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Protein Kinase B Alpha (PKBα) Stimulates the Epithelial Sodium Channel (ENaC) Heterologously Expressed in *Xenopus laevis* Oocytes by Two Distinct Mechanisms

Alexei Diakov, Viatcheslav Nesterov, Marianna Mokrushina, Robert Rauh and Christoph Korbmacher

Institut für Zelluläre und Molekulare Physiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen

Key Words

Ion channel regulation • Phosphorylation • Surface expression• Patch-clamp • Protein kinase B α • Serum- and glucocorticoid-inducible kinase isoform 1 • *Xenopus laevis* oocytes

Abstract

Kinases contribute to the regulation of the epithelial sodium channel (ENaC) in a complex manner. For example, SGK1 (serum- and glucocorticoid-inducible kinase type 1) enhances ENaC surface expression by phosphorylating Nedd4-2, thereby preventing ENaC retrieval and degradation. An additional mechanism of ENaC activation by SGK1 involves an SGK consensus motif (^{616}RSRYWS^{621}) in the C-terminus of the channel's α-subunit. This consensus motif may also be a target for ENaC regulation by protein kinase B α (PKBα) known to be activated by insulin and growth factors. Therefore, we investigated a possible role of PKBα in the regulation of rat ENaC heterologously expressed in *Xenopus laevis* oocytes. We found that recombinant PKBα included in the pipette solution increased ENaC currents in outside-out patches by about 4-fold within 15-20 min. Replacing the serine residue S621 of the SGK consensus motif by an alanine (S621A) abolished this stimulatory effect. In co-expression experiments active PKBα but not catalytically inactive PKBα significantly increased ENaC whole-cell currents and surface expression by more than 50 % within 24 hours of co-expression. Interestingly, this stimulatory effect was preserved in oocytes expressing ENaC with the S621A mutation. We conclude that the acute stimulatory effect of PKBα involves a specific kinase consensus motif in the C-terminus of the channel's α-subunit. In contrast, the increase in channel surface expression caused by co-expression of PKBα does not depend on this site in the channel and is probably mediated by an effect on channel trafficking.

Introduction

Regulation of the epithelial sodium channel (ENaC) in the aldosterone sensitive distal nephron (ASDN) is critically important for body sodium homeostasis and hence

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Prof. Dr. med. Christoph Korbmacher
Institut für Zelluläre und Molekulare Physiologie
Waldstr. 6, 91054 Erlangen (Germany)
Tel. +49-9131-8522301, Fax +49-9131-8522770
E-Mail christoph.korbmacher@physiologie2.med.uni-erlangen.de

for the control of arterial blood pressure [1-2]. ENaC is the rate-limiting step for Na^+ reabsorption and a main target of hormones involved in the regulation of renal salt excretion e.g. aldosterone, angiotensin II, vasopressin, insulin and insulin-like growth factor-1 (IGF-1). The investigation of intracellular signalling pathways involved in mediating the effects of these hormones on ENaC may help to understand abnormalities in salt and water homeostasis and arterial blood pressure.

Insulin stimulates renal Na^+ retention and K^+ excretion in man and in laboratory animals [3]. Insulin and IGF-1 receptors have been detected along the mammalian renal tubular system including the cortical collecting duct [3-4] indicating that both hormones may regulate ENaC in renal epithelia. Insulin and/or IGF-1 have been reported to stimulate ENaC activity by increasing cell surface density of ENaC [5-8], but also by increasing the open probability of Na^+ channels already present in the plasma membrane [9-10]. At present it is unclear which of the two effects is predominant and whether this may vary depending on the physiological condition. It has also been suggested that the stimulatory effect of insulin involves direct phosphorylation of channel subunits and possibly additional more indirect actions of kinases [11-12].

Although involvement of insulin and IGF-1 signalling in regulation of Na^+ transport has been intensively studied, it is not yet fully understood. Data from amphibian and mammalian renal cell lines indicated that insulin and IGF-1 stimulate Na^+ transport *via* a signalling cascade that involves sequential phosphorylation and activation of phosphoinositide 3-kinase (PI3K), 3-phosphoinositide-dependent protein kinase (PDK1) and serum- and glucocorticoid-inducible kinase type 1 (SGK1) [13-15]. The rather mild renal salt wasting phenotype of SGK1 deficient-mice [16], however, points to a certain degree of redundancy in the SGK1-dependent signalling pathway. In this context, a possible role of the closely related protein kinase B α (PKBα) (called also Akt1) has to be considered [15]. In its kinase domain this insulin-dependent kinase is more then 50% homologous to SGK1 [17]. Using an *in vitro* assay with synthetic peptide substrates, it was shown that PKBα specificity requirements are similar to those of SGK1. Both kinases phosphorylate serine and threonine residues located in RxRxxS/T motifs [18-19]. A putative SGK/PKB consensus motif (^{616}RSRYWS^{621}, numeration according to rat sequence) is localized in the C-terminus of the α-subunit of ENaC next to the second transmembrane domain and is well conserved within all mammals. We have previously reported that the acute stimulatory effect of SGK1 on rat ENaC in outside-out membrane patches critically depends on the serine residue S621 in this putative consensus site [20]. It was also shown that SGK1 and PKBα can phosphorylate the same proteins, for example GSK3 (glycogen synthase kinase 3) [21] and the transcription factor FOXO3a (forkhead box O3a) [22] both *in vitro* and when they are over-expressed in cultural cells. Thus, PKBα and SGK1 may have some common physiological substrates. Interestingly, data obtained in Fisher rat thyroid cells indicate that both PKBα and SGK1 are components of an insulin signalling pathway that increases Na^+ absorption by upregulating membrane expression of ENaC *via* a regulatory system that involves inhibition of Nedd4-2 [23]. In contrast, it was reported that PKBα does not increase ENaC function under basal conditions or after stimulation with aldosterone, insulin, or AVP in A6 renal epithelial cells [24]. Thus, additional experiments are needed to further explore a possible regulatory effect of PKBα on ENaC function.

In the present study we investigated the effect of PKBα on ENaC heterologously expressed in *Xenopus laevis* oocytes. In outside-out patches we tested the acute effect of recombinant PKBα on ENaC activity. Moreover, we studied the effects of PKBα co-expression on ENaC whole-cell currents and surface expression in comparison with those of SGK1. Furthermore, we determined the role of the channel's putative SGK consensus motif [20] in mediating an effect of PKBα on ENaC.

Materials and Methods

cDNA clones

Full-length cDNAs for rat wild-type α-, β- and γENaC [25] and for α_{S621A}ENaC [20] were in pGEM-HE vector. FLAG-tagged rat βENaC [26] was in pSD5 vector. HA-tagged rat βENaC [27] was in pGEM-HE vector. The full-length cDNA encoding the human constitutively active PKBα (T308D, S473D; N-terminally HA-tagged) or inactive PKBα (T308A, S473A; N-terminally HA-tagged) and active SGK1 (S422D) were in pGHJ vector [28] and were generously provided by Prof. Florian Lang (Tübingen, Germany). Linearised plasmids were used as templates for cRNA synthesis using T7 (α-, α_{S621A}-, β-, β_{HA}- and γENaC; PKBα and SGK1) or SP6 (β_{FLAG}ENaC) RNA polymerases (mMessage mMachine, Ambion, Austin, TX, USA).

Isolation of oocytes, two-electrode voltage-clamp experiments, and surface expression assay

Defolliculated stage V-VI oocytes were obtained from ovarian lobes of adult female *Xenopus laevis* in accordance with the principles of German legislation, with approval by the animal welfare officer for the University of Erlangen-Nürnberg,

and under the governance of the state veterinary health inspectorate. Animals were anesthetized in 0.2 % MS222 (Sigma, Taufkirchen, Germany) and ovarian lobes were obtained by a small abdominal incision. Isolation of oocytes and two-electrode voltage-clamp experiments were performed essentially as described previously [20, 29-31]. Oocytes were injected with cRNA using 0.1 ng of cRNA per ENaC subunit per oocyte alone or together with 0.5 ng per oocyte of active PKBα, inactive PKBα, or active SGK1. To prevent Na^+ overloading [32], injected oocytes were incubated in low sodium modified Barth's solution (in mM: 1 NaCl, 40 KCl, 60 NMDG-Cl, 0.4 $CaCl_2$, 0.3 $Ca(NO_3)_2$, 0.8 $MgSO_4$, and 10 HEPES adjusted to pH = 7.4 with HCl) supplemented with 100 U/ml sodium penicillin and 100 µg/ml streptomycin sulphate. Unless stated otherwise, oocytes were studied two days after injection. To obtain current-voltage (*I/V*) plots, voltage pulse protocols were performed using consecutive 400 ms step changes of the clamped potential from -60 to -120 mV up to +40 mV in 20 mV increments. Amiloride-sensitive current (ΔI_{amt}) was determined by subtracting the current values (using the last 50 ms of the pulse traces) recorded in the presence of amiloride (2 µM) from those recorded prior to its addition. ENaC surface expression was determined using a chemiluminescence assay and a βENaC construct with a FLAG tag inserted into its extracellular loop as described previously [27, 33].

Outside-out patch-clamp recordings

Current recordings from outside-out membrane patches were performed as described previously [20, 34-35] using conventional patch-clamp technique. Patch pipettes were pulled from borosilicate glass capillaries and had a tip diameter of about 5-7 µm after fire polishing. Pipettes were filled with K-gluconate pipette solution (in mM: 90 K-gluconate, 5 NaCl, 2 Mg-ATP, 2 EGTA, and 10 mM HEPES adjusted to pH = 7.28 with Tris). Seals were routinely formed in a low sodium NMDG-Cl bath solution (in mM: 95 NMDG (N-methyl-D-glucamine)-Cl, 1 NaCl, 4 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES adjusted to pH 7.4 with Tris). In this bath solution the pipette resistance averaged about 3 MΩ. In NaCl bath solution the NMDG-Cl was replaced by 95 mM NaCl. Outside-out patches were routinely held at a holding pipette potential of -70 mV which was close to the calculated reversal potential of Cl^- (E_{Cl} = -77.2 mV) and K^+ (E_K = -79.4 mV) under our experimental conditions with experiments performed at room temperature (~23° C). The current traces were sampled at 800 Hz and filtered at 200 Hz. *I/V* plots were obtained from voltage-step protocols using consecutive 70 ms step changes of the clamp potential from -70 to -120 mV up to +40 mV in 20 mV increments (PULSE, HEKA, Lambrecht, Germany). The average current values reached during the last 15 ms of the voltage steps were used for the *I/V* plots.

Western blot analysis

Oocytes expressing $\alpha\beta_{HA}\gamma$ENaC alone or in combination with $_{HA}$PKBα-active or $_{HA}$PKBα-inactive were lysed in lysis buffer containing 500 mM NaCl, 5 mM EDTA, 50 mM Tris-Cl, pH 7.4, and a protease inhibitor cocktail ('Complete Mini EDTA-free' protease inhibitor cocktail tablets, Roche Diagnostics, Mannheim, Germany). The lysates were centrifuged for 10 min at 3000 *g* and supernatants were incubated with 1% Triton X-100 and 1% Igepal CA-630 (Sigma) on ice for 20 min. 4 x Sample buffer was added to 25 µg of total protein. All samples were boiled for 5 min at 95°C and subjected to 10% SDS-PAGE. After separation, proteins were transferred to PVDF membranes by semi-dry electroblotting and probed with a monoclonal rat anti-HA antibody (Roche Diagnostics) diluted to 1 : 1000. Horseradish peroxidase-labelled secondary goat anti-rat antibody was purchased from Dianova (Hamburg, Germany) and used at a dilution of 1 : 20000. Chemiluminescence signals were detected using ECL plus (Amersham, GE Healthcare, UK).

Chemicals

Constitutively active PKBα (as a recombinant MBP-PKBα fusion protein containing human PKBα residues 140-480 with T308D, S473D mutations) was purchased from New England BioLabs Inc. (Frankfurt/Main, Germany) as 1000 units vials in 100 µl stock solution containing 50 mM NaCl, 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol (DTT) and 50% glycerol. PKBα pipette solution was freshly prepared on the day of the experiment by adding 8 µl of the PKBα stock solution to 1 ml of the K-gluconate pipette solution giving a final PKBα concentration of 80 U/ml. To preserve PKBα activity, the pipette solution was supplemented with 1 mM DTT (Sigma). Control experiments were performed using heat-inactivated PKBα which had been incubated at 68° C for 45 minutes. Okadaic acid (OA) was purchased from Sigma and was dissolved in DMSO as a stock solution with a concentration of 0.1 mM. Amiloride hydrochloride (Sigma) was added from an aqueous 10 mM stock solution.

Statistics

Data are presented as mean values ± S.E.M; *n* indicates the number of individual oocytes; N indicates the number of different batches of oocytes used. Appropriate versions of Student's *t* test were used. Significance was accepted for $p<0.05$. *, **, and *** represent *p* values smaller than 0.05, 0.01, and 0.001, respectively.

Results

PKBα stimulates ENaC in outside-out patches

We previously have shown that recombinant constitutively active SGK1 included in the pipette solution can stimulate ENaC currents in outside-out membrane patches from *Xenopus laevis* oocytes heterologously expressing rat ENaC [20]. Since the substrate specificity of PKBα is similar to that of SGK1, we hypothesized that PKBα can also stimulate ENaC. To test this hypothesis, we investigated the effect of recombinant constitutively active PKBα (80 U/ml) on ENaC currents in outside-out patches excised from oocytes expressing wild-type rat αβγENaC.

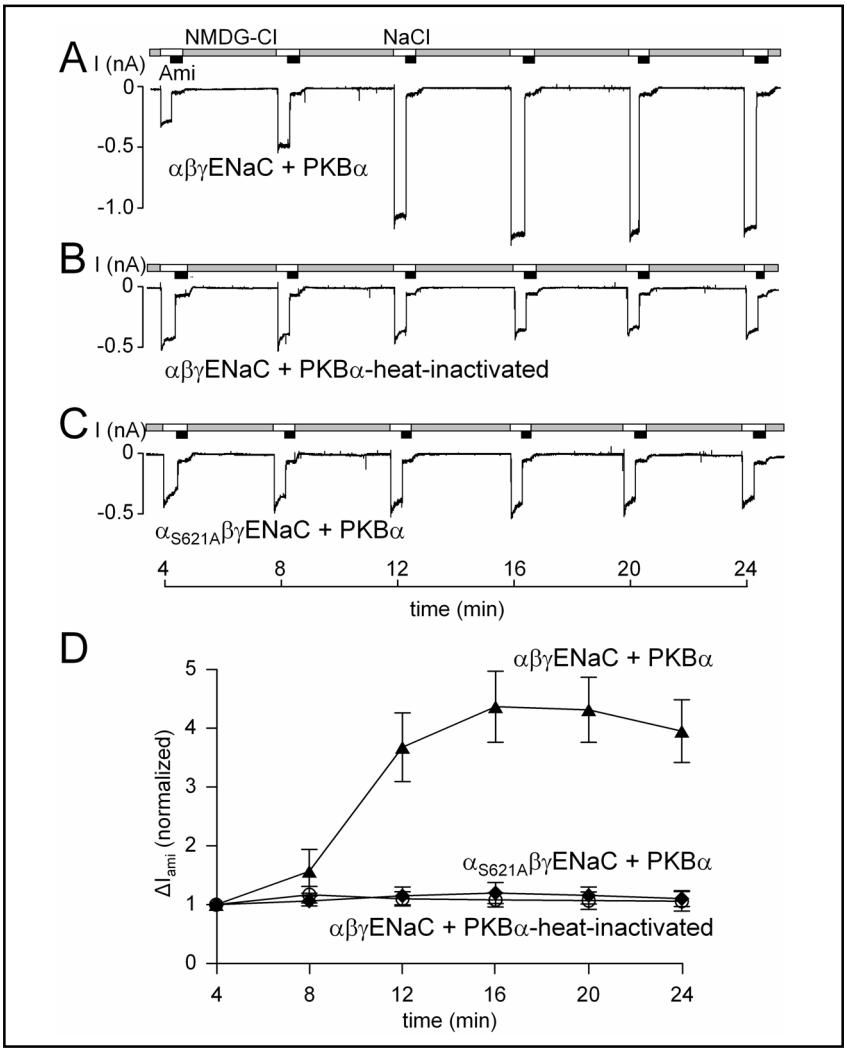

Fig. 1. In outside-out patches stimulation of ENaC by recombinant PKBα is abolished by heat-inactivating PKBα and by mutating the SGK/PKB consensus motif in αENaC (S621A). Representative current traces recorded in outside-out patches are shown in A-C. Using patches from oocytes expressing αβγENaC, constitutively active recombinant PKBα (80 U/ml) (A) or heat-inactivated enzyme (PKBα-heat-inactivated) (B) was included in the pipette solution. In C, an outside-out patch was excised from a $\alpha_{S621A}\beta\gamma$ENaC expressing oocyte with active PKBα in the pipette solution. The holding potential (V_{hold}) was –70 mV. As indicated by the bars, bath solution was changed from a low Na^+ (NMDG-Cl; [Na^+] = 1 mM) to a normal Na^+ containing solution (NaCl; [Na^+] = 95 mM) without or with amiloride (Ami, 2 μM). Average normalized amiloride-sensitive current (ΔI_{ami}) data from similar experiments as shown in A-C are summarized in D (αβγENaC + PKBα: n=8; αβγENaC + PKBα-heat-inactivated: n=5; $\alpha_{S621A}\beta\gamma$ENaC + PKBα: n=6).

As illustrated by the representative current traces in Figure 1 (A, B, and C) typical amiloride-sensitive currents could be recorded in outside-out patches from ENaC-expressing oocytes. To reduce spontaneous channel rundown during the recording, outside-out patches were maintained in NMDG-Cl bath solution for most of the time [36]. In this solution only a minimal inward current was detectable at a holding potential of -70 mV. In contrast, periodic changes to NaCl bath solution revealed sizeable inward currents consistent with a current component carried by Na^+ influx *via* ENaC. This current component was largely inhibited by the application of 2 μM amiloride, a concentration known to specifically inhibit ENaC. Using this protocol, the amiloride-sensitive sodium current (ΔI_{ami}) was repeatedly determined to monitor ENaC activity over time.

As shown in Figure 1A and summarized in Figure 1D, recombinant PKBα included in the pipette solution increased ΔI_{ami} about 4-fold within 15-20 minutes. This stimulatory effect of PKBα on ΔI_{ami} is similar to that previously reported for SGK1 [20]. In contrast, heat-inactivated PKBα failed to stimulate ENaC (Fig. 1B, D) which demonstrates that the stimulatory effect requires catalytically active PKBα. We have previously shown that the serine residue S621 in the α-subunit of ENaC is essential for mediating channel activation by SGK1 [20]. To test whether this residue is also important for mediating the stimulatory effect of PKBα on ENaC, we performed recordings in outside-out patches obtained from oocytes expressing mutant $\alpha_{S621A}\beta\gamma$ENaC in which S621 of the α-subunit was replaced by alanine. No stimulatory effect of PKBα was observed on mutant $\alpha_{S621A}\beta\gamma$ENaC (Fig. 1C, D). In summary, we have shown that catalytically active PKBα can stimulate ENaC in outside-out patches and that this effect critically depends on the presence of S621 in the C-terminus of αENaC.

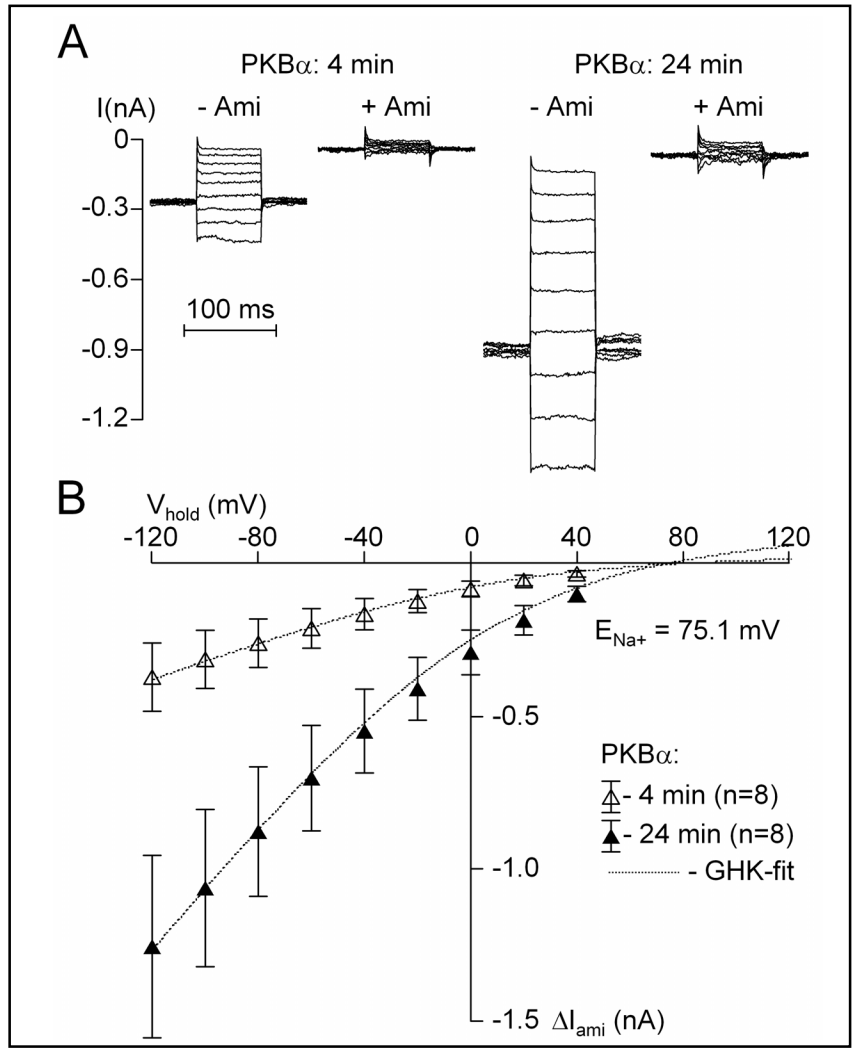

Fig. 2. PKBα does not change ion selectivity of activated ENaC. A, overlay current traces were derived from voltage step protocols performed during experiments as shown in Fig. 1A with active PKBα in the pipette solution, in the absence (-Ami) and in presence (+Ami) of amiloride, 4 min (PKBα: 4 min) and 24 min (PKBα: 24 min) after patch excision. ΔI_{ami} values obtained from 8 similar experiments were used to construct average *I/V* plots shown in B. Vertical bars represent SEM values. The dotted line represents a GHK-fit of the data for a Na^+ selective channel with a predicted Na^+ equilibrium potential (E_{Na}) of 75.1 mV.

Stimulation of ENaC by PKBα does not alter the selectivity of the channel

In experiments like those illustrated in Figure 1 brief voltage step protocols were routinely performed shortly before and after amiloride application to determine *I/V* relationships. In the traces shown in Figure 1 the current responses resulting from these voltage step protocols were omitted for clarity. In Figure 2 overlay current traces from representative voltage step protocols are shown on an expanded time scale. They were recorded with active PKBα in the pipette solution 4 minutes and 24 minutes after patch excision in the absence and presence of amiloride in the bath solution. Subtracting traces recorded in the presence of amiloride from corresponding traces recorded in its absence allows the calculation of ΔI_{ami} values at different holding potentials. Figure 2 B shows average *I/V* plots of ΔI_{ami} 4 minutes and 24 minutes after patch excision from experiments with PKBα in the pipette solution. A Goldman-Hodgkin-Katz (GHK) fit of these data reveals that ΔI_{ami} is highly Na^+ selective as expected for a current mediated by ENaC. Importantly, the *I/V* plots indicate that stimulation of the currents by PKBα does not shift the reversal potential, which suggests that the stimulated currents remain highly Na^+ selective. Thus, stimulation by PKBα does not alter the selectivity of ENaC.

Stimulation of ENaC by the phosphatase inhibitor okadaic acid is abolished by mutating the putative SGK consensus motif in the channel's α-subunit

Our results indicate that PKBα-mediated activation of ENaC in outside-out patches critically depends on the S621 residue in the C-terminus of αENaC. This suggests that phosphorylation of this residue is involved in PKBα-mediated ENaC activation. However, our findings do not

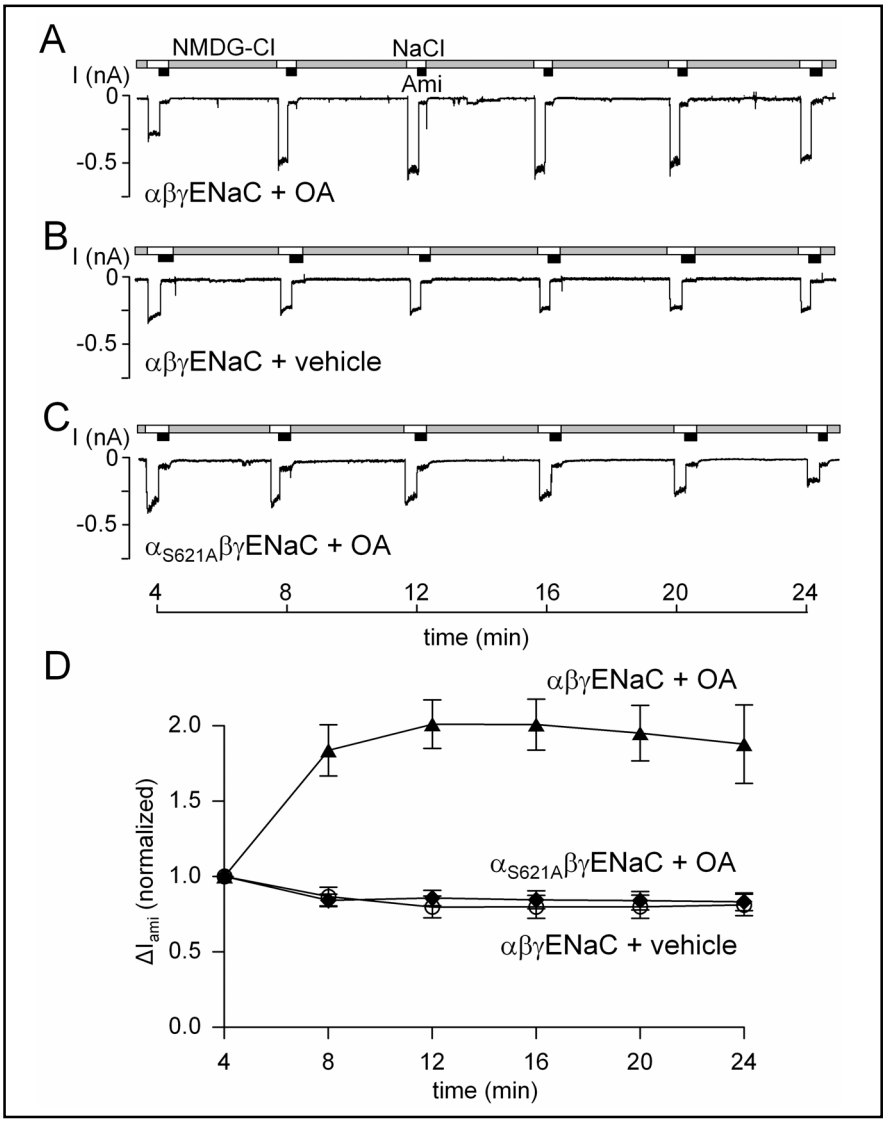

Fig. 3. The S621A mutation in the α-subunit prevents ENaC stimulation by okadaic acid in outside-out patches. Representative current traces in outside-out patches from oocytes expressing αβγENaC (A, B) or α_{S621A}βγENaC (C) are shown. Phosphatase inhibitor okadaic acid (OA, 100 nM) was included in the pipette solution in A and C. In B, vehicle (DMSO, 0.7 µl/ml) was included in the pipette solution as negative control. Average normalized ΔI_{ami} data from similar experiments as shown in A, B and C are summarized in D (αβγENaC + OA: n=5; αβγENaC + vehicle: n=5; α_{S621A}βγENaC + OA: n=6).

prove a direct phosphorylation of this site by PKBα. Indeed, we have previously shown that the nonspecific phosphatase inhibitor okadaic acid (OA) has a similar stimulatory effect on ENaC currents in outside-out patches as SGK1 [20]. Thus, inhibition of an endogenous phosphatase by OA may favour ENaC activation by an endogenous kinase present in the patch. To test whether the stimulatory effect of OA also depends on the S621 residue in the channel's α-subunit, we performed the experiments illustrated in Figure 3. We confirmed our previous finding [20] that including OA (100 nM) in the pipette solution stimulates ΔI_{ami} in outside-out patches from αβγENaC expressing oocytes (Fig. 3A, D). In contrast, in control experiments with vehicle (DMSO) in the pipette solution ΔI_{ami} did not increase over time (Fig. 3B, D). Importantly, the αS621A mutation abolished the stimulatory effect of OA on ENaC (Fig. 3C, D). These findings indicate that an endogenous kinase present in the patch can stimulate ENaC and that this stimulation most likely involves phosphorylation of the S621 residue in the channel's α-subunit. This raises the possibility that the stimulatory effect of SGK1 and PKBα may not require direct phosphorylation of the channel by these kinases but may be mediated by an activation or inhibition of an endogenous kinase or phosphatase, respectively. In any case our findings with OA confirm the functional importance of the S621 residue as a likely phosphorylation site for ENaC activation.

Co-expression of constitutively active PKBα stimulates ENaC whole-cell currents by increasing channel surface expression

We and others have previously shown that co-expression of SGK1 increases ENaC whole-cell currents

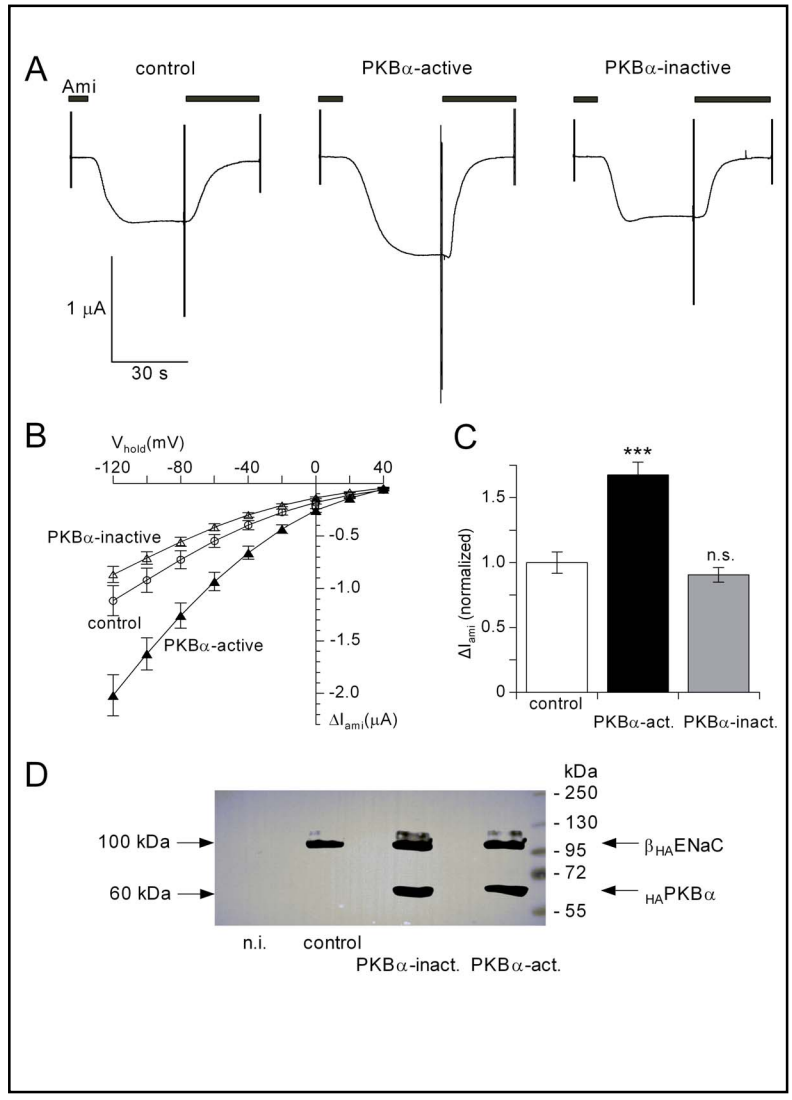

Fig. 4. Co-expression of constitutively active PKBα increases ENaC whole-cell current. Representative whole-cell current traces shown in A were recorded at a holding potential of –60 mV in oocytes expressing αβγENaC (control), αβγENaC and active PKBα (PKBα-active), or αβγENaC and inactive PKBα (PKBα-inactive). Representative averaged *I/V* plots (n=14, for all plots) measured in one single batch of oocytes are shown in B: control (open circles), PKBα-active (filled triangles), PKBα-inactive (open triangles). C, averaged normalized ΔI_{ami} values obtained from similar experiments using three different batches of oocytes (mean ± SEM; n=39; n.s., not significant). In D, representative western blot analysis is shown to detect PKBα and βENaC protein expression in oocytes using an anti-HA antibody. Oocytes used for the western blot were from the same batch as those used for the current measurements shown in Fig. 4B. No HA-signal was detected in non-injected oocytes. In oocytes expressing ENaC with an HA tagged β-subunit we observed a prominent band at ~100 kDa and a weaker band at ~110 kDa (lane 2, Fig. 4D) which correspond to the non-glycosylated and glycosylated forms of βENaC, respectively [43]. In addition to these βENaC signals a third band with a molecular weight of ~60 kDa appeared in oocytes expressing either HA tagged inactive PKBα (lane 3, Fig. 4D) or HA tagged active PKBα (lane 4, Fig. 4D). The size of this signal is in a good agreement with the molecular weight of PKBα [18]. The intensity of the signals for active and inactive PKBα was similar which indicates that the lack of effect of inactive PKBα cannot be attributed to a low expression level. Similar results were obtained in two additional western blot experiments.

in *Xenopus laevis* oocytes by increasing channel surface expression [33, 37-39]. To investigate whether co-expression of PKBα also stimulates ENaC whole-cell currents, we expressed ENaC alone or in combination with constitutively active PKBα or inactive PKBα. Amiloride-sensitive whole-cell currents were assessed using the two-electrode voltage-clamp technique one day after cRNA injection. Representative whole-cell current recordings from individual oocytes are shown in Figure 4 A. They illustrate that ΔI_{ami} was larger in an oocyte co-expressing ENaC and active PKBα than in oocytes from the same batch but expressing ENaC alone or in combination with inactive PKBα. Data from similar recordings obtained in a single batch of oocytes are summarized in Figure 4B as average *I/V* plots using ΔI_{ami} values measured at different holding potentials. Similar results were obtained in three batches of oocytes and demonstrated that co-expression of active PKBα stimulated ΔI_{ami} on average by more than 50 % (Fig. 4C). This stimulatory effect of PKBα depends on its kinase activity because the constitutively inactive construct of PKBα did not affect ENaC currents. Expression of active as well as inactive PKBα was confirmed by western blot analysis (Fig. 4D).

To investigate whether PKBα co-expression stimulates ENaC whole-cell currents by increasing channel surface expression, we determined ENaC surface expression with a semiquantitative chemiluminescence assay using a tagged channel with a FLAG-epitope in the extracellular loop of the β-subunit as previously described [33]. Co-expression of PKBα increased both, ΔI_{ami} (Fig. 5A) and channel surface expression (Fig. 5B). For comparison we also performed co-expression experiments with constitutively active

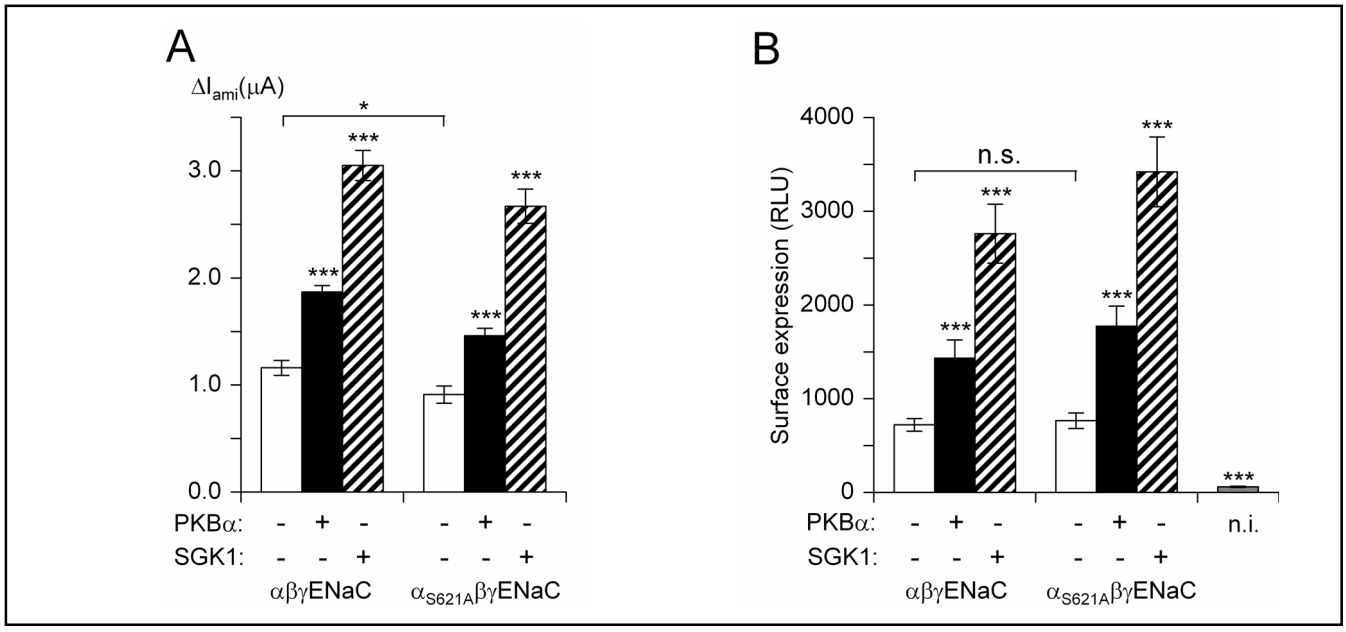

Fig. 5. Stimulation of ENaC whole-cell currents and surface expression by PKBα and SGK1 is preserved in oocytes expressing α_{S621A}-mutant ENaC. Amiloride-sensitive whole-cell currents were measured as described in Fig. 4. A FLAG-tagged β-subunit was used to detect ENaC surface expression in a chemiluminescence assay. Oocytes expressed ENaC with an intact (αβγENaC) or a mutated SGK/PKB-consensus motif ($\alpha_{S621A}\beta\gamma$ENaC) with or without co-expression of active PKBα or active SGK1 as indicated. ΔI_{ami} and surface expression values are shown in A (N=4, n=49-54, per column) and B (N=4, n=69-85, per column), respectively.

SGK1 (SGK1-S422D) known to increase ENaC surface expression [33, 39]. In these experiments, we confirmed the well known stimulatory effect of co-expressed SKG1 on ΔI_{ami} (Fig. 5A) and surface expression (Fig. 5B). The stimulatory effect of SGK1 was more prominent than that of PKBα but qualitatively similar. Taken together the data indicate that the detected increase in channel surface expression can fully account for the stimulatory effects of PKBα and SGK1 on ΔI_{ami} in co-expression experiments.

The stimulatory effect of co-expressed PKBα is independent of the serine residue S621 in the C-terminus of αENaC

We investigated whether the stimulatory effect of co-expressed PKBα depends on the S621 residue in the C-terminus of αENaC shown to be critically important for the acute stimulatory effect of recombinant PKBα on ENaC in outside-out patches (see above). ΔI_{ami} in $\alpha_{S621A}\beta\gamma$ENaC expressing oocytes was not much smaller than ΔI_{ami} in αβγENaC expressing oocytes (Fig. 5A). This argues against a relevant baseline activation of ENaC by constitutive channel phosphorylation at the residue S621. Importantly, in oocytes expressing mutant $\alpha_{S621A}\beta\gamma$ENaC the stimulatory effect of co-expressed PKBα on ΔI_{ami} (Fig. 5A) and channel surface expression (Fig. 5B) was similar to that observed in oocytes expressing wild-type ENaC. This indicates that the serine residue S621 is not essential for mediating the stimulatory effect of co-expressed PKBα. We also demonstrated that the stimulatory effect of co-expressed constitutively active SGK1 (Sgk1-S422D) on ΔI_{ami} and channel surface expression was fully preserved in oocytes expressing $\alpha_{S621A}\beta\gamma$ENaC which is in good agreement with previous reports [40-41]. Taken together, our data indicate that the stimulatory effect of co-expressed PKBα and SGK1 on ENaC is mediated by a different mechanism than the acute stimulatory effect of the recombinant kinases on ENaC in outside-out patches.

Discussion

The key findings of the present study are the following: i) PKBα can activate ENaC in outside-out patches and in intact oocytes; ii) the acute stimulatory effect of PKBα in outside-out patches critically depends on the residue S621 within a putative SGK/PKB consensus motif in the C-terminus of the channel's α-subunit; iii) in contrast, in co-expression experiments

the stimulatory effect of PKBα on ENaC is independent of S621 and is caused by an increase in channel surface expression. These findings indicate that PKBα can stimulate ENaC by two distinct mechanisms, one probably involving direct channel phosphorylation at a specific site, the other involving an effect on channel trafficking.

The SGK/PKB consensus site (^{616}RSRYWS^{621} in rat αENaC) located C-terminally close to the second transmembrane domain of αENaC is highly conserved in mammals [20]. In the present study we demonstrate that the second serine residue in this motif (S621) is critically important for mediating the stimulatory effect of PKBα and of OA on ENaC in outside-out patches. We previously have shown that this serine S621 is also essential for mediating the ENaC activating effect of SGK1 under similar experimental conditions [20]. In excised outside-out patches insertion of new channels into the patch membrane is unlikely to occur. Therefore, the observed stimulatory effect of PKBα, like that of SGK1 [20], is probably caused by an activation of channels already present in the patch membrane. Taken together our findings underline the importance of the S621 residue as a likely phosphorylation site to mediate rapid activation of ENaC in the plasma membrane. However, at present it remains unclear whether PKBα and SGK1 directly phosphorylate the channel at this site or whether they modify the activity of endogenous kinases or phosphates present in the patch, thereby favoring spontaneous channel phosphorylation at this site. Our experiments with OA argue in favor of this latter hypothesis since the stimulatory effect of OA is also dependent on the S621 residue.

Interestingly, in co-expression experiments the stimulatory effect of PKBα and of SGK1 on ENaC currents was preserved in channels with a S621A mutation in the α-subunit. The latter finding is in good agreement with previous reports [40-41]. We also showed that co-expression of PKBα or of SGK1 increased surface expression of both, wild-type ENaC and αS621A mutant ENaC. Thus, the effects of PKBα and of SGK1 on channel trafficking appear to be independent of the putative SGK/PKB phosphorylation site. Moreover, the finding that stimulation of ΔI_{ami} by co-expressed kinases is fully explained by an increase in ENaC surface expression indicates that channel phosphorylation at the residue S621 does not contribute to the stimulatory effect of PKBα or SGK1 in intact oocytes. One interpretation would be a highly active membrane associated phosphatase system masking PKBα or SGK1 action at the channel phosphorylation site. Our observation that ENaC currents were similar in $\alpha_{S621A}\beta\gamma$ENaC expressing oocytes to those in αβγENaC expressing oocytes is in good agreement with this interpretation. Moreover, it suggests that baseline channel phosphorylation at S621 does not readily occur in intact oocytes and probably does not play a major role in determining baseline ENaC activity in the oocyte expression system. In this context, it is of interest that in preliminary experiments exposure of intact oocytes to OA in a concentration of 100 nM or 1 μM (V.N. unpublished observation) failed to stimulate ENaC whole-cell currents. This is in contrast to the robust stimulatory effect of 100 nM OA on ENaC currents observed in outside-out patches with OA included in the pipette solution [20]. As shown in our present study, the stimulatory effect of OA in outside-out patches is abolished by mutating the critical phosphorylation site S621 which indicates that phosphatase inhibition by OA activates ENaC by favouring channel phosphorylation at this site. The lack of effect of OA on ENaC whole-cell currents may result from insufficient diffusion of OA across the plasma membrane. However, this explanation is not very likely since OA is thought to be membrane permeable and was applied from the outside in a concentration that was up to ten times higher than the concentration used in the pipette solution. Thus, in intact oocytes ENaC activation by phosphorylation at S621 seems to be prevented by mechanisms that are presently unknown. This could explain both, the lack of effect of OA in intact oocytes, and the finding that the stimulatory effect of co-expressed PKBα and SGK1 is not larger in oocytes expressing wild-type ENaC than in oocytes expressing αS621A mutant ENaC.

Our finding that co-expression of PKBα stimulates ENaC is in contrast to findings obtained in cultured A6 *Xenopus laevis* renal epithelial cells [24]. In A6 cells overexpression of constitutively active PKBα had no effect on ENaC activity and did not modify the stimulation of ENaC by insulin, aldosterone or AVP. In contrast, overexpression of SGK1 increased ENaC activity in A6 cells by about 10-fold. Interestingly, the SGK/PKB consensus site in the C-terminus of αENaC is absent in amphibians [20]. Thus, an acute stimulatory effect of PKBα *via* this phosphorylation site cannot be expected to occur in A6 cells. However, our findings indicate that the stimulatory effect of PKBα co-expression on ENaC surface expression is independent of this phosphorylation site. Thus, at present

we cannot reconcile the lack of effect of PKBα overexpression on ENaC in A6 cells with our observation that co-expression of PKBα increases ENaC currents in *Xenopus laevis* oocytes by increasing channel surface expression. Furthermore, it is unclear at present whether co-expression of PKBα increases ENaC surface expression in oocytes by increasing channel insertion or by inhibiting channel retrieval. It has been suggested that co-expression of SGK1 reduces ENaC retrieval by the following mechanism: Co-expressed SGK1 phosphorylates Nedd4-2, thereby preventing a close interaction of its WW-domains with the PY-motifs of ENaC required for Nedd4-2 mediated channel retrieval [13-14, 42]. However, it has been reported that the stimulatory effect of co-expressed SGK1 is preserved in oocytes expressing C-terminally truncated ENaC lacking all PY-motifs. This finding was interpreted as an indication that SGK1 is favoring channel insertion rather than inhibiting Nedd4-2 mediated ENaC retrieval [37]. Thus, the precise mechanism by which SGK1 and PKBα may affect ENaC trafficking remains to be elucidated.

In summary, PKBα can stimulate ENaC by two distinct pathways possibly involving additional kinases/phosphatases and complex effects on channel trafficking. The acute stimulatory effect of recombinant PKBα depends on a critical serine residue (S621) in the putative PKB/SGK consensus motif in the α-subunit of ENaC. In contrast, the more delayed stimulatory effect of co-expressed PKBα is mediated by an increased surface expression of ENaC which is independent of the S621 phosphorylation site and involves an effect on channel trafficking. Insulin is a potent activator of PI3K. So far the stimulatory effect of insulin on Na^+ transport has been attributed to the activation of SGK1 which is dependent on PI3K activity [13]. However, insulin also activates PKBα in a PI3K-dependent manner. Our finding that PKBα can stimulate ENaC indicates that insulin enhances ENaC activity not only *via* the activation of SGK1 but in addition by an activation of PKBα. Thus, activation of ENaC by PKBα may be relevant for insulin induced stimulation of ENaC *in vivo*.

Abbreviations

Amiloride-sensitive current (ΔI_{ami}); Epithelial sodium channel (ENaC); Protein kinase B α (PKBα); Serum- and glucocorticoid-inducible kinase isoform 1 (SGK1).

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