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Structure-function relationship of the sodium channel rat brain
IIA

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University of Hawaii, 1993
Structure-Function Relationship
of the Sodium Channel Rat Brain IIA

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION
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Abstract

Sodium channels are voltage-gated transmembrane proteins propagating electrical signals in neurons, heart and skeletal muscle cells. Cloning and subsequent expression in heterologous systems has yielded structure-function information about wildtype and mutant Na channels. The protein's α subunit has four homologous and highly conserved domains (I-IV), each consisting of six transmembrane segments. The fourth segment (S4) of each domain carries evenly spaced and positively charged amino acids and has been proposed to function as voltage sensor. The patch clamp technique was used to investigate Na channel mutations that substitute or screen a II4 charge in excised membrane patches from Xenopus oocytes expressing channels encoded by wildtype rat brain II A (RBIIA) or single-point mutation cDNAs (K859Q or L860F). Since Na channels were expressed from singular mRNA, they are presumed to represent a single, homogeneous population. However, the initial characterization of wildtype Na currents revealed an unexpected behavior: After excision of membrane patches from the cytosolic environment, there was a unidirectional and time-dependent transition in channel inactivation from slow to fast kinetics, paralleled by alterations in voltage dependence. This suggests that a single sodium Na channel can adopt at least two distinguishable gating modes, whose equilibrium may be modified by biochemical processes. Moreover, Na channel characteristics were affected by modification of the molecular structure in the point mutations K859Q and L860F. Both mutants induce similar shifts in the current-voltage relationship and L860F affects the valence of activation. In addition, steady-state inactivation curves and kinetic rates of activation and inactivation differ considerably. These results challenge the notion that S4 segments exclusively control the activation of Na channels. Rather, it seems that specific locations within the protein may affect multiple features of Na channel function.
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I. Introduction

1. Biophysical Properties of Sodium Channels

*Background*

The sodium channel, together with the outwardly rectifying potassium channel, was one of the first voltage-sensitive channels whose biophysical properties were described in detail using the voltage clamp technique (Hodgkin & Huxley, 1952a; Hodgkin & Huxley, 1952b; Hodgkin & Huxley, 1952c; Hodgkin & Huxley, 1952d; Hodgkin et al., 1952). The sodium channel is a transmembrane protein responsible for the rapid rising phase of the action potential in electrically excitable cells like neurons, neuroendocrine and muscle cells. Like most cells, electrically excitable cells maintain high potassium and low sodium on the cytosolic side of the membrane creating a cell-specific resting potential which is maintained by an ion pumping Na\(^+\)-K\(^+\)-ATPase. The sodium channel responds to adequate membrane potential changes by conducting sodium ions down their electrochemical gradient into the cell, thereby increasing the overall membrane permeability to sodium ions on a time scale of milliseconds.

Sodium currents recorded under voltage clamp show a biphasic behavior during depolarizing voltage steps (e.g., to 0 mV) from a negative holding potential (e.g., -100 mV) (Fig. 1). Currents are characterized by a steep rising phase and a subsequent rapid decay. This behavior is due to inherent properties of Na channels, which quickly open (activate) in response to depolarization. Activated channels quickly inactivate despite maintained depolarization of the membrane. Repolarization to holding potential also closes open channels (deactivation). A
brief, rapidly decaying inward current can be detected after repolarization. This current, called 'tail current', arises from non-inactivated channels which are returning to their closed conformation. Thus, the phenotype of sodium currents reflects successive transitions of voltage-dependent and time-dependent conformational changes - or states - in the channel protein.

Careful studies of voltage-sensitivity and time course of sodium currents, together with various pharmacological treatments have suggested many hypo-

![Figure 1: Functional states of sodium channel gating](image)

Schematic diagram of voltage-dependent state transitions from closed to activated, inactivated, and closed. Channels are closed at a holding potential of -100 mV. They are rapidly activated by depolarization and inactivate despite maintained positive potentials. Repolarization restores the channels in a closed state from which they can be activated again.
eses concerning functional sodium channel structures in native systems. The channel has been postulated to have molecular structures creating a selectivity filter preferentially selective for sodium ions (Chandler & Meves, 1965; Hille, 1971; Hille, 1972), one (or more) voltage sensor(s) that register the electrical field across the membrane to gate the channel (Armstrong, 1981; Armstrong, 1992), and intracellular structures involved in channel inactivation which are sensitive to tryptic or chemical treatment (Rojas & Armstrong, 1971; Eaton et al., 1978; Starkus et al., 1993).

**Modelling sodium channel gating**

Voltage-sensitivity and time course of sodium channel gating has traditionally been described by the Hodgkin & Huxley model (Hodgkin & Huxley, 1951b; Hodgkin & Huxley, 1952a; Hodgkin & Huxley, 1952b; Hodgkin & Huxley, 1952c; Hodgkin & Huxley, 1952d; Hodgkin, et al., 1952) using a kinetic scheme of four independent first-order reactions representing four independent and identical, charged particles physically controlling channel activation and inactivation. The hypothesis inferred these charged particles to act as molecular voltage sensors, where activation would be voltage-controlled by three separate protein configuration changes, "m-gates", and inactivation by a single parallel process, the "h-gate". It was predicted that displacement of m-gates would be associated with charge movement across the membrane: at the onset of a test pulse to open the channels and after repolarization of the membrane to close the channels. It was further hypothesised that tail current kinetics would be three times faster than the closing charge movement, as all three m-gates necessary to open the channel would have to return to resting position, but only one m-gate would be enough to close the channel.
Later work succeeded in measuring capacitive currents, evoked by voltage-dependent charge movement across the membrane, and defined these transients as "gating currents" (I_g ON and I_g OFF, respectively) (Armstrong & Bezanilla, 1974; Armstrong & Bezanilla, 1975). It could be shown that depolarization of the membrane indeed caused a gating current preceding sodium influx; however, tail current kinetics were not three times faster than the OFF gating current, but in fact had a similar time course. This refuted the model of the sodium channel being gated by identically charged and independent particles. Additional evidence showed, that ON gating currents are composed of three, dissimilar kinetic components: a fast component preceding sodium influx, an intermediate and a slow component coinciding with the rising phase of macroscopic sodium currents and maximal activation of channels, respectively. This implied that sodium channels are gated by non-identical particles (Armstrong & Gilly, 1979; Starkus et al., 1981). The suggestion was that the particles act independently - but are energetically coupled - so that transitions between channel states appear to follow a quasi-sequential order (Armstrong & Gilly, 1979; Rayner & Starkus, 1989).

Energetic coupling between channel states (open, inactivated, closed) is described using the Boltzmann equation of statistical mechanics:

\[ \frac{1}{1 + \exp^{-z(V_m - V_{1/2})/kT}} \]

which gives the relative probabilities of finding a particle in state 1 (e.g., closed) or in state 2 (e.g., open) at equilibrium depending on the energy difference between these states. Thus, looking at a simplistic kinetic model of channel activation from closed (C) to open (O)

(closed) C ⇔ O (open),

4
the magnitude of gating charge needed to open the channel can be calculated from the steepness of the voltage dependence of gating, using the Boltzmann function. The transition from C to O is a conformational change which is accompanied by the movement of gating charges of the valence $z$ from the inner membrane surface to the outer. The energy at which the probability distribution between the two states is 0.5 (external energy input is absent), is the midpoint voltage $V_{1/2}$ and a measurement of the energy difference between two states. The probability distribution between the two states also depends on the external energy input $V_m$, the membrane potential. The time required to switch from an open to a closed state is determined by the energy requirement of the transition in question. The whole system is dependent on the ambient temperature $T$ (in Kelvin) and is scaled with the Boltzmann constant $k$.

**Inactivation properties**

Sodium channels not only activate and deactivate depending on voltage, they also feature an additional, mainly voltage-independent process which actively occludes the open channel. This process has been termed inactivation. The single, parallel "h-gate" of inactivation proposed by Hodgkin and Huxley (Hodgkin & Huxley, 1952c; Hodgkin & Huxley, 1952d), assumed a separate voltage sensor for channel inactivation - and thus separate voltage sensitivity of the inactivation mechanism. In 1973 Armstrong et. al. showed that sodium channel inactivation is sensitive to proteases. Exposing the cytoplasmic side of the squid giant axon to pronase removed inactivation, and activated channels stayed open for long times closing only after repolarization of the membrane to holding potential. This supported the original idea of a physically distinct and independent mechanism for inactivation. However, subsequent evidence from macroscopic
currents (Armstrong & Bezanilla, 1977; Bezanilla & Armstrong, 1977; Goldman & Kenyon, 1982) and single channel studies (Aldrich & Stevens, 1984; Cota & Armstrong, 1989) showed inactivation voltage sensitivity to be dependent and coupled to the voltage sensitivity of activation and not at all an independent process.

A "ball-and-chain" model of inactivation was developed (Armstrong & Bezanilla, 1977) postulating an inactivation gate composed of a cytosolic peptide ball and an amino acid chain connecting it to the main protein. The authors hypothesized that, as activation of the channel is accompanied by gating charge movement, it makes a "receptor site" accessible for the inactivation ball at the inner mouth of the channel pore. Subsequent binding of the inactivation gate to its receptor would occlude the pore.

The inactivation mechanism described above has to be separated from an additional phenomenon occurring in sodium channel inactivation named "slow Na channel inactivation". This kind of inactivation occurs on a time frame of at least 100 ms to several minutes (Narahashi, 1964), as opposed to the "fast inactivation" event occurring within a few milliseconds (see above). Fast and slow inactivation can also be separated pharmacologically, as treatments that remove fast inactivation (enzymes, toxins) do not remove slow inactivation (Chandler & Meves, 1970; Narahashi, 1974; Almers et al., 1983). Thus fast and slow inactivation are two independent processes which are probably controlled by different channel structures. Additional evidence supporting this hypothesis comes from cloned shaker potassium channels. Here, the two kinds of inactivation have been termed N-type and C-type inactivation, where the amino group terminus of the protein is involved with fast inactivation, while the carboxyl terminus appears to be involved in slow
inactivation processes (Choi et al., 1991; Hoshi et al., 1991). In addition, the N-type inactivation is sensitive to the K channel blocker tetraethylammonium (TEA) when applied internally. The C-type inactivation however is insensitive to internal TEA but is affected by the presence of external TEA (Hoshi, et al., 1991).

To summarize, sodium channel function is complex. Distinct functional mechanisms - activation, deactivation, fast and slow inactivation, - can be detected and separated by voltage sensitivity and/or pharmacology. To correlate those functions with respective structural entities within the channel protein is the interest of current research on sodium channels.

2. Molecular Properties of Sodium Channels

Much of our current understanding of molecular functions of membrane proteins comes from studies in heterologous expression systems. The *Xenopus* oocyte expression system was originally used to study various aspects of gene expression and control (Gurdon et al., 1971). In 1982, Miledi and co-workers reported expression of tissue-extracted mRNA from various ion channel types and receptors (Miledi, 1982; Miledi & Sumikawa, 1982). *Xenopus* oocytes have also been proven to be a reliable expression system for mRNA encoded by cDNA (Fig. 4). Using electrophysiological techniques, the system has been employed for structure-function studies not only in sodium channel but also other cloned voltage-gated and ligand-gated ion channels (e.g., potassium and calcium channels, nicotinic acetylcholine and glutamate receptors) (Mishina et al., 1984; Timpe et al., 1988; Hollmann et al., 1989; Hoshi et al., 1990; Lopez et al., 1991; Yool & Schwarz, 1991).
Molecular channel structure

Advances in molecular biological techniques made it possible to purify the Na channel protein of electric eel electroplax and deduce its nucleic acid sequence (Noda et al., 1984). Since then several sodium channels have been cloned from rat brain (RBI, RBII, RBIIA and RBIII), from rat heart (RHI) and from rat skeletal muscle (μI) (Noda et al., 1986; Salkoff et al., 1987; Auld et al., 1988; Kayano et al., 1988; Loughney et al., 1989; Ramaswami & Tanouye, 1989; Rogart et al., 1989; Trimmer et al., 1989). The α subunit of rat brain sodium channels, a glycoprotein of 260 kD, co-purifies with two β subunits of 36 kD and 33 kD. The individual α subunits alone are sufficient to yield voltage-dependent currents when expressed in heterologous systems like *Xenopus* oocytes (Noda et al., 1986; Auld, et al., 1988; Suzuki et al., 1988; Trimmer, et al., 1989) or Chinese Hamster Ovary (CHO) cells (Scheuer et al., 1990; West et al., 1992).

Hydropathy plots suggest that the Na channel protein consists of four homologous regions of 225 to 325 amino acids each (domains I to IV, Fig. 2), where highest homology is observed in the six putative transmembrane spanning segments (S1 to S6) within each domain. The fourth transmembrane segment of each protein domain carries evenly spaced basic amino acids (arginine [R] and lysine [K]) (Fig. 3), which may allow it to function as a voltage sensor. The S4 segment of sodium channels shows a high degree of conservation in the nucleic sequence both across species and within other types of voltage-dependent ion channels (e.g., calcium channels), suggesting high genetic pressure on structural features of voltage-gated ion channels (Table 1). Structure-function studies of the sodium channel by introducing judicious point mutations into the cDNA and searching for altered
biophysical properties in expressed mutant channels has indeed indicated IS4 to be the primary voltage sensor of the protein (Stühmer et al., 1989).

**Table 1: Amino Acid Sequence in S4 segments of three ion channel types**

Listed are amino acid sequences in homologous S4 segments of the potassium (K) channel and domains I to IV of sodium (Na) and calcium (Ca) channel. Shaded letters indicate positively charged amino acids (arginine [R] and lysine [K]).

<table>
<thead>
<tr>
<th>Ion Channel</th>
<th>Amino Acid Sequence (Single Letter Code)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>LA I L R V I R L V R V F R I F K L S R H S K G L Q</td>
</tr>
<tr>
<td>Na (I)</td>
<td>VS A L R T F R V L R A L K T I S V I P G L K T I V</td>
</tr>
<tr>
<td>Ca (I)</td>
<td>V K A L R A F R V L R P L R L V S V P S L Q V V L</td>
</tr>
<tr>
<td>Ca (II)</td>
<td>I S V L R C I R L L R L F K I T K Y W T S L S N L V</td>
</tr>
<tr>
<td>Na (III)</td>
<td>L G A I K S L R T L R A L R P L R A L S R F E G M R</td>
</tr>
<tr>
<td>Ca (III)</td>
<td>I S V V K I L R V L R V L R P L R A I N R A K G L K</td>
</tr>
<tr>
<td>Ca (IV)</td>
<td>E S A R I S S A F F R L F R V M R L I K L L S R A E</td>
</tr>
</tbody>
</table>

**Models of activation based on molecular channel structure**

Two major models have been proposed as to the putative gating function of the S4 transmembrane segments: the "sliding helix model" (Catterall, 1986) and the "propagating helix" model (Guy & Conti, 1990). In the "sliding helix" model, the S4 segments are proposed to have an α-helical conformation which is stabilized by ion pair formation between positive charges of the S4 segments and negatively charged amino acids from the surrounding α-helices of the S1, S2 and S3 segments. Depolarization breaks the electrostatic interaction allowing the helix to slide verti-
cally and form a new set of ion pairs. This conformation change is suggested to initiate sequential conformational changes in the other domains of the protein leading to channel activation. The "propagating helix" model proposes the S4 segment to be in part $\alpha$-helical and in part a $\beta$-sheet at resting potentials. Voltage changes induce a transition from $\alpha$-helical to $\beta$-sheet and bending of the S4 segment to move the $\alpha$-helical part across the membrane.

**Figure 2: Primary structure of the sodium channel $\alpha$ subunit**

Schematic of the sodium channel $\alpha$ subunit with homologous domains I through IV, each with six putative transmembrane spanning $\alpha$ helices. Extracellular and intracellular linker segments are shown as loops. Some regions of functional relevance of the protein have been identified: voltage sensing regions in S4 (●), putative inactivation site (●), phosphorylation sites of protein kinase A (●) and protein kinase C (●), and scorpion toxin (ScTx) binding sites. Basic, acidic and neutral amino acids are marked (●), (●) and (●) respectively; extracellular glycosylation is indicated (●).

**Correlating structure and function**

Further structure-function studies have revealed effects of mutations in the cytoplasmic linker region between domain III and IV on channel inactivation
(Stühmer et al., 1989; Moorman et al., 1990) whereas lysine$^{1422}$ and alanine$^{1714}$, located at the carboxyl end of the cytosolic linker between domain II/III and III/IV, respectively (Fig 2), alter ion selectivity in favor of calcium if changed to a negatively charged glutamic acid (Heinemann et al., 1992). Studies with antibodies suggest the same cytoplasmic linker to be involved in channel modulation by protein kinase C (PKC) (Numann et al., 1991), whereas protein kinase A (PKA) sites are located in the linker between domain I and II (Li et al., 1992) (Fig. 2).

3. Hypothesis

The structure of the fourth segment of each sodium channel domain - carrying evenly spaced basic amino acids - led to the suggestion that the S4 segments serve as voltage sensors of sodium channel activation. So far, only S4 of domain I (S4I) was demonstrated to act as voltage sensor (Stühmer et al., 1989). On the other hand, double mutations (Stühmer et al., 1989) and a neutral mutation (Auld et al., 1990) in S4 of domain II have both been shown to affect the voltage-sensitivity of activation. The techniques used to study sodium channels expressed in *Xenopus* oocytes have had major shortcomings: using the two-electrode voltage clamp left the membrane under poor voltage control thus introducing clamp-induced artifacts. This problem can be avoided through the patch clamp technique in the cell-attached conformation. However, with this method the channels are still subjected to biochemical modulation by the oocyte. The prediction is that studying sodium channels in an excised patch, a technique which allows for accurate voltage control and renders the sodium channels independent of the cell's biochemical machinery, will lead to a finer resolution of effects induced by single-point mutations in S4 and may prove this segment to be involved in channel activation.
Figure 3: Point mutations In II S4

Schematic view of the sodium channel α subunit structure emphasizing the location of the mutation K859Q and L860F in the S4 segment of domain II.
Aim and approach

The scope of this study was to use the patch clamp technique to investigate basic biophysical properties of Na channel mutations which substitute or screen II S4 charge in excised membrane patches from *Xenopus* oocytes expressing channels encoded by wildtype rat brain IIA (RBIIA) or single-point mutation cDNAs (K859Q or L860F) (Fig. 3). The presumption was that activation properties were expected to be altered if the S4 segment of domain II was involved in the voltage-sensing process. Indeed, the findings of this study show that the two mutations K859Q and L860F induce congruent shifts in the current-voltage relationship and L860F affects the steepness of activation (Chapter IV). In addition, steady-state inactivation curves and kinetic rates of activation and inactivation differ considerably. Further results, as described in Chapter III, suggest that a single sodium Na channel can adopt at least two distinguishable gating modes, whose equilibrium is modified by biochemical processes. The results of this study challenge the notion that S4 segments exclusively control the voltage dependence and kinetics of Na channel activation. Rather, it seems that specific locations within the protein may affect multiple features of Na channel function.
II. Methods

1. Molecular Biology

Sodium channel cDNA from rat brain IIA and the mutants K859Q and L860F served as template for standard *in vitro* transcription to yield mRNA for microinjection in *Xenopus* oocytes (Fig. 4). *Escherichia coli* strain JM109 was transformed with the plasmids pVA2580, K859Q or L860F (kindly provided by Alan L. Goldin) following standard CaCl₂ transformation procedures (Sambrook et al., 1989). The plasmids (Bluescript KS+) carry full-length sodium channel cDNA as inserts. The inserts are flanked by T7 and SP6 mRNA polymerase promoters for *in vitro* transcription using T7 or SP6 mRNA polymerase. Large quantities of cDNA were obtained growing single, successfully transformed *E. coli* colonies in Terrific Broth (per liter: 12 g Bacto-tryptone, 24 g Bacto-yeast extract, 4 ml glycerol, 0.17 M KH₂PO₄ and 0.72 M K₂HPO₄). Plasmid DNA extraction was achieved by following standard alkaline lysis with PEG purification (Sambrook et al., 1989). Concentration of plasmid DNA was controlled spectrophotometrically and the final concentration of DNA was adjusted to 1 μg/μl. To prepare plasmid DNA for *in vitro* transcription the DNA was linearized with the restriction enzyme ClaI (Sambrook et al., 1989). RBIIA and K859Q cDNA could be transcribed using T7 mRNA polymerase, whereas SP6 polymerase had to be used for L860F due to the inverse orientation of the cDNA inserts in the plasmid. *In vitro* transcription followed a standard protocol according to the transcription kit used (Promega). The final concentration of mRNA could not be determined exactly, as no radioactive labeled ³²P-CTP could be used in this study. For this reason, the original concentration of cDNA of 1 μg/μl served as reference point. The final concentration of mRNA,
however, depended in part on degradation levels by RNAase and successful incorporation of the CAP GpppG necessary for in vivo translation in the *Xenopus* oocyte. mRNA was stored at -20 °C for one day maximally before it was injected into oocytes. Long term storage was done at -80 °C to avoid degradation of mRNA.

2. Oocyte Expression System

Oocytes for mRNA injection were obtained from laboratory-reared female *Xenopus laevis* (Nasco Biologicals). The frogs were anesthetized in 0.1% tricaine removal of ovaries

![Diagram of the main experimental procedures](image)

**Figure 4: Methods**

Schematic of the main experimental procedures employed in heterologous expression of sodium channels in *Xenopus* oocytes. For details see text.
methanesulfonate (Sigma) for at least half an hour before aseptically removing small portions of the ovaries. To isolate the cells and remove the follicular layer, oocytes were enzymatically treated by bathing them for 1 to 2 h in Ca\(^{2+}\)-free oocyte-Ringer (OR-2) containing (in mM): NaCl 82.5, KCl 2, MgCl\(_2\) 1, Hepes 5, pH 7.5, supplemented with 2 mg/ml collagenase (Boehringer-Mannheim). Individual oocytes, bathed in Ca\(^{2+}\)-free OR-2, were injected with 46 nl of mRNA-containing solution using an automatic nanoinjector (Drummond Scientific). Afterwards, the oocytes were incubated at 16 °C. Na channel expression was assessed by two-electrode voltage clamp and proved sufficient for macro-patch recordings between days four and seven after microinjection of mRNA. To prepare oocytes for patch clamping, their vitelline membrane was manually removed after exposure to a hyperosmotic solution containing (in mM): K-glutamate 200, KCl 20, MgCl\(_2\) 1, EGTA 10, Hepes 10, pH 7.4.

3. Two-Electrode Voltage-Clamp

Expression testing of oocytes was performed using a conventional two-electrode voltage clamp (TEV; Dagan). In the voltage clamp, the membrane potential is held constant while the membrane permeability is changing. The transmembrane potential is measured as the difference between an intracellular voltage electrode and a reference electrode in the bath. A feedback amplifier is used to adjust for the voltage changes caused by conductance changes such as to keep the voltage at the desired value. This is accomplished by current injection through a second intracellular electrode from the output of the feedback amplifier. The voltage-clamp amplifier was controlled by a PC 386 using the data acquisition program Clampex (Axon Instruments). A TL1 DMA interface (Axon Instruments) con-
verted analog and digital signals. A test pulse in the form of a ramp with gradual potential changes from a holding potential of -100 mV to +100 mV applied over 200 ms was used to determine levels of channel expression. Fine-tipped microelectrodes were fabricated on a horizontal puller (Sutter) and had resistances of 7-10 MΩ for the current electrode and 10-20 MΩ for the voltage electrode when filled with 3 M KCl.

4. Patch-Clamp Technique

The patch clamp amplifier uses a current-voltage converter circuit to convert currents at the tip of a single, wide-tipped glass electrode to an analog voltage signal which is then available for further processing (Hamill, et al., 1981). Patch electrodes (aluminosilicate, Sutter Instruments) were prepared essentially as described (Corey & Stevens, 1983; Rae & Levis, 1984). Electrodes were pulled in five stages to result in pipette tips with resistances between 0.8 and 1.5 MΩ. Pipette tips were coated with Sylgard (Dow Corning) to reduce capacitative noise and heat-polished on a microforge (Narishige) to smooth the edges of the tips. The pipette solution contained (in mM): NaCl 96, KCl 4, CaCl₂ 1.8, MgCl₂ 1, Hepes 5, pH 7.4. The bath solution contained (in mM): NaCl 9.6, KCl 88, CaCl₂ 1, MgCl₂ 1, EGTA 11, Hepes 5, pH 7.4.

The inside-out patch configuration was used to record macroscopic sodium currents in excised patches from Xenopus oocytes. To this end, patch pipettes were abruptly pulled back from the oocyte membrane after a seal in the gigohm range between the pipette and the cell membrane of the oocyte was formed (Fig. 4). The tip of the glass electrode was brought as close to the bath surface as possible to minimize capacitive currents.
5. Data Acquisition and Analysis

Oocytes which had at least 2 µA whole-cell current were chosen for patch-clamp recording. Data acquisition was made with an EPC-9 patch-clamp amplifier controlled by an ITC-16 interface (Instrutech) and a Macintosh Quadra 700 computer running Pulse software (HEKA). All experiments were performed at room temperature (20-24 °C). Data are from three to seven oocytes out of at least two separate injection batches and two to five patches were obtained from each oocyte. All data records were acquired by averaging four sweeps and low-pass filtered with a Bessel filter at a cutoff frequency of 11.3 kHz (-3 dB). A P/4 procedure was used to subtract linear leak and capacitive currents (Armstrong & Bezanilla, 1974). Throughout, the number n refers to patches unless indicated otherwise. Statistical significance was evaluated by Student's t-test or paired t-test, where appropriate.

To analyse the voltage dependence of sodium currents, peak values of current records were determined by twelfth order polynomial fits with the analysis program PulseFit (HEKA) to compensate for variances introduced by source noise (originating from the amplifier, the pipette holder or pipette and patch). Data were then exported and further processed with the analysis program IGOR (WaveMetrics). To determine the current-voltage I(V) relationship, peak currents of the activation protocol were plotted as a function of voltage and fitted to the sum of a linear term and a Boltzmann function:

\[ g_{\text{max}} \cdot (V_m - E_{Na}) \cdot (1/1 + \exp^{-z(V_m - V_{1/2})/kT}) \]

where \( g_{\text{max}} \) is the maximal conductance, \( V_m \) the test potential, \( E_{Na} \) the reversal potential, \( z \) the valence, \( V_{1/2} \) the voltage at half-maximal activation, \( k \) the Boltzmann constant, and \( T \) the absolute temperature (Fig. 5).
Figure 5: Current-voltage relationship of sodium currents

Peak Na currents are plotted as a function of voltage. The I(V) curve was fitted using the product of a Boltzmann function and a linear term to estimate half-activation $V_{1/2}$, valence $z$, maximal conductance $g_{max}$, and reversal potential $E_{Na}$.

Figure 6 illustrates how channel activation $F(V)$ and steady-state inactivation $s_{\infty}$ were assessed. Stimulation protocols to determine the voltage dependence of channel activation were composed of a 200 ms fixed prepulse to -150 mV to remove residual steady-state inactivation, followed by a 10 ms test pulse to variable potentials (-60 or -80 mV to +60 mV in 10 mV steps). Protocols for steady-state
inactivation had variable prepulse potentials of 200 ms followed by a test pulse of
10 ms to a fixed test potential of 0 mV. Prepulse potentials sufficient to define the
voltage dependence of $s_\infty$ ranged from -160 to -30 mV. The conductance at specific
potentials was calculated according to:

$$g = I / (V_m - E_{Na})$$

where $g$ is conductance, $I$ is current, $V_m$ is membrane potential, and $E_{Na}$ the reversal potential. The values of conductance $g$ obtained in this way were normalized to

![Inactivation and Activation Diagram](image)

Figure 6: Activation and inactivation properties of sodium currents

The stimulation protocol for obtaining activation and inactivation curves is shown schematically. For activation curves ($F(V)$), peak Na currents are plotted as a function of the test pulse voltage. For inactivation curves ($s_\infty$), peak Na currents are plotted as a function of the prepulse voltage. The curves were fitted using a Boltzmann function to estimate half-activation/inactivation voltages $V_{1/2}$ and valences $z$. 

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the maximal value $g_{\text{max}}$ and plotted against voltage to obtain the $F(V)$ relationship. $F(V)$ curves and peak currents of the steady-state inactivation protocol were subjected to a similar analysis and subsequently fitted by a Boltzmann equation of the form:

$$1 + \exp^{-z(V_m - V_{1/2})/kT}$$

Kinetic analysis of Na currents was performed to determine time constants and their respective intercepts for activation ($a$) and inactivation ($h_1$, $h_2$). The rising

![Figure 7: Kinetic analysis of sodium currents](image)

To estimate the kinetic parameters of activation ($a$) and inactivation ($h$), sodium currents were fitted by the sum of three exponential functions. In this example, RBIIA Na current was elicited by a test pulse of -10 mV (grey trace). Individual exponential time courses are indicated by black lines. Inactivation is described by at least two exponentials with time constants of $\tau_{h1} \sim 100 \mu$s and $\tau_{h2} \sim 2$ ms.
phase and the biphasic current decay were approximated by the sum of three exponential functions fitted to current traces according to:

\[ (A_a \cdot \exp \frac{-t}{\tau_1}) + (A_{h1} \cdot \exp \frac{-t}{\tau_{h1}}) + (A_{h2} \cdot \exp \frac{-t}{\tau_{h2}}) + A_{\text{offset}} \]

where \( A \) equals the respective intercepts of the time constants \( \tau \), \( t \) is time, and \( A_{\text{offset}} \) the baseline (Fig. 7). Na currents were categorised into fast or slow inactivation gating depending on the relative amplitude of the intercept \( \tau_{h2} \) and peak current. Amplitude ratios \( h_{2/\text{peak}} \) of <0.4 and >0.6 were assigned to fast or slow kinetic behavior, respectively.
III. Kinetic Mode Switch of Sodium Channel RBIIA

1. Introduction

Sodium channels are voltage-gated transmembrane proteins responsible for propagation of electrically encoded signals in neurons, heart and skeletal muscle cells. In native membranes, sodium channel properties have been studied in detail using the voltage-clamp method in different arrangements like axial wire, two-electrode voltage clamp, and patch clamp. The results of this work allow for the current concept of three functional states the channel can occupy: closed, activated and inactivated. Typical gating behavior of macroscopic sodium currents upon depolarizing voltage steps reflects the successive transition of voltage- and state-dependent conformational changes in the protein. Voltage-sensitivity and time course of transitions vary with the preparation but typically both activation and inactivation are nearly complete within a few milliseconds. In some cells channel inactivation was found to be much delayed (Patlak & Ortiz, 1985; Patlak & Ortiz, 1986; Huguenard et al., 1988), indicating that Na channels can adopt different gating modes. Furthermore, modulation of inactivation can be induced experimentally by diverse agents (Armstrong et al., 1973; Vassilev et al., 1989; Starkus, et al., 1993), excising the channels from the cellular environment (Nilius, 1988; Kirsch & Brown, 1989), or enzymatic regulation by kinases (Numann et al., 1991).

Advances in molecular biological techniques made it possible to purify the Na channel protein and deduce its nucleic acid sequence (Noda et al., 1984; Noda et al., 1986; Salkoff et al., 1987; Auld et al., 1988; Kayano et al., 1988; Loughney et al., 1989; Ramaswami & Tanouye, 1989; Rogart et al., 1989; Trimmer et al., 1989). In rat brain sodium channels I, II, III and IIA the α subunit, a glycoprotein of 260 kD,
co-purifies with two \( \beta \) subunits of 36 kD and 33 kD. The individual \( \alpha \) subunits alone are sufficient to yield voltage-dependent currents when expressed in heterologous systems (Noda et al., 1986; Auld et al., 1988; Suzuki et al., 1988; Trimmer et al., 1989). Measured in Xenopus oocytes with TEV or cell-attached macro-patch the channels show unusually slow inactivation rates as compared to channels induced by injection with poly (A\(^+\)) rat brain mRNA (Auld et al., 1990) or co-expression of the \( \alpha \) and \( \beta 1 \) subunit (Isom et al., 1992; Patton et al., 1993). However, channel gating in Chinese hamster ovary (CHO) cells expressing only the \( \alpha \) subunit is comparable to native channel behavior under whole-cell conditions (Scheuer et al., 1990; West et al., 1992).

Macroscopic Na currents in excised macro-patches with inside-out configuration from Xenopus oocytes expressing rat brain IIA (RBIIA) were investigated. Under cell-free conditions, a unidirectional and time-dependent transition in channel inactivation from slow to predominantly fast kinetics was observed, caused by a change in the equilibrium between at least two distinguishable gating modes. The fast inactivation rates in excised macro-patches compare to channel behavior in native systems. Thus the unusual slow inactivation found in TEV experiments and cell-attached macro-patches may be due, in part, to modulatory effects of the oocyte system on Na channels.

2. Results

*Patch excision induces transition from slow to fast gating kinetics*

Figure 8 illustrates a typical example of kinetic alterations in Na currents recorded from inside-out macro-patches excised from Xenopus oocytes expressing
the α subunit of RBIIA. Initially, immediately after patch excision, families of Na currents evoked by depolarizing voltage steps showed fast activation followed by a rather slow inactivation over a period of several milliseconds. Subsequent application of the same voltage protocol to the same patch induced Na currents with gradually changing kinetics, yielding apparently accelerated inactivation. In this patch, the transition from slow to fast inactivation gating was virtually complete after 18 min. An increase in maximum peak current could be observed in this and other experiments (4 of 10 patches).

Figure 8: Macroscopic sodium currents in oocyte excised macro-patches

Currents from oocytes injected with RBIIA mRNA at time zero (upper panel) and 18 min. after patch excision (lower panel). Time zero designates the first current family, acquired immediately after patch excision. Holding potential was -100 mV. A conditioning prepulse to -150 mV for 200 ms preceded a 10 ms test pulse to remove slow inactivation. Test potentials ranged from -60 mV to +60 mV (upper panel) and from -80 mV to +60 mV (lower panel). Test pulse frequency was 0.5 Hz.

Kinetic analysis of sodium currents revealed that in all patches examined the inactivation time course was composed of at least two distinguishable compo-
ments that could be approximated by double-exponential functions with time constants differing by about tenfold (e.g., $\tau_{h1} = \sim150$ $\mu$s and $\tau_{h2} = \sim2$ ms, see also Fig. 12). Kinetic modulation, as shown in Fig. 8, involves a relative decrease in the inter-

![Graph A]

![Graph B]

**Figure 9: Time-dependence of the modal transition in excised patches**

(A) Each data point represents the peak amplitude (open symbols) and slow component intercept (filled symbols) of currents at a test potential of -10 mV (corresponding symbols represent data from one patch). Peak current amplitudes were corrected for background current. The amplitudes for the slow component were derived from three-exponential fits. All amplitudes in a given patch were then normalized relative to the respective amplitude determined immediately after patch excision. (B) The raw data traces represent currents evoked by test pulses to -10 mV, recorded from the same patch at time zero and 18 min after patch excision.
cept of the time constant $h_2$ normalized to the measured peak current for each data trace. Depending on the relative amplitude of intercept $\tau_{h_1}$ and peak current, Na currents with amplitude ratios $h_2/\text{peak}$ of <0.4 and >0.6 will be referred to as showing predominantly fast or slow kinetic behavior, respectively. Figure 9 depicts the time-dependent change of $\tau_{h_1}$ and $\tau_{h_2}$ component amplitudes in four patches. The slow amplitude decreased to about 20% of its original value within 10 to 30 min (8 of 10 patches), while peak current increased slightly (4 of 10 patches) or remained stable. In 2 patches the $h_2/\text{peak}$ ratio remained virtually unaltered over time.

The relative proportion of initial fast and slow intercepts could vary between patches. The proportion of patches that started off with predominantly slow kinetics was 70% (7 of 10 patches). Consecutively drawn patches from the same oocyte could have either fast or slow characteristics from the start, with no predictable pattern. However, in no case there was evidence for conversion from macroscopic fast into slow kinetic behavior.

**Slow and fast Na currents differ in voltage-sensitivity**

Next, the current-voltage relationships as well as the voltage-dependence of activation and steady-state inactivation from Na currents showing predominantly fast or slow gating kinetics were assessed. Figure 10 illustrates that channel activation and peak amplitude of slow gating is shifted to more positive potentials by 9 mV as compared to fast (Fig. 10A), whereas neither the reversal potentials for fast and slow kinetics (+45 ± 3.3 mV and +48 ± 2.2 mV) nor the valences (3.2 ± 0.27 and 3.5 ± 0.25) differ significantly (means ± S.D., n = 5 and 7, respectively). Figure 10B shows the steady-state activation and inactivation characteristics of fast and slow mode Na currents. The peak currents have been normalized for maximal conduc-
Figure 10: Voltage dependence of activation and steady-state inactivation

(A) Normalized mean peak amplitudes are plotted against test potential with corresponding mean I(V) fits of fast (●) and slow (○) modes. Midpoints of activation are $V_{1/2(1)} = -34 \pm 3 \text{ mV}$ (mean ± S.D., $n = 5$) and $V_{1/2(2)} = -25 \pm 2 \text{ mV}$ (mean ± S.D., $n = 7$) [P <0.005]. Valences $z$ differ slightly with 97% certainty where $z_1 = 3.2$ and $z_2 = 3.5$.

(B) Data represent the normalized mean peak amplitudes of fast (●, ■) and slow mode (○, □). F(V) data were determined from peak amplitudes and plotted as a function of variable test pulses (preceded by prepulses to -150 mV for 200 ms). Half-maximal open probabilities of channels are $V_{1/2(1)} = -35 \pm 7 \text{ mV}$ (mean ± S.D., $n = 5$) and $V_{1/2(2)} = -25 \pm 5 \text{ mV}$ (mean ± S.D., $n = 7$) [P <0.001]. The $s_{\infty}$ data were determined from a constant test pulse to 0 mV, plotted as a function of variable prepulses (200 ms duration). Boltzmann fits yielded half-maximal availabilities of channels of $V_{1/2(1)} = -102 \pm 5 \text{ mV}$ (mean ± S.D., $n = 5$) and $V_{1/2(2)} = -78 \pm 7 \text{ mV}$ (mean ± S.D., $n = 3$). These parameters were used to compute the fits in the graph.

(C) Kinetics of Na currents evoked by test pulses to 0 mV from holding potentials of -100 mV (a) and -150 mV (b). See text for details.
tance and expressed as fraction of open channels $F(V)$. When compared to fast Na currents, half-maximal activation and inactivation of slow currents are shifted to the right along the voltage axis by 10 mV and 24 mV, respectively ($n = 5-7$).

**Holding potential can be used to select for slow gating channels**

The data presented in Fig. 10B show that the $s_{in}$ curve for fast gating channels lies about 20 mV to the left of the $s_{in}$ curve for slow gating channels, so that a significant selective suppression of fast gating channels can be expected at holding potentials more positive than about -130 mV. Thus, although excised patches were held at -100 mV (see Methods) all protocols were preceded by a 200 ms conditioning pulse to -150 mV, to simulate holding at that potential. Fig. 10C demonstrates the effects of this procedure on the kinetics of test pulse sodium currents. Trace $a$ shows the current recorded following a direct step from -100 mV holding potential to 0 mV test potential. Three exponential fitting shows that this current is predominantly slow, with no detectable $\tau_{h1}$ kinetic. By contrast, trace $b$ shows a current from this same patch obtained following a 200 ms prepulse to -150 mV. This unscaled record shows an almost two-fold larger peak current and a marked $\tau_{h1}$ component. However, the $h2$ intercept is essentially identical to the peak current seen in trace $a$. Prepulses to potentials between -150 and -100 (data not shown here) demonstrate the progressive suppression of the fast inactivating channels at these intermediate potentials.

This finding clarifies the difference between the fast inactivation kinetics seen in this study and the predominantly slow inactivation rates reported for rat brain IIA channels expressed in *Xenopus* oocytes by Auld et al. (1988, 1990), using
a holding potential of -100 mV but without conditioning hyperpolarizing prepulses.

*Equilibrium changes between gating modes do not affect time constants*

The question occurred whether the observed shifts in voltage-sensitivity and kinetics could be due to two discrete modal states of Na channels. To this end, a concurrent three-exponential analysis of fast and slow current traces was performed to assess the time constants of the rising phase $\tau_a$ and the two decaying phases $\tau_{h1}$ and $\tau_{h2}$ (see Methods).

![Figure 11: Three-exponential fits to fast and slow sodium currents](image)

Examples of current records at time zero and after 18 min after patch excision. Holding potential was -100 mV, a 200 ms prepulse to -150 mV preceded the test pulse to -10 mV. Superimposed curves are three-exponential fits to the data. For average values of time constants see text.
The traces in Figure 11 illustrate currents evoked by test pulses to -10 mV and are identical to the records shown in Figure 9. Besides the peak current augmentation and the reduced h₂ intercept of the slow inactivation, no kinetic variances can be detected at this test potential. Time constants as measured in fast (f) or slow (s) currents, did not show statistically significant differences in activation (τₐ) or inactivation (τ₁,τ₂) at -10 mV. For this potential, activation time constants were τₐ(f) = 103 ± 17 μs and τₐ(s) = 124 ± 35 μs (means ± S.D., n = 5 and 7, respectively). Inactivation time constants for fast currents were τ₁(f) = 144 ± 23 μs and

Figure 12: Voltage dependence of activation and inactivation time constants

Fast mode (●) and slow mode (○) time constants for activation τₐ and the two components of inactivation τ₁ and τ₂ as a function of test potential. Each point represents values of mean time constants ± S.E.M. of fast and slow Na currents (7 and 3 current families, respectively). Note the different time scales of τ₁ and τ₂.
\[ \tau_{h2(0)} = 2.1 \pm 1.2 \text{ ms}, \text{ and for slow currents they were } \tau_{h1(s)} = 134 \pm 39 \mu\text{s and } \tau_{h2(s)} = 3.7 \pm 1.2 \text{ ms}. \]

To determine whether this result applies to the entire voltage range of inward currents, traces at different test potentials were analyzed. Figure 12 compares the voltage-dependence of each respective time constant. Overall, comparison of fast and slow gating rates did not reveal significant differences. The apparent difference of the time constant \( \tau_{h2} \) at test potentials more negative than 0 mV may be due to differences in the F(V) characteristics; at negative potentials slow kinetic amplitudes are less prominent (see Fig. 10B) and expected to render larger variations. On the other hand, analysis of a higher number of patches, recorded at higher bandwidths than used in this study, may prove \( \tau_{h2} \) to be statistically significant. It could be that currents activate slightly faster in fast mode than in slow mode, although this point cannot yet be substantiated by statistical evaluation.

*Amplitudes of slow mode intercepts are voltage dependent*

Presuming that inactivation occurs primarily only in open channels, i.e., as a sequential process (Armstrong & Bezanilla, 1977; Goldman & Kenyon, 1982; Zagotta et al., 1990), then peak current is scarcely affected by inactivation. Hence the \( h_2 \) intercept should reflect peak current of the slow mode channel fraction. The intercepts of \( h_2 \) in a total of 10 current families (from 5 patches) that showed a sizeable slow component were determined, to assess a possible voltage-dependence of the slow gating mode, regardless of whether the data traces had been classified as fast or slow currents. Within each current family, the amplitudes of \( h_2 \) at a given potential were normalized to the peak amplitude of the \( h_2 \) intercept. It can be seen
Figure 13: Current-voltage relationship of the slow inactivating component $\tau_{h2}$

Intercepts of $\tau_{h2}$ (●) as derived from three-exponential fits (10 current families) and current-voltage relationship of slow mode Na currents (○, identical to the data set in Fig. 3A) plotted as a function of test potential. Amplitudes of $h_2$ were normalized to the maximum amplitude.

that the amplitude of $h_2$ reaches a peak at -10 mV and decreases again (Fig. 13), closely paralleling the I(V) relationship seen for slow Na currents.

3. Discussion

Macroscopic sodium currents in excised patches from Xenopus oocytes injected with the α subunit of rat brain Na channel IIA show time-dependent and unidirectional transitions between at least two gating modes. Current-voltage relationship of activation, fraction of open channels as well as steady-state inactivation are modified during such transitions. It seems likely that the equilibrium between
at least two gating modes of the $\alpha$ subunit determines the phenotype of macroscopic currents, since kinetic analysis does not reveal significant differences between slow and fast inactivation rates. The conclusion is that the two modes discerned in this study are conferred by two states of Na channels whose interconversion is regulated by an as yet unknown mechanism that might involve cytosolic factors.

Modal gating of Na channels also occurs in native systems such as heart muscle cells (Patlak & Ortiz, 1985), frog skeletal muscle (Patlak & Ortiz, 1986) or neurons (Huguenard et al., 1988). Distinct gating modes also were reported in Xenopus oocyte studies of rat brain III or rat skeletal muscle µI channels for whole-oocyte and single-channel recordings (Johe et al., 1990; Moorman et al., 1990; Zhou et al., 1991). In single-channel cell-attached patch recordings, rat brain III channels sometimes switch between slow and fast mode, whereas µI channels will settle in fast mode regardless of the initial mode after patch formation. It should be pointed out that the terms "fast" and "slow" are used in rather arbitrary ways in different studies. Thus, the time constant for fast inactivation observed in µI single channel recordings corresponds approximately to the rate classified as slow mode. However, Zhou et al. (1991) mention that the open probability history of fast gating µI channels revealed an additional, even faster mode which could not be resolved in their ensemble averages, but which could correspond to the phenomenon termed fast mode in this study. Clearly, at least two kinetic modes with different time scales can be encountered depending on channel type and experimental conditions.

Patch excision seems to influence critically the equilibrium between gating modes of Na channels expressed in Xenopus oocytes. In cardiac cells, the slow inac-
tivating component becomes more prominent after patch excision and the chan-
nels fluctuate between two major modes (Nilius, 1988), quite in contrast to rat
brain, where excision tends to speed inactivation (Kirsch & Brown, 1989). Patch
excision-induced alterations in modal gating indicates the involvement of the cyto-
solic milieu in controlling mode changes. For example, G proteins have been
reported to modulate Na channels (Schubert et al., 1989) and pertussis toxin-
induced reduction of Na currents is counteracted by addition of the human $G_{p, \alpha_4}$.3
subunit in the epithelial cell line A6 (Cantiello et al., 1989). Modulatory effects of
redox states have been reported for voltage-gated ion channels, affecting inactiva-
tion of A-type K channels (Ruppersberg et al., 1991) as well as amplitudes of Ca$^{2+}$-
dependent K currents (Post et al., 1993).

Another important regulatory mechanism might be provided by phospho-
rylation of ion channels. Na channels possess multiple phosphorylation sites for
both protein kinase A (PKA) and protein kinase C (PKC). Rapid cAMP-dependent
protein phosphorylation by PKA reduces ensemble peak sodium currents in
excised inside-out patches from transfected CHO cells (Li et al., 1992). Moderate
activation of PKC by phorbol ester slows Na current inactivation and stronger acti-
vation reduces ensemble peak sodium current in RBIIA-transfected CHO cells
(Numann et al., 1991). Therefore, phosphorylation seems a very plausible candi-
date for stabilizing distinct gating modes in individual Na channels.

The observed results in excised patches of this study - the time-dependent
increase in peak current and the modal switch speeding current decay - possibly
reflect gradual loss of channel phosphorylation. The degree of phosphorylation
may determine the channel's preferential gating behavior, converting consecu-
tively from non-conducting under heavy phosphorylation, to slow mode upon
Figure 14: Proposed mechanism for mode transition

The diagram illustrates the hypothetical shift from fast inactivating Na channels in the unphosphorylated state to slowly inactivating at moderate phosphorylation, and eventually to non-activatable upon heavy phosphorylation. The enzymes involved and the resulting phenotypes of Na currents are indicated. See text for details.
partial dephosphorylation, and eventually to preferentially fast mode upon complete dephosphorylation (Fig. 14). This hypothesis could explain both the present observations of unidirectional mode switching from slow to fast in excised patches and observations of fluctuating mode shifts in cell-attached patches (Joho et al., 1990; Moorman et al., 1990; Zhou et al., 1991), where kinases and phosphatases could regulate the intricate level of modal gating. However, fluctuation of gating modes has also been reported to occur in excised patches (Moorman et al., 1990). If this phenomenon was also related to phosphorylation-dephosphorylation events, one would have to postulate the presence of parts of the biochemical machinery to sustain channel modulation even in excised patches. Indications that this might indeed be the case comes from enzymatic modulation of muscarinic K channels in excised patches (Kim et al., 1989). The results of the present work would then seem compatible with the notion that the preferentially visited gating mode of Na channels is dependent on the incidental presence of specific enzymes in the channel vicinity.

Loss of phosphorylation of specific amino acids could be phosphatase-dependent. Evidence for possible involvement of membrane-associated kinases or phosphatases with patchy distribution in the oocyte membranes comes from the fact that excised patches from the same oocyte could start off in either fast or slow mode. The fact that some patches started off and remained in slow mode during the recording session would indicate the dependence on phosphatase present in the excised patch. Phosphatase-independent or use- and voltage-dependent dephosphorylation has not been addressed in this study but is a speculative possibility. Zhou et al. (1991) and Krafte et al. (1990) reported speeding of inactivation in oocyte currents depending on test-pulse frequency.
The perspective of modal gating in Na channels complicates structure-function studies of the protein. Do mutations that shift voltage-sensitivity or slow the decay of macroscopic current reflect alterations in the respective functional structures of channel activation and inactivation, or have structures been influenced involved in setting the mode of gating? Further complication arises from the shift in the steady-state inactivation curve, as sodium currents will predominantly reflect slow mode characteristics with holding potentials more positive than -100 mV (see Fig. 10C). This partially explains prior observations of unusually slow inactivation properties of whole-cell Na currents in *Xenopus* oocytes expressing the α subunit (Auld et al., 1990). It seems unavoidable that gating modes be considered in structure-function analyses of sodium channels until the underlying mechanism can be experimentally controlled.

Kinetic mode switching may be of importance in developmental processes. The β1 subunit normally co-purifies with the α subunit in adult rat brain Na channels (Messner & Catterall, 1985) but may be expressed differentially at various stages of embryonic development (McHugh-Sutkowski & Catterall, 1990). Coexpression of α and β subunits speeds inactivation and increases sodium peak currents (Isom et al., 1992), while shifting activation and steady-state inactivation to more negative values (Patton et al., 1993). Although the mechanism underlying these changes is not known, it appears compatible with switching from slow to fast gating mode. It is conceivable that in native systems the β1 subunit, when associated with the α subunit, protects part of the modulation sites which are otherwise exposed to phosphorylation or other regulatory mechanisms at early stages of development.
Modulation of modal gating of Na channels could have profound physiological implications. Na channels determine the threshold for action potential generation and influence the frequency of neuronal firing in somata and axonal hills. Due to differing voltage-sensitivities, the threshold may be determined according to the quantitative distribution of channel gating types. The effect of modal gating may be equally important for the duration of membrane potential changes in cell bodies, which is influenced by Na channel density and open probability. Furthermore, alterations in membrane potential may change Ca$^{2+}$ influx affecting various cellular functions. Variation of Na channel modal gating at such crucial points as synapses could affect regulatory mechanisms of transmitter release.
IV. Point Mutations in IIS4 affect Sodium Channel Gating

1. Introduction

Sodium channels are principally responsible for the rising phase of electrogenic action potentials in nerve and muscle. The tetrameric structure of the membrane-spanning protein has been deduced (Noda et al., 1984; Auld et al., 1988; Kayano et al., 1988) and properties of activation and inactivation gating (Stühmer et al., 1989; Vassilev et al., 1989; West et al., 1992) and selectivity (Heinemann et al., 1992) have been attributed to various regions of the protein. The fourth membrane-spanning segment in each domain contains a high proportion of positively charged residues with, typically, a lysine or arginine in every third position. The S4 segment in the first domain (IIS4) has been shown to be associated with activation gating by site-directed mutagenesis (Stühmer et al., 1989); charge deletion or reversal altered the voltage sensitivity of channel activation as measured by changes in the slope and midpoint of $g(V)$ curves. On the other hand, that study reported little effect on channel gating by single charge deletions of IIS4, whereas double mutations that included both IIS4 charge deletions and IIS4 deletions paired with IS4 deletions did shift the voltage sensitivity of channel activation to a greater extent than the IS4 deletion alone. By contrast, a neutral mutation (L860F) in the IIS4 segment at a position adjacent to the fourth charge affected the voltage sensitivity of channel gating by producing a $+20$ mV shift in the peak of the $I(V)$ curve without a detectable change in valence (Auld et al., 1990).
In the attempt to resolve these findings, macroscopic currents of wildtype rat brain IIA (RBIIA) and mutant sodium channels were recorded in excised patches from *Xenopus* oocytes. The charge deletion (K859Q) and the adjacent conservative mutation (L860F) in the second domain S4 membrane-spanning region differentially altered voltage sensitivity and kinetics. Analysis of voltage dependence was confined to Na currents with fast inactivation kinetics, although RBIIA and K859Q (but not L860F) also showed proportional shifts between at least two gating modes rendering currents with fast or slow inactivation kinetics, respectively. Compared to RBIIA, the midpoint of the F(V) curve was shifted in both K859Q and L860F to more positive potentials, yet this shift was not associated with a corresponding change in the voltage dependence of time constants for activation ($\tau_a$) or inactivation ($\tau_{hl}$, $\tau_{ho}$). Similarly, the steady-state inactivation curve of L860F but not K859Q shifted in hyperpolarizing direction. These results challenge the notion that S4 segments exclusively control the activation of Na channels. Rather, it seems that specific locations within the protein may affect multiple features of Na channel function.

2. Results

*Sodium currents in excised macro-patches*

Figure 15 depicts typical examples of Na currents recorded from inside-out macro-patches excised from *Xenopus* oocytes expressing the $\alpha$ subunit of RBIIA (A), K859Q (B) and L860F (C). Current families of RBIIA and K859Q evoked by depolarizing voltage steps could have either fast (left) or rather slow inactivation kinetics (right). The proportion of patches exhibiting slow Na currents was 70% for RBIIA (7 of 10) and 27% for K859Q (6 of 22). L860F displayed only fast inactivation
A  
slow kinetics

fast kinetics

RBIIA

B

K859Q

C

L860F

Figure 15: Macroscopic sodium currents in oocyte excised macro-patches

Current families recorded in excised macro-patches from oocytes injected with mRNA coding for RBIIA, K859Q and L860F. Inactivation kinetics of RBIIA (A) and K859Q (B) could have predominantly fast or slow kinetics, whereas L860F (C) displayed only fast kinetics. Holding potential was -100 mV. A conditioning prepulse to -150 mV for 200 ms preceded a 10 ms test pulse to remove slow inactivation. Test potentials were -60 mV to +60 mV (A, B) and -80 mV to +60 mV (C).
kinetics in this study. The overall appearance of inactivation kinetics in current traces is based on the equilibrium between at least two distinguishable gating modes, as observed previously in macroscopic currents from excised macro-patches from *Xenopus* oocytes (Chapter III). Only currents which could be categorized as showing fast kinetic behavior (see methods) were considered for further analysis here, so as to segregate voltage shifts induced by changes in gating mode from those induced by the mutations.

*Mutations in IIS4 affect voltage-sensitivity*

Figure 16 illustrates the current-voltage relationship (Fig. 16A) and the voltage-dependence of activation (Fig. 16B) from Na currents of RBIIA (●) and mutants K859Q (○) and L860F (□). A 200ms prepulse to -150 mV preceded each test pulse to remove any residual steady-state inactivation occurring at holding potential (-100 mV). Channel activation and peak amplitude of both K859Q and L860F is shifted to more positive potentials by about 20 mV as compared to RBIIA (Fig. 16A) [P <<0.001, Student's t-test], whereas reversal potentials do not change (RBIIA: +45 ± 3.3 mV, K859Q: +48 ± 1.8 mV and L860F: +47.6 ± 1.2; means ± S.D., n = 5, 14 and 14, respectively). Figure 16B shows the steady-state activation characteristics of RBIIA and mutant Na currents. The peak currents have been normalized for maximal conductance and expressed as fraction of open channels F(V). The midpoint $V_{1/2}$ of the F(V) curve is indicative of the probability that 50% of channels will open at that test potential. The slope of the F(V) curve suggests the effective valence of channel activation. When compared to RBIIA Na currents, half-maximal activation of both K859Q and L860F is shifted to the right along the voltage axis by about 20 mV (same n as in Fig. 16A) [P <<0.001]. Only L860F resulted in a statistically significant reduction in the slope valence as compared to
Figure 16: Voltage dependence of activation and fraction of open channels

(A) Normalized mean peak amplitudes and mean I(V) fits of RBIIA (●), K859Q (○) and L860F (□) (n = 5, 14 and 14, respectively). Activation midpoints are $V_{1/2}^{(RBIIA)} = -34 \pm 3$ mV, $V_{1/2}^{(K859Q)} = -16 \pm 7$ mV and $V_{1/2}^{(L860F)} = -14 \pm 3$ mV (means ± S.D., respectively). (B) F(V) data plotted as a function of variable test pulses (conditioning prepulse: -150 mV, duration: 200 ms) for RBIIA (●), K859Q (○) and L860F (□). Half-maximal open probabilities are $V_{1/2}^{(RBIIA)} = -35 \pm 7$ mV, $V_{1/2}^{(K859Q)} = -13 \pm 11$ mV and $V_{1/2}^{(L860F)} = -13 \pm 3$ mV. Valences are $z_{RBIIA} = 3.2 \pm 0.3$, $z_{K859Q} = 2.9 \pm 0.3$ and $z_{L860F} = 2.3 \pm 0.2$. 
RBIIA ($z_{\text{RBIIA}} = 3.2 \pm 0.27$, $z_{\text{K859Q}} = 2.9 \pm 0.3$ and $z_{\text{L860F}} = 2.3 \pm 0.2$; mean ± S.D.) [P << 0.001 for L860F]. Comparing the mutants with each other shows that the valence for activation differs at a probability level of P << 0.001.

Fast inactivation and slow inactivation have been shown to be two independent processes, which can be separated pharmacologically (Rudy, 1978; Heggeness & Starkus, 1986; Quandt, 1987), and are differentially selected for depending on the prepulse length (Ruben et al., 1992). To assess fast inactivation ($h_\infty$), short prepulses of few millisecond duration (2 to 10 ms) precede the test pulse. To reach equilibrium of slow inactivation ($s_\infty$), the prepulses have to be substantially longer (>50 ms). Increasing prepulses durations from a few milliseconds to several hundred milliseconds can shift the midpoint of the inactivation curve to more negative potentials (e.g., Ruff et al., 1987).

Fast inactivation ($h_\infty$) and steady-state inactivation ($s_\infty$) from Na currents of RBIIA and mutants K859Q and L860F was assessed using pulse protocols with variable prepulse potentials of 5 ms and 200 ms length, respectively. The prepulse was followed by a 10 ms test pulse to 0 mV. Figure 17 illustrates the steady-state inactivation curves for all three channel species. Half-maximal availability of RBIIA (●) and K859Q (O) channels coincide in midpoint values, whereas L860F (□) is shifted by 9 mV to more negative potentials as compared to RBIIA [P < 0.003] (n = 5, 8 and 12, respectively). Valences of the $s_\infty$ curves are not affected. Not illustrated are fast inactivation data for RBIIA, K859Q, and L860F, where the midpoint voltages of RBIIA and L860F are not statistically different, but K859Q is shifted to the right by 7 mV [P < 0.02] ($V_{1/2\text{RBIIA}} = -78 \pm 9$ mV, $V_{1/2\text{K859Q}} = -71 \pm 4$ mV, and $V_{1/2\text{L860F}} = -82 \pm 5$ mV, n = 4, 5, and 10, respectively). The valences are similar to those of channel activation ($z_{\text{RBIIA}} = 3 \pm 0.5$, $z_{\text{K859Q}} = 2.3 \pm 0.2$ and $z_{\text{L860F}} = 2 \pm 0.27$).
Figure 17: Steady-state inactivation curves of RBIIA, K859Q, and L860F

Data show the mean peak amplitudes of RBIIA (●), K859Q (○), and L860F (□), as indicated in the graph. The data were determined from a constant test pulse to 0 mV and plotted as a function of variable conditioning prepulses (duration: 200 ms). Half-maximal availabilities, as determined by Boltzmann fits to individual data sets, are $V_{1/2\text{(RBIIA)}} = -102 \pm 5$ mV, $V_{1/2\text{(K859Q)}} = -103 \pm 9$ mV, and $V_{1/2\text{(L860F)}} = -111 \pm 5$ mV (means ± S.D., n = 5, 8, and 12, respectively). Valences are $z_{\text{RBIIA}} = 2.5 \pm 0.4$, $z_{\text{K859Q}} = 2.5 \pm 0.4$ and $z_{\text{L860F}} = 2.8 \pm 0.5$. These parameters were used to compute the superimposed fits in the graph.

Mutants differentially affect activation time constants

Kinetic analysis of current traces from RBIIA and mutant channels was performed to assess whether the shifts in voltage-sensitivity were caused by shifts in channel kinetics. Concurrent three-exponential analysis of individual traces rendered time constants for the rising phase $\tau_a$ and the two decaying phases $\tau_{h1}$ and $\tau_{h2}$ (see Methods).
Figure 18 shows activation time constants of RBIIA (●), K859Q (○) and L860F (□) as a function of test potential (n = ten current families each) recorded at room temperature (20-24 °C). K859Q activates slower than RBIIA, whereas L860F is faster at more negative potentials \( P < 0.006 \) and \( P < 0.03 \), respectively; assessed by Student's paired t-test. At this point it cannot be evaluated if L860F activates faster than RBIIA at -40 to -20 mV, as the cutoff frequency used in this study limits the resolution of very fast time constant values. Resorting to evaluation of time constants at -10 mV, only activation time constants of K859Q showed significant differences as compared to RBIIA \( P < 0.001 \) with \( \tau_a(\text{RBIIA}) = 103 \pm 17 \mu s \), \( \tau_a(\text{K859Q}) = 150 \pm 40 \mu s \) and \( \tau_a(\text{L860F}) = 108 \pm 20 \mu s \) (means ± S.D., n = 10, 20 and 14, respectively).

**Figure 18: Voltage dependence of the activation time constant \( \tau_a \).**

Time constants \( \tau_a \) for activation of RBIIA (●), K859Q (○) and L860F (□) plotted as function of test potential. Each point represents values of mean time constants ± S.E.M. (10 current families each).
Also, comparing activation time constants of K859Q and L860F across voltage reveals that they differ significantly \( P < 0.01 \).

Voltage dependence of inactivation time constants \( \tau_{h1} \) and \( \tau_{h2} \) for RBIIA (●), K859Q (○) and L860F (□) is shown in Figure 19A and 19B, respectively. Time constants \( \tau_{h1} \) of K859Q are slower than RBIIA with \( P < 0.05 \) (\( n = \) ten current families each), whereas \( \tau_{h1} \) of L860F is not altered. However, looking at the test potential of -10 mV only (with higher \( n \) for the mutants), neither K859Q nor L860F shows significant differences of the time constant \( \tau_{h1} \) as compared to RBIIA (\( \tau_{h1(\text{RBIIA})} = 144 \pm 23 \) ms, \( \tau_{h1(\text{K859Q})} = 176 \pm 50 \) ms and \( \tau_{h1(\text{L860F})} = 163 \pm 33 \) ms; means ± S.D., \( n = 10, 20 \) and 14, respectively). Likewise, comparing the voltage dependence of inactivation time constants \( \tau_{h1} \) of K859Q with L860F shows that L860F activates faster than K859Q (\( P < 0.01 \)), but this cannot be confirmed looking at -10 mV only.

**Figure 19: Voltage dependence of inactivation time constants \( \tau_{h1} \) and \( \tau_{h2} \)**

Time constants \( \tau_{h1} \) and \( \tau_{h2} \) for the two inactivation components of RBIIA (●), K859Q (○) and L860F (□) plotted as function of test potential. Each point represents values of mean time constants ± S.E.M. (10 current families each).
No statistically significant differences could be detected for the voltage
dependence of \( \tau_{h2} \) comparing RBIIA and mutants or mutants with each other (Fig.
19B; \( n = \) ten current families each). Equally, time constants at -10 mV are \( \tau_{h2(\text{RBIIA})} = 2.8 \pm 1.5 \text{ ms} \), \( \tau_{h2(\text{K859Q})} = 2.8 \pm 1.5 \text{ ms} \) and \( \tau_{h2(\text{L860F})} = 2 \pm 1 \text{ ms} \) with no observed sta-
tistical difference (means \( \pm \text{ S.D.}, \) same \( n \) as for \( \tau_{h1} \) at -10 mV). Overall, shifts in the
F(V) curves of K859Q and L860F are not correlated to respective changes in the
voltage dependence of time constants (Table 2). Although both mutants cause F(V)
to be shifted in depolarizing direction, K859Q activates much slower and L860F, at
negative potentials, faster than RBIIA.

Table 2: Parameters of RBIIA, K859Q and L860F
The table lists the parameters (\( \pm \text{ S.D.} \)) evaluated for RBIIA, K859Q and L860F. Shaded numbers
for K859Q and L860F indicate statistically significant differences to RBIIA.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wildtype</th>
<th>K859Q</th>
<th>L860F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation</td>
<td>( V_{1/2} )</td>
<td>-35 ( \pm ) 7 mV</td>
<td>-16 ( \pm ) 7 mV</td>
</tr>
<tr>
<td></td>
<td>( z )</td>
<td>3.2 ( \pm ) 0.27</td>
<td>2.9 ( \pm ) 0.3</td>
</tr>
<tr>
<td>( 3_w )</td>
<td>( V_{1/2} )</td>
<td>-102 ( \pm ) 5 mV</td>
<td>-103 ( \pm ) 9 mV</td>
</tr>
<tr>
<td></td>
<td>( z )</td>
<td>2.5 ( \pm ) 0.36</td>
<td>2.5 ( \pm ) 0.4</td>
</tr>
<tr>
<td>( h_w )</td>
<td>( V_{1/2} )</td>
<td>-78 ( \pm ) 9 mV</td>
<td>-71 ( \pm ) 4 mV</td>
</tr>
<tr>
<td></td>
<td>( z )</td>
<td>3.0 ( \pm ) 0.5</td>
<td>2.3 ( \pm ) 0.2</td>
</tr>
<tr>
<td>time constant (at -10 mV)</td>
<td>( \tau_{a} )</td>
<td>103 ( \pm ) 17 ms</td>
<td>150 ( \pm ) 40 ms</td>
</tr>
<tr>
<td></td>
<td>( \tau_{h1} )</td>
<td>144 ( \pm ) 23 ms</td>
<td>176 ( \pm ) 50 ms</td>
</tr>
<tr>
<td></td>
<td>( \tau_{h2} )</td>
<td>2.8 ( \pm ) 1.5 ms</td>
<td>2.8 ( \pm ) 1.5 ms</td>
</tr>
</tbody>
</table>

3. Discussion
The results show that: (i) neutralization of the fourth charge in II\( S4 \) at posi-
tion 859 changes the F(V) curve midpoint by +20 mV and decreases the rates of
both activation and fast inactivation; (ii) replacement of a leucine with an apparently neutral phenylalanine at the 860 position shifts the F(V) curve by +20 mV, decreases effective valence of activation, changes the s∞ curve by -10 mV, and increases the rate of activation. Thus, despite the similar effects on the F(V) curve of charge neutralization and an adjacent conservative mutation, these mutations have opposite effects on the rates of activation and inactivation, and differing effects on steady-state inactivation and activation valence.

A charge neutralization and a conservative mutation similarly affect activation voltage sensitivity.

Both K859Q and L860F mutations shift the activation curve some 20 mV in the depolarizing direction. In addition, L860F decreases the effective valence of activation by as much as 0.9e in comparison to wildtype RBIIA, whereas K859Q did not cause a significant change in valence. The right-shift imposed on the activation voltage sensitivity by L860F has been previously noted by Auld et al. (1990). However, the resolution of their data, obtained with two-electrode voltage clamp, did not distinguish the decrease in activation valence. Using cell-attached macropatches, Stühmer et al. (1989) noted similar shifts in the midpoint of the activation curve with K859Q/K862Q double mutations and K226Q/K859Q/K862Q triple mutations. The triple mutation gave the greatest effect whereas K862Q alone did not produce a voltage shift in the activation curve. Although the study by Stühmer et al. (1989) did not include data on K859Q alone, their results in combination with the present study indicate that IIS4 and, more specifically, K859 (but not K862) are important determinants of activation voltage sensitivity.
The results of this study are similar to those of Tytgat et al. (1993) who reported that, in RCK1 channels, neutralization of the 5th positive charge in the S4 putative membrane spanning region caused a positive shift in the activation curve. The 5th charge in K channels is homologous to the 859 position in II54 of the sodium channel. Tytgat et al., (1993) did not observe a change in activation valence with the RCK1 K5I mutation and therefore concluded that the 5th charge is not an activation voltage sensor. If homology in function as well as structure is assumed to exist between K channels and Na channels, then by extension it is possible that the lysine at position 859 in RBIIA should not directly participate in activation gating.

Similarly, it is not yet clear whether the II54 components studied here directly control activation gating by functioning as a voltage sensor, or indirectly exert control over gating through interactions with other components of the channel protein. The marginal effect produced by neutralization of the 859 charge might indicate a voltage sensing role if confirmed in a larger experimental series. However, the coincident effects of the L860F mutation suggests other possible interpretations. Of these, interactions with other gating-control components seems the most parsimonious explanation.

Effects of mutations on steady-state and fast inactivation voltage sensitivity

Fast and slow inactivation may be readily distinguished by their characteristically different pharmacological sensitivities. The enzymatic agents that selectively remove fast inactivation (Rudy, 1978; Heggeness and Starkus, 1986; Quandt, 1987) can alter the voltage sensitivity of slow inactivation; yet in the presence of these agents, slow inactivation remains intact. Thus, it should be re-emphasized
that fast and slow inactivation are separate processes (Adelman and Palti, 1969; Narahashi, 1974; Ruben et al., 1992) mediated by pharmacologically distinct mechanisms (Rudy, 1978). However, the steady-state inactivation measured by $s_\infty$ curve protocols is, presumably, a mixture of fast and slow inactivation, whose relative contributions are dependent on prepulse duration. For purely practical reasons, in the present investigation, prepulse durations of 5 msec were used to study fast inactivation and 200 msec to approximate the true steady-state of these inactivation processes. Although 200 ms is the maximum time that one can safely expose excised macro-patches to extreme prepulse voltages, 200 ms may not be long enough to reach a fully equilibrated distribution.

The L860F mutation moves the 200 msec $s_\infty$ curve by -10 mV relative to RBIIA. This is particularly remarkable given the observation that the L860F mutation shifts the activation curve by +20 mV. It has previously been hypothesized that slow inactivation is a parallel process coupled to activation (Ruben et al., 1992; Starkus et al., in press). The present results support this interpretation insofar as slow inactivation contributes to the $s_\infty$ curve. If slow inactivation were a direct consequence of channel activation (like fast inactivation, see Armstrong and Bezanilla, 1977; Aldrich et al., 1983), then one would expect that both activation and steady-state inactivation curves to be shifted in the same direction and to be affected similarly by mutations that influence either one or the other process. By contrast, the results presented here independently support the concepts of parallel voltage sensitivity for steady-state inactivation and some form of coupling between activation and steady-state inactivation, since L860F differentially affects both activation and steady-state inactivation parameters. The $s_\infty$ curve for K859Q approximately superimposes with that of RBIIA. By the same argument, this observation suggests
that the K859Q mutation may also alter the coupling between activation and steady-state inactivation (with a -80 mV change in midpoint from the F(V) curve to the s∞ curve, as compared to a -60 mV midpoint shift for RBIIA), but to a lesser extent than L860F (with a -90 mV change in midpoint).

Since neither of the mutations studied here appreciably alters the valence of steady-state inactivation, it seems unlikely that IIS4 could be the voltage sensor for steady-state inactivation. Thus, the present results suggest that interactions between the voltage sensor for activation and that for steady-state inactivation might be linked through IIS4, and that this link has been altered by the L860F mutation and to a lesser extent by K859Q.

The h∞ curves (following a 5 msec prepulse) for RBIIA and L860F approximately superimpose with midpoints at about -80 mV. One may speculate that this is coincidental since inactivation midpoints are dependent upon prepulse duration. Whereas the midpoint of voltage sensitivity for RBIIA shifts between the F(V) extreme of ca. -35 mV and the steady-state extreme of ca. -102 mV, the L860F midpoints shift between ca. -13 mV and ca. -111 mV (see Table 2 and below). Therefore, the midpoints of the RBIIA and L860F curves must coincide at some prepulse duration between no prepulse and change of holding potential. Thus, some preliminary data (not shown) indicate, that the h∞ curve for L860F lies to the right of that for RBIIA with prepulse durations shorter than 5 msec, and to the left with prepulse durations longer than 5 msec. By contrast, the midpoint of the h∞ curve for K859Q is at about -70 mV. Although significantly different from the RBIIA and L860F h∞ midpoints, the +10 mV shift is not unexpected. As discussed above, K859Q voltage sensitivity undergoes a -80 mV shift from the F(V) position to the steady-state, compared to the -90 mV shift for L860F.
Implications of kinetic changes imposed by mutations

A time-dependent transition from a predominantly slow inactivation mode to a predominantly fast inactivation mode in wildtype sodium channels has been demonstrated (see Chapter III; Fleig et al., submitted; cf. Joho et al., 1990; Moor-man, et al., 1990; Zhou et al., 1991). Those observations are extended by the study of mutant Na channels insofar as to note that K859Q shows similar, time-dependent changes in the relative contribution of fast and slow inactivation rates as RBIIA. In contrast, L860F channels have thus far only been observed to have primarily fast inactivation kinetics. However, it is important to note that inactivation of L860F channels does indeed show two well-separated kinetic components whose rates are comparable to those seen in wildtype channels. Thus, both $\tau_{h1}$ and $\tau_{h2}$ are readily apparent in L860F, and the major difference between these versus wildtype channels is that the vast majority of L860F channels inactivate via the fast pathway. No time-dependent transitions in the inactivation gating mode of L860F channels were observed.

Only fast mode channels have been compared for the purposes of the present study. Although the sample rate used to collect data did not allow very high resolution of activation rates, the difference between $\tau_a$ for K859Q and $\tau_a$ for RBIIA and L860F is substantial over a wide range of test potentials. One interpretation of the relative slowness of K859Q channels is that IIS4 participates directly in channel gating and that the neutralization of a charge results in a less-responsive voltage sensor, both in terms of time- and voltage dependence.

By contrast, L860F channels activate slightly faster than RBIIA. These differences were only observed over a limited range of test potentials, but the resolution
of activation kinetics was limited by sample rate. At higher sample rates and/or slower kinetics (e.g., at low temperatures) one might find that L860F is faster than RBIIA and K859Q over a wider range of test potentials.

This difference between the activation kinetic effects of K859Q as opposed to L860F could suggest that the two mutations might influence activation gating in different ways: K859Q through direct alteration of IIS4 voltage sensitivity and L860F through indirect interactions with other gating segments (e.g., IS4). The relatively small increase in activation rate by L860F, and the limited test potential range over which its affect was visible does not provide a particularly strong case for this argument. However, additional data at higher sample rates should clarify this hypothesis.

All the channel-types studied here show two well-separated and distinguishable rates of inactivation. The apparent slowing of both $\tau_{h1}$ and $\tau_{h2}$ in K859Q relative to RBIIA and L860F can be largely accounted for by the corresponding decrease in its activation rate. Thus, both $\tau_{h1}$ and $\tau_{h2}$ seem to represent different forms of fast inactivation whose voltage dependence and kinetics may be expected to follow that of activation.

**Conclusions**

How can an apparently neutral mutation shift the F(V) curve 20 mV in the depolarizing direction, the so curve in the hyperpolarizing direction, and increase the rates of activation and fast inactivation? How can another mutation similarly shift the F(V) curve 20 mV in the depolarizing direction, yet not affect the so curve and actually decrease activation and inactivation rates? The dual and disparate effects of L860F and K859Q on the voltage sensitivities of activation and inactiva-
tion suggest that IIS4 might be both a voltage sensor for activation and be linked to the voltage sensitive components that control slow inactivation. This is a profound set of effects which could explain previous observations that L860F expression is typically lower than RBIIA (Auld et al., 1990), since those studies assessed L860F from a holding potential of -100 mV at which well over half the channels would be slow inactivated and thus unavailable for opening.

The range of effects we see on kinetics and voltage sensitivity points to independent control of these parameters at the structural level. In a system of first order reactions, midpoint is determined by relative well-depth, valence is determined by particle charge, and reaction rate is determined by barrier height. Our present findings suggest that these reaction parameters may be manipulated in complex ways via mutations of a single residue. It seems unlikely that different S4 segments are voltage sensors for single, identifiable, physiological parameters. Rather the S4 segments represent a complexly coupled system of voltage sensitive elements (see also Auld, et al., 1990; Tytgat et al., 1993). It thus appears that physiologically identifiable parameters derive from the interactive properties of many structural components in the sodium channel protein.
V. Synopsis

The prevailing concept about Na channel function comes from a long tradition of seminal electrophysiological studies in many different preparations. In their classical studies, Hodgkin and Huxley established a theory in which Na channel gating could be described by 3 separate "m" particles of activation and a parallel "h" particle of inactivation. Work of the 70's and early 80's identified fast inactivation as a sequential process (rather than a parallel one), with only minimal independent voltage sensitivity. However, additional studies established a slow, voltage sensitive component of inactivation. This clarified the pharmacological separation between fast and slow inactivation.

The recent developments in molecular cloning and high resolution patch-clamp recording allowed identification of functional sites within the Na channel responsible for its electrophysiological behavior. In the 80's, the activation of Na channels was attributed to the S4 segment of the channel protein. More recent work with K channel monomers further clarified that fast inactivation seems to be an independent "N-terminus ball-and-chain" mechanism whereas slow inactivation is a "C-terminus" mechanism of a different sort. This leaves the S4 segments as solely activation determining (except, perhaps, for the concept that the inactivation binding site is accessible only in open channels, and hence appears sequential to activation). These concepts have been picked up and applied to Na channels, thus suggesting a primarily "activation" role for the sodium channel S4 segments. Together, these studies have shaped a view of Na channel function which seems to leave little space for surprises. Nevertheless, the present study reveals some
unexpected results, which suggest that Na channel function may be more complex than had been anticipated.

The main objective of the present study was to determine the effects of point mutations in a putative activation segment of RBIIA sodium channels. To this end, wildtype and mutant Na channels were expressed in *Xenopus* oocytes. While the *Xenopus* oocyte expression system is well-suited for expression of wildtype and mutant Na channels, it bears some severe methodical limitations in the sense that conventional two-electrode voltage-clamp techniques will yield erroneous estimates of current kinetics. This is mainly due to the oocyte's size. To study Na currents at high temporal resolution, a novel approach had to be taken, i.e., to record Na currents in excised macro-patches; the present results constitute the first investigation of expressed Na channels in a cell-free membrane environment.

The experimental results of this study have been subdivided into two sections. The first part deals with the observed mode shift in Na channel kinetics, which may be attributed to "biochemical modulation" of Na channels, while the second part emphasizes what might be considered "molecular modulation", i.e., the effects of introducing artificial modifications of the channel protein itself by site-directed mutations of Na channels in the region of interest. Biochemical modulation of Na channels bears important implications for Na channel function in the physiological context, as Na currents influence membrane excitability, action potential duration, and neuronal firing frequency. Molecular modulation is important for understanding Na channel properties at the molecular level. Altering the structure of Na channels and monitoring the ensuing functional changes may reveal relevant functional features not only of the protein under investigation but also for the large family of voltage-gated ion channels.
For a given preparation, Na current kinetics are relatively fixed with little room for modulation. It is recognized that inactivation may be composed of fast and slow processes, where slow inactivation occurs at very long time scales of hundreds of milliseconds, while fast inactivation takes place within a few milliseconds. Fast inactivation is thought to be a singular process obeying first order kinetics. Only few studies have hinted at multiple components of fast inactivation.

The results of this study clearly identify and characterize multiple components of fast inactivation of Na channels. Na currents of RB1IA show two distinguishable inactivation time courses with time constants $\tau_{h1}$ (~100 $\mu$s, fast mode) and $\tau_{h2}$ (~2 ms, slow mode). Excision of membrane patches revealed a surprising behavior of Na currents, manifesting itself in a kinetics shift from slow to fast inactivation. The amplitude $h2$ decreases within a few minutes of patch excision, while the time constants $\tau_{h1}$ and $\tau_{h2}$ remain nearly constant. At the same time, activation and steady-state inactivation shift to more negative potentials. These results imply that sodium channels can adopt at least two gating modes.

Since mode shifts were unidirectional and likely due to the removal of the channels from the cytoplasmic environment, equilibrium between slow and fast gating modes of Na channels appears to be modified by cytosolic factors. Hypothetical candidates for conferring kinetic regulation of Na channels are kinases and phosphatases, as Na channels are endowed with numerous putative phosphorylation sites for both protein kinase C and protein kinase A. Future structure-function studies with point mutations introduced into these phosphorylation sites may clarify their role in modal behavior of Na channels.
If modal behavior were a physiological feature of Na channels in native cells, it would undoubtedly have important functional consequences, as the contribution of fast inactivating channels to the total current is favored at negative resting potentials. A less obvious but intriguing conjecture may be drawn from the analogous kinetic effects of co-expressing α and β subunits, which results in accelerated Na current kinetics. It is tempting to speculate and test for the possibility that the β subunit of the sodium channel may be involved in conferring a mode shift.

Although not surprising for a complex three-dimensional structure such as the Na channel protein, the results obtained with the molecularly modified Na channels challenge the generally held notion of S4 segments being exclusively a voltage sensing region. From the data presented here, functional domains in the Na channel protein seem not strictly localized, since not only activation but also fast and slow inactivation, as well as kinetic properties, are affected by mutations in IIS4. Thus, the mutants K859Q and L860F reduce the valence of activation and shift the current-voltage relationship to positive values. However, steady-state inactivation is affected differentially by the mutants, where K859Q data correlate with RBIIA, but the L860F curve is shifted to more negative potentials. Furthermore, the voltage dependence of channel kinetics does not correlate with the observed shifts in the voltage sensitivity of activation; while both mutants shift the activation curve to positive potentials, K859Q kinetics are slower and those of L860F are faster.

Thus, providing evidence that τa and τh1 shift together is consistent with a mechanism in which τh1 is primarily determined by activation rate. Additionally, the presented evidence that IIS4 can affect activation valence is again consistent
with a broad view that S4 segments (other than just IS4) are involved in control of activation parameters. Interestingly, the point mutations have equal effects on F(V) midpoints but different effects on valence (although both are reductions) and even more different effects on kinetics. Thus, these aspects of activation are apparently "independent" in the sense that they do not seem necessarily correlated in a consistent fashion. This is a very curious finding in one sense but quite reasonable in terms of first-order reaction kinetics (where the barrier height is the principle determinant of the rate, the relative well depth controls the midpoint, and the particle charge determines the slope valence). Hence, single residue changes may differentially affect these "independent" parameters. On the other hand, the demonstration that "s∞" is affected by activation (and in the opposite direction to the effect on the F(V) curve) is something quite different since it suggests that single sites on a given S4 segment can link functional parameters from seemingly independent functional systems.

Together, these results imply that the positions K859 and L860 of the IIS4 segment are not only involved in channel activation, where they can influence parameters like midpoint and slope of the F(V) curves, but exert other functions as well, some of which appear to involve inactivation. The most intriguing function may be to coordinate independent functions like activation and slow inactivation. If such unity is uncoupled by a single-point mutation, the quasi-sequential order of channel transitions is bound to be disturbed.
VI. References


