Proc Natl Acad Sci USA;* Mizuno H and Nishida E., (2001) *Genes Cells*), the protein kinase Raf (Mizuno H and Nishida E., (2001) *Genes Cells*), mammalian mitogen-activated protein kinase (BMK1) (Hayashi M. et al., (2001) *J Biol Chem*), mitogen-activated protein kinase (MKK1) (Davies SP. et al., (2000) *Biochem J*; Mizuno H and Nishida E., (2001) *Genes Cells*), stress-activated protein kinase-2 (SAPK2, p38 kinase) (Bell LM. et al., (2000) *J Biol Chem*; Chen S. et al., (2004) *Hypertension*; Waldegger S. et al., (2000) *Cell Physiol Biochem*) and phosphatidylinositol (PI) 3-kinase.

Follicle-stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-induced kinase (SGK): evidence for a kinase-independent signaling by FSH in granulosa cells, cAMP (Gonzalez-Robayna IJ. et al., (2000) *Mol Endocrinol*; Klingel K. et al., (2000) *Am J Physiol Gastrointest Liver Physiol*) and p53 (Maiyar AC. et al., (1996) *J Biol Chem*; Maiyar AC. et al., (1997) *Mol Endocrinol*).

Moreover, SGK1 transcription is stimulated by an increased cytosolic Ca^{2+} concentration (Klingel K. et al., (2000) *Am J Physiol Gastrointest Liver Physiol*) and by nitric oxide (Turpaev K. et al., (2005) *Free Radic Biol Med*). SGK1 transcript levels are increased by ischemia of brain (Nishida Y. et al., (2004) *Brain Res*) and kidney (Feng Y. et al., (2006) *Kidney Blood Pressure Res*). SGK1 expression is decreased during rejection of transplanted kidneys (Velic A. et al., (2005) *Am J Transplant*).

Similar to its isoforms SGK2 and SGK3, SGK1 is activated by insulin and growth factors via phosphatidylinositol 3-kinase and the 3-phosphoinositide-dependent kinase PDK1. SGKs activate ion channels (e.g.: ENaC, TRPV5, ROMK, Kv1.3, KCNE1/KCNQ1, GluR1, GluR6), carriers (e.g., NHE3, GLUT1, SGLT1, EAAT1–5), and the Na^+-K^+-ATPase. They regulate the activity of enzymes (e.g., glycogen synthase kinase-3, ubiquitin ligase Nedd4–2, phosphomannose mutase-2) and transcription factors (e.g., forkhead transcription factor FKHRL1, α-catenin, nuclear factor kB).

Leukocyte SGK1 transcript levels are enhanced by treatment with dialysis (Friedrich B. et al., (2005) *Nephrol Dial Transplant*). A striking increase of SGK1 expression is observed during wound healing (Iyer V. et al., (1999) *Science*) and in fibrosing tissue, such as diabetic nephropathy (Kumar J. M et al., (1999) *J Am Soc Nephrol*), glomerulonephritis (Friedrich B. et al., (2002) *Kidney Blood Press Res*), liver cirrhosis (Fillon S. et al., (2002) *Cell Physiol Biochem*), fibrosing pancreatitis (Klingel K. et al., (2000) *Am J Physiol Gastrointest Liver Physiol*), Crohn's disease (Waldegger S. et al., (1999) *Gastroenterology*), lung fibrosis and cardiac fibrosis (Vallon V. et al., (2006) *J Mol Med*). SGK1 gene transcription is stimulated by DNA damage through p53 and activation of extracellular signal-regulated kinase (ERK1/2) (Mizuno H. et al., (2001) *Genes Cells*; You H. et al., (2004) *Proc Natl Acad Sci USA*), and is also upregulated after neuronal injury (Imaizumi K. et al., (1994) *Brain Res*), neuronal excitotoxicity (Hollister R. et al., (1997) *Neuroscience*), and neuronal challenge by exposure to microgravity (David S. et al., (2005) *J Neurosci*).

The promoter of the rat SGK1 gene carries several putative and confirmed transcription factor binding sites including those for the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR), the progesterone receptor (PR), the vitamin D receptor (VDR), the retinoid X receptor (RXR), the farnesoid X receptor (FXR), the sterol regulatory element binding protein (SREBP), PPARγ, the cAMP response element binding protein (CREB), the p53 tumor suppressor protein, the Sp1 transcription factor, the activating protein 1 (AP1), the activating transcription factor 6 (ATF6), the heat shock factor (HSF), reticuloendotheliosis viral oncogene homolog (c-Rel) and nuclear factor κB (NFκB), signal transducers and activators of transcription (STAT), the TGF-α-dependent transcription factors SMAD3 and SMAD4, and forkhead activin signal transducer (FAST) (Firestone G. et al., (2003) *Cell Physiol Biochem*). The regulation of SGK1 transcript levels is fast; appearance and disappearance of SGK1 mRNA require circa 20 min (Waldegger S. et al., (1997) *Proc Natl Acad Sci USA*).

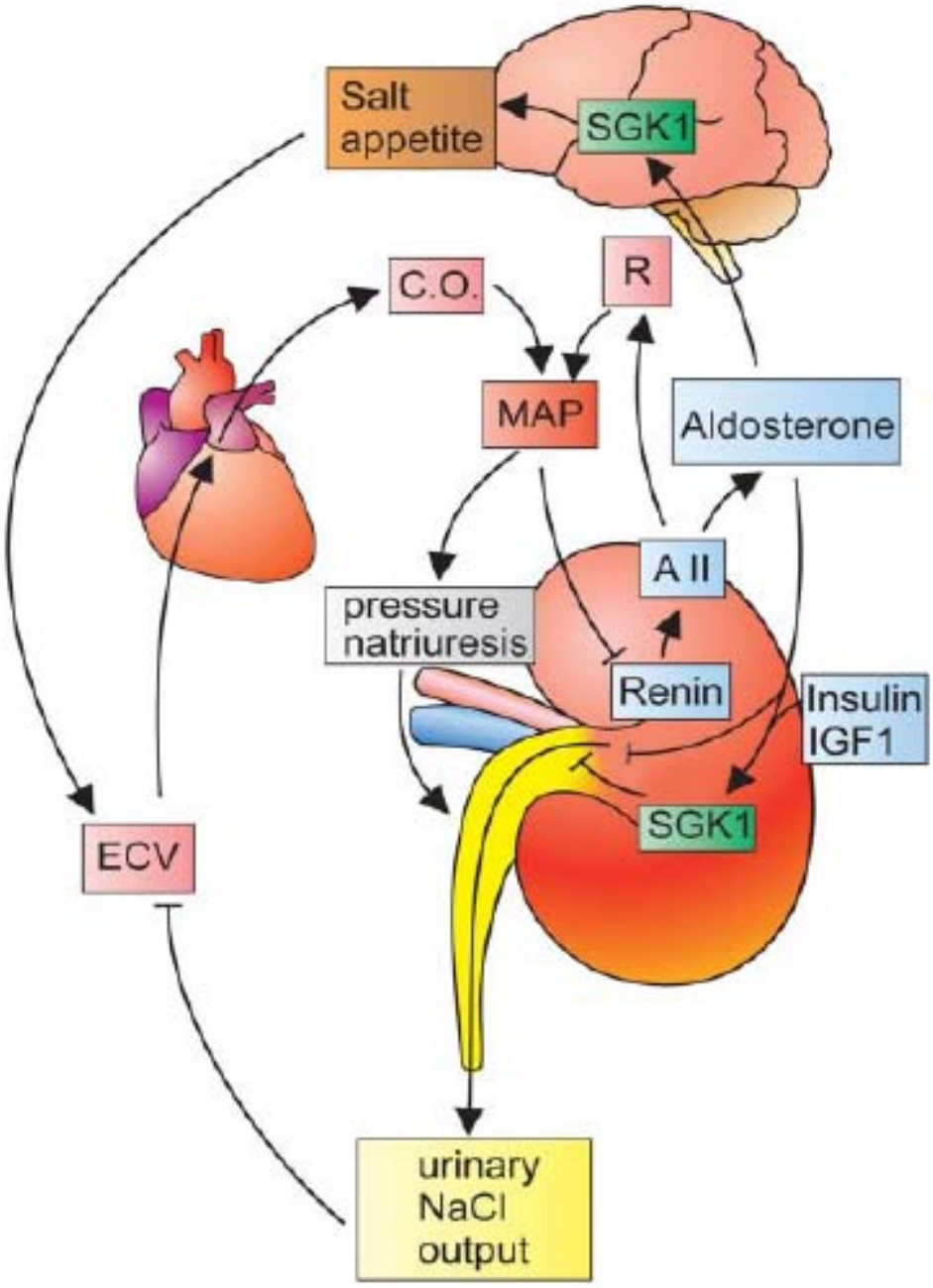

Figure nr. 3 - Dual role of SGK1 in the maintenance of salt homeostasis and blood pressure.

SGK1 plays a dual role in the regulation of salt balance, i.e., in the stimulation of both renal Na^+ reabsorption and salt appetite. SGK1 contributes to aldosterone- and insulin-induced stimulation of renal Na^+ reabsorption. The increased extracellular fluid volume (ECV) enhances the cardiac output (C.O.), thus increasing mean arterial blood pressure (MAP). The enhanced blood pressure leads to pressure natriuresis and thus secondarily increases renal salt excretion, eventually counteracting renal salt retention. A II, angiotensin II; R, total peripheral vascular resistance.

SGK1 participate in the regulation of transport, hormone release, neuroexcitability, cell proliferation, and apoptosis. SGK1 contributes to Na^+ retention and K^+ elimination of the kidney, mineralocorticoid stimulation of salt appetite, glucocorticoid stimulation of intestinal Na^+/H^+ exchanger and nutrient transport, insulin-dependent salt sensitivity of blood pressure and salt sensitivity of peripheral glucose uptake, memory consolidation, and cardiac repolarization.

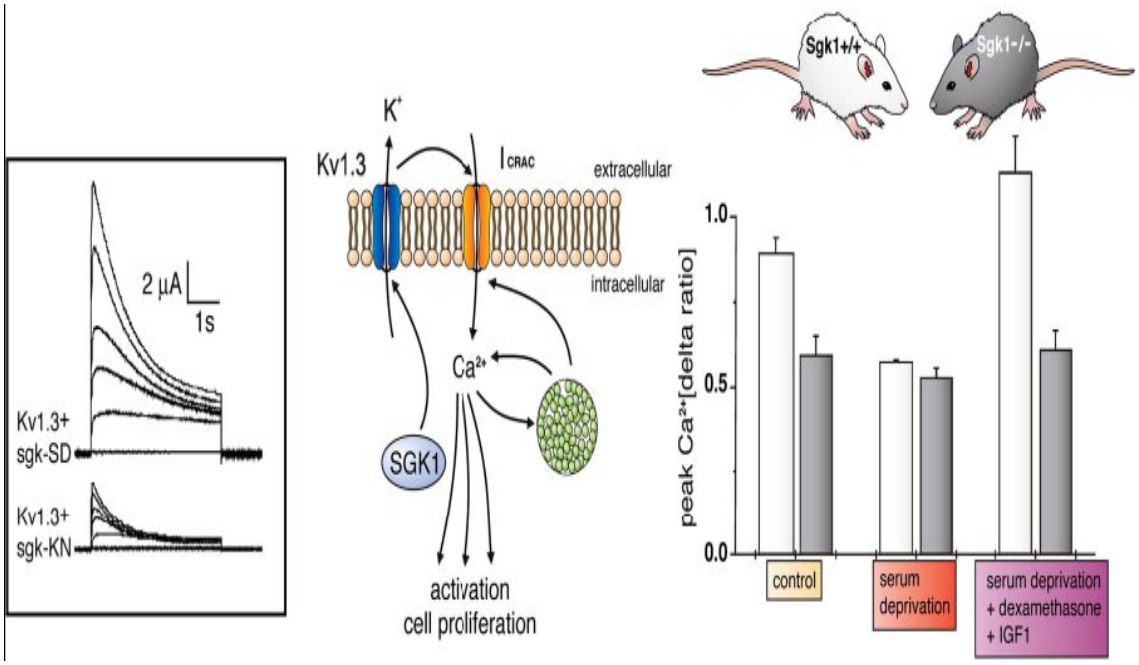

Figure nr. 4 - *Middle*: putative role of SGK1 in the regulation of K^+ and Ca^{2+} channels required for stimulation of cell proliferation.

Mitogenic factors stimulate the Ca^{2+} release-activated Ca^{2+} channel *I*CRAC. Ca^{2+} entry through this channel is highly sensitive to cell membrane potential, which is maintained by K^+ channels. The stimulation of the voltage-sensitive K^+ channel Kv1.3 by SGK1 may serve to maintain the cell membrane polarization and thus sustain oscillating Ca^{2+} entry through *I*CRAC. *Left*: current generated by depolarization of HEK cells overexpressing constitutively active S^{422}DSGK1 (sgk-SD) or the inactive K^{127}NSGK1 mutant (sgk-KN). *Right*: peak Ca^{2+} concentration after Ca^{2+} entry in Ca^{2+}-depleted fibroblasts from wild-type mice (*sgk1*$^{+/+}$, open bars) or SGK1 knockout mice (*sgk1*$^{-/-}$, solid bars) fibroblasts. Ca^{2+} entry in *sgk1*$^{-/-}$ fibroblasts is not sensitive to serum deprivation or to dexamethasone plus IGF-I (Shumilina E. et al, (2005) *J Cell Physiol*).

A common SGK1 gene variant is associated with increased blood pressure and body weight. SGK1 may thus contribute to metabolic syndrome. SGK1 may further participate in tumor growth, neurodegeneration, fibrosing disease, and the sequelae of ischemia. SGK3 is required for adequate hair growth and maintenance of intestinal nutrient transport and influences locomotive behavior. In conclusion, SGK1 cover a wide variety of physiological functions and may play an active role in a multitude of pathophysiological conditions. There is little doubt that further targets will be identified that is modulated by the SGK and that further SGK-dependent *in vivo* physiological functions and pathophysiological conditions will be defined.

1.3.2. The SGK1 Isoforms SGK2 and SGK3

There are two paralogs of SGK1, SGK2 and SGK3/CISK, which share 80% amino acid identity with SGK1 and with each other in their catalytic domains (Kobayashi T. et al., (1999) *Biochem J*; Liu D. et al., (2000) *Curr Biol*; Dai F. et al., (1999) *Genomics*).

SGK3 is expressed in all tissues tested thus far and is particularly high in the embryo, adult heart and spleen. Expression of SGK2 is most abundant in epithelial tissues including kidney, liver, pancreas, and presumably choroid plexus of the brain. The subcellular distribution may be nuclear and cytoplasmic, as SGK2 and SGK3 contain a similar nuclear localization signal sequence as SGK1.

The gene encoding human SGK1 has been localized to chromosome 6q23, whereas the gene encoding SGK2 is localized to chromosome 20q12. SGK3 is identical to the cytokine-independent survival kinase CISK (Liu D. et al., (2000) *Curr Biol*).

The three enzymes differ in the region N-terminal of the C-terminal catalytic domain: SGK2 contains a relatively short N terminus (98 amino acids), with no discernable domain, whereas SGK3 has a longer N terminus (162 amino acids) comprising a phox homology (PX) domain (Xu J. et al., (2001) *Cell Biol*).

PX domains were originally found as conserved domains in the p40phox and p47phox subunits of the neutrophil NADPH oxidase (phox) superoxide-generating complex (Ponting CP., (1996) *Protein Sci*). These domains are part of many proteins involved in intracellular protein trafficking, such as the sorting nexins. PX domains are phosphoinositide-binding domains that appear to be important for localization of these proteins to membranes (especially endosomes) enriched in phosphoinositides. In this respect, these domains resemble other domains such as the pleckstrin homology (PH), FYFE, FERM and ENTH domains. PKB/Akt contains a PH domain in its N terminus, which is important for PKB/Akt activation by phosphoinositide-3 kinases (PI-3Ks). This domain enables the colocalization with the 3-phosphoinositide-dependent protein kinase-1 (PDK1), which is known to phosphorylate and activate PKB/Akt. Similarly, SGK3's PX domain is involved in SGK3 localization and activity: It is necessary for phosphoinositide binding, endosomal localization, and proper kinase activity. Moreover, structural studies indicate that it may play a role in dimerization of the kinase. With respect to their physiological role(s), it has been shown *in vitro* that both the SGK2 and SGK3 enzymes have the same phosphorylation consensus as SGK1 (and PKB/Akt), namely R-X-R-X-X-(S/T). It is likely, however, that other factors, such as surrounding amino acids, subcellular localization, or cofactors are important for the specificity of and functional differences between the enzymes. For example, in *Xenopus* A6 cells, only SGK1 and not the coexpressed PKB modulates the activity of the epithelial Na^+ channel (ENaC) (Arteaga MF. et al., (2005) *Am J Physiol Renal Physiol*).

The role of SGK2 has mainly been studied in heterologous expression systems such as *Xenopus laevis* oocytes or HEK293 cells and with respect to numerous transport and channel proteins. These studies revealed that SGK2 can stimulate the activity of K^+ channels such as the voltage- gated K^+ channel Kv1.3 (Gamper et al., (2002) *Pflügers Arc,*; Henke et al., (2004) *J Cell Physiol*), Na^+-K^+-ATPase (Henke G. et al., (2002) *Kidney Blood Press Res*), KCNE1 (Embark et al., (2003) *Pflügers Arch*), ENaC (Friedrich et al., (2003) *Pflügers Arch*), the glutamate transporter EEAT4 (Böhmer et al., (2004) *Biochem Biophys Res Commun*), and the glutamate receptors GluR6 (Strutz-Seebohm et al., (2005) *J Physiol*) and GluR1 (Strutz-Seebohm et al., (2005a) *J Physiol*). All of these transport proteins are also stimulated in the same cellular systems by SGK1, SGK3, and/or PKB; hence, the physiological relevance of these findings has to be considered with caution. To define more precisely the role of SGK2, it will be necessary to carry out additional studies, using more relevant cell or animal systems and knocking down SGK2 by either RNA interference protocols or by gene inactivation. SGK3/CISK, which is better characterized than SGK2, was identified in a screen for antiapoptotic genes (Liu et al., (2000) *Curr Biol*) and found to act downstream of the PI-3K pathway and in parallel with PKB/Akt. Moreover, it was demonstrated to phosphorylate and inhibit Bad (a proapoptotic protein) and FKHRL1, a proapoptotic transcription factor. Knockout (ko) mice have been generated; these mice are viable and fertile and have normal Na^+ handling and glucose tolerance, as opposed to the KO mice of SGK1 or PKB/Akt2 (McCormick et al., (2004) *Mol Biol Cell*; Garofalo et al., (2003) *J Clin Invest*; Wulff et al., (2002) *J Clin Invest*). However, they display after birth a defect in hair follicle development, a defect preceded by disturbances in the β-catenin/Lef1 gene regulation (McCormick et al., (2004) *Mol Biol Cell*). Like SGK2, SGK3 has been implicated in the regulation of numerous

transporters and channels, including K^+ channels (Gamper et al., (2002) *Pflügers Arch*; Henke et al., (2004) *J cell Physiol*); Embark et al., (2003) *Pflügers Arch*), Na^+-K^+-ATPase (Henke G. et al., (2002) *Kidney Blood Press Res*), the glutamate transporter EEAT1 (Boehmer et al., (2003) *Cardiovasc Res*), the cardiac voltage-gated Na^+ channel SCN5A (Boehmer et al., (2003) *J Neurochem*), ENaC (Friedrich et al., (2003) *Pflügers Arch*), Na^+-dicarboxylate cotransporter 1 (Böhmer et al., (2004) *Biochem Biophys Res Commun*), the chloride channel ClCa/barttin (Embark et al., (2004) *Pflügers Arch*), the epithelial Ca^{2+} channel TRPV5 (Embark et al., (2004) *Pflügers Arch*), the Na^+-phosphate cotransporter NaPi2b (Palmada M. et al., (2004) *Cell Physiol Biochem*), the amino acid transporter ASCT2 (Palmada M. et al., (2005) *Cell Physiol Biochem*), GluR1, and GluR6 (Strutz-Seebohm et al., (2005); Strutz-Seebohm et al., (2005a) *J Physiol*). For the same reasons mentioned above for SGK2, additional studies on SGK3 will be necessary to evaluate the physiological relevance of these findings.

1.4. Tissue distribution of SGK isoforms

SGK isoforms are expressed in numerous tissues and cell lines. Among the three kinases, SGK1 (Waldegger et al., (1997) *Proc Natl Acad Sci USA*; Kobayashi et al., (1999) *Biochem J*) and SGK3 show the broadest distribution, with expression in many tissues including the brain, placenta, lung, liver, pancreas, kidney, heart and skeletal muscle. In situ hybridization studies localized SGK1 mRNA in several epithelial and/or nonepithelial cells within the brain (Wärntges et al., (2002) *Cell Physiol Biochem*; Nishida et al., (2004) *Brain Res*; Tsai et al., (2002) *Proc Natl Acad Sci USA*; Stichel et al., (2005) *Eur J Neurosci*; Gonzalez-Nicolini A. and McGinty F., (2002) *Brain Res Gene ExpPatterns*), eye (Rauz et al., (2003) *Exp Eye Res*; Rauz et al., (2003a) *Invest Ophtalmol Vis Sci*), lung (Wärntges et al., (2002) *Cell Physiol Biochem*) liver (Fillon et al., (2002) *Comp Biochem Physiol A Mol Integr Physiol*), ovary (Alliston et al., (2000) *Endocrinology*), pancreas (Klingel et al., (2000) *Am J Physiol Gastrointest Liver Physiol*), intestine (Waldegger et al., (1999) *Gastroentherology*) and kidney (Chen et al., (1999) *Proc Natl Acad Sci USA*; Friedrich et al., (2002) *Kidney Blood Press res*; Huber et al., (2001) *Pflügers Archive*).

SGK1 mRNA expression is established very early in embryonic development, as indicated by in situ hybridizations on whole-mount preparations of mouse embryo (Lee et al., (2001) *Mech Dev*). By embryonic day (E) 8.5, SGK1 is already highly expressed in the decidua and yolk sac. By days E9.5– E12.5 it is found in the developing heart, eye, and lung, and it becomes highly expressed by days E13.5–E16.5 in the brain choroid plexus, kidney distal tubules, bronchi/bronchiole, adrenal glands, liver, thymus, and intestine (Lee et al., (2001) *Mech Dev*). In contrast to SGK1 and SGK3, SGK2 reveals a more restricted distribution and is highly abundant only in the liver, kidney, and pancreas, where it is found in two different SGK2 species, referred to as SGK2α and SGK2β (Kobayashi et al., (1999) *Biochem J*). SGK isoform expression varies also between cell lines cultured *in vitro*. Similar to its expression pattern *in vivo*, SGK1 is broadly expressed in cultured cells and is readily detectable in, for example, hepatoma cells, fibroblasts and mammary tumor cells (Webster et al., (1993) *J Biol Chem*; Kobayashi et al., (1999) *Biochem J*). By contrast, SGK2 mRNA is expressed in hepatoma cells but not in fibroblasts, whereas SGK3 is found in fibroblasts but not in hepatoma cells. Remarkably, all three SGK isoforms are expressed in cells derived from the renal cortical collecting duct (Naray-Fejes-Toth et al., (2004) *Proc Natl Acad Sci USA*).

In mammals, SGK1 is expressed in virtually all tissues tested (Waldegger S. et al., (1997) *Proc Natl Acad Sci USA*). However, transcript levels vary profoundly among different

cell types of any given tissue, such as brain, eye, lung, kidney, liver, intestine, pancreas, and ovary. Moreover, typical expression patterns are found during embryonic development.

1.5. Stimuli of SGK

SGK1 is under transcriptional control of numerous stimuli. SGK1 is activated by insulin and growth factors via phosphatidylinositol 3-kinase and the 3-phosphoinositide-dependent kinase PDK1. Like SGK1, the mRNA encoding SGK3 is expressed in all tissues examined, but SGK2 mRNA is only present at significant levels in liver, kidney and pancreas and, at lower levels, in the brain. The levels of SGK2 mRNA and SGK3 mRNA are not increased by stimulation with serum or dexamethasone. SGK2 and SGK3 are activated *in vitro* by PDK1, albeit more slowly than SGK1, and their activation is accompanied by the phosphorylation of Thr^{193} and Thr^{253} respectively. Like SGK1, SGK2 and SGK3 are activated 5-fold by H2O2, to a smaller extent by insulin-like growth factor-1 (2- fold) than SGK1 (5-fold).

1.6. Regulation of SGK kinase activity

To become functional, the SGK protein kinases require activation by phosphorylation, which is accomplished through a signaling cascade involving PI 3-kinase, the 3-phosphoinositide (PIP3)-dependent kinase PDK1, and a yet unidentified but also PIP3-dependent kinase that has been referred to as PDK2 or "hydrophobic motif" (H-motif) kinase (Collins BJ. et al., (2003) *EMBO J*; Frodin M. et al., (2002) *EMBO J*; Kobayashi T. et al., (1999) *Biochem J*; Mora A. et al., (2004) *Semin Cell Dev Biol*; Nilsen T. et al., (2004) *J Biol Chem*; Park J. et al., (1999) *EMBO J*). PIP3 is degraded by the phosphatase and tensin homolog PTEN (Lian Z. et al., (2005) *Oncogene;* Oudit GY. et al., (2004) *J Mol Cell Cardiol*; Sulis ML. et al., (2003) *Trends Cell Biol*), which thus disrupts PI 3-kinasedependent activation of the SGKs. Maximal stimulation of SGK1 activity requires the PDK1-dependent phosphorylation at ^{256}Thr within the activation loop (T-loop) and phosphorylation at ^{422}Ser in the hydrophobic motif at its COOH terminus by PDK2/H-motif kinase (Kobayashi T. et al., (1999) *Biochem J*; Park J. et al., (1999) *EMBO J*). The PDK1-mediated SGK1 phosphorylation is facilitated when ^{422}Ser is already phosphorylated.

Phosphorylation of SGK1 at ^{422}Ser promotes SGK1 binding to the PDK1 interacting fragment (PIF)-binding pocket and phosphorylation at ^{256}Thr by PDK1 (Biondi RM. et al., (2001) *EMBO J*). An alternate mechanism of SGK1 activation by PDK1 involves the scaffold protein Na^+-H^+ exchanger regulating factor 2 (NHERF2). NHERF2 mediates the assembly of SGK1 and PDK1 via its PDZ domains and PIF consensus sequence (Chun J. et al., (2003) *J Biochem Tokyo*). NHERF2 interacts with the PDZ binding motif of SGK1 through its first PDZ domain and with PIF-binding pocket of PDK1 through its PIF tail. The formation of the ternary complex facilitates the phosphorylation of SGK1 on ^{256}Thr in its T-loop by PDK1 (Chun J. et al., (2003) *J Biochem Tokyo*).

Most recent evidence suggests that the activation of SGK1 by PDK1 may indirectly involve the serine/threonine kinase WNK1 (with no lysine kinase 1) (Xu BE. et al., (2005) *Proc Natl Acad Sci Usa*). It is well established that insulin-like growth factor I (IGF-I) enhances SGK1 activity in a PI3-kinase-dependent manner via PDK1. Recent evidence

suggested a role of WNK1 in the activation of SGK1 by IGF-I (Xu BE. et al., (2005) *J Biol Chem*). According to this evidence, IGF-I induces SGK1 activity by stimulating WNK1 phosphorylation at ^{58}Thr, a site that is phosphorylated by protein kinase B (PKB/Akt). The PI3-kinase-dependent step in the activation of SGK1 by IGF-I was thus suggested to be the PDK1-dependent activation of PKB/Akt and the subsequent phosphorylation of WNK1 at ^{58}Thr (Xu BE. et al., (2005) *J Biol Chem*). Neither the catalytic activity nor the kinase domain but the NH2-terminal 220 residues of WNK1 are required for activation of SGK1 (Xu BE. et al., (2005) *J Biol Chem*). WNK1 binds SGK1 directly but does not phosphorylate it, suggesting that WNK1 serves as a scaffold protein to assemble other molecules required for maximal SGK1 activation. Its phosphorylation at ^{58}Thr by PKB-Akt may induce binding of accessory proteins or a conformational change in SGK1 that stimulates the kinase. However, further experimental evidence is needed to elucidate how WNK1 phosphorylation promotes SGK1 activation. SGK2 and SGK3 may similarly be activated by PDK1 and PDK2/H-motif kinase. The equivalent phosphorylation sites for SGK2 and SGK3 are predicted to be at ^{193}Thr-^{356}Ser and ^{253}Thr-^{419}Ser, respectively, but this requires further investigation. The kinases are also regulated by WNK1, although to a lesser extent than SGK1 (Xu BE. et al., (2005) *J Biol Chem*).

Replacement of the serine at position 422 by aspartate, in the human SGK, leads to the constitutively active S^{422}DSGK1 (Kobayashi T. et al., (1999) *Biochem J*), whereas replacement of lysine at position 127, within the ATP-binding region required for enzymatic activity, with asparagine leads to the inactive K^{127}NSGK1 (Kobayashi T. et al., (1999) *Biochem J*). Analogous mutations in the human SGK2 and SGK3 lead to the constitutively active S^{356}DSGK2 and S^{419}DSGK3 and the constitutively inactive K^{64}NSGK2 and K^{191}NSGK3. In part through the PI3-kinase pathway, SGK1 is activated by insulin (Kobayashi T. et al., (1999) *Biochem J*), IGF-I (Hayashi M. et al., (2001) *J Biol Chem*; Kobayashi T. et al., (1999) *Biochem J*), hepatic growth factor (HGF) (Shelly C. et al., (2002) *J Cell Sci*) and follicle stimulating hormone (FSH) (Richards JS. et al., (2002) *Mol Endocrinol*). SGK1 can be activated by bone marrow kinase/extracellular signal-regulated kinase 5 (BK/ERK5) or by p38α. The kinases do not phosphorylate SGK1 at ^{256}Thr but at ^{78}Ser, which is outside the catalytic domain (Hayashi M. et al., (2001) *J Biol Chem*; Meng F. et al., (2005) *Am J Physiol Cell Physiol*). How this phosphorylation activates SGK1 is not known. SGK1 can also be activated by an increase of cytosolic Ca^{2+} activity, an effect presumably mediated by calmodulin - dependent protein kinase kinase (CaMKK) (Imai S. et al., (2003) *Life Sci*). Moreover, the small G protein Rac1 activates SGK1 via a PI3-kinase-independent pathway (Shelly C. et al., (2002) *J Cell Sci*). Additional activators of SGK1 include neuronal depolarization (Kumari S. et al., (2001) *Brain Res*), cAMP (Kumari S. et al., (2001) *Brain Res*; Perrotti N. et al., (2001) *J Biol Chem*; Thomas CP. et al., (2004) *Am J Physiol Lung Cell Mol Physiol*), lithium (Kumari S. et al., (2001) *Brain Res*), oxidation (Kobayashi T and Cohen P., (1999) *Biochem J*; Prasad N. et al., (2000) *Biochemistry*) and adhesion to fibronectin (Shelly C. et al., (2002) *J Cell Sci*). Similar to SGK1, SGK2 and SGK3 are activated by oxidation, insulin, and IGF-I through a signaling cascade.

1.7. Degradation of SGKs

SGK1 is rapidly degraded, with a half-life of 30 min (Brickley DR. et al., (2002) *J Biol Chem*). Ubiquitination of SGK1 labels the kinase for degradation by the proteasome (Brickley DR. et al., (2002) *J Biol Chem*). SGK1 degradation may be mediated by the ubiquitin ligase Nedd4–2 (neuronal precursor cells expressed developmentally downregulated) (Zhou R.et al., (2005) *J Biol Chem*). Nedd4–2 contains a series of tryptophan-rich sequences (WW motifs) that interact with a proline-tyrosine PY motif present in its target proteins. SGK1 bears such a PY motif. Overexpression of Nedd4–2 decreases steady state levels of SGK1 in a dose-dependent manner by increasing SGK1 ubiquitination (presumably within the first 60 NH2-terminal amino acids) and subsequent degradation in the 26S proteasome. Conversely, silencing of Nedd4–2 by RNA interference, or loss of the NH2-terminal amino acids, abrogates the ubiquitination and thus increases the half-life of SGK1. The effect of Nedd4–2 apparently requires phosphorylation of the ubiquitin ligase by SGK1, as SGK1 degradation is reduced by a phosphorylation site-deficient Nedd4–2 mutant (Nedd4–2S/T-A) or by SGK1 inhibition. Accordingly, active SGK1 favors its own degradation, thus contributing to the limitation of its action (Zhou R. et al., (2005) *J Biol Chem*).

1.8. Influence of SGK on renal function

SGKs activate ion channels (e.g. ENaC, TRPV5, ROMK, Kv1.3, KCNE1/KCNQ1, GluR1, GluR6), carriers (e.g. NHE3, GLUT1, SGLT1, EAAT1–5), and the Na^+-K^+-ATPase. They regulate the activity of enzymes (e.g. glycogen synthase kinase-3, ubiquitin ligase Nedd4–2, phosphomannose mutase-2) and transcription factors (e.g. forkhead transcription factor FKHRL1). The functional significance of SGK1, SGK2, and SGK3 is still far from understood. Notably, all three kinases are potent regulators of ion channel activity, transport, and transcription (Bhargava A. and Pearce D., (2004) *Trends Endocrinol Metab*; Fillon S. et al., (2001) *Comp Biochemi Physiol Mol Integr Physiol*). Functional analysis of gene-targeted mice lacking SGK1 (Wulff P. et al., (2002) *J Clin Invest*) and SGK3 (McCormick JA. et al., (2004) *Moll Biol Cell*) provided insight into the functional significance of SGK1- and SGK3-dependent regulation of physiological functions. Interestingly, neither knockout of SGK1 or SGK3, nor knockout of both SGK1 and SGK3 leads to a severe phenotype, suggesting that neither SGK1 nor SGK3 is required for survival. Closer inspection of the renal physiology of those mice discloses, however, multiple physiological deficits pointing to the broad functional role of these kinases.

The role of SGK1 in renal Na^+, K^+ and Ca^{2+} reabsorbtion will be discussed.

a) Role of SGK1 in aldosterone dependent Na^+ reabsorption

Although SGK isoforms are expressed in various tissues and cell types, the role of SGK1 in aldosterone-dependent regulation of Na^+ homeostasis is the best-studied function of these kinases with respect to epithelial ion transport. The kidneys play a pivotal role in the maintenance of Na^+ homeostasis. Urinary Na^+ excretion must be tightly regulated to maintain a constant extracellular volume during varying dietary Na^+ intake and extrarenal Na^+ losses. The final control of renal Na^+ excretion is achieved by the ASDN i.e. the late distal convoluted tubule, the connecting tubule and the cortical as well as the medullary collecting ducts (CCD and MCD respectively) (Loffing J. et al., (2001) *Am J Physiol Renal Physiol*).

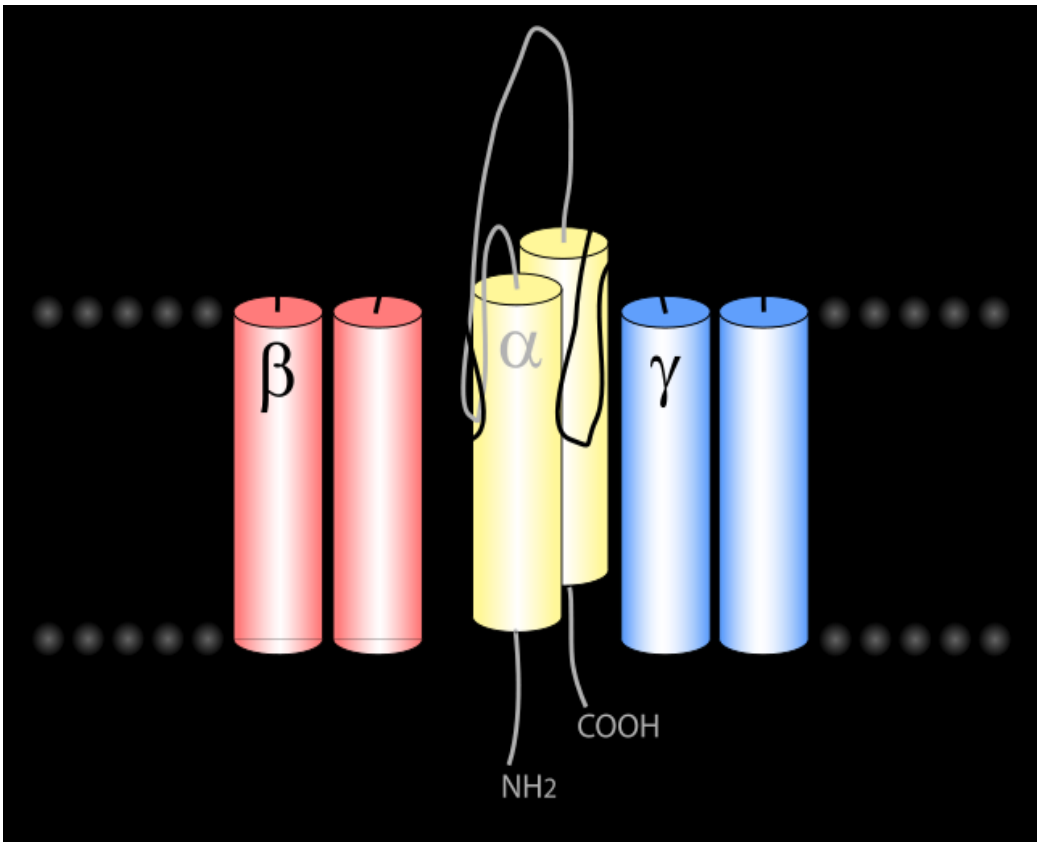

Figure nr. 5 - Schematic picture of an ENaC (the second α-subunit is omitted for clarity)**.**

Transepithelial Na^+ transport in these segments is accomplished by Na^+ entry into the epithelial cells via the epithelial Na+ channel (ENaC) in the luminal membrane and by exit of Na^+ through the Na^+, K^+-ATPase in the basolateral plasma membrane. ENaC represents the rate limiting step in this process and is highly regulated (Kellenberger S. and Schild L., (2002) *J Physiol*). It is composed of three subunits (α, β and γ) (Canessa CM. et al., (1994) *Nature*; Lingueglia et al., (1993) *FEBS Lett*; Lingueglia et al., (1994) *J Biol Chem*) with a stoichiometry of 2α1β1γ (Firsov et al., (1998) *EMBO J*), although other stoichiometries have also been proposed (octa- or nonamers) (Eskandari et al., (1999) *J Biol Chem*; Snyder et al., (1998) *J Biol Chem*). Its subunits have a similar topology, with two transmembrane domains, one extracellular loop, and two cytoplasmic ends (Renard et al., (1994) *J Biol Chem*; Canessa CM. et al., (1994) *Nature*; Snyder et al., (1994) *PMID*). Each subunit also contains, at its C-terminal end, a PY-motif (P-P-X-Y, where P is a proline, Y a tyrosine, and X any amino acid), which is known as protein: protein interaction motifs that can interact with tryptophan (W)-rich WW domains (Chen HI. and Sudol M., (1995) *Proc Natl Acad Sci USA*; Staub O. and Rotin D., (1996) *Am Physiol Soc*).

The importance of these PY-motifs for ENaC regulation has been recognized by the findings that most cases of Liddle's syndrome, K^+-ATPase (Zecevic M. et al., (2004) *Pflügers Arch*; Setiawan I. et al., (2002) *Pflügers Arch*) with SGK1 profoundly increases the activity of both Na^+- transporting proteins. Likewise, SGK2 and SGK3 stimulate ENaC (Friedrich B. et al., (2003) *Pflügers Arch*) and Na^+- K^+-ATPase (Henke G. et al., (2002) *Kidney Blood Press Res*).

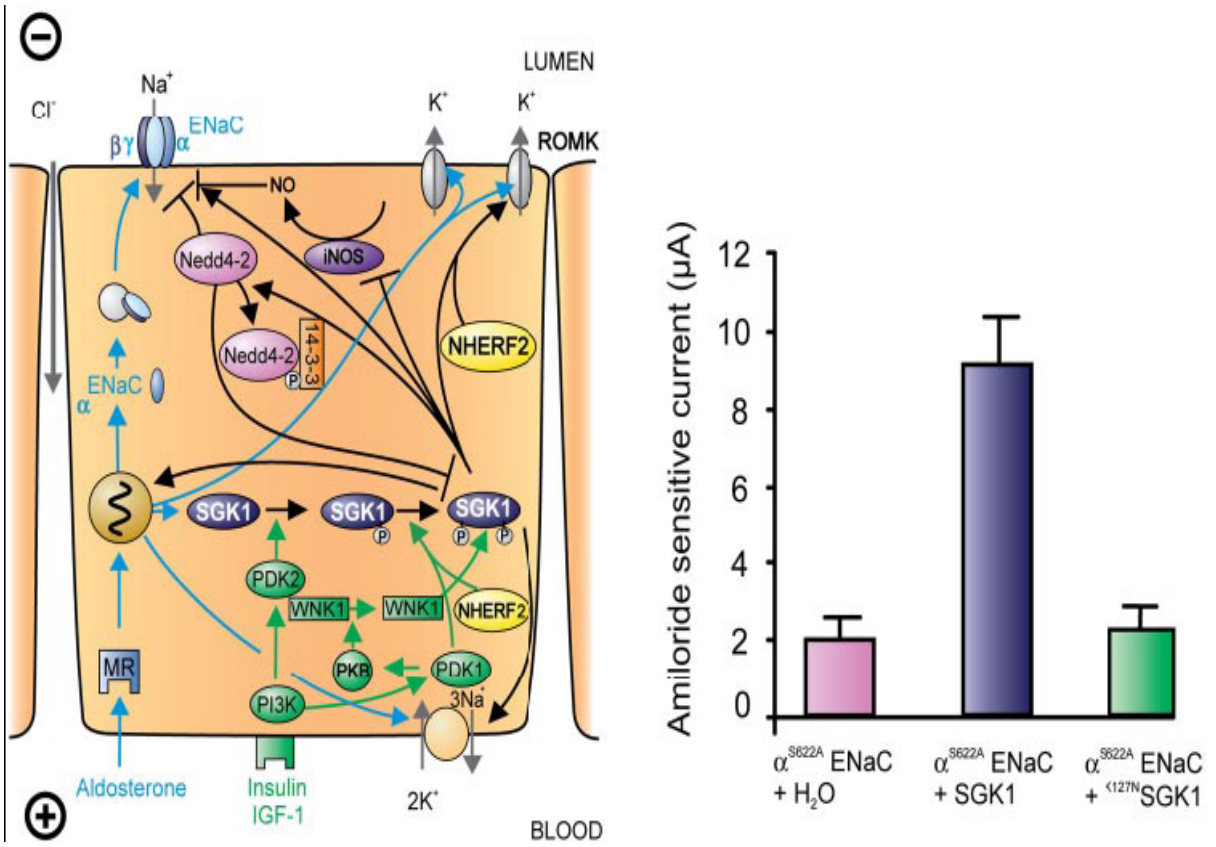

Figure nr. 6 - *Left*: model for the Serum- and Glucocorticoid-inducible Kinase-1 (SGK1)-dependent regulation of Na^+ reabsorption and K^+ secretion in the aldosterone-sensitive distal nephron.

Aldosterone binds to mineralocorticoid receptors (MR) and stimulates the expression of SGK1, α-epithelial Na^+ channel (αENaC), renal outer medullary K^+ channel (ROMK), and the Na^+-K^+-ATPase. αENaC associates with constitutive β- and γ-subunits to form fully active ENaC. SGK1 can be phosphorylated on ^{422}Ser by insulin or insulin-like growth factor I (IGF-I) through a signaling cascade involving phosphatidylinositol 3-kinase (PI3K) and an unknown kinase (PDK2?/hydrophobic motif kinase). Phosphorylated ^{422}Ser allows binding of PDK1 and/or NHERF2 with subsequent phosphorylation of SGK1 at ^{256}Thr. PDK1 might activate SGK1 indirectly through phosphorylation of WNK1 kinase. The mechanism of SGK1 activation by WNK1 is yet unknown but does not require SGK1 phosphorylation. Activated SGK1 increases Na^+ reabsorption in part by phosphorylation of the ubiquitin ligase Nedd4–2, allowing binding of the chaperone 14–3-3 to phosphorylated ^{444}Ser. This interaction prevents Nedd4–2-mediated ubiquitination of the ENaC-PY motif and thus internalization and degradation of ENaC. SGK1 further stimulates ENaC by upregulation of transcription, by direct phosphorylation of the channel protein and by inhibition of the inducible nitric oxide synthase (iNOS). In addition to its effect on ENaC, SGK1 stimulates the Na^+-K^+-ATPase and K^+ channels including ROMK. *Right*: arithmetic means ± SE of ENaC-induced currents in *Xenopus* oocytes coexpression experiments showing that coexpression of wild-type SGK1 but not of the inactive mutant K127NSGK1 leads to stimulation of an ENaC mutant lacking the SGK1 phosphorylation consensus sequence (^{S622A}ENaC).

The stimulatory effect of SGK1 on ENaC is related both to an increased number of channels in the plasma membrane (Lang F. et al., (2000) *Proc Natl Acad Sci USA*; Loffing J. et al., (2001) *Am J Physiol Renal Physiol*; Alvarez de la Rosa D. et al., (1999) *J Biol Chem*) and an activation of channels already present in the membrane (Diakov A. and Korbmacher C., (2004) *J Biol Chem*). The first effect likely involves the action of Nedd4-2, as there are several consensus phosphorylation motifs (2–3 depending on the splice variant) on Nedd4-2 and a PY-motif on SGK1 that may serve as a binding site for Nedd4-2.

In *Xenopus* oocytes, SGK1 induces Nedd4-2 phosphorylation on two of these phosphorylation sites (primarily Ser^{444}, but also Ser^{338}) (Embark HM. et al., (2004) *Cell Physiol Biochem*; Palmada M. et al., (2004) *Am J Physiol Gastrointest Liver Physiol*; Debonneville C. et al., (2001) *EMBO J*), which decreases the interaction of Nedd4-2 with ENaC and finally leads to an enhanced expression and activity of ENaC at the cell surface (Debonneville C. et al., (2001) *EMBO J*).

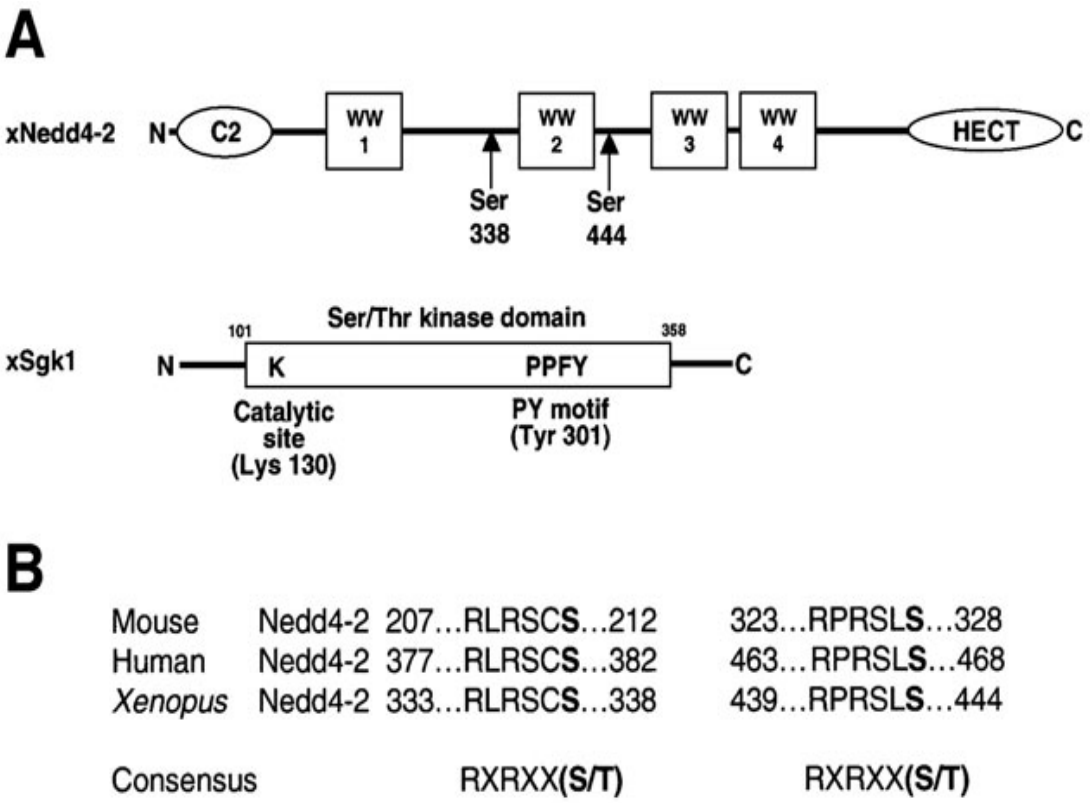

Figure nr. 7 - Schematic view of Nedd4-2 and Sgk1.
(**A**) Scheme of *Xenopus* Nedd4-2 with the consensus phosphorylation sites and *Xenopus* Sgk1 with the indication of the catalytic domain, the catalytically essential Lys130 and the PY motif. (**B**) Conserved consensus phosphorylation sites in mouse, human and *Xenopus* Nedd4-2.

This inhibitory effect of SGK1 on Nedd4-2 likely involves 14-3-3 proteins as phosphorylation of Ser4[44] in Nedd4-2 creates a possible binding site for such proteins, an inherited form of salt-sensitive hypertension are caused by mutations in the genes encoding *β*- and *γ* -ENaC (Hansson JH. et al., (1995) *Proc Natl Acad Sci USA*; Shimkets RA. et al., (1994) *Cell*). These mutations invariably cause either the deletion or the mutation of the PY-motifs on these subunits. When such Liddle channels are expressed in heterologous systems, increases in both the density at the cell surface and the open probability of ENaC are observed (Firsov et al., (1996) *Proc Natl Acad Sci USA*; Snyder PM. et al., (1994) *Cell*; Schild et al., (1995) *Proc Natl Acad Sci USA*; Schild et al., (1996) *EMBO J)*. Loffing and his coworkers, has demonstrated that these PY-motifs are the binding sites for ubiquitin-protein ligases of the Nedd4/Nedd4-like family (Kamynina E. et al., (2001) *EMBO J*) and particularly of Nedd4-2 (Kamynina E. et al., (2001) *EMBO J*; Snyder PM. et al., (2004) *J Biol Chem*).

It is thought that Nedd4-2 binds via its WW domains with the PY-motifs of ENaC and ubiquitylates ENaC on its own *α* and *γ* subunits, consequently leading to the internalization and degradation of ENaC in the endosomal/lysosomal system (Snyder PM. et al., (2004) *J Biol Chem*). In Liddle's syndrome, this mechanism is impaired owing to the inactivation of a PY-motif, causing the accumulation of ENaC at the plasma membrane (Kamynina E. and Staub O., (2002) *Am J Physiol Renal Physiol*). The activity of ENaC and the Na^+, K^+-ATPase is tightly regulated by aldosterone and by SGK1 (Vallon V. et al., (2005) *Am J Physiol Regul Integr Comp Physiol*; Bhargava A. et al., (2004) *Trends Endocrinol Metab*).

Experiments in heterologous expression systems (i.e., *X. laevis* oocytes) revealed that coexpression of either ENaC or Na^+ (consensus: R-S-X-pS-X-P) increases SGK stimulation. Indeed, in *X. laevis* oocytes, SGK1 increases the binding of 14-3-3 to Nedd4-2 in a phosphorylation-dependent manner, a dominant-negative 14-3-3 mutant profoundly attenuates SGK1-dependent stimulation of ENaC, and overexpression of the 14-3-3 protein impairs Nedd4-2-dependent ubiquitylation of ENaC (Ichimura T. et al., (2005) *J Biol Chem*).

In addition to this indirect action of SGK1 on ENaC cell surface abundance, it was proposed that SGK1 can directly interact with ENaC (Wang J. et al., (2001) *Am J Physiol Renal Physiol)* and increase ENaC channel activity by phosphorylating the *α*-ENaC subunits (Diakov A. and Korbmacher C., (2004) *J Biol Chem*). Diakov and Korbmacher used outside-out membrane patches of *X. laevis* oocytes expressing rat ENaC to demonstrate that addition of recombinant, constitutively active SGK1 directly stimulates ENaC currents two- to threefold. An alanine mutation of the serine residue in the SGK1 consensus R-X-R-X-X-S phosphorylation motif abolishes the stimulatory effect on ENaC in this experimental setting.

Experiments in native *Xenopus* A6 cells expressing endogenous SGK1 and ENaC further confirmed that the action of SGK1 on ENaC is complex and likely involves (*a*) increases in the subunit abundance in the plasma membrane and (*b*) activation of channels already in the plasma membrane combined with an increase in ENaC open probability (Alvarez de la Rosa D. et al., (2004) *J Physiol*). However, in this model the stimulatory effect on ENaC channel activity cannot be explained by a direct SGK1-dependent phosphorylation of *α*-ENaC because *Xenopus α*-ENaC does not contain the SGK1 consensus phosphorylation motif. That direct phosphorylation of ENaC at the SGK1 consensus site is not essential for ENaC activation is also supported by data from Lang and coworkers (Lang F. et al., (2000) *Proc Natl Acad Sci USA*; Friedrich B. et al., (2002) *Kidney Blood Press Res*) that showed that channels with a serine-to-alanine mutation within the consensus site of *α*-ENaC are still rigorously upregulated by coexpression of SGK1 in *Xenopus* oocytes.

NDRG-2, which is an aldosterone-induced protein in the ASDN, is another target of SGK1 (Boulkroum S. et al., (2002) *J Biol Chem;* Murray JT. et al., (2004) *Biochem J*). Although the functional role of NDRG-2 in the ASDN is not known, this protein may also have some function in the SGK1-dependent signaling cascade related to Na^+ transport. As an aldosterone-induced protein, SGK1 is thought to mediate at least some of the physiological effects of aldosterone on ENaC and Na^+, K^+-ATPase. The stimulatory effect of aldosterone (or of dexamethasone) on SGK1 expression has now been firmly documented in several studies on various *in vitro* and *in vivo* systems, including *Xenopus* A6 cells (Bhargava A. et al., (2004) *Trends Endocrinol Metab*), primary rabbit CCD cells (Narey-Fejes-Toth et al., (1999) *J BiolChem)*, mouse inner MCD cells (Gumz et al., (2003) *Am J Physiol Renal Physiol*), mouse mpkCCDcl4 (Flores SY. et al., (2005) *J Am Soc Nephrol*), mouse M1 cells (Helms MN. et al., (2003) *Am J Physiol Renal Phyisol*) and mouse and rat kidneys (Chen et al., (1999) *Proc Natl Acad Sci USA*; Loffing et al.,(2001) *Am J Physiol Renal Physiol*; Bhargava A. et al., (2001) *Endocrinology*). Corticosteroids rapidly (within 30 minutes) induce SGK1 at the mRNA and/or protein levels. This induction precedes or at least coincides with enhanced phosphorylation of Nedd4-2 (Flores SY. et al., (2005) *J Am Soc Nephrol*), the activation of transepithelial Na^+ transport in cultured renal epithelia (Naray-Fejes-Toth A. et al., (1999) *J Biol Chem*; Bhargava A. et al., (2004) *Trends Endocrinol Metab*; Flores SY. et al., (2005) *J Am Soc Nephrol*), and reduced renal Na^+ secretion in intact animals (Bhargava A. et al., (2001) *Endocrinology*).

At least part of the stimulatory effect of aldosterone on SGK1 appears to be mediated by activation of the MR, as indicated by findings in primary rabbit collecting duct cells *in vitro* (Narey-Fejes-Toth et al., (1999) *J Biol Chem*) and kidneys *in vivo* (Bhargava A. et al., (2001) *Endocrinology*). Consistently, physiologically relevant concentrations of aldosterone are sufficient to significantly induce SGK1 mRNA in the renal cortex and outer medulla (Muller OG. et al., (2003) *J Am Soc Nephrol*). The physiological importance of aldosterone in SGK induction is also supported by the fact that dietary Na^+ restriction, which physiologically increases plasma aldosterone, induces SGK1 mRNA in the renal cortex (Farjah M. et al., (2003) *Hypertension*). The aldosterone-dependent induction of SGK1 occurs specifically in the ENaC-positive cells of the ASDN, whereas SGK1 expression in other nephron portions such as the thick ascending limb or the proximal tubule is not increased by aldosterone. Likewise, the high level of expression of SGK1 in the renal papilla is not further stimulated by aldosterone, suggesting that SGK1 expression at this site is controlled by factors other than aldosterone. The renal papilla plays an important role for the urinary concentration mechanism, and the cells in the renal papilla can be exposed to a large variation in extracellular osmolarity depending on the requirements for diuresis to antidiuresis. SGK1 expression is strongly modulated by osmotic cell shrinkage and swelling (Waldegger S. et al., (1997) *Proc Natl Acad Sci USA*; Rozansky DJ. et al., (2002) *Am J Renal Physiol*), and it is therefore conceivable that SGK1 participates in the functional adaptation of the renal papilla cells to fluctuation of extracellular osmolarity.

Consistent with this notion, recent data suggest that SGK1 mediates the osmotic induction of the type A natriuretic peptide receptor (NPR-A) in rat inner MCD cells (Chen S. et al., (2004) *Hypertension*). Aldosterone also controls SGK1 expression in the distal colon (Coric CM. et al., (2004) *Am J Physiol Gastrointest Liver Physiol*; Bhargava A. et al., (2001) *Endocrinology*). Aldosterone-dependent Na^+ reabsorption at this site may help to limit extrarenal Na^+ losses during conditions of dietary Na^+ restriction. Transepithelial Na^+ transport is achieved mainly by epithelial cells that are situated at the tips of colonic crypts and that express high levels of ENaC (Coric CM. et al., (2004) *Am J Physiol Gastrointest Liver Physiol*) and SGK1 (Waldegger S. et al., (1999) *Gastroenterology*; Coric CM. et al., (2004) *Am J Physiol Gastrointest Liver Physiol*). In spite of these data pointing to aldosterone-dependent regulation of ENaC via SGK1, recent Western blot and immunohistochemical studies on rat kidney and colon, which reported no or rather modest aldosterone-dependent induction of SGK1 at the protein level, were interpreted to question the significance of aldosterone-dependent induction of SGK1 for ENaC-mediated Na^+ transport regulation (Coric CM. et al., (2004) *Am J Physiol Gastrointest Liver Physiol*). Support for a functional significance of SGK1 in regulation of transepithelial Na^+ transport comes from experiments in *X. laevis* A6 cells and in mouse M1 CCD cells.

Transfection of A6 or M1 cells with SGK1 leads to an increase in transepithelial Na^+ transport, whereas transfection of a dominant-negative "kinase-dead" SGK1 mutant or an antisense SGK1 transcript abolishes dexamethasone- and/or insulin-dependent regulation of transepithelial Na^+ transport (Alvarez de Rosa D. et al., (2003) *J Physiol*; Faletti CJ. et al., (2002) *Am J Physiol Cell Physiol*). Likewise, the use of interfering RNA to knockdown SGK1 expression in A6 cells results in a significant reduction in SGK1 protein levels and a ~50% reduction in dexamethasone-induced short-circuit currents (Bhargava A. et al., (2001) *Endocrinology*). Consistent with these *in vitro* findings, experiments in SGK1 KO ($sgk1^{-/-}$) mice supported the importance of SGK1 for aldosterone-dependent regulation of renal Na^+ transport (Wulff et al., (2002) *J Clin Invest*). Under a standard diet, the KO mice have

unaltered Na^+ excretion as compared to their wildtype littermates. However, plasma aldosterone levels are significantly increased in *sgk1*$^{-/-}$ mice, suggesting extracellular volume contraction.

Under dietary Na^+ restriction, activated compensatory mechanisms are no longer sufficient to keep the mice in Na^+ balance, and mice disclosed significant loss in renal NaCl and in body weight. Experiments on collecting ducts perfused *ex vivo* revealed significantly lower transepithelial amiloride-sensitive potential differences, consistent with a reduced Na^+ transport activity in the CCD. Although apical localization of ENaC was seen in both Na^+-restricted *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice, the apical localization of ENaC is inappropriately low in the *sgk1*$^{-/-}$ mice given the several fold higher plasma aldosterone levels in the KO mice.

Nevertheless, these data, together with the rather mild phenotype of *sgk1*$^{-/-}$ mice, as compared to the much more severe and life-threatening phenotypes of MR or ENaC ko mice, suggest that (*a*) aldosterone-dependent control of ENaC function does not solely rely on the induction and activation of SGK1 and (*b*) some redundancy exists in the signal transduction pathway that controls ENaC activity. Consistent with these ideas, Loffing and his coworkers found significant phosphorylation of the SGK1 target Nedd4-2 in mouse mpkCCDcl4 cells *in vitro* and in rat collecting ducts *in vivo* in the absence of any aldosterone and detectable SGK1 protein expression (Flores SY. et al., (2005) *J Am Soc Nephrol*). In addition to aldosterone-dependent regulation of renal Nareabsorption, SGK1 appears to be involved also in the regulation of aldosterone-induced salt appetite. *Sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice show a similar salt intake under standard conditions. Treatment with the synthetic aldosterone analogue deoxycorticosterone-acetate (DOCA) increases Na^+ intake much more in *sgk1*$^{+/+}$ mice than in *sgk1*$^{-/-}$ mice. The underlying mechanism for the reduced mineralocorticoid-induced salt intake is unclear (Vallon V. et al., (2005) *Am J Physiol Regul Integr Comp Physiol*).

b) Role of SGK1 in renal K^+ secretion

Aside from its stimulatory effect on renal Na^+ reabsorption, aldosterone has strong kaliuretic action. Renal K^+ secretion also takes place in the ASDN and is likely mediated by the renal outer medullary K^+ channel ROMK. ROMK is coexpressed with ENaC in the ASDN cells, and Na^+ reabsorption via ENaC provides the necessary driving force for K^+ secretion. Consistently, pharmacological inhibition (i.e., by amiloride) or genetic loss of function (i.e., pseudohypoaldosteronism (PHA) type 1) of ENaC lower renal K^+ secretion and predispose one to hyperkalemia.

It remains unresolved whether the kaliuretic effect of aldosterone is entirely secondary to the activation of ENaC-mediated Na^+ reabsorption or whether aldosterone directly regulates ROMK function.

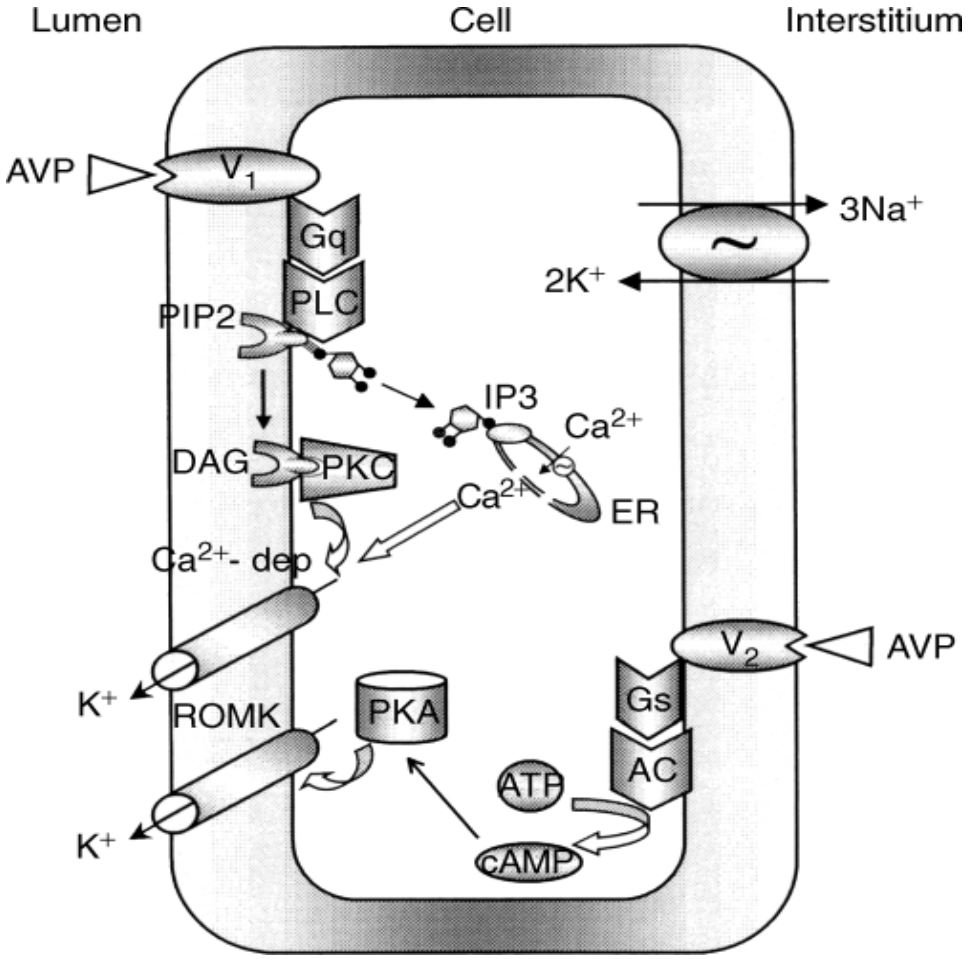

Figure nr. 8 - Schematic drawing of cell signalling mechanisms of luminal (V1 receptors) and basolateral (V2 receptors) action of arginine vasopressin (AVP).
The role of V1 receptors and their signalling are derived from the present studies. The role of V2 receptors in ROMK channel stimulation is based on the studies of Cassola, Giebisch and Wang.

Patch-clamp studies on rat CCDs found no measurable effect of acute aldosterone administration on K^+ channel number, open probability, or conductance (Palmer LG. et al., (1994) *Am J Physiol*). However, some data suggested that aldosterone induces renal K^+ secretion already at aldosterone concentrations that do not exhibit any measurable effect on urinary Na^+ excretion (Bhargava et al., (2001) *Endocrinology)*. Moreover, high K^+ intake increases ROMK activity more efficiently in intact rats than in adrenalectomized animals, suggesting that aldosterone may have at least a permissive effect on ROMK activation (Palmer LG. et al., (1994) *Am J Physiol*).

Consistent with a possible role of aldosterone in ROMK regulation, recent studies in heterologous expression systems advocated a regulatory action of aldosterone-induced SGK1 on ROMK cell surface activity and abundance (Palmada M. et al., (2003) *Biochem Biophys Res Commun*). The regulatory role of SGK1 with regard to ROMK may be indirect via increased interaction with the Na^+, H^+ exchanger–regulating factor 2 (NHERF2) (Palmada M. et al., (2003) *Biochem Biophys Res Commun*) or direct via increased phosphorylation of ROMK at a serine residue within the canonical SGK1 consensus phosphorylation motif (Yoo D. et al., (2003) *J Biol Chem*).

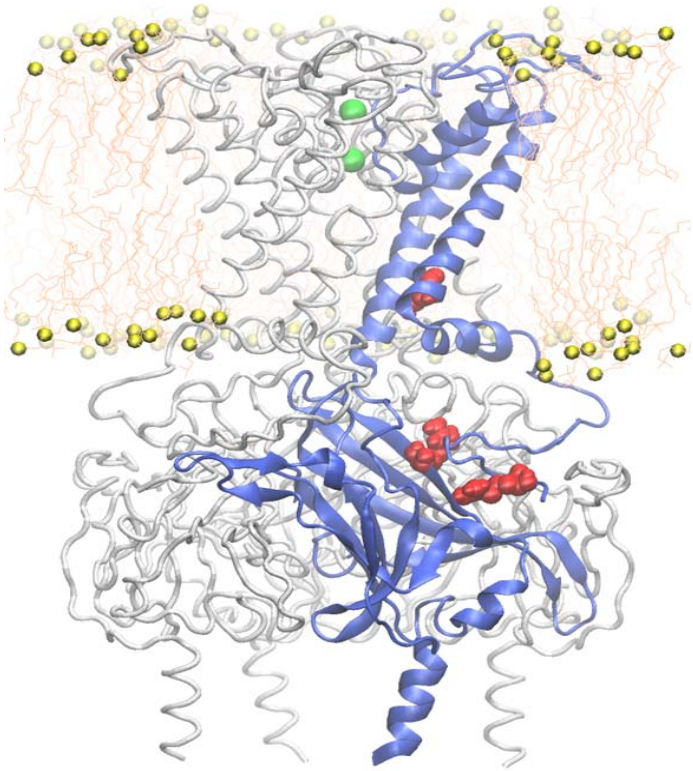

Figure nr. 9 - Homology model of Kir1.1 (ROMK)

The *in vivo* significance of SGK1 in regulation of renal K^+ transport was recently analyzed in SGK1 KO mice. These mice indeed show a disturbed adaptation to an acute and chronic K^+ load, but, as indicated by electrophysiological and immunohistochemical data obtained from these mice after a chronic potassium load, this maladaptation likely is related to altered ENaC (or Na^+, K^+-ATPase) activity in the ASDN cells rather than to inhibition of ROMK cell surface targeting or activity (Huang DY. et al., (2004) *J Am Soc Neprhol*).

c) Role of SGK1 in renal Ca^{2+} secretion

Almost all physiologic processes depend, in one way or another, on calcium. From muscle contraction, nervous system function to intracellular signalling, calcium is essential. It is critical to maintain blood calcium concentrations within a tight normal range. Deviations above or below the normal range frequently lead to serious diseases. There are three major pools of calcium in the body:

- **Intracellular calcium:** a large majority of calcium within cells is sequestered in mitochondria and endoplasmic reticulum. Intracellular free calcium concentrations fluctuate greatly, from roughly 100 nM to greater than 1 µM, due to release from cellular stores or influx from extracellular fluid. These fluctuations are integral to calcium's role in intracellular signaling, enzyme activation and muscle contractions.
- **Calcium in blood and extracellular fluid:** roughly half of the calcium in blood is bound to proteins. The concentration of ionized calcium in this compartment is normally almost invariant at approximately 1 mM or 10000 times the basal concentration of free calcium within cells. Also, the concentration of phosphorus in blood is essentially identical to that of calcium.
- **Bone calcium:** a vast majority of body calcium is in bone. Within bone, 99% of the calcium is tied up in the mineral phase, but the remaining 1% is in a pool that can rapidly exchange with extracellular calcium.

Three organs participate in supplying calcium to blood and removing it from blood when necessary:

- The **small intestine** is the site where dietary calcium is absorbed. Importantly, efficient absorption of calcium in the small intestine is dependent on expression of a calcium-binding protein in epithelial cells.
- **Bone** serves as a vast reservoir of calcium. Stimulating net resorption of bone mineral releases calcium and phosphate into blood, and suppressing this effect allows calcium to be deposited in bone.
- The **kidney** is critically important in calcium homeostasis. Under normal blood calcium concentrations, almost all of the calcium that enters glomerular filtrate is reabsorbed from the tubular system back into blood, which preserves blood calcium levels. If tubular reabsorption of calcium decreases, calcium is lost by excretion into urine.

The following table summarizes body responses to changes in calcium:

	Calcium Deprivation	Calcium Loading
Parathyroid hormone	Secretion stimulated	Secretion inhibited
Vitamin D	Production stimulated by increased parathyroid hormone secretion	Synthesis suppressed due to low parathyroid hormone secretion
Calcitonin	Very low level secretion	Secretion stimulated by high blood calcium
Intestinal absorption of calcium	Enhanced due to activity of vitamin D on intestinal epithelial cells	Low basal uptake
Release of calcium and phosphate from bone	Stimulated by increased parathyroid hormone and vitamin D	Decreased due to low parathyroid hormone and vitamin D
Renal excretion of calcium	Decreased due to enhanced tubular reabsorption stimulated by elevated parathyroid hormone and vitamin D; hypocalcaemia also activates calcium sensors in loop of Henle to directly facilitate calcium reabsorption	Elevated due to decreased parathyroid hormone-stimulated reabsorption.
Renal excretion of phosphate	Strongly stimulated by parathyroid hormone; this phosphaturic activity prevents adverse effects of elevated phosphate from bone resorption	Decreased due to hypoparathyroidism
General Response	Typically see near normal serum concentrations of calcium and phosphate due to compensatory mechanisms. Long term deprivation leads to bone thinning (osteopenia).	Low intestinal absorption and enhanced renal excretion guard against development of hypocalcaemia.

Control of calcium uptake in the kidney:

PTH also enhances Ca^{2+} reabsorption in the kidney at the ascending loop of Henle and the distal convoluted tubule by increasing the active uptake of calcium by Ca^{2+}-ATPase and a Na^{+}-Ca^{2+} antiporter. Calcium excretion rate is reduced. PTH has little effect on modulating calcium fluxes in the proximal tubule where 65% of the filtered calcium is reabsorbed, by being coupled to the transport of solutes such as sodium and water. Active Ca^{2+} reabsorption takes place in the distal convoluted tubule (DCT) and connecting tubule (CNT) of the kidney. PTH receptors have been detected throughout the kidney, as well as in the actively Ca^{2+} transporting tubules DCT and CNT. About 20% of filtered calcium is reabsorbed in the cortical thick ascending limb of the loop of Henle (CTAL) and 15% in the distal convoluted tubule (DCT). Here PTH also binds to the PTHR and again by a cyclic AMP-mediated mechanism, enhances calcium reabsorption. In the CTAL, this appears to occur by increasing the activity of the Na^{+}-K^{+}-$2Cl^{-}$ cotransporter that drives NaCl reabsorption and also stimulates paracellular calcium and magnesium reabsorption.

The extracellular calcium sensor is also resident in the CTAL and can respond to increased ECF calcium by activating phospholipase A2, reducing the activity of the Na^{+}-K^{+}-$2Cl^{-}$ cotransporter and of an apical K^{+} channel, and diminishing paracellular calcium and magnesium reabsorption. Consequently raised ECF calcium antagonizes the effect of PTH in this region of the kidney and ECF calcium can regulate its own homeostasis.

In the DCT, PTH can also influence transcellular calcium transport. This is a multistep process involving transfer of luminal Ca^{2+} into the renal tubule cell via the transient receptor potential channel (TRPV5) or ECaC (epithelial calcium channel), translocation of Ca^{2+} across the cell from apical to basolateral surface a process involving proteins such as calbindin-D28K and finally active extrusion of Ca^{2+} from the cell into the blood via a Na^{+}-Ca^{2+} exchanger, designated NCX1 and the Ca^{2+} -ATPase pump. PTH markedly stimulates Ca^{2+} reabsorption in the DCT primarily by augmenting NCX1 activity via a cyclic AMP-mediated mechanism.

Transient receptor potential channels (TRPs)

The mammalian TRP channels encode a family of about 30 ion channel proteins. This superfamily consists of seven diverse groups structurally similar to the originally found *Drosophila* TRP and they differ in ion selectivities, modes of activation and physiological functions.

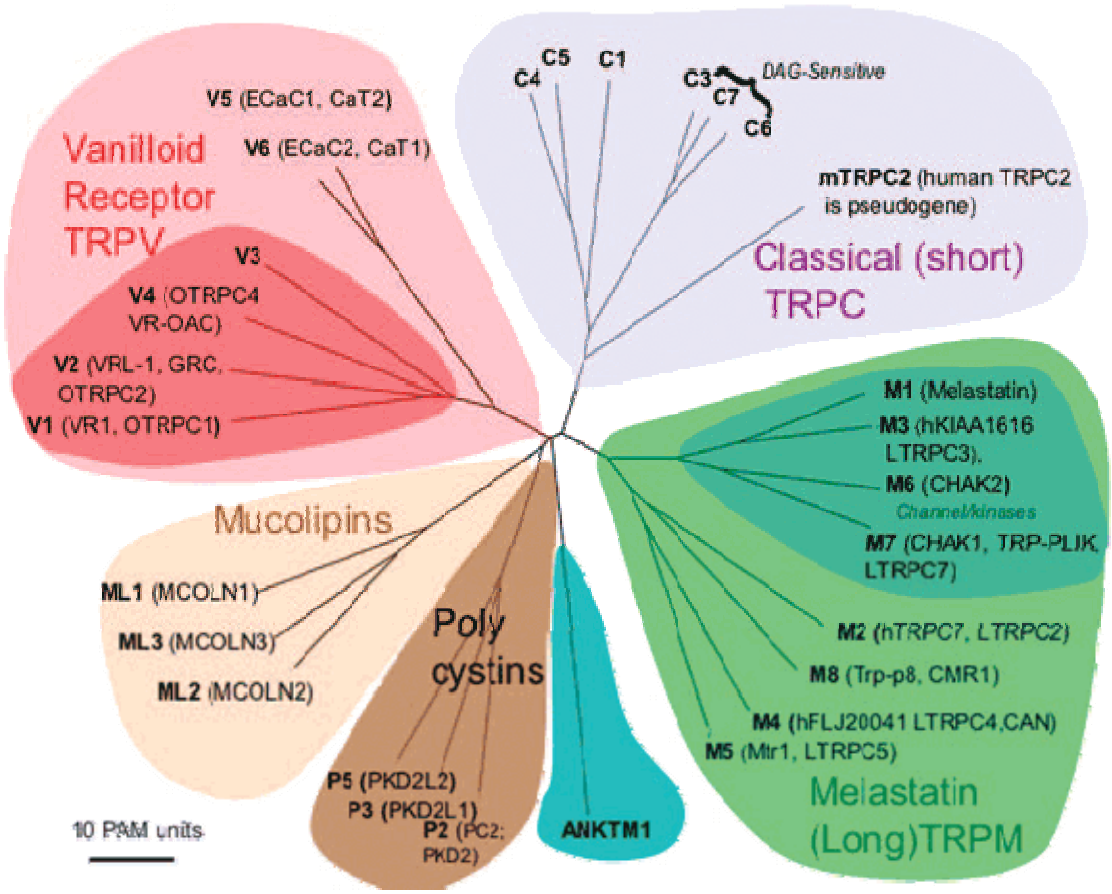

Figure nr. 10 - TRP family with its subgroups.
The vanilloids, the classical, the melastatin, the mucolipins (TRPML) and the polycystins (TRPP) (members of TRPN, TRPA not shown).

TRP proteins are expressed predominantly in the nervous system and are of particular importance in sensory physiology. (Montell C. et al., (2005) *Sci STKE*).

In each subfamily are three to eight members. (Hönderop JG. et al., (2005) *Physiol Rev*). The reason to identify mammalian TRPs is to characterize those channels that might account for highly Ca^{2+} selective Ca^{2+} entry mechanism in nonexcitable cells, referred to as store-operated Ca^{2+} entry (SOCE). SOCE is interesting, due to association of these modes of Ca^{2+} entry with processes ranging from T cell activation to apoptosis, cell proliferation, fluid secretion and cell migration.(Montell C. et al., (2005) *Sci STKE.*). The TRP superfamily can be divided into two structurally different groups (Clapham DE. et al., (2003) *Pharmacol Rev*):

- *Group 1:* TRPC, TRPV, TRPM, TRPN, TRPA. They share substantial sequence identity in the transmembrane domains.
- *Group 2:* TRPP and TRPML. They have low sequence similarity and a large extracellular loop between the first and the second transmembrane domains.

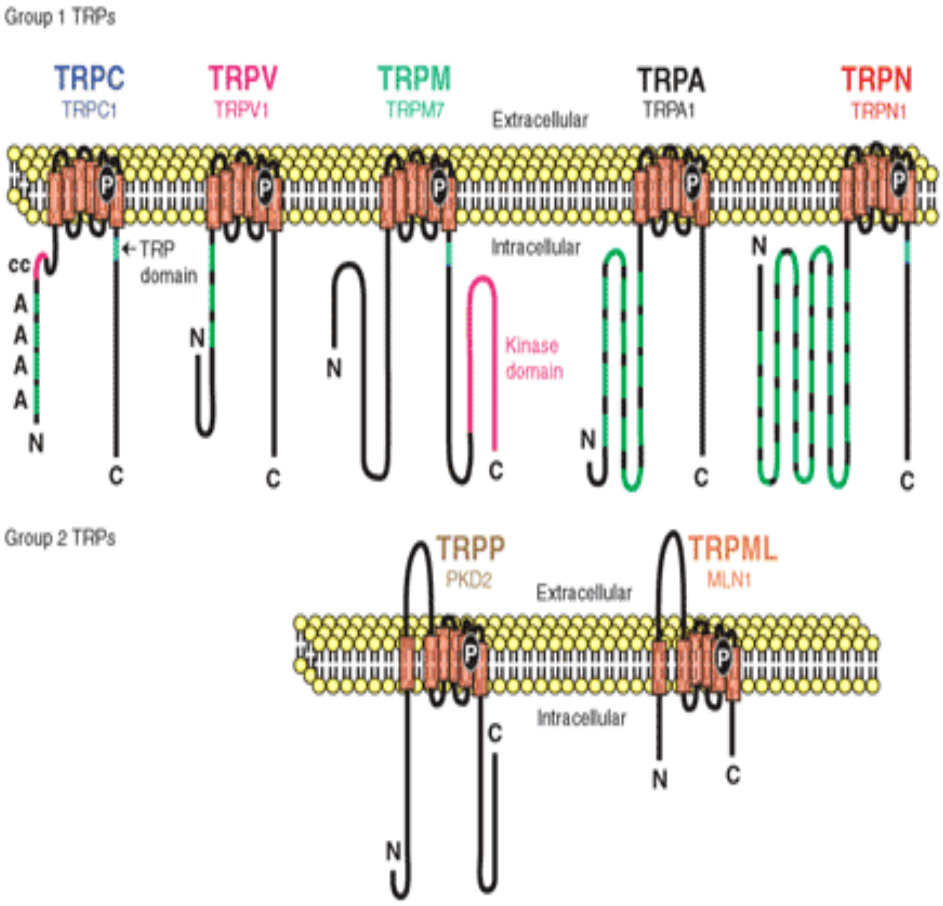

Figure nr. 11 - The seven TRP subfamilies.
Representatives of the different subfamilies are indicated at the top and bottom, respectively. Several domains are indicated: ankyrin repeats (A), coiled coil domain (cc), protein kinase domain (TRPM6/7 only), transmembrane segments, and the TRP domain.

TRP proteins are supposed to form 6 membrane-spanning segments whereas the pore region is formed by a hydrophilic region between S5 and S6 forms. The N- and C-termini are located intracellular. The N-terminus often contains ankyrin repeats as well as a coiled-coil domain, which are suspected to bind with other proteins or the cytoskeleton. Especially they are thought to be needed for TRP protein interaction because a functional channel contains 4 TRP subunits, either similar or different ones, to form homo- or heteromers.

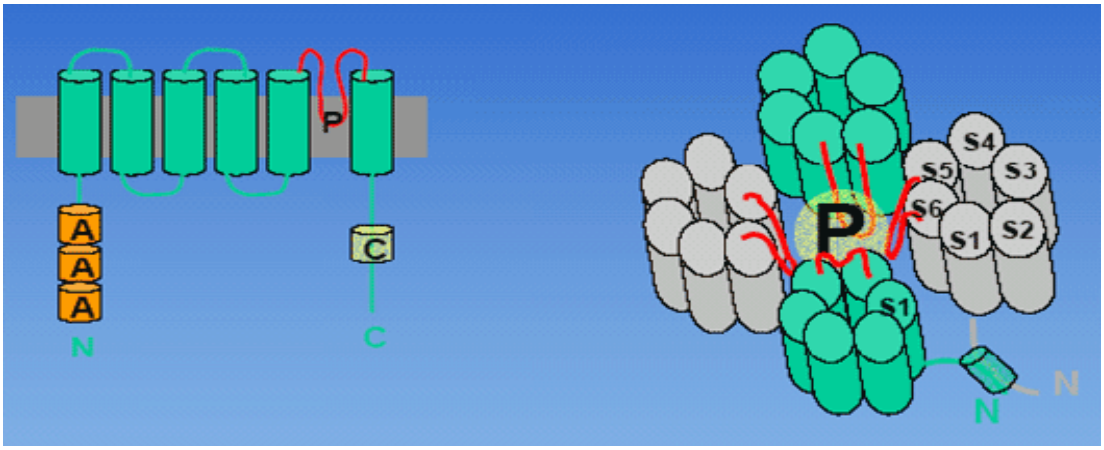

Figure nr. 12 - The quaternary structure of TRP channels allows homo- or heteromeric configurations.
Left: TRP channel subunit, right: structure of functional TRP channel.

TRPV (vanilloid) family has six mammalian members grouped into three subfamilies. These proteins contain three to five ankyrin repeats and share ~25% amino acid identity to TRPC proteins (Montell C. et al, (2005) *Sci STKE*). TRPV1-TRPV4 form poor selective cation channels and are sensitive to heat (Hellwig N. et al., (2005) *J Cell Sci*). TRPV5 and TRPV6 are phylogenetically closely related Ca^{2+} selective channels (PCa: PNa > 100) expressed in epithelia of kidney and intestine and exhibit a constitutive activity (Clapham DE. et al., (2003) *Pharmacol Rev*). Both proteins become permeable to monovalent cations in the absence of divalent cations. It was proposed that TRPV6 may be the highly Ca^{2+}-selective, store-operated channels, referred to CRAC but several biophysical properties of TRPV6 are distinct from those of ICRAC (Kahr H. et al., (2004) *J Physiol*). Nevertheless there remains still the option that TRPV5/ TRPV6 may be subunits of CRAC channels.

TRPM (long TRPC, melastatin) family is composed of eight members. They share ~20% identity, have a TRP domain and contain ankyrin repeats at the N-terminus which is longer than that of TRPCs and TRPVs (Fleig A. et al., (2004) *Novartis Found Symp*).

Transient receptor potential (TRP) cation channel subfamily V, members 5 and 6 (TRPV5 and TRPV6) have recently been postulated to be the molecular gatekeepers facilitating Ca^{2+} influx in these tissues and are members of the TRP family, which mediates diverse biological effects ranging from pain perception to male aggression. Genetic ablation of TRPV5 in the mouse allowed us to investigate the function of this novel Ca^{2+} channel in maintaining the Ca^{2+} balance. Here, we demonstrate that mice lacking TRPV5 display diminished active Ca^{2+} reabsorption despite enhanced vitamin D levels, causing severe hypercalciuria. In vivo micropuncture experiments demonstrated that Ca^{2+} reabsorption was malfunctioning within the early part of the distal convolution, exactly where TRPV5 is localized. In addition, compensatory hyperabsorption of dietary Ca^{2+} was measured in TRPV5 knockout mice. Furthermore, the knockout mice exhibited significant disturbances in bone structure, including reduced trabecular and cortical bone thickness. These data demonstrate the key function of TRPV5 in active Ca^{2+} reabsorption and its essential role in the Ca^{2+} homeostasis.

In humans, the daily dietary Ca^{2+} intake is less than 1000 mg, of which only 30% is absorbed in the intestinal tract. This percentage is significantly enhanced during growth, pregnancy, and lactation by increased levels of circulating 1.25-$(OH)_2D_3$. Although there is continuous turnover of bone mass, there is no net gain or loss of Ca^{2+} from bone in a young and healthy individual. This indicates that healthy adults excrete a maximum of 300 mg Ca^{2+} in the urine to balance the intestinal Ca^{2+} uptake and that the remaining 98% of the Ca^{2+} filtered in the glomeruli is reabsorbed along the nephron. The molecular mechanism responsible for Ca^{2+} absorption in the small intestine and the kidney was elusive for a long time.

The cloning of transient receptor potential (TRP) cation channel subfamily V, member 5 (TRPV5; originally called ECaC1) from vitamin D–responsive rabbit renal epithelial cells and TRP cation channel subfamily V, member 6 (TRPV6; originally called CaT1) from rat duodenum has ignited research into transcellular Ca^{2+} (re)absorption at the molecular level. Mammals harbor at least 21 genes of the so-called TRP channels, whose functions remain mostly unknown. TRPV5 has been implicated as the Ca^{2+} influx channel in the process of vitamin D–responsive active Ca^{2+} reabsorption in the kidney. In comparison, the TRPV5 homolog TRPV6, which displays an amino acid sequence identity of about 75% to TRPV5, has been postulated to be the Ca^{2+} influx channel facilitating Ca^{2+} absorption in enterocytes. TRPV6 is ubiquitously expressed and has been implicated as part of the capacitative Ca^{2+}

entry mechanism and, therefore, intracellular Ca^{2+} signaling. TRPV5 and TRPV6 are Ca^{2+}-selective channels that belong, together with the temperature-activated vanilloid receptors, to the TRPV subfamily. Genomic cloning has demonstrated that TRPV5 and TRPV6 form a unique pair of novel Ca^{2+} channels that are transcribed from distinct genes juxtaposed on human chromosome 7q35.

As shown in *Xenopus* oocytes, SGK1 and SGK3 activate the renal epithelial Ca^{2+} channel TRPV5 by enhancing channel abundance in the plasma membrane, an effect again requiring cooperation with NHERF2 (Embark HM. et al., (2004) *Cell Physiol Biochem*; Palmada M. et al., (2005) *Cell Physiol Biochem*). The TRPV5 C-tail interacts in a Ca^{2+}-independent manner with NHERF2. Deletion of the second, but not the first, PDZ domain in NHERF2 abrogates the stimulating effect of SGK1 on TRPV5 protein abundance (Palmada M. et al., (2005) *Cell Physiol Biochem*).

2. The aim of these studies

The aim of these studies was to establish the influence of SGK kinases on renal function of Na^+, K^+ and Ca^{2+} channels at mice, a comparison between the normal mice wildtype and genetically modified mice knock-outs.

Ca^{2+} homeostasis is a function of intestinal Ca^{2+} absorption and renal Ca^{2+} excretion (Hönderop et al., (2000) *Curr Opin Nephrol Hypertens*; Hönderop JG. et al., (2002) *Annu Rev Physiol*). Filtered Ca^{2+} is reabsorbed in the proximal tubule, loop of Henle, distal convoluted tubule, and connecting tubule. In the proximal tubule and loop of Henle, Ca^{2+} transport is accomplished by both transcellular and paracellular transport (Friedman PA. et al., (1995) *Physiol Rev*). In both segments, Ca^{2+} reabsorption is largely a function of Na^+ reabsorption. Fine-tuning of renal Ca^{2+} excretion is achieved in the distal convoluted and connecting tubule, which reabsorbs Ca^{2+} exclusively through the transcellular pathway (Hönderop JG. et al., (2000) *Curr Opin Nephrol Hypertens*; Hönderop JG. et al., (2002) *Annu Rev Physiol*). The rate-limiting step of Ca^{2+} transport in those nephron segments is the Ca^{2+} entry across the apical cell membrane via the epithelial Ca^{2+} channel TRPV5/ECaC1 (Hönderop JG et al., (2000) *J Biol Chem*; Hönderop JG. et al., (2002) *Biochem Biophys Res Commun*).

Ca^{2+} is extruded across the basolateral plasma membrane by a Ca^{2+}ATPase and a Na^+/Ca^{2+} exchanger (Hönderop JG. et al., (2000) *Am J Physiol Renal Physiol*). *In vitro* experiments disclosed the ability of the serum and glucocorticoid-inducible kinase 1 (SGK1) to regulate TRPV5 (Embark HM. et al., (2004) *Cell Physiol Biochem*; Palmada M. et al., (2005*) Cell Physiol Biochem*). It does so, at least partially, by enhancing the channel protein abundance within the cell membrane. For this effect, SGK1 requires the cooperation of the Na^+/H^+ exchanger regulating factor 2 (NHERF2) (Embark HM. et al., (2004) *Cell Physiol Biochem*; Palmada M. et al., (2005) *Cell Physiol Biochem*). NHERF1 and NHERF2 are known to modulate the targeting and trafficking of several proteins including TRPV5 into the plasma membrane (Baccarini M. et al., (2005), *FEBS Lett*; Bell LM. et al., (2000), *J Biol Chem*; Bhargava A and Pearce D., (2004) *Trends Endocrinol Metab*). Transcription of SGK1 is upregulated by several hormones and mediators including glucocorticoids (Alliston TN. et al., (2000), *Endocrinology*; Bhalla V. et al., (2005) *Mol Endocrinol*; Bhargava A. et al. (2001) *Endocrinology*), mineralocorticoids (Akutsu N. et al. (2001) *MolEndocrinol*; Bahr V. et al. (2002) *Exp Clin Endocrinol Diabetes;* Berger S. et al., (1999) *Proc Natl Acad Sci USA*) and 1.25-dihydroxycholecalciferol ($1.25(OH)_2D_3$) (Abbasi S. et al., (2005) *J Biol Chem*).

The kinase is activated by insulin-like growth factor 1 (IGF1) and insulin through the phosphatidylinositol - 3(PI3) kinase and phosphoinositide-dependent kinase 1 (PDK1) (Abriel H. et al., (2000) *FEBS Lett*; Abriel H. et al., (1999) *J Clin Invest*; Alessi DR. et al., (1997) *Curr Biol*; Alonso L. et al., (2005) *J Cell Biol*; Asher C. et al., (2003) *Biochem Biophys Acta*; Auld GC. et al., (2005) *Biochem J*; Bednarski E. et al., (1998) *Exp Neurol*).

To explore the functional role of SGK1 in the regulation of renal Ca^{2+} transport, clearance experiments were performed in SGK1 knockout mice ($sgk1^{-/-}$) and their wild-type littermates ($sgk1^{+/+}$).

To explore the role of SGK1 in DOCA-induced renal fibrosis, we implanted continuous-release DOCA pellets (2.4 mg/day) into gene-targeted mice lacking SGK1 (*sgk1*$^{-/-}$) and their wild-type littermates (*sgk1*$^{+/+}$). The mice were exposed to 1% saline in drinking water and analyzed by blood pressure measurements, metabolic cages, and renal histology.

SGK1 was originally discovered as a gene genomically upregulated by glucocorticoids (Firestone GL et al., (2003) *Cell Physiol Biochem*) and was later found to be a mineralocorticoid-inducible gene (Ali S. et al., (2003) *Cell Physiol Biochem;* Alvarez de la Rosa D. et al., (2005) *Am J Physiol Cell Physiol*; Aronzon A. et al., (2005) *Otol Neurotol*; Baltaev R. et al., (2005) *Pflügers Arch*; Böhmer C. et al., (2003) *J Neurochem*; Böhmer C. et al., (2003) *Cardiovasc Res*; Boulkroun S. et al., (2002). *J Biol Chem*; Brennan FE and Fuller PJ., (2000) *Mol Cell Endocrinol*; Brooks DE. et al., (1997) *Am J Vet Res*). SGK1 is expressed in the aldosterone-sensitive distal nephron (Akutsu N. et al., (2002) *MolEndocrinol;L* Böhmer C. et al., (2003) *J Neurochem*) and coexpression of SGK1 markedly enhances activity of the epithelial Na^+ channel (ENaC) heterologously expressed in *Xenopus* oocytes (Abriel H. et al., (1999) *J Clin Invest*, Alvarez de la Rosa D. et al., (1999) *J Biol Chem*; Aronzon A. et al., (2003) *Otol Neurotol*; Böhmer C. et al., (2006) *J Neurochem*; Böhmer C. et al., (2003) *Cardiovasc Res*; Brunet A. et al., (2001) *Mol Cell Biol*) and A6 cells (Faletti CJ et al., (2002) *Am J Physiol Cell Physiol*), an effect at least partially due to phosphorylation of the ubiquitin ligase Nedd4-2 (Asher C. et al., (2003) *Biochem Biophys Acta*; Brickley DR. et al., (2002) *J Biol Chem*), which reduces the affinity of the enzyme to the target protein (Abriel H. et al., (1999) *J Clin Invest*; Böhmer C. et al., (2006) *J Neurochem*; Brunet A. et al., (2001) *Mol Cell Biol*).

As a result, SGK1 inhibits ubiquitination of the ENaC and, thus, leads to enhanced ENaC protein abundance in the cell membrane. Also, SGK1 has been suggested to stimulate ENaC activity by direct phosphorylation of the carrier protein (Auld GC. et al., (2005) *Biochem J*). SGK1 also enhances the activity of other renal transport systems, including the apical renal outer medullary K^+ (ROMK) channel (Böhmer C. et al., (2004) *Biochem Biophys Res Commun*; Busjahn A. and Luft FC., (2003) *Cell Physiol Biochem*), the Na^+-K^+-ATPase (Bhargava A. et al., (2001) *Endocrinology;* Bramham CR. and Messaoudi E., (2005) *Prog Neurobiol*; Busjahn A. et al., (2004) *Cell Physiol Biochem*) , the Na^+-K^+-$2Cl^-$ cotransporter NKCC2 (Böhmer C. et al., (2006) *J Neurochem*), the epithelial Ca^{2+} channel TRPV5, the Na^+-H^+ exchanger NHE3 and the K^+ channel KCNE1 (Bednarski E. et al., (1998) *Exp Neurol*).

The stimulatory effect of SGK1 on the ENaC (Berridge MJ. et al., (2000) *Nat Rev Mol Cell Biol*), the Na^+-K^+-ATPase (Bhargava A. et al., (2001) *Endocrinology*; Bramham CR. et al., (2005) *Prog Neurobiol*; Busjahn A. et al., (2004) *Cell Physiol Biochem*) and the epithelial Ca^{2+}channel TRPV5 (Böhmer C. et al., (2000) *Cell Physiol Biochem*) in *Xenopus* oocytes is shared by its isoforms SGK3, which has been discovered by homology screening (Blazer-Yost BL. et al., (1998) *Am J Physiol Cell Physiol*) and as "cytokine-independent survival kinase" (Böhmer C. et al., (2004) *Biochem Biophys Res Commun*). In contrast to SGK1, SGK3 appears not to be a transcriptional target of glucocorticoids or serum (Blom IE et al., (2002) *Matrix Biol*).

All three kinases are, however, activated by insulin-like growth factor I and insulin through phosphatidylinositol 3-kinase and phosphoinositide dependent kinase 1 (Abbasi S. et al., (2005) *J Biol Chem*; Abriel H. et al., (2000)b *FEBS Lett*; Bahr V. et al., (2005) *Exp Clin Endocrinol Diabetes*; Bhalla V. et al., (2005) *Mol Endocrinol*; Bistrup C. et al., (2005) *Acta Physiol Scand*; Blazer-Yost BL. et al., (1999) *Am J Physiol Cell Physiol;* Boini KM. et al., (2006) *Diabetes*). Accordingly, SGK1 is considered to participate in the regulation of renal Na^+ excretion by aldosterone, insulin, and insulin-like growth factor I (Alliston TN. et al., (2000) *Endocrinology*; Almeida RD. et al., (2005) *Cell Death Differ*; Alonso L. et al., (2005) *J Cell Biol*; Bureau I. et al., (1999) *J Neurosci*).

The role of SGK1 in renal Na^+ reabsorption is illustrated by the phenotype of the SGK1-knockout (*sgk1*$^{-/-}$) mouse, which shows normal Na^+ excretion and blood pressure when fed a normal-salt diet but does not show a sufficient decrease in renal Na^+ excretion when fed a salt-deficient diet (Wulff P. et al., (2002) *J Clin Invest*). However, the mild impairment of renal Na^+ retention in the *sgk1*$^{-/-}$ mouse differs from the severe salt wasting of mineralocorticoid receptor- knockout mice (Berger S. et al., (1998) *Proc Natl Acad Sci USA*) and the lethality of ENaC-knockout mice (Hummler E. et al., (1996) *Nat Genet*). Moreover, there was no evidence for renal salt wasting in the SGK3-knockout (*sgk3*$^{-/-}$) mouse (McCormick JA. et al., (2004) *Mol Biol Cell*). Thus the following question arises: Does SGK1 could fully replace SGK3 in *sgk3*$^{-/-}$ mice, and does SGK3 partially replace SGK1 in *sgk1*$^{-/-}$ mice? The present study was performed to explore whether animals lacking both SGK1 and SGK3 would be viable or would suffer from severe salt wasting.

3. Material

3.1. Equipment

Accucheck Sensor Comfort (Roche Diagnostics, Manheim, Germany)
Balance (Sartorius, Göttingen, Germany)
BioPhotometer Eppendorf (Eppendorf, Wesseling-Berzdorf, Germany)
Blood pressure computerized data acquisition system (PowerLab 400 and Chart 4, AdInstruments, Colorado Springs, USA)
Blood gas analyzer (Eschweiler System 2000, Kiel, Germany)
Centrifuge 5417 R (Eppendorf, Hamburg, Germany)
Electrometric titration (Chloridometer 6610, Eppendorf, Germany)
Electrophoresis chamber (BioRad, München, Germany)
Flame photometry (AFM 5051, Eppendorf, Hamburg, Germany)
Gamma Counter (Perkin Elmer, Massachusetts, USA)
Kodak film (Sigma-Aldrich, Hannover, Germany)
LightCycler System (Roche Diagnostics, Manheim, Germany)
MagNa Lyser (Roche Diagnostics, Mannheim, Germany)
Magnetstirrer KMO2 basic IKAMAG (IKA, Staufen, Germany)
Metabolic cages (Techniplast, Hohenpeissenberg, Germany)
Multireactiontubes (Eppendorf, Hamburg, Germany)
MultiChanelPipet (Eppendorf, Hamburg, Germany)
Multilevel counter (Victor 1420, PerkinElmer, Boston, USA)
Petri dishes (Greiner Bio-one, Frickenhausen, Germany)
Pipettes (Eppendorf, Hamburg, Germany)
Pipette tips (Carl Roth, Karlsruhe, Germany)
Spectronic GENESYS 6 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc. Massachusets, USA)
SpeedVac SVC 100 (Savant Life Sciences, Bath, UK)
Sterile PS-tube 4.5 ml 12.4/75 MM (Greiner bio-one, Frickenhausen, Germany)
Sterile filters (Millipore, Cork, Ireland)
Syringes, Omnifix-H, 1ml (Braun, Melsungen, Germany)
Ultracentrifuge (Beckman Coulter, Krefeld, Germany)
UV-cuvettes 8.5mm (Plastiband, Antwerp, Belgium)
Vortex (Labnet Abimed, Langenfeld, Germany)
Waterbath (Labortechnik, Seelbach, Germany)

3.2. Material

Sgk1$^{+/+}$ and *sgk1*$^{-/-}$ mice (n=30 each, 5-7 moths old)

Sgk1$^{+/+}$ and *sgk1*$^{-/-}$ mice (n=12 each, 6-9 moths old)

Sgk1 $^{+/+}$/sgk3$^{+/+}$ and *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice (n=9 each, 12-16 months old)

3.3. Kits

Albumin determination kit (microfluoral, Progen, Heidelberg, Germany)

Aldosterone kit-IMMUNOTECH, Abeckman Coulter Company (Demeditec, Kiel, Germany)

cDNA kit (Promega, Mannheim, Germany)

Creatinine determination kit - creatinine PAP (Lehmann, Berlin, Germany)

Gamma-B 1.25-Dihydroxy vitamin D (IDS, Boldon, UK)

Glucose kit: gluco-quant® (Roche Diagnostics, Mannheim, Germany)

Inorganic Phosphate (Roche Diagnostics, Mannheim, Germany)

Intact parathormone ELISA kit (Immunotopics, San Clemente, CA, USA)

Magnesium determination kit (Roche Diagnostics, Mannheim, Germany)

Master SybrGreen I mix (Roche Molecular Biochemicals, Mannheim, Germany)

Mouse intact PTH Elisa kit (Immunotopics, California, USA)

Plasma leptin ELISA kit (Linco, St. Charles, USA)

RNA kit (RNeasy Mini Kit, Qiagen, Hilden, Germany)

RNA kit PAXgene System (Qiagen, Hilden, Germany)

Vasopressin kit ADH (IBL, Hamburg, Germany).

3.4. Chemicals

Acetic acid 4% (Sigma-Aldrich, Hannover, Germany)

Acrylamide/Bysacrylamide (Carl Roth, Karlsruhe, Germany)

Agarose-Gel-Elektrophorese-Chamber (Biorad, München, Germany)

Aqua ad injectabili (Ampuwa, Niefern, Germany)

Ammoniumpersulphate (Carl Roth, Karlsruhe, Germany)

Azotic acid (Sigma-Aldrich; Hannover, Germany)

Beta-Mercaptoethanol (Sigma-Aldrich; Hannover, Germany)

BenchMark prestained protein ladder (Invitrogen, California, USA)

Biorad protein assay solution (BioRad, München, Germany)

Chlorhidric acid (Carl Roth, Karlsruhe, Germany)

Complete Protease Inhibitor (Roche Diagnostics, Mannheim, Germany)

Diethylether (Carl Roth, Karlsruhe, Germany)

DOCA pellets (50 mg, Innovative Research of America, Sarasota, Florida, USA)

EDTA (Sigma-Aldrich, Hannover, Germany)

EGTA (Sigma-Aldrich, Hannover, Germany)

Ethanol absolute (99%) (Carl Roth, Karlsruhe, Germany)

Ethidiumbromid (Sigma-Aldrich, Hannover, Germany)

Glycin (Merck, Darmstadt, Germany)

Glycerol (Sigma-Aldrich, Hannover, Germany)

Glucose (Merck, Darmstadt, Germany)

Inactin (Sigma-Aldrich, Hannover, Germany)

Ketamine (Sigma-Aldrich, Hannover, Germany)

Magnesiumchloride (Sigma-Aldrich; Hannover, Germany)

Methanol absolute (99%) (Carl Roth, Karlsruhe, Germany)

Milk powder (Carl Roth, Karlsruhe, Germany)

Mouse albumin standard (Sigma, Taufkirchen, Germany)

Natriumacetat (Sigma-Aldrich, Hannover, Germany)

Natriumchloride (Sigma-Aldrich, Hannover, Germany)

Nitrocellulose membrane (Schnell&Schlechker, Wiesbaden, Germany)

Nitrogen liquide (Linde, Wiesbaden, Germany)

Proteinase K (Boehringer Ingelheim, Manheim, Germany)

Sterilium (Carl Roth, Karlsruhe, Germany)

SDS (Sigma-Aldrich, Hannover, Germany)

TEMED (Carl Roth, Karlsruhe, Germany)

Trisbuffer (pH 8.5) (Sigma-Aldrich, Hannover, Germany)

Triton X-100 (Roche Diagnostics, Mannheim, Germany)

Tris-base (Sigma-Aldrich, Hannover, Germany)

Tween 20 (Böhringer Ingelheim, Manheim, Germany)

3.5. Diets (Altromin, Heidenau, Germany)

Standard diet C1310/1314 [0.24% Na+, 0.71% K+, 0.95% Ca2+ (wt/wt)]

Control diet C1000 [0.24% Na+, 0.71% K+, 0.95% Ca2+ (wt/wt)]

Low-salt diet C1036 [0.015% Na+, 0.71% K+, 0.95% Ca2+ (wt/wt)]

Low-Ca2+ diet C1031 [0.24% Na+, 0.71% K+, 0.08% Ca2+ (wt/wt)]

3.6. Anti mouse monoclonal antibodies

Alexa Fluor 488 (Invitrogen, Karlsruhe, Germany)

Antirabbit Alexa 546 antibodies (Invitrogen, Karlsruhe, Germany)

Anti - TRPV5 (Alpha Diagnostics, San Antonio, Texas, USA)

Blocking peptide for TRPV5 (Alpha Diagnostics, San Antonio, Texas, USA).

Calbindin D-28K (Swant, Bellizona, Switerzland)

HRP-conjugated antimouse secondary antibody (Santa Cruz Biotechnology, Heidelberg, Germany)

GAPDH antibody (Santa Cruz Biotechnology, Heidelberg, Germany)

GAPDH antibody (Search-LC, Heidelberg, Germany)

3.7. Software

Blood pressure computerized data acquisition software (PowerLab 400 and Chart 4, Colorado Springs, Colorado Springs, USA)

Data link version 1.0.0 (Herbert &Scheneider Software &CAM, Siglingen, Germany)

GraphPad Instat version 3.05 (GraphPad Software Inc., San Diego, USA)

Magellan version 3.11 (Tecan GmbH, Crailsheim, Germany)

Microsoft Windows 95 (Microsoft GmbH., Remscheid, Germany)

Office (Excel, Word, PowerPoint) (Germany)

Quantity One version 4.3.0 (BioRad, München, Germany)

Sigma plot version 7.0 (Systat Software Inc., Erkrath, Germany)

4. Methods

4.1. Generation of sgk1$^{-/-}$ mice

Mice deficient in SGK1 (*sgk1*$^{-/-}$) were generated and bred as previously described (Huang et al., (2004) *J Am Soc Nephol*; Wulff et al., (2002) *J Clin Invest*; Huang et al., (2004) *J Am Soc Nephol*). In brief, a conditional targeting vector was generated from a 7-kb fragment encompassing the entire transcribed region on 12 exons. The neomycin resistance cassette was flanked by two loxP sites and inserted into intron 11. Exons 4–11, which code for the sgk1 kinase domain, were "floxed" by inserting a third loxP site into intron 3. A clone with a recombination between the first and third loxP site (type I recombination) was injected into C57BL/6 blastocytes. Male chimeras were bred to C57BL/6 and 129/SvJ females. Heterozygous SGK1-deficient mice were backcrossed to 129/SvJ wild-type mice (Charles River, Sulzfeld, Germany) for ten generations and then intercrossed to generate homozygous SGK1 knockout mice (*sgk1*$^{-/-}$) and their wild type littermates (*sgk1*$^{+/+}$). Male and female *sgk1*$^{-/-}$ mice were used in the studies and compared with littermate *sgk1*$^{+/+}$ mice of the respective gender. Mice were genotyped by PCR (Huang Y. et al., (2004) *J Am Soc Nephol*; Wulff P. et al., (2002) *J Clin Invest*).

All animal experiments were conducted according to the guidelines of the American Physiological Society as well as the German law for the welfare of animals and were approved by local authorities.

For evaluation of renal Ca^{2+} handling experiments were made on 5- to 7-month-old homozygous *sgk1* knockout mice and their age- and sex-matched wild-type littermates. Mice were maintained on control diet (C1314, Altromin, Heidenau, Germany) prior to the experiment. To induce mineralocorticoid excess, wild-type (*sgk1*$^{+/+}$) and *sgk1* knockout (*sgk1*$^{-/-}$) mice (6–9 months old, n=12 each) were treated with DOCA (2.4 mg/day) + 1% NaCl in drinking water over 12 weeks. Throughout the entire study, mice had free access to a standard mouse diet (C1310 - Altromin, Heidenau, Germany). The study was completed in 9 animals from each group.

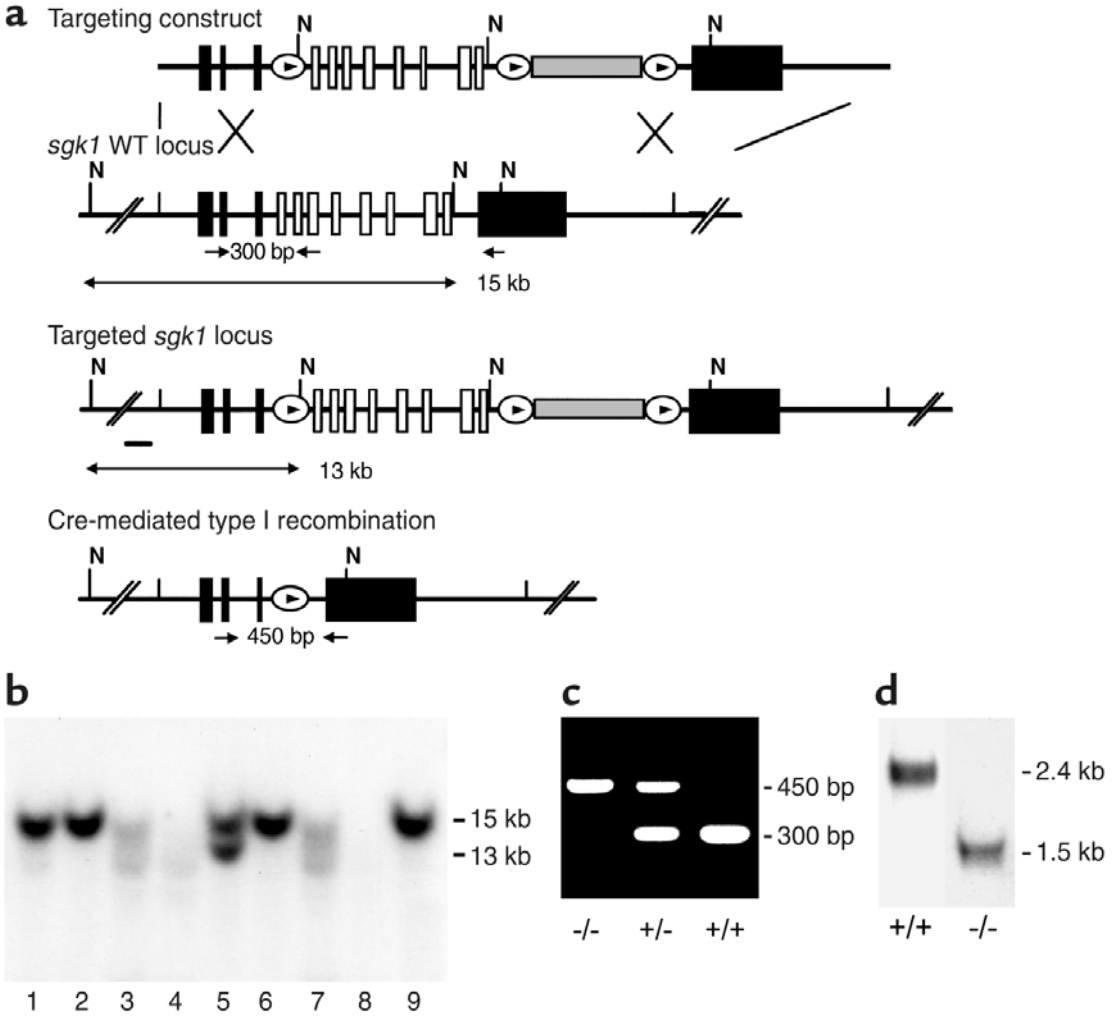

Figure nr. 13 - Generation of *sgk1*$^{-/-}$ mice.

(**a**) Targeting strategy. The neomycin resistance cassette (gray box) was flanked by two loxP sites (ovals) and inserted into intron 11. Exons 4–11, which code for the Sgk1 kinase domain (open boxes), were "floxed" by inserting a third loxP site into intron 3. N indicates NheI restriction sites, and the small black bar indicates the external 5′ probe used for Southern blot analysis. Expected fragment sizes of the wild-type and targeted *sgk1* locus are also indicated. One homologously recombined ES cell clone was transiently transfected with Cre recombinase, and a clone that had undergone recombination between the first and the third loxP site (type I recombination) was chosen for injection. Arrows below the gene indicate PCR primers used for genotyping. Numbers between the arrows indicate the size of the amplified fragments. Crossed bars below **(a)** indicate homologous recombination. (**b**) Southern blot of NheI-digested genomic DNA from ES cell clones after gene targeting hybridized with a 5′ external probe (black bar in **a**). Lane 5 shows a targeted ES cell line. (**c**) Genotyping by PCR of genomic tail DNA of homozygous ($^{-/-}$) and heterozygous ($^{-/+}$) *sgk1*-deficient mice and wild-type mice ($^{+/+}$) using a mix of three specific primers (arrows in **a**). (**d**) Autoradiograph of Northern blot analysis of Sgk1-specific transcripts in $^{+/+}$ and $^{-/-}$ mice. The deletion of the kinase domain from the genome results in a size reduction of 0.9 kb at the mRNA level in *sgk1*$^{-/-}$ mice.

Gene-targeted mice deficient in SGK1 (*sgk1*$^{-/-}$) and SGK3 (*sgk3*$^{-/-}$) (McCormick A. et al., (2004) *Mol Biol Cell*) were crossed, and the offspring were genotyped by PCR on tail DNA using neomycin resistance specific primers as previously described. Mice were reproduced by heterozygous crossing. The genetic background of the animals was a mix of Sv/J129 and C57BL/6.

4.2. Metabolic cages

For evaluation of renal excretion, both *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice were placed individually in metabolic cages (Techniplast, Hohenpeissenberg, Germany) for 24-h urine collection (Vallon V. et al., (2003) *Neprhon Physiology*) and had free access to drinking water or saline and the respective diet. The inner wall of the metabolic cages was siliconized and urine was collected under water-saturated oil.

Experiments were made on 5- to 7-month-old homozygous SGK1 knockout mice and their age- and sex-matched wild-type littermates. The mice were exposed to either a control diet (C1310: 0.2% Na^+, 1% K^+, 0.9% Ca^{2+}, Altromin, Heidenau, Germany), high-salt treatment (C1310 plus 1% NaCl in drinking water), or a low-calcium diet (C1031: 0.24% Na^+, 0.7% K^+, 0.08% Ca^{2+}, Altromin) with or without supplement of 1% NaCl in drinking water. Mice were maintained on control diet prior to the experiment. Before and throughout the experiments, mice were allowed free access to tap water or saline as indicated. To assess the effects of diuretics on renal clearance, mice were placed individually in metabolic cages without access to food or water. Urine was collected for 3 h after the intraperitoneal injection of furosemide (Lasix, 20 µg/gbw) with or without additional acetazolamide (Diamox, 50 µg/gbw).

To induce mineralocorticoid excess, wild-type (*sgk1*$^{+/+}$) and SGK1 knockout (*sgk1*$^{-/-}$) mice (6–9 months old, n=12 each) were treated with DOCA (2.4 mg/day) plus 1% NaCl in drinking water over 12 weeks. Drug delivery was achieved by repeated subcutaneous implantation of 21-day-release DOCA pellets in the neck area (Vallon V. et al., (2005) *Am J Physiol Regul Integr Comp Physiol*) during superficial general anesthesia (intraperitoneal midazolam 5 mg/kg plus ketamin 50 mg/kg), which was partially antagonized by flumazenil (0.5 mg/kg i.p.) afterwards. Prior to the pellet implantation, (control period), the mice had free access to plain tap water. After the implantation, the tap water was replaced by 1% NaCl (high salt). Throughout the entire study, mice had free access to a standard mouse diet (C1310 - Altromin, Heidenau, Germany). The study was completed in nine animals from each group.

For the evaluation of renal function of mice lacking both *sgk1* and *sgk3*, experiments were carried out on 12 to 16 months-old homozygous double (SGK1/SGK3)-knockout animals (*sgk1*$^{-/-}$/ *sgk3*$^{-/-}$, 4 females and 5 males) and their wild-type littermates (*sgk1*$^{+/+}$/*sgk3*$^{+/+}$, 4 females and 5 males) and, for comparison, age-matched SGK1- knockout animals (*sgk1*$^{-/-}$) and their wild-type littermates (*sgk1*$^{+/+}$).

The mice were maintained on a standard diet and tap water before the experiment. For evaluation of renal excretion, *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ and *sgk1*$^{-/-}$/ *sgk3*$^{-/-}$ mice were placed individually in metabolic cages over five consecutive days (Techniplast, Hohenpeissenberg, Germany) for 24-h urine collection. They had free access to tap water and a control diet (C1000, 0.24% Na^+ (wt/wt); Altromin, Heidenau, Germany) for 2 days followed by a low-salt diet (C1036, 0.015% Na^+ (wt/wt); Altromin) for another 3 days. The inner wall of the metabolic cages was siliconized, and urine was collected under water-saturated oil.

Metabolic cages protocol

A. Assembly

1. Clean all parts very carefully, especially the grid, food and bottle holder and the separation conus.
2. Siliconize the metall grid from both sides, the inner surfaces of the pyramidal conus and the ring which is attached to the separation conus. Before siliconizing the separation conus, put two paper towels into the lower narrow part and only siliconize the upper broad part. Remove any silicone from the lower part with 70% ethanol.
3. Put all parts together, make sure that all parts are functioning properly and that nothing is broken.

B. Maintenance

1. Put all twelve fresh urine collecting tubes in the red rack and fill 150µl water saturated oil into them then add the grid.
2. Number and weigh the appropriate number of green top cups, number and date the appropriate number Petri dishes, get enough long pellets for food holders (i.e. at least double the appropriate number, 4 to 4.5 cm long pieces).
3. Remove water bottle holder, close cage with one of the numbered plates and take the bottle out of the holder. Weigh it and refill with fresh tab water or any other solution specified in the experimental protocol every second day. Before you weigh the bottle to put it back into the holder, tap it on the palm of your hand to make sure that no air bubble is closing the metal nipple and is preventing water from coming down. Weigh the bottle with the metal nipple downwards in a horizontal position on the scales. Put the bottle back into the holder and do the same thing with the second bottle. Make sure that the rubber plate is at the front of the two bottles. Now attach rubber strap.
4. Dismantle lower part of the first metabolic cage and put the round Plexiglas plate in, to prevent the loss of feces or urine during collection. Collect any adhering urine from the separation and pyramidal conuses. Put adhering feces into feces collector. Take out the conus part carefully and remove the ring at the end of the conus. Take out the urine collecting tube, put urine into green top cup and weigh again. Put feces into Petri dish. Put the ring, and both collecting tubes into a cage filled with hot water, to pre-clean them. Clean separation and pyramidal conuses with cold water and the small brush. Use a fresh ring and collecting tubes to put the metabolic cage together again.
5. After having done this with all twelve cages start to exchange the food, take care of any food inside the cage or fallen into one of the collection tubes.
6. Weigh the animal. If the mouse looses any urine or stool while being weighed, put it into the respective collector.

7. As the last step, weigh the drinking bottle and put it back into the holder in an upright position, if using the holder in the two bottle mode use a rubber ring to strap the bottles tightly into the holder, put into metabolic cage and attach collector for lost drinking water. Do not forget to put 200 µl of water saturated oil into this collector.
8. Finally make a visual check that every cage looks the same and that nothing was forgotten.
9. Clean the feces and urine collector with the big brush and hot water.
10. Close petridishes with two stripes of tape; put them into a plastic bag and in the –20 °C Freezer.
11. Take one ml of urine (or everything if less), spin it down at 14000 rpm for 5 min. in a small Eppendorf centrifuge and put it in a labeled Eppendorf and then into the –20 °C as well.

C. Trouble shouting

1. Make sure bottles are labeled correctly.
2. Put them into the holder in an upright position, attach rubber strap in the same position.
3. If there is urine in the feces collector, measure it and mark the respective result in the list.
4. If there is feces in the urine collector tube mark the respective result.
5. If a mouse spills drinking water inside the cage, the pair of mice should be exchanged against another one.

4.3. Blood, fecal and urinary concentrations

To obtain blood specimens, animals were lightly anesthetized with diethyl ether (Roth, Karlsruhe, Germany) and approximately 150 µl of blood was withdrawn into heparinized capillaries by puncturing the retro-orbital plexus. Plasma and urinary concentrations of Na^+, K^+ and Ca^{2+} were measured by flame photometry (AFM 5051, Eppendorf, Germany). Plasma and urinary creatinine concentrations were measured by using an enzymatic colorimetric method (Creatinine PAP, Labortechnik, Berlin, Germany). Plasma intact parathormone levels were measured by using an ELISA kit (Immunotopics, San Clemente, California, USA) and plasma $1.25(OH)_2D_3$ levels were measured with a RIA kit (IDS, Boldon, UK) according to the manufacturer's instructions. Urinary albumin was measured fluorometrically using an albumin determination kit (microfluoral Progen, Heidelberg, Germany) on a multilabel counter (Victor 1420, Perkin Elmer, Boston, Massachussets, USA) according to the manufacturer's instructions. Standard curves were generated with mouse albumin (Sigma Aldrich, Taufkirchen, Germany) and measurements were performed within the linear range 0–156 mg/l.

Urine, feces, plasma electrolytes measurement protocol

1. Mix the following standard and zero solutions:
   a.**Serum Zero**: 1000 µl *Li Dilution Solution* - 1:100 from stock solution in the fridge- and 20µl ddH_20 (Ampuwa) make five Eppendorfs (Dilution 1+50)
   b.**Serum Standard**: 1000 µl *Li Dilution Solution* and 20µl Serum Standard Solution (please take an aliquot, do not take it directly out of the bottle) make at least five Eppendorfs (Dilution 1+50)
   c.**Urine Zero**: 1000 µl *Li Dilution Solution* and 5 µl ddH_20 (Ampuwa) make five Eppendorfs (Dilution 1+200)
   d.**Urine Standard**: 1000 µl Li *Dilution Solution* and 5µl Urine Standard Solution (please take an aliquot, do n0,ot take it directly out of the bottle) make at least five Eppendorfs (Dilution 1+200)
2. Calibrate with zeros and standards
3. Measure samples

Measurement range: Na-60-180 mmol/l; K- 1-9 mmol/l; Ca- 1-5 mmol/l
Measurement range: Na- 10- 300 mmol/l; K- 5-200 mmol/l

Feces preparation protocol

Dry the feces sample at around 80° C for about 3 hour and take the dry weight. Add 5 ml of 0.75 *M* HNO_3 (5.2 ml acid in 94.8 ml distilled water) to the feces in a falcon tube of 15 ml capacity and process for 48 hours to give a homogenous creamy mass (processing involves shaking of the sample for 12 hours on an electric shaker and keeping in a water bath at 50 °C for another 12 hours alternately and finally vortexing on a minishaker for 2 minutes to hasten homogenization). Then centrifuge the sample at 4500 rpm for 10 minutes and collect 1 ml of the supernatant. Centrifuge the supernatant again at 14000 rpm in an Eppendorf centrifuge for 5 min. Take supernatant and store at -20 °C until analysis. Thaw the sample before analysis and take 10 µl of the diluted sample for analysis. Apply the dilution factor during calculations.

4.4. Blood pressure

The tail cuff method has the advantage to be noninvasive and can provide reproducible results of systolic blood pressure if those precautions are taken into account (Kurtz et al., (2005) *Arterioscler Tromb Vasc Biol*). Systolic arterial blood pressure was determined by the tail-cuff method (IITC, model 179, Woodland Hills, California, USA) before, 7 weeks and 12 weeks following the initiation of DOCA/high-salt treatment. As reviewed recently, (Meneton P et al., (2000) *J Am Soc Neprhol*), the tail cuff approach to determine arterial blood pressure requires certain precautions to reduce the stress of the animals, including appropriate training of the mice over multiple days and adequate prewarming to dilate the tail artery. The animals were placed in a heated chamber at an ambient temperature of 28–30°C for 15 minutes and from each animal 10–20 blood pressure traces were recorded in one session per day. The readings from 3 days were then averaged to obtain a mean blood pressure. All recordings and data analyses were done using a computerized data acquisition system and software (PowerLab 400 and Chart 4). All measurements were done by one person during a defined time (between 2 and 4 P.M.)

4.5. TRPV5 and calbindin D-28K protein abundance

Total protein was isolated from kidneys obtained from untreated *sgk1*$^{+/+}$ (n=6) and *sgk1*$^{-/-}$ mice (n=6). Kidneys were removed and immediately frozen in liquid nitrogen. For calbindin D-28K the tissue was then homogenized in a lysis buffer containing 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% Triton X-100, 1% sodium deoxycholate, 1% sodium dodecyl sulfate, and a protease inhibitor cocktail (1 per 10 ml, Complete Mini EDTA-free). The homogenates were centrifuged at approximately 7000×g at 4°C for 15 minutes and the supernatant was removed and used for Western blotting.

For TRPV5 the nuclear and mitochondrial debris were cleared by centrifugation at 7000×g for 15 min at 4°C. The supernatant was subjected to ultracentrifuge spin (100000×g for 1 h using Rotor 70.1 Ti, Beckman). The pellet from this spin was considered as a membrane fraction and was solubilized by use of lysis buffer containing 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% Triton X-100, 1% sodium deoxycholate, 1% sodium dodecyl sulfate, and protease cocktail inhibitor (1 per 10 ml, Roche).

Total protein was determined by BioRad protein assay (BioRad, München, Germany). Total proteins (75 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS PAGE) (10% Tris–glycine), transferred to nitrocellulose membranes, blocked for 1 h in blocking buffer (5% fat-free milk in PBS containing 0.1% Tween), and incubated overnight at 4°C with a monoclonal anti-calbindin D-28K antibody diluted 1:500 in a blocking buffer (Swant, Bellizona, Switzerland) and a rabbit polyclonal anti-TRPV5 antibody (diluted 1:500 in blocking buffer). After incubation with a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody, the bands were visualized with enhanced chemoluminescence (ECL) according to the manufacturer's instructions. For calbindin D-28K homogenates were also probed with a primary glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology, Heidelberg, Germany) as a loading control. Densitometric analysis of calbindin D-28K and TRPV5 was performed with Quantity One software and normalized using GAPDH. For TRPV5 in addition, a specific blocking peptide, with the 20 amino acid sequence near the cytoplasmic C terminus of rat CaT-2/ECaC1, was applied in parallel (Alpha Diagnostics).

Measurement for total proteins (Bradford assay) protocol

1. Dilute Bradford Reagent in water 1:5 volumes
2. Use 2µl Sample and 1000µl diluted Bradford reagent
3. Vortex and add volume in 1 ml cuvette
4. Measure sample with UV cuvette in Biophotometer

Western Blot Protocol

1. Prepare stacking gel and resolving gel in Western blot system
2. Running step:
- prepare samples
- add samples on the gel according to concentration
- run gel in running buffer
3. Transfer step:
- prepare sandwich for transfer (1 sponge, 1 paper, 1 nitrocellulose membrane, 1 gel, 1 paper, 1 sponge)
- add sandwich in transfer system
- add transfer buffer and run transfer
4. Determine the transfer of proteins fromm gel on membrane with Ponceau staining
5. Blocking step:
- block for non-specific binding in PBST plus non-saturated milk 5 % for 1-2 hours
6. Add first Antibody:
- leave overnight at 4° C on rotating system
7. Wash 4-5 times with PBST buffer
8. Add second Antibody:
- leave 1-2 hours at room temperature on rotating system
9. Wash 4-5 times with PBS Buffer plus 0.15% Tween
10. Develop with ECL solution on film
11. Analyse gel with Biorad Quantity One.

Preparation of Brush Border Membrane Vesicles (BBMV) protocol

BBMV were prepared from whole mouse kidney, jejunum and ileum using the Mg^{2+} precipitation technique as described previously (Biber B. et al., (1981) *Biochem Biophys Acta*). The small intestines were rinsed with ice-cold saline solution and opened longitudinally. Briefly, the mucosa was scraped off with a glass slide in a buffer containing (in mM) 250 Sucrose, 20 Tris (pH 7.5), 5 EGTA and a protease inhibitor cocktail (Roche, Mannheim, Germany). The suspension was homogenized with an Omnimixer for 1 minute. $MgCl_2$ was added to the homogenate to a final concentration of 10 mM. The suspension was stirred on ice for 10 minutes and then centrifuged at 1600×*g* for 15 min. The plasma membranes retained in the supernatant were collected by centrifugation at 20000×*g* for 30 min. The resultant pellet was suspended in a pH 7.4 buffer consisting of (in mM) 125 Sucrose, 10 Tris (pH 7.5), 2.5 EGTA, 2.5 $MgSO_4$. This suspension was homogenized with 50 up-down strokes with a glass homogenizer and centrifuged at 20000×*g* for 30 minutes. The final pellet, containing the purified BBMV, was homogenized by passing the suspension through 25- and 28-gauge needles and solubilised. All the steps were carried out at 4°C. After the final suspension, samples were frozen at -80°C for later use. Membrane protein was assessed as described by Bradford.

4.6. TRPV5 and calbindin D-28K mRNA expression

Total RNA of kidneys from *sgk1*$^{+/+}$ (n=12 for TRPV5, n=7 for calbindin D-28K) and *sgk1*$^{-/-}$ mice (n=12 for TRPV5, n=7 for calbindin D-28K) was stabilized and extracted by use of the PAXgene System (Qiagen, Germany). Subsequently, 1 µg of total RNA was reverse transcribed to cDNA by using the reverse transcription system (Promega, Mannheim, Germany) with oligo(dT) primers according to the manufacturer's protocol.

To determine TRPV5 and calbindin D-28K mRNA levels, quantitative real-time PCR with the LightCycler System (Roche) was established. PCR reactions for TRPV5 and calbindin D-28K were performed in a final volume of 20 µl containing 2 µl cDNA, 3 mM $MgCl_2$, 0.5 µM of both primers, 2 µl cDNA Master SybrGreen I mix (Roche), and diethylprocarbonate (DEPC)-treated water. The transcript levels of the housekeeping gene GAPDH were also determined for each sample by use of a commercial primer kit (Search-LC, Heidelberg, Germany). PCR reactions for GAPDH were performed in a final volume of 20 µl containing 2 µl cDNA, 2 µl primer mix (Search-LC), 2 µl cDNA Master SybrGreen I mix (Roche), and 14 µl DEPC-treated water. The target DNA was amplified during 35 cycles at 95°C for 10 s, 68°C for 10 s and 72°C for 16 s each with a temperature transition rate of 20°C/s and a secondary target temperature of 58°C with a step size of 0.5°C. Melting curve analysis was performed at 95°C for 0 s, 58°C for 10 s, and 95°C for 0 s to determine melting temperatures of primer dimers and the specific PCR products. Melting curve analysis confirmed the amplified products, which were then separated on 1.5% agarose gels to confirm the expected size. Finally, results were calculated as a ratio of the target versus housekeeping gene GAPDH transcripts. The following intron-spanning primers were used for TRPV5 (GenBank accession no. NM-001007572.1) and calbindin D-28K (GenBank accession no: NM-009788) were used TRPV5 - forward f1-5′-CGTTGGTTCTT ACGGGTTGAAC-3′, reverse r1-3′-GTTT GGAGAAC CACAGAGCCTCTA-5′; calbindin D-28K - forward f1-5′ AACT GACA GAGATGGCCAGGTTA-3′; reverse r1-3′- TGAA CTCTTTC CCACA CATTTT GAT-5′.

4.7. SGK2 mRNA expression

Automated disruption and homogenization of frozen renal tissue from *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ (*n*=5) and *sgk1*$^{-/-}$/ *sgk3*$^{-/-}$ (n=5) mice was performed using the MagNa Lyser (Roche Diagnostics, Mannheim, Germany). Cleared cell lysate was transferred for further RNA purification (RNeasy Mini Kit, Qiagen, Hilden, Germany). Subsequently, 1 µg of total RNA was reverse transcribed to cDNA utilizing the reverse transcription system (Bioscience) with oligo(dT) primers according to the manufacturer's protocol. To determine murine SGK2 (mSGK2) mRNA levels, quantitative real-time PCR was carried out with the LightCycler System (Roche Diagnostics). PCR for mSGK2 were performed in a final volume of 20 µl containing 2 µl of cDNA, 2.4 µl of $MgCl_2$ (3 µM), 1 µl of primer mix (0.5 µM both primers), 2 µl of cDNA Master SybrGreen I mix (Roche Molecular Biochemicals, Mannheim, Germany), and 12.6 µl of diethylpyrocarbonate-treated water. The transcript levels of the housekeeping gene GAPDH were determined for each sample using a commercial primer kit (Search LC, Heidelberg, Germany). PCR for GAPDH were performed in a final volume of 20 µl containing 2 µl of cDNA, 2 µl of primer mix (Search LC), 2 µl of cDNA Master SybrGreen I mix and 14 µl of diethylpyrocarbonate treated water. The target DNA was amplified during 35 cycles at 95°C for 10 s, 68°C for 10 s and 72°C for 16 s, each with a temperature transition rate of 20°C/s and a secondary target temperature of 58°C with a step size of 0.5°C. Melting

curve analysis was performed at 95°C at 0 s, 58°C at 10 s and 95°C at 0 s to determine melting temperatures of primer dimers and the specific PCR products. Melting curve analysis confirmed the amplified products, which were then separated on 1.5% agarose gels to confirm the expected size (413 bp). Finally, results were calculated as a ratio of target gene to housekeeping gene GAPDH transcripts. The following primers for mSGK2 (GenBank accession no. NM - 013731) were used: 5'-CCA CAG ACT TTG ATT TCC TC-3' (forward) and 3'-GGC AGT CCA AGA GAA TGT T-5' (reverse). The primers showed no overlap with murine SGK1 (mSGK1, Gen- Bank accession nr. NM-011361) and murine SGK3 (mSGK3, GenBank accession nr. NM-133220).

4.8. Immunohistochemistry

For immunohistochemistry, kidneys from *sgk1*$^{+/+}$ (n=4) and *sgk1*$^{-/-}$ mice (n=4) were fixed with 4% paraformaldehyde/ 0.1 M sodium phosphate buffer (pH 7.2) via retrograde perfusion over an indwelling aortic catheter and kept in buffered 4% paraformaldehyde for another 4 h. After immersion in 30% sucrose for 48 h, 12-µm cryostat sections were taken. After a brief rinse, the sections were incubated for 1 h in normal goat serum with 0.3% Triton X- 100. All the following washes and incubations were performed in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 and 1% dimethyl sulfoxide. Without rinsing, polyclonal (rabbit) antibodies against TRPV5 (Alpha Diagnostics) diluted 1:100 and monoclonal (mouse) anti-calbindin D-28K (Swant) diluted 1:1000 were applied together in a moist incubation chamber for 12 h at 4°C. After three washes at 10 min each, sections were incubated with goat anti-mouse Alexa 488 (for calbindin D-28K detection) and antirabbit Alexa 546 (for TRPV5 detection) secondary antibodies (Molecular Probes, diluted 1: 400 for 1.5 h). Controls were performed by omitting either one or both primary antibodies. After coverslipping with Mowiol, the slides were analyzed on a Zeiss LSM 510 Axioplan 2 confocal microscope equipped with a 40x oil immersion lens (NA 1.3). By use of the multitrack function, individual fluorochromes were scanned with laser excitation at 488 and 543 nm separately with appropriate filter sets to avoid cross talk. Controls were scanned with identical laser excitation and filter settings. In addition, transmitted light images were recorded.

4.9. Morphology

Fixed kidneys were stored in 4% paraformaldehyde/0.1 M sodium phosphate buffer. After the determination of kidney weight and volume, the kidneys were dissected into 1-mm thick slices perpendicular to the longitudinal axis. Using area-weighted sampling, 10 small pieces of the kidney cortex were selected for embedding in epon araldite. Semithin (1 µm) sections were prepared and stained with methylene blue/basic fuchsine. All remaining kidney slices were embedded in paraffin yielding one representative section of each slice for morphometric and stereological investigations. Four-micrometer sections were cut and stained with hematoxylin/eosin (HE), periodic acid-Schiff stain (PAS) and Sirius red as fibrous tissue stain.

Semiquantitative, morphometric, and stereological measurements

All semiquantitative, morphometric, and stereological measurements were performed in a blinded manner by an observer who was unaware of the study protocol.

Indices of renal damage (glomerulosclerosis, tubulointerstitial, and vascular damage).

The glomerulosclerosis index as a parameter of progression of renal failure was determined on PAS-stained paraffin sections according to a previously described scoring system (scores of 0 to 4) for each animal as the arithmetic mean of assessing 100 glomeruli (Wolf G. et al., (2005) *Kidney Int*). The tubulointerstitial damage index, i.e., tubular atrophy, dilatation, casts, interstitial inflammation, and fibrosis, was assessed as previously described on PAS - stained paraffin sections at a magnification of 100× (Wolf G. et al., (2005) *Kidney Int*). The vascular damage score, i.e., mild/moderate/severe wall thickening, lumen obliteration, and fibrinoid necrosis, was determined using a semiquantitative scoring system (score 0–4) (Wolf G. et al., (2005) *Kidney Int*).

Mesangiolysis, i.e., dissolution of the mesangial matrix with capillary dilation due to damage to mesangial or endothelial cells or the mesangial matrix, was determined on PAS - stained paraffin sections and graded in 100 systematically subsampled glomeruli per animal using a scoring system (0–4). The resulting index in each animal was expressed as the mean of all scores obtained.

Glomerular geometry (paraffin sections, light microscopy, various magnifications)

Briefly, glomerular geometry was analyzed as follows: Volume density (V_V) of glomeruli and area density of glomerular tuft (A_{AT}) were measured by point counting according to $P_P=A_A=V_V$ at a magnification of 400x on HE sections (P_P, point density; A_A, area density) (Schwarz U. et al., (1998) *Kidney Int*). Total area of glomerular tuft (AT) was then determined as $A_T=A_{AT}\times A_{Cortex}$. The number of glomeruli per volume (NV) and the volume density (VV) of glomeruli were obtained using the formula $N_V = k/\beta \text{ x } N_A^{1.5} V_V^{0.5}$, with k=1.1 and β=1.382. The total number of glomeruli was derived from the total volume of the renal cortex and the number of glomeruli per cortex volume: $N_{glom}=N_V\times V_{Cortex}$. The mean glomerular tuft volume was determined according to $v= \beta/k \text{ x } A^{1.5}_T$, with β=1.382 and k=1.1.

Glomerular cells and capillaries (semithin sections, magnification 1000x)

Glomerular cell number and volume were analyzed in semithin sections with a 100-point eyepiece for point-counting at a magnification of 1000× (oil immersion) as previously described (Wolf G. et al., (2005) *Kidney Int*). Glomerular cell numbers (podocytes, mesangial cells, and endothelial cells) were calculated in at least 30 glomeruli for each animal, from cell density per volume (Nc_V) and volume density of the respective cell type (Vc_V) according to the formula $Nc_V = k/\beta \text{ x } Nc^{1.5}_A = Vc^{0.5}_V$, with k=1 and β=1.5 for podocytes and β=1.4 for mesangial and endothelial cells. The respective cell volumes were calculated according to the equation $Vc=Vc_V \times V_{glom}$.

4.10. Data analysis and statistics

Unpaired t-test, or ANOVA as appropriate, has been employed to analyze for significant differences in the respective parameters between wild type and knockout mice. Data are provided as means ± SEM (standard error deviation), where n represents the number of independent experiments. Only results with $p<0.05$ were considered statistically significant.

5. Results

Characterization of *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice under normal and low Ca^{2+} diet

As evident from immunohistochemistry (Figure nr.14), TRPV5 and calbindin D-28K were colocalized in distal convoluted tubules and connecting tubules. The TRPV5 abundance in connecting tubules and to a lesser extent in distal convoluted tubules was weaker in knockout mice (*sgk1*$^{-/-}$) than in their wild-type littermates (*sgk1*$^{+/+}$). After the mice were given a Ca^{2+}-deficient diet, TRPV5 abundance increased in both *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice, clearly pointing to SGK1-independent regulation of TRPV5.

In Western blot analysis, the membrane protein level of TRPV5 was again lower in *sgk1*$^{-/-}$ than in *sgk1*$^{+/+}$ mice (optical density 0.17 ± 0.03 in *sgk1*$^{-/-}$ vs 0.25 ± 0.03 in *sgk1*$^{+/+}$; n=8 animals each, p<0.03 at paired comparison; Figure nr. 15).

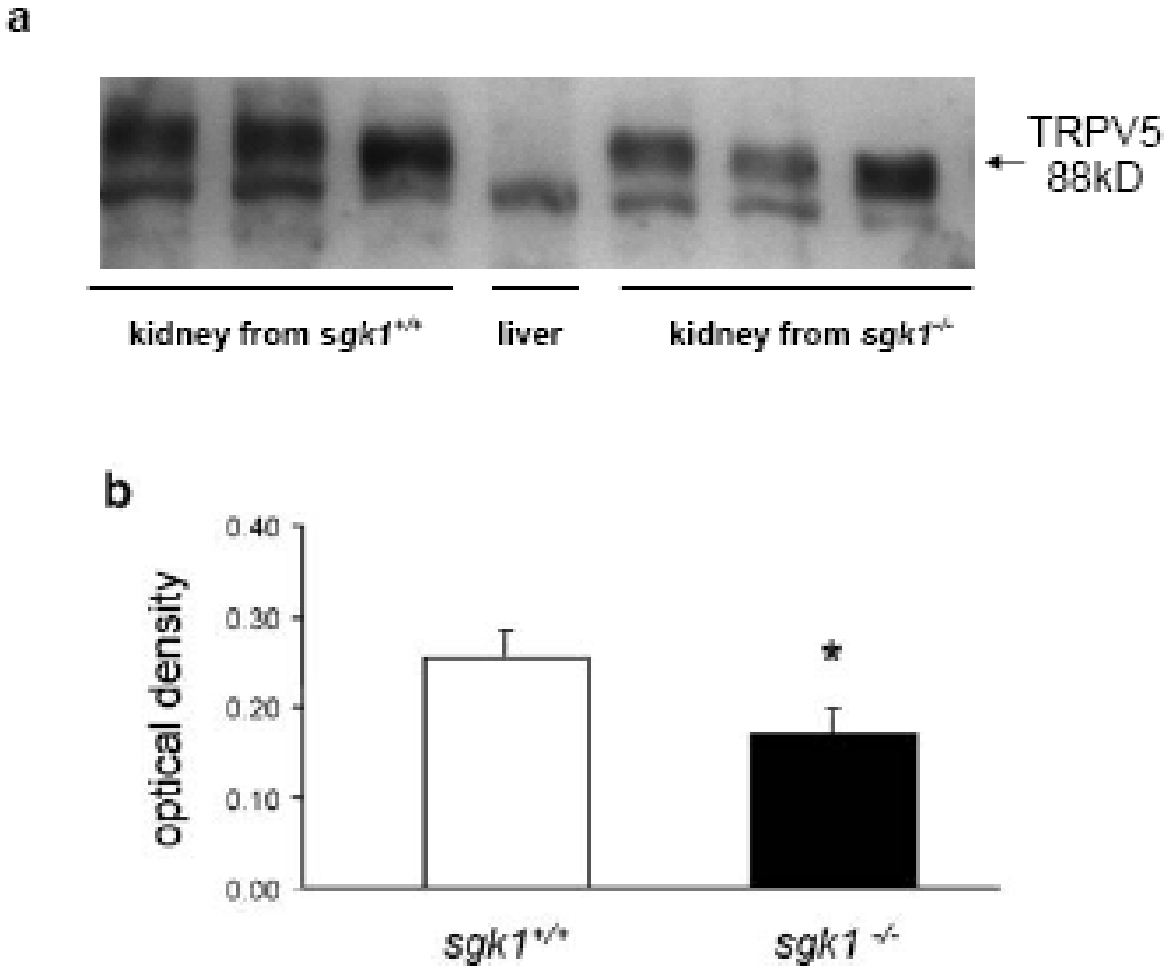

Figure nr. 15 - Western blot analysis of TRPV5 in membranes under control conditions in *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice.

a) Original Western blot showing membrane expression of TRPV5 in kidney of knockout and wild-type animals and its absence in liver tissue.

b) Arithmetic means of the optical density±SEM (n=8 each). *p<0.05, indicates statistically significant difference between genotypes.

Cytosolic calbindin D-28K expression was significantly smaller in *sgk1*$^{-/-}$ than in *sgk1*$^{+/+}$ mice (Figure nr.16). No significant differences were observed between *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice under the control diet in the plasma concentrations of intact parathyroid hormone (33.1 ± 5.4 pg/ml in sgk1$^{+/+}$ vs. 31.9 ± 4.9 pg/ml in *sgk1*$^{-/-}$; n=12 animals each) and 1.25$(OH)_2D_3$ (147 ± 19 pmol/l in *sgk1*$^{+/+}$ vs. 147 ± 22 pmol/l in *sgk1*$^{-/-}$; n=12 animals each). Thus, the reduced abundance of membrane TRPV5 and cytosolic calbindin D-28K protein in *sgk1*$^{-/-}$ mice were not attributable to hormonal differences.

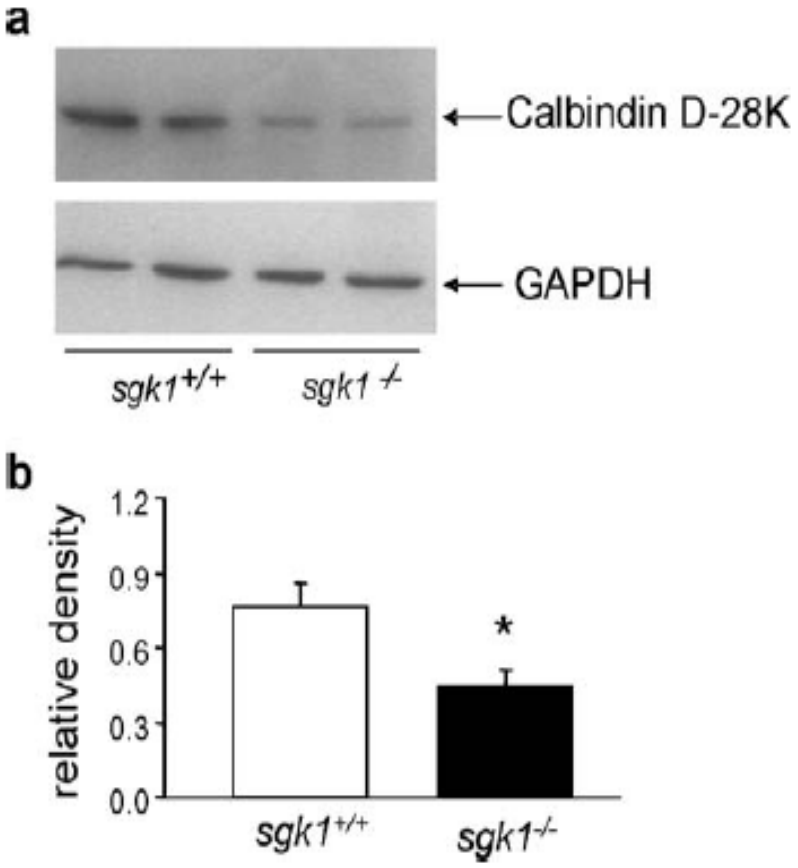

Figure nr. 16 - Western blot analysis of cytosolic calbindin D-28K protein abundance under control conditions in *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice.

a. Original Western blots.

b. Arithmetic means of the GAPDH-normalized relative density ± SEM (n=6 each). *p<0.05, indicates statistically significant difference between genotypes.

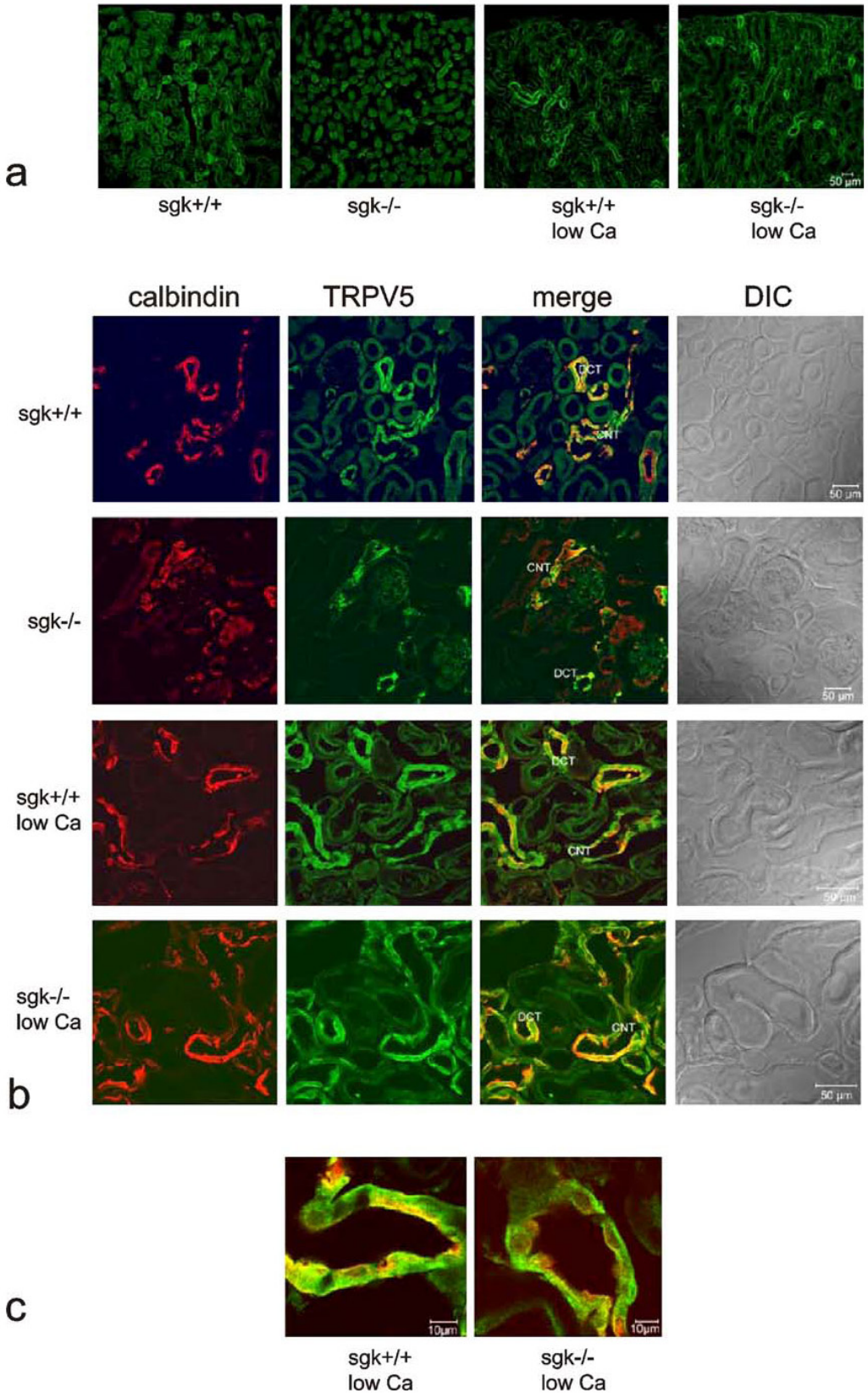

Figure nr. 14 - Immunohistochemistry of TRPV5 (n=4) and calbindin D-28K (n=4) abundance in distal tubular segments under control diet and low-Ca^{2+} diet in *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice.

a. Overview of TRPV5 staining in *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mouse kidneys.

b. TRPV5 and calbindin D-28K colocalization in the distal convoluted tubule and connecting tubule.

c. High-power confocal images of TRPV5 immunoreactivity in distal tubules reveals its distribution over the entire cell cytoplasm in *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice.

Quantitative real-time PCR measurements did not reveal statistically significant differences between the genotypes in the levels of mRNA encoding TRPV5 (ratio of TRPV5 mRNA over GAPDH mRNA: 0.63 ± 0.08 in *sgk1*$^{+/+}$ vs. 0.59 ± 0.05 in *sgk1*$^{-/-}$; n=12 animals each) or calbindin D-28K (ratio of calbindin D-28K mRNA over GAPDH mRNA: 20.1 ± 3.0 in *sgk1*$^{+/+}$ vs. 17.8 ± 2.4 in *sgk1*$^{-/-}$; n=7 animals each).

The apparently low TRPV5 protein abundance in the cell membranes of *sgk1*$^{-/-}$ mice is expected to impair tubular Ca^{2+} reabsorption and thus favor calciuria. However, under the control diet the opposite was observed, i.e., absolute and fractional urinary excretion of Ca^{2+} was significantly lower in *sgk1*$^{-/-}$ than in *sgk1*$^{+/+}$ mice (Figure nr. 17, Table nr. 1).

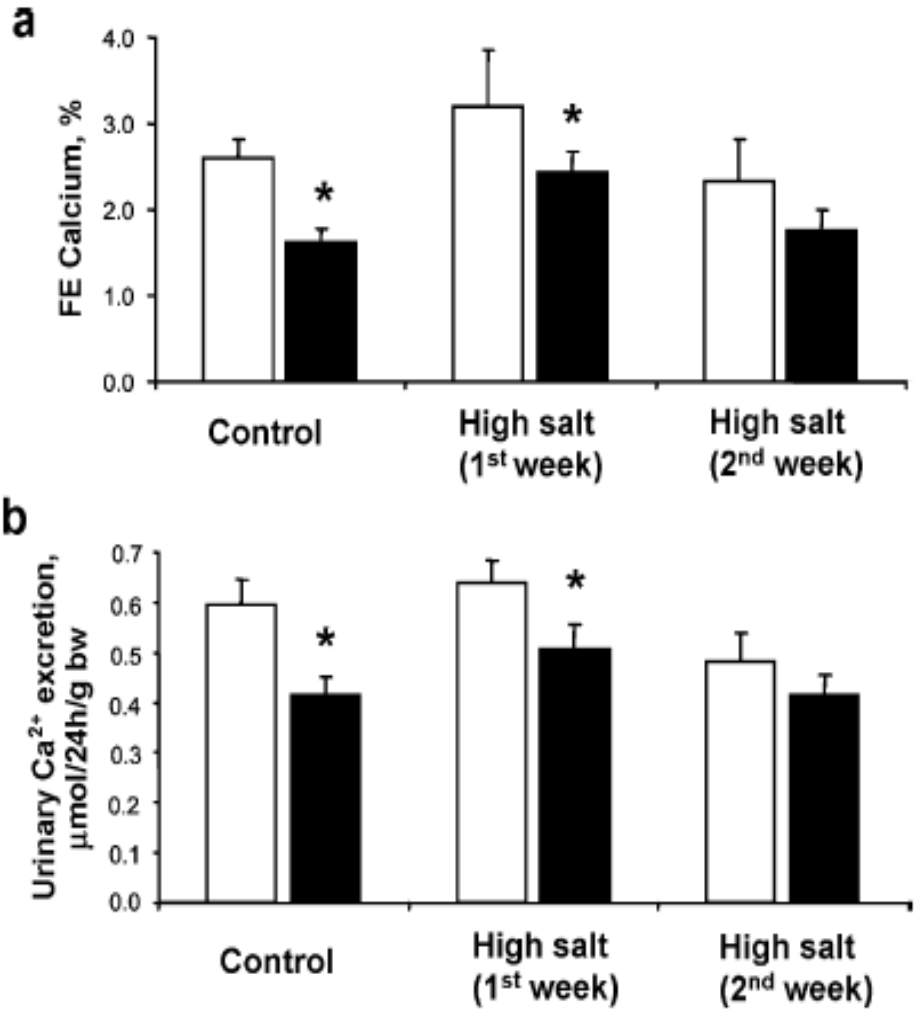

Figure nr. 17 - Fractional excretion and renal Ca^{2+} excretion in *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice under control diet and with free access to tap water or 1% saline.

Arithmetic means ± SEM of fractional Ca^{2+} excretion (FE, %), and daily urinary excretion of Ca^{2+} (µmol/24 h/ g bw) in *sgk1*$^{-/-}$ (black bars) and in *sgk1*$^{+/+}$ mice (white bars) under control diet and with free access to tap water (left, n=30 animals each) or 1% saline after 1 week (middle, n=18 animals each) and after 2 weeks (right, n=6 animals each). #p<0.05, indicates statistically significant difference between diets; *p<0.05, indicates statistically significant difference between genotypes.

	Tap water (n=30)		1% saline			
			1 st week (n=18)		2 nd week (n=6)	
	sgk1$^{+/+}$	**sgk1$^{-/-}$**	**sgk1$^{+/+}$**	**sgk1$^{-/-}$**	**sgk1$^{+/+}$**	**sgk1$^{-/-}$**
Body Weight (g)	26.7±0.7	26.5±0.6	27.6±0.7	27.0±0.8	26.8±0.4	25.4±0.5
Food intake (mg/g BW)	153±4	155±4	154±4	156±5	162±4	166±3
Fluid Intake (µl/24h/ g BW)	211±11	200±6	229±8	247±15$^{\#}$	227±15	229±8$^{\#}$
Hematocrit (%)	54.9±0.5	52.1±0.5	*n.d.*	*n.d.*	53.4±0.7	49.2±0.5$^{\#}$
[Na^+]plasma (mM)	153±2	152±2	*n.d.*	*n.d.*	153±2	154±1
[K^+]plasma (mM)	4.5±0.1	4.6±0.1	*n.d.*	*n.d.*	4.1±0.1	4.9±0.4
[Ca^{2+}]plasma (mM)	2.29±0.03	2.27±0.03	*n.d.*	*n.d.*	2.30±0.07	2.30±0.06
Urinary flow (µl/24h /g BW)	111±9	92±6*	120±5	143±13	126±12	131±9
Urine Na^+ (µmol/24h/ g BW)	15.3±0.8	14.9±0.8	44.0±2.5	44.8±2.9	39.3±2.5	41.7±1.5
Urine K^+ (µmol/24h/ g BW)	33.5±1.1	32.9±1.1	31.3±0.8	32.1±1.1	31.1±0.9	34.4±0.9
Urine Ca^{2+} (µmol/24h/g BW)	0.59±0.05	0.41±0.03	0.64±0.04	0.50±0.05	0.48±0.05	0.41±0.04
Creatinine clearance (µl/min /g BW)	6.3±0.4	7.1±0.5	6.1±0.6	5.9±0.3	7.4±1.4	7.8±0.5
Fractional excretion Na^+ (%)	1.04±0.05	0.93±0.04	2.79±0.32	2.50±0.16	2.47±0.23	2.46±0.16
Fractional excretion K^+ (%)	77.4±3.7	69.1±4.7	83.8±9.6	81.7±3.2	73.0±9.7	74.1±4.8
Fractional excretion Ca^{2+} (%)	2.6±0.2	1.6±0.2	3.2±0.7	2.4±0.2	2.3±0.5	1.8±0.2

Table nr. 1 - Effect of high salt intake on body weight, food and fluid intake, plasma concentrations and urinary excretion of electrolytes.

Body weight, food and fluid intake**,** hematocrit, Na^+, K^+ and Ca^{2+} concentrations in plasma, urinary flow, renal excretions of Na^+, K^+ and Ca^{2+}, creatinine clearance and fractional renal excretions of Na^+, K^+ and Ca^{2+} in both *sgk1*$^{-/-}$ and in *sgk1*$^{+/+}$ mice under control diet at free access to tap water (n=30 each) or 1% saline 1st week (n=18 each) or 2nd week (n=6 each). Arithmetic means ± SEM (number of animals studied); # ($p<0.05$) indicates statistically significant difference between control and high salt diet; * ($p<0.05$) indicates statistically significant difference between genotypes (*n..d.* - not determined.).

Under the chosen experimental conditions, the differences of renal Ca^{2+} elimination had little influence on the Ca^{2+} balance, as in both genotypes renal excretion was less than 2% of the calculated dietary Ca^{2+} intake (*sgk1*$^{+/+}$: 825 ± 43 µmol/24h/g bw, *sgk1*$^{-/-}$: 775 ± 47 µmol/24h/g bw, n=6 animals) and fecal Ca^{2+} excretion (*sgk1*$^{+/+}$: 773 ± 24 µmol/24 h/g bw, *sgk1*$^{-/-}$: 709 ± 26 µmol/24h/g bw, n=6 animals).

Under a Ca^{2+}-deficient diet, renal elimination of Ca^{2+} decreased significantly in both *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice (Table nr. 2, Figure nr. 19). The ability of the *sgk1*$^{-/-}$ mice to decrease urinary Ca^{2+} output again points to SGK1- independent regulation of renal tubular Ca^{2+} reabsorption. However, the decrease of absolute and fractional excretion upon exposure to the Ca^{2+}-deficient diet was blunted in the *sgk1*$^{-/-}$ mice.

Thus, the Ca^{2+}-deficient diet dissipated the differences of absolute and fractional Ca^{2+} excretions between *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice. Replacement of tap water in the drinking bottle with 1% saline led to the expected increase in urinary Na^{+} output (Table nr. 2), which was paralleled by a moderate increase in urinary Ca^{2+} output (Figure nr.18, Table nr. 2). During salt loading, the urinary excretion of Ca^{2+} remained slightly lower in *sgk1*$^{-/-}$ than in *sgk1*$^{+/+}$ mice (Figure nr. 17, Table nr. 2).

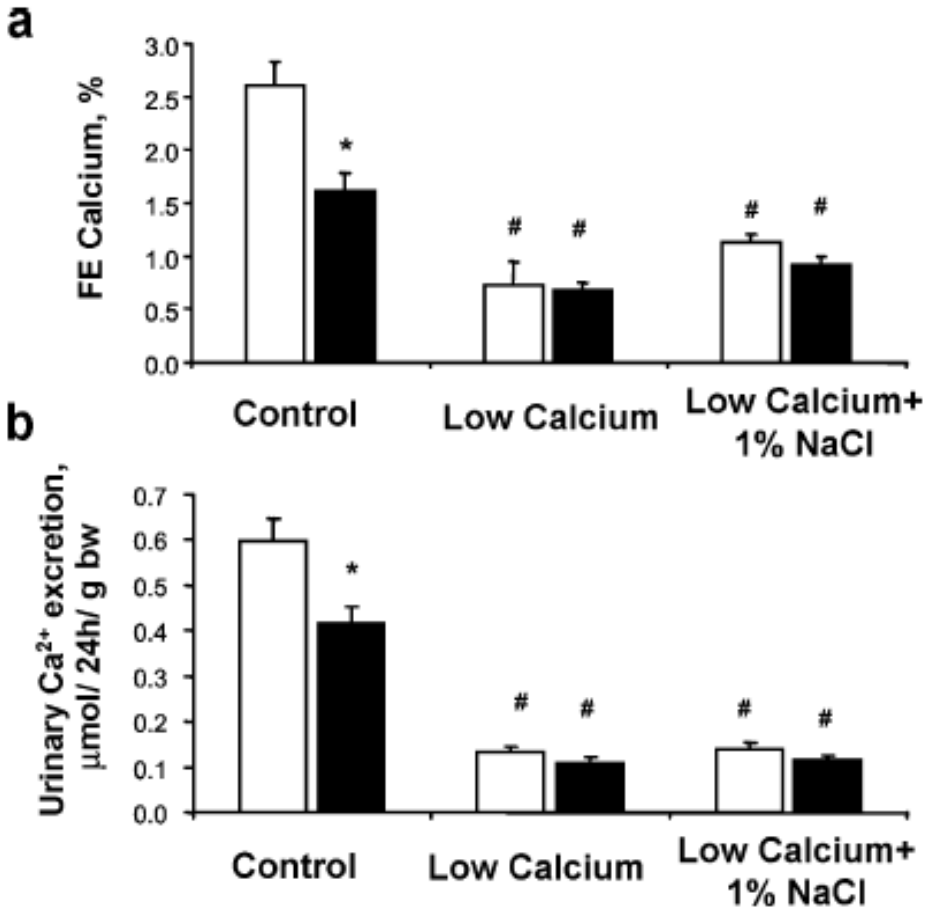

Figure nr. 18 - Renal Ca^{2+} excretion in *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice under control diet and low-Ca^{2+} diet.

Arithmetic means ± SEM of fractional Ca^{2+} excretion (FE, %) and daily urinary excretion of Ca^{2+} (µmol/24h/gbw) in *sgk1*$^{-/-}$ (black bars) and in *sgk1*$^{+/+}$ mice (white bars) under control diet (left, n=30 animals each) and under low Ca^{2+} diet (middle and right, n=6 animals each). #p<0.05, indicates statistically significant difference between diets; *p<0.05, indicates statistically significant difference between genotypes.

	Control diet tap water (n=30)		Low Ca^{2+} diet+ tap water (n=6)		Low Ca^{2+} diet + 1%NaCl (n=6)	
	$sgk1^{+/+}$	$sgk1^{+/+}$	$sgk1^{+/+}$	$sgk1^{-/-}$	$sgk1^{-/-}$	$sgk1^{-/-}$
Body Weight (g)	26.7±0.7	25.7±1.4	25.7±1.3	24.9±1.5	24.6±1.7	26.5±0.6
Food intake (mg/g BW)	153±4	167±7	167±4	171±6	171±7	155±4
Fluid Intake (µl/24h/ g BW)	210±10	187±14	237±21	205±12	159±16#	199±6
Hematocrit (%)	54.9±0.4	54.4±0.8	54.2±0.9	51.6±1.1	52.7±0.7	52.1±0.5*
$[Na^+]$plasma (mM)	153±2	155±1	173±2#	166±1	154±2	152±1
$[Ca^{2+}]$plasma (mM)	2.29±0.04	2.07±0.03#	2.41±0.06	2.29±0.08	1.98±0.05#	2.27±0.03
$[K^+]$plasma (mM)	4.45±0.12	4.45±0.21	4.84±0.20	4.33±0.09	4.44±0.14	4.55±0.09
Urinary flow (µl/24h /g BW)	111±9	91±5	114±10	99±8	75±10	92±6
Urine Na^+ (µmol/24h//g BW)	15.3±0.8	13.8±0.5	32.3±1.9	30.1±2.0	14.7±1.1	14.9±0.8
Urine K^+ (µmol/24h /g BW)	33.5±1.1	24.3±0.7	23.0±0.6	23.6±1.0	24.8±1.8	32.9±1.1
Urine Ca^{2+} (µmol/24h/ g BW)	0.59±0.05	0.13±0.01	0.14±0.02	0.12±0.01	0.11±0.01	0.41±0.03
Creatinine clearance (µl/min/ g BW)	5.8±0.5	7.5±0.9	6.5±0.9	6.1±0.9	5.5±0.3	6.4±0.5
Fractional excretion Na^+ (%)	1.04±0.05	1.99±0.15#	5.78±0.82#	6.22±1.10#	2.35±0.26#	0.93±0.04
Fractional excretion K^+ (%)	77.4±3.8	123.7±7.0#	159.3±24.4#	175.6±29.1#	139.0±16.1#	69.1±4.7*
Fractional excretion Ca^{2+} (%)	2.6±0.2	1.4±0.1#	1.7±0.2	1.7±0.3	1.3±0.1	1.6±0.1*

Table nr. 2 - Effect of low-calcium diet on body weight, food and fluid intake, plasma concentrations and urinary excretion of electrolytes.

Body weight, food and fluid intake, hematocrit, Na^+, K^+ and Ca^{2+} concentrations in plasma, urinary flow rate, renal excretions of Na^+, K^+ and Ca^{2+}, creatinine clearance and fractional renal excretions of Na^+, K^+ and Ca^{2+} in both $sgk1^{-/-}$ and in $sgk1^{+/+}$ mice under control (n=30 animals each) and low-Ca^{2+} diet (n=6 animals each) with free access to tap water or 1% saline. Fecal Ca^{2+} excretion was measured in n=6 animals each. Values are arithmetic means ± SEM. N.d. - Not determined; #$p<0.05$, statistically significant difference between diets; *$p<0.05$, statistically significant difference between genotypes.

To explore the sensitivity of renal Ca^{2+} excretion to inhibition of Na^+ reabsorption, the NKCC2 inhibitor furosemide (20 µg/g bw) was applied with or without the carboanhydrase inhibitor acetazolamide (50 µg/g bw). Furosemide alone increased renal Ca^{2+} excretion in *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice and abolished the difference of Ca^{2+} excretion between genotypes (Table nr. 3). The additional administration of acetazolamide did not further increase natriuresis or calciuria (Table nr. 3).

	Control (n=12)		**Furosemide (n=12)**		**Furosemide+ Acetazolamide (n=12)**	
	sgk1$^{+/+}$	**sgk1$^{-/-}$**	**sgk1$^{+/+}$**	**sgk1$^{-/-}$**	**sgk1$^{+/+}$**	**sgk1$^{-/-}$**
Urine Na^+ (µmol/mg crea)	710±54	868±57	3224±857$^{\#}$	4227±710$^{\#}$	3356±307$^{\#}$	2989±246$^{\#}$
Urine K^+ (µmol/mg crea)	1905±118	1973±155	1909±180	2519±239	1594±92	1879±84*
Urine Ca^{2+} (µmol/mg crea)	30±3	22±2	65±22	64±16$^{\#}$	38±4	40±4$^{\#}$

Table nr. 3 - Effect of furosemide and furosemide plus acetazolamide on urinary electrolyte excretion.

Renal excretions of Na^+, K^+ and Ca^{2+} (µmol/mg creatinine) in both *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice (n=12 animals each) under control diet with free access to tap water or after intraperitoneal injection with 20 µg furosemide/g bw with or without additional 50 µg acetazolamide/g bw. Excretion of electrolytes was expressed as ratio between the urinary concentration of the respective electrolyte and urinary creatinine concentration. Values are arithmetic means ± SEM #p<0.05, statistically significant difference between diets; *p<0.05, statistically significant difference between genotypes.

Characterization of *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice under DOCA/high salt

Prior to treatment, fluid intake was similar in gene-targeted mice lacking functional SGK1 (*sgk1*$^{-/-}$) and in their wildtype littermates (*sgk1*$^{+/+}$) (Table nr. 4). Treatment with DOCA/high salt led to a marked increase of fluid and Na^+ intake in both genotypes. Three weeks after the initiation of DOCA/high salt treatment, the fluid intake increased by 3.0 ± 0.2 - fold (n=9) in *sgk1*$^{-/-}$ mice, and by 11.1 ± 1.9 - fold (n=9) in *sgk1*$^{+/+}$ mice. Fluid intake increased further in *sgk1*$^{+/+}$ mice during treatment and was significantly larger in *sgk1*$^{+/+}$ mice than in *sgk1*$^{-/-}$ mice throughout the DOCA/high salt treatment (Figure nr. 19).

After 12 weeks of treatment, body weight had increased significantly (p<0.05) more in the *sgk1*$^{+/+}$ mice (+4.6 ± 0.9 g) than in the *sgk1*$^{-/-}$ mice (+1.7 ± 1.0 g). The fluid and food intake and the respective Na^+ content allowed an approximate estimate of Na^+ intake (Table nr. 4), which was again significantly larger in *sgk1*$^{+/+}$ mice than in *sgk1*$^{-/-}$ mice under DOCA/high salt treatment.

	Water		DOCA 1% NaCl (week 12)	
	$sgk1^{+/+}$	**$sgk1^{-/-}$**	**$sgk1^{+/+}$**	**$sgk1^{-/-}$**
Body Weight (g)	26.2 ± 1.04	26.6 ± 1.4	31.0 ± 1.1 #	29.3 ± 1.2 #
Fluid Intake (ml/24h)	5.7 ± 0.4	5.6 ± 0.5	80.8 ± 15.8 #	13.8 ± 3.9 *, #
Food Intake (g/24h)	3.5 ± 0.2	3.6 ± 0.2	4.9 ± 0.2 #	4.2 ± 0.2
Daily Na^+ Intake (µmol/24h)	280 ± 29	313 ± 21	296441 ± 6737 #	6349 ± 1699 *, #
Daily Ca^{2+} Intake (µmol/24h)	723 ± 75	809 ± 55	1090 ± 43	950 ± 41
Daily K^+ Intake (µmol/24h)	824 ± 85	922 ± 62	1243 ± 49	1082 ± 47
Hematocrit (%)	54 ± 1	53 ± 1	51 ± 1	51 ± 1
[Na^+]plasma (mM)	151 ± 2	151 ± 2	141 ± 6	137 ± 5
[K^+]plasma (mM)	4.3 ± 0.2	4.4 ± 0.3	3.2 ± 0.3	3.4 ± 0.2
[Ca^{2+}] plasma (mM)	2.4 ± 0.07	2.4 ± 0043	2.3 ± 0.07	2.3 ± 0.05

Table nr. 4 - Effect of DOCA/high salt treatment prior and 12 weeks after treatment on urinary electrolyte excretion.

Body weight, intake of fluid, food, Na^+, Ca^{2+} and K^+ **,** hematocrit, Na^+, Ca^{2+} and K^+ concentrations in plasma prior to and 12 weeks following DOCA / 1% NaCl treatment. * indicates significant difference between *$sgk1^{-/-}$* and *$sgk1^{+/+}$*, # indicates significant difference to respective baseline (water) value.

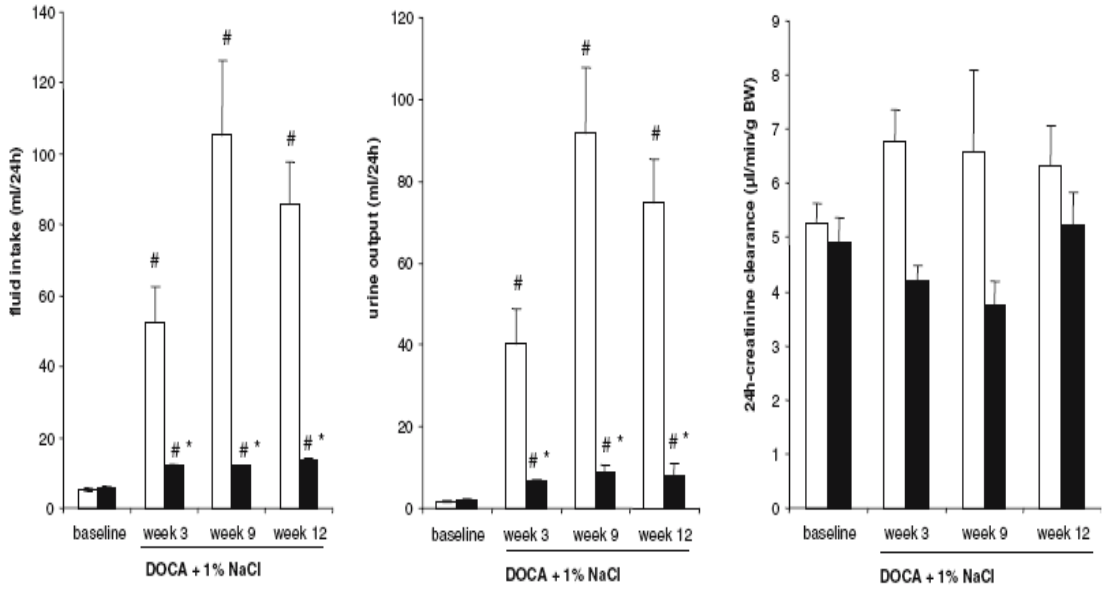

Figure nr. 19 - Fluid intake, urinary flow, and 24-h-creatinine clearance prior to and following DOCA/high salt treatment.

Arithmetic means ± SEM of fluid intake (left panel), urinary output (middle panel), and 24-hcreatinine clearance (right panel) in SGK1 knockout mice (*$sgk1^{-/-}$*, closed bars, n=9) and their wild-type littermates (*$sgk1^{+/+}$*, open bars, n=9) prior to (baseline) and following 3-, 9- and 12-week treatments with DOCA/high salt. * indicate significant difference between *$sgk1^{-/-}$* and *$sgk1^{+/+}$*, # indicate significant difference from respective baseline value.

The treatment was followed by hypokalemia in both genotypes (Table nr. 4). Twenty-four-hour-creatinine clearance and urinary output were similar in both genotypes prior to the treatment (Figure nr. 19). DOCA/high salt treatment tended to increase creatinine clearance in *sgk1*$^{+/+}$ mice and tended to decrease creatinine clearance in *sgk1*$^{-/-}$ mice, with effects not reaching statistical significance. Within 3 weeks of DOCA/high salt treatment, the urinary volume increased significantly by 24.8 ± 5.0-fold in *sgk1*$^{+/+}$ mice and 3.9 ± 0.4-fold (n=9) in *sgk1*$^{-/-}$ mice. In *sgk1*$^{+/+}$ mice, urinary volume increased further within 9 and 12 weeks (Figure nr. 19).

Moreover, the DOCA/high salt treatment significantly increased the fractional excretion of Na^+ and Ca^{2+} and tended to increase the fractional excretion of K^+ in both genotypes (Figure nr. 20).

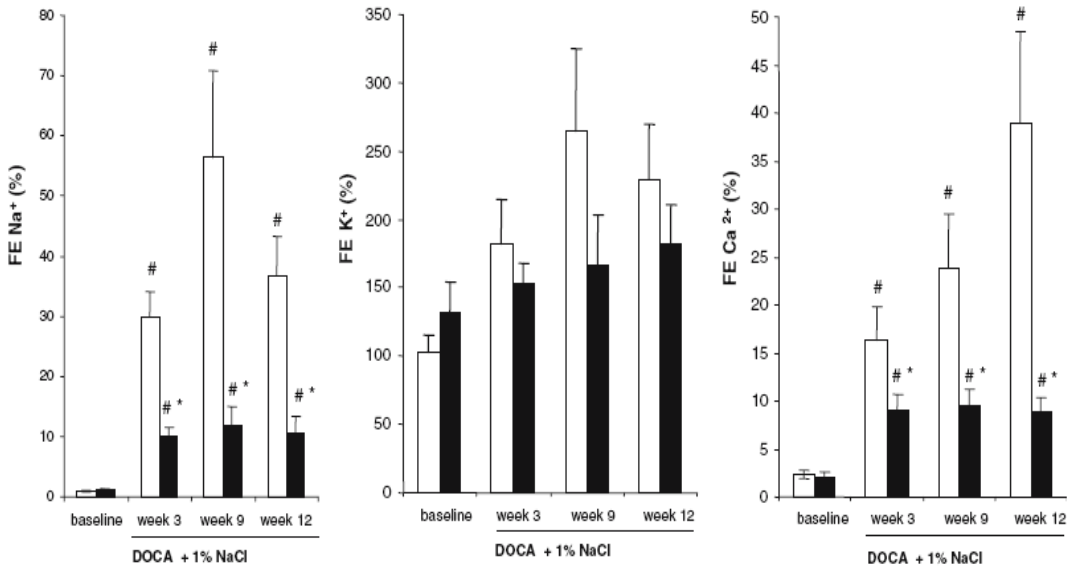

Figure nr. 20 - Fractional excretions of Na^+, K^+ and Ca^{2+} prior to and following DOCA/high salt treatment.

Arithmetic means ± SEM of fractional excretions of Na^+ (FENa, left panel), K^+ (FEK, middle panel) and Ca^{2+} (FECa, right panel) in *sgk1*$^{-/-}$ mice (closed bars, n=9) and *sgk1*$^{+/+}$ mice (open bars, n=9) prior to (baseline) and following 3-, 9-, and 12-week treatments with DOCA/high salt. * indicate significant difference between *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$, # indicate significant difference from respective baseline value.

The fractional Na^+ excretion was similar in both genotypes prior to the treatment (Figure nr. 19). Within 3 weeks of DOCA/high salt treatment, the fractional Na^+ excretion increased 42.7 ± 8.8-fold in *sgk1*$^{+/+}$ mice and 14.5 ± 3.6-fold (n=9) in *sgk1*$^{-/-}$ mice. The increase of fractional Na^+ excretion was significantly larger in *sgk1*$^{+/+}$ mice than in *sgk1*$^{-/-}$ mice. The increase of fractional K^+ excretion tended to be larger in *sgk1*$^{+/+}$ mice, a difference, however, not reaching statistical significance (Figure nr. 20). Under baseline conditions, the fractional Ca^{2+} excretion was similar in *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice. Within 3 weeks of DOCA/high salt treatment, the fractional Ca^{2+} excretion increased 11.5 ± 3.7-fold in sgk1$^{+/+}$ mice and 6.0 ± 1.3-fold (n=9) in *sgk1*$^{-/-}$ mice. As a consequence, fractional Ca^{2+} excretion was larger in *sgk1*$^{+/+}$ mice than in *sgk1*$^{-/-}$ mice. Because in *sgk1*$^{+/+}$ mice fluid intake increased further within 9 weeks of DOCA/high salt treatment, the fractional excretion of Na^+ similarly increased further (Figure nr. 20). The additional natriuresis was paralleled by additional calciuria (Figure nr. 20).

Prior to the treatment of the mice with DOCA/high salt, blood pressure was similar in *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice (Figure nr. 21). DOCA/high salt treatment for 7 weeks led to a statistically significant increase of systolic blood pressure in both genotypes (Figure nr. 21), pointing to SGK1-independent effects of mineralocorticoids on blood pressure. However, within the following weeks of continued DOCA/high salt treatment, systolic blood pressure

increased further in *sgk1*$^{+/+}$ mice but not in *sgk1*$^{-/-}$ mice. Thus, 12 weeks after beginning of DOCA/high salt treatment, blood pressure was significantly higher in *sgk1*$^{+/+}$ mice than in *sgk1*$^{-/-}$ mice.

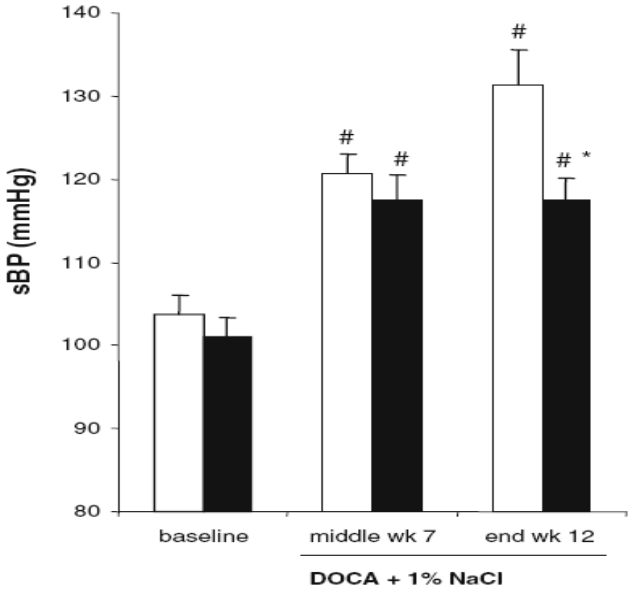

Figure nr. 21 - Blood pressure prior to and following DOCA/high salt treatment.
Arithmetic means ± SEM of systolic blood pressure (sBP) in *sgk1*$^{-/-}$ mice (closed bars, n=9) and *sgk1*$^{+/+}$ mice (open bars, n=9) prior to (baseline) and following 7- and 12-week treatments with DOCA/high salt. * indicates significant difference between *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$, # indicate significant difference from respective baseline value.

Urinary albumin excretion as an indicator of glomerular hyperpermeability was markedly increased following treatment of *sgk1*$^{+/+}$ mice with DOCA/high salt (Figure nr. 22).

The increase of albuminuria was almost absent in *sgk1*$^{-/-}$ mice, even though the increase of systemic blood pressure was similar up to 7 weeks of treatment of *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice. Thus, SGK1 is required for the development of severe albuminuria following DOCA/high salt treatment.

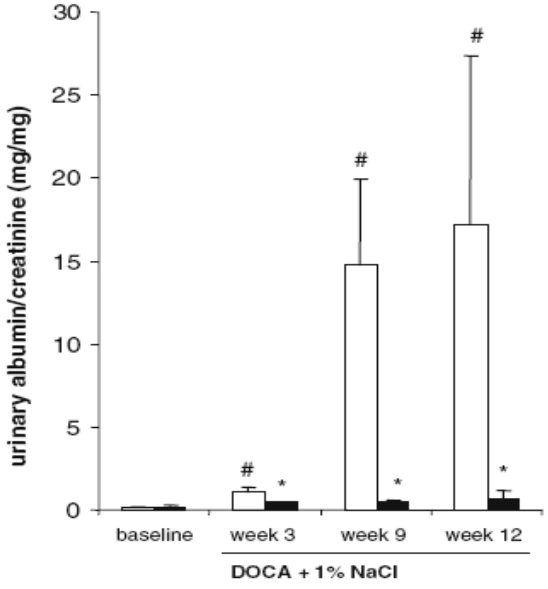

Figure nr. 22- Urinary albumin excretion prior to and following DOCA/high salt treatment.
Arithmetic means ± SEM of urinary albumin excretion in *sgk1*$^{-/-}$ mice (closed bars, n=9) and *sgk1*$^{+/+}$ mice (open bars, n=9) prior to (baseline) and following 3-, 9-, and 12-week treatments with DOCA/high salt. * indicate significant difference between *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$, # indicate significant difference from respective baseline value.

Kidney weight significantly increased following DOCA/high salt treatment in *sgk1*$^{+/+}$ mice only (Figure nr. 23).

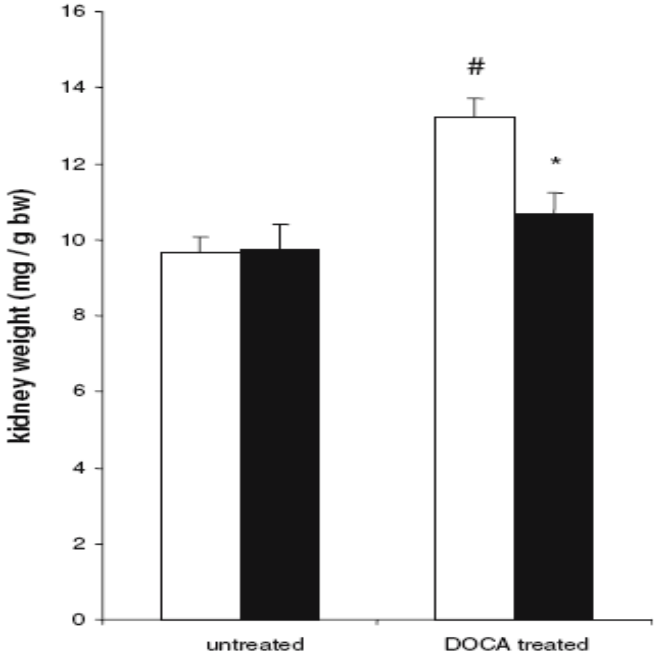

Figure nr. 23 - Kidney weights following DOCA/high salt treatment.
Arithmetic means ± SEM of kidney weights from DOCA/high salt treated *sgk1*$^{+/+}$ (open bars) and *sgk1*$^{-/-}$ mice (closed bars, n=9 each), as well as from untreated age- and gender-matched *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice (n=8 each). * indicates significant difference between *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice, #indicates significant difference from untreated mice.

Histological analysis revealed a marked influence of DOCA/high salt treatment on the morphology of kidneys from *sgk1*$^{+/+}$ mice (Figure nr. 24). The treatment was followed by marked glomerular enlargement with segmental glomerulosclerosis and tubulointerstitial fibrosis in *sgk1*$^{+/+}$ mice, effects clearly blunted in *sgk1*$^{-/-}$ mice (Tables nr. 5 and 6).

Parameter	***sgk1*$^{+/+}$ (n=5)**	***sgk1*$^{-/-}$ (n=5)**	**P-value**
glomerular volume in µm³	440 ± 23	363 ± 23	P=0.045
glomerular sclerosis index (GSI)	2.21 ± 0.26	1.85 ± 0.16	P=0.27
mesangial sclerosis index (MSI)	0.62 ± 0.07	0.85 ± 0.14	P=0.18
tubulointerstitial sclerosis index (TSI)	1.24 ± 0.42	0.15 ± 0.09	P=0.036
vascular sclerosis index (VSI)	0.67 ± 0.03	0.65 ± 0.08	P=0.83

Table nr. 5 - Histomorphological data after 12 weeks of DOCA/high salt treatment (paraffin sections).
Arithmetic means ± SEM of kidney parameteres from DOCA/high salt treated *sgk1*$^{+/+}$ (open bars) and *sgk1*$^{-/-}$ mice (closed bars, n=9 each). * indicates significant difference between *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice, #indicates significant difference from untreated mice.

Stereological analysis of semithin sections revealed no difference in glomerular capillarization between *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice. The number of podocytes and endothelial cells per glomerulus was also not significantly different between the two groups (Table nr. 6). Mesangial cell number, however, was significantly higher in *sgk1*$^{-/-}$ mice compared to *sgk1*$^{+/+}$ mice.

Parameter	*sgk1*$^{+/+}$ (n=5)	*sgk1*$^{-/-}$ (n=5)	p-value
mesangial cell number per glomerulus	283 ± 15	351 ± 20	p<0.05
endothelial cell number per glomerulus	188 ± 19	188 ± 18	n.s.
podocyte number per glomerulus	127 ± 5	119 ± 5	n.s.
mesangial cell volume (μm^3)	88 ± 8	80 ± 3	n.s.
endothelial cell volume (μm^3)	78.5 ± 3	80 ± 2	n.s.
podocyte volume (μm^3)	281 ± 46.1	325 ± 21	n.s.
length density of glomerular capillaries (mm/mm^3)	10438 ± 2022	11566 ± 2067	n.s.

Table nr. 6 - Glomerular cells and capillaries after 12 weeks of DOCA/high salt treatment (semithin sections).

Arithmetic means ± SEM of kidney parameters from DOCA/high salt treated *sgk1*$^{+/+}$ (open bars) and *sgk1*$^{-/-}$ mice (closed bars, n=5 each). * indicates significant difference between *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice, #indicates significant difference from untreated mice.

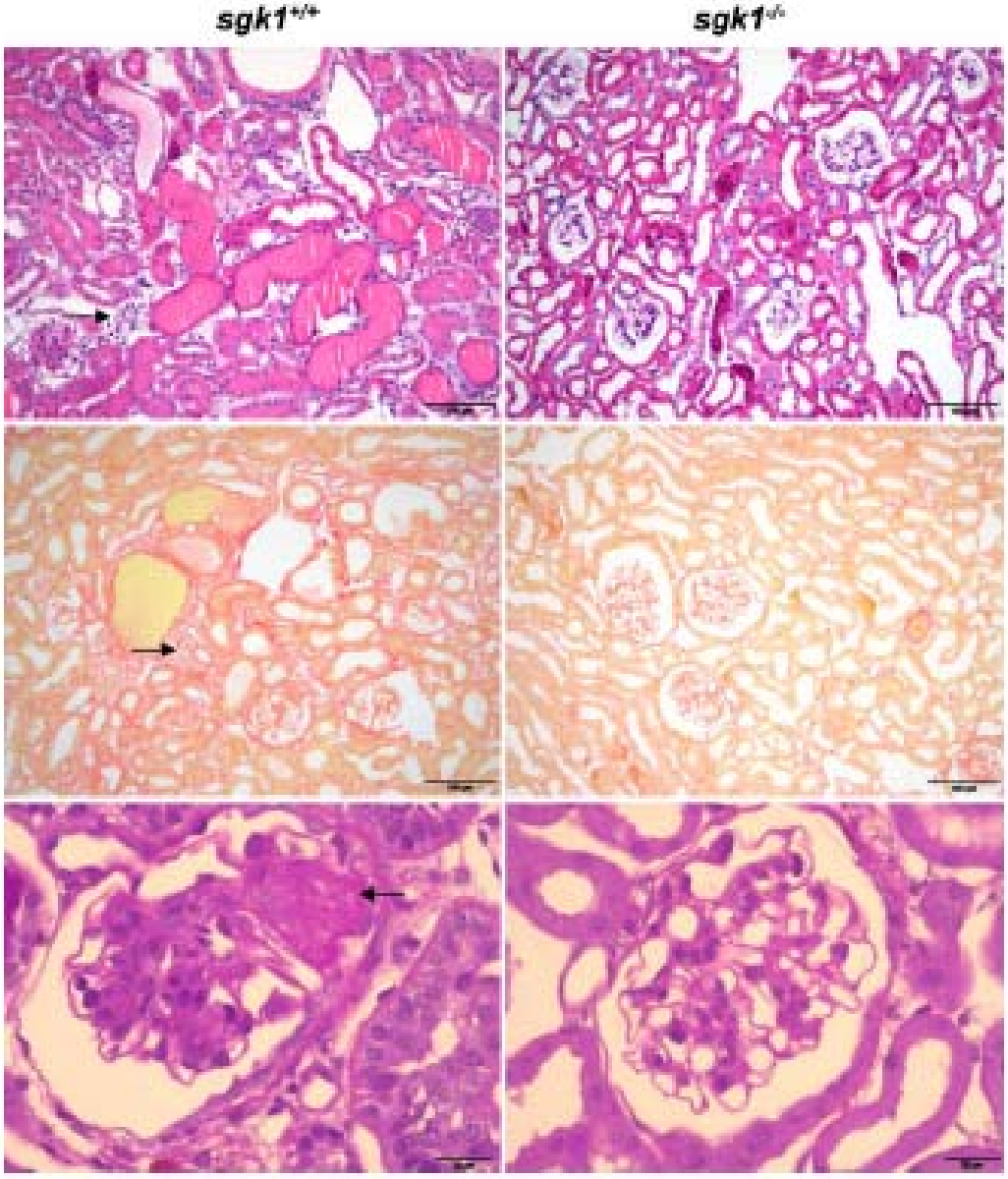

Figure nr. 24 - Morphology of kidneys following DOCA/high salt treatment.

HE (upper and lower panels) and Sirius red fibrous staining (middle panel) of renal tissue from *sgk1*$^{-/-}$ (right) and *sgk1*$^{+/+}$ mice (left) following a 12-week treatment with DOCA/high salt. As indicated by arrows, marked segmental glomerulosclerosis and tubulointerstitial inflammation with interstitial fibrosis is seen in *sgk1*$^{+/+}$ and not in *sgk1*$^{-/-}$ mice.

Characterization of *sgk1*$^{-/-}$/ *sgk3*$^{-/-}$ mice

As shown in Figure nr. 25, hair growth was markedly delayed in *sgk1*$^{-/-}$/ *sgk3*$^{-/-}$ mice. The time course of hair growth of the *sgk1*$^{-/-}$/ *sgk3*$^{-/-}$ mice was virtually identical to that of the SGK3-knockout mouse (McCornick JA. et al., (2004) *Mol Biol Cell*).

Figure nr. 25 - Hair growths on *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ and *sgk1*$^{-/-}$/*sgk3*$^{-/-}$.
Representative photographs of hair growth on serum- and glucocorticoid-inducible kinase SGK1 and SGK3 double-knockout (*sgk1*$^{-/-}$/ *sgk3*$^{-/-}$) (right) mice and their wild-type littermates (*sgk1*$^{+/+}$/*sgk3*$^{+/+}$) (left) 10 wk after birth.

After birth, body weight gain was slightly slower in (*sgk1*$^{-/-}$/*sgk3*$^{-/-}$) than in wildtype (Figure nr. 25), but after 12 weeks both groups attained an almost identical body weight. Thus, at the time of the experiments, body weight was not significantly different between *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ and *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice (Table nr. 7). The low-salt diet did not significantly alter body weight (Table nr. 7). Food intake per body weight was slightly, but significantly, greater in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ than in *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ mice fed the control ($p<0.0084$) and low-salt ($p<0.0013$) diets (Table nr. 7). Food intake tended to increase after the low-salt diet, but the change did not reach statistical significance ($p< 0.055$ for *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ and $p< 0.057$ for *sgk1*$^{-/-}$/*sgk3*$^{-/-}$).

Fluid intake in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice similarly tended to increase after a salt-deficient diet and tended to be greater in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ than in *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ mice; again, the differences did not reach statistical significance. In *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice fed the low-salt diet, fluid intake was significantly increased (Table nr. 8).

	Control diet		Low salt diet	
	$sgk1^{+/+}/sgk3^{+/+}$	**$sgk1^{-/-}/sgk3^{-/-}$**	**$sgk1^{+/+}/sgk3^{+/+}$**	**$sgk1^{-/-}/sgk3^{-/-}$**
Body Weight (g)	27.4 ± 0.8 (27)	26.3 ± 0.6 (24)	27.8 ± 1.7 (9)	25.8 ± 1.3 (9)
Food intake (mg/g BW)	103.4 ± 5.6	125.1 ± 5.5*	118.7 ± 4.8 (24)	143.8 ± 4.3 (9)*
Fecal dry weight (mg/g BW)	11.9 ± 1.0 (9)	15.1 ± 0.9 (9)*	11.2 ± 0.6 (9)	12.5 ± 0.6 (9)#
Fluid Intake (µl/24h g BW)	194 ± 29 (27)	234 ± 29 (22)	122 ± 11 (9)	326 ± 94 (9)
Urinary flow (µl/24 h g BW)	79.2 ± 16.2 (27)	88.8 ± 16.9 (23)	46.5 ± 5.4 (9)	76.3 ± 13.1 (8)
Hematocrit (%)	49.9 ± 0.6 (9)	50.7 ± 1.0 (9)	50.1 ± 0.7 (9)	50.0 ± 0.9 (9)
$[Na^+]$plasma (mM)	141.7 ± 1.1 (9)	143.2 ± 1.6 (9)	141.3 ± 1.2 (9)	143.1 ± 0.5 (9)
$[K^+]$plasma (mM)	4.1 ± 0.2 (9)	4.2 ± 0.2 (9)	4.3 ± 0.1 (9)	4.5 ± 0.1 (9)
Creatinine clearance (µl/min/g BW)	5.4 ± 0.4 (9)	5.0 ± 0.5 (9)	4.6 ± 0.5 (9)	5.2 ± 9.5 (8)
Urine Na^+ (µmol/24h g BW)	5.49 ± 0.57 (27)	7.21 ± 0.69 (22)*	0.4 ± 0.1 (9)#	1.18 ± 0.18 (8)*#
Urine K^+ (µmol/24h g BW)	14.8 ± 1.7 (27)	17.6 ± 1.6 (22)	11.7 ± 1.3 (9)	17.6 ± 1.3 (7)*
Fecal Na^+ (µmol/24h g BW)	4.0 ± 0.6 (9)	4.6 ± 0.2 (9)	0.9 ± 0.2 (9)#	0.6 ± 0.1 (9)#
Fecal K^+ (µmol/24h g BW)	1.9 ± 0.2 (9)	2.5 ± 0.2 (9)	2.2 ± 0.3 (9)	2.1 ± 0.3 (9)
Fractional excretion Na^+ (%)	0.54 ± 0.07 (9)	0.79 ± 0.09 (9)*	0.04 ± 0.01 (9)	0.12 ± 0.03 (8)*
Fractional excretion K^+ (%)	42.0 ± 6.2 (9)	57.3 ± 5.8 (9)	40.0 ± 5.0 (9)	67.1 ± 9.6 (8) *

Table nr. 7 - Physiological parameters for *$sgk1^{-/-}/sgk3^{-/-}$* vs. *$sgk1^{+/+}/sgk3^{+/+}$* mice fed control and low-salt diets.

Body weight, food and fluid intake**,** hematocrit, Na^+ and K^+ concentrations in plasma, fecal weight, fecal and renal excretions of Na^+ and K^+, creatinine clearance and fractional renal excretions of Na^+ and K^+, in both *$sgk1^{-/-}/sgk3^{-/-}$* and in *$sgk1^{+/+}/sgk3^{+/+}$* mice under control and low salt diet. Arithmetic means ± SEM (number of animals studied); # $p<0.05$ control diet vs. low salt diet; * $p<0.05$ *$sgk1^{-/-}/sgk3^{-/-}$* vs. *$sgk1^{+/+}/sgk3^{+/+}$*.

Fecal dry weight was significantly higher in *$sgk1^{-/-}/sgk3^{-/-}$* mice fed the control diet; this difference disappeared when these animals were fed the low-salt diet (Table nr.7). As expected, fecal Na^+ excretion significantly decreased in *$sgk1^{+/+}/sgk3^{+/+}$* ($p<0.0002$) and *$sgk1^{-/-}/sgk3^{-/-}$* ($p<0001$) mice fed the low-salt diet. However, no significant differences were observed in fecal Na^+ excretion between *$sgk1^{+/+}/sgk3^{+/+}$* and *$sgk1^{-/-}/sgk3^{-/-}$* mice fed either diet. Fecal K^+ excretion was similar in *$sgk1^{+/+}/sgk3^{+/+}$* and *$sgk1^{-/-}/sgk3^{-/-}$* mice fed either diet (Table nr. 7).

No significant differences were observed in hematocrit between *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ and *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice fed either diet or between the control diet and the low-salt diet in either genotype (Table nr.8). Similarly, plasma concentrations of Na^+ and K^+ were not significantly modified by the diet in either genotype and were virtually identical in *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ and *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice fed both diets (Table nr. 7). Plasma K^+ concentration tended to increase on initiation of the low-salt diet and tended to be higher in *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ than in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice (Table nr. 7). None of these apparent differences reached statistical significance.

	Control diet		**Low salt diet**	
	sgk1$^{+/+}$	**Sgk1$^{-/-}$**	**sgk1$^{+/+}$**	**sgk1$^{-/-}$**
Body Weight (g)	30.1±0.7	32.8±1.6	27.8±0.6$^{\#}$	29.9±1.5$^{\#}$
Food intake (mg/g BW)	120±8	116±6	111±5	83±5
Fecal dry weight (mg/g BW)	10.5±0.7	10.4±0.6	11.6±0.9	8.6±0.7
Fecal Na^+ (µmol/24h g BW)	1.5±0.2	1.6±0.1	2.2±0.3	1.5±0.2
Fecal K^+ (µmol/24h g BW)	1.5±0.3	1.9±0.2	2.7±0.4	1.6±0.3
Fluid Intake (µl/24h g BW)	187±27	162±13	286±29$^{\#}$	302±46$^{\#}$
Urinary flow (µl/24 h g BW)	79±23	48±5	126±22	122±33$^{\#}$
Hematocrit (%)	51.5±1.2	51.2±0.5	57.1±1.3$^{\#}$	58.1±1.1$^{\#}$
$[Na^+]_{plasma}$ (mM)	148±0.8	148±0.5	146±1.8	146±0.7
$[K^+]_{plasma}$ (mM)	4.5±0.1	4.6±0.2	5.3±0.2$^{\#}$	5.1±0.3
24h-Creatinine clearance (µl/min/g BW)	5.0±0.4	5.6±1.2	2.6±0.2$^{\#}$	2.9±0.2$^{\#}$
Urine Na^+ (µmol/24h g BW)	8.0±0.5	6.7±0.4	0.28±0.05$^{\#}$	0.51±0.09$^{\#*}$
Urine K^+ (µmol/24h g BW)	13.1±0.9	11.3±0.7	13.0±0.9	10.3±0.4
Fractional excretion Na^+ (%)	0.79±0.07	0.67±0.08	0.05±0.01$^{\#}$	0.09±0.02
Fractional excretion K^+ (%)	40.5±4.7	36.0±4.3	68.9±6.2$^{\#}$	50.2±1.9$^{\#}$

Table nr. 8 - Physiological parameters for *sgk1*$^{-/-}$ vs. *sgk1*$^{+/+}$ mice fed control and low-salt diets.

Body weight, food and fluid intake, hematocrit, Na+ and K+ concentrations in plasma, fecal weight, fecal and renal excretions of Na+ and K+, creatinine clearance and fractional renal excretions of Na^+ and K^+, in both sgk1$^{-/-}$ and in sgk1$^{+/+}$ mice under control and low salt diet. Arithmetic means ± SEM of n=8 animals studied; # $p<0.05$ control diet vs. low salt diet; * $p<0.05$ sgk1$^{-/-}$ vs. sgk1$^{+/+}$.

Plasma aldosterone concentration was significantly ($p<0.01$) higher in $sgk1^{-/-}/sgk3^{-/-}$ than in $sgk1^{+/+}/sgk3^{+/+}$ mice fed the standard diet (Figure nr. 26). Over 7 days, plasma aldosterone concentration markedly increased in $sgk1^{-/-}/sgk3^{-/-}$ mice fed the low-salt diet ($p<0.01$). The effect of the low-salt diet on plasma aldosterone concentration did not reach statistical significance ($p<0.17$) in $sgk1^{+/+}/sgk3^{+/+}$ mice. Plasma aldosterone concentration was also higher in $sgk1^{-/-}$ than in $sgk1^{+/+}$ mice fed the control diet (71 ± 35 vs. 42 ± 12 pg/ml; n =5 each) and the low-salt diet (4515 ± 949 vs. 3395 ± 350 pg/ml; n =5 each).

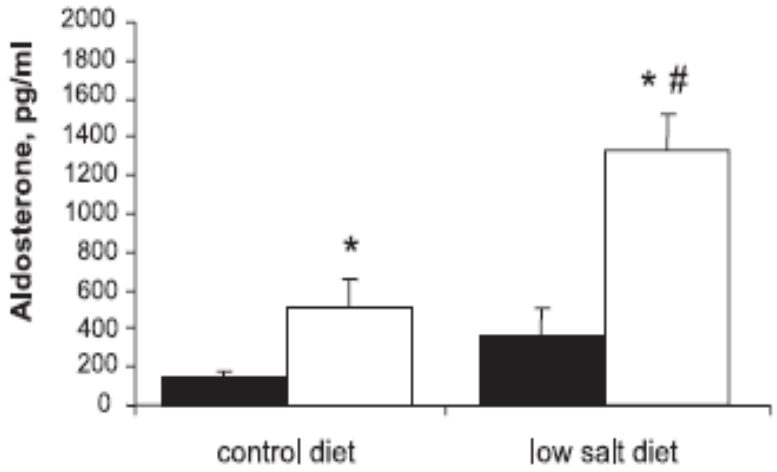

Figure nr. 26 - Plasma aldosterone concentrations in $sgk1^{-/-}/sgk3^{-/-}$ and $sgk1^{+/+}/sgk3^{+/+}$

Plasma aldosterone concentrations in $sgk1^{-/-}/sgk3^{-/-}$ (open bars) and $sgk1^{+/+}/sgk3^{+/+}$ (solid bars) mice fed a control or a low-salt diet for 7 days. Values are means ± SE (n=7–8 animals in each group). *significantly different ($p<0.05$) from $sgk1^{+/+}/sgk3^{+/+}$. # significantly different ($p< 0.05$) from control diet.

No significant differences were observed in creatinine clearance and urinary flow rate between $sgk1^{-/-}/sgk3^{-/-}$ and $sgk1^{+/+}/sgk3^{+/+}$ mice fed either diet or between the control and the low-salt diet in either genotype (Table nr. 6). Urinary sodium excretion decreased significantly in $sgk1^{+/+}/sgk3^{+/+}$ ($p<0.0002$) and $sgk1^{-/-}/sgk3^{-/-}$ ($p<0.0001$) mice fed the low-salt diet. Under control ($p<0.05$) and low-salt ($p<0.005$) diet conditions, urinary Na^+ excretion was significantly higher in $sgk1^{-/-}/sgk3^{-/-}$ than in $sgk1^{+/+}/sgk3^{+/+}$ mice (Table nr. 7). The fractional Na^+ excretion was again significantly decreased by the low-salt diet in $sgk1^{+/+}/sgk3^{+/+}$ ($p<0.0001$) and $sgk1^{-/-}/sgk3^{-/-}$ ($p<0.0001$) mice. Fractional Na^+ excretion was significantly higher in $sgk1^{-/-}/sgk3^{-/-}$ than in $sgk1^{+/+}/sgk3^{+/+}$ mice fed either diet.

Absolute and fractional urinary K^+ excretion tended to be higher in $sgk1^{-/-}/sgk3^{-/-}$ than in $sgk1^{+/+}/sgk3^{+/+}$ mice, a difference reaching statistical significance when the animals were fed the low-salt diet ($p<0.03$). The low-salt diet did not significantly influence absolute or fractional K^+ excretion in $sgk1^{+/+}/sgk3^{+/+}$ mice, whereas it tended to increase K^+ excretion in $sgk1^{-/-}/sgk3^{-/-}$ mice (Table nr.7).

Blood pressure was significantly ($p<0.05$) lower in $sgk1^{-/-}/sgk3^{-/-}$ than in $sgk1^{+/+}/sgk3^{+/+}$ mice fed the control diet (103 ± 4 vs. 114 ± 3 mmHg; Figure nr.27). Neither the low-salt (2 wk) nor the high-salt diet significantly influenced systemic blood pressure. Blood pressure remained significantly lower in $sgk1^{-/-}/sgk3^{-/-}$ than in $sgk1^{+/+}/sgk3^{+/+}$ mice fed low-salt ($p<0.05$) and high-salt ($p<0.05$) diets (Figure nr. 27). In $sgk1^{-/-}$ mice fed the control diet, blood pressure was similarly lower than in $sgk1^{+/+}$ mice (106 ± 4 vs.121 ± 4 mmHg; n =8 each, $p<0.05$).

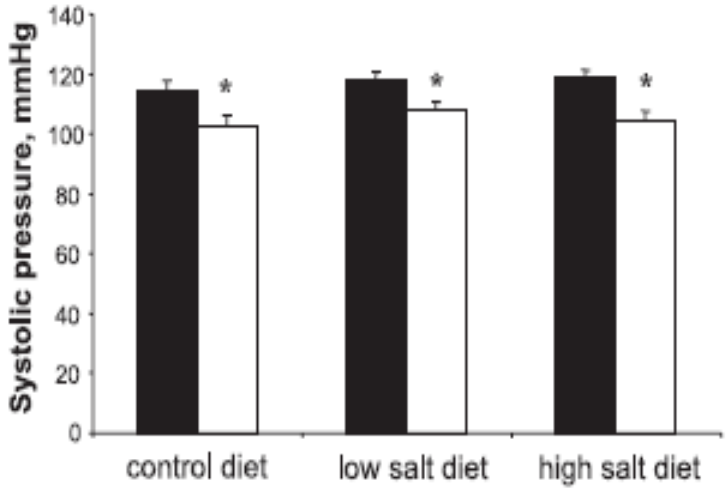

Figure nr. 27 - Systolic blood pressure in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ and *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ under different diets.

Systolic blood pressure in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ (open bars) and *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ (solid bars) mice fed a control diet, a low-salt diet, or a high-salt diet for 14 days. Values are means SE (n = 8 animals in each group). *Significantly different (P <0.05) from *sgk1*$^{+/+}$/*sgk3*$^{+/+}$.

Quantitative real-time PCR of liver and kidney did not reveal statistically significant differences between the genotypes in the levels of mRNA encoding SGK2 (ratio of SGK2 mRNA to GAPDH mRNA) in liver or kidney (Figure nr.28).

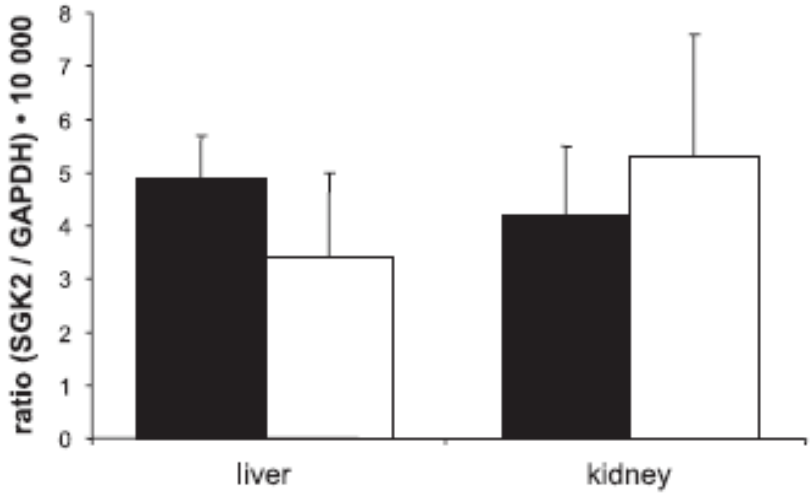

Figure nr. 28 - SGK2 transcript levels in liver and kidney from *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ and *sgk1*$^{+/+}$/*sgk3*$^{+/+}$.

SGK2 transcript levels in liver and kidney from *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ (open bars) and *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ (solid bars) mice shown as ratio of SGK2 mRNA to GAPDH mRNA (i.e., mRNA encoding SGK2) in liver and kidney. Values are means ± SE (n = 4–5 animals in each group).

6. Discussion

Characterization of *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice under normal and low Ca^{2+} diet

The present study reveals that under the control diet the abundance of TRPV5 in the cell membrane of the distal nephron, particularly in connecting tubules, is lower in mice lacking functional SGK1 (*sgk1*$^{-/-}$) than in their wildtype littermates (*sgk1*$^{+/+}$). The difference was evident only in the membrane fraction but not in the transcript levels. This observation could be taken as evidence of SGK1-dependent regulation of TRPV5 membrane trafficking in this nephron segment, confirming the *in vitro* capacity of SGK1 to enhance TRPV5 protein abundance and activity in the cell membrane (Embark HM. et al., (2004) *Cell Physiol Biochem*; Palmada M. et al., (2005) *Cell Physiol Biochem*). However, the decrease in TRPV5 activity is expected to cause calciuria. Similarly, the decrease in calbindin D-28K protein abundance would be expected to decrease renal tubular Ca^{2+} reabsorption and thus to enhance renal Ca^{2+} elimination. The opposite is observed, i.e., the absolute and fractional excretion of Ca^{2+} is lower in *sgk1*$^{-/-}$ than in *sgk1*$^{+/+}$ mice despite normal plasma calcitriol and parathyroid hormone concentrations in both genotypes. The relative anticalciuria of the *sgk1*$^{-/-}$ mice may be the result of the extracellular volume contraction, which is reflected in those mice by enhanced plasma aldosterone concentration even under standard NaCl intake (Wulff P. et al., (2002) *J Clin Invest*).

SGK1 mediates part of the stimulating effect of aldosterone on the renal epithelial Na^+ channel (ENaC) (Pearce D. et al., (2003) *Cell Physiol Biochem*; Verrey F. et al., (2003) *Cell Physiol Biochem*) and thus the lack of SGK1 leads to impaired Na^+ reabsorption in the terminal nephron segments (Wulff P. et al., (2002) *J Clin Invest*). As a result, the Na^+ reabsorption in the proximal tubule and loop of Henle are enhanced. Micropuncture experiments documented the increase in proximal tubular Na^+ reabsorption in *sgk1*$^{-/-}$ mice (Wulff P. et al., (2002) *J Clin Invest*).

Ca^{2+} reabsorption in the proximal tubule and loop of Henle parallels the Na^+ reabsorption (Friedman PA. et al., (1995) *Physiol Rev*) and is thus expected to be enhanced in *sgk1*$^{-/-}$ as compared to *sgk1*$^{+/+}$ mice. Indeed, the difference in Ca^{2+} excretion between *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice is dissipated after treatment with the NKCC2 inhibitor furosemide, which is expected to blunt not only Na^+ but also Ca^{2+} reabsorption in the loop of Henle. The disappearance of the difference between *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice after furosemide treatment thus points to enhanced tubular Ca^{2+} reabsorption in the furosemide-sensitive thick ascending limb of the loop of Henle from *sgk1*$^{-/-}$ mice. Acetazolamide did not augment natriuresis or calciuria and may not have been able to substantially inhibit proximal tubular electrolyte reabsorption. It appears that the anticalciuria of *sgk1*$^{-/-}$ mice is at least partly due to enhanced Ca^{2+} reabsorption in the loop of Henle. It should be kept in mind, however, that the use of diuretics provides only circumstantial evidence for the localization of altered renal tubular Ca^{2+} transport.

The role of SGK1 in renal Ca^{2+} handling is reminiscent of its role in renal K^+ elimination (Huang DY. et al., (2004) *J Am Soc Nephrol*). In contrast to *sgk1*$^{+/+}$ mice, *sgk1*$^{-/-}$ mice are unable to rapidly excrete an acute potassium load and their ability to eliminate K^+ during a K^+-rich diet is impaired. Again, it is at least partly due to the impaired increase in ENaC activity, which impedes renal K^+ elimination. In conclusion, despite decrease in TRPV5 abundance in the connecting tubule, SGK1 knockout mice are anticalciuric presumably due to a decrease in extracellular volume and enhancement of Na^+ and Ca^{2+} reabsorption upstream of the aldosterone-sensitive distal nephron including the loop of Henle.

Characterization of *sgk1*$^{-/-}$ *and sgk1* $^{+/+}$ mice under Doca/high salt

The present observations reveal similarities and differences in the renal effects of mineralocorticoids on gene-targeted mice lacking functional SGK1 (*sgk1*$^{-/-}$) and their wild-type littermates (*sgk1*$^{+/+}$). These observations allow some conclusions as to the functional role of SGK1 in the mineralocorticoid-dependent regulation of renal function and the development of renal damage during mineralocorticoid and high salt excess. As observed earlier (Vallon V. et al., (2005) *Am J Physiol Regul Integr Comp Physiol*), mineralocorticoid excess leads to excessive salt intake and, hence, renal salt excretion in *sgk1*$^{+/+}$ mice, an effect blunted in *sgk1*$^{-/-}$ mice. The enhanced salt intake and subsequent renal Na^+ excretion in *sgk1*$^{+/+}$ mice results from SGK1-dependent stimulation of salt appetite (Vallon V. et al., (2005) *Am J Physiol Regul Integr Comp Physiol*). The differences of salt intake by *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice lead to the respective alterations of renal salt excretion concealing subtle differences of renal tubular Na^+ transport regulation (Wulff P. et al., (2002) *J Clin Invest*). In a previous study focusing on the role of SGK1 in the regulation of salt appetite (Vallon V. et al., (2005) *Am J Physiol Regul Integr Comp Physiol*), the mineralocorticoid excess had been maintained only for 3 weeks, and no attempts had been made to assess the effect of chronic mineralocorticoid excess on the integrity of the kidney, the focus of the present study.

Fractional urinary K^+ excretion tended to be lower in *sgk1*$^{-/-}$ mice, which may relate to the stimulating effect of SGK1 on the renal outer medullary K^+ channel ROMK1 (Yoo D. et al., (2003) *J Biol Chem*; Yun CC. et al., (2002) *J Am Soc Nephrol*) or other renal K^+ channels leading to the impaired renal potassium elimination by the *sgk1*$^{-/-}$ mice (Huang DY. et al., (2004) *J Am Soc Nephrol*). Moreover, the calciuria appears to be blunted in *sgk1*$^{-/-}$ mice, again reflecting the tendency of the *sgk1*$^{-/-}$ mice to retain calcium (Sandulache D. et al., (2005) *Pflügers Arch*). Most importantly, the present observations disclose a crucial role of SGK1 in the development of albuminuria and renal tubulointerstitial fibrosis during mineralocorticoid and salt excess. SGK1 may be effective, in part indirectly, due to the stimulation of salt appetite (Vallon V. et al., (2005) *Am J Physiol Regul Integr Comp Physiol*), which leads to excessive salt intake in *sgk1*$^{+/+}$ mice and subsequent excessive increase of blood pressure.

Hypertension is known to induce albuminuria and renal fibrosis (Fiebeler A. et al., (2005) *Circulation*; Kang N. et al., (2002) *J Mol Med*; Markmann A. et al., (2005) *Cell Physiol Biochem*; Shindo T. et al., (2002) *J Mol Med*). However, in the early stages of mineralocorticoid excess, blood pressure was similar in both genotypes but severe albuminuria occurred only in the wild-type mice. The effect of mineralocorticoid excess on albuminuria could thus have been, at least in part, more direct. Albuminuria in turn favors the development of renal fibrosis in part by upregulation of transforming growth factor beta (TGFβ) receptors (Wolf G. et al., (2004) *Kidney Int*). SGK1 is a transcriptional target of TGFβ (Lang F. et al., (2000) *Proc Natl Acad Sci USA*; Waldegger S. et al., (1999) *Gastroenterology*) and was suspected to participate in the machinery eventually leading to the stimulation of matrix protein deposition during fibrosing disease (Lang F. et al., (2000) *Proc Natl Acad Sci USA*; Friedrich B. et al., (2002) *Kidney Blood Press Res*; Waldegger S. et al., (1999) *Gastroenterology*; Wärntges S. et al., (2002) *Cell Physiol Biochem*; Fillon S. et al., (2002) *Cell Physiol Biochem*; Klingel K. et al., (2000) *Am J Physiol Gastrointest Liver Physiol*).

It was, however, only recently that the functional significance of SGK1 in fibrosis became apparent (Vallon V. et al., (2006). *J Mol Medicine*; Feng Y. et al., (2005) *Cell Physiol Biochem*). SGK1 stimulates the transcription factor NFκB (Zhang L. et al., (2005) *Cancer Res*), which in turn stimulates the expression of connective tissue growth factor (CTGF) (Blom IE. et al., (2002) *Matrix Biol*). Mineralocorticoids increase the release of CTGF (Moussad EE. et al., (2000) *Mol Genet Metab*), which stimulates matrix protein formation

(Brigstock DR. et al., (2003) *J Endocrinol*; Ihn H. et al., (2002) *Curr Opin Rheumatol*). Most recently, it was shown that the upregulation of CTGF in cardiac tissue required the presence of SGK1 (Vallon V. et al., (2006) *J Mol Medicine*).

Gene array further suggested the SGK1-dependent upregulation of plasminogen activator inhibitor-1 (Vallon V. et al., (2006) *J Mol Medicine*), a further signaling molecule participating in the regulation of fibrosis (Coffman TM. et al., (1998) *Am J Physiol*; Edgtton KL. et al., (2004) *Kidney Int*; Kanasaki K. et al., (2003) *J Am Soc Nephrol*; Okada H. et al., (2004) *J Am Soc Nephrol*). SGK1 transcription is similarly increased in diabetic nephropathy (Feng Y. et al., (2005) *Cell Physiol Biochem*; Lang F. et al., (2000) *Proc Natl Acad Sci USA*; Lang F. and Cohen P. (2001) *Sci STKE* 2001: RE17; Kumar JM. et al., (1999) *J Am Soc Nephrol*) and glomerulonephritis (Friedrich B. et al., (2002) *Kidney Blood Press Res*) and the present observations could indicate that SGK1 plays an active role in the pathophysiology of those diseases as well.

SGK1 has been shown to be upregulated by excessive glucose concentrations and to participate in the stimulation of fibronectin formation by glucose (Feng Y. et al., (2005) *Cell Physiol Biochem*; Lang F. et al., (2000) *Proc Natl Acad Sci USA*). Interestingly, the overexpression of SGK1 did not significantly increase the formation of the matrix protein fibronectin at normal extracellular glucose concentrations but markedly increased the fibronectin formation at excessive extracellular glucose concentrations (Feng Y. Et al., (2005) *Cell Physiol Biochem*). Apparently, matrix protein formation is not triggered by increased expression of SGK1 alone, but requires the concerted action with further glucose- or mineralocorticoid-dependent mechanisms.

Irrespective of the mechanisms involved, the present observations disclose the enhanced susceptibility to the fibrosing effects of mineralocorticoids plus high salt in the presence of SGK1. The observations could indicate that individuals with increased SGK1 activity could be at an enhanced risk of developing fibrosing disease. Some 3–5% of Caucasians carry a gene variant of SGK1, which is associated with increased blood pressure (Busjahn A. et al., (2002) *Hypertension*; von Wowern F. et al., (2005) *Kidney Int*), increased body weight (Dieter M. et al., (2004) *Obes Res*), and a decreased QT interval (Busjahn A. et al., (2004) *Cell Physiol Biochem*), effects correlating with effects of SGK1 on the renal epithelial Na^+ channel ENaC (Chen SY. et al., (1999) *Proc Natl Acad Sci USA*; Naray-Fejes-Toth A. et al., (1999) *J Biol Chem*; Diakov A. and Korbmacher C., (2004) *J Biol Chem*), the renal and intestinal glucose transporter SGLT1 (Busjahn A. et al., (2004) *Cell Physiol Biochem*), and the cardiac K^+ channel KCNE1/KCNQ1 (Busjahn A. et al. (2004) *Cell Physiol Biochem*).

It is tempting to speculate that those individuals would be more prone to develop fibrosis following exposure to mineralocorticoid and salt excess.

Characterization of *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice

The present observations show that *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice are viable and display a phenotype reflecting properties of *sgk1*$^{-/-}$ and *sgk3*$^{-/-}$ mice. On the one hand, the mice display the same delay of hair growth described previously for *sgk3*$^{-/-}$ mice (McCormick JA. et al., (2004) *Mol Biol Cell*). Moreover, similar to *sgk3*$^{-/-}$ mice (McCormick JA. et al., (2004) *Mol Biol Cell*) they tend to be smaller, despite significantly greater food intake. The discrepancy may be due to moderately impaired intestinal nutrient absorption in the intestine, similar to that shown in *sgk3*$^{-/-}$ mice (Sandu C. et al., (2005) *Pflügers Arch*).

On the other hand, the *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice display a moderate impairment of renal Na^+ retention previously described in *sgk1*$^{-/-}$ mice (Wulff P. et al., (2002) *J Clin Invest*).

This impairment is evident because of enhanced renal excretion of Na^+, particularly in animals fed a low-salt diet, the enhanced plasma aldosterone concentration, and the decreased blood pressure under anesthesia (Wulff P. et al., (2002) *J Clin Invest*). The enhanced plasma

aldosterone concentration partially overrides the lack of SGK1 and apparently prevents hypotension in non-anesthetized animals. The moderate salt wasting of the *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice is similar to that of *sgk1*$^{-/-}$ mice, but by far less severe than that of mice lacking functional mineralocorticoid receptors (Berger S. et al., (1998) *Proc Natl Acad Sci USA*) or mice lacking a functional ENaC (Hummler E. et al., (1996) *Nat Genet*). Mice without mineralocorticoid receptors suffer from severe renal salt wasting (Berger S. et al., (1998) *Proc Natl Acad Sci USA*), and ENaC knockout mice are not viable (Hummler E. et al., (1996) *Nat Genet*).

The mild salt wasting of *sgk1*$^{-/-}$ mice is consistent with SGK1-independent regulation of renal Na^+ reabsorption (Wulff P. et al., (2002) *J Clin Invest*). In theory, the lack of severe salt wasting could have been due to the partial functional compensation by SGK3 in *sgk1*$^{-/-}$ mice. The present observations clearly demonstrate that this is not the case. Instead, the salt wasting in the *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice is not substantially more severe than that in *sgk1*$^{-/-}$ mice. Thus it appears safe to conclude that SGK3 does not play a major role in renal Na^+ reabsorption and that it does not account for the SGK1-independent regulation of the ENaC.

The third isoform, SGK2, similarly stimulated the ENaC in vitro (Friedrich B. et al., (2003) *Pflügers Arch*) and thus, is a further candidate for the SGK1-independent stimulation of the ENaC. However, SGK2 transcript levels are not upregulated in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice. Even though the influence of SGK1 on renal salt retention is only moderate, it may well play a significant role in deranged blood pressure regulation.

Enhanced SGK1 expression has been observed in the salt-sensitive Dahl rat (Farjah M. et al., (2003) *Hypertension*), and moderately enhanced blood pressure is observed in individuals carrying a variant of the SGK1 gene, affecting as many as 5% of unselected Caucasians (Busjahn A. et al., (2002) *Hypertension*). In the same individuals, increased body mass index (Dieter M. et al., (2004) *Obes Res*) and a shortening of the Q-T interval (Busjahn A. et al., (2002) *Hypertension*; Busjahn A and Luft FC., (2003) *Cell Physiol Biochem*) have been observed. The increased body mass index may be partially due to enhanced stimulation of the intestinal glucose transporter SGLT1 (Dieter M. et al., (2004) *Obes Res*), the accelerated cardiac repolarization due to enhanced activation of the cardiac K^+ channel KCNE1 (Busjahn A. et al., (2004) *Cell Physiol Biochem*; Embark HM. et al., (2004) *Cell Physiol Biochem*). Thus excessive stimulation of carriers and channels by SGK1 could account for obesity, hypertension, and shortened cardiac action potential. SGK1 has previously been shown to regulate the ROMK1 channel (Yun CC et al., (2002) *J Biol Chem*). Moreover, SGK1-dependent ENaC activity is expected to depolarize the apical cell membrane of principal cells, thus favoring K^+ secretion. Presumably, both effects lead to slightly impaired renal K^+ elimination by the *sgk1*$^{-/-}$ mice (Huang DY. et al., (2004) *J Am Soc Nephrol*). Despite enhanced plasma aldosterone concentration, plasma K^+ concentration is not lower in the *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice. However, neither plasma concentration nor renal excretion of K^+ is significantly different between *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ mice and *sgk1*$^{+/+}$/*sgk3*$^{+/+}$ mice. The phenotype of a gene-targeted mouse is influenced by the genetic background.

We have been unable to breed *sgk1*$^{-/-}$ mice on a pure C57BL/6 background (unpublished observations). To minimize the bias of variable genetic background, comparisons were always made between littermates. The observations that hair phenotype is not as pronounced in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ as in *sgk3*$^{-/-}$ mice and that salt wasting is not as severe in *sgk1*$^{-/-}$/*sgk3*$^{-/-}$ as in *sgk1*$^{-/-}$ mice indicate that there is little overlap of SGK1 and SGK3 in these functions. Thus, even though there is considerable overlap of targets (Lang F. et al., (2003) *Cell Physiol Biochem*; Pearce D., (2003) *Cell Physiol Biochem*) and regulation (Lang F and Cohen P., (2001) *Sci STKE*), the kinases are apparently not serving identical functions. Additional experiments are needed to elucidate whether the lack of SGK1 and SGK3 has additive effects on other functions. If so, those functions are not required for viability, or they are sufficiently maintained in the absence of SGK1 and SGK3 that the mice do not show gross functional deficits.

7. References

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Diana Maria Sandulache
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Curriculum Vitae

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Education:

11.2003 – present	"Eberhard Karls" University of Tübingen, Faculty of Biology, **Ph.D. Student**
11.2002 – 03.2003	"Albert Ludwigs" University of Freiburg, Faculty of Biology, **Master in Biology** with Socrates Scholarship
09.2001 – 07.2003	"Alexandru Ioan Cuza" University of Iaşi, Faculty of Biology, Molecular Genetics Department, **Master in Biology** Dissertation: "*In vivo and in vitro aspects of chromosomal formula behavior of Papaver somniferum L.*" Graduation results: 9 RO (=1.4 DE)
09.1994 – 07.2000	"Gr. T. Popa" University of Medicine and Pharmacy, Iaşi, Faculty of Medical Bioengineering **Medical Bioengineer** Specialization - "Bioactive substances and Medical Biotechnology", full time Dissertation: *"In vitro behavior and the cariotype of Papaver somniferum L. species"* Graduation results: 9.62 RO (=1.2 DE)
09.1990 – 07.1994	"Petru Rares" High School, Piatra Neamţ, Profile: Mathematics-Physics, full-time, **High School Graduation** Exam results: 8.13 RO (=1.9 DE)

Trainings:

11.2003-present	**Institute for Physiology I, Eberhard Karls University, Tübingen, Germany** Ph.D. Student
08.1999-09.1999	**"Centre hospitalier de Roanne", "Long séjour" clinic, Roanne, France** Biomedical summer training **"Institute de formation en soins infirmières", Roanne, France** Biomedical summer training
08.1996-09.1996	**"Micromedica" Chemistry Laboratory, Piatra Neamţ, Romania**
08.1998-09.1998	Monitoring of biochemistry analysis, Biomedical summer training
08.1997-09.1997	**General Hospital, Piatra Neamţ, Romania** Biomedical summer training
07.1997-08.1997	**"Europharm", "Plafar"- pharmaceutical companies, Braşov, Romania** Biomedical summer training

Courses:

06.2003-07.2003	Centre Culturel Français, Iaşi, Romania "Course de français – Niveau I"
11.2002-03.2003	"Albert Ludwigs" Universität, Freiburg, Germany "Deutsch als Fremdsprache"
10.2002-10.2002	Goethe Zentrum, Iaşi, Romania "Deutsch – Grundstufe I "
12.2000-12.2000	University "Alexandru Ioan Cuza", Iaşi, Romania "Toefl" preparation course

Languages:

	Read	***Write***	***Speak***
English	Excellent	Excellent	Excellent
German	Good	Acceptable	Acceptable
French	Good	Acceptable	Acceptable

TOEFL: 263 Points from 270 maximum-Computer test, 6 from 6 to Essay
GRE: 390 point verbal, 710 points quantitative, 530 points analytical

Publications: **For complete list, please see the attached file – "Publications list"**

Conferences:

03.2006 "Deutsche Physiologische Gesellschaft" Congress, München, Germany
Poster presentation: " Involvement of sgk1 in volume retention induced by the PPARγ-agonist pioglitazone"

03.2005 "Deutsche Physiologische Gesellschaft" Congress, Göttingen, Germany
Poster presentation: "Renal calcium handling in SGK 1 knock-out mouse"

05.2000 3rd "Medical Bioengineering Student Symposium", Iaşi, Romania
Presentation: "*In vitro* behavior and the cariotype of *Papaver Somniferum L.* species"
Organizer and participant

Organizational skills and competences:

03.1998 - present Founding member of "Bioengineering Students Association", Iaşi, Romania
- raising founds, donations and obtaining sponsorships

05.1998 1st "Medical Bioengineering Student Symposium", Iaşi, Romania
Organizer of the first Symposium

01.2002-04.2002 Collaboration with Center of Development, Recruitment and Management of Organizations (CDRMO) in general communication programs with the press, visual/audio media for medical personnel

Social skills and competences:

Long-term accumulated experience in living and working with other people, in multicultural environments, in positions where communication is important and situations where teamwork is essential
High capacity to work under pressure, good organizer, analytical mind, capacity of synthesis

Artistic skills and competences:

Dancing, Reading

Work experience:

11.2003-12..2006 **Institute for Physiology I, Tübingen, Germany**

Natur Wissenschaftliche Assistentin - Ph.D. student

Driving License: class 3 (known also as B class)
Present activity: Ph.D. Student
Computer Skills: Excel, PowerPoint, Word, Quantity one, Magellan, Sigma plot, Graph Pad Instat 3, Data Link.

Publications List

Diana Maria Sandulache
Ph.D. Student

Already published:

1. **Sandu C., Artunc F., Grahammer F., Sandulache D., Metzger M., Just L., Skutella T., Rexhepaj R., Friedrich B., Risler T., Wulff P., Kuhl D., Lang F.** -*„Role of the Serum and Glucocorticoid inducible Kinase SGK1 in glucocorticoid stimulation of gastric acid secretion*", European Journal of Physiology, February, 2007
2. **Nasir O., Artunc F., Sandulache D., Jahovic N., Boini M. K., Saeed A., Kambal M. A., Lang F.** – *„Effects of Gum Arabic (Acacia Senegal) on water and electrolyte balance in healthy mice"* , Journal of Renal Nutrition, March, 2007
3. **Rexhep R., Grahammer F., Völkl H., Remy C., Wagner C., Sandulache D., Artunc F., Henke G., Srinivas N., Capasso G., Alessi D., Lang F.** – *„Reduced intestinal and renal amino acid transport in PDK1 hypomorphic mice",* FASEB Journal, November 20, 2006
4. **Artunc F., Amann K., Nasir O., Friedrich B., Sandulache D., Jahovic N., Risler T., Vallon V., Wulff P., Kuhl D., Lang F.** – **„***Blunted DOCA/high salt induced albuminuria and renal tubulointerstitial damage in gene-targeted mice lacking SGK1"*, Journal of Molecular Medicine, September 8, 2006
5. **Artunc F., Rexhepaj R., Voelkl H, Grahammer F., Remy C., Sandulache D., Nasir O., Wagner C.A., Alessi D.R., Lang F.** – *„Impaired intestinal and renal glucose transport in PDK1 hypomorphic mice",* American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, June 1, 2006
6. **Sandulache D., Grahammer F., Artunc F., Henke G., Hussain A., Nasir O., Mack A., Friedrich B., Vallon V., Wulff P., Kuhl D., Palmada M., Lang F.** – *„Renal Ca(2+) handling in SGK1 knockout mice",* Pflugers Archive, May 10, 2006. (Note: Abstract published also in „Deutsche Physiologische Gesellschaft Congress" booklet, Göttingen, March, 2005)
7. **Grahammer F., Artunc F., Sandulache D., Rexhepaj R., Friedrich B., Risler T., McCormick J.A., Dawson K., Wang J., Pearce D., Wulff P., Kuhl D., Lang F.** –*„Renal function of gene-targeted mice lacking both SGK1 and SGK3",* American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, April, 2006
8. **Sandulache D., Bara I. Ioan** – *„In vivo and in vitro aspects of the behavior of chromosomal formula of Papaver somniferum L."***,** master thesis, Faculty of Biology Archive, University 'Al.I.Cuza', Iasi, Romania, June, 2003
9. **Sandulache D., Pavel A.** – *„In vitro behavior and the cariotype of Papaver somniferum L. species"*, diploma thesis, University of Medicine Archive,Univ. of Medicine, Iasi, Romania, June, 2000 (Note: also presented at 3rd "Medical Bioengineering Student Symposium", Iasi, Romania, 2000)

Submitted for review:

1 **Artunc F., Sandulache D., Nasir O., Friedrich B., Beier N., Dicks E., Pötzsch S., Scholz W., Risler T., Kuhl D., Lang F.** – *„Contribution of the serum and glucorticoid inducible kinase to the volume retention following treatment with the PPARγ agonist pioglitazone"* , Journal of the American Society of Nephrology (JASN), 2006
(Note: Abstract already published in „Deutsche Physiologische Gesellschaft Congress" booklet, March 2006, München)