isolate prevalence need to be carried out before the biological significance of the present results can be fully assessed. However, all six virus isolates from the New Guinea populations that we examined had a mutation specifically affecting the dominant EBNA4 epitope for A11-restricted CTL responses. Such viruses may have enjoyed a biological advantage in host populations where the HLA-A11 gene frequency is unusually high. At the same time, the polyclonal nature of the EBV-induced CTL responses would ensure that A11 epitope–loss isolates of EBV remain subject to CTL recognition through other less immunogenic epitopes so that a stable, but more relaxed, virus-host balance could still be achieved.

Observations on the pathogens malaria and human immunodeficiency virus (HIV) have provided an indication that HLA-restricted CTL responses may influence the evolution of the host-pathogen relation (*19–21*). That influence may be seen in the case of malaria as determining the distribution of HLA alleles retained in the population at risk (*19, 20*) and in the case of HIV as driving virus evolution in the infected host (*21*). Our results suggest that by examining human populations with limited HLA polymorphism, one may be able to address the same issues in the context of viruses (such as EBV) that are widespread, genetically stable, and not life-threatening to the immunocompetent host.

REFERENCES AND NOTES

1. D. Moss, A. Rickinson, J. Pope, *Int. J. Cancer* **23**, 618 (1978).
2. L. E. Wallace, A. B. Rickinson, M. Rowe, M. A. Epstein, *Nature* **297**, 413 (1982).
3. R. Gavioli *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5862 (1992).
4. R. Murray *et al.*, *J. Exp. Med.* **176**, 157 (1992).
5. R. Khanna *et al.*, *ibid.*, p. 169.
6. Single letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
7. R. Gavioli *et al.*, *J. Virol.* **67**, 1572 (1993).
8. U. Zimber *et al.*, *Virology* **154**, 56 (1986).
9. M. G. Masucci, unpublished observations.
10. P. Morris *et al.*, in *Histocompatibility Testing 1972*, J. Dausset and J. Colombani, Eds. (Munksgaard, Copenhagen, 1973), pp. 267–274.
11. K. Bhatia, C. Jenkins, M. Prasad, G. Koki, J. Lombange, *Hum. Biol.* **61**, 45 (1989).
12. S. Serjeantson, in *The Colonization of the Pacific. A Genetic Trail*, A. V. S. Hill and S. W. Serjeantson, Eds. (Clarendon, Oxford, 1989), vol. 7, pp. 120–173.
13. A 578-bp DNA fragment comprising the coding sequence for the 416–424 EBNA4 epitope was amplified by the polymerase chain reaction with biotinylated oligonucleotide primers. The biotinylated products were separated with Dynabeads M280–streptavidin (Dynal, Oslo, Norway) and denatured with 0.1 M NaOH. The single-stranded template was annealed at 65°C for 5 min with 5 pmol of the sequencing primers in 10 mM tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, and bovine serum albumin (0.1 mg/ml). The reactions were cooled at 37°C and incubated for 10 min. After further incubation for 30 min with 50 nM dATP, dCTP, dGTP, and fluore-dUTP (Boehringer Mannheim, Germany) and 5 U of T7 DNA polymerase (Pharmacia, Uppsala, Sweden), 6 mM dithiothreitol, and 45 mM NaCl were added. The reactions were then divided into four portions, mixed with one of the four dideoxynucleotides, and incubated for 5 min at 37°C. An equal volume of deionized formamide containing Blue dextran (5 mg/ml) was added, and the samples were loaded onto a 0.5-mm-thick 6% polyacrylamide–7 M urea gel and run on an A.L.F. DNA sequencer (Pharmacia, Uppsala, Sweden).
14. J. Sample *et al.*, *J. Virol.* **64**, 4084 (1990).
15. R. Salter and P. Cresswell, *EMBO J.* **5**, 943 (1986).
16. M. G. Masucci *et al.*, *Int. Immunol.* **4**, 1283 (1992).
17. Q.-J. Zhang, R. Gavioli, G. Klein, M. G. Masucci, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2217 (1993).
18. M. Davis and P. Björkman, *Nature* **334**, 395 (1989).
19. A. V. S. Hill *et al.*, *ibid.* **352**, 595 (1991).
20. A. V. S. Hill *et al.*, *ibid.* **360**, 434 (1992).
21. R. E. Phillips *et al.*, *ibid.* **354**, 453 (1991).
22. G. Miller and M. Lipman, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 190 (1973).
23. J. Pope, *Aust. J. Exp. Biol. Med. Sci.* **46**, 643 (1968).
24. G. Lenoir, M. Vuillaume, C. Bonnardel, in *Burkitt's Lymphoma. Human Cancer Model*, G. Lenoir, G. O'Connor, C. Olweny, Eds. (IARC Scientific, Lyon, 1985), vol. 60, pp. 309–318.
25. C. M. Rooney *et al.*, *J. Natl. Cancer Inst.* **77**, 681 (1986).
26. M. Rowe *et al.*, *Int. J. Cancer* **35**, 435 (1985).
27. P. A. Pizzo, I. T. Magrath, S. K. Chattopadhyay, R. J. Biggar, P. Gerber, *Nature* **272**, 629 (1978).
28. D. T. Rowe *et al.*, *EMBO J.* **5**, 2599 (1986).
29. U. K. Laemmli, *Nature* **227**, 680 (1970).
30. M. Rowe *et al.*, *J. Virol.* **63**, 1031 (1989).
31. S. Torsteinsdottir *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5620 (1986).
32. C. Hirs and S. Timasheff, Eds., *Enzyme Structure* (Academic Press, New York, 1977), vol. 47, pp. 501–617.
33. B. Dumas, *Clin. Chem.* **21**, 1159 (1975).
34. J. Colombani *et al.*, *Tissue Antigens* **20**, 161 (1982).

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Extracellular Access to the Na,K Pump: Pathway Similar to Ion Channel

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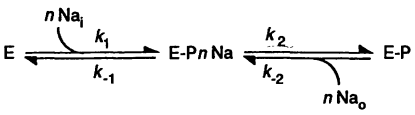
In each normal Na,K pump cycle, first three sodium and then two potassium ions are transported; in both cases, the ions become temporarily occluded in pump conformations that isolate them from internal and external solutions. A major charge movement occurs during sodium translocation and accompanies the deocclusion of sodium ions or their release to the cell exterior, or both. The nature of the charge movement was examined by measurement of the unidirectional sodium-22 efflux mediated by Na_i-Na_o exchange (Na_i and Na_o are internal and external sodium ions) in voltage-clamped, internally dialyzed squid giant axons in the absence of potassium; in this way the pump activity was restricted to the sodium-translocation pathway. Although electroneutral, the Na_i-Na_o exchange was nevertheless voltage-sensitive: increasingly negative potentials enhanced its rate along a saturating sigmoid curve. Such voltage dependence demonstrates that the release and rebinding of external sodium is the predominant charge-moving (hence, voltage-sensitive) step, suggesting that extracellular sodium ions must reach their binding sites deep in the pump molecule through a high-field access channel. This implies that part of the pump molecule is functionally analogous to an ion channel.

To probe the charge-moving steps in the Na-translocation pathway, we measured the voltage sensitivity of ^{22}Na efflux mediated by the electroneutral Na_i-Na_o exchange mode (*1*) of the pump. Pumped ^{22}Na efflux and current were determined in voltage-clamped, internally dialyzed squid giant axons as the components that are sensitive to dihydrodigitoxigenin (H_2DTG), a rapidly reversible specific inhibitor of the Na,K pump (*2, 3*). Pump-mediated ^{22}Na efflux was almost doubled by hyperpolarization from 0 to −60 mV (Fig. 1A). The accompanying pump currents were barely detectable at −60 mV and were still very small at 0 mV (Fig. 1B), confirming a predominance (>97% at −60 mV and ≥90% at 0 mV) of the electroneutral mode of Na_i-Na_o exchange.

Because the principal voltage-sensitive step is believed to occur late in the Na exit pathway (*4, 5*), the kinetic scheme below is sufficient to account for the influence of membrane potential on electroneutral Na_i-Na_o exchange:



(1)

where n is the apparent molecularity (6) of the interaction between Na_i or Na_o and each pump molecule (nonphosphorylated, E; phosphorylated, E-P). Voltage sensitivity arises because the first-order (7) rate constants k_i and k_{-i} (i = 1 or 2) may be exponential functions of membrane poten-

tial: as the potential becomes more negative, they may decrease $\{k_i = k_i^0 \exp[(1 - \delta_i)z_iU]\}$ or increase $[k_{-i} = k_{-i}^0 \exp(-\delta_i z_i U)]$ (*8*) with a steepness governed by z_i, which corresponds to a valence or equivalent charge, and by a symmetry factor δ_i ($0 \leq \delta_i \leq 1$), which apportions voltage dependence between forward and reverse transitions (*5*). Because Na_i-Na_o exchange obligatorily includes transitions through both steps in both directions, it must vanish if any of the four rate constants falls to zero or if either k_{-1} or k_2 becomes large enough to kinetically trap all the enzyme at one end of the reaction sequence. Thus, in general, the rate of any electroneutral exchange that involves charge movement is expected to show a bell-shaped voltage dependence, declining to zero at extreme positive and negative potentials (Table 1) (*9*).

We tested the voltage dependence of Na_i-Na_o exchange–mediated ^{22}Na efflux against these predictions. Because the background, H_2DTG-insensitive ^{22}Na efflux was practically unaffected by membrane potential over the entire range explored, the sharp reduction of ^{22}Na efflux accompanying a positive voltage shift from 0 to +30 mV, before addition of H_2DTG, and subsequent asymptotic increase in response to stepwise hyperpolarization to −75 mV (Fig. 2A) could be attributed to changes in the rate of Na_i-Na_o exchange. If the plateau that is approached near −50 mV, and apparently extends as far as −90 mV (Figs. 2A and 3B), were to be maintained at even more negative potentials, then voltage sensitivity of all rate constants other than k_{-2} could be ruled out (Table 1) [indeed, a least squares fit to the data at 400 mM Na_o (Fig. 3B) that permited voltage sensitivity of both k_2 and k_{-2} yielded a value of 0.95 for δ_2, indistinguishable from 1.0].

The sigmoid shape of the flux-voltage curve (Fig. 3B) therefore leads us to conclude that voltage sensitivity resides predominantly, if not exclusively, in the backward pseudo first-order rate constant $k_{-2} = k_{-2}^0 \exp(-zU)$ (Na_o rebinding), whereas the forward rate constant, k_2 (Na release), and all preceding rate constants (k_1 and k_{-1} in scheme 1) appear to be virtually independent of voltage. Because k_{-2}, but not k_2, is a function of the Na concentration ([Na]) at the binding site, this apparent asymmetry is readily explained if the Na_o-binding reaction occurs within the transmembrane electric field (Fig. 2B), so that the probability of each external Na ion binding to the pump is multiplied by a Boltzmann coefficient, $\exp(-\lambda U)$ [λ is the fraction of membrane voltage dropped between the external medium and the binding site (*10*)], as if the local [Na] at the binding site varies with membrane potential. In other words, extracellular Na ions must approach their binding sites through a narrow, high-field access channel in the pump molecule. No influence of voltage on Na release is observed because ion migration along the access channel, although voltage sensitive, is expected to occur orders of magnitude faster than the reaction that limits Na release (rate constant k_2).

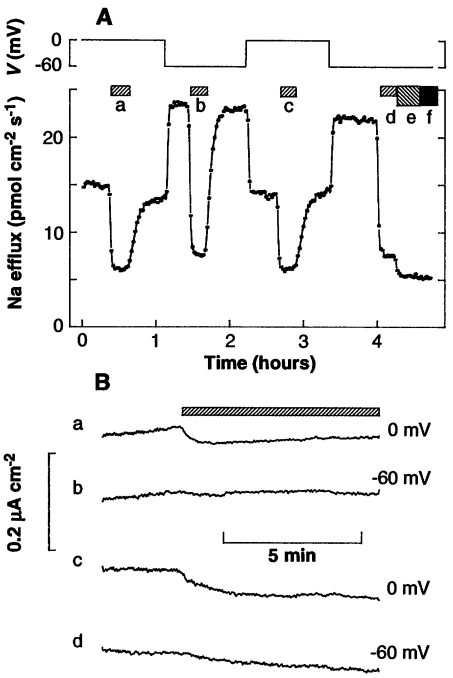


Fig. 1. Reversible stimulation of electroneutral Na_i-Na_o exchange by 60-mV hyperpolarization. (**A**) Addition of 10 μM dihydrodigitoxigenin (H_2DTG) (hatched bars) reduced ^{22}Na efflux into 400 mM Na, K-free seawater by only 8.8 (a) and 7.7 pmol cm^{-2} s^{-1} (c) at 0 mV, but by 15.7 (b) and 16.2 pmol cm^{-2} s^{-1} (d) at −60 mV. Subsequent exposure to 100 μM H_2DTG (e) and then 100 μM ouabain (a supramaximal dose) (f) demonstrates that 10 μM H_2DTG inhibited ~90% of total steroid-sensitive ^{22}Na efflux. Temperature, 21.0°C. (**B**) Small shifts in holding current (ΔI) upon application of 10 μM H_2DTG at 0 and −60 mV, from the experiment in (A). Values for ΔI were −0.035 and −0.043 μA cm^{-2} at 0 mV, reflecting inhibition of a small residual outward pump current, which was probably mediated by electrogenic $3Na_i$-$2Na_o$ exchange (*26*). At −60 mV, ΔI was even smaller, −0.010 and −0.013 μA cm^{-2}, despite the larger H_2DTG-sensitive ^{22}Na efflux.

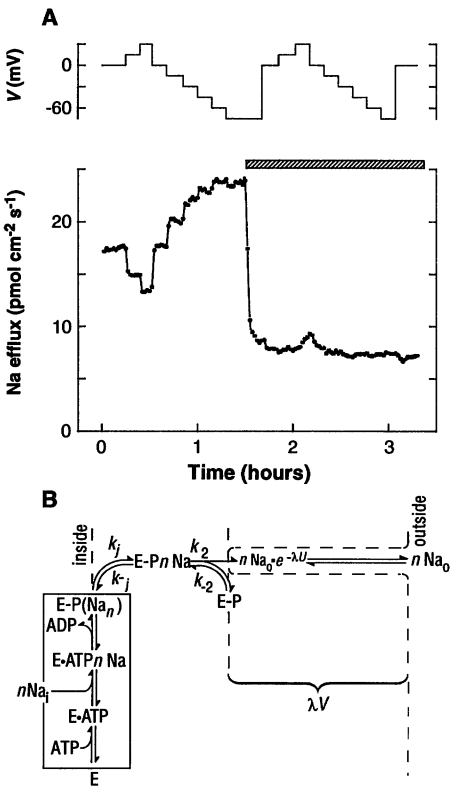


Fig. 2. Saturable voltage dependence of ^{22}Na efflux mediated by Na_i-Na_o exchange. (**A**) The membrane potential was varied between +30 and −75 mV in 15-mV increments (upper line) before and during exposure (hatched bar) to 100 μM H_2DTG, which caused no measurable ΔI (not illustrated). Temperature, 21.0°C. (**B**) Access-channel model for electroneutral Na_i-Na_o exchange dictated by the saturable voltage dependence of ^{22}Na efflux in (A). The simplified scheme lumps together enzyme forms (box) involved in the ATP- and Na_i-binding, phosphorylation, and occlusion reactions because $[Na]_i$, [ATP], and [ADP] were held constant in these experiments and because charge translocation is thought to accompany or follow deocclusion of the Na ions (*4, 5*). This model reduces to scheme 1 when the rate constants k_1, k_{-1}, and k_2 are voltage independent (k_1 and k_{-1} represent lumped values for all rate constants up to and including k_j and k_{-j}); the pseudo first-order reverse rate constant k_{-2} alone is voltage-sensitive ($\delta_2 = 1$) because external Na ions reach their binding sites a fractional distance λ into the membrane field, yielding $k_{-2} = k'_{-2}[Na]_o^n \exp(-n\lambda U)$, where k'_{-2} is a voltage-independent second-order rate constant (*27*).

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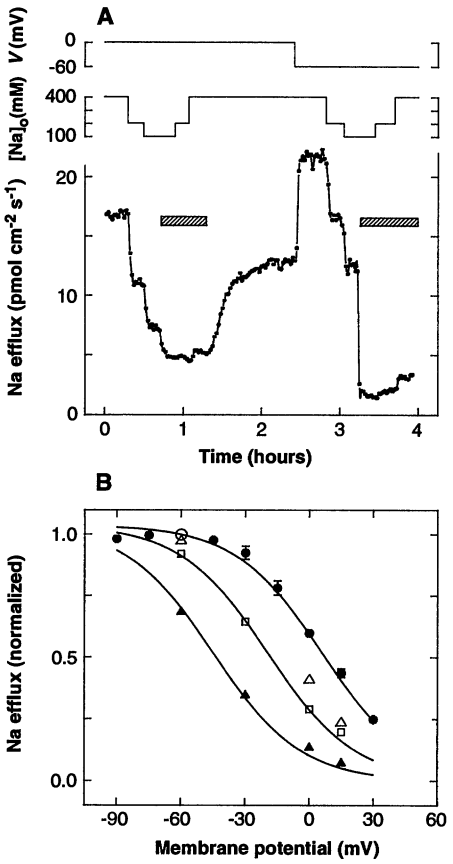

Fig. 3. Voltage-dependent stimulation of Na_i-Na_o exchange by Na_o. (**A**) The ^{22}Na efflux at 100, 200, and 400 mM Na_o, tested at 0 and −60 mV, without and with 100 μM H_2DTG (hatched bars). Temperature, 25.1°C. (**B**) Summarized relative flux-voltage data at four $[Na]_o$ from nine axons. Voltage dependence of H_2DTG-sensitive ^{22}Na efflux at 400 mM Na_o (●), normalized to the flux at −60 mV (○), from four runs in three axons (including that in Fig. 2A) at 20.9° to 21.4°C. In three of the runs, a repeat of the voltage steps after washout of H_2DTG allowed interpolation of flux values so we could correct for the slight rundown (≤5% per hour). Six other axons [including that in (A)] yielded, with the protocol of (A), the data at 100 (▲), 200 (□), and 300 (△) mM Na_o: four axons provided data at −60 mV, three at −30 mV, four at 0 mV, and one at +15 mV. For each axon, at a given voltage, the H_2DTG-sensitive ^{22}Na efflux values at the various $[Na]_o$ were normalized to the flux at 400 mM Na_o at that voltage. Interpolation permitted us to correct for rundown (~14 to 30% per hour at the higher temperatures, 23.0° to 25.2°C, in these Na_o dependence experiments) the flux values at 400 mM Na_o used for each normalization. All these individually normalized (by axon and by potential) data sets, including the 400 mM Na_o data, were simultaneously fit by least squares to the expression for unidirectional Na efflux for the model in Fig. 2B, $\Phi = \Phi_{max}/\{1 + [K^0_{0.5} \exp(\lambda U)]^n/[Na]^n_o\}$, and constrained to 1.0 at the reference point (V = −60 mV, $[Na]_o$ = 400 mM), yielding the three curves. For clarity, we omitted the curve (but not the data) at $[Na]_o$ = 300 mM. Least squares parameter values (± SD) were n = 1.80 ± 0.13, λ = 0.69 ± 0.04, and $K^0_{0.5}$ = 335 ± 8 mM.

The binding of Na ions within the membrane field should make a rise in $[Na]_o$ or a negative shift of membrane potential kinetically equivalent (*11*) because both enhance Na_o binding. This prediction was verified. Three $[Na]_o$ levels were tested without and with H_2DTG, first at 0 mV and then at −60 mV (Fig. 3A). The influence of $[Na]_o$ on ^{22}Na efflux through the pump was strong at both potentials, whereas the background ^{22}Na efflux (pump-inhibited by H_2DTG) was relatively independent of $[Na]_o$. At 0 mV, the pump-mediated ^{22}Na efflux was activated almost linearly by Na_o, whereas the larger efflux at −60 mV increased nonlinearly with $[Na]_o$ and approached saturation near 400 mM Na_o. A fit of the simplest access channel model (Fig. 2B) to all the data yielded a value of 0.69 for λ and showed that, as predicted, doubling $[Na]_o$ caused a parallel positive shift of the flux-voltage curve by an amount $(RT/\lambda F)$ ln2, which equals 26 mV in this instance (Fig. 3B) (*12*).

Voltage dependence of the apparent affinity for an ion has been observed previously for the proton pump (*13*), Na-Ca exchanger (*14*), and Na,K pump (*15*); also, access channel, or "ion well" (*5, 11, 13*), mechanisms were postulated. However, in general, voltage-induced shifts of apparent affinity for activation of electrogenic transport are, by themselves, insufficient to distinguish voltage dependence of the penultimate (or earlier) step from that of the final ion release and rebinding step (*16*). Kinetic analysis of the voltage dependence of an electroneutral exchange permitted that distinction to be made here, as also made recently for the red blood cell anion exchanger (*17*).

The strong apparent asymmetry of the voltage sensitivity echoes that of the relaxation rates of transient pump charge movements induced by voltage jumps, also in K-free solution (*18–20*). Asymmetric voltage sensitivity of rate constants is also a central feature of the simplest kinetic models that mimic the effect of voltage on the inhibition of forward Na,K exchange by Na_o (*21, 22*). Enhanced binding of Na_o on hyperpolarization thus readily accounts for the ability of Na_o or hyperpolarization not only to accelerate electroneutral Na_i-Na_o exchange (described here) and backward Na,K cycling (*19, 23*) but also to inhibit forward Na,K cycling (*2, 21*).

The fits to the data (Fig. 3B) indicate that more than half the transmembrane voltage drops between the exterior and the Na binding sites, implying that the Na,K pump molecule incorporates a structure functionally analogous to an ion channel. Whether it bears any relation to the ouabain-sensitive channels formed when Na,K pumps are exposed to palytoxin (*24*) or when bilayers are treated with partially purified Na,K pumps (*25*) remains to be determined.

Table 1. Predicted behavior, at extreme negative ($V = -\infty$) or positive ($V = +\infty$) membrane potentials, of the magnitude of the rate constants k_1, k_{-1}, k_2, and k_{-2} and of the relative unidirectional Na_i-Na_o exchange flux (Φ_{rel}) for scheme 1, calculated from

$$\Phi_{rel} = [1/k_1 + 1/k_{-1} + 1/k_2 + 1/k_{-2} + k_{-1}/(k_1k_2) + k_2/(k_{-1}k_{-2})]^{-1}$$

Symmetry factor δ_i stands for δ_1 (when $z_1 > 0$ and $z_2 = 0$) or δ_2 (when $z_1 = 0$ and $z_2 > 0$).

Symmetry factor	$z_1 > 0, z_2 = 0$			$z_1 = 0, z_2 > 0$		
	k_1	k_{-1}	Φ_{rel}	k_2	k_{-2}	Φ_{rel}
			$V = -\infty$			
$\delta_i = 0$	0	k^0_{-1}	0	0	k^0_{-2}	0
$0 < \delta_i < 1$	0	∞	0	0	∞	0
$\delta_i = 1$	k^0_1	∞	0	k^0_2	∞	finite
			$V = +\infty$			
$\delta_i = 0$	∞	k^0_{-1}	finite	∞	k^0_{-2}	0
$0 < \delta_i < 1$	∞	0	0	∞	0	0
$\delta_i = 1$	k^0_1	0	0	k^0_2	0	0

REFERENCES AND NOTES

1. F. Cornelius, *Biochim. Biophys. Acta* **1071**, 19 (1991).
2. R. F. Rakowski, D. C. Gadsby, P. De Weer, *J. Gen. Physiol.* **93**, 903 (1989).
3. Giant axons from the squid, *Loligo pealeii*, were internally dialyzed and voltage clamped, and ^{22}Na efflux and holding current were measured continuously as described (*2*). The standard artificial seawater was K- and Cl-free and contained 400 mM sodium isethionate, 75 mM calcium sulfamate, 1 mM 3,4-diaminopyridine, 5 mM tris-Hepes (pH = 7.7), and 0.2 μM tetrodotoxin. In seawaters with lowered [Na], *N*-methyl-*D*-glucamine (NMG) sulfamate replaced Na isethionate. The K- and Cl-free internal solution contained 43.7 mM Na Hepes, 91 mM NMG Hepes, 150 mM glycine, 50 mM phenylpropyltriethylammonium sulfate [to block K channels; C. M. Armstrong, *J. Gen. Physiol.* **58**, 413 (1971)], 5 mM tris ATP, 5 mM tris adenosine disphosphate (ADP), 15 mM Mg Hepes, 5 mM dithiothreitol, 1.25 mM Na_5-diadenosine pentaphosphate (to inhibit adenylate kinase), 0.1 mM sodium atractyloside (to inhibit mitochondrial nucleotide transport), and 2.5 mM BAPTA [1,2 bis(2-aminophenoxy) ethane *N,N,N',N'*-tetraacetic acid] [to minimize Na-Ca exchange; R. DiPolo and L. Beaugé, *J. Gen. Physiol.* **90**, 505 (1987)]. Osmolality of all solutions was 925 to 935 mos*m* kg^{-1}. Temperature was 21° to 25°C. The H_2DTG was added to seawater from a 0.1 M stock solution in dimethyl sulfoxide (DMSO). In controls, 0.1% DMSO did not alter ^{22}Na efflux or holding current. Further experiments confirmed that, under these conditions, as expected for electroneutral Na_i-Na_o exchange, the H_2DTG-sensitive ^{22}Na efflux was sensitive to removal of intracellular Mg or ADP [P. De Weer, *J. Gen. Physiol.* **56**, 583 (1970); I. M. Glynn and J. F. Hoffman, *J. Physiol. London* **218**, 239 (1971)] and was activated by relatively high $[Na]_o$ (Fig. 3) [P. J. Garrahan and I. M. Glynn, *ibid.* **192**, 159 (1967)].
4. P. De Weer, D. C. Gadsby, R. F. Rakowski, *Annu. Rev. Physiol.* **50**, 225 (1988).
5. P. Läuger, *Electrogenic Ion Pumps* (Sinauer, Sunderland, MA, 1991).
6. The apparent molecularity is experimentally determined as a Hill coefficient; it need not have the same numerical values for Na_i and Na_o.
7. k_1 and k_{-2} are pseudo first-order rate constants that incorporate $[Na]_i^n$ and $[Na]_o^n$, respectively.
8. Here, $U = VF/RT$, where V is membrane potential,

F is the Faraday constant, R is the gas constant, and T is absolute temperature; k_i^0 and k_{-i}^0 define values at zero membrane potential; z_i is known to be positive for Na translocation (*4, 5*).

9. As shown in Table 1, Na_i-Na_o exchange flux is predicted to saturate at extreme positive potentials if $\delta_1 = 0$ (only k_1 affected by potential) and extreme negative potentials if $\delta_2 = 1$ (only k_{-2} affected by potential) but to decline toward zero at extreme positive and negative potentials for all other values of δ_i. It can also be shown that (i) ^{22}Na efflux–voltage plots must be bell-shaped (flux vanishes at the voltage extremes) in all cases in which both steps are voltage sensitive ($z_1 > 0$ and $z_2 > 0$), regardless of the values of δ_i, and (ii) insertion of electroneutral transitions between steps one and two does not alter the results in Table 1, but (iii) insertion of a voltage-sensitive step, irrespective of the value of its δ, again causes the exchange to cease at both voltage extremes. In other words, only when k_{first} (Na_i binding) or k_{-last} (Na_o rebinding) is the sole voltage-sensitive step in the reaction chain will saturating exchange rates be observed at extreme potentials; all other models predict bell-shaped flux-voltage plots.
10. Voltage independence of k_1 and k_{-1} implies that no charge movement accompanies translocation of nNa ions through the remaining $(1 - \lambda)V$, presumably because equal but opposite charges are supplied by the ion binding sites (*4, 5*).
11. P. Mitchell, *Theor. Exp. Biophys.* **2**, 159 (1969).
12. The model in Fig. 2B yields a Hill equation for unidirectional Na efflux, $\Phi = \Phi_{max}/(1 + K$ [illegible] where Φ_{max} is unidirectional efflux at [illegible] or hyperpolarization and $K_{0.5}$ is the voltage-dependent, half-activating $[Na]_o$ given by $K_{0.5} = K_{0.5}^0 \exp(\lambda U)$, where $K_{0.5}^0$ is the value at 0 mV.
13. O. S. Andersen, J. E. N. Silveira, P. R. Steinmetz, *J. Gen. Physiol.* **86**, 215 (1985).
14. L. Lagnado, L. Cervetto, P. A. McNaughton, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4548 (1988); D. W. Hilgemann, D. A. Nicoll, K. D. Philipson, *Nature* **352**, 715 (1991).
15. R. F. Rakowski, L. A. Vasilets, J. LaTona, W. Schwarz, *J. Membr. Biol.* **121**, 177 (1991); L. A. Vasilets *et al.*, *J. Biol. Chem.* **266**, 16285 (1991); W. Stürmer, R. Bühler, H.-J. Apell, P. Läuger, *J. Membr. Biol.* **121**, 163 (1991).
16. In scheme 1, if hyperpolarization were to increase the ratio k_{-1}/k_1, the steady-state concentration of the intermediate E-PnNa would decline, and so the apparent affinity for Na_o would be enhanced.
17. M. L. Jennings, R. K. Schulz, M. Allen, *J. Gen. Physiol.* **96**, 991 (1990).
18. M. Nakao and D. C. Gadsby, *Nature* **323**, 628 (1986); D. C. Gadsby, M. Nakao, A. Bahinski, G. Nagel, M. Suenson, *Acta Physiol. Scand.* **146**, 111 (1992); R. F. Rakowski, *J. Gen. Physiol.* **101**, 117 (1993).
19. A. Bahinski, M. Nakao, D. C. Gadsby, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3412 (1988).
20. Although the voltage-induced transient pump currents (*18, 19*) relax relatively slowly (~40 s^{-1} at 0 mV and 20°C), they are still readily explained by our model (Fig. 2B) provided that the rate constants for occlusion and deocclusion (k_1 and k_{-1}) and for Na_o rebinding and release (k_{-2} and k_2) have appropriate magnitudes. According to our model, a voltage jump is expected to cause a virtually instantaneous charge movement (obscured by the membrane-capacitance current) as Na ions migrate along the access channel in response to the field change. The ensuing redistribution of enzyme intermediates subsequent to Na_o rebinding or release would induce a much slower, hence detectable, charge transient reflecting further Na-ion movement along the access channel, now rate-limited by the conformational changes [P. Läuger and H.-J. Apell, *Biochim. Biophys. Acta* **944**, 451 (1988)].
21. M. Nakao and D. C. Gadsby, *J. Gen. Physiol.* **94**, 539 (1989).
22. P. De Weer, in *Regulation of Potassium Transport Across Biological Membranes*, L. Reuss, G. Szabo, J. M. Russell, Eds. (Univ. of Texas Press, Austin, 1990), pp. 5–28.
23. P. De Weer, D. C. Gadsby, R. F. Rakowski, in *The Na^+,K^+-Pump, Part A: Molecular Aspects*, J. C. Skou, J. G. Nørby, A. B. Maunsbach, M. Esmann, Eds. (Liss, New York, 1988), pp. 421–434.
24. S. Y. Kim, C. H. Wu, L. Béress, in *The Sodium Pump: Recent Developments*, J. H. Kaplan and P. De Weer, Eds. (Rockefeller Univ. Press, New York, 1991), pp. 505–508.
25. B. M. Anner, *Biochim. Biophys. Acta* **832**, 335 (1985).
26. The small pump current at 0 mV is unlikely to reflect Na,K exchange because K contamination of the nominally K-free seawater was ~25 μM whereas, under these conditions, the half-activating $[K]_o$ is 4 mM (*2*), but it is presumably mediated by electrogenic $3Na_i$-$2Na_o$ exchange [K. H. Lee and R. Blostein, *Nature* **285**, 338 (1980); H.-J. Apell, V. Häring, M. Roudna, *Biochim. Biophys. Acta* **1023**, 81 (1990)]. In all such experiments, H_2DTG-sensitive ^{22}Na efflux and ΔI averaged 14.0 ± 1.1 pmol cm^{-2} s^{-1} and −0.010 ± 0.003 μA cm^{-2} (±SEM, $n = 7$), respectively, at −60 mV, and 9.5 ± 0.6 pmol cm^{-2} s^{-1} and −0.034 ± 0.003 μA cm^{-2} ($n = 8$) at 0 mV, indicating that even at 0 mV, electrogenic $3Na_i$-$2Na_o$ exchange could have contributed, on average, ≤10% to the H_2DTG-sensitive ^{22}Na efflux.
27. The model is reduced to scheme 1 by setting $k_{-2}^0 = k'_{-2}[Na]_o^n$ and $z_2 = n\lambda$.
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Developmental Regulation of Neural Response to FGF-1 and FGF-2 by Heparan Sulfate Proteoglycan

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Murine neural precursor cells and cell lines derived from them are stimulated by members of the heparin-binding fibroblast growth factor (FGF) family. The activity of FGF is regulated by heparan sulfate proteoglycans (HSPGs), and this interaction is an essential prerequisite for the binding of growth factor to the signal transducing receptors. Messenger RNA for FGF-2 was detectable in the neuroepithelium at embryonic day 9, and the HSPGs produced by these cells at this time preferentially bound FGF-2. However, at embryonic day 11, when messenger RNA for FGF-1 was first detectable, there was a switch in the binding specificity of the HSPG to FGF-1. Thus, a single species of HSPG undergoes a rapid, tightly controlled change in growth factor–binding specificity concomitant with the temporal expression of the FGFs.

The mammalian central nervous system is developmentally derived from the cells of the neural tube. The heparin-binding growth factors FGF-1 and FGF-2 are potent developmental regulators of the proliferation, migration, and differentiation of murine neural precursor cells (*1*). FGF activity is regulated in part by heparin analogs such as the glycosaminoglycan (GAG) heparan sulfate (*2*). HSPGs are a highly diverse group of macromolecules, each of which consists of a core protein to which highly sulfated GAG side chains of heparan sulfate are covalently attached (*3*). They are ubiquitous constituents of mammalian cell surfaces and of most extracellular matrices, including the specialized basal laminae that surround neural tissue (*2, 4*). FGF binding to HSPGs appears to offer protection against proteolytic degradation, thus creating a reservoir of growth factor in tissues (*5*). More recent studies have demonstrated that HSPG-FGF interaction is an essential prerequisite for the presentation and subsequent binding of growth factor to signal transducing receptors (*6*). However, the timing of expression of the FGFs and HSPGs and the interactions between them during the early proliferative phases of brain development remain largely unknown.

To determine the precise timing of the expression of FGF-1 and FGF-2 during neural development, we performed specific amplification of the mRNA sequences for these factors on mesencephalic and telencephalic neuroepithelial tissue at embryonic days 9, 10, 11, and 13 (E9, E10, E11, and E13) by polymerase chain reaction (PCR). The two factors were expressed in different temporal patterns: FGF-2 mRNA was readily detectable at E9 and throughout subsequent ages, but FGF-1 mRNA was detectable only from E11 on (Fig. 1). Immunohistochemical studies with antibodies to FGF support this differential pattern of expression (*7*). A similar differential expression of the FGFs was observed in a cell line originally derived from E10 neuroepithelium, 2.3D (*1*). At low cell density, this cell line expressed only FGF-2 mRNA. In contrast, when these cells were maintained

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