Ile481 from the Guinea-Pig α -subunit Plays a Major Role in the Activation of ENaC by cpt-cAMP

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Key Words

ENaC • Amiloride • cpt-cAMP • Extracellular activation
• *Xenopus* oocyte

Abstract

The epithelial sodium channel (ENaC) is the major rate-limiting step for vasopressin and aldosterone sensitive Na^+ reabsorption across kidney epithelia. Recently, ENaC activity was shown to be modulated by extracellular factors such as proteases, Na^+ ion and several other elements. However, the molecular mechanisms of these actions remain unclear. We and others have shown that ENaC composed of the guinea-pig α -subunit (αgp), and the $\beta\gamma$ rat subunits ($\beta\gamma\text{r}$) could be activated by cpt-cAMP, a cAMP analogue, through a mechanism not involving the cAMP-PKA pathway. In the present study, we confirmed by patch-clamp experiments on *Xenopus* oocytes that the number of open channels increased by 2.4-fold after cpt-cAMP exposure. In order to characterize the extracellular domain involved in this activation, we generated α -subunit chimera's harboring different portions of the extracellular loop of the αgp and αr . Using two-electrode voltage-clamp, we established that Tyr456-Ser532 from the

αgp confers sensibility to cpt-AMP. Then, by site-directed mutagenesis, we have isolated Ile481 as a major residue for cpt-cAMP-dependant activation. Taken together, these experiments provide evidence of an extracellular-ligand stimulating ENaC. They also contribute to the further understanding of the structure-function relationship of this channel.

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Introduction

The epithelial sodium channel (ENaC) is the rate-limiting step for sodium transport in many reabsorbing epithelial like the distal nephron [1], distal colon [2], air ways [3] and the secretory ducts of several exocrine glands [4]. ENaC belongs to the DEG/ENaC family of ion channels and contains three homologous subunits (α, β, γ) [5]. Each shares the same topology corresponding to two transmembrane-spanning domains, a short intracellular N- and C-termini and a large extracellular loop ($\approx 70\%$ of their size). The structure of the ENaC channel is still under debate. A stoichiometry of four ($2\alpha 1\beta 1\gamma$) [6, 7] and nine ($3\alpha 3\beta 3\gamma$) subunits [8] was proposed. Recently, Jasti and colleagues determined the crystal structure of ASIC1, a neuronal acid-sensing DEG/

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ENaC channel [9]. Thus, there is presently good evidence that ENaC, like ASIC1, appears to be a trimeric channel containing 1α , 1β and 1γ subunits [10].

In native epithelia, ENaC activity is tightly regulated by several hormones such as vasopressin and aldosterone [4]. Interestingly, vasopressin produces a rapid increase of Na^+ reabsorption in amphibian epithelia (toad bladder, frog skin and A6 cells) [11, 12] and in rabbit collecting tubule [13, 14] but not in mammalian distal colon or urinary bladder [4]. Several studies have demonstrated that vasopressin-dependent activation can be mimicked by permeable cAMP analogues and activators of adenylate cyclase [15]. However, the underlying molecular mechanisms responsible of this modulation in ENaC activity are not completely understood and remain a matter of controversies. Lines of evidence suggest that different types of regulatory mechanisms could be involved: i) a raise in the number of cell surface ENaC from subapical membrane vesicles ii) an activation of quiescent channels already present in the apical membrane iii) an increase in channel open probability [16, 17]. Interestingly, rat or *Xenopus* ENaC could not be stimulated by cAMP and its analogues when expressed in *Xenopus* oocyte [18]. However, Schnizler et al. [19] have shown that the ENaC α -subunit from the guinea-pig (α_{gp}) possessed the intrinsic ability to be activated by 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate (cpt-cAMP) when co-expressed with the rat β and γ subunits ($\beta\gamma\text{r}$). We have previously reported that this heterologous channel was not activated by intracellular perfusion of cAMP or its membrane permeable analogue in cut-open oocyte [20]. In addition, the cAMP pathway did not seem to be involved since, the addition of PKI 6-22, a PKA inhibitor, did not influence the amiloride-sensitive currents. Taken together, these results strongly suggest the presence of an interaction between cpt-cAMP and the extracellular part of α_{gp} ENaC.

To date, several factors were identified to modulate the activity of ENaC through direct interaction with the extracellular domain: extracellular Na^+ itself by a phenomenon named "self-inhibition" [21, 22], serine-proteases [23, 24], sulfonylurea glibenclamide [25] and cpt-cAMP as described above. Self-inhibition is linked to a extracellular Na^+ sensing site and has rapid kinetics, with a time constant of ≈ 0.3 s. As the kinetics are too fast for the internalization of the channels, it has been supposed that this phenomenon was associated to a decrease of the open related probability of ENaC [21]. Several recent studies have determined the role of structural elements by observing the effects of site-

directed mutations in the extracellular loop of ENaC subunits on self-inhibition [26, 27]. Numerous evidences have shown that ENaC can be activated by the proteolytic treatment of soluble serine-proteases (trypsin) or by the members of the channel activating proteases (membrane-bound serine-proteases) [28, 29]. The proteolysis of ENaC subunits at specific sites within the extracellular domains of α and γ subunits is now believed to play a key role in activating the channel by increasing its open probability [30, 31]. While the stimulation of ENaC channels by glibenclamide is fast and reversible in outside-out patches, also suggesting an extracellular site of action [25]. However, the binding sites within the extracellular domain of ENaC and the mechanisms by which extracellular factors have their effects are still mainly unknown. An interaction with the large extracellular domain of ENaC is potentially an important regulatory mechanism for the activity of this channel. Better knowledge in this area will help to understand the role of the large extracellular domain of this channel.

Our objective in the present study was to determine the portion of the α_{gp} extracellular loop implicated in the activation of ENaC by cpt-cAMP. To do so, we used two-electrode voltage-clamp technique and patch-clamp in the outside-out configuration, combined with a molecular biology approach. Here we show that the effect of cpt-cAMP on ENaC formed by α_{gp} and $\beta\gamma\text{r}$ involves an increase in the $N\text{Po}$ (the product of P_o and the total number of channels N). Furthermore, an Ile residue at position 481 in α_{gp} is playing a major role in this activation.

Materials and Methods

Chimeric α -subunits and site-directed mutagenesis

For each chimera (Fig. 2A), two restriction sites were introduced into α_{gp} cDNA at homolog positions into αr cDNA by PCR and inserted into the pSD5 vector (Invitrogen, Burlington, ON, Canada) as previously described [32]. Site-directed mutagenesis was performed using the pfu turbo DNA polymerase kit according to manufacturer's instructions (Stratagene, La Jolla, CA, USA). The parental cDNAs were degraded using *DpnI* methylated specific restriction enzyme (Promega, San Luis Obispo, CA, USA). All mutations and chimeric constructions were confirmed by sequencing (University Core DNA Services, Calgary, AB, Canada).

*Expression of ENaC in *Xenopus laevis* oocyte*

In vitro transcribed cRNA of wild-type or mutant α , α_{gp} subunits and chimeric constructions were co-injected with cRNA of $\beta\gamma\text{r}$ ENaC into stage V/VI *Xenopus* oocytes (2 ng of

Fig. 1. Effect of cpt-cAMP on macroscopic and single-channel currents. (A) Original recording of inward currents from *Xenopus* oocytes expressing α gpb β rr (left panel) and α r β rr (right panel). The currents sensitive to amiloride were measured at -60 mV before and after a 3 min exposure to cpt-cAMP. (B) A representative patch-clamp recording in outside-out configuration of *Xenopus* oocyte expressing α gpb β rr ENaC at -60 mV. A downward deflection indicates current flowing into the pipette and dashed lines represent the current level when all sodium channels were closed. Addition of amiloride (200 nM) to the bath (extracellular side) solution reduced the activity of the sodium channels by more than half. This effect was quickly reversible. Application of cpt-cAMP (100 μ M) in the external solution induced a rapid stimulation of the channel. Seven parts of the recording (a-g) are extended to display details of the channel activity without or with amiloride and cpt-cAMP. (C) The mean of normalized N.Po from 10 independent experiments are reported. The effect of amiloride is shown followed by the normalized NPo after 1 and 3 min of cpt-cAMP perfusion. * $P=0.016$; § $=0.027$; §§ $=0.007$ (versus control, using a paired t-test).

cRNA of each subunit in 50 nl total) as described earlier [33]. Injected oocytes were incubated at 18.5°C (Fisher Scientific, Ottawa, ON, Canada) in a low- Na^+ modified Barth's solution that contained (in mM): NaCl 10, KCl 2.0, CaCl_2 0.7, MgCl_2 0.8, $\text{Ca}(\text{NO}_3)_2$ 0.33, N-methyl-D-glucamine (NMDG)-Cl 80, NMDG-HEPES 10.0, pH 7.4. The low- Na^+ modified Barth's was chosen to prevent excessive Na^+ loading during the time needed for ENaC expression. Electrophysiological experiments were performed 1 or 2 days after cRNA injection.

Single-channel recordings

Before patch-clamp experiments the oocytes were placed for 3–5 min at room temperature (22–25°C) in a hypertonic medium (475 mOsm) with the following composition (in mM): K-aspartate 200, KCl 20, MgCl_2 1, EGTA 10, HEPES-Na 10, pH 7.4. The vitelline membrane was removed from the cell using fine forceps [34]. The oocytes were then transferred to the recording chamber. Single-channel currents were measured in the excised outside-out patch configuration at room temperature at the holding potential of -100mV according to Hamill et al. [35]. Patch pipettes with a resistance of 8–12 M Ω were made of borosilicate glass (WPI, Sarasota, FL, USA) and pulled in two stages with a PP-83 vertical puller (Narishige, Tokyo, Japan) and fire-polished with Micro Forge MF 830 (Narishige, Tokyo, Japan). The pipette solution contained

(in mM): 75 CsF, 17 NMDG, 10 EGTA and 10 HEPES, pH 7.4, titrated with HEPES acid. The composition of the perfusion and bath solutions was (in mM): 100 LiCl, 0.4 CaCl_2 , 10 NMDG-HEPES (pH 7.4), 5 BaCl_2 and 10 tetraethyl-ammonium chloride. The effect of 200 nM amiloride (Sigma-Aldrich Oakville, ON, Canada) in the extracellular solution was used to identify the presence of ENaC. By convention, negative (downwards) single-channel currents corresponded to Li^+ flux from the extracellular to the intracellular side of the membrane and the intracellular potential corresponded to the pipette potential (V_{pip}). Extracellular solutions flowed under gravity through RSC-200 rapid solution changer lines at room temperature (BioLogic Science Instruments, Knoxville, TN, USA). Single-channel currents were recorded using a MultiClamp 700B amplifier, filtered at 200 Hz and digitized at 1 kHz using a Digidata 1440A interface analogue-to-digital converter and AxoScope software (Axon Instruments, Sunnyvale, CA, USA). Single-channel currents data were analyzed using PClamp10 Software (Axon Instruments, Sunnyvale, CA, USA). The number of open channels (the product of P_o and the total number of channels N) was calculated as $N \cdot P_o = I_{\text{Na}} / i_{\text{Na}}$, where i_{Na} is the unitary current measured as the peak-to-peak interval in the current amplitude histogram and I_{Na} corresponded to the current due to ENaC. I_{Na} was measured in 0.5 to 1 min recordings as the difference between the mean of total current and the current in

Fig. 2. Determination of the extracellular region of α gp involved in activation of ENaC by cpt-cAMP. (A) Schematic representation of α ENaC where the wild-type (wt) α gp (black) and α r (grey) and the different chimeras are shown. The α gp aa fragments introduced in each chimera are illustrated. The two transmembrane domains are represented by cylinders. (B) The amiloride-sensitive currents of each chimera co-expressing $\beta\gamma$ r were measured at -60 mV before and after cpt-cAMP perfusion (100 μ M). Chimera IV was significantly activated by cpt-cAMP, $*P \leq 0.001$ versus α r (unpaired t-test). The positive control (α gp $\beta\gamma$ r) had a mean increase of I_{Na} of 2.28-fold ± 0.05 ($n=152$), the negative control (α r $\beta\gamma$ r) had an I_{Na} of 1.13-fold ± 0.01 ($n=131$). All the absolute values of currents before cpt-cAMP perfusion were similar.

the absence of any open channel (the latter defined from recordings in the presence of 10 μ M amiloride).

Electrophysiological measurements in whole oocytes

Amiloride-sensitive currents were measured using the two-electrode voltage-clamp technique (TEVC) with a Dagan TEV voltage-clamp apparatus (Dagan, Minneapolis, MN, USA), at room temperature at a holding potential of -60 mV in a solution containing (in mM): 100 Na-gluconate, 0.4 $CaCl_2$, 0.8 $MgCl_2$, 5 $BaCl_2$, 10 tetraethyl-ammonium chloride (TEA-Cl) and 10 NMDG-HEPES (pH 7.4). Low Cl^- concentration and K^+ channel blockers (Barium and TEA, Sigma-Aldrich Oakville, ON, Canada) were used to reduce the background membrane conductance. Extracellular solutions flowed under gravity at flow rate of 6 ml/min. The currents signals were filtered at 20 Hz using the internal filter of the Dagan apparatus (Dagan, Minneapolis, MN, USA) and continuously recorded and sampled at 1000 Hz with Pclamp 10 Software (Axon Instruments, Sunnyvale, CA, USA).

Statistics

Statistical analysis was performed with SigmaStat version 2.03 (Systat Software Inc. San Jose, CA, USA). All results are expressed as the mean $\pm$ SEM and statistical significance was evaluated by either unpaired or paired Student's t-test as indicated in the text, $P < 0.05$ was considered significant.

Results

Membrane permeable cAMP activates the ENaC channel containing the α -subunit from the guinea-pig: effect in single channel recording

In Fig. 1A are shown original recordings using the TEVC technique on *Xenopus* oocytes. In the left panel, we confirmed that the Na^+ currents driven by the ENaC channels produced by the expression of the α gp combined with $\beta\gamma$ r (α gp $\beta\gamma$ r) are stimulated by cpt-AMP. However, when α r was expressed with $\beta\gamma$ r (α r $\beta\gamma$ r), cpt-cAMP perfusion had no effect (right panel). Similar results were previously reported and confirmed the activating effect of cpt-cAMP on the ENaC channel harbouring the α gp in our system [19, 20].

To understand the mechanism of this activation, we performed outside-out recordings on oocytes co-injected with α gp $\beta\gamma$ r. As shown in Fig. 1B, a low concentration of external amiloride (200 nM) induced a reversible decrease of the N.Po by $47 \pm 8\%$ and confirmed the outside-out configuration. Also, addition of 100 μ M cpt-cAMP to the solution bathing the extracellular surface of

		460		470		480		490		500		510	
gp	(456)	YKLQGA	FSS	DSL	GCF	NKCR	KPC	NV	TI	YKLS	SAGY	SRWP	SVTSQDWIFOMLSLQNNY
rat	(485)	YKLQGA	FSL	DSL	GCF	SKCR	KPC	SV	IN	YKLS	SAGY	SRWP	SVKSQDWIFEMLSLQNNY
		520		530		540		550		560		570	
gp	(516)	RNGV	AN	LNI	YF	KEL	NY	RT	NSE	SP	SV	TM	VSLLSNLGSQWSLWFGSSVLSVVE
rat	(545)	RNGV	AK	LN	IF	KEL	NY	KT	NSE	SP	SV	TM	VSLLSNLGSQWSLWFGSSVLSVVE
													MAEFMFDLL
													MAADVIFDLL

Fig. 3. Sequence alignment analysis of the C-terminal region of the extracellular domain of α gp and α r. The aa sequence of α gp (Genbank accession number: AJ249296) extracellular region contained in chimera IV was analyzed against the corresponding sequence from α r (Genbank accession number: NM_031548). The mismatching aa between the two species are in grey shade, whereas the second transmembrane domain is black shade.

Fig. 4. The effect of cpt-cAMP perfusion on oocytes co-expressing α r subunit mutants with β rr. The amiloride-sensitive currents were measured at -60 mV before and after 100 μ M cpt-cAMP perfusion. Among the mutants tested co-expressed with β rr (n=11-40), only α r Asn481Ile conferred a superior response to cpt-cAMP. *P=0.001 versus α r wt (n=131) (unpaired t-tests). The absolute values of currents before cpt-cAMP perfusion were similar among the controls and mutants.

the membrane increased the N.Po by 2.69-fold $\pm$ 0.43 after 1 min and by 4.12-fold $\pm$ 1.21 after 3 min. This activation was similar in magnitude to that observed in macroscopic currents (Fig. 1A) and occurred without influencing the single-channel conductance.

Determination of the extracellular region involved in the activation of ENaC by a cAMP analogue

Previously, we have shown that the sensitivity to cpt-cAMP of α gp was not conferred by the intracellular N- and C- terminal domains [20], we thus focused our investigation on the extracellular region. To do so, we have generated four α -chimeras where different portions of the α r were systematically replaced with the homologous sequence from α gp (schematized in Fig. 2A). Each chimera was then expressed in *Xenopus* oocytes,

in combination with brgr. The effects of cpt-cAMP on macroscopic currents were monitored by the TEVC technique. The amiloride-sensitive currents absolute values generated by α r β rr and α gp β rr ENaC were similar, 2168 $\pm$ 85 nA and 2302 $\pm$ 86 nA, respectively. Absolute values of currents generated by the α -subunit chimeras co-expressed with β rr were not significantly different, they corresponded to 2604 $\pm$ 155 nA (chimera I), 2374 $\pm$ 200 nA (chimera II), 2571 $\pm$ 140 nA (chimera III) and 2068 $\pm$ 144 nA (chimera IV). Our results in Fig. 2B demonstrate that cpt-cAMP activation of the ENaC channel is not mediated by the aa 12-456 of the α gp since chimeras I, II and III induced an effect similar to the control (α r β rr). However, the amiloride-sensitive currents generated by the chimera enclosing the α gp C-terminal segment of the extracellular loop (chimera IV) significantly increased by 1.84-fold $\pm$ 0.06 (n=41) under

Fig. 5. Role of Ile481 in the activation of the ENaC channel by cpt-cAMP. The amiloride-sensitive currents were measured at -60 mV before and after cpt-cAMP perfusion (100 μ M) on the various point-mutated α -subunits co-expressed with β yr. Substitution Ile481Asn decreased the sensitivity of α gp to cpt-cAMP and abolished the sensitivity of chimera IV. This aa swapping in chimera I, II and III, increased their sensitivity to cpt-cAMP. * $P=0.001$ (unpaired t-tests). The absolute values of currents before cpt-cAMP perfusion were similar among the respective controls and mutants.

cpt-cAMP exposure. The chimera IV contained α gp specific aa from 456 to 622, within this fragment, residues Tyr456 to Ser559 are part of the extracellular loop (see Fig. 3).

Determination of the specific amino acid involved in cpt-cAMP activation of the ENaC channel

At the level of the extracellular loop enclosed in chimera IV we have found eleven mismatches between the guinea-pig and rat sequences (Fig. 3). To evaluate the potential role played by the α gp specific aa, we have generated the latter eleven swapping mutants in α r. The TEVC technique was used to study each mutant co-expressed with β yr. The results shown in Fig. 4 establish that among the eleven α r mutants generated, only the α r-Asn481Ile conferred cpt-cAMP sensitivity ($n=40$). To confirm the role played by this residue in cpt-cAMP-dependent activation, we substituted this Ile481 by an Asn residue in α gp (α gp Ile481Asn) and co-expressed it with β yr. This site-mutation over the corresponding Asn481 from the α r sequence considerably inhibited cpt-cAMP stimulation (Fig. 5) with a current ratio of $1.48\text{-fold} \pm 0.07$ ($n=26$). We have also generated this Asn481Ile substitution in chimera IV and the resulting chimera (chimera IV-Ile481Asn) exhibited ENaC channels that were completely resistant to cpt-cAMP. Next, we evaluated if the substitution of an Ile at position 481 could create a stimulating effect on ENaC channels otherwise unaffected by cpt-cAMP. The Asn481Ile substitution on the chimeras III and II co-expressed with the β yr

produced current ratios of $1.97\text{-fold} \pm 0.08$ ($n=20$) and $2.02\text{-fold} \pm 0.09$ ($n=12$), respectively (Fig. 5). However, this site-mutation on chimera I, was unable to generate a similar fold-activation by cpt-cAMP, the currents ratio were $1.55\text{-fold} \pm 0.05$ ($n=14$). This partial activation might reflect an important role played the conformation of the C-terminal portion of the extracellular loop specific to α gp.

Discussion

We and others have previously reported that a membrane permeable cAMP analogue, cpt-cAMP, was able to increase the activity of the ENaC channel generated by the expression of α gp and β yr subunits in *Xenopus* oocytes [19, 20, 36]. We have also established that intracellular factors were not key players in this up-regulation, strongly suggesting that the extracellular domain of the channel was involved. Accordingly, in the *Xenopus* oocyte system, the ENaC channel activation could not be triggered by intracellular perfusion of cAMP or its permeable analogue. The intracellular perfusion of PKA-inhibitors was also unable to block the stimulating effect of cpt-cAMP.

In this report, we have identified the portion of the extracellular loop responsible of the cpt-cAMP activation. To do so, we have generated chimeras of α -subunits harboring different fragments of cpt-cAMP-sensitive (α gp) and cpt-cAMP-resistant (α r). In TEVC experiments, the absolute values of currents produced

by each of the four chimeras and controls were similar. However, their sensitivity to the cpt-cAMP was significantly different. Notably, the sodium currents generated by the chimera IV and α_{gp} in the presence of cpt-cAMP raised by almost 2-fold compared to the control (α_r). This specific portion of cpt-cAMP-sensitive α -subunit contained 11 mismatches when compared to the cpt-cAMP-resistant α -subunit. By site-directed mutagenesis, we have identified and confirmed that Ile481 played a major role in cpt-cAMP activation. Furthermore, substitutions of Ile481 in the cpt-cAMP-resistant chimeras increased significantly their cpt-cAMP sensitivity. Likewise, swapping this important Ile residue by an Asn in cpt-cAMP-sensitive α -subunits (α_{gp}) considerably decreased the activating effect of cpt-cAMP on the ENaC channels.

Among the regulation factors implicated in ENaC activity, several hormones and growth factors were identified as activators of sodium transport in mammalian and amphibian epithelial cell and tissues through the activation of the cAMP-PKA pathway [4, 12-14]. However, the underlying mechanisms are poorly understood and highly dependent on the cellular context and specie. Additional factors are needed in the *Xenopus* system in order to the stimulatory actions of cAMP-PKA to occur. Our present work raises further questions as to the possibility that the membrane permeable cAMP analogue could stimulate the ENaC channel by a differential mechanism in the *Xenopus* oocytes.

As stated above, the up-regulation of ENaC channels by cpt-cAMP is most likely mediated by an extracellular interaction. This makes cpt-cAMP part of the newly identified small molecules able to regulate ionic channels members of the DEG/ENaC family. Recently, another research team has identified the compound S3969, as a new activator of ENaC channels *via* a specific interaction in the extracellular moiety of the channel [37]. They also reported that this small molecule increases open probability through interactions requiring the extracellular domain of the β -subunit. Based on our results in excised patch outside-out configuration on oocyte expressing

$\alpha_{gp}\beta\gamma_{rat}$, cpt-cAMP activation was not correlated to internalization of the channels, but with an increase in the open probability of channels present at the plasma membrane.

Meanwhile, many unknown factors still remain in the regulation that we have uncovered. The replacement of the α -subunit from the guinea-pig, which shares ~75% homology with other mammalian α ENaC, generated an ENaC channel sensitive to cpt-cAMP. The site-directed mutagenesis of the Ans for an Ile residue in the cpt-cAMP-resistant α -subunit (α_r), induced a significant activation of amiloride-sensitive currents. However, this substitution alone was unable to produce a full-activation similar to the cpt-cAMP-sensitive α -subunit (α_{gp}). This partial dependency on the gp α -subunit is most likely due to structural differences of the C-terminal end of the extracellular loop of the α_{gp} .

In summary, we have shown evidences of a regulatory site located in the extracellular loop of the guinea-pig α -subunit. Moreover, we have shown that Ile481 is a major residue for this up-regulation of amiloride-sensitive currents by a membrane permeable cAMP analogue. Thus, this study contributes to the further understanding of the structure-function relationship of the channel and the complex regulation of ENaC. Our results could also be significant for other members of the family of DEG/ENaC ionic channels.

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