



**HAL**  
open science

## Beta-scorpion toxin effects suggest electrostatic interactions in domain II of voltage-dependent sodium channels.

Massimo Mantegazza, Sandrine Cestèle

► **To cite this version:**

Massimo Mantegazza, Sandrine Cestèle. Beta-scorpion toxin effects suggest electrostatic interactions in domain II of voltage-dependent sodium channels.: Electrostatic interactions between segments IIS2, IIS3 and IIS4 of Na<sup>+</sup> channel.. The Journal of Physiology, 2005, 568 (Pt 1), pp.13-30. 10.1113/jphysiol.2005.093484 . inserm-00381745

**HAL Id: inserm-00381745**

**<https://inserm.hal.science/inserm-00381745>**

Submitted on 19 Jun 2009

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# **$\beta$ -scorpion toxin effects suggest electrostatic interactions in domain II of voltage-dependent sodium channels.**

Massimo Mantegazza <sup>2</sup> and Sandrine Cestèle <sup>1,2</sup>

**Running title:** Electrostatic interactions between segments IIS2, IIS3 and IIS4 of Na<sup>+</sup> channel.

**Keywords:** sodium channels, activation, toxins

<sup>1</sup> CNRS FRE 2738, Université de la Méditerranée, Laboratoire d'Ingénierie des Protéines, IFR Jean Roche, Faculté de Médecine Nord, Bd Pierre Dramard, 13916 Marseille Cedex 20, France

<sup>2</sup> Dipartimento di Neurofisiopatologia, Istituto Neurologico Besta, Via Temolo 4, 20126 Milano, Italy

**Corresponding author:**

Dr. Sandrine Cestèle

Laboratoire d'Ingénierie des Protéines, CNRS FRE 2738,

Université de la Méditerranée, IFR Jean Roche,

Faculté de Médecine Nord, Bd Pierre Dramard,

13916 Marseille Cedex 20, France

e-mail : [cestele.s@jean-roche.univ-mrs.fr](mailto:cestele.s@jean-roche.univ-mrs.fr)

Phone : (+39) 022 394 611

Fax : (+ 39) 022 394 619

## **SUMMARY**

$\beta$ -scorpion toxins specifically modulate the voltage dependence of sodium channel activation by acting through a voltage-sensor trapping model. We used mutagenesis, functional analysis and the action of  $\beta$ -toxin as tools to investigate the existence and role in channel activation of molecular interactions between the charged residues of the S2, S3 and S4 segments in domain II of sodium channels. Mutating to arginine the acidic residues of the S2 and S3 transmembrane segments in domain II, or making charge-reversal mutation of the two outermost gating charges of the IIS4 voltage-sensor, shifts the voltage-dependence of channel activation to more positive potentials and enhances the effect of  $\beta$ -scorpion toxin. Thus, mutations of acidic residues in IIS2 and IIS3 segments are able to promote voltage sensor trapping in a way that is similar to the mutations of the arginines in the IIS4 segment. In order to disclose the network of interactions among acidic and basic residues we performed functional analysis of charge-inversion double mutants: our data suggest that the first arginine of the voltage-sensor S4 in domain II (R850) interacts specifically with E805, D814 and E821 in the S2 and S3 segments, whereas the second arginine (R853) only interacts with D827 in the S3 segment. Our results suggest that the S2, S3 and S4 segments in domain II form a voltage-sensing structure, and that molecular interactions between the charged residues of this structure modulate the availability of the IIS4 voltage-sensor for trapping by  $\beta$ -toxins. They also provide unique insights into the molecular events that occur during channel activation, as well as into the structure of the channel.

## INTRODUCTION

Voltage-gated sodium channels are responsible for the voltage-dependent increase in sodium permeability and play a major role in initiating and propagating action potentials in excitable cells (Hodgkin & HUXLEY, 1952). They are transmembrane proteins composed of a pore forming  $\alpha$  subunit that can be associated with smaller subunits  $\beta$ 1,  $\beta$ 2,  $\beta$ 3 and  $\beta$ 4 (Catterall, 2000; Yu *et al.*, 2003). The  $\alpha$  subunit consists of four homologous domains (I-IV), each containing six transmembrane segments (S1-S6) and one re-entrant segment (SS1/SS2) connected by internal and external polypeptide loops (Yu *et al.*, 2003). The pore is formed by the transmembrane segments S5 and S6 and the membrane re-entrant segment SS1 and SS2 (Noda *et al.*, 1989; Terlau *et al.*, 1991; Heinemann *et al.*, 1992; Ragsdale *et al.*, 1994). The voltage-dependent gating of sodium channels is controlled by the S4 segments, which contain one positively charged residue at every third position and move outward in response to depolarisation; they therefore act as intrinsic voltage sensors to initiate activation (Catterall, 1986; Guy & Seetharamulu, 1986; Stuhmer *et al.*, 1989; Yang & Horn, 1995; Yang *et al.*, 1996; Yang *et al.*, 1997; Mitrovic *et al.*, 1998; Cha *et al.*, 1999; Chanda & Bezanilla, 2002).

The function of voltage-dependent sodium channels is strongly altered by various groups of neurotoxins (Cestèle & Catterall, 2000), whose high affinity and specificity make them unique tools to get new insights into channel structure and function.  $\beta$ -scorpion toxins are particularly useful to understand and unmask the molecular basis of sodium channel activation (Cestèle *et al.*, 1998; Cestèle *et al.*, 2001). Indeed,  $\beta$ -scorpion toxins bind to receptor site 4, reduce the peak sodium current and shift the voltage dependence of activation to more negative potentials (Cestèle *et al.*, 1998). Identification of molecular determinants for binding of toxins that modify activation provide important information about the mechanisms of channel gating and the structure involved in this process. Attempts to localize the receptor site of  $\beta$ -scorpion toxins on sodium channels have been realised using the fact that they shift the voltage-dependence of activation on neuronal and skeletal sodium channels but not on cardiac sodium channels. Using chimeras between cardiac and skeletal muscle channels, it has been shown that domain II of skeletal muscle sodium channels is required for the effect of  $\beta$ -scorpion toxins on the voltage-dependence of activation (Marcotte *et al.*, 1997). This data strongly suggest that  $\beta$ -scorpion toxins bind to domain II of sodium channels. Moreover, chimeric sodium channels in which the amino acid residues within each of the 16 extracellular loops of the

Na<sub>v</sub>1.2 brain sodium channels  $\alpha$  subunit had been converted to those in the cardiac isoform revealed that chimeras of the S1-S2 and S3-S4 extracellular loops in domain II had altered  $\beta$ -toxin binding affinity. The strongest effect was obtained with the chimera G845N in the IIS3-S4 loop which not only reduces the affinity of the toxin for its receptor site but transfers specifically all the functional effects that  $\beta$ -scorpion toxins have on cardiac sodium channels to the Na<sub>v</sub>1.2 sodium channels (Cestèle *et al.*, 1998). According to these data, it has been suggested that  $\beta$ -scorpion toxins bind to the extracellular loops in domain II of sodium channels and their effect on gating is described by a voltage-sensor trapping model (Cestèle *et al.*, 1998; Cestèle *et al.*, 2001). In fact,  $\beta$ -scorpion toxins shift the voltage-dependence of activation to more negative potentials only after a strong depolarising pre-pulse, thus indicating that their effect depends on channel activation. The voltage-sensor trapping model predicts that  $\beta$ -scorpion toxin first binds to the channel and partially inhibits the current and then, as the IIS4 segment moves outward during activation, the toxin binds to newly accessible residues in the IIS3-S4 loop and in the extracellular end of the IIS4 segment, thus trapping IIS4 in an outward, activated position. Therefore, voltage-sensor trapping favours sodium channel activation and causes the negative shift in the voltage dependence of activation characteristic of  $\beta$ -scorpion toxins. Arguments in favour of the voltage-sensor trapping model include the following ones: 1- chemical modification of R850C in the IIS4 segment by MTS reagents shifts the activation to more negative potentials as the  $\beta$ -toxin (Mitrovic *et al.*, 1998; Cestèle *et al.*, 2001); 2- chemical modification of R853C by MTSEA alter the voltage dependence of activation and  $\beta$ -toxin action on sodium channels (Cestèle *et al.*, 2001); 3- mutations in domain II alter  $\beta$ -toxin affinity and/or effect on sodium channels (Marcotte *et al.*, 1997; Cestèle *et al.*, 1998; Cestèle *et al.*, 2001) ; 4- mutation G845N transfers to Na<sub>v</sub>1.2 the typical effects of  $\beta$ -scorpion toxins on the cardiac channel (Cestèle *et al.*, 1998).

Our previous study (Cestèle *et al.*, 2001) demonstrated that neutralizing the two outermost arginine residues of the IIS4 voltage sensor markedly enhances the effect of  $\beta$ -scorpion toxin on sodium channels, because of increased IIS4 segment accessibility from the extracellular side of the membrane. As it has been shown that basic amino acid residues in the S4 segment of K<sup>+</sup> channels interact with acidic residues in the S2 and S3 segments (Papazian *et al.*, 1995; Planells-Cases *et al.*, 1995; Seoh *et al.*, 1996; Tiwari-Woodruff *et al.*, 1997; Tiwari-Woodruff *et al.*, 2000), we predicted that the two

outermost arginine of the IIS4 segment could be involved in similar electrostatic interactions that may stabilize the voltage sensor within the membrane. Thus, mutations of acidic residues that interact with the arginines of the IIS4 could modify the availability of the IIS4 segment for  $\beta$ -scorpion toxin trapping. The selective interactions of the charged residues could therefore be disclosed by studying the effects of their mutations on  $\beta$ -scorpion toxin activity, whose action, according to the voltage-sensor trapping model, depends on the availability of IIS4 segment. Moreover, the possible electrostatic interactions between the S2, S3 and S4 segments in domain II of voltage-gated sodium channels could be specific and distinct from the ones observed in potassium channels. Therefore, their study could disclose important aspects of the molecular topology and structure-function relationships of sodium channels.

In this paper, we show that mutation of acidic amino acid residues of the S2 and S3 segments in domain II alter sodium channel gating, which suggests that the S2, S3 and S4 segments in domain II may constitute a voltage-sensing structure for voltage-gated sodium channels as has been shown for potassium channels (Planells-Cases *et al.*, 1995; Seoh *et al.*, 1996). Furthermore, our results indicate that mutation of the acidic amino acid residues of IIS2 and IIS3 segments enhances the effect of  $\beta$ -scorpion toxin on activation, finding that is consistent with the voltage sensor-trapping model. Finally, using a charge-reversal mutation strategy and analysing the functional effect of  $\beta$ -scorpion toxin, we suggest the possible existence of electrostatic interactions between the two outermost arginines of the IIS4 segment and the four acidic amino acid residues of S2 and S3 segments in domain II.

## **METHODS**

### *Materials*

$\beta$ -scorpion toxin C<sub>ss</sub> IV was purified from the venom of *Centruroides suffusus suffusus* (Martin et al., 1987). The restriction endonucleases were purchased from New England Biolabs, and the other molecular biology reagents from Promega. The MC1061 *Escherichia coli* bacterial strain was purchased from Invitrogen. The human embryonic kidney tsA-201 cells, a derivative of HEK-293 cells expressing the simian virus (SV-40) large antigen, were purchased from ECACC (UK). cDNAs encoding rat Na<sub>v</sub>1.2a  $\alpha$  subunits in the pCDM8 vector and  $\beta$ 1 subunit in the pEYFP-N1 vector were used for transfection.

### *Site-directed mutagenesis*

The R850E, R853E, E805R, D814R, E821R and D827R mutations were introduced by PCR amplifying an 800-bp cDNA fragment from the XmaI to the SphI site, that was subcloned into the Na<sub>v</sub>1.2a cDNA in pCDM8. In order to facilitate the screening of the clones, a silent restriction site was introduced along with the mutations: a silent HpaII site for R850E and R853E, a silent XmnI site for E805R and D814R, and a silent BamHI site for E821R and D827R. Double mutations E805R/R850E, E805R/R853E, D814R/R850E, D814R/R853E, E821R/R850E, E821R/R853E, D827R/R850E and D827R/R853E were introduced by PCR using R850E or R853E cDNA as template, and the PCR fragments were subcloned as describe above. The clones were screened using the silent restriction sites associated with the respective mutation. All of the clones were sequenced through the entire PCR fragment.

### *Transient expression in tsA-201 cells.*

The tsA-cells were maintained at 37°C in 5% CO<sub>2</sub> in DMEM/F12 medium (GIBCO/BRL/Life Technologies) supplemented with 10% FBS (GIBCO/BRL/Life Technologies), 20  $\mu$ g/ml penicillin, and 10  $\mu$ g/ml streptomycin (Sigma). They were transiently co-transfected using calcium phosphate precipitation (Jordan et al., 1996) with cDNAs for the channel  $\alpha$  subunit and for the  $\beta$ 1 subunit tagged with YFP (in the vector pEYFP-N1, Clontech) in a molar ratio of 1:2. The successfully transfected cells were detected by fluorescence microscopy.

### *Electrophysiological recording and analysis*

Whole-cell sodium currents were recorded from tsA-201 cells expressing Na<sub>v</sub>1.2a wild-type or mutant sodium channel  $\alpha$  subunits. The external recording solution consisted of the following (in mM): 110 NaCl, 10 Cs-HEPES, 2 KCl, 1 CaCl<sub>2</sub>, 80 glucose, pH 7; the internal recording solution consisted of the following (in mM): 90 CsF, 15 CsCl, 10 Cs-HEPES, 10 EGTA, 70 glucose, pH 7. Recordings were obtained at room temperature using a Multiclamp 700A patch-clamp amplifier (Axon Instruments, Inc.) and were usually started 5 min after rupture of the membrane patch to allow intracellular dialysis with the pipette solution. Current signals were filtered at 10KHz, capacitive currents were minimized by means of the amplifier circuitry and 80-85% series resistance compensation was routinely used. The remaining linear leak and capacitance currents were subtracted online using a P/4 subtraction paradigm. Css IV was dissolved in the extracellular recording bath solution at the final concentration. All experiments were performed at room temperature. Conductance-voltage curves were derived from peak sodium current versus test voltage (I-V) curves according to  $G=I/(V-V_R)$  where I is the peak current, V is the test voltage, and V<sub>R</sub> is the apparent reversal potential. Normalized conductance-voltage curves were fit with a Boltzmann relationship of the form  $1/\{1+\exp[(V_{1/2}-V)/k]\}$  or with the sum of two such expressions, where V<sub>1/2</sub> is the voltage for half-maximal activation and k is a slope factor with the dimensions of millivolts. No perfusion of the toxin was used to record the I/V curves because the amount of natural toxin that we can use is limited. Therefore, in all these experiments the toxin was directly dissolved in the recording bath solution and conductance-voltage curves were normalized in control and in the presence of toxin.

## RESULTS

### *Effects of the $\beta$ -scorpion toxin C<sub>ss</sub> IV on wild-type Na<sub>v</sub>1.2a sodium channels.*

The effects of the  $\beta$ -scorpion toxin on Na<sub>v</sub>1.2a sodium channels are depicted in Figure 1. We have measured the sodium current elicited by a strong depolarisation to 0 mV and the sodium current elicited by a test pulse to a negative membrane potential of -60 mV following a 1-ms depolarisation to +60 mV (Fig. 1B), in the absence or in the presence of toxin. As shown in Figure 1, C<sub>ss</sub> IV caused a reduction in the peak sodium current (Fig. 1A) and elicited a current at -60 mV (Fig. 1C). Both effects are concentration dependent but only the activation at negative potentials requires a 1-ms depolarisation to very positive membrane potentials. As shown in Figures 1A and 1C, using 500 nM of C<sub>ss</sub> IV does not produce a larger reduction of peak sodium current or a larger current amplitude at -60 mV than with 200nM of toxin. Therefore, 200 nM of toxin represents a saturating concentration regarding the toxin effect on Na<sub>v</sub>1.2 sodium channels. As previously described for the wild-type Na<sub>v</sub>1.2a channels (Cestèle *et al.*, 1998; Cestèle *et al.*, 2001), treatment with 50 nM of the  $\beta$ -scorpion toxin C<sub>ss</sub> IV had no effect on channel activation, as measured by the conductance-voltage curve at a holding potential of -100 mV (Fig. 1D). However, in the presence of a 1-ms prepulse to +60 mV, 50 nM C<sub>ss</sub> IV induced a negative shift in the voltage dependence of activation of 18% of the channels (Fig. 1D, Table 1). A higher concentration of toxin (200 nM) had no effect on the voltage-dependence of activation in the absence of the depolarising prepulse (data not shown) but caused a complete shift to more negative potentials of the conductance-voltage curve when the prepulse was applied prior the test pulse (Fig. 1D). Therefore, C<sub>ss</sub> IV has two sequential effects on Na<sub>v</sub>1.2 sodium channels: the first one corresponds to the reduction in the peak sodium current and the second one, which requires prior depolarisation and depends on channel activation, corresponds to the negative shift in the voltage dependence of activation, which is responsible for the appearance of sodium current at negative membrane potentials. The effect on sodium channel activation has been proposed to be due to the trapping by the toxin of the IIS4 segment in an outward position which favours activation at negative potentials (Cestèle *et al.*, 1998). Therefore, the ability of  $\beta$ -scorpion toxins to trap the IIS4 segment during activation makes them unique and powerful tools to monitor the movement within the membrane and the availability of the IIS4 segment from the extracellular side of the membrane.

*Effects of Css IV on mutants in IIS4 segment of Na<sub>v</sub>1.2a sodium channels.*

To investigate the possible molecular interactions between the IIS4 voltage sensor and the acidic residues of the IIS2 and IIS3 segments, we constructed mutants in which the charged residues of the S2, S3 and S4 segments were mutated to the opposite charge. First, the two outermost arginines of the IIS4 segment were mutated to a glutamic acid: R850E and R853E (Fig. 2). In control conditions (Figs. 3A, 3B and Table 1), analysis of the voltage-conductance relationships for the wild-type and mutant channels showed a positive shift in the activation curve of 12.6 mV for R850E and 4.9 mV for R853E and a reduction in the steepness of the curve. Comparison of the measurements of the steady-state inactivation (Fig. 3C, Table 2) for Na<sub>v</sub>1.2a, R850E and R853E showed that the mutations do not significantly change the voltage of half maximal inactivation despite the difference in voltage dependence of activation. These data are similar to the ones we observed when the two outermost arginines of the IIS4 segment were mutated in glutamine or cysteine (Cestèle *et al.*, 2001) and underline the specific role that the positive charges of the IIS4 segment play in activation.

As in the case of the neutralization of the two outermost arginine by glutamine or cysteine (Cestèle *et al.*, 2001), mutation of R850 and R853 to a glutamic acid strongly alters the effect of Css IV: the voltage-dependence of 19% of the R850E channels was shifted to more negative potentials even in the absence of a pre-pulse and 59% of the channels in the presence of the pre-pulse to +50 mV (Figs. 4A, 4B, Table 1); the voltage dependence of all the R853E channels was shifted by 10.6 to 12.8 mV even in the absence of the depolarising pre-pulse to +50 mV (Figs. 4C, 4D, Table 1). The ability of Css IV to induce a negative shift in the voltage dependence of activation even in the absence of the pre-pulse to +50 mV suggests that toxin binding by itself is sufficient to stabilize the IIS4 voltage-sensor in an outward/activated position at the resting membrane potential. Evidently, mutation of the two outermost arginine residues of the IIS4 segment to either a glutamine, a cysteine or a glutamic acid residue favours voltage-sensor trapping by  $\beta$ -scorpion toxins. The ability to favour the activated position is modulated by point mutations in domain II: at resting membrane potential, the voltage sensor IIS4 can be fully deactivated (as for the wild type), partially activated (as for the mutant R850E) or fully activated (as for the mutant R853E). Therefore,  $\beta$ -scorpion toxins represent unique tools to track changes in the equilibrium for the IIS4 voltage sensor.

*Effect of mutation of acidic residues of the IIS2 and IIS3 segment on Css IV activity on rat brain Na<sub>v</sub>1.2a sodium channels.*

It has been shown for K<sup>+</sup> channels that basic residues in the S4 segment interact with acidic residues in the S2 and S3 segments. These electrostatic interactions represent structural constraints that can stabilize the S4 segments and impede their motion (Papazian *et al.*, 1995; Planells-Cases *et al.*, 1995; Tiwari-Woodruff *et al.*, 1997; Tiwari-Woodruff *et al.*, 2000; Li-Smerin *et al.*, 2000). In order to examine the possible interactions of R850 and R853 with acidic residues localized in the S2 and S3 segments of sodium channels, we also constructed mutants in which the acidic residues of these segments were mutated to arginine (Fig. 2). According to our hypothesis, if the negative charged residues of the S2 and S3 segments in domain II are involved in electrostatic interactions with R850 or R853 in the IIS4 segment, their mutation should have the same consequences regarding the  $\beta$ -scorpion toxin effect on activation than the mutation of R850 and R853. The disruption of one electrostatic interaction should modify the availability of the IIS4 segment within the membrane and therefore, it should favour the activated position of the voltage sensor.

In control conditions, comparison of the conductance-voltage relationship of the wild type Na<sub>v</sub>1.2a and mutant channels (Figs. 5A, 5B, Table 3) showed that there was a positive shift of 5.4 to 12.6 mV in the voltage dependence of activation of E805R, D814R and D827R, and a reduction of the steepness of the voltage-conductance curves. These data are comparable with those described above for R850E and R853E. In the opposite, the mutant E821R did not show significant change in the voltage dependence of activation. This indicates that mutating the E805, D814 and D827 amino acid residues alters the voltage-dependent properties of activation. As shown in Figure 5C, analysis of the steady-state inactivation for E805R, D814R, E821R and D827R showed that the mutant E821R is the only one to shift the inactivation curve to more positive potentials by 6.9 mV (Table 2). This underlines the specific role of the acidic residues E805, D814 and D827 (segments IIS2 and IIS3) in activation, and suggests that the S4 segment may not be the only segment governing the voltage-dependent activation of sodium channels. Therefore, our results suggest the existence of a voltage-sensing structure formed by S2, S3 and S4 segments in domain II of sodium channels.

Analysis of the consequences of the mutation of the acidic residues of the IIS2 and IIS3 segments on Css IV activity is presented in Figure 6. Compared to the effect of 50 nM of Css IV on wild-type Na<sub>v</sub>1.2a channels, the effect of  $\beta$ -toxin is strongly altered. Treatment

with 50 nM Css IV induced a 21 to 31% negative shift in the voltage-dependence of activation of the E805R and D814R mutant channels in the IIS2 segment (Figs. 6A and B) and of the E821R mutant channel in the IIS3 segment (Fig. 6C) even in the absence of the depolarising pre-pulse to +50 mV (Table 3). Furthermore, in the presence of the pre-pulse, the fraction of toxin-modified channels was 62% for E805R, 44% for D814R and 53% for E821R (Table 3). Thus, the effect of Css IV on the three mutants E805R, D814R and E821R was much greater than its effect on the wild-type Na<sub>v</sub>1.2a channels, and comparable to the one observed with R850E mutant channels (Figs. 4C, 6A, 6B and 6C; Table 3). More remarkably, Css IV induced a complete shift in the voltage dependence of activation of D827R channels, without or with the depolarising pre-pulse to +50 mV (Fig. 6D, Table 3). This effect is identical to the one described for R853E mutant channels. The mutation of the acidic residues of IIS2 and IIS3 segments or of the two outermost gating charges of the IIS4 voltage-sensor therefore have the same effect on the action of Css IV: i.e. they shift the equilibrium for the voltage sensor in domain II and favour its activated position. The ability of Css IV to induce a negative shift in the voltage-dependence of activation even in the absence of the depolarising pre-pulse suggests that the mutations E827R and R853E allow the IIS4 segment to adopt an outward position at the resting membrane potential which favours voltage sensor trapping by the toxin. All together, these results indicate that the negatively charged residues in IIS2 and IIS3 segments, and the two outermost gating charges of the IIS4 voltage sensor, play an identical role regarding the effect of Css IV on sodium channels, and therefore suggest their possible role in modifying the conformation of the IIS4 segment to favour its activated position which increases its availability for  $\beta$ -toxin trapping.

#### *Effect of $\beta$ -scorpion toxin on charge-inversion double mutants*

In order to identify pairs of interacting residues, we designed charge-inversion double mutants between S2 and S4 segments or between S3 and S4 segments in domain II. In fact, electrostatic interactions are involved in specifying a particular fold of a protein and charges reversal mutations provide insight into the conformations in which these residues are in the closest proximity (Tiwari-Woodruff *et al.*, 2000) and may give a conformation to the channel close to the native one.

We constructed eight charge-inversion double mutants but, unfortunately, no current was detected for four of them (E805R/R850E, E805R/R853E, D827R/R850E, D827R/R853E) and their functional analysis in voltage-clamp experiments was not possible. Possible

reasons for this absence of currents are that the mutant channels lose their plasma membrane targeting and undergo degradation, or may not be conductive despite correct plasma membrane targeting. Preliminary binding experiments (data not shown) using the radiolabeled Css IV toxin and membranes prepared from tsA201-cells transfected with the charge-inversion double mutant sodium channels showed specific binding for all the tested mutants except D827R/R850E and D827R/R853E, thus indicating that these are not targeted to the plasma membrane, whereas the lack of current in E805R/R850E and E805R/R853E indicates that these mutations give the channel a conformation that prevents conduction.

The properties of the other mutants (D814R/R850E, D814R/R853E, E821R/R850E and E821R/R853E), were analysed in control and in the presence of Css IV. We found that they shifted the voltage-dependence of activation to more positive potentials and reduced the steepness of the curves (Figs. 7A and 7B, Table 4). Therefore, these double mutants favour the resting state of the sodium channels. Moreover, the double charge-reversal mutations produce positive shifts in the voltage dependence of activation that are greater than the single charge reversal mutations. Thus, even if the double charge-reversal mutants have the same number of positive and negative charges than the wild type channels, our data point out that the localisation of the charges within the membrane is important to respond to changes in membrane depolarisation and gives to the channel its voltage-dependent properties.

Analysis of the steady-state inactivation curves of these double mutants did not reveal any major changes in half-maximal inactivation potentials in comparison with wild-type sodium channels (Fig. 7C, Table 2). These effects are consistent with a decrease in voltage sensitivity due to the modifications of the charges in the voltage sensor. However,  $\beta$ -scorpion toxin can reveal electrostatic interactions of the mutated charges. In fact, swapped interacting charges should favour the deactivated position of the IIS4 segment, whereas swapped non-interacting charges should conversely favour its partially or fully activated position and therefore increase its availability for  $\beta$ -scorpion toxin trapping.

Treatment with 50 nM of Css IV did not induce a positive shift in the voltage-dependence of D814R/R850E (Fig. 8A) or E821R/R850E (Fig. 8B), unlike the single mutations D814R (Fig. 6B), E821R (Fig. 6C) and R850E (Fig. 4C). Furthermore, even in the presence of the depolarising pre-pulse to +50 mV, the D814R/R850E and E821R/R850E mutants abolished the effect of the toxin on sodium channel activation (Figs. 8A and 8B). In order to exclude the hypothesis that the lack of effect of Css IV on the double charge

reversal mutations is due to a lack of binding of the toxin to its receptor site, we measured the peak sodium current elicited by a test pulse at 0 mV. Indeed, we have shown that  $\beta$ -scorpion toxins have two sequential effects on wild-type sodium channels: the toxin binds to the channel and induces a reduction in peak sodium current, and then, after depolarisation, which allow the toxin to bind to newly accessible residues on the IIS4 voltage sensor, it shifts the activation to more negative potentials. As Scatchard analysis of binding experiments, realised under depolarised conditions, have shown that  $\beta$ -toxins bind to a single class of receptor sites (Cestèle *et al.*, 1998), measurements of the current at 0mV gives information about the receptor site occupancy. As shown in Figure 8C, application of 50 nM, 200 nM or 500 nM of toxin reduces the peak sodium current on D814R/R850E and D821R/R850E sodium channels as on wild type  $\text{Na}_v1.2$  sodium channels (Figs. 8C). 500 nM of C<sub>ss</sub> IV does not produce a larger reduction of peak sodium current, indicating that 200nM of toxin represent a saturating concentration also for the mutant D814R/R850E and D821R/R850E channels. Therefore, the double charged reversal mutations do not alter the binding of the toxin to its receptor site on the channel. Moreover, we measured the current elicited by a test pulse at -60 mV following a depolarisation to +60 mV in the presence of high concentration of toxin (200 nM) that produces a complete shift of the conductance voltage curve of the wild type sodium channels (Fig. 1A). As shown in Figure 8, in the presence of 200 nM of the toxin, a test pulse to -60 mV following a depolarisation to +60 mV resulted in a substantial sodium current for the wild-type sodium channel (Fig. 8D) and no current for the double mutant D814R/R850E (Fig. 8E) and E821R/R850E (Fig. 8F) even if the length of the prepulse to +60 mV was as long as 30 ms (data not shown). The same experiments were done in the presence of 500 nM of toxin, and no current was activated for the double mutant D814R/R850E and E821R/R850E (data not shown). Moreover, to exclude the hypothesis that the absence of current at -60 mV is due to unbinding of the toxin (i.e. untrapping of the IIS4 segment) during the interval between the prepulse to +60 mV and the test pulse to -60mV, we reduced the time between the two test pulses. No current was recorded for the mutants D814R/R850E and E821R/R850E, even if the interval between the two test pulses was as short as 5ms and the amplitude of the current activated at -60mV for the wild type sodium channels was unchanged (data not shown). Therefore, the lack of activation at negative membrane potentials that we observed for the double mutant D814R/R850E and E821R/R850E is not caused by the unbinding of the toxin. All

together, these experiments suggest that the  $\beta$ -toxin C<sub>ss</sub> IV is bound to the channel but that the double charge reversal mutations alter the availability of the IIS4 segment for voltage-sensor trapping by the toxin and favour the deactivated state of the IIS4 segment. Therefore, despite the effect of C<sub>ss</sub> IV on the single D814R, E821R and R850E mutants, the double charge-inversion mutants prevent voltage-sensor trapping by  $\beta$ -scorpion toxins. These results favour the hypothesis of the existence of electrostatic interactions between R850E and D814R and E821R, which stabilize the IIS4 voltage-sensor within the membrane (i.e. favour the deactivated state) and reduce the probability that this segment adopts a conformation favourable for voltage-sensor trapping by  $\beta$ -scorpion toxins (i.e. activated conformation). In order to verify that the absence of voltage sensor trapping, which we observed for the double reversal mutants D814R/R850E and E821R/R850E, is the consequence of an electrostatic interaction between R850E and D814R or E821R, we studied the effect of the toxin on the double charge neutralizing mutants D814C/R850Q and E821C/R850Q. Indeed, these mutants give the opportunity to check if the combination of the two mutations at these specific positions is responsible for the lack of effect of the toxin on the double charge reversal mutants or if this effect is specific to the nature of the amino acid used for the mutations. As for the charge reversal mutants, these mutations shift the voltage-dependence of the channels to positive potentials (Table 4), but 50 nM of C<sub>ss</sub> IV strongly shifts the voltage-dependence of activation to more negative membrane potentials in the presence of the depolarising prepulse (Figs. 8G, 8H). These data exclude the hypothesis that a strong positive shift in the voltage-dependence of activation of sodium channels is sufficient to alter the ability of the  $\beta$ -scorpion toxin to trap the IIS4 segment in an outward position. Moreover, our results show that double charge neutralization mutations do not alter voltage-sensor trapping but favour the activated position of the voltage sensor. Our data reinforce the hypothesis that the double charge-reversal mutations restore electrostatic interactions between the S2, S3 and S4 segments of the sodium channels that prevent trapping of the IIS4 segment by the toxin.

Remarkably, in the case of the D814R/R853E (Fig. 9A) and E821R/R853E mutants (Fig. 9B), treatment with 50 nM of C<sub>ss</sub> IV completely shifted activation to more negative potentials regardless of the presence of the depolarising pre-pulse to +50 mV. This effect was identical to that observed in the case of R853E (Fig. 4C) and D827R (Fig. 6D). Moreover, although the mutant R853E, D814R/R853E and E821R/R853E channels have

different voltage dependences of activation ( $V_{1/2}$  values ranging from  $-18$  to  $-26$  mV, Tables 1 and 4), their modification by C<sub>ss</sub> IV shifted their voltage dependences to approximately the same position on the voltage axis ( $V_{1/2}$  values ranging from  $-34$  to  $-39$  mV, Tables 1 and 4). Therefore, as we have previously suggested (Cestèle *et al.*, 2001), once sodium channels have been successfully modified by the toxin, they adopt a similar toxin-modified conformation regardless of the mutation. Evidently, the addition of the mutation D814R or E821R to the mutation R853E does not modify the ability of the R853E channels to enhance voltage-sensor trapping by  $\beta$ -scorpion toxins, and therefore does not inhibit the ability of C<sub>ss</sub> IV binding to give the channel an outward/activated position at resting membrane potentials. Thus, the charges swapped in these double mutants do not interact and consequently their swapping does not alter the conformation of the IIS4 voltage-sensor which remains available for trapping by  $\beta$ -toxins.

## DISCUSSION

*Evidence for a voltage-sensing structure in domain II of voltage-gated sodium channels.*

The voltage-dependent activation process of sodium channels is mainly supported by the outward translocation of several positive charges across the membrane (Hodgkin & HUXLEY, 1952; Armstrong, 1981). The S4 segments have been identified as the voltage sensors and it has been shown that they move outward in response to the changes in membrane potential that initiate channel activation (Catterall, 1986; Guy & Seetharamulu, 1986; Yang & Horn, 1995; Yang *et al.*, 1996; Yang *et al.*, 1997; Chanda & Bezanilla, 2002). Structure-function studies on sodium channels have highlighted that their voltage sensors have discrete functions. The voltage sensor in domain IV is implicated specifically in inactivation (Stuhmer *et al.*, 1989; Chen *et al.*, 1996; Kontis & Goldin, 1997; Kontis *et al.*, 1997; Cha *et al.*, 1999; Chanda & Bezanilla, 2002; Chanda *et al.*, 2004) whereas the voltage sensors of the three other domains are specifically involved in voltage dependent activation (Cha *et al.*, 1999; Chanda & Bezanilla, 2002; Chanda *et al.*, 2004). Therefore, modifications of one of the voltage sensors in domain I, II or III directly modify the activation process of the channel. Moreover, the mutation of the positive charges of the S4 segment in domain II reduces the steepness of the voltage dependence of activation (Stuhmer *et al.*, 1989) and the total gating charges of the channel (Sheets & Hanck, 2003), therefore decreasing its voltage sensitivity. Thus, the mutation of the positive charges stabilizes the resting state of the channel relative to the open state and the decrease in the number of positive charges in IIS4 voltage sensor implies stronger depolarisation to push the IIS4 segment outward (Cestèle *et al.*, 2001).

Analysis of the voltage dependence of gating of four mutant sodium channels in which the negative charged amino acid residues in the IIS2 and IIS3 segments have been replaced by an arginine indicates that the voltage-dependence of activation of three of them is strongly altered. The E805R, D814R and D827R mutants shift the voltage dependence of Na<sub>v</sub>1.2a sodium channels in the positive direction, reduce the steepness of the voltage-conductance curve, and thus favour the resting state of the channel. Furthermore, their effects on activation varies from one mutant to another, which indicates that the acidic residues of S2 and S3 segments in domain II are not functionally equivalent. These data are similar to those obtained using sodium channels with mutations of the gating charges of the IIS4 segment (Cestèle *et al.*, 2001). Our data show that the negatively charged residues of the S2 and S3 segments in domain II modulate sodium channel activation because their mutation alters the voltage dependence of

activation. Therefore, our data suggest that S2, S3 and S4 segments in domain II have a cooperative role in activation of sodium channels and may constitute a voltage-sensing structure. Moreover, the negative charged residues in the S2 and S3 segments as well as the positive charged residues in the IIS4 segment are well conserved among the different sodium channels isoforms, which suggests that this voltage sensing structure is a common feature for the voltage-dependent sodium channels. These results are in agreement with data concerning K<sup>+</sup> channels, in which it has been shown that the S4 segment is not solely responsible for gating charges movement but that S2 and S3 segments may be part of the voltage sensing structure (Planells-Cases *et al.*, 1995; Seoh *et al.*, 1996). This interpretation of our data seems to be the most plausible as it is in accordance with the results obtained for K<sup>+</sup> channels. However, we can not exclude that the negative charges of the IIS2 and IIS3 segment are not a physical part of the voltage sensor as we suggest above but that their mutation rather modulate in an allosteric manner the voltage sensor IIS4.

*Voltage-sensor trapping is enhanced by charge-reversal mutation in the S2, S3 and S4 segments.*

On wild-type sodium channels,  $\beta$ -scorpion toxin Css IV induce a partial shift of the activation curve, after a strong depolarising pre-pulse (Cestèle *et al.*, 1998; Cestèle *et al.*, 2001). The biphasic curves for activation in the presence of  $\beta$ -scorpion toxin are proposed to be due to the trapping of the IIS4 segment of a fraction of sodium channels in an outward position by binding the extracellular end of the IIS4 segment to the previously bound toxin in its receptor site, which includes the IIS3-S4 extracellular loop (Cestèle *et al.*, 1998). Interestingly, although the voltage sensors of the domains I, II and III are involved in voltage-dependent activation (Cha *et al.*, 1999; Chanda & Bezanilla, 2002), the specific binding of  $\beta$ -scorpion toxin in domain II (Cestèle *et al.*, 1998; Cestèle *et al.*, 2001) or the chemical modification of cysteine in the IIS4 voltage-sensor (Mitrovic *et al.*, 1998; Cestèle *et al.*, 2001) is sufficient to modulate specifically the voltage dependence of sodium channel activation.

Functional analysis of the charge-reversing mutants E805, D814, E821, E827, R850 and R853 shows that  $\beta$ -scorpion toxin action is strongly enhanced. The substitution of the two outermost arginine of the IIS4 segment (R850 and R853) by glutamine or cysteine gives identical results (Cestèle *et al.*, 2001). As the mutation of E805, D814, E821, E827, R850

and R853 favours the resting state of the channels by shifting their voltage dependence of activation toward more positive potentials (Stuhmer *et al.*, 1989; Cestèle *et al.*, 2001; Izhar *et al.*, 2004), the increase of  $\beta$ -scorpion toxin activity on these mutants can not be explained by a general enhancement of activation. Therefore, binding of  $\beta$ -scorpion toxin on the mutants E805, D814, E821, E827, R850 and R853 favours the activated position of the IIS4 voltage sensor, reduces the energy barrier for trapping the IIS4 segment in its outward position by binding of  $\beta$ -scorpion toxin while increasing the electrostatic force required to drive the IIS4 segment outward on depolarisation.

Another hypothesis able to explain the increase of  $\beta$ -toxin activity would be a change in toxin affinity for its receptor site induced by the mutations. However, binding experiments for the mutants R850C/Q and R853C/Q show that these mutations do not alter the affinity of the toxin C<sub>ss</sub> IV for the channels (Cestèle *et al.*, unpublished data). Moreover, the  $\beta$ -scorpion toxin receptor site is localized within the S1-S2 and S3-S4 extracellular loops in domain II (Cestèle *et al.*, 1998; Cestèle *et al.*, unpublished data) and therefore, the amino acid residues E805, D814, E821 and E827 in the S2 and S3 segments in domain II are not involved in the formation of the receptor site. Thus, the increase in  $\beta$ -toxin activity that we observed for these double mutants is not correlated to an increase in toxin affinity for its receptor site.

To explain our data, we proposed that R850 and R853 may interact with acidic residues localized in the S2 and S3 segments of sodium channels. We predicted the existence of electrostatic interactions between the positive residues of IIS4 and negatively charged residues localized in S2 and S3 segments in domain II because such structural constrains have been demonstrated for K<sup>+</sup> channels (Papazian *et al.*, 1995; Planells-Cases *et al.*, 1995; Tiwari-Woodruff *et al.*, 1997; Tiwari-Woodruff *et al.*, 2000; Li-Smerin *et al.*, 2000). The specific effect of  $\beta$ -toxin on the sodium channel activation makes it a unique and powerful tool for dissecting the molecular events occurring during the activation process of voltage-dependent sodium channels. We show that charge-reversal mutations of the negatively charged amino acid residues of the IIS2 and IIS3 segments not only modify the voltage-dependent gating of sodium channels, but also enhance the effect of  $\beta$ -scorpion toxin on sodium channels in a similar way to that we have described for the mutation of the two outermost arginine of the IIS4 segment. The fact that mutation of charged amino acid residues in different transmembrane segments in domain II have the same consequences on the voltage-dependence of activation and on the effect of  $\beta$ -

scorpion toxin action strongly suggests that they play a similar and/or a cooperative role in the voltage-sensing structure of sodium channels. Thus, acidic amino acid residues of the IIS2 and IIS3 segments and gating charges of the IIS4 voltage-sensor could have electrostatic interactions similar to the ones described for K<sup>+</sup> channels. Mutations of the charged amino acid residues in the voltage-sensing structure may abolish such electrostatic interactions and reduce the kinetic barrier for S4 movement within the membrane leading to greater availability of the IIS4 segment and favouring voltage-sensor trapping by  $\beta$ -scorpion toxins because IIS4 segment is more often available for toxin binding, even at negative membrane potentials where the channel is not activated. An alternative interpretation of our data would be that the mutations we introduced in domain II produce broad conformational changes in the sodium channel that alter  $\beta$ -scorpion toxin binding and/or action. This seems unlikely because we have to assume that several mutations in three different segments in domain II (S2, S3 and S4) have the same consequences regarding  $\beta$ -toxin action on sodium channels. This interpretation of our data would involve complex modulation of  $\beta$ -toxin receptor site and multiple ad hoc assumptions are required to explain our data.

*The action of  $\beta$ -scorpion toxins on double charge-inversion mutants discloses molecular interactions among charged residues in S2, S3 and S4 segments in domain II.*

The conclusion presented above is supported by our results obtained using the double charge-inversion mutants. The effects of  $\beta$ -scorpion toxin on these mutants, depending on the availability of IIS4, give unique insights into the electrostatic interactions occurring within the voltage-sensing structure and the molecular mechanism of sodium channel activation. Electrostatic interactions are involved in specifying a particular fold of a protein (Tiwari-Woodruff *et al.*, 2000) and charge-reversal mutations, which cause dramatic changes in local electrostatic potential in comparison with the wild-type protein, help to reveal the conformations in which these residues are in the closest proximity. As it has been shown in binding experiments that  $\beta$ -scorpion toxins bind to a single class of receptor site, our experiment measuring the reduction of the peak current at 0 mV gives information about the receptor site occupancy. Indeed, previous data and unpublished results have shown that molecular determinants involved in the formation of the receptor site for  $\beta$ -scorpion toxin are mainly localized in the S1-S2 and S3-S4 extracellular loops in domain II (Marcotte *et al.*, 1997b; Cestèle *et al.*, 1998). Our data show that  $\beta$ -scorpion

toxin is bound to the double mutants D814R/R850E and E821R/R850E channels with an affinity comparable to the one of the wild-type, but has no effect on the voltage-dependence of activation. Thus, the double charge-reversal mutations prevent specifically the voltage-sensor trapping of the IIS4 segment, but do not affect the ability of the toxin to bind to the channel. Moreover, the double charge-neutralization mutants (D814C/R850Q and E821C/R850Q) do not alter the ability of the  $\beta$ -scorpion toxin C<sub>ss</sub> IV to trap the IIS4 segment and induce a positive shift in the voltage-dependence of activation. Therefore, the double charge-reversal mutants D814R/R850E and E821R/R850E give a conformation to the channel that allows  $\beta$ -scorpion toxin binding but prevents specifically voltage-sensor trapping. Thus, our data can be interpreted in terms of electrostatic interactions between the first outermost arginine R850 of the IIS4 segment and D814 in IIS2 and E821 in IIS3 segments. A stable electrostatic interaction occurs between the two mutated amino acid residues, which gives the voltage-sensing structure a conformation that makes the IIS4 segment unfavourable for voltage-sensor trapping by  $\beta$ -scorpion toxins and favours the deactivated position of the IIS4 voltage sensor.

We have interpreted our data in terms of electrostatic interactions between the acidic residues of the IIS2 and IIS3 segments and basic residues of the IIS4 segment. However, our data could be accommodated by complex conformational changes induced by the mutations that could result in an allosteric modulation of the receptor site of  $\beta$ -scorpion toxin on sodium channels. This interpretation seems to be unlikely as it implies first, that mutations in different part of the channel (IIS2, IIS3 and IIS4 segments) induce conformational changes that have the same consequences on  $\beta$ -toxin action, and second, that the combination of two mutations (D814R, E821R or R850E) that have a positive effect on  $\beta$ -scorpion toxin action, produce a negative allosteric modulation. Moreover, all the single mutations (charge-reversal or charge-neutralizing) favour the activated state of the channel and it seems unlikely that combination of two charge-reversal or two charge-neutralizing mutations have distinct consequences on  $\beta$ -toxin action. Thus, although this interpretation of our data is possible, it implies multiple complex assumptions.

On the contrary, the activity of C<sub>ss</sub> IV on the double D814R/R853E and E821R/R853E mutants is identical to that described for R853E mutant channels. The movement of the IIS4 segment in the R853E mutant is therefore not affected by the additional D814 or E821 mutation: the enhanced effect of C<sub>ss</sub> IV in these double mutant is due to the greater

availability of the IIS4 segment induced by the mutation of R853. Thus, our data indicate that D814 and E821 interact specifically with R850.

We can only make hypothesis concerning the possible involvement of E805 and D827 residues in electrostatic interactions with the two outermost arginine of the IIS4 segment, because the double mutants involving these residues failed to generate currents. Preliminary binding experiments (data not shown) show specific binding for E805R/R850E and E805R/R853E, which indicate the ability of those mutants to be targeted to the plasma membrane. The lack of current with these double mutants indicate that they create a channel conformation that prevents current conduction. In the other hand, no specific binding was detected for the double mutants D827R/R850E and D827R/R853E indicating that those mutants are not targeted to the plasma membrane.

However, comparison of the effect of  $\beta$ -scorpion toxin on E805R and D827R with the one on R850E and R853E provides information about the possible electrostatic interactions occurring within them. The fact that R850E and R853E differently enhance the effect of  $\beta$ -scorpion toxin on sodium channels makes the interpretation of the E805R and D827R data more specific. We show that E805R and R850E identically alter the activity of  $\beta$ -toxin on sodium channels because their activation curves can be superimposed in the absence or presence of the depolarising pre-pulse. The mutation of E805 or R850 thus has the same consequences on the effect of  $\beta$ -scorpion toxin on sodium channels and it is possible that E805 interacts with R850. On the other hand, both D827R and R853E modified the effect of  $\beta$ -scorpion toxin on sodium channels in the same manner: the activation curve is completely shifted in the absence or in the presence of the depolarising pre-pulse. As D827R and R853E mutants lead to the same modification of  $\beta$ -toxin activity on sodium channels, we conclude that D827 is involved in an electrostatic interaction with R853 in the native channels. Abolishing this interaction by mutating D827 or R853 unlocks the IIS4 segment, which becomes more accessible for  $\beta$ -scorpion toxin to bind even at negative membrane potentials. Thus, as illustrated in Fig. 10A, our data indicate that R850 interacts with three negative residues in the wild-type sodium channels: E805 and D814 in IIS2 and E821 in IIS3 segments. It is likely that the interaction of R850 with each of these negatively charged residues occurs at different membrane potentials and accompanies the conformation changes involved in voltage-dependent activation. Another set of experiments is required to determine the sequence of these interactions during the voltage-dependent activation of sodium channels. Our data also suggest the existence of an electrostatic interaction of

R853 in IIS4 with D827 in IIS3, and underline its key role in stabilizing the voltage-sensing structure within the membrane: voltage-sensor trapping and the resulting negative shift of activation occur with the D827R and R853E mutants in the absence of a depolarising pre-pulse.

*Divergence between sodium and potassium channels.*

Recently, the crystal structure of a bacterial voltage-dependent  $K^+$  channel has given evidence for the existence of a “voltage-sensor paddle” formed by the packing of the C-terminal part of the S3 segment and the S4 segment (Jiang et al., 2003a). The same authors have shown that the voltage-sensor paddles move a large distance across the membrane from inside to outside when the channel opens (Jiang et al., 2003b). The voltage-sensor paddle model is controversial and do not support a large number of experimental data of the litterature. The effect of the mutations of the acidic residues of the S2 and S3 segments on voltage dependence of activation and on the effect of  $\beta$ -scorpion toxin suggests that in sodium channels these negative charges have a role in voltage sensing and that the IIS4 may interact with the negative charges of IIS2 and of the N-terminal part of IIS3 during its movements across the membrane, decreasing in this way the energetic of the translocation (Ahern & Horn, 2004). As illustrated in Figure 10, comparison of our data with the ones obtained with  $K^+$  channels (Papazian *et al.*, 1995; Planells-Cases *et al.*, 1995; Seoh *et al.*, 1996; Tiwari-Woodruff *et al.*, 1997; Tiwari-Woodruff *et al.*, 2000) reveals a different set of electrostatic interactions between S2, S3 and S4 segments and suggest that the molecular basis for channel activation is specific to  $K^+$  or  $Na^+$  channels. Moreover, only three of the negative charges that we have studied are aligned with the ones of Shaker potassium channels. The negative charges are very well conserved in sodium channels, except E821 that is present only in the neuronal isoforms. Also, D814 may be localised in the S2-S3 linker rather than in the IIS2 segment, but as for the potassium channels, this linker may be localised in the membrane and therefore interact with the IIS4 segment of the channel. It is not surprising that our data are different in comparison with the ones obtained for potassium channels, as sodium and potassium channels display a large number of differences. For example, potassium channels are comprised of four subunits whereas sodium channels is a single polypeptide with four homologous domains. The difference in the primary structure between the four domains confers discrete functions. Therefore, our data disclose properties that are unique to the domain II of sodium channels. An other difference is that

in contrast with potassium channels, the mutation of the positive charges in the IIS4 segment does not prevent expression of the channels and the positive charges involved in electrostatic interactions in sodium and potassium channels are different. Therefore, our results stress out the specific properties of domain II of sodium channels.

*Conclusions.*

Taken together, the results of our experiments indicate the existence of a voltage-sensing structure in voltage-dependent sodium channels composed of the S2, S3 and S4 segments in domain II. As the negative charges of IIS2 and IIS3 as well as the positive charges of IIS4 segment are conserved in all sodium channel subtypes, we suggest that this voltage sensing structure is present in all of them. Our data provide unique insights into the structure of sodium channels, and reveal molecular details about the conformational changes occurring during their voltage-dependent activation.

## REFERENCES

- Ahern, C. A. & Horn, R. (2004). Stirring up controversy with a voltage sensor paddle. *Trends Neurosci.* **27**, 303-307.
- Armstrong, C. M. (1981). Sodium channels and gating currents. *Physiol Rev.* **61**, 644-683.
- Catterall, W. A. (1986). Molecular properties of voltage-sensitive sodium channels. *Annu.Rev.Biochem.* **55**, 953-985.
- Catterall, W. A. (2000). From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. *Neuron* **26**, 13-25.
- Cestele, S. & Catterall, W. A. (2000). Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie* **82**, 883-892.
- Cestèle, S., Qu, Y., Rogers, J. C., Rochat, H., Scheuer, T., & Catterall, W. A. (1998). Voltage sensor-trapping: enhanced activation of sodium channels by beta-scorpion toxin bound to the S3-S4 loop in domain II. *Neuron* **21**, 919-931.
- Cestèle, S., Scheuer, T., Mantegazza, M., Rochat, H., & Catterall, W. A. (2001). Neutralization of gating charges in domain II of the sodium channel alpha subunit enhances voltage-sensor trapping by a beta-scorpion toxin. *J.Gen.Physiol* **118**, 291-302.
- Cha, A., Ruben, P. C., George, A. L., Jr., Fujimoto, E., & Bezanilla, F. (1999). Voltage sensors in domains III and IV, but not I and II, are immobilized by Na<sup>+</sup> channel fast inactivation. *Neuron* **22**, 73-87.
- Chanda, B., Asamoah, O. K., & Bezanilla, F. (2004). Coupling interactions between voltage sensors of the sodium channel as revealed by site-specific measurements. *J.Gen.Physiol* **123**, 217-230.
- Chanda, B. & Bezanilla, F. (2002). Tracking voltage-dependent conformational changes in skeletal muscle sodium channel during activation. *J.Gen.Physiol* **120**, 629-645.
- Chen, L. Q., Santarelli, V., Horn, R., & Kallen, R. G. (1996). A unique role for the S4 segment of domain 4 in the inactivation of sodium channels. *J.Gen.Physiol* **108**, 549-556.
- Guy, H. R. & Seetharamulu, P. (1986). Molecular model of the action potential sodium channel. *Proc.Natl.Acad.Sci.U.S.A* **83**, 508-512.

Heinemann, S. H., Terlau, H., Stuhmer, W., Imoto, K., & Numa, S. (1992). Calcium channel characteristics conferred on the sodium channel by single mutations. *Nature* **356**, 441-443.

Hodgkin, A. L. & HUXLEY, A. F. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J.Physiol* **117**, 500-544.

Izhar, K., Lior, C., Nicolas, G., Dalia, G., & Michael, G. (2004). Conversion of a scorpion toxin agonist into an antagonist highlights an acidic residue involved in voltage sensor trapping during activation of neuronal Na<sup>+</sup> channels. *FASEB J.* **18**, 683-689.

Jiang, Y., Lee, A., Chen, J., Ruta, V., Cadene, M., Chait, B. T., & MacKinnon, R. (2003a). X-ray structure of a voltage-dependent K<sup>+</sup> channel. *Nature* **423**, 33-41.

Jiang, Y., Ruta, V., Chen, J., Lee, A., & MacKinnon, R. (2003b). The principle of gating charge movement in a voltage-dependent K<sup>+</sup> channel. *Nature* **423**, 42-48.

Jordan, M., Schallhorn, A., & Wurm, F. M. (1996). Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res.* **24**, 596-601.

Kontis, K. J. & Goldin, A. L. (1997). Sodium channel inactivation is altered by substitution of voltage sensor positive charges. *J.Gen.Physiol* **110**, 403-413.

Kontis, K. J., Rounaghi, A., & Goldin, A. L. (1997). Sodium channel activation gating is affected by substitutions of voltage sensor positive charges in all four domains. *J.Gen.Physiol* **110**, 391-401.

Li-Smerin, Y., Hackos, D. H., & Swartz, K. J. (2000). alpha-helical structural elements within the voltage-sensing domains of a K(+) channel. *J.Gen.Physiol* **115**, 33-50.

Marcotte, P., Chen, L. Q., Kallen, R. G., & Chahine, M. (1997a). Effects of Tityus serrulatus scorpion toxin gamma on voltage-gated Na<sup>+</sup> channels. *Circ.Res.* **80**, 363-369.

Marcotte, P., Chen, L. Q., Kallen, R. G., & Chahine, M. (1997b). Effects of Tityus serrulatus scorpion toxin gamma on voltage-gated Na<sup>+</sup> channels. *Circ.Res.* **80**, 363-369.

Martin, M. F., Perez, L. G., El Ayeb, M., Kopeyan, C., Bechis, G., Jover, E., & Rochat, H. (1987). Purification and chemical and biological characterizations of seven toxins from the Mexican scorpion, *Centruroides suffusus suffusus*. *J.Biol.Chem.* **262**, 4452-4459.

- Mitrovic, N., George, A. L., Jr., & Horn, R. (1998). Independent versus coupled inactivation in sodium channels. Role of the domain 2 S4 segment. *J.Gen.Physiol* **111**, 451-462.
- Noda, M., Suzuki, H., Numa, S., & Stuhmer, W. (1989). A single point mutation confers tetrodotoxin and saxitoxin insensitivity on the sodium channel II. *FEBS Lett.* **259**, 213-216.
- Papazian, D. M., Shao, X. M., Seoh, S. A., Mock, A. F., Huang, Y., & Wainstock, D. H. (1995). Electrostatic interactions of S4 voltage sensor in Shaker K<sup>+</sup> channel. *Neuron* **14**, 1293-1301.
- Planells-Cases, R., Ferrer-Montiel, A. V., Patten, C. D., & Montal, M. (1995). Mutation of conserved negatively charged residues in the S2 and S3 transmembrane segments of a mammalian K<sup>+</sup> channel selectively modulates channel gating. *Proc.Natl.Acad.Sci.U.S.A* **92**, 9422-9426.
- Ragsdale, D. S., McPhee, J. C., Scheuer, T., & Catterall, W. A. (1994). Molecular determinants of state-dependent block of Na<sup>+</sup> channels by local anesthetics. *Science* **265**, 1724-1728.
- Seoh, S. A., Sigg, D., Papazian, D. M., & Bezanilla, F. (1996). Voltage-sensing residues in the S2 and S4 segments of the Shaker K<sup>+</sup> channel. *Neuron* **16**, 1159-1167.
- Sheets, M. F. & Hanck, D. A. (2003). Molecular action of lidocaine on the voltage sensors of sodium channels. *J.Gen.Physiol* **121**, 163-175.
- Stuhmer, W., Conti, F., Suzuki, H., Wang, X. D., Noda, M., Yahagi, N., Kubo, H., & Numa, S. (1989). Structural parts involved in activation and inactivation of the sodium channel. *Nature* **339**, 597-603.
- Terlau, H., Heinemann, S. H., Stuhmer, W., Pusch, M., Conti, F., Imoto, K., & Numa, S. (1991). Mapping the site of block by tetrodotoxin and saxitoxin of sodium channel II. *FEBS Lett.* **293**, 93-96.
- Tiwari-Woodruff, S. K., Lin, M. A., Schulteis, C. T., & Papazian, D. M. (2000). Voltage-dependent structural interactions in the Shaker K(+) channel. *J.Gen.Physiol* **115**, 123-138.
- Tiwari-Woodruff, S. K., Schulteis, C. T., Mock, A. F., & Papazian, D. M. (1997). Electrostatic interactions between transmembrane segments mediate folding of Shaker K<sup>+</sup> channel subunits. *Biophys.J.* **72**, 1489-1500.

Yang, N., George, A. L., Jr., & Horn, R. (1996). Molecular basis of charge movement in voltage-gated sodium channels. *Neuron* **16**, 113-122.

Yang, N., George, A. L., Jr., & Horn, R. (1997). Probing the outer vestibule of a sodium channel voltage sensor. *Biophys.J.* **73**, 2260-2268.

Yang, N. & Horn, R. (1995). Evidence for voltage-dependent S4 movement in sodium channels. *Neuron* **15**, 213-218.

Yu, F. H., Westenbroek, R. E., Silos-Santiago, I., McCormick, K. A., Lawson, D., Ge, P., Ferreria, H., Lilly, J., DiStefano, P. S., Catterall, W. A., Scheuer, T., & Curtis, R. (2003). Sodium channel beta4, a new disulfide-linked auxiliary subunit with similarity to beta2. *J.Neurosci.* **23**, 7577-7585.

## **ACKNOLEGMENTS**

We are grateful to Dr. W.A. Catterall (Department of Pharmacology, University of Washington, Seattle, USA) for his generous gift of the Na<sub>v</sub>1.2a clone and to Prof. R. Guieu (CNRS FRE 2738, Marseille, France), Prof. G. Avanzini and Dr. S. Francheschetti (Department of Neurophysiopathology, Istituto Neurologico Besta, Milan, Italy) for their constant support. We thank Dr. M.F. Martin-Eauclaire (CNRS FRE 2738, Marseille, France) for purifying the β-scorpion toxin C<sub>ss</sub> IV, Dr. B. Céard (CNRS FRE 2738, Marseille, France) for DNA sequencing, Dr. P. Scalmani and Dr. E. Armatura (Department of Neurophysiopathology, Istituto Neurologico Besta, Milano, Italy) for their help.

This work was supported by the CNRS and the Pierfranco and Luisa Mariani Foundation.

**Table 1:** Voltage Dependence of activation of Na<sub>v</sub>1.2 and mutant sodium channels

	<b>V1<sub>1/2</sub> (mV)</b>	<b>k1 (mV)</b>	<b>A1</b>	<b>V2<sub>1/2</sub> (mV)</b>	<b>k2 (mV)</b>	<b>A2</b>	<b>n</b>
<b>Na<sub>v</sub>1.2a-Control</b>	-31.6 ± 0.3	6.3 ± 0.3					9
<b>Na<sub>v</sub>1.2a-NP-Css IV 50 nM</b>	-31.4 ± 0.5	6.3 ± 0.5					6
<b>Na<sub>v</sub>1.2a-Pre-Css IV 50 nM</b>	-30.2 ± 1.6	6.1 ± 1.1	0.82 ± 0.08	-64.1 ± 2.9	6.1 ± 2.5	0.2 ± 0.2	6
<b>Na<sub>v</sub>1.2a-Pre-Css IV 200 nM</b>	-51.6 ± 0.6	8.9 ± 0.5					3
<b>R850E-Control</b>	-18.9 ± 0.4	8.4 ± 0.3					6
<b>R850E-NP-Css IV 50 nM</b>	-14.9 ± 1.2	8.3 ± 1.2	0.8 ± 0.2	-46.7 ± 3.6	13.1 ± 2.4	0.2 ± 0.01	7
<b>R850E-Pre-Css IV 50 nM</b>	-16.7 ± 0.9	6.1 ± 0.8	0.39 ± 0.02	-51.1 ± 0.8	8.5 ± 0.6	0.6 ± 0.02	7
<b>R853E-Control</b>	-26.6 ± 0.6	8.8 ± 0.6					14
<b>R853E-NP-Css IV 50 nM</b>	-37.3 ± 0.7	10.7 ± 0.7					7
<b>R853E-Pre-Css IV 50 nM</b>	-39.5 ± 0.9	9.4 ± 0.8					6

**Table 2:** Voltage dependence of inactivation of Na<sub>v</sub>1.2 and mutant sodium channels

	<b>V1<sub>1/2</sub> (mV)</b>	<b>k (mV)</b>	<b>n</b>
<b>Na<sub>v</sub>1.2</b>	-63.1 ± 0.3	8.1 ± 0.3	8
<b>R850E</b>	-63.9 ± 0.3	7.2 ± 0.3	4
<b>R853E</b>	-65.8 ± 0.8	9.3 ± 0.7	13
<b>E805R</b>	-60.5 ± 1.5	9.9 ± 1.3	10
<b>D814R</b>	-61.8 ± 0.4	6.3 ± 0.3	7
<b>E821R</b>	-54.4 ± 9.4	9.4 ± 0.6	11
<b>D827R</b>	-64.2 ± 1.1	8.2 ± 0.9	11
<b>D814R/R850E</b>	-60.3 ± 0.7	9.6 ± 0.6	7
<b>D814R/R853E</b>	-62.9 ± 0.9	8.2 ± 0.8	10
<b>E821R/R850E</b>	-65.6 ± 0.3	7.5 ± 0.2	9
<b>E821R/R853E</b>	-58.7 ± 0.5	9.1 ± 0.5	4

**Table 3:** Voltage dependence of activation of mutants in the IIS2 and IIS3 segments

	V1 $\frac{1}{2}$ (mV)	k1 (mV)	A1	V2 $\frac{1}{2}$ (mV)	K2 (mV)	A2	n
E805R-Control	-21.6 $\pm$ 0.3	8.2 $\pm$ 0.3					15
E805R-NP-Css IV 50 nM	-16.1 $\pm$ 1.3	6.6 $\pm$ 0.9	0.79 $\pm$ 0.11	-49.4 $\pm$ 1.9	10.1 $\pm$ 1.7	0.21 $\pm$ 0.08	18
E805R-Pre-Css IV 50 nM	-15.7 $\pm$ 1.6	4.3 $\pm$ 1.3	0.37 $\pm$ 0.08	-46.1 $\pm$ 2.9	8.9 $\pm$ 1.3	0.62 $\pm$ 0.8	20
D814R-Control	-26.2 $\pm$ 0.3	9.2 $\pm$ 0.2					7
D814R-NP-Css IV 50 nM	-22.9 $\pm$ 2.2	6.9 $\pm$ 2.6	0.71 $\pm$ 0.10	-45.2 $\pm$ 1.9	13.9 $\pm$ 1.6	0.28 $\pm$ 0.01	5
D814R-Pre-Css IV 50 nM	-23.9 $\pm$ 2.1	7.9 $\pm$ 1.9	0.55 $\pm$ 0.07	-52.3 $\pm$ 1.9	11.1 $\pm$ 1.7	0.44 $\pm$ 0.01	12
E821R-Control	-29.8 $\pm$ 0.3	5.6 $\pm$ 0.3					7
E821R-NP-Css IV 50 nM	-26.6 $\pm$ 2.2	5.9 $\pm$ 3.2	0.67 $\pm$ 0.05	-51.4 $\pm$ 3.2	14.2 $\pm$ 2.8	0.32 $\pm$ 0.02	7
E821R-Pre-Css IV 50 nM	-24.4 $\pm$ 2.1	6.1 $\pm$ 1.4	0.46 $\pm$ 0.14	-55.1 $\pm$ 0.8	11.1 $\pm$ 0.7	0.53 $\pm$ 0.01	9
D827R-Control	-18.9 $\pm$ 0.3	9.2 $\pm$ 0.2					13
D827R- NP-Css IV 50 nM	-28.6 $\pm$ 0.6	11.9 $\pm$ 0.6					11
D827R-Pre-Css IV 50 nM	-30.3 $\pm$ 0.7	10.1 $\pm$ 0.7					11

**Table 4:** Voltage-dependence of charge-inversion double mutants

	<b>V1<sub>1/2</sub> (mV)</b>	<b>k (mV)</b>	<b>n</b>
<b>D814R/R850E-Control</b>	-15.3 ± 0.3	7.9 ± 0.2	8
<b>D814C/R850Q-Control</b>	-14.8 ± 0.1	7.9 ± 0.1	7
<b>D814R/R850E-NP-CssIV 50 nM</b>	-14.7 ± 0.5	9.2 ± 0.4	8
<b>D814R/R850E-Pre-CssIV 50 nM</b>	-15.1 ± 0.7	7.9 ± 0.7	8
<b>D814R/R853E-Control</b>	-21.4 ± 0.4	8.9 ± 0.3	7
<b>D814R/R853E-NP-CssIV 50 nM</b>	-32.2 ± 0.7	11.6 ± 0.6	4
<b>D814R/R853E-Pre-CssIV 50 nM</b>	-38.1 ± 0.6	10.2 ± 0.5	16
<b>E821R/R850E-Control</b>	-18.5 ± 0.3	7.7 ± 0.2	10
<b>E821C/R850Q-Control</b>	-15.9 ± 0.1	9.6 ± 0.3	8
<b>E821R /R850E-NP-CssIV 50 nM</b>	-19.1 ± 0.3	9.5 ± 0.3	10
<b>E821R /R850E-Pre-CssIV 50 nM</b>	-19.1 ± 0.3	9.5 ± 0.3	14
<b>E821R/R853E-Control</b>	-18.5 ± 0.3	8.1 ± 0.3	5
<b>E821R /R853E-NP-CssIV 50 nM</b>	-32.9 ± 0.7	12.2 ± 0.6	7
<b>E821R /R853E-Pre-CssIV 50 nM</b>	34.8 ± 0.6	11.0 ± 0.5	9

## FIGURE LEGEND

### Figure 1:

Effects of the  $\beta$ -scorpion toxin C<sub>ss</sub> IV on wild-type Na<sub>v</sub>1.2 sodium channels. (A) Current recorded in a representative cell in response to the test pulse to 0 mV in control (plain line) or during perfusion with 50nM (dot line) or 200nM (dash line) of C<sub>ss</sub> IV. Traces were normalized to the peak current in control. (B) Test pulse protocol for obtaining the data shown in panels (A) and (C). A test pulse to 0 mV was applied from a holding potential of -100 mV. This was followed 60 ms later by a 1 ms conditioning pulse to +60 mV, a second period of 60 ms at the holding potential, and a 15 ms test pulse to -60 mV. This protocol was applied every 5s. (C) Currents recorded in response to the test pulse to -60 mV in control or during perfusion with 50 or 200 nM of C<sub>ss</sub> IV. (D) Conductance-voltage relationships in control (closed circles) in the presence of 50 nM of C<sub>ss</sub> IV without the prepulse to +50mV (open circles) or in the presence of 50 nM of C<sub>ss</sub> IV (open triangles) or 200 nM (filled squares) with a +50 mV, 1-ms prepulse that preceded the test pulse by 60 ms for wild-type Na<sub>v</sub>1.2a.

### Figure 2:

Schematic representation of the transmembrane folding topology of the domain II in the  $\alpha$  subunit of voltage dependent sodium channels. The localization of the residues in transmembrane S2, S3 and S4 studied in this paper are shown.

### Figure 3:

Effect of the mutation of the two outermost arginine R850 and R853 of the IIS4 segment of rat brain Na<sub>v</sub>1.2a sodium channels. (A) Current traces for Na<sub>v</sub>1.2a, R850E and R853E in control conditions elicited by a family of depolarizing pulses from -100 mV holding potentials to voltages ranging from -90 to -20 mV in 5mV step. (B) Voltage dependence of activation (B) and inactivation (C) for wild type Na<sub>v</sub>1.2a, R850E and R853E. Conductance-voltage were determined as described in materials and methods. Inactivation curves were determined using 30 ms-long prepulses to the indicated potentials followed by a test pulse to -10mV.

**Figure 4:**

Beta-scorpion toxin activity on R850E and R853E sodium channels. Conductance-voltage relationships in control (closed circles) or in the presence of 50 nM of C<sub>ss</sub> IV without (open circles) or with (open triangles) a +50 mV, 1-ms prepulse that preceded the test pulse by 60 ms for R850E (A) and R853E (C) sodium channels. Sodium current traces evoked by a test pulse to -65 mV before and after a prepulse to +50 mV in the presence of 50 nM C<sub>ss</sub> IV for R850E (B) and R853E (D) sodium channels. Traces were normalized to the peak current.

**Figure 5:**

Voltage dependence of activation of Na<sub>v</sub>1.2a sodium channels with mutations of the acidic residues of the S2 and S3 segments in domain II. (A) Current traces for E805R, D814R and E821R and D827R in control conditions elicited by a family of depolarizing pulses from -100 mV holding potentials to voltages ranging from -90 to -20 mV in 5mV step. Voltage dependence of activation (B) and inactivation (C) for wild type Na<sub>v</sub>1.2a, E805R, D814R, E821R and D827R.

**Figure 6:**

Activity of  $\beta$ -scorpion toxin on mutants sodium channels E805R, D814R, E821R and D827R. Comparison of the conductance-voltage relationships in the absence (closed circles) or in the presence of 50 nM C<sub>ss</sub> IV without (open circles) or with (open triangles) a +50 mV, 1-ms prepulse that preceded the test pulse by 60 ms for E805R (A), D814R (B), E821R (C) and D827R (D) sodium channels.

**Figure 7:**

Effect of the charge-inversion double mutants D814R/R850E, D814R/R853E, E821R/R850E and E821R/R853E of rat brain Na<sub>v</sub>1.2a sodium channels. (A) Current traces for the charge-inversion double mutants activated by depolarisations in 5-mV increments from -90 to -20 mV. Voltage dependence of activation (B) and inactivation (C) for D814R/R850E, D814R/R853E, E821R/R850E and E821R/R853E.

**Figure 8:**

Effect of the charge-inversion double mutants on C<sub>ss</sub> IV activity on rat brain Na<sub>v</sub>1.2a sodium channels. Voltage dependence of activation for D814R/R850E (A) and E821R/R850E (B) in control (closed circles) or in the presence of 50 nM C<sub>ss</sub> IV without (open circles) or with (open triangles) a +50 mV, 1-ms prepulse that preceded the test pulse by 60 ms. (C) Percentage of peak current reduction in the presence of 50 nM, 200 nM or 500 nM of toxin. Currents traces recorded in response to a test pulse to -60 mV in the absence or in the presence of 200 nM of C<sub>ss</sub> IV for wild type Na<sub>v</sub>1.2 (D), D814R/R850E (E) and E821R/R850E (F) sodium channels. Voltage dependence of activation for D814C/R850Q (G) and E821C/R850Q (H) in control (closed circles) or in the presence of 50 nM C<sub>ss</sub> IV (open triangles) with a +50 mV, 1-ms prepulse that preceded the test pulse by 60 ms.

**Figure 9:**

Effect of the charge-inversion double D814R/R853E and E821R/R853E mutants on C<sub>ss</sub> IV activity on rat brain Na<sub>v</sub>1.2a sodium channels. Voltage dependence of activation for D814R/R853E (A) and E821R/R853E (B) in control (closed circles) or in the presence of 50 nM C<sub>ss</sub> IV without (open circles) or with (open triangles) a +50 mV, 1-ms prepulse that preceded the test pulse by 60 ms.

**Figure 10:**

Schematic representation of electrostatic interactions between charged residues in segments S2, S3 and S4 in domain II of sodium channels (A) or potassium channels (B)

Figure 1

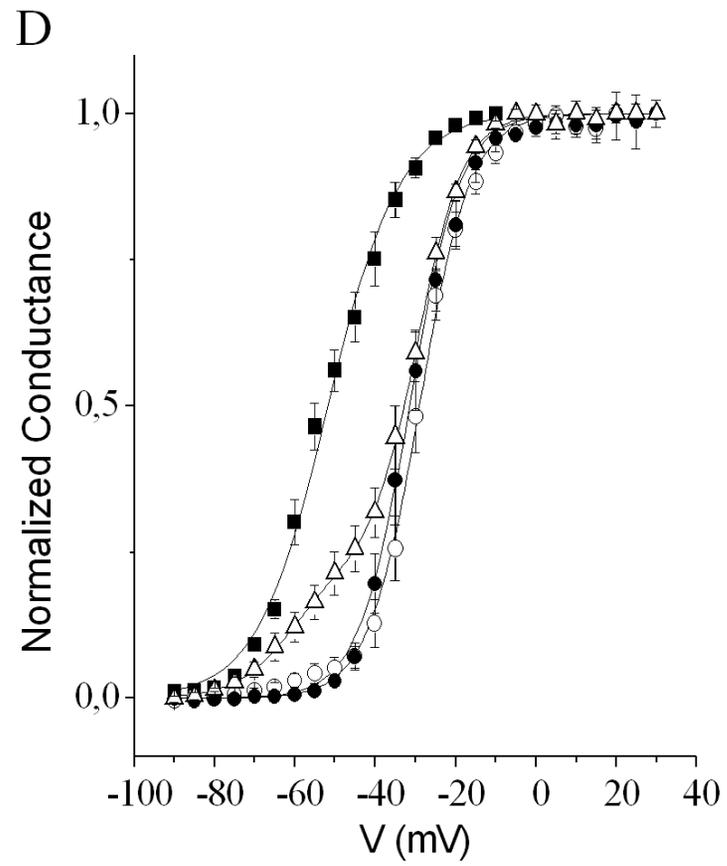
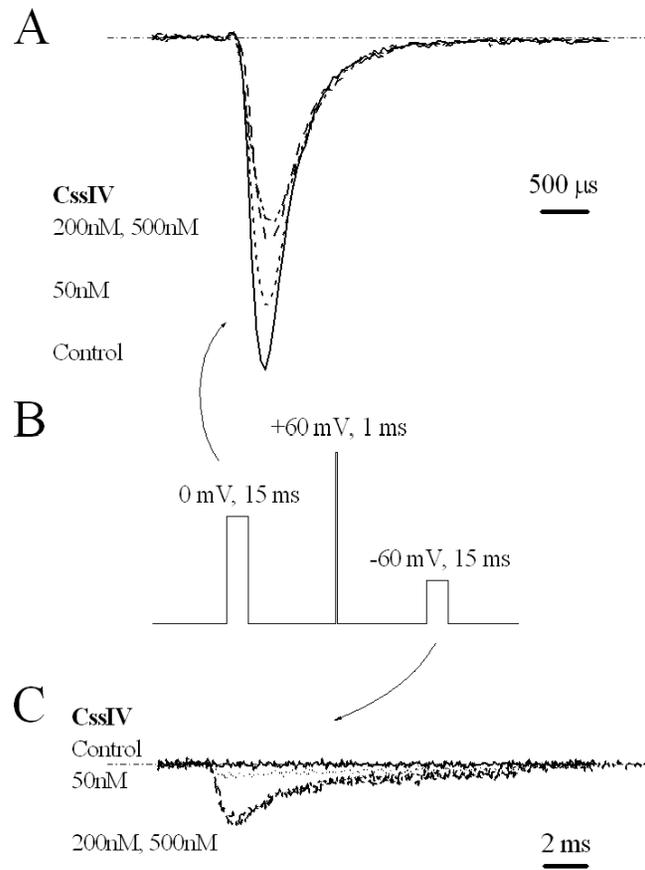


Figure 2

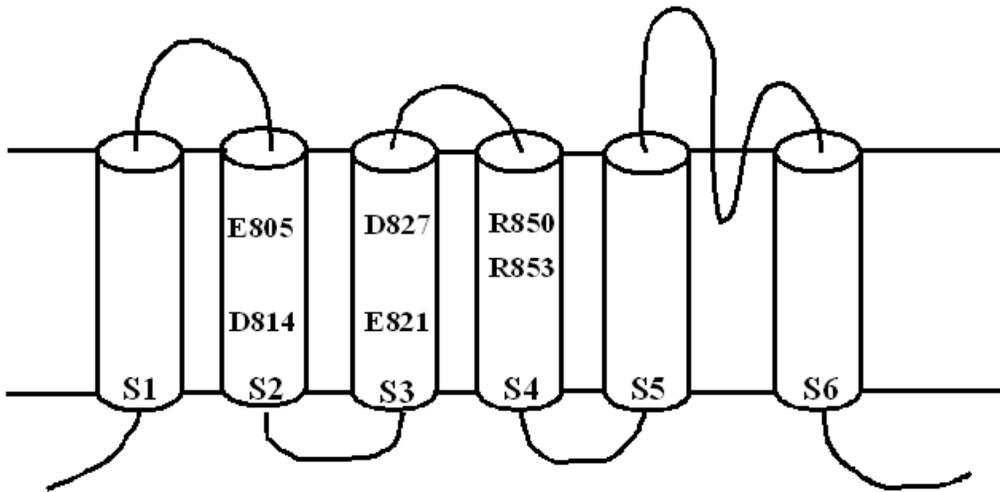


Figure 3

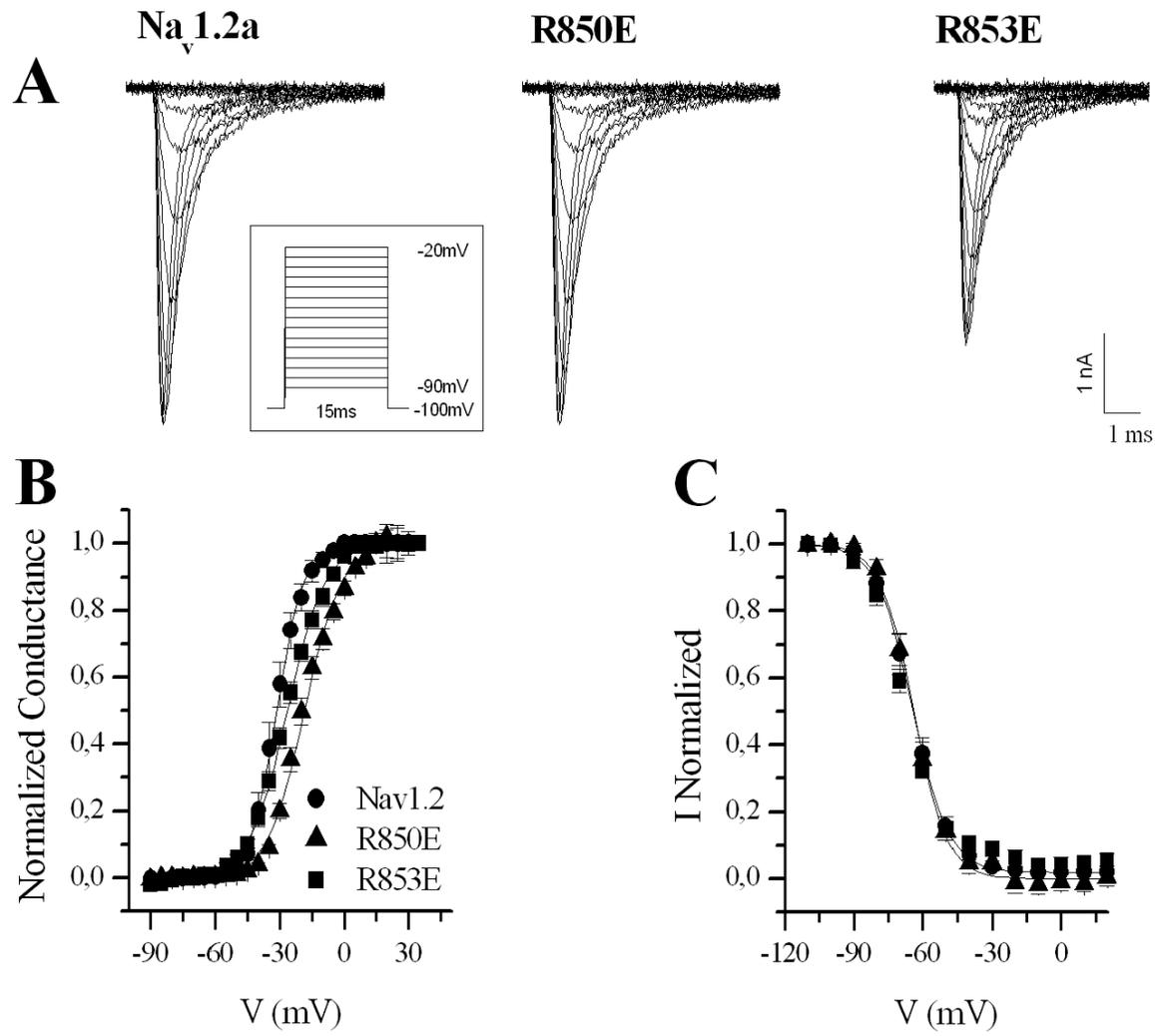


Figure 4

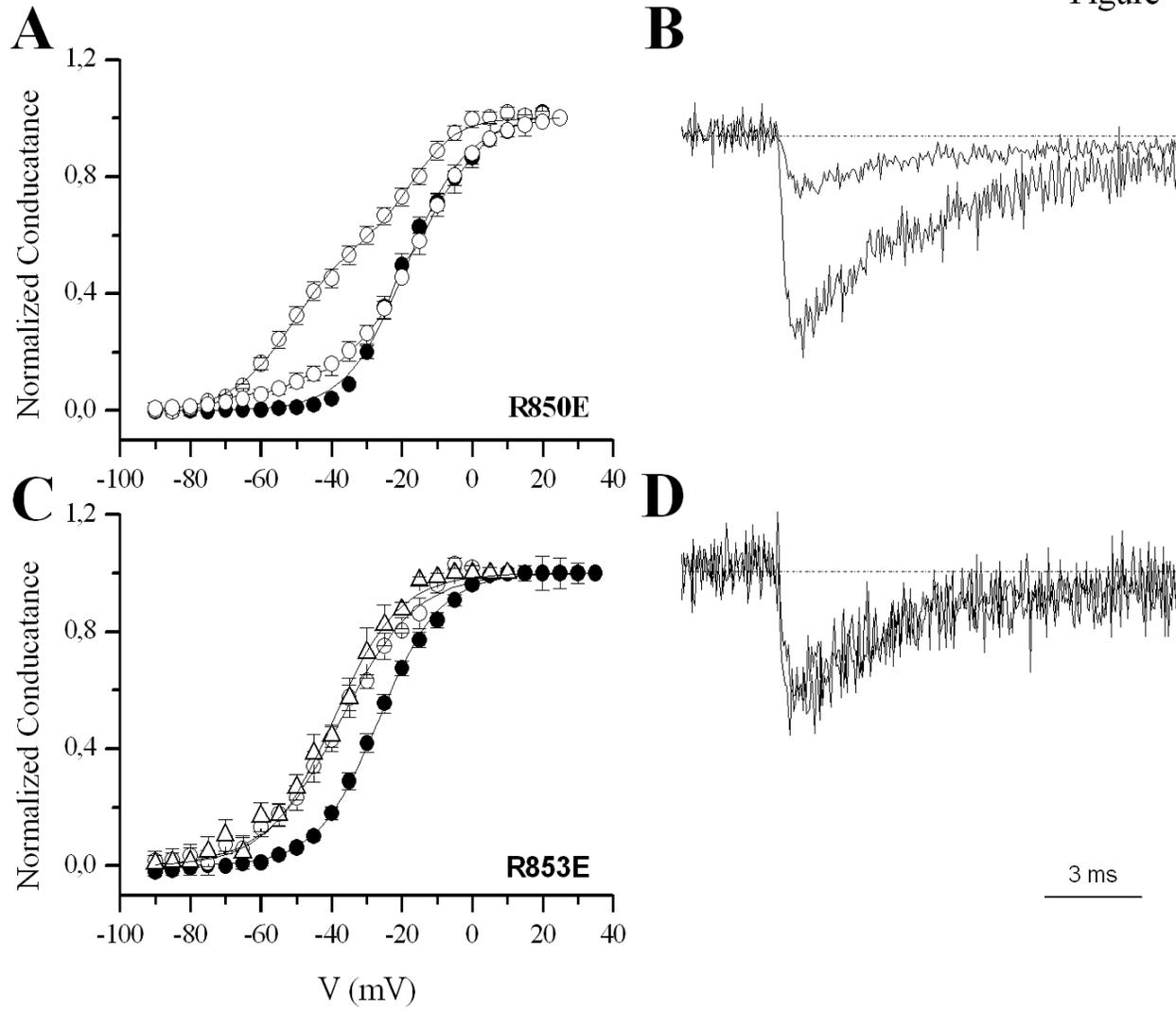


Figure 5

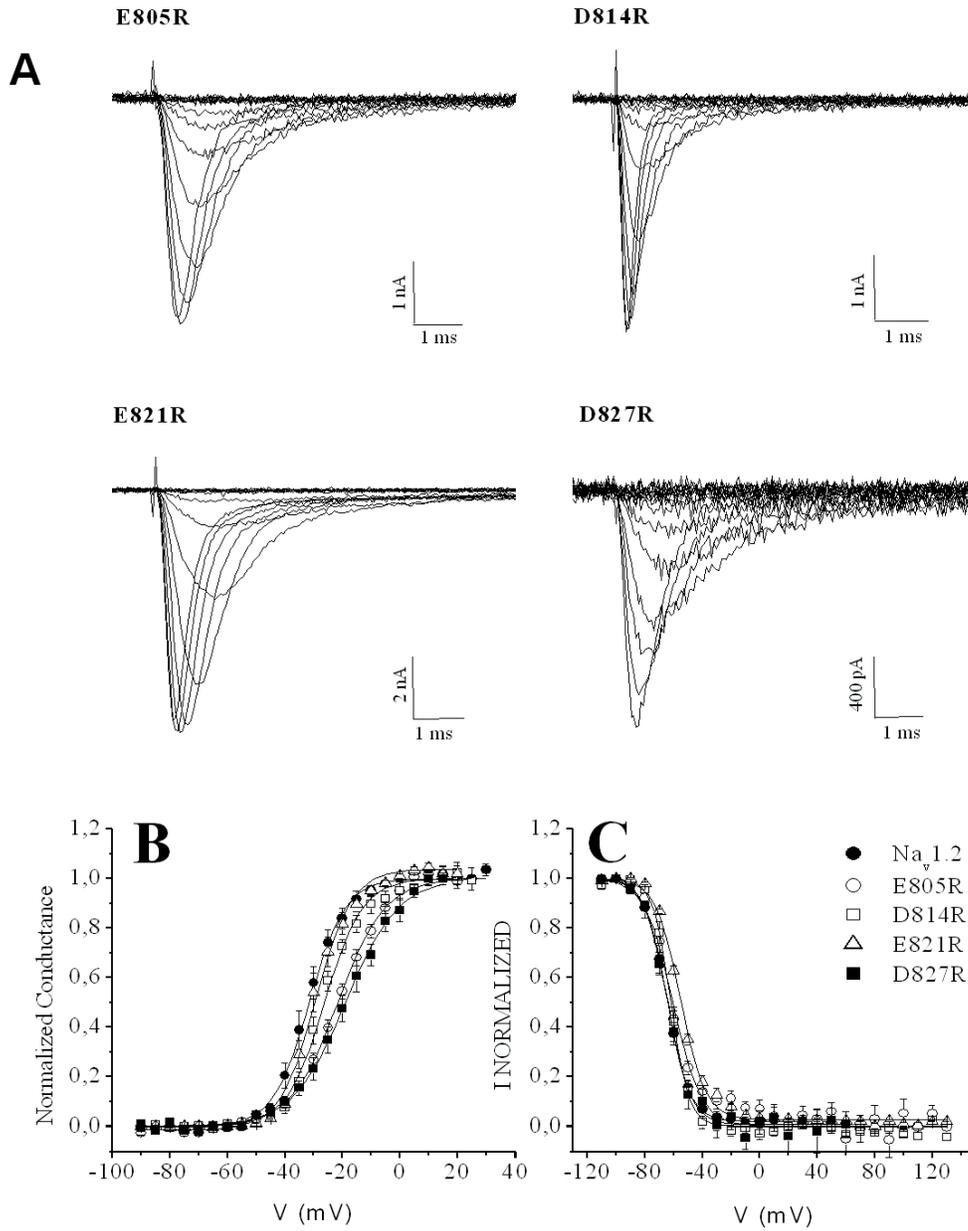


Figure 6

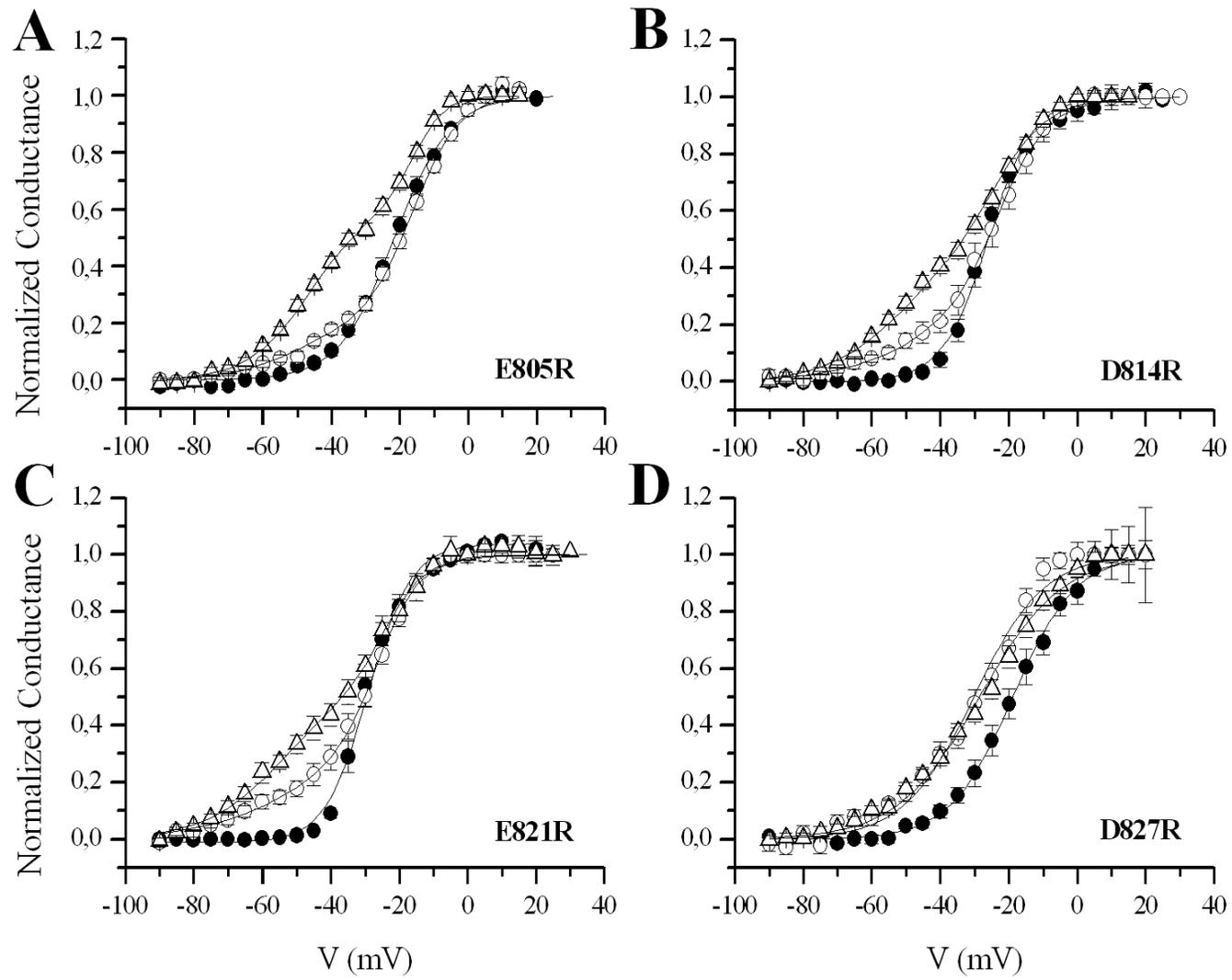


Figure 7

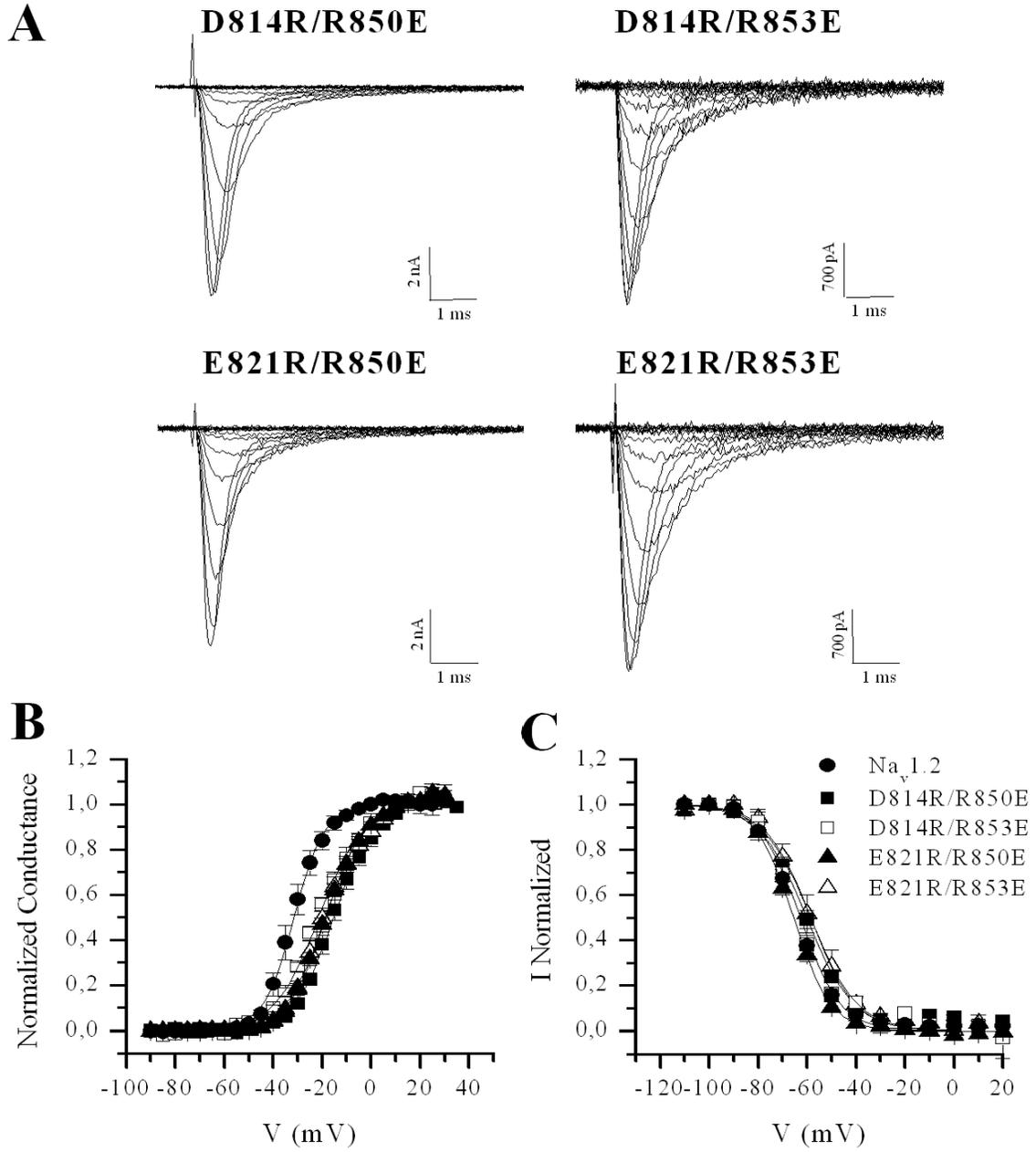


Figure 8

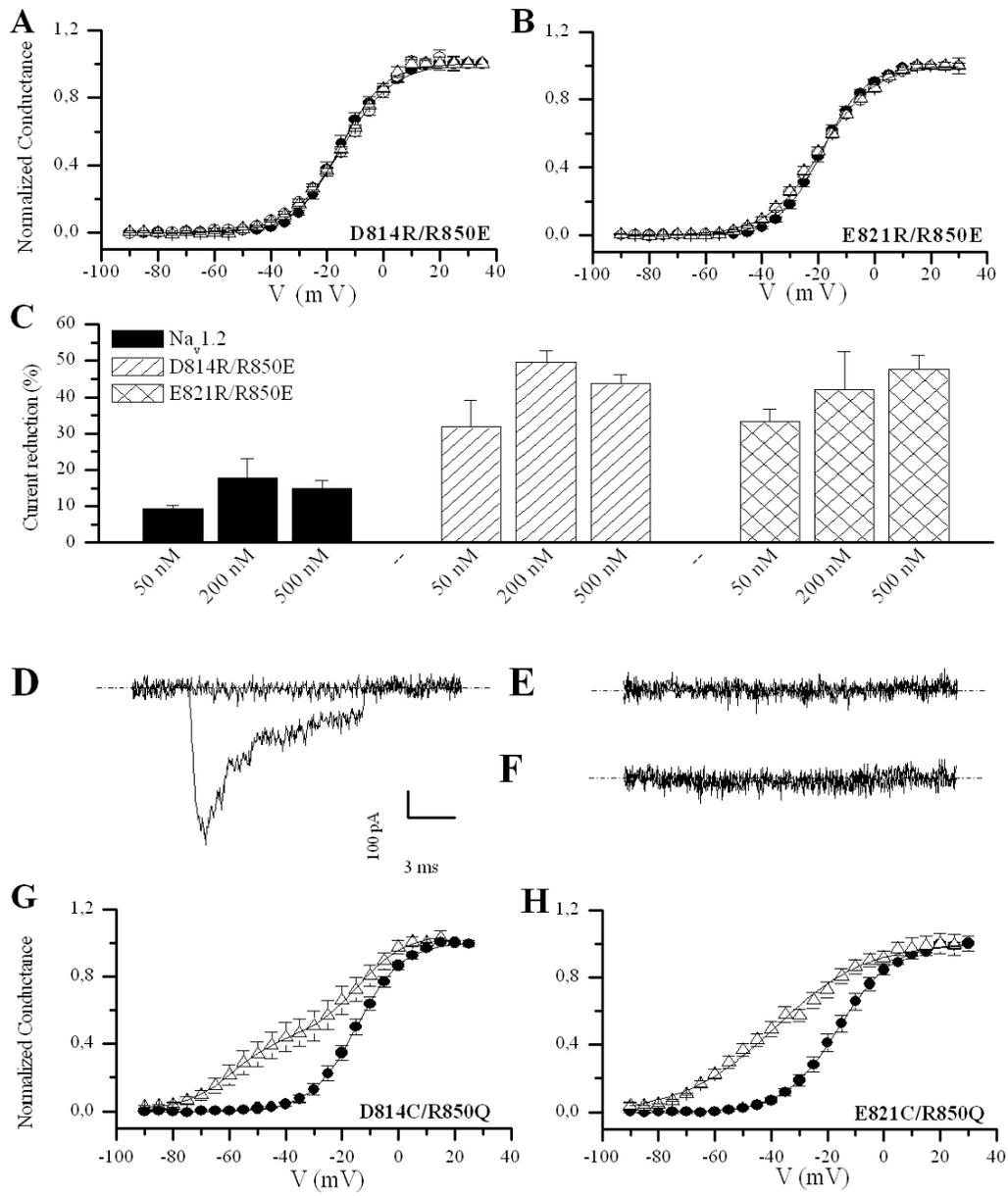


Figure 9

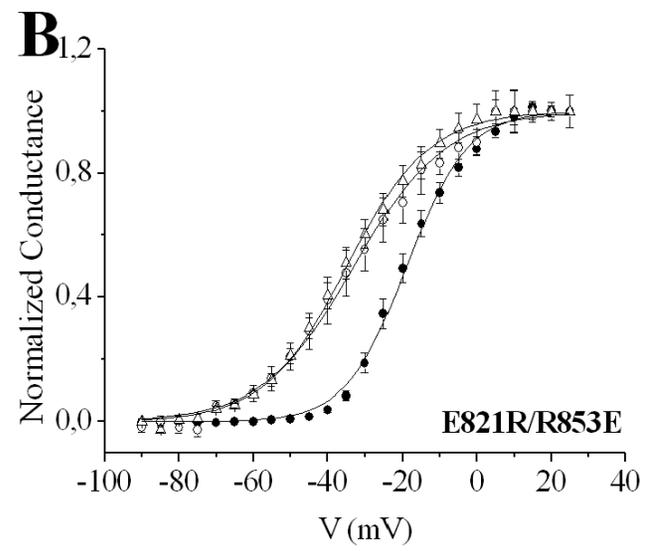
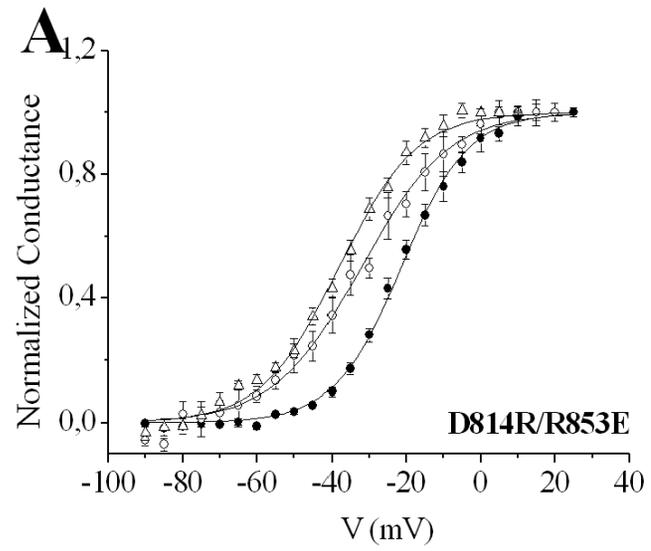


Figure 10

