Synergistic Activation of ENaC by Three Membrane-bound Channel-activating Serine Proteases (mCAP1, mCAP2, and mCAP3) and Serum- and Glucocorticoid-regulated Kinase (Sgk1) in *Xenopus* Oocytes

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ABSTRACT Sodium balance is maintained by the precise regulation of the activity of the epithelial sodium channel (ENaC) in the kidney. We have recently reported an extracellular activation of ENaC-mediated sodium transport (I_{Na}) by a GPI-anchored serine protease (mouse channel–activating protein, mCAP1) that was isolated from a cortical collecting duct cell line derived from mouse kidney. In the present study, we have identified two additional membrane-bound serine proteases (mCAP2 and mCAP3) that are expressed in the same cell line. We show that each of these proteases is able to increase I_{Na} 6–10-fold in the *Xenopus* oocyte expression system. I_{Na} and the number (N) of channels expressed at the cell surface (measured by binding of a FLAG monoclonal I^{125}-radioiodinated antibody) were measured in the same oocyte. Using this assay, we show that mCAP1 increases I_{Na} 10-fold ($P < 0.001$) but N remained unchanged ($P = 0.9$), indicating that mCAP1 regulates ENaC activity by increasing its average open probability of the whole cell (wcP_o). The serum- and glucocorticoid-regulated kinase (Sgk1) involved in the aldosterone-dependent signaling cascade enhances I_{Na} by 2.5-fold ($P < 0.001$) and N by 1.6-fold ($P < 0.001$), indicating a dual effect on N and wcP_o. Compared with Sgk1 alone, coexpression of Sgk1 with mCAP1 leads to a ninefold increase in I_{Na} ($P < 0.001$) and 1.3-fold in N ($P < 0.02$). Similar results were observed for mCAP2 and mCAP3. The synergism between CAPs and Sgk1 on I_{Na} was always more than additive, indicating a true potentiation. The synergistic effect of the two activation pathways allows a large dynamic range for ENaC-mediated sodium regulation crucial for a tight control of sodium homeostasis.

KEY WORDS: epithelial sodium channel • amiloride • serum glucocorticoid–regulated kinase 1 (Sgk1) • aldosterone • NEDD4

INTRODUCTION

Sodium balance, extracellular volume, and blood pressure are maintained by the tight control of the activity of the epithelial sodium channel (ENaC)* (Verrey et al., 2000). We have recently identified a membrane-bound serine protease that acts as channel-activating protease, namely CAP1 (Vallet et al., 1997, 1998; Vuagniaux et al., 2000). This regulation defines a novel extracellular signaling pathway, which appears to be highly conserved throughout evolution from *Xenopus* (xCAP1) (Vallet et al., 1997) to mouse (Vuagniaux et al., 2000), rat (Adachi et al., 2001), and human (Yu et al., 1995). CAP1 activates ENaC through its extracellular serine protease activity, as evidenced by patch-clamp experiments (Chraibi et al., 1998). The activation of ENaC by CAP1 can be mimicked by external addition of trypsin, showing that both CAP1 and trypsin act via the same pathway. Aprotinin, an inhibitor of serine proteases, can block this effect (Vallet et al., 1997; Vuagniaux et al., 2000). In epithelial cell lines, like the *Xenopus* kidney cell line (A6), ENaC appears to be constitutively activated through the presence of endogenously expressed serine proteases, since basal transepithelial Na^+ transport cannot be further activated by addition of trypsin (Vallet et al., 1997). However, the base line Na^+ transport can be inhibited by the addition of aprotinin on the apical side of the cell and this inhibition can be reversed by the addition of trypsin (Vallet et al., 1997). In *Xenopus* kidney cells, up to 90% of the amiloride-sensitive electrogenic Na^+ transport can be blocked by aprotinin (50 μM) (Vallet et al., 1997), whereas the mouse $mpkCCD_{cl4}$ cell line (derived from the cortical collecting duct; Bens et al., 1999) appears to be only 50% sensitive to aprotinin (Vuagniaux et al., 2000). These findings suggest that ENaC activation is achieved by either a constitutive, serine protease–independent mechanism or, alternatively, that the activation depends on more than one serine protease with different sensitivity to

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**Abbreviations used in this paper:* ASDN, aldosterone-sensitive distal nephron; ENaC, epithelial sodium channel; mCAP, mouse channel–activating protease; TTSP, type 2 transmembrane serine protease.

aprotinin and that acts in combination within the same cell. The mechanism by which serine proteases like CAP1 activates ENaC is not yet understood. CAP1 leads to a very substantial increase in the open probability of the Na^+ channel, whereas the number of channels at the cell surface is either unchanged (Vallet et al., 1997) or even diminished (Vuagniaux et al., 2000).

The activity of ENaC is tightly controlled by hormones, including aldosterone and vasopressin (for review see Verrey et al., 2000). Components of the aldosterone-dependent signaling pathway have been identified recently. Aldosterone rapidly induced the expression of Sgk1 kinase (serum- and glucocorticoid-regulated kinase), a member of the PKB-AKT family of serine-threonine kinases (Webster et al., 1993). When coexpressed in *Xenopus laevis* oocytes, Sgk1 stimulates ENaC activity by 2–3-fold (Chen et al., 1999; Naray-Fejes-Toth et al., 1999). In *Xenopus* oocytes, Sgk1 increases cell surface expression of ENaC without changing its open probability (Alvarez de La Rosa et al., 1999; Loffing et al., 2001). Recently, it has been demonstrated that the phosphorylation of Nedd4-2, an ubiquitin protein ligase, by Sgk1 may regulate epithelial sodium channel cell surface expression in the *Xenopus* oocyte system (Debonneville et al., 2001). It was further demonstrated that the phosphorylation of Nedd4-2 decreases its affinity for ENaC, thereby diminishing ENaC endocytosis and/or degradation (Debonneville et al., 2001; Snyder et al., 2002). These data provided evidence for the missing link between aldosterone binding to its receptor, transcription activation of an aldosterone-induced protein (Sgk1) and the molecular mechanism leading to an increased cell surface expression of ENaC.

The aims of the present study were twofold: (a) to test the interaction between two regulatory pathways that can activate ENaC through extracellular signaling via CAPs and intracellular signaling via Sgk1, and (b) to identify further putative membrane-bound serine proteases able to activate ENaC; in particular, to search for an aprotinin-resistant protease that could account for the pharmacological data observed in the mouse CCD cell line.

We demonstrate here the existence of three channel-activating proteases, namely mouse channel–activating protease, mCAP1, mCAP2, and mCAP3 within the same kidney cell. Whereas mCAP2 is inhibited by aprotinin as well as mCAP1, mCAP3 is not significantly inhibited by this serine protease inhibitor. Each of these membrane-bound serine proteases increases ENaC-mediated I_{Na} and potentiates the effect mediated by Sgk1.

MATERIALS AND METHODS

Identification and Isolation of Full-length Clones

Mouse CAP2: Partial mCAP2 sequence was identified by RT-PCR using degenerated oligonucleotides and total RNA extracted from $mpkCCD_{cl4}$ cells as described previously (Vuagniaux et al., 2000). The primers used were as follows: D1 (sense) 5′-AA(AG)TT(CT)CCITGGCA(AG)GT-3′, nt +118 to +134; D2 (antisense) 5′-CC(AG)CA(CT)TC(AG)TCICCCCA-3′, nt +743 to +727; D3 (antisense) 5′-CCIGC(AG)CA(AGT)ATCAT (AG)TC-3′, nt +629 to +613; according to xCAP1 (5′ and 3′). To obtain a full length clone, rapid amplification of 5′ and 3′ ends (RACE) has been performed (Vuagniaux et al., 2000). For the 5′-RACE, successively after mCAP2-specific reverse primers were used: RC1 (5′-ACAGGCAAGGATGGAGAGT-3′, nt position +812 to +794) and adaptor-specific primer dC-5R (5′-GCATGCTCGAGCGGCCGCAACCCCCCCCCCCCCCCCCC-3′), and RC2 (5′-GATGCTCCCACCACAGAC-3′, nt position +696 to +679) and the adaptor-specific primer 5R (5′-GCATGCTCGAGCGGCCGCAAC-3′), according to the manufacturer's instructions (Life Technologies). For 3′-RACE, reverse transcription was performed, using mCAP2-specific primers RC3 (5′-GATGAGGTGCTTGTCCCAG-3′, nt position +937 to +955) and an oligo(dt) adaptor primer (5′-CGAGATCTATGCGGCCGCTTTTTTTTTTTTTTT-3′). Full-length clone of mCAP2 was obtained by PCR on total RNA extracted from distal colon using S2 (5′-TAGGAGGATTACCAAAGCAG-3′, nt position −20 to −1) and AS2 (5′-ATGCCGTAGGTAGGTCTGAA-3′). 33 cycles were run consisting of 30 s at 94°C, 30 s at 54°C, and 1 min 30 s at 72°C. After the last cycle, elongation was allowed to proceed for 7 min at 72°C using Taq polymerase (Roche). PCR products were purified by gel electrophoresis and, following extraction, cloned into pT7Blue vector (Novagen) and sequenced. The nucleotide sequence reported for mCAP2 in this paper has been submitted to the National Institutes of Health database under EMBL/GenBank/DDBJ accession no. AY043240.

Mouse CAP3: By analyzing a SAGE library (serial analysis of genome sequences; Robert-Nicoud et al., 2001) and EST database, a SAGE tag (GATCAAAGAGCACA) corresponding to the recently cloned mouse epithin (AF042822) had been identified (Kim et al., 1999). Based on sequence information, RT-PCR was performed on RNA of $mpkCCD_{cl4}$ cells using primers S3 (5′-GACCACGCGTCTGAGACC-3′, nt position −38 to −20) and AS3 (5′-GACAGTTGGAAGCAGCTCTC-3′, nt position +2868 to +2849). 10 cycles were run, each consisting of 15 s at 94°C, 30 s at 58°C, and 2 min at 68°C, followed by 20 cycles with continuously increasing elongation times of each 20 s at 68°C using Taq polymerase (Expand High Fidelity PCR system; Roche). PCR product was gel purified, extracted, cloned into pT7Blue vector, and named mCAP3.

Northern Blot Analysis

Total RNA was prepared from whole mouse kidney and confluent $mpkCCD_{cl4}$ cells as described. 20 μg RNA were run on a 0.8% denaturing glyoxal agarose gel and blotted onto nylon membranes (Hybond-N; Amersham Biosciences). Membranes were hybridized with randomly primed ^{32}P-labeled probes for rENaC (Canessa et al., 1994), mCAP1 (512 bp: nt +163 to +674), mCAP2 (472 bp: nt +643 to +1115), mCAP3 (603 bp: nt +2442 to +3045).

Electrophysiological Experiments in Xenopus Oocytes

For functional expression studies, cDNA for mCAP1, mCAP2, mCAP3, and mouse Sgk1 (provided by O. Staub, Institute of Pharmacology and Toxicology, Lausanne, Switzerland) were subcloned into pSDS expression vector and in vitro transcribed, as described previously (Canessa et al., 1994). Purified cRNA were injected into stage V/VI *Xenopus* oocytes. Routinely, oocytes were injected with 0.33 ng of each cRNA coding for the rat α-, β- and

γ-ENaC subunits in the presence or absence of varying concentrations of mCAP1, mCAP2, and mCAP3 and incubated in a modified Barth (low sodium) solution, as described (Firsov et al., 1996). Oocytes were incubated overnight in modified Barth saline solution in the presence or absence of 100 μg/ml aprotinin. This protocol insures that intracellular sodium is low at the beginning of the measurement and independent of the level of ENaC expression at the cell surface. Electrophysiological measurements were performed 1–2 d after cRNA injection using the two-electrode voltage-clamp technique and one bath electrode, allowing corrections for serial resistance. The oocytes were exposed to 2 μg/ml trypsin during 2–3 min and amiloride-sensitive Na^+ current (I_{Na}) was measured in the presence of 120 mM of Na^+ in frog Ringer's solution with 5 μM amiloride at a holding potential of −100 mV.

Measurement of N and I_{Na} within the Same Oocyte

Cell surface expression has been performed as described (Firsov et al., 1996). Briefly, 1 ng of tagged rat α-, β-, and γ-ENaC subunits was expressed in oocytes either alone or with 4 ng (mCAP1), 8 ng (mCAP2) or 2 ng (mCAP3) of each CAP and/or 4 ng Sgk1 cRNA. The density of the channel was then calculated by binding of iodinated anti-FLAG monoclonal antibody (M2Ab*) (Sigma-Aldrich). The binding assay was performed at 4°C, and I_{Na} was measured at room temperature as described. To deduce the whole cell P_o (wcP_o), we have used a macroscopic approach, as described (Firsov et al., 1997) in which the starting point is the equation, derived from Ohm's law:

$$I_{Na} = g_{Na} \cdot N \cdot P_o \cdot (E - E_{Na}), \quad (1)$$

where (a) I_{Na} is the macroscopic amiloride-sensitive current that measures the total activity of all channels expressed in a cell. (b) g_{Na} is the single channel conductance measured by patch clamp in independent experiments. In the presence of sodium it is 5 and 8 pS in the presence of Li (Canessa et al., 1994). (c) ($E - E_{Na}$) is assessed by the measurement of membrane potential and the reversal potential for sodium. For practical purposes, $E - E_{Na}$ can be set constant by clamping the membrane at −100 mV or higher, allowing the measurement of changes in P_o in function of the various experimental conditions. (d) N is the total number of channel molecules (active and inactive) expressed at the cell surface. The binding assay permits to quantitate the total number of channel molecules at the cell surface, independently of their function. Having determined with good accuracy I_{Na}, g_{Na}, $E - E_{Na}$, and N, one can deduce a whole-cell P_o (wcP_o) that reflects the activity of all channels expressed in an intact whole cell. The main advantage of this macroscopic approach is that it takes into account all channels expressed at the cell surface, whatever their activity. Our method is therefore distinct from a microscopic approach that is the classical measurement of P_o in membrane patches (mpP_o). This is performed by determining the overall $N \cdot P_o$ of all the active channels and the number of the channels N present in the patch membrane.

Statistical Analysis

All results are reported as means ± SEM. Comparing independent sets of data, unpaired *t* tests were used to determine significance. For the measurement of N and I_{Na}, a minimum of 3–6 batches of oocytes, 5–10 oocytes per batch, each batch from different animals, were used for each experimental condition. Experiment 1, "ENaC + H_2O" vs. "ENaC + mCAP1" vs. "ENaC + Sgk1" vs. "ENaC + mCAP1 + Sgk1"; experiment 2, "ENaC + H_2O" vs. "ENaC + mCAP2" vs. "ENaC + Sgk1" vs. "ENaC + mCAP2 + Sgk1"; experiment 3, "ENaC + H_2O" vs. "ENaC + mCAP3" vs. "ENaC + Sgk1" vs. "ENaC + mCAP3 + Sgk1."

RESULTS

Identification of Two Membrane-bound Serine Proteases in the $mpkCCD_{cl4}$ Cell Line

mCAP2. By using degenerated oligos to the previously isolated serine protease from *Xenopus* xCAP1 and RACE technique, we identified mCAP2, which encodes a 435–amino acid protein. Analysis of the amino acid sequence and the hydrophobicity plot reveals a type II–oriented membrane-bound serine protease with a predicted transmembrane segment (V30–151) and an extracellular COOH terminus containing a serine protease catalytic domain with the classical catalytic triad at H243, D288, and S385 and with a potential proteolytic activation cleavage site at R202V203 (Fig. 1 A). Two cysteines at position C194 and C308 are predicted to connect the prodomain to the main serine protease domain via a disulfide bond at the extracellular surface of the cell (Hooper et al., 2001). The extracellular domain has two potential N-glycosylation sites (N128 and N176) that are used when mCAP2 is expressed in the *Xenopus* oocyte system (unpublished data). The catalytic domain is preceded by a low density lipoprotein receptor class A domain (LDLR, F60-V91) and a group A scavenger receptor domain (SRCR, V102-G142) (Fig. 1 A). The serine protease domain shares homology with mouse trypsinogen precursor (49%), xCAP1 (45%), mCAP1 (43%), and 80% with a human orthologue hTMPRSS4 (Wallrapp et al., 2000) (Fig. 1 B).

mCAP3. By analysis of the transcriptome of an $mpkCCD_{cl4}$ cell line library by SAGE (Robert-Nicoud et al., 2001) and EST database analysis, we identified a serine protease we termed mCAP3. mCAP3 is identical to the recently cloned mouse epithin (Kim et al., 1999). At the time we started this project, epithin cDNA sequence had not been published. To underline the functional relevance of this serine protease, we name the third serine protease throughout our paper mouse channel–activating protein three (mCAP3). Analysis of the primary sequence revealed the presence of a transmembrane domain with predicted type II orientation (Fig. 1 A). COOH terminus contained the serine protease domain with the catalytic triad (H656, D711, and S805) and a potential activation cleavage site at R605V606. The catalytic domain was preceded by two complement factor IR-urchin embryonic growth factor-bone orphogenetic proteins (CUBs) (C214-F331 and C340-Y444) and four LDLR (C453-R487, C488-S524, C525-N563 and C567-L603) domains (Fig. 1 A) (Kim et al., 1999). mCAP3 shares 47% homology with the mouse trysino-

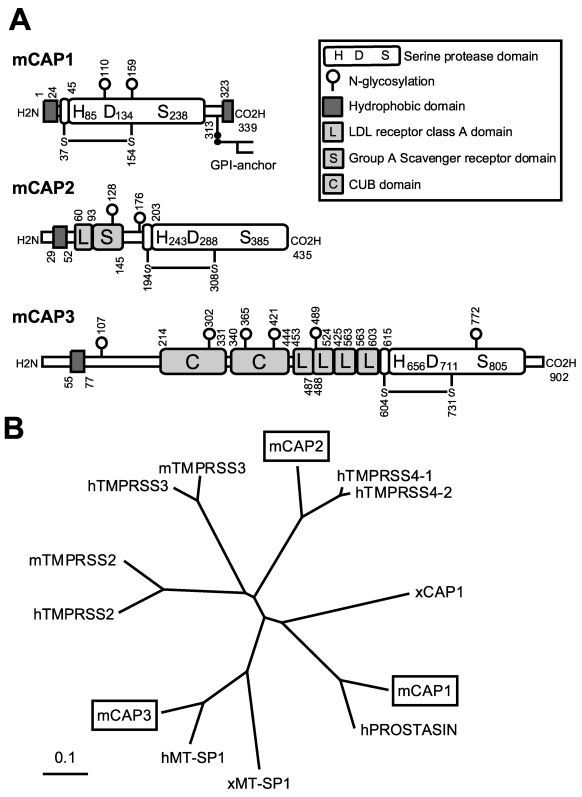

FIGURE 1. mCAP1, 2, and 3 are membrane-bound serine proteases. (A) Structural properties of the three mCAPs (mCAP1, mCAP2, and mCAP3). The amino acid sequence of each protein was scanned using ProfileScan algorithm to confirm the presence of domains indicated. Numbers delineate the location of each domain. (B) A representative phylogenetic tree of the GPI-anchored mCAP1 and type II transmembrane mCAP2 and mCAP3 serine protease family proteins. Multiple alignment was generated by the clustal W program (mCAP1, AAG1705; hProstasin, Q16651; xCAP1, AAB969054; mCAP2, AY043240; hTMPRSS4-1, CAC60389; hTMPRSS4–2; MT-SP2, Q9NRS4; mTMPRSS2; epitheliasin, AAF97867; hTMPRSS2, AAC51784; mTMPRSS3, CAC83350; hTMPRSS3, P57727; mCAP3; epithin, AAD02230; hMT-SP1, AAF00109; xMT-SP1, BAB08218). (C) Amino acid alignment of the serine protease catalytic domain of mCAP1, mCAP2, and mCAP3 with mouse trypsinogen precursor (1–246), was performed using Pileup program (Genetic Computer Group). Gray and black boxes indicate similar and identical residues, respectively. The catalytic triad (H, D, and S, asterisks), the amino acids implicated in the P1 substrate specificity (closed circle), and the position for Na^+ sensibility (open circle) are indicated.

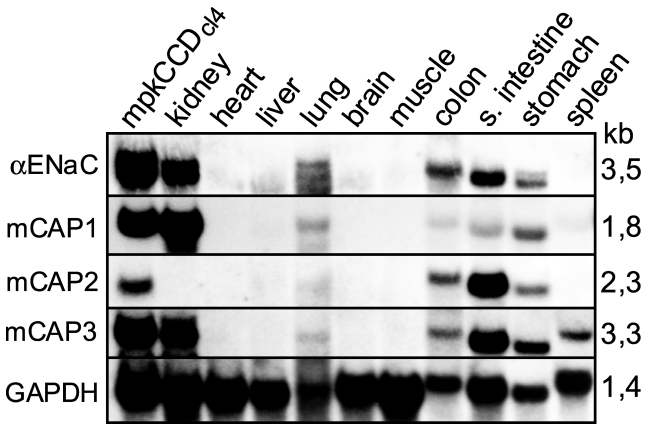

FIGURE 2. Coexpression of all three serine proteases with ENaC. Multiple tissue Northern blot containing 20 μg of RNA/lane were hybridized with cDNA fragments of the rat αENaC, mouse CAP1, CAP2, CAP3, and GAPDH genes, as described in MATERIALS AND METHODS. mRNA transcript lengths are indicated.

gen, 49% with xCAP1, 40% with mCAP2 and 83% with the human orthologue hMT-SP1 (Takeuchi et al., 1999) (or Matriptase; Lin et al., 1999) (Fig. 1 B).

Primary sequence comparison of the catalytic domain of mCAP1, mCAP2, mCAP3, and trypsin revealed the presence of an aspartate (mCAP1, D232; mCAP2, D379; mCAP3, D799) at the S1 site (Schechter and Berger, 1967) and 2 glycines at the entry of the S1 pocket (mCAP1, G259 and G269; mCAP2, G405 and G415; and mCAP3, G827 and G837) which direct the specificity of serine proteases (Perona and Craik, 1995, 1997) (Fig. 1 C). This suggests that all three serine proteases are specific for the positively charged P1 amino acids. The presence of a proline residue (mCAP1, P268; mCAP2, P414; and mCAP3, P836) instead of a tyrosine residue at the Na^+-induced allosteric regulation of catalytic activity site in serine proteases suggests that the catalytic activity of three serine proteases is not sensitive to extracellular Na^+ concentration variations (Dang and Di Cera, 1996) (Fig. 1 C).

Northern blot analysis showed that mCAP1, mCAP2, mCAP3, and αENaC subunit are expressed, as expected, in mpkCCD_{c14} cell line. Whereas mCAP3 expression was expressed in the cell line and the kidney at a level similar to that of mCAP1, mCAP2 expression was low and not detectable in the whole kidney (Fig. 2). The three membrane-bound proteases and the αENaC subunit were also expressed in epithelia known to express an amiloride-sensitive sodium transport (lung and colon). A similar pattern of expression was also found in small intestine and stomach. In these tissues, the role of ENaC and/or CAPs remains to be established.

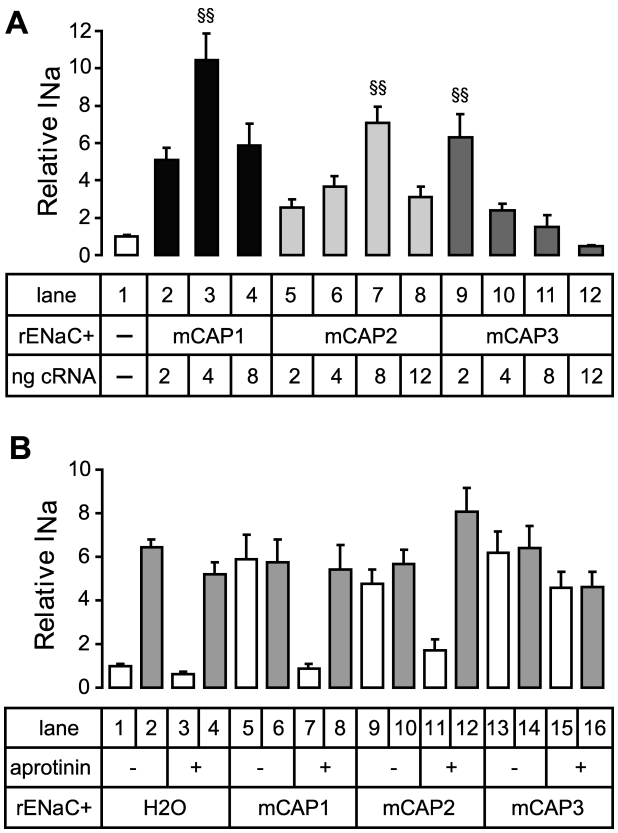

FIGURE 3. Functional analysis of mCAP1–3 in *Xenopus* oocytes. (A) Comparison of the effect of mCAP1, mCAP2, and mCAP3 on I_{Na} in *Xenopus* oocytes. Oocytes were injected with rat ENaC subunits in the presence of either water (open bar, lane 1) or increasing amounts (2, 4, and 8 or 12 ng) of mCAP1 (closed bar, lanes 2–4), mCAP2 (light gray bar, lanes 5–8), or mCAP3 (dark gray bars, lanes 9–12). §§, $P < 0.01$ versus conditions with other amounts of the same cRNA protease. $n \geq 12$ measured oocytes. (B) Effect of preincubation with aprotinin (lanes 3, 4, 7, 8, 11, 12, 15, 16) or perfusion of trypsin (gray bars, lanes 2, 4, 6, 8, 10, 12, 14, 16) on oocytes injected with either rENaC and water (lane 1–4) or rENaC together with 4 ng mCAP1 (lane 5–8), 8 ng mCAP2 (lane 9–12), and 2 ng mCAP3 (lane 13–16). $n \geq 12$ measured oocytes.

ENaC Activation by mCAP1, mCAP2, and mCAP3 in Xenopus Oocytes

In the *Xenopus* oocytes, ENaC activity is recorded as an amiloride-sensitive Na^+ current (I_{Na}). As shown in Fig. 3 A (lanes 1–4), mCAP1 increased ENaC activity 5–10-fold. Increasing amounts of cRNA coding for mCAP1 were tested and a maximal effect was observed with 4 ng mCAP1. mCAP2 and mCAP3 (Fig. 3, lanes 5–8 and 9–12) were also able to increase ENaC activity by 4–8-fold, the maximal effect for mCAP2 was observed with 8 ng of injected cRNA and for mCAP3 with only 2 ng cRNA. At higher concentrations of mCAP3 (4–12 ng), ENaC activation was inhibited, ($P < 0.01$) presumably by competition for the translational machinery. Consequently, in all further experiments we used 4 ng (mCAP1), 8 ng (mCAP2), and 2 ng (mCAP3) that induce maximal activation of ENaC.

Trypsin can activate the I_{Na} (Fig. 3 B, lane 2 vs. 1). The activity of ENaC can be partially blocked by incubating the oocytes in the presence of the serine protease inhibitor aprotinin (lane 3), suggesting that ENaC has been weakly activated by endogenous serine proteases. This block can be overcome by further addition of trypsin in the medium (lane 4). Activation of ENaC in the presence of mCAP1 (lane 5) cannot be further increased by trypsin (lane 6), whereas preincubation in the presence of aprotinin inhibits ENaC activity to base line level (lane 7), which can then be restored by trypsin (lane 8). In the same assay, one can observe that mCAP2 similarly activates ENaC (lanes 9–12). mCAP3 was also able to fully activate ENaC (lane 13), to a level that was not further increased by trypsin (lane 14). Unlike mCAP1 or mCAP2, this effect was aprotinin resistant (lane 15) and could not be further increased by perfusion of trypsin (lane 16). These data indicate that the mCAP3 aprotinin binding site, unlike that of mCAP1 and mCAP2, has a low affinity for the inhibitor. mCAP3 is therefore a good candidate for being a serine protease responsible for the aprotinin-resistant component observed in $mpkCCD_{c14}$ cells.

Functional Interaction between CAPs and Sgk1 in the Xenopus Oocyte System

To test whether mouse CAPs and Sgk1 interact functionally, oocytes injected with rENaC FLAG-tagged subunits (rENaCf) were coexpressed either individually or with each protease (mCAP), or with Sgk1 or both together. If the two activation pathways are fully independent of each other but have a final common effector (ENaC), a true potentiation should be observed, i.e., any synergistic effect should be more than additive. In Table I (mCAP1-Skg1), Table II (mCAP2-Sgk1), Table III (mCAP3 + Sgk1), the data are presented in absolute values, allowing to quantify the synergism observed.

mCAP1 and Sgk1

When rENaCf was coexpressed with mCAP1 (Table I), N did not change ($P < 0.9$) (column a) whereas I_{Na} (column b) increased 11-fold ($P < 0.001$) compared with rENaCf expressed alone. When rENaCf was coexpressed with Sgk1, we observed a 1.6-fold in N ($P < 0.01$) and a 2.6-fold increase in I_{Na} ($P < 0.001$). When Sgk1 and mCAP1 were coexpressed, I_{Na} increased 9.1-fold ($P < 0.001$) and N by only 0.3-fold ($P < 0.05$) when compared with oocytes injected with Sgk1 alone. If the effect of the two pathways was just additive, their combined effect would not exceed the sum of the two cRNA given alone. For I_{Na} it should not exceed 13 μA per oocyte, but we do observe 24 μA/oocyte. Thus, the data indicate a strong and highly significant ($P < 0.001$) potentiation between mCAP1 and Sgk1 for the

TABLE I

Effect of Sgk1 and CAP1 on ENaC-mediated Sodium Current (I_{Na}) and Cell Surface Expression (N) Measured by Monoclonal Antibody Binding to Flagged αβγENaC

cRNA injection	n (oocyte)	N (*f*mole/oocyte)	Before trypsin treatment		After trypsin treatment		I_{Na}/N − tryp/I_{Na}/N + tryp
			I_{Na}	I_{Na}/N	I_{Na}	I_{Na}/N	
		a	b	c	d	e	e/c
			μA/oocyte	*μA/f mole*	*μA/oocyte*	*μA/f mole*	
ENaC + H_2O	40	0.085 ± 0.007	1.1 ± 0.2	14.2 ± 1.0	6.2 ± 1.0	84.1 ± 12.3	5.9 ($P < 0.01$)
ENaC + mCAP1	35	0.085 ± 0.010	11.9 ± 1.5	182.6 ± 24.3	11.4 ± 1.5	175.9 ± 23.3	1.0 (ns)
ENaC + Sgk1	31	0.139 ± 0.014	2.7 ± 0.4	21.7 ± 2.9	12.0 ± 1.6	102.9 ± 14.2	4.7 ($P < 0.01$)
ENaC + Sgk1 + mCAP1	26	0.186 ± 0.015	24.5 ± 1.8	162.8 ± 21.2	24.4 ± 1.7	149.3 ± 14.1	0.9 (ns)

I_{Na} and N were measured in the same oocyte as described in MATERIALS AND METHODS. 26–40 oocytes from a minimum of 5 different batches were measured for each experimental condition.

t test results for N: control vs. CAP1, $P < 0.9$; control vs. Sgk1, $P < 0.001$; control vs. Sgk1 + mCAP1, $P < 0.001$; mCAP1 vs. Sgk1, $P < 0.001$; mCAP1 vs. Sgk1 + mCAP1, $P < 0.001$; Sgk1 vs. Sgk1 + mCAP1, $P < 0.02$.

t test results for I_{Na}: control vs. CAP1, $P < 0.001$; control vs. Sgk1, $P < 0.001$; control vs. Sgk1 + mCAP1, $P < 0.001$; mCAP1 vs. Sgk1, $P < 0.001$; mCAP1 vs. Sgk1 + mCAP1, $P < 0.001$; Sgk1 vs. Sgk1 + mCAP1, $P < 0.001$.

t test results for I_{Na}/N: control vs. CAP1, $P < 0.001$; control vs. Sgk1, $P < 0.03$; control vs. Sgk1 + mCAP1, $P < 0.001$; mCAP1 vs. Sgk1, $P < 0.001$; mCAP1 vs. Sgk1 + mCAP1, $P < 0.55$; Sgk1 vs. Sgk1 + mCAP1, $P < 0.001$.

I_{Na} effect, whereas the synergism for the N effect was small (though significant) and less than additive. To verify that mCAP1 was expressed for its maximal effect, we tested the effect of external trypsin. As shown in Table I, trypsin (column d) was able to increase baseline I_{Na} only in ENaC or ENaC + Sgk1–injected oocytes. By contrast, trypsin did not elicit any further increase in I_{Na} when mCAP1 was expressed. These data indicate that mCAP1 has already fully activated ENaC expressed at the surface of the oocyte. I_{Na}/N (column c) is a parameter from which one can deduce the average open probability in a whole cell wP_o (see MATERIALS AND METHODS and DISCUSSION). As shown in Table I, mCAP1 increased I_{Na}/N by 13-fold ($P < 0.001$), Sgk1 alone had a minimal effect (1.5-fold; $P < 0.05$), mCAP1 and Sgk1 lead to an 11-fold increase in I_{Na}/N ($P < 0.001$), not significantly different from stimulation observed with CAP1 alone. Upon activation by trypsin (column e), I_{Na}/N increased 5.9-fold in controls ($P < 0.001$) and 4.7-fold in Sgk1 injected oocytes ($P < 0.001$). No further significant increase in I_{Na}/N was observed in mCAP1 or mCAP1 + Sgk1–coinjected oocytes.

mCAP2 and Sgk1

When rENaCf was coexpressed with mCAP2 (Table II), N did not change ($P = 0.6$) (column a), whereas I_{Na} (column b) increased 8.4-fold ($P < 0.001$) compared with rENaCf expressed alone. When rENaCf was coexpressed with Sgk1, we observed a 1.6-fold increase in N ($P < 0.01$) and a 2.4-fold increase in I_{Na} ($P < 0.001$). When Sgk1 and CAP2 were coexpressed, I_{Na} increased 7.6-fold ($P < 0.001$) and N by 1.4-fold ($P < 0.06$) when compared with oocytes injected with Sgk1 alone. If the effect of the two pathways was just additive, their combined effect would not exceed the sum of the two cRNA given alone. For I_{Na} it should not exceed 5.4 μA/oocyte, but we do observe 9.1 μA/oocyte. Thus, the data indicate a strong and highly significant ($P < 0.001$) potentiation between mCAP2 and Sgk1 for the I_{Na} effect, whereas the synergism for the N effect did not reach the level of significance ($P < 0.06$) and was less than additive. To verify that mCAP2 was expressed maximally, we tested the effect of external trypsin. As shown in Table II, trypsin (column d) was able to increase baseline I_{Na} only in ENaC or ENaC + Sgk1–injected oocytes. By contrast, trypsin did not elicit any further increase in I_{Na} when mCAP2 was expressed. These data indicate that mCAP2 has already fully activated ENaC expressed at the surface of the oocyte. As shown in Table II (column d), mCAP2 increased I_{Na}/N by ninefold ($P < 0.001$), Sgk1 alone had a small effect (2.4-fold; $P < 0.05$), and mCAP2 and Sgk1 led to a 10-fold increase in I_{Na}/N ($P < 0.001$), not significantly different from the stimulation observed with mCAP2 alone ($P < 0.76$). Upon activation by trypsin (column e), I_{Na}/N increased 7.3-fold in controls ($P < 0.001$) and 4.4-fold in Sgk1-injected oocytes ($P < 0.001$). No further significant increase in I_{Na}/N was observed in mCAP2 or mCAP2 + Sgk1–coinjected oocytes.

mCAP3 and Sgk1

When rENaCf was coexpressed with mCAP3 (Table III), N did not change ($P < 0.7$) (column a), whereas I_{Na} (column b) increased 6.8-fold ($P < 0.001$) compared with rENaCf expressed alone. When rENaCf was coexpressed with Sgk1, we observed a 1.8-fold increase in N ($P < 0.001$) and a 3.6-fold increase in I_{Na} ($P < 0.001$).

TABLE II

Effect of Sgk1 and CAP2 on ENaC-mediated Sodium Current (I_{Na}) and Cell Surface Expression (N) Measured by Monoclonal Antibody Binding to Flagged αβγENaC

cRNA injection	n (oocyte)	N (*f*mole/oocyte)	Before trypsin treatment		After trypsin treatment		I_{Na}/N − tryp/I_{Na}/N + tryp
			I_{Na}	I_{Na}/N	I_{Na}	I_{Na}/N	
		a	b	c	d	e	e/c
			μA/oocyte	*μA/f mole*	*μA/oocyte*	*μA/f mole*	
ENaC + H_2O	32	0.065 ± 0.008	0.5 ± 0.1	10.1 ± 1.5	3.9 ± 0.9	73.3 ± 13.8	7.3 ($P < 0.01$)
ENaC + mCAP2	28	0.070 ± 0.007	4.2 ± 0.9	90.0 ± 23.0	4.9 ± 0.9	94.2 ± 20.0	1.0 (ns)
ENaC + Sgk1	26	0.105 ± 0.016	1.2 ± 0.1	24.1 ± 7.2	6.4 ± 0.7	106.2 ± 18.9	4.4 ($P < 0.01$)
ENaC + Sgk1 + mCAP2	19	0.151 ± 0.018	9.1 ± 1.3	80.7 ± 14.8	12.4 ± 1.1	108.4 ± 16.3	1.3 (ns)

I_{Na} and N were measured in the same oocyte as described in MATERIALS AND METHODS. 19–32 oocytes from a minimum of 5 different batches were measured for each experimental condition.

t test results for N: control vs. CAP2, $P < 0.6$; control vs. Sgk1, $P < 0.02$; control vs. Sgk1 + mCAP2, $P < 0.001$; mCAP2 vs. Sgk1, $P < 0.04$; mCAP2 vs. Sgk1 + mCAP2, $P < 0.001$; Sgk1 vs. Sgk1 + mCAP2, $P < 0.06$.

t test results for I_{Na}: control vs. CAP2, $P < 0.001$; control vs. Sgk1, $P < 0.001$; control vs. Sgk1 + mCAP2, $P < 0.001$; mCAP2 vs. Sgk1, $P < 0.001$; mCAP2 vs. Sgk1 + mCAP2, $P < 0.003$; Sgk1 vs. Sgk1 + mCAP2, $P < 0.001$.

t test results for I_{Na}/N: control vs. CAP2, $P < 0.001$; control vs. Sgk1, $P < 0.04$; control vs. Sgk1 + mCAP2, $P < 0.001$; mCAP2 vs. Sgk1, $P < 0.01$; mCAP2 vs. Sgk1 + mCAP2, $P < 0.8$; Sgk1 vs. Sgk1 + mCAP2, $P < 0.001$.

When Sgk1 and mCAP3 were coexpressed, I_{Na} increased 7.3-fold ($P < 0.001$) and N by only 0.14-fold ($P = 0.4$) compared with oocytes injected with Sgk1 alone. If the effect of the two pathways was just additive, their combined effect would not exceed the sum of the two cRNAs given alone. For I_{Na}, it should not exceed 5.2 μA/oocyte, but we do observe 13.2 μA/oocyte. Thus, the data indicate a strong and highly significant ($P < 0.001$) potentiation between mCAP3 and Sgk1 for the I_{Na} effect, whereas the synergism for the N effect was significant but small and less than additive. To verify that mCAP3 was expressed maximally, we tested the effect of external trypsin. As shown in Table III, trypsin (column d) was able to increase baseline I_{Na} only in ENaC or ENaC + Sgk1–injected oocytes. By contrast, trypsin did not elicit any further increase in I_{Na} when mCAP3 was expressed alone or with Sgk1. These data indicate mCAP3 has already fully activated ENaC expressed at the surface of the oocyte. As shown in Table III, mCAP3 increased I_{Na}/N by 9.4-fold ($P < 0.001$), Sgk1 alone had a small effect (twofold ($P < 0.01$), and mCAP3 and Sgk1 led to 7.6-fold increase in I_{Na}/N ($P < 0.001$), not significantly different from stimulation observed with CAP3 alone ($P < 0.14$). Upon activation by trypsin (column e), I_{Na}/N increased 6.7-fold in controls ($P < 0.001$) and 5.3-fold in Sgk1-injected oocytes ($P < 0.001$). No further significant increase in I_{Na}/N was observed in mCAP3 or mCAP3 + Sgk1–coinjected oocytes.

In summary, qualitatively, the effects of mCAP1, mCAP2, or mCAP3 are remarkably similar. Since base-

TABLE III

Effect of Sgk1 and CAP3 on ENaC-mediated Sodium Current (I_{Na}) and Cell Surface Expression (N) Measured by Monoclonal Antibody Binding to Flagged αβγENaC

cRNA injection	n (oocyte)	N (*f*mole/oocyte)	Before trypsin treatment		After trypsin treatment		I_{Na}/N − tryp/I_{Na}/N + tryp
			I_{Na}	I_{Na}/N	I_{Na}	I_{Na}/N	
		a	b	c	d	e	e/c
			μA/oocyte	*μA/f mole*	*μA/oocyte*	*μA/f mole*	
ENaC + H_2O	34	0.067 ± 0.008	0.5 ± 0.1	9.3 ± 1.4	2.9 ± 0.5	62.8 ± 12.1	6.7 ($P < 0.01$)
ENaC + mCAP3	15	0.061 ± 0.013	3.4 ± 0.5	87.6 ± 20.1	4.4 ± 0.6	101.9 ± 17.5	1.2 (ns)
ENaC + Sgk1	28	0.122 ± 0.015	1.8 ± 0.3	18.6 ± 2.7	8.7 ± 1.1	99.2 ± 15.4	5.3 ($P < 0.01$)
ENaC + Sgk1 + mCAP3	30	0.140 ± 0.014	13.2 ± 1.1	141.3 ± 20.7	15.7 ± 1.5	150.8 ± 18.4	1.1 (ns)

I_{Na} and N were measured in the same oocyte as described in MATERIALS AND METHODS. 15–34 oocytes from a minimum of 5 different batches were measured for each experimental condition.

t test results for N: control vs. CAP3, $P < 0.7$; control vs. Sgk1, $P < 0.001$; control vs. Sgk1 + mCAP3, $P < 0.001$; mCAP3 vs. Sgk1, $P < 0.01$; mCAP3 vs. Sgk1 + mCAP3, $P < 0.001$; Sgk1 vs. Sgk1 + mCAP3, $P < 0.4$.

t test results for I_{Na}: control vs. CAP3, $P < 0.001$; control vs. Sgk1, $P < 0.001$; control vs. Sgk1 + mCAP3, $P < 0.001$; mCAP3 vs. Sgk1, $P < 0.003$; mCAP3 vs. Sgk1 + mCAP3, $P < 0.001$; Sgk1 vs. Sgk1 + mCAP3, $P < 0.001$.

t test results for I_{Na}/N: control vs. CAP3, $P < 0.001$; control vs. Sgk1, $P < 0.002$; control vs. Sgk1 + mCAP3, $P < 0.001$; mCAP3 vs. Sgk1, $P < 0.001$; mCAP3 vs. Sgk1 + mCAP3, $P < 0.1$; Sgk1 vs. Sgk1 + mCAP3, $P < 0.001$.

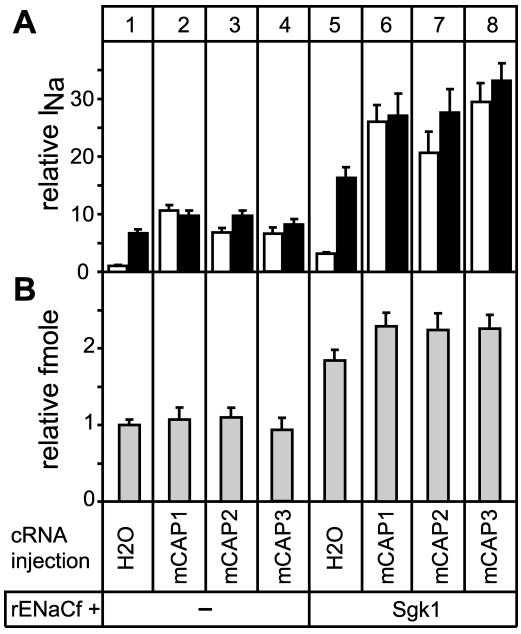

FIGURE 4. Synergistic activation of mCAP1–3 and Sgk1 on ENaC activity. (A) Oocytes were injected with cRNA coding either for α, β, and γFLAG-tagged rENaC subunits (rENaCf) and water (lane 1) or rENaCf together with mCAP1 (lane 2), mCAP2 (lane 3), mCAP3 (lane 4), Sgk1 (lanes 5–8), or both Sgk1 and mCAP1 (lane 6), Sgk1 and mCAP2 (lane 7), and Sgk1 and mCAP3 (lane 8). I_{Na} was measured in absence (open bar) and after perfusion of trypsin (closed bar) and normalized to I_{Na} in oocytes injected with rENaC alone. $n \geq 15$ measured oocytes per experimental condition taken from Tables I–III. (B) Normalized oocyte cell surface expression of rENaCf in oocytes experiments described in A. Water, lane 1; mCAP1, lane 2; mCAP2, lane 3; mCAP3, lane 4; Sgk1, lane 5; mCAP1 + Sgk1, lane 6; mCAP2 + Sgk1, lane 7; mCAP3 + Sgk1, lane 8. ***, $P < 0.001$ versus rENaCf + water.

line I_{Na} varies by a factor of two from experiment to experiment (see column b, ENaC-injected oocytes of each table), comparisons between CAPs are also shown in Fig. 4 and summarized as relative changes normalized to ENaC and/or water-injected oocytes. It is evident that, when corrected for the different baseline values, the effects of each CAP are also quantitatively very similar.

DISCUSSION

Membrane-bound Serine Proteases: a Novel Signaling Cascade in Epithelial Cells?

In this study, we describe the identification of three distinct mammalian membrane-bound serine proteases that are present in the same cell type. mCAP1 is a GPI-anchored protein, whereas mCAP2 and mCAP3 belong to the type 2 transmembrane serine proteases (TTSPs) family (Hooper et al., 2001). A few members of membrane-bound serine proteases have been so far biochemically characterized or their cell surface localization has been demonstrated (Hooper et al., 2001). Based on a conserved disulfide bond linking the pro- and the catalytic domains, one can predict that TTSPs are active in their membrane-bound form. Interestingly, some TTSPs have a high degree of autocatalytic activity and the targeting of the TTSPs to the apical membrane of an epithelial cell involves its proteolytic domain (Zheng et al., 1999; Hooper et al., 2001). Recently, a recombinant Kunitz-type serine protease inhibitor, which is similar to aprotinin, was tested in human bronchial epithelial cells and shown to efficiently block ENaC-mediated Na^+ transport in the lung (Bridges et al., 2001). Homology screening of a human airway epithelial cDNA library identified human prostasin (the orthologue of mCAP1) and TMPRSS2, a homologue of mCAP2 or the human TMPRSS4 (Donaldson et al., 2002). These authors demonstrated that prostasin increased ENaC activity, whereas TMPRSS2 had a strong antagonist effect in the *Xenopus* oocyte system. In our screen, we were not able to identify epitheliasin, the mouse homologue of TMPRSS2, in the $mpkCCD_{c14}$ cell line. In our study, mCAP2 clearly has an agonist effect on ENaC activation, which is very similar to that of mCAP1 or prostasin. It is not clear, at present time, what the structural difference is between the two proteases that might explain this striking functional difference. Our present working hypothesis is that mCAP1–3 are coexpressed at the apical membrane of, for example, kidney cells, and exert their effect either together on channel activation or by a catalytic cascade similar to the serine protease cascade of the blood clotting system. For xCAP1, we have reported recently the importance of the GPI anchor for the activation of ENaC at the plasma membrane (Vallet et al., 2002), but the role of the transmembrane domains of mCAP2 or mCAP3 in ENaC activation has not yet been studied. How and in which sequence each of the serine proteases may activate ENaC is not known.

Control of Cell Surface Expression of ENaC and its Open Probability by Two Distinct and Synergistic-signaling Pathways Extends the Dynamic Range of Channel Regulation

The *Xenopus* oocyte expression system allows to quantify cell surface expression of epithelial sodium channels and to determine the channel open probability in a whole cell (wcP_o) (Firsov et al., 1996, 1997) (see MATERIALS AND METHODS). According to the single channel measurement after trypsin activation reported by Chraibi et al. (1998), one can make the assumption that gNa remains unchanged under our experimental conditions. Under a voltage clamp set constant at −100 mV, one can now get a fairly good estimate of the wcP_o and how it is affected by our experimental maneuvers. Under low sodium incubation, we reported a I_{Na}/N of 4.82 mA/fmole (Firsov et al., 1996). We can calculate a

wcP_o of around 0.018, assuming a heterotetrameric channel (Firsov et al., 1998). In the present study, we injected three times less ENaC cRNA, leading to a lower cell surface expression of ENaC (N varies from 0.06–0.08 fmole/oocyte [present study] vs. 0.5 fmole/oocyte [Firsov et al., 1998]) but with a relatively high current (I_{Na}, 0.5–1 μA/oocyte with a I_{Na}/N varying between 9 and 14 μA/fmole (See Table I–III). From these data, one can calculate a wcP_o of 0.05. For ENaC, the estimation of P_o in membrane patches (mpP_o) has been reported to be extremely variable (Palmer and Frindt, 1988; Palmer and Frindt, 1996). In native membranes from salt-repleted rat, no channel activity was detectable. It is likely that this observation is due to the lack of sensitivity of the patch method to detect channels with very low P_o or very few channels with higher P_o. In native membrane from salt-depleted animals with high plasma aldosterone levels, mpP_o ranged from 0.05 to 0.9 with an average value of 0.5 (Pacha et al., 1993). Due to the wide distribution of spontaneous P_o and the slow transitions between open and closed states, the quantitation of the number of active channels in the membrane patch is difficult, leading to overestimation of P_o. This overestimation could be experimentally documented by direct comparisons of the two methods for a mutant channel of the β subunit (G37S) leading to a 50% decrease in Po (Firsov et al., 1997). The alternative wcP_o method used in the present study is therefore a useful quantitative approach to assess the effect of CAP or Sgk1 on P_o in an intact cell.

mCAP1, mCAP2, and mCAP3 Increase wcP_o without Changing N

Upon CAP stimulation, it is evident that the wcP_o of ENaC can reach very high values (up to 0.65 for mCAP1), which cannot be further increased by external trypsin. None of the three CAPs tested changed the cell surface expression of ENaC. It would appear that this signaling pathway is unique in controlling the gating of the channel from an external site, which is trypsin sensitive (Chraibi et al., 1998). The molecular mechanisms remain elusive. It has been suggested that the γ subunit was a substrate for CAP (Masilamani et al., 1999) but, so far, no biochemical experimental evidence has supported this hypothesis. A second important issue is to know whether CAPs are regulated by hormones or other factors. In our experimental systems, we have not been able to demonstrate any effect of aldosterone on the mRNA abundance of either of the three CAPs expressed in mpkCCD cells (unpublished data). It is, however, interesting to note a recent article reporting that aldosterone increased in vitro the expression of mCAP1/prostasin mRNA and protein in a kidney cell line (M1) and in vivo in adrenalectomized rats (Narikiyo et al., 2002).

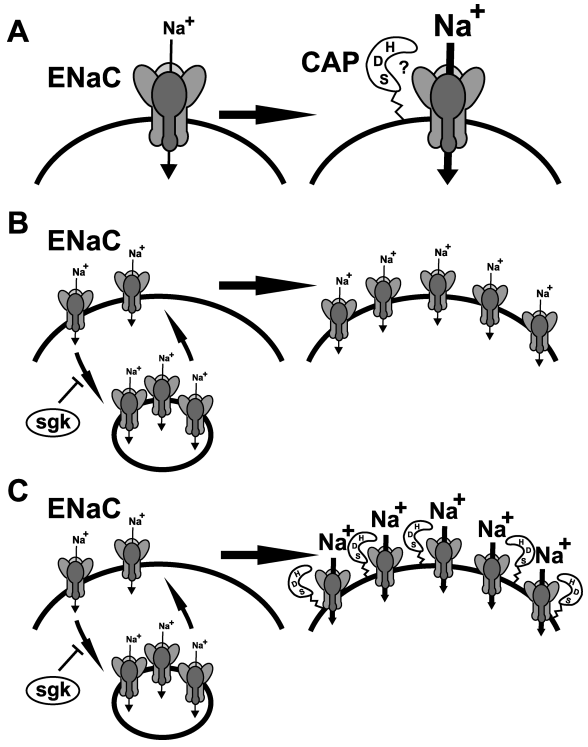

FIGURE 5. Model of CAPs and Sgk1 action on ENaC. (A) CAPs increase the open probability of ENaC channels. The substrate of CAPs may be ENaC or protein(s) associated with ENaC. (B) Model of Sgk1 action on ENaC. Sgk1 principally increases the number of active channels at the cell surface by diminishing the removal of the channels from the cell surface and by increasing its P_o. (C) CAPs proteases and Sgk1 act synergically on the channel open probability and cell surface expression.

Dual Effect of Sgk1 on N and wcP_o

Our data clearly indicate that Sgk1 increases N significantly in three large and independent series of experiments (Table I–III and Fig. 4), confirming previous reports showing an over twofold increased expression of αβγ-FLAG ENaC measured by ^{125}I-protein G binding to the anti-FLAG monoclonal antibody (Alvarez de La Rosa et al., 1999), with no evidence of an effect on P_o measured in membrane patches. More recently, it has been shown that Sgk1 induced a proportional increase in I_{Na} and in N (about threefold) in the *Xenopus* oocyte system, suggesting that the effect is entirely mediated by a change in ENaC cell-surface expression (Loffing et al., 2001) that may occur in vivo only in a restricted part of the aldosterone-sensitive distal nephron (proximal ASDN). Our data differ somewhat from these two studies in the sense that we do observe a small but highly significant and highly reproducible effect of Sgk1 on I_{Na}/N (1.52, $P < 0.001$, Table I; 2.4, $P < 0.001$ Table II; and 2.0, $P < 0.001$) that is consistently higher than that observed on N (Table III), suggesting that Sgk1 has an additional effect on wcP_o. The reasons for that differ-

ence are not clear, but our experimental protocol differs significantly from those of the two previous studies in the sense that we have measured I_{Na} and N in the same individual oocyte, allowing a better quantitation of any effect on N and/or P_o. On the other hand, values of mpP_o reported in one study were 0.83 for ENaC-injected oocytes and 0.87 for ENaC + Sgk1–injected oocytes (Alvarez de La Rosa et al., 1999). As discussed above, such high baseline values may not reflect the physiological regulation of ENaC in a kidney cell and the technique may underestimate the number of silent or weakly active channels in the membrane. Interestingly, we reported previously that the Liddle mutation has also a dual effect on N and P_o (Firsov et al., 1996), very similar to the effect of Sgk1 reported here. Since Sgk1 is proposed to mediate its effects on ENaC through the phosphorylation of Nedd4–2 (Debonneville et al., 2001), which binds to the PPXY motif of ENaC, which is precisely the consensus for the Liddle mutation, it would make sense that the Sgk1-Nedd4-2-ENaC–signaling cascade involves a dual mechanism of ENaC activation.

CAP and Sgk1: Synergism of Potentiation

This paper demonstrates a synergism between the two signaling pathways in the oocyte expression system (Figs. 4 and 5). mCAP1, mCAP2, or mCAP3 have no effect on cell surface expression of ENaC, but increase wcP_o by 7–10-fold. On the other hand, Sgk1 increases the number of channels expressed at the cell surface by twofold, without changing wcP_o. When the two signaling pathways are activated together, a 20–30-fold increase is observed. The synergism is therefore more than additive and is a true potentiation. The possible mechanisms for this synergism has not yet been studied. It will be interesting to examine whether mCAPs can bind directly to ENaC by protein–protein interaction through their extracellular (mCAP1, mCAP2, or mCAP3) and/or through their cytoplasmic domains (mCAP2 or mCAP3). Another possible mechanism would imply a protein–protein interaction between Sgk1 and the intracellular COOH termini of mCAP2 or mCAP3 as a potential target for phosphorylation. It remains to demonstrate that the synergism we describe here operates in vivo in the principal cell of CCD in response to aldosterone. Gene inactivation of Sgk1 (Wulff et al., 2001) and mCAP1 (Rubera et al., 2002), mCAP2, and mCAP3 will help to dissect the respective role of the two signaling pathways. Despite large variations in salt intake, the kidney is able to maintain sodium balance and the extracellular volume within narrow margins, an important factor in the control of blood pressure. The ASDN plays a key role in adjusting sodium reabsorption to diet intake that may vary from 1 g NaCl/d to 40 g/d. The fine control of ENaC through the control of its cell surface expression and its open probability by aldosterone acting, on one hand, on an intracellular signaling cascade and, on the other hand, on a serine protease that controls gating from the extracellular compartment, should provide the large range required for rapid and long term regulation of sodium transport in ASDN.

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REFERENCES

Adachi, M., K. Kitamura, T. Miyoshi, T. Narikiyo, K. Iwashita, N. Shiraishi, H. Nonoguchi, and K. Tomita. 2001. Activation of epithelial sodium channels by prostasin in Xenopus oocytes. *J. Am. Soc. Nephrol.* 12:1114–1121.

Alvarez de La Rosa, D.A., P. Zhang, A. Naray-Fejes-Toth, G. Fejes-Toth, and C.M. Canessa. 1999. The serum and glucocorticoid kinase sgk increases the abundance of epithelial sodium channels in the plasma membrane of Xenopus oocytes. *J. Biol. Chem.* 274: 37834–37839.

Bens, M., V. Vallet, F. Cluzeaud, L. Pascual-Letallec, A. Kahn, M.E. Rafestin-Oblin, B.C. Rossier, and A. Vandewalle. 1999. Corticosteroid-dependent sodium transport in a novel immortalized mouse collecting duct principal cell line. *J. Am. Soc. Nephrol.* 10:923–934.

Bridges, R.J., B.B. Newton, J.M. Pilewski, D.C. Devor, C.T. Poll, and R.L. Hall. 2001. Na+ transport in normal and CF human bronchial epithelial cells is inhibited by BAY 39-9437. *Am. J. Physiol.* 281:L16–L23.

Canessa, C.M., L. Schild, G. Buell, B. Thorens, Y. Gautshi, J.-D. Horisberger, and B.C. Rossier. 1994. The amiloride-sensitive epithelial sodium channel is made of three homologous subunits. *Nature.* 367:463–467.

Chen, S.Y., A. Bhargava, L. Mastroberardino, O.C. Meijer, J. Wang, P. Buse, G.L. Firestone, F. Verrey, and D. Pearce. 1999. Epithelial sodium channel regulated by aldosterone-induced protein sgk. *Proc. Natl. Acad. Sci. USA.* 96:2514–2519.

Chraibi, A., V. Vallet, D. Firsov, S.K. Hess, and J.D. Horisberger. 1998. Protease modulation of the activity of the epithelial sodium channel expressed in Xenopus oocytes. *J. Gen. Physiol.* 111:127–138.

Dang, Q.D., and E. Di Cera. 1996. Residue 225 determines the Na(+)-induced allosteric regulation of catalytic activity in serine proteases. *Proc. Natl. Acad. Sci. USA.* 93:10653–10656.

Debonneville, C., S.Y. Flores, E. Kamynina, P.J. Plant, C. Tauxe, M.A. Thomas, C. Münster, A. Chraïbi, J.H. Pratt, J.-D. Horisberger, et al. 2001. Phosphorylation of Nedd4-2 by Sgk1 regulates epithelial Na+ channel cell surface expression. *EMBO J.* 20: 7052–7059.

Donaldson, S.H., A. Hirsh, D.C. Li, C.D. Holloway, R.C. Boucher, J. Chao, and S.E. Gabriel. 2002. Regulation of the epithelial sodium channel by serine proteases in human airways. *J. Biol. Chem.* 277:8338–8345.

Firsov, D., I. Gautschi, A.M. Merillat, B.C. Rossier, and L. Schild. 1998. The heterotetrameric architecture of the epithelial sodium channel (ENaC). *EMBO J.* 17:344–352.

Firsov, D., S. Gruender, L. Schild, and B.C. Rossier. 1997. Quantita-

tion of cell surface expressed epithelial sodium channel: a novel approach to determine the channel open probability in a whole cell. *Nova Acta Leopold*. 302:13–22.

Firsov, D., L. Schild, I. Gautschi, A.M. Merillat, E. Schneeberger, and B.C. Rossier. 1996. Cell surface expression of the epithelial Na channel and a mutant causing Liddle syndrome: a quantitative approach. *Proc. Natl. Acad. Sci. USA*. 93:15370–15375.

Hooper, J.D., J.A. Clements, J.P. Quigley, and T.M. Antalis. 2001. Type II transmembrane serine proteases. Insights into an emerging class of cell surface proteolytic enzymes. *J. Biol. Chem.* 276: 857–860.

Kim, M.G., C. Chen, M.S. Lyu, E.G. Cho, D. Park, C. Kozak, and R.H. Schwartz. 1999. Cloning and chromosomal mapping of a gene isolated from thymic stromal cells encoding a new mouse type II membrane serine protease, epithin, containing four LDL receptor modules and two CUB domains. *Immunogenetics*. 49: 420–428.

Lin, C.Y., J. Anders, M. Johnson, Q.A. Sang, and R.B. Dickson. 1999. Molecular cloning of cDNA for matriptase, a matrix-degrading serine protease with trypsin-like activity. *J. Biol. Chem.* 274: 18231–18236.

Loffing, J., M. Zecevic, E. Feraille, B. Kaissling, C. Asher, B.C. Rossier, G.L. Firestone, D. Pearce, and F. Verrey. 2001. Aldosterone induces rapid apical translocation of ENaC in early portion of renal collecting system: possible role of SGK. *Am. J. Physiol.* 280: F675–F682.

Masilamani, S., G.H. Kim, C. Mitchell, J.B. Wade, and M.A. Knepper. 1999. Aldosterone-mediated regulation of ENaC alpha, beta, and gamma subunit proteins in rat kidney. *J. Clin. Invest.* 104:R19–R23.

Naray-Fejes-Toth, A., C. Canessa, E.S. Cleaveland, G. Aldrich, and G. Fejes-Toth. 1999. sgk is an aldosterone-induced kinase in the renal collecting duct - Effects on epithelial Na+ channels. *J. Biol. Chem.* 274:16973–16978.

Narikiyo, T., K. Kitamura, M. Adachi, T. Miyoshi, K. Iwashita, N. Shiraishi, H. Nonoguchi, L.-M. Chen, K.X. Chai, J. Chao, and K. Tomita. 2002. Regulation of prostasin by aldosterone in the kidney. *J. Clin. Invest.* 109:401–408.

Pacha, J., G. Frindt, L. Antonian, R.B. Silver, and L.G. Palmer. 1993. Regulation of Na channels of the rat cortical collecting tubule by aldosterone. *J. Gen. Physiol.* 102:25–42.

Palmer, L.G., and G. Frindt. 1988. Conductance and gating of epithelial Na channels from rat cortical collecting tubule. Effects of luminal Na and Li. *J. Gen. Physiol.* 92:121–138.

Palmer, L.G., and G. Frindt. 1996. Gating of Na channels in the rat cortical collecting tubule - effects of voltage and membrane stretch. *J. Gen. Physiol.* 107:35–45.

Perona, J.J., and C.S. Craik. 1995. Structural basis of substrate specificity in the serine proteases. *Protein Sci.* 4:337–360.

Perona, J.J., and C.S. Craik. 1997. Evolutionary divergence of substrate specificity within the chymotrypsin-like serine protease fold. *J. Biol. Chem.* 272:29987–29990.

Robert-Nicoud, M., M. Flahaut, J.M. Elalouf, M. Nicod, M. Salinas, M. Bens, A. Doucet, P. Wincker, F. Artiguenave, J.D. Horisberger, et al. 2001. Transcriptome of a mouse kidney cortical collecting duct cell line: effects of aldosterone and vasopressin. *Proc. Natl. Acad. Sci. USA*. 98:2712–2716.

Rubera, I., E. Meier, G. Vuagniaux, A.-M. Merillat, F. Beermann, B.C. Rossier, and E. Hummler. 2002. A conditional allele at the mouse channel activating protease 1 (Prss8) gene locus. *Genesis*. 32:173–176.

Schechter, I., and A. Berger. 1967. On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun*. 27:157–162.

Snyder, P.M., D.R. Olson, and B.C. Thomas. 2002. SGK modulates Nedd4-2-mediated inhibition of ENaC. *J. Biol. Chem.* 277:5–8.

Takeuchi, T., M.A. Shuman, and C.S. Craik. 1999. Reverse biochemistry: use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-type serine protease in epithelial cancer and normal tissue. *Proc. Natl. Acad. Sci. USA*. 96:11054–11061.

Vallet, V., A. Chraibi, H.P. Gaeggeler, J.-D. Horisberger, and B.C. Rossier. 1997. An epithelial serine protease activates the amiloride-sensitive sodium channel. *Nature*. 389:607–610.

Vallet, V., J.-D. Horisberger, and B.C. Rossier. 1998. Epithelial sodium channel regulatory proteins identified by functional expression cloning. *Kidney Int*. 54:S109–S114.

Vallet, V., C. Pfister, J. Loffing, and B.C. Rossier. 2002. Cell surface expression of the channel activating protease xCAP1 is required for activation of ENaC in the Xenopus oocyte. *J. Am. Soc. Nephrol.* 13:588–594.

Verrey, F., E. Hummler, L. Schild, and B.C. Rossier. 2000. Control of Na+ transport by aldosterone. *In* The Kidney: Physiology and Pathophysiology. D.W. Seldin and G. Giebisch, eds. Lippincott Williams and Wilkins, Philadelphia. 1441–1471.

Vuagniaux, G., V. Vallet, N.F. Jaeger, C. Pfister, M. Bens, N. Farman, N. Courtois-Coutry, A. Vandewalle, B.C. Rossier, and E. Hummler. 2000. Activation of the amiloride-sensitive epithelial sodium channel by the serine protease mCAP1 expressed in a mouse cortical collecting duct cell line. *J. Am. Soc. Nephrol.* 11: 828–834.

Wallrapp, C., S. Hahnel, F. Muller-Pillasch, B. Burghardt, T. Iwamura, M. Ruthenburger, M.M. Lerch, G. Adler, and T.M. Gress. 2000. A novel transmembrane serine protease (TMPRSS3) overexpressed in pancreatic cancer. *Cancer Res*. 60:2602–2606.

Webster, M.K., L. Goya, Y. Ge, A.C. Maiyar, and G.L. Firestone. 1993. Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. *Mol. Cell. Biol.* 13:2031–2040.

Wulff, P., V. Vallon, D.Y. Huang, I. Pfaff, K. Klingel, D. Kauselmann, H. Volkl, F. Lang, and D. Kuhl. 2001. Deficient salt retention in the SGK1 knockout mouse. *J. Am. Soc. Nephrol.* 12:44A.

Yu, J.X., L. Chao, and J. Chao. 1995. Molecular cloning, tissue-specific expression, and cellular localization of human prostasin mRNA. *J. Biol. Chem.* 270:13483–13489.

Zheng, X., D. Lu, and J.E. Sadler. 1999. Apical sorting of bovine enteropeptidase does not involve detergent-resistant association with sphingolipid-cholesterol rafts. *J. Biol. Chem.* 274:1596–1605.