



# On the slowly rising phase of the sodium gating current in the squid giant axon

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High-resolution records of the sodium gating current in the squid giant axon demonstrate the existence of a slowly rising phase that is first apparent at pulse potentials slightly below zero, and becomes increasingly pronounced at more positive potentials. At +80 mV the current reaches its peak with a delay of 30  $\mu$ s at 10 °C. It is suggested that this current is generated by the first two steps labelled R $\rightarrow$ P and P $\rightarrow$ A in the S4 units of all four domains of the series-parallel gating system, activating the channel before its opening by the third steps A $\rightarrow$ B in domains I, II and III in conjunction with hydration. The kinetics of the slowly rising phase can only be explained by the incorporation of an appropriate degree of voltage-dependent cooperativity between the S4 voltage-sensors for their two initial transitions.

**Keywords:** sodium gating current; squid axon

## 1. INTRODUCTION

The sodium gating current in the squid giant axon as described by Armstrong & Gilly (1979) showed that although after small depolarizations the current rose quickly and decayed smoothly, the application of large test pulses yielded records that displayed a slowly rising phase and a delayed plateau. It was subsequently claimed by Stimers *et al.* (1987) that the rising phase was the result of incomplete compensation for the Schwann cell series resistance in setting up the voltage clamp, and could be abolished by bathing the axon in a hyperosmotic solution. However, investigations by Keynes *et al.* (1990) in which the  $R_s$  compensation was adjusted as well as possible for critical damping, and could not be improved by adjusting the external osmolarity, have fully confirmed the genuineness of the slowly rising phase. We present here an analysis of further experimental data from the files of Keynes *et al.* (1990) that throw an important new light on the kinetics of the sodium gating system.

## 2. METHODS

### (a) Experimental recording

Giant axons from *Loligo forbesi* were dissected and mounted in an air-gap voltage-clamp chamber with a central 5 mm recording region and 6 mm guard regions. Porous dialysis tubing was threaded inside the axon, through which a solution controlling the internal ionic composition flowed under gravity feed. The axon was bathed in a solution maintained at a constant temperature that was circulated through the chamber by a peristaltic pump. Ionic and gating currents were recorded under voltage-clamp conditions by the methods described by Keynes *et al.* (1990, 1992) and Forster & Greeff (1990). The data now described here and previously unpublished, were taken from their file numbered L24oct.008 and others listed in the figure legends and table 1.

### (b) Model fitting

The model adopted for these studies was a further development of the system described by Keynes (1994), consisting of the four S4 voltage-sensors operating in parallel as screw-helices, each of them occupying one of four or five discrete states as determined by the electric field. For purposes of computation, the forward rate constants  $k_f$  increasing with a more positive potential, and corresponding backward rate constants  $k_b$  were expressed in the conventional manner as

$$k_f(V) = k_{eq} \exp(z\beta(V - V_{eq})FR^{-1}T^{-1}) \quad (1)$$

and

$$k_b(V) = k_{eq} \exp(-z(1 - \beta)(V - V_{eq})FR^{-1}T^{-1}), \quad (2)$$

where  $k_{eq}$  is the rate constant at equilibrium, when  $k_f = k_b$ .  $z$  is the charge of the gate that traverses the entire membrane potential,  $\beta$  is the symmetry factor (the fraction of the total drop of potential across the membrane that the gate traverses before reaching the activation energy peak),  $V$  is the absolute membrane potential, and  $V_{eq}$  is the membrane potential at equilibrium.  $F$ ,  $R$  and  $T$  have their usual thermodynamic significances. The total gating current for all transitions is given by

$$I_g(t) = \sum z_{ij}(k_{ij}P_i(t) - k_{ji}P_j(t)) \quad (3)$$

where  $z_{ij}$  is the charge for the transition,  $P_i(t)$  and  $P_j(t)$  are the probabilities that the channel is in states  $i$  or  $j$ , and  $k_{ij}$  and  $k_{ji}$  are the rate constants from equations (1) and (2) including the coefficients shown in figure 6.

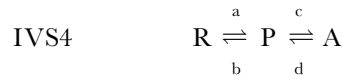
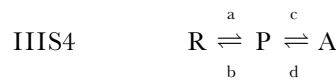
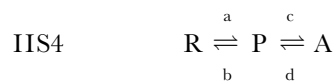
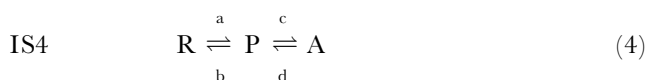
It has been found by Hirschberg *et al.* (1995) from careful determinations of the voltage dependence of the steady-state open probability in non-inactivating sodium channels expressed in oocytes that the total displacement of charge for a single channel is 12  $e_0$ . Measurements for  $K^+$  channels give very similar values (Schoppa *et al.* 1992). From the evidence discussed by Keynes (1994) and Keynes & Elinder (1998) it is clear that

the opening of a channel involves sequences of voltage-dependent transitions similar in nature in all four of the S4 segments, so that if equally distributed, three transitions carrying a charge of  $1 e_0$  would take place in each S4 voltage-sensor, as is confirmed by an analysis of the data plotted in figure 5. For the 11 experiments that provided the data presented in table 1a of this paper, the average transfer of charge between  $-140$  and  $+80$  mV was  $30.6 \pm 1.1$  nC.cm $^{-2}$ , or close to  $31$  nC.cm $^{-2}$  for traversing the whole field. By the application of fluctuation analysis to cut-open squid axons, Bekkers *et al.* (1986) estimated the density of channels as  $180 \times 10^8$  cm $^{-2}$ , whence the charge for opening a single channel in squid would be  $10.7 e_0$ , in reasonable agreement with Hirschberg *et al.* (1995). In considering our intact rather than cut-open axons, the channel density needed for correct scaling of the experimental data was accordingly worked out in each case from the observed charge on the basis that it corresponded to  $12 e_0$  per channel.

For a simple chemical reaction the system would not be expected to be far from symmetrical (Bockris & Reddy 1970). For all of the transitions, the electric field across the short hydrophobic pore at the centre of the membrane (Yang *et al.* 1996) is effectively the same, and the symmetry factor  $\beta$  was assumed throughout to be 0.5. But it should be noted that, as seen below, the introduction of cooperativity may modify the operative value of  $\beta$ .

The computations for finding the least squares best fit of the selected model to families of experimental data were restricted for each pulse to the currents recorded before the channel had begun to open, which could be safely assumed to be generated by the first two transitions, with no contribution from the third one taken by Keynes & Elinder (1998) to be responsible for the actual opening. The settling time of the voltage-clamp was within  $10 \mu\text{s}$ , and the traces often displayed an initial blip arising from the gating current component  $I_{\text{gl}}$  (Keynes *et al.* 1990) that relaxed with a time constant around  $20 \mu\text{s}$  (Forster & Greeff 1992), and was most clearly recorded with the sampling period reduced to  $3 \mu\text{s}$  as in figure 32a of Keynes (1994). With  $5 \mu\text{s}$  sampling, the size of the blip varied somewhat with the precise setting of the compensation for the Schwann cell series resistance,  $R_s$ , which was always apt to change with time during the experiments. In retrospect, it may also be suggested that the saturation of  $I_{\text{gl}}$  at large positive potentials reported by Forster & Greeff (1992) should probably be attributed to its being in effect swallowed up at  $+40$  mV and above by the slowly rising phase of the main part of the gating current, as may be seen to occur in figure 1a. To allow for the settling time of the voltage clamp, true zero time was taken as  $10 \mu\text{s}$  after the start of the pulse, and to minimize errors introduced by the tail of  $I_{\text{gl}}$  in fitting the initial slow rise of the gating current the following  $10 \mu\text{s}$  were excluded from the fit.

First an  $N$ -dimensional space where there were  $N-1$  free parameters was scanned to identify local minima, and the parameters found for these minima were then inserted as initial values in a search for better solutions. This iterative procedure was halted when a change of each free parameter by 0.01% of its value did not improve the fit. The model was computed by a simple Euler integration for the system of transitions shown in equation (4), which describes the kinetics of the changes in state of four S4 voltage-sensors operating in parallel and each occupying states R (resting), P (primed) or A (activated), thus:



where a, b, c and d are rate constants described by equations (1) and (2).

The integration step was chosen always to be at least ten times shorter than the inverse of the largest rate constant involved. The shortest step was  $0.1 \mu\text{s}$ . A personal computer (CPU 80486 or Pentium) was used, and the programs were written in BASIC.

### 3. RESULTS

#### (a) *Records of the sodium gating current*

Figure 1a shows a low-noise family of gating currents for test pulses rising from  $-57$  to  $+83$  mV. After adjustment of the  $R_s$  compensation for critical damping, the clamp potential had settled down after  $10 \mu\text{s}$ , and for the test pulses to between  $-57$  and  $-27$  mV, the relaxation of the gating current was reasonably well fitted from the initial peak by the sum of two exponentials. However, for the pulse to  $-17$  mV a small delayed rise can clearly be seen to come in just after the start of the relaxation, and with larger pulses the slowly rising phase became increasingly prominent, reaching its peak with a roughly constant delay of about  $30 \mu\text{s}$ . Very similar observations had been reported by Armstrong & Gilly (1979), who also pointed out that for the smallest depolarizations no slowly rising phase was detectable, and that for larger depolarizations it definitely preceded the actual opening of the channels. This is confirmed by figure 1b, which shows that the rise of  $I_{\text{Na}}$  for a pulse to  $+23$  mV, after subtracting the gating current as in fig. 13b of Keynes (1994), only began around  $75 \mu\text{s}$  after the start of the test pulse.

The less well averaged records seen in figure 2 reinforce the argument by showing that for large negative test pulses the current reaches its peak immediately, in marked contrast to the delay of about  $50 \mu\text{s}$  at  $5^\circ\text{C}$  in arriving at the initial peak for large positive pulses.

It was reported by Keynes *et al.* (1990) and Keynes (1994) that the same pulses applied after a 20 ms inactivating prepulse to  $+3$  mV followed by a 0.5 ms recovery gap at  $-80$  mV yielded the smaller type of response illustrated in figure 3a. They called this 'the non-inactivating gating current  $I_{\text{g,n}}$ ', but it would be more accurately described as the gating current generated by the system in the earliest stage of recovery. Figure 3b shows how for pulses to  $+3$  and  $+83$  mV, the initial rises of current were the same with or without the prepulse, but the slowly rising fractions were eliminated, in each case halving the total transfer of charge. This behaviour results from the relatively slow recovery of the gating current from inactivation, often termed its immobilization, which as seen in figure 3c, and in figs 28, 35 and 45 of Keynes (1994), for a return to  $-80$  mV has a time constant of 2.4 ms at  $10^\circ\text{C}$ ,

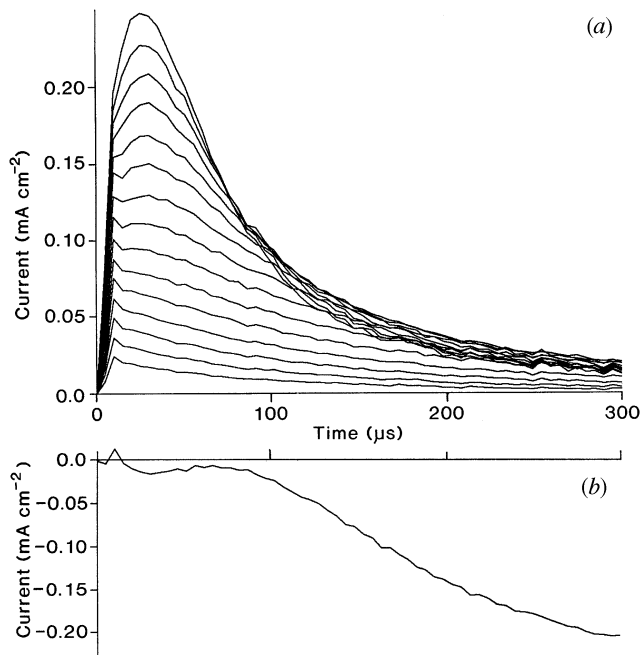


Figure 1. (a) Superimposed family of gating currents recorded from an axon dialysed with 350 mM TMAF, and bathed in Tris SW +1  $\mu$ M TTX. Test pulses  $-57$  to  $+83$  mV in steps of 10 mV. Holding potential  $-80$  mV. Back reference pulses  $-147$  to  $-177$  mV. Sampling period 5  $\mu$ s per point. Bessel filter bandwidth 100 kHz. Temperature 10  $^{\circ}$ C. Number of sweeps averaged was 32. (b) Initial rise of  $I_{Na}$  after subtraction of  $I_g$  recorded similarly for a test pulse to  $+23$  mV in another axon bathed in 1/5 Na Tris SW. Data files L24oct.008 and L27oct.s05 of R. D. Keynes, N. G. Greeff and I. C. Forster.

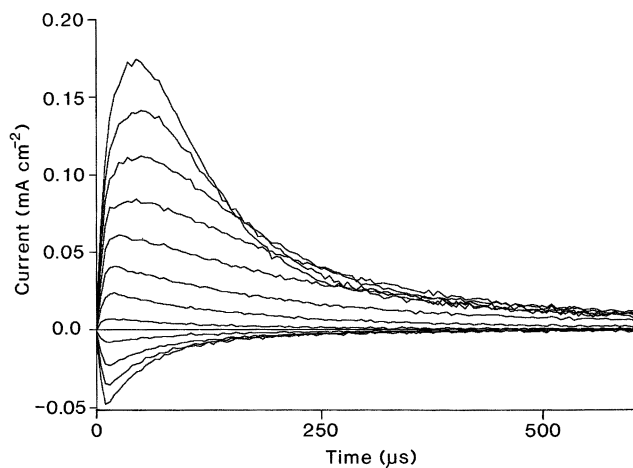


Figure 2. Superimposed family of gating currents recorded from an axon dialysed with 350 mM TMAF, and bathed in Tris SW +1  $\mu$ M of TTX. Test pulses  $-147$  to  $+64$  mV in steps of 19.2 mV. Holding potential  $-80$  mV. Back reference pulses  $-147$  to  $-176$  mV. Sampling period 5  $\mu$ s per point. Bessel filter bandwidth 100 kHz. Temperature 5  $^{\circ}$ C. Number of sweeps averaged was four. Data file I16nov.004 of R. D. Keynes, N. G. Greeff, I. C. Forster and J. M. Bekkers.

or 5 ms at 5  $^{\circ}$ C, and delays the reappearance of the slowly rising phase until well over 1 ms has elapsed.

Although the effect on  $R_s$  compensation of bathing the axons in hyperosmotic solutions that had been reported by Stimers *et al.* (1987) could readily be confirmed, imperfect

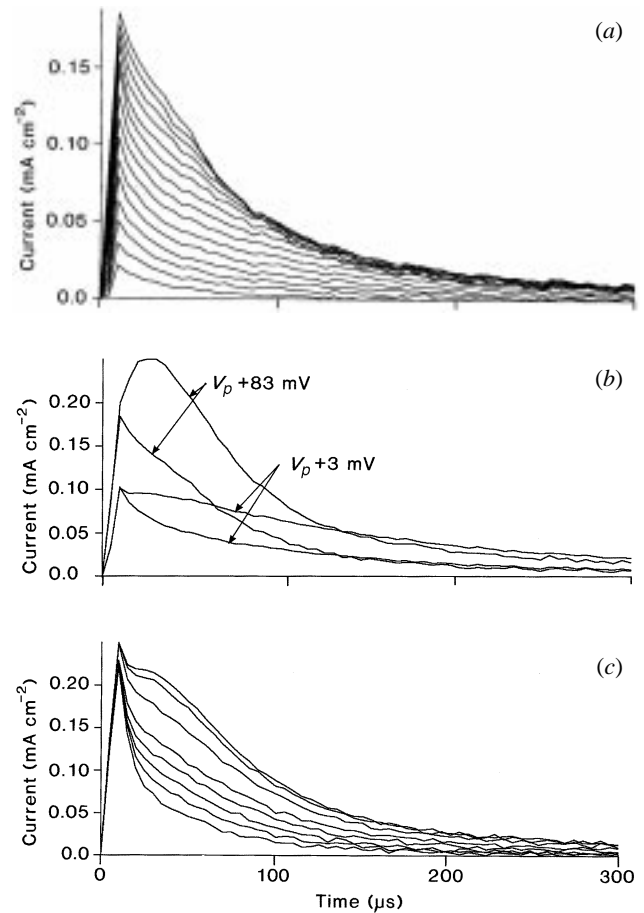


Figure 3. (a) Test pulses as in figure 1a applied after an inactivating prepulse to  $+3$  mV followed by 0.5 ms recovery at  $-80$  mV. (b) Superimposed records for the pulses to  $+3$  and  $+83$  mV from figures 1a and 3a. (c) Records of the gating current for a pulse to  $+63$  mV applied after a 20 ms inactivating prepulse to  $+3$  mV followed by recovery at the holding potential of  $-80$  mV for 0.05, 0.1, 0.2, 0.5, 1, 2.5, 5 and 10 ms. Temperature 10  $^{\circ}$ C. A total of eight sweeps were averaged. Other conditions as for figure 1a. Data file L04nov.009 of R. D. Keynes, N. G. Greeff and I. C. Forster.

compensation could not account for the slowly rising phase clearly visible in figures 1a and 2, because the settling time of the voltage clamp in our experiments was not more than 10  $\mu$ s, and since with the sampling period reduced to 3  $\mu$ s the earliest and very rapidly relaxing component of the gating current described by Forster & Greeff (1992) could be well recorded, there was no reason to question the accuracy of our records of the slowly rising phase. A part explanation for the conclusions of Stimers *et al.* (1987) may be provided by the experiment illustrated in figure 4, which shows clearly how arrival at the final shoulder of the slowly rising phase at the end of the initial plateau was increasingly delayed by application of a negative prepulse preceding the test pulse. The same phenomenon may be seen in fig. 13c of Keynes (1994), and can also be observed in fig. 2 of Taylor & Bezanilla (1983), although their results are plotted on a longer timescale, and on this occasion they were not applying any  $R_s$  compensation. At the holding potential of  $-70$  mV used by Stimers *et al.* (1987), the delay in reaching the peak of the slowly rising phase would have been appreciably less obvious than it was at  $-80$  mV as in figures 1a and 2.

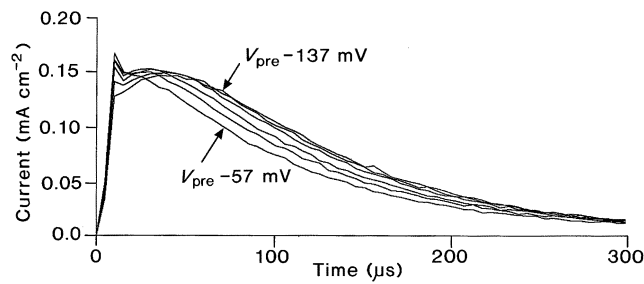


Figure 4. Superimposed family of gating currents recorded from an axon dialysed with 350 mM TMAF +200 mM sucrose, and bathed in 1/5 Na Tris SW +1  $\mu$ M TTX. 20 ms negative prepulses to  $-57$ ,  $-67$ ,  $-77$ ,  $-97$ ,  $-117$  and  $-137$  mV were followed by a test pulse to  $+23$  mV. Holding potential  $-80$  mV. Back reference pulses  $-147$  to  $-177$  mV. Sampling period  $5 \mu$ s per point. Bessel filter bandwidth 100 kHz. Temperature  $10^\circ\text{C}$ . Number of sweeps averaged was 32. Data file L27oct.002 of R. D. Keynes, N. G. Greeff and I. C. Forster.

Furthermore, there was appreciable variation between individual axons in the amplitude of the slowly rising phase, and the failure of Keynes *et al.* (1990) to report its existence can be attributed to its relatively small size in data file L07nov.007, as may be seen in fig. 1 of their paper and fig. 32a of Keynes (1994).

Figure 5 shows typical semi-logarithmic plots against pulse potential of the relaxation time constants  $\tau_2$  and  $\tau_3$  obtained by fitting double exponentials to the gating current family of figure 2 and another one. The slower component was invariably large enough to rule out an acceptable fit by a single exponential. As reported by Keynes *et al.* (1990) and Keynes (1994),  $\tau_3$  was roughly five times greater than  $\tau_2$  for potentials between  $-150$  and  $-30$  mV, when the slowly rising phase of the gating current was absent, and the fits could be started within  $15 \mu$ s of applying the test pulse. Though for potentials between  $-80$  and  $-30$  mV, the steepness of both the  $\tau$  curves was not far from the  $e$ -fold decline in 48 mV that for an electrically symmetrical system would correspond to a transfer of unit charge, for more negative pulses the slopes were appreciably reduced. However, this is the expected consequence of the occurrence of negative cooperativity between the voltage-sensors over this potential range that will be described below. For potentials above  $-30$  mV, when the channels began to open, the advent of the slowly rising phase resulted in a delay in reaching the peak of the gating current that made it impossible to fit double exponentials without considerably delaying their start. However, the semi-logarithmic plots of the time constants for positive potentials, starting the fits  $120 \mu$ s after applying the test pulse, then fell on excellent straight lines that in figure 5 declined  $e$ -fold in 59 mV for  $\tau_2$ , and in 48 mV for  $\tau_3$ . In nine experiments of this kind done at  $5^\circ\text{C}$ , in which  $\tau_2$  was fitted for pulses up to  $+100$  mV, the mean ( $\pm$ s.e.) of the slope for  $\tau_2$  was  $53 \pm 1$  mV, whereas for  $\tau_3$  fitted between  $+50$  and  $+100$  mV it was  $49 \pm 3$  mV. In six similar experiments at  $10^\circ\text{C}$ , the slopes for an  $e$ -fold change were  $58 \pm 3$  mV for  $\tau_2$  and  $51 \pm 4$  mV for  $\tau_3$ . These figures are consistent with a charge of  $1 e_0$  and an approximately symmetrical field, though for  $\tau_3$  they might suggest that there was a slight change in asymmetry corresponding to an increase in the effective charge. It has

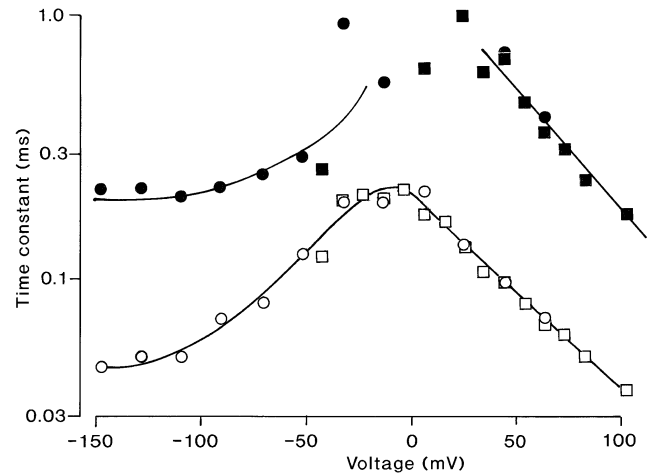


Figure 5. Semi-logarithmic plots of the voltage-dependence of the time constants of the rapidly relaxing (open symbols) and slowly relaxing (filled symbols) components of the sodium gating current at  $5^\circ\text{C}$ . Between  $-150$  and  $-20$  mV double exponentials were fitted to the experimental data starting at a point  $15 \mu$ s after application of the test pulse. Above  $-20$  mV the initial peak was delayed, and the fits were therefore started at the point where the relaxation was fastest. Circles show results from the experiment illustrated in figure 2. Squares show results from data file K09oct.009, when conditions were the same except that the axon was dialysed with 350 mM of CsF.

nevertheless to be remembered that the currents observed experimentally are not the product of a single transition, but of an appreciable number that are coupled sequentially. Their voltage-dependence is inevitably liable to some distortion from the cooperativity between the voltage-sensors that has to be invoked to account for the slowly rising phase of the current, and this could account for the reduced slope displayed by  $\tau_2$  for potentials below  $-80$  mV and also for the small increase in the slope of  $\tau_3$  for the largest test pulses. Although the rapidly relaxing component of the current generated by the two transitions that preceded the channel opening had declined to about one-third before starting the fits, the fitted charge arose mainly from the  $R \rightarrow P$  and  $P \rightarrow A$  transitions in domains I, II, III and IV. The  $\tau_3$  component can be attributed to the  $R \rightarrow P$  and  $P \rightarrow A$  transitions in all four domains that were most slowed down by the negative cooperativity, added to the third and slower  $A \rightarrow B$  transitions in domains I, II and III, and the  $A \rightarrow I$  transition of IVS4 (Keynes & Elinder 1998). Hence it may safely be concluded that in all four domains the charge carried by each of the three successive transitions is close to  $1 e_0$ .

#### (b) *The kinetics of the slowly rising phase*

It was pointed out by Armstrong & Gilly (1979), as is now generally accepted, that in a sequentially coupled system the occurrence of a slowly rising phase indicates that the first transition from the most closed state to the next one must be slower or less charged than the subsequent transitions. Thus, in equation (4) the forward rate constants (a) for the  $R \rightleftharpoons P$  steps would be slower than the forward rate constants (c) for the  $P \rightleftharpoons A$  steps. However, it is necessary in addition to explain how it is that the slowly rising phase makes its presence clear only for test

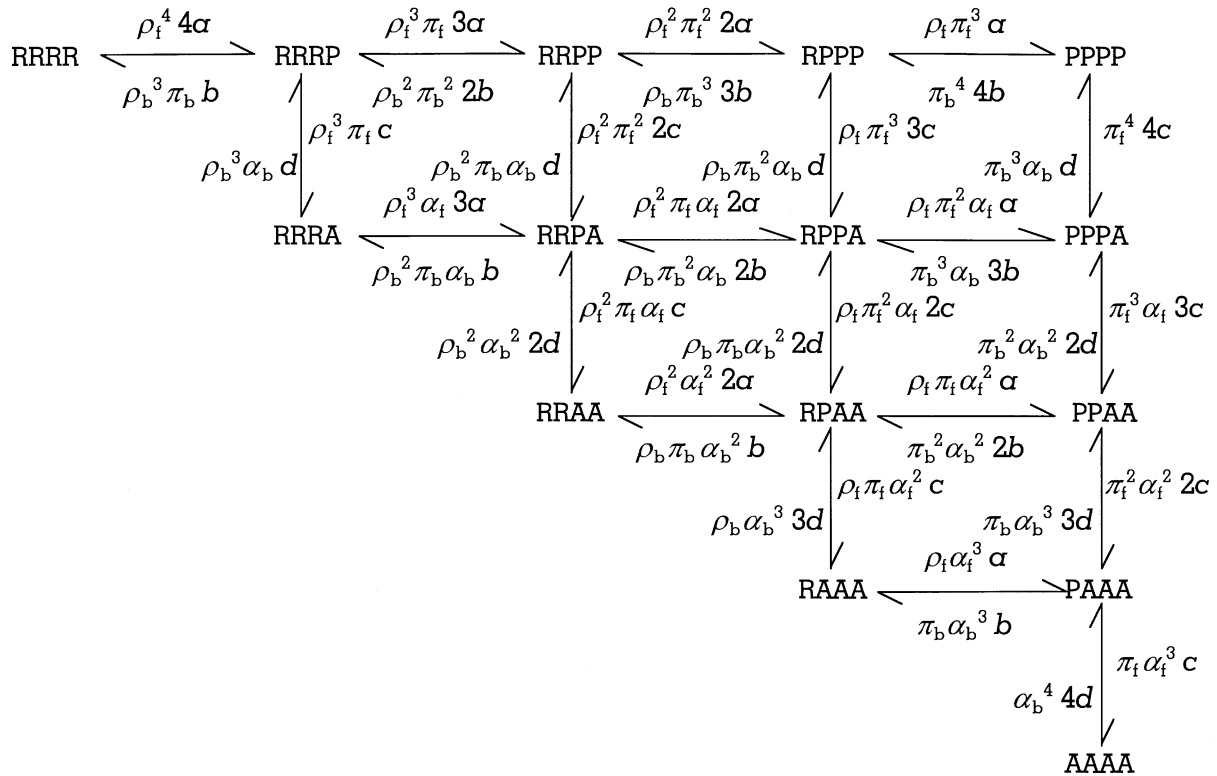


Figure 6. Extended state diagram for computation of the kinetics of a series-parallel system in which four S4 voltage-sensors arranged side-by-side make transitions between three states R, P and A. For the present purpose the S4 units are assumed to be identical, with forward and backward rate constants a and b for R $\rightleftharpoons$ P, and c and d for P $\rightleftharpoons$ A, expressed as in equations (1) and (2). The cooperativity factors  $\rho_f$ ,  $\rho_b$ ,  $\pi_f$ ,  $\pi_b$ ,  $\alpha_f$  and  $\alpha_b$  were derived as described in the text.

potentials above about  $-17$  mV, and is entirely absent for large negative potentials. Although by suitable adjustment of the equilibrium potentials ( $V_{eq}$ ), symmetry factors ( $\beta$ ) and rate constants ( $k_{eq}$ ) for four equal and independent two-step transitions it might be possible in theory to fit the observed families of gating currents, this could not be reconciled with the values of time constants plotted in figure 5, requiring that the symmetry factors should be 0.2 rather than 0.5, and failing to predict at all correctly either the gating currents for large negative pulses shown in figure 2, or the effect of changing the holding potential shown in figure 4.

We therefore decided to investigate the behaviour of the 15-state model constituted by four three-state S4 voltage-sensors arranged in parallel as in the extended state diagram illustrated in figure 6, when there is cooperativity between the neighbouring sensors so that their movements are not wholly independent and each may be slightly speeded up or slowed down by the others. If it is assumed that one S4 affects the others equally, there will be 12 possible cooperativity factors, because there are three different positions (R, P and A) and four different transitions (R $\rightarrow$ P, P $\rightarrow$ R, P $\rightarrow$ A and A $\rightarrow$ P) to be taken into account. To make the system more tractable, it could reasonably be assumed that one S4 being in a particular position affects the forward transitions R $\rightarrow$ P and P $\rightarrow$ A to equal extents, as also the backward transitions P $\rightarrow$ R and A $\rightarrow$ P. This left us with the six cooperativity factors denoted  $\rho_f$ ,  $\pi_f$ ,  $\alpha_f$ ,  $\rho_b$ ,  $\pi_b$  and  $\alpha_b$  in the state diagram shown in figure 6. If, as seems most likely,  $\rho_f = \alpha_b$ ,  $\pi_f = \pi_b$  and  $\alpha_f = \rho_b$ , a maximum of three cooperativity factors

remain to be fitted. The alternative, that  $\rho_f = \rho_b$ ,  $\pi_f = \pi_b$  and  $\alpha_f = \alpha_b$  was also tried, but with less success.

We first examined the behaviour of the system if the cooperativity factors were taken to be voltage-independent. If the symmetry factor  $\beta$  was fixed at 0.5, no good fits could be obtained at all, even if six parameters were permitted to be free, namely  $V_{eq}$  for R $\rightleftharpoons$ P, henceforth written as  $V_{RP}$ , and similarly  $V_{PA}$ ,  $k_{RP}$  and  $k_{PA}$ , together with two cooperativity factors  $CF_1$  and  $CF_2$  as above, noting that  $CF_3$  could be described as a function of the other parameters. If  $\beta$  was allowed to be free, fairly good fits for the family of gating currents shown in figure 1 could be obtained, but the predictions for negative gating currents and the effect of negative prepulses were not acceptable.

The alternative was to suppose that the cooperativity factors were voltage-dependent. This could suitably be expressed by the equation

$$CF_x = \exp((V - V_{eq})/s_x), \tag{5}$$

where  $s_x$  is a slope factor. The next step had to be a further reduction of the number of free parameters, and because of the structural uniformity of the screw-helices it seemed reasonable to set the equilibrium potentials to be the same for the transitions R $\rightleftharpoons$ P and P $\rightleftharpoons$ A and for equation (5). This reduced the free parameters to six, that is  $k_{RP}$ ,  $k_{PA}$ ,  $V_{eq}$ ,  $s_1$ ,  $s_2$  and  $s_3$ . In preliminary tests  $k_{RP}$  then turned out to be very similar in size to  $k_{PA}$ , whereas  $s_1$  (the slope factor for  $\rho_f$  and  $\alpha_b$ ),  $s_2$  (for  $\pi_f$  and  $\pi_b$ ), and  $s_3$  (for  $\alpha_f$  and  $\rho_b$ ) were similar in size but not in sign,  $s_1$  and  $s_2$  being negative, and

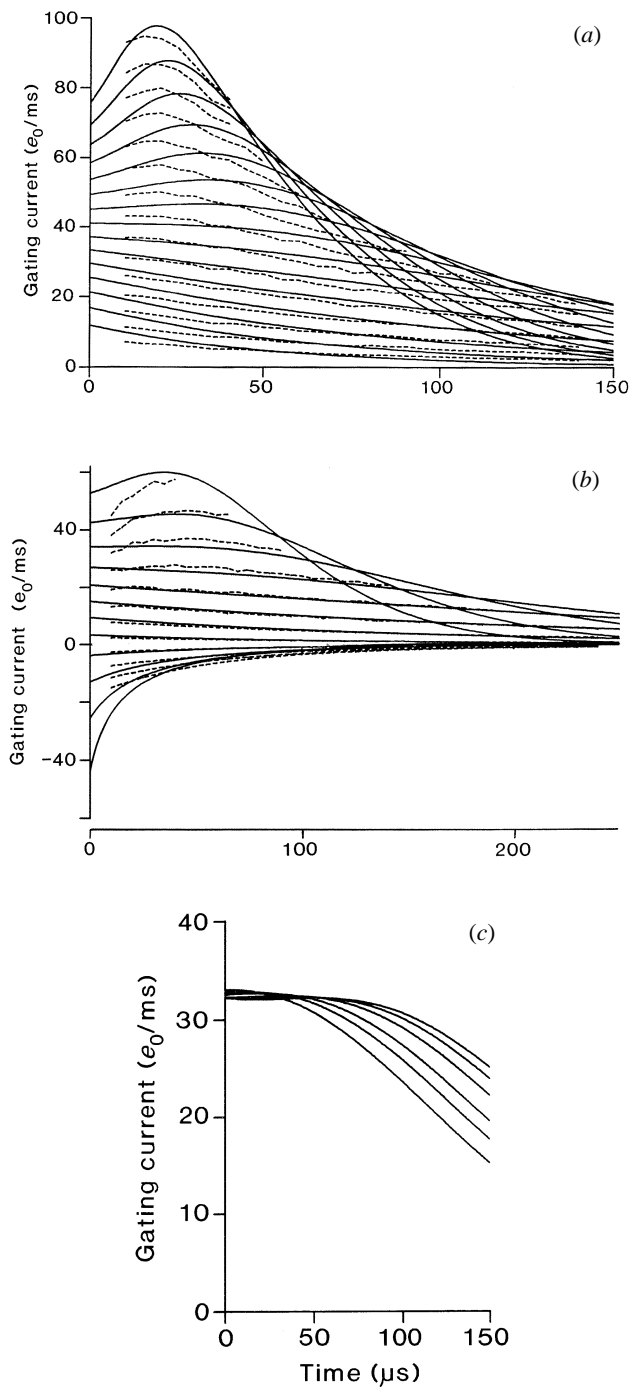


Figure 7. Least-squares best fits, plotted as continuous lines, of experimentally observed gating currents for the period before the channel begins to open, plotted as dashed lines. (a) Families of data for L24oct.008 as seen in figure 1a. (b) Families of data for I16nov.004 as seen in figure 2. (c) Simulations of the effect of pulses to +23 mV following negative prepulses to potentials between  $-57$  and  $-137$  mV as seen in figure 4, using the mean parameters shown in table 1b.

$s_3$  being positive. Taking advantage of these equalities, the free parameters finally remaining to be fitted were  $k_{eq}$ ,  $V_{eq}$  and the common slope, either positive or negative. The resulting best fits for L24oct.008 and I16nov.004 are illustrated in figure 7a,b, and the parameters for 17 families of gating currents recorded from 13 different axons are shown in table 1.

Although the fits were far from perfect, the time-courses of the computed gating currents varied with pulse potential in a qualitatively correct fashion in figure 7a and b, as they did in figure 7c when negative prepulses were applied. We were unable to devise any expression for the cooperativity factors, or any relaxation of the constraints that were imposed on the number of free parameters, that would perform better. Nor was there any obvious way of allowing for the variation in properties between the individual voltage-sensors that has been revealed by the studies of Kontis *et al.* (1997), in which neutralization of the fourth of the positive charges in IS4 and IIS4 shifted the voltage dependence of activation appreciably in the positive direction, whereas in IIIS4 the shift was negative and in IVS4 it was absent.

For the axons dialysed both with Cs and with TMA, it was clear that the nature of the dialysis had no significant effect on the fitted parameters, nor on the total transfer of charge. The overall means for the experiments in table 1a done at  $5^\circ\text{C}$  were  $k_{eq}=4.8\text{ ms}^{-1}$ ,  $V_{eq}=-39\text{ mV}$ , and slope factor = 320 mV. The means for the experiments done at  $10^\circ\text{C}$  were  $k_{eq}=9.1\text{ ms}^{-1}$ ,  $V_{eq}=-30\text{ mV}$ , and slope factor = 260 mV. Hence, as was expected, the equilibrium rate constant was strongly temperature-dependent, with a  $Q_{10}$  of the order of three as noted by Keynes (1994). The slight differences between the equilibrium potentials and the slope factors are probably attributable to the fact that the lower end of the potential range was  $-150\text{ mV}$  for the families of table 1a, but only  $-60\text{ mV}$  for the families of table 1b.

#### 4. DISCUSSION

It cannot be doubted that the slowly rising phase that we have shown in records of the sodium gating current in the squid giant axon to be responsible for the delay in arrival at the peak, and the consequent broadening of the peak, is a genuine phenomenon. Furthermore, it can be reasonably well simulated by our postulate of the occurrence of some voltage-dependent cooperativity between neighbouring S4 units for the transitions  $R\rightleftharpoons P$  and  $P\rightleftharpoons A$ . However, the questions arise as to what is the physical basis of such a voltage-dependent cooperativity, and whether it plays a physiological role of importance in other types of sodium channel.

Our central finding was that the occurrence of voltage-dependent cooperativity between the S4 voltage-sensors for their first two transitions, rather than voltage-independent cooperativity as suggested by Zagotta *et al.* (1994) in modelling the gating of  $K^+$  channels, was essential to explain the slowly rising phase of the gating current. This might conceivably arise from interactions between dipoles carried by the S4 units. Referring to the data in table 1a, the mean slope factor of 320 mV corresponds to a charge of  $0.075 e_0$  traversing the entire field, or a change in the symmetry factor  $\beta$  for the various transitions. For instance the transition  $PPPA\rightarrow PPAA$  shown in figure 6 has a cooperativity  $\pi_f^3 \alpha_f$ , which results in a change in  $\beta z$  from 0.5 to  $0.5+3(-0.075)+1(+0.075)=0.35$ . The cooperativity for the backward transition  $PPAA\rightarrow PPPA$  is  $\pi_b^2 \alpha_b^2$ , which results in a change of  $(1-\beta)z$  from 0.5 to 0.8, so that the operative gating charge of the  $PPPA\rightleftharpoons PPAA$  transitions becomes  $1.15 e_0$ . Hence the operative gating charge within

Table 1. Values for the equilibrium rate constants ( $k_{eq}$ ) and potentials ( $V_{eq}$ ), and for the cooperativity slope factors ( $s$ ) that gave least squares best fits to families of gating currents

data file no.	dialysis	channel density ( $\times 10^8 \text{ cm}^{-2}$ )	$k_{eq}$ ( $\text{ms}^{-1}$ )	$V_{eq}$ (mV)	slope factor (mV)
<b>(a) Experiments done at 5 °C</b>					
I14nov.016	TMA	163	5.6	-38	320
I16nov.004	TMA	189	5.6	-39	270
I16nov.011	TMA	171	4.6	-40	290
I20nov.010	Cs	142	5.4	-46	330
I20nov.014	TMA	170	4.0	-41	360
I27nov.001	Cs	139	3.4	-44	390
I27nov.002	Cs	139	4.9	-30	350
I27nov.004	Cs	139	3.5	-44	390
I27nov.006	TMA	150	3.8	-35	350
I27nov.014	TMA	184	4.5	-40	310
K09oct.009	Cs	165	7.3	-32	210
mean (TMA) $\pm$ s.e.		171 $\pm$ 6	4.7 $\pm$ 0.3	-39 $\pm$ 1	320 $\pm$ 10
mean (Cs) $\pm$ s.e.		145 $\pm$ 5	4.9 $\pm$ 0.7	-39 $\pm$ 3	330 $\pm$ 20
overall mean $\pm$ s.e.		159 $\pm$ 6	4.8 $\pm$ 0.3	-39 $\pm$ 2	320 $\pm$ 20
<b>(b) Experiments done at 10 °C</b>					
L11oct.006	TMA	172	7.5	-29	210
L13oct.001	TMA	174	8.6	-31	220
L20oct.008	TMA	184	7.8	-30	320
L24oct.008	TMA	166	9.5	-33	230
L03nov.001	TMA	174	8.9	-31	240
L07nov.007	TMA	136	12.0	-27	340
mean (TMA) $\pm$ s.e.		168 $\pm$ 7	9.1 $\pm$ 0.7	-30 $\pm$ 1	260 $\pm$ 20

Note that the slope factors for  $\rho_b$ ,  $\pi_b$ ,  $\pi_h$  and  $\alpha_b$  are negative. Files I20nov.010 and .014, and I27nov.001, .002, .004 and .006 were successive runs on the same axon.

the system of figure 6 might vary between 0.55  $e_0$  at the RRRR side and 1.45  $e_0$  at the AAAA side. This is borne out by the flattening off of the semi-logarithmic plots of the time constants  $\tau_2$  and  $\tau_3$  seen in figure 5 when the potential falls below -80 mV, though above the equilibrium potential the picture is considerably complicated by the additional gating current generated by the A $\rightarrow$ B transitions, and between +20 and +100 mV the slopes of the plots do correspond to a charge close to 1  $e_0$ .

In their high-resolution studies of the sodium gating current in crayfish giant axons, Starkus & Rayner (1991) found no evidence for any broadening of the initial peak of the gating current as large as that seen in squid, and the same is true for the frog node of Ranvier (Meves & Pohl 1990), although in both cases a slowly relaxing component that followed the fast one was sometimes seen. The gating current of rat brain sodium channels expressed in oocytes is hard to measure with similar time resolution, but the records of Conti & Stühmer (1989) do not appear to exclude the existence of a small rising phase. Only in the gating current records of Ichikawa *et al.* (1991) from the squids *Doryteuthis bleekeri* and *Loligo budo*, and of Armstrong & Gilly (1979) from *Loligo pealii*, would there appear to be a slowly rising component comparable in magnitude with that of the specimens of *Loligo forbesi* (and sometimes of *Loligo vulgaris*) on which we have worked. This might suggest that the type of cooperative interaction between neighbouring S4  $\alpha$ -helices that we have invoked

to explain our observations may be peculiar to squid. Nevertheless, it has been concluded by Stefani *et al.* (1994) in their close examination of the kinetics of the gating currents in *Shaker B* K<sup>+</sup> channels that a rising phase is present, so that although the phenomenon is not prominent in many species it may in fact be widespread.

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