GEOGRAPHICAL DISTRIBUTION AND INACTIVATION KINETICS IN INTERNALLY PERFUSED *MYXICOLA* GIANT AXONS

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ABSTRACT In some preparations the time constant of Na current inactivation determined with two pulses (τ_c) is larger over some range of potentials than that determined from the current decay during a single pulse (τ_h), while in others $\tau_c(V)$ and $\tau_h(V)$ are the same. *Myxicola* giant axons obtained from specimens collected from coastal waters of northeastern North America display a $\tau_c - \tau_h$ difference under all conditions we have tested. In these axons $\tau_c(V)$ and $\tau_h(V)$ are unchanged by reduction of Na current density, addition of K-channel blockers, or internal perfusion. Specimens of the same species, *Myxicola infundibulum*, collected from a different geographical location, the south coast of England, have been studied under internal perfusion with K as the major cation internally, with reduced external Na concentration and in the presence of K-channel blockers. In these axons $\tau_c(V)$ and $\tau_h(V)$ approximately superpose, raising the possibility that dramatic differences in Na current kinetics may not necessarily reflect basic differences in the organization of the Na channel gating machinery.

INTRODUCTION

In some preparations the time constant of Na current inactivation as determined with one pulse (τ_h) or two pulse (τ_c) methods differs when compared at the same potential with τ_c being larger especially at more negative conditioning potentials. This is the case for the giant axons of *Myxicola* (Goldman and Schauf, 1973; Goldman and Kenyon, 1982), lobster (Oxford and Pooler, 1975), and the crab, *Cancer*, (Connor, 1976), for cultured heart cells (Ebihara and Johnson, 1980), and for neuroblastoma cells as determined by recording currents through single Na channels with the patch clamp method (Nagy and Hof, 1984). A $\tau_c - \tau_h$ difference has also been reported for Ca currents recorded from *Helix* neurons (Akaike et al., 1978) and muscle fibers from the stick insect, *Carusius*, (Ashcroft and Stanfield, 1982). In other preparations, for example the giant axons of squid (Bezanilla and Armstrong, 1977; Gillespie and Meves, 1980) and crayfish (Bean, 1981) and in frog myelinated fibers (Chiu, 1977), τ_c and τ_h are the same over the whole potential range examined. The observation of a $\tau_c - \tau_h$ difference is of interest as of itself it rules out models in which activation and inactivation are independent and appear as a product. There are other inactivation kinetic differences between preparations. For example, a delay in the development of inactivation, which indicates a precursor process, is well developed in crayfish (Bean, 1981) and *Myxicola* (Goldman and Kenyon, 1982; Goldman and Ebert, 1984) axons, but very small in squid (Gillespie and Meves, 1980).

All of the *Myxicola* experiments showing the $\tau_c - \tau_h$ difference (see above and also Schauf and Davis, 1975; Schauf et al., 1976; Bullock and Schauf, 1978; Schauf and Bullock, 1978) were done on specimens collected from coastal waters of northeastern North America. We report here that specimens of the same species, *Myxicola infundibulum*, collected from a different geographical location, the southern coast of England, do not show any significant $\tau_c - \tau_h$ difference when studied under the same conditions as for the previous studies.

METHODS

Myxicola infundibulum were obtained either from Marine Research Associates, St. Andrews, New Brunswick, Canada or from the Laboratory of the Marine Biological Association of the United Kingdom, Citadel Hill, Plymouth, United Kingdom. Results from animals from these two sources are presented separately and identified as deriving from either North American *Myxicola* or Plymouth *Myxicola*. On examination we were unable to distinguish animals from the two sources either when intact or dissected open.

Methods for preparing and electrically recording from all axons were as in Binstock and Goldman (1969). Normal artifical sea water (ASW) had the following composition: 440 mM Na, 10 mM Ca, 50 mM Mg, 560

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mM Cl, 5 mM Tris (Tris (hydroxymethyl) aminomethane; Sigma Chemical Co., St. Louis, MO), pH 8.0 ± 0.1. Temperature was 5° ± 0.5°C. All potentials are reported as absolute membrane potential (inside minus outside) and have been corrected for liquid junction potentials as described by Ebert and Goldman (1975). Internal perfusion was established using the KCl-axoplasm dispersal method of Goldman and Kenyon (1979). Standard internal perfusate had the following composition: 410 mM K, 50 mM F, 360 mM glutamate, 1 mM HEPES (*N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid; Calbiochem-Behring Corp., San Diego, CA), 4 mM EGTA (ethyleneglycol-bis-(β-amino-ethyl ether) *N*, *N'*-tetra acetic acid; Sigma Chemical Co.), 145 mM sucrose, pH 7.30 ± 0.05. Internal perfusates always had an osmotic pressure within 5% of that of ASW as determined by osmometer (osmette A, Precision System Inc., Sudbury, MA).

All voltage clamp observations were made using compensated feedback to reduce errors produced by the series resistance, R_s. The new, faster, voltage clamp used in the Plymouth axon experiments also included an adjustable differentiating circuit in the R_s compensation loop to reduce the phase shift produced by R_s compensation, and so improve the clamp settling time. In practice feedback compensation was first adjusted to the maximum possible without the clamp going into oscillation, to about two-thirds of the measured R_s (Goldman and Schauf, 1972), the differentiating circuit was then activated, and R_s compensation increased somewhat further. This will leave ~1–3 Ωcm² of residual uncompensated R_s (Ebert and Goldman, 1976; Binstock et al., 1975). Clamp settling time was ~20 μs. To further reduce R_s errors all observations were made in bathing media with the Na concentration reduced, by substitution with Tris, to ½ that in ASW. The largest net inward Na currents encountered in these experiments were ~0.65 mA/cm² for a maximum displacement in membrane potential produced by R_s of 2 mV and generally well <1 mV.

Pulses sent to the voltage clamp were formed by a PDP 11/34 computer (Digital Equipment Corp., Maynard, MA). Membrane current records were lightly filtered (100 KHz) with a four pole Bessel filter (ITHACO 4302 dual electronic filter, ITHACO Inc., Ithaca, NY), digitized at a 10 or 20 μs sampling interval with 12-bit accuracy using a Datel ADC-EH12B3 analog to digital converter (Datel Systems, Inc., Canton, MA), and stored on floppy disks for later analysis. Na currents were extracted by repeating, for each axon, the entire voltage clamp protocol in the presence of 1 μM tetrodotoxin (TTX, Calbiochem-Behring Corp.) and subtracting the two sets of records with the aid of the PDP 11/34.

Methods for the North American *Myxicola* perfused experiments were very similar. As for all the results discussed here, R_s compensation was also used, and the maximum displacement in membrane potential produced by the residual uncompensated R_s in the reduced Na ASW used was again 2 mV, ranging down to <1 mV. Na currents were again extracted with TTX subtraction. For these experiments pulses sent to the voltage clamp were also formed by the PDP 11/34, but the analysis was from photographic records. To perform data analysis in the same way for both North American and Plymouth experiments, all of the Plymouth τ_h and τ_c determinations reported here were made on (TTX subtracted) current records printed out on an Hewlett-Packard X-Y recorder equipped with a 17012C point plotter (model 7034A; Hewlett-Packard Co., San Diego, CA), proceeding as with photographic records. For the Plymouth axon experiments peak Na current values during the test pulses in the τ_c determinations were also read with the aid of the 11/34 by first setting cursors, digitally filtering many times between these limits, and computing the maximum value of the smoothed record. Values were indistinguishable with the two methods.

Experiments on all axons were done in the presence of 2 mM 3,4-diaminopyridine (Aldrich Chemical Co., Milwaukee, WI) in the external bathing medium to reduce the K conductance (Kirsch and Narahashi, 1978). The 3,4-diaminopyridine solutions were made fresh each day and the pH carefully checked. In addition, for some experiments 40 mM tetraethylammonium Br (TEA; Eastman Kodak Co., Rochester, NY) was added to the internal perfusate. There were no differences in any of the results presented here with or without TEA present. Holding potentials were always about −100 mV. τ_c determinations were done as described by Goldman and Kenyon (1982) with a 5-ms gap between

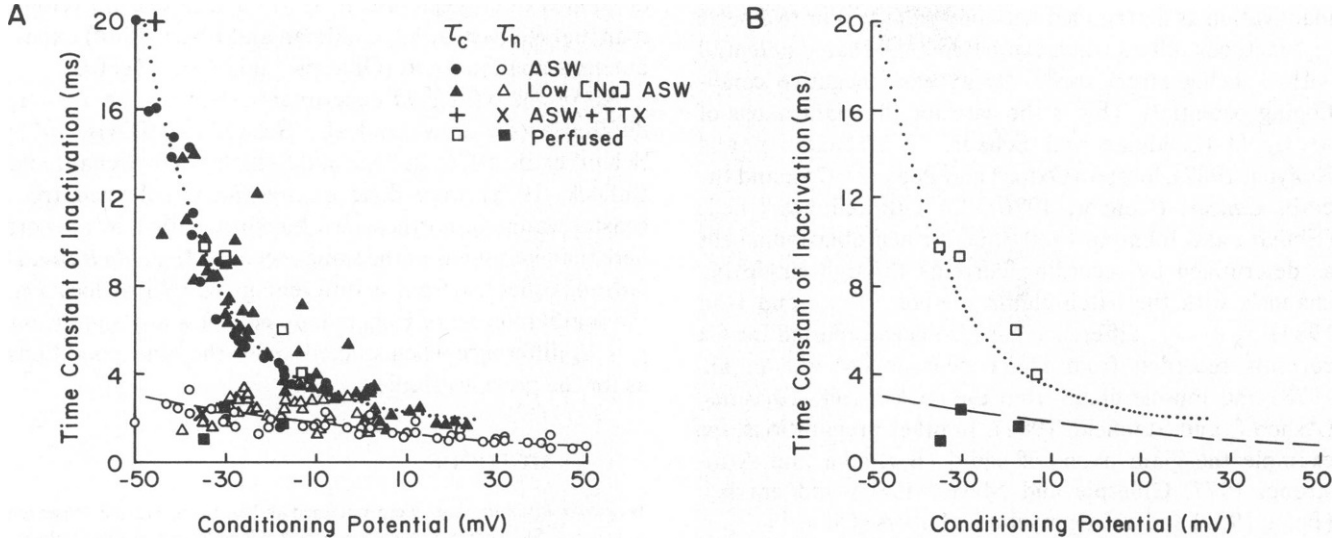

FIGURE 1 Na current inactivation time constants determined with one (τ_h) and two-pulse methods (τ_c) as a function of membrane potential. Data obtained from North American *Myxicola infundibulum*. (*A*) The filled circles and the open circles are τ_c and τ_h, respectively, determined in full Na ASW with no K-channel blocker (data from Goldman and Schauf, 1972 and 1973. The + and × are τ_c and τ_h, respectively, determined in the presence of TTX (data from Goldman and Hahin, 1978). The filled and open triangles are τ_c and τ_h, respectively, determined in either ⅓ or ¼ Na ASW and in the presence of 2 mM 3,4-diaminopyridine (data from Goldman and Kenyon, 1982). The open and filled squares are τ_c and τ_h, respectively, determined in ½ Na ASW, in the presence of 2 mM 3,4-diaminopyridine and under internal perfusion with K as the major internal cation (this paper). Under all conditions the $\tau_c - \tau_h$ difference is evident. Temperature, 5°C throughout. Dotted and dashed curves drawn by eye. (*B*) τ_c (open squares) and τ_h (filled squares) data from the internally perfused experiments of part *A* now shown separately. Dotted and dashed curves are the same as in part *A*.

conditioning and test pusles. 15 s were allowed between each voltage clamp pulse to minimize the effects of slow inactivation (Rudy, 1981).

RESULTS AND DISCUSSION

Fig. 1 *A* presents pooled values of the Na inactivation time constants, τ_c and τ_h, from North American *Myxicola*. The filled and open circles indicate τ_c and τ_h values, respectively, obtained on intact axons in full Na ASW and with no K-channel blocker. These are the original values of Goldman and Schauf (1972, 1973). The filled and open triangles indicate τ_c and τ_h obtained also on intact axons, but in ⅓ or ¼ Na ASW and in the presence of 2 mM 3,4-diaminopyridine, and are the values reported by Goldman and Kenyon (1982). The + (τ_c) and × (τ_h) are values obtained with peak test and conditioning pulse currents now reduced with TTX to 100 $\mu A/cm^2$ and 70 $\mu A/cm^2$, respectively (Goldman and Hahin, 1978). In all cases experiments were conducted under R_s compensation, Na currents were extracted using TTX subtraction and temperature was 5°C. For all experimental conditions inactivation time constants determined with one and two pulse methods lie on the same τ_c(V) (dotted curve) and τ_h(V) (dashed curve) functions, respectively.

A wide range of Na current densities was encountered in these experiments. For the τ_h determinations indicated by the open circles, considering only values at and negative to −20 mV, where the $\tau_c - \tau_h$ differences is substantial, mean peak Na current, I_{Na}, was 0.458 mA/cm^2 with a range of 0.105–0.900 mA/cm^2. For the corresponding determinations indicated by the open triangles, mean peak I_{Na} was only 0.087 mA/cm^2 with a range of 0.018–0.220 mA/cm^2. Similarly, for the τ_c determinations indicated by the filled circles, mean peak I_{Na} during the unconditioned test pulse was 0.831 mA/cm^2 (range 0.600–1.065 mA/cm^2). For those indicated by the filled triangles it was 0.373 mA/cm^2 (range 0.0033–0.908 mA/cm^2), while τ_c(V) and τ_h(V) are not significantly different for the two sets of measurements. This insensitivity of the inactivation kinetics to substantial changes in I_{Na} density indicates that these data have not been significantly affected by voltage clamp errors that depend on the current magnitude, i.e., R_s and spatial stability. Correspondingly, when compared directly on the same axons, reducing I_{Na} current density fourfold and fivefold was without effect on either τ_h (Goldman and Hahin, 1978), τ_c or inactivation delay (Goldman and Kenyon, 1982).

τ_c(V) and τ_h(V) are also unchanged by internal perfusion. Fig. 2 *A* shows a τ_c determination at a conditioning potential of −17.5 mV from a North American preparation internally perfused with a K glutamate-KF medium, as described in Methods, in reduced Na ASW, with R_s compensation and in the presence of 2 mM 3,4-diaminopyridine. τ_c (indicated by the solid curve) was 6.07 ms, consistent with intact axon values. Inactivation delay was 0.52 ms (shown on an expanded time scale in the *inset*) also in agreement with intact axon values (see Fig. 6 of Goldman and Kenyon, 1982). τ_h for this determination was 1.67 ms, again consistent with intact axon values.

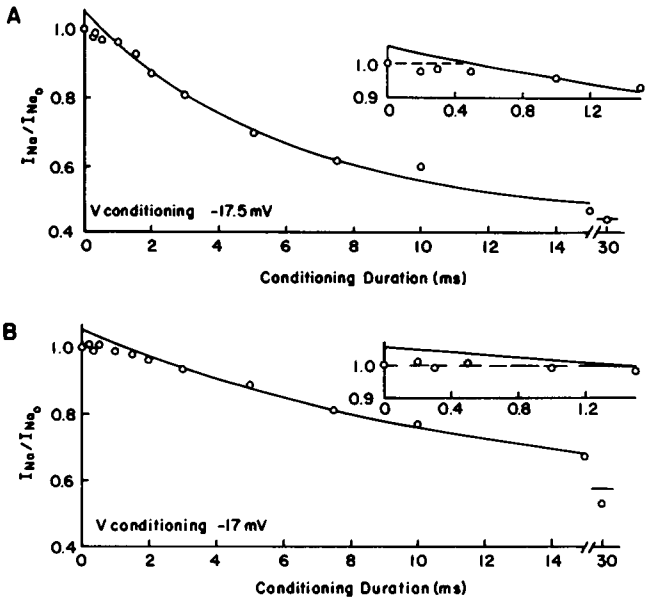

FIGURE 2 Time course of inactivation development in an internally perfused North American (part *A*) and a Plymouth (part *B*) *Myxicola* axon. The perfusion medium and method were the same in both cases. For both determinations peak I_{Na} during a test pulse (10.5 mV in part *A* and 8 mV in part *B*) is shown as a function of the duration of a conditioning pulse (−17.5 mV in part *A* and −17 mV in part *B*). Gap width 5 ms, 2 mM 3,4-diaminopyridine, and 5°C throughout. The solid curves are simple exponentials with time constants (τ_c) of 6.07 ms (*A*) and 12.15 ms (*B*). *Insets* present the first 1.5 ms of the determinations on an expanded time scale and illustrate how the inactivation delays were determained (time at which the unconditioned peak current value intersects the exponential). Delays are 0.52 (*A*) and 1.37 ms (*B*).

Collected τ_c and τ_h values from perfused North American axons are shown as the open and filled squares, respectively, together with the pooled intact axon data in Fig. 1 *A* and again separately in Fig. 1 *B*. The dashed and dotted curves in Fig. 1 *B* are the same curves fitted by eye to the inact axon data of Fig. 1 *A*. Collected perfused axon values are in good agreement with the intact τ_c(V) and τ_h(V) functions, and accordingly also display a substantial $\tau_c - \tau_h$ difference. Inactivation delays in perfused preparations were also in agreement with those in intact axons (not illustrated). Peak I_{Na} during the conditioning pulses from which τ_h values were determined ranged from 0.074 to 0.130 mA/cm^2, and that during the unconditioned test pulses for the τ_c determinations ranged from 0.167 to 0.574 mA/cm^2. This agreement between intact and perfused preparations indicates that the presence or absence of a $\tau_c - \tau_h$ difference in different species is not attributable to such methodological differences. The squid (Bezanilla and Armstrong, 1977; Gillespie and Meves, 1980) and myelinated (Chiu, 1977) axon studies were done on perfused and cut preparations, respectively.

Under all experimental conditions in North American *Myxicola* τ_c is clearly $>\tau_h$ for negative potentials, showing

nearly no overlapping values for potentials negative to −10 mV, and reaching differences of sixfold and more at very negative levels. With all the treatments we have tried (reducing I_{Na} density, adding K-channel blockers, internally perfusing) the $\tau_c(V)$ and $\tau_h(V)$ functions appear to be unchanged. $\tau_c(V)$ and $\tau_h(V)$ in North American *Myxicola* are therefore reasonably well defined and highly reproducible. The results of Fig. 1 have been collected over an 11 year period using, then, a number of different recording chambers, internal electrodes and external ground and guard electrode assemblies with no obvious effect on either $\tau_c(V)$ or $\tau_h(V)$.

The rest of the results presented in this paper have all been obtained on Plymouth *Myxicola*, internally perfused with methods and perfusates identical to those for the North American axons, using R_s compensation, with reduced Na ASW, in the presence of 2 mM 3,4-diaminopyridine, and at 5° ± 0.5°C. Fig. 2 *B* presents a τ_c determination from a Plymouth axon. Conditioning potential was −17, nearly identical to that for the North American axon determination in Fig. 2 *A*. τ_c (indicated by the solid curve) was 12.15 ms and the inactivation delay was 1.37 ms (see *inset*). τ_h for this determination was 11.66 ms, nearly identical to τ_c. Both τ_c, τ_h, and inactivation delay are well larger than the corresponding values from North American axons at this potential (compare with Fig. 1 above and Fig. 6 of Goldman and Kenyon, 1982), with τ_h more than threefold larger than the largest τ_h value seen at any potential in North American *Myxicola*.

Pooled τ_c and τ_h values from Plymouth axons are presented in Fig. 3. Over the whole potential range examined τ_c is somewhat and τ_h is well greater than the corresponding values from North American *Myxicola* even though the measurements were taken at the same temperature. Inactivation delay, which is a measure of activation kinetics (Goldman and Kenyon, 1982; Goldman and Ebert, 1984), is also larger in Plymouth as compared to North American preparations (not illustrated). For the Plymouth τ_h determinations peak inward I_{Na} ranged from 0.094 to 0.570 mA/cm² with a mean of 0.281 mA/cm². Mean peak I_{Na} during the unconditioned test pulses for the τ_C determinations was 0.609 mA/cm² (range 0.272–0.653 mA/cm²). These values are well within the range encountered in the pooled North American axon experiments of Fig. 1 *A*.

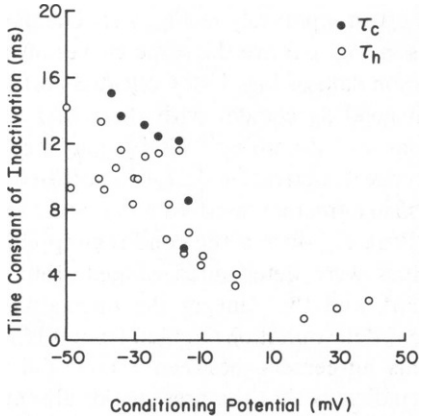

FIGURE 3 Na current inactivation time constants, τ_c (filled circles) and v_h (open circles), as a function of membrane potential. Data obtained from Plymouth *Myxicola infundibulum*. Experiments were done under internal perfusion with identical perfusates as for Fig. 1, in the presence of 2 mM 3,4-diaminopyridine (and in some cases 40 mM TEA internally), in ½ Na ASW, and at 5°C. There is no clear $\tau_c - \tau_h$ difference.

Particularly clear is the absence of any significant $\tau_c - \tau_h$ difference in the Plymouth axons even when compared over a potential range of −15 to −35 mV, where the difference is unambiguous in North American axons. The relative agreement between τ_c and τ_h in Plymouth *Myxicola* is even more evident when values for both parameters from the same axon are compared at the same potential. We have six such determinations from five axons over a potential range of −14 to −34 mV. The results are presented in Table I. In these experiments τ_c ranged from 1.04 to 1.32 times $>\tau_h$. τ_c and τ_h are, therefore, nearly the same in these axons. By contrast, Table II presents τ_c and τ_h values again from the same axons, compared at the same potential, but now from North American *Myxicola*. In three comparisons from three different axons τ_c ranged from 3.64 to 8.86 times $>\tau_h$.

Schauf and Bullock (1978) reported that internal dialysis with Cs abolishes the $\tau_c - \tau_h$ difference in North American *Myxicola* by selectively decreasing $\tau_c(V)$ so that it superposes with $\tau_h(V)$, τ_h itself being unaffected by internal Cs. We have no observations on Cs perfused North American *Myxicola*. However, internal Cs has no effect on either τ_c or τ_h in Plymouth *Myxicola* (Goldman, unpublished manuscript). There is, then, no appreciable $\tau_c - \tau_h$ difference of any sort in Plymouth *Myxicola* under any experimental conditions examined.

The $\tau_c - \tau_h$ difference is seen in some, but not in other preparations. Its presence cannot be attributed to any obvious voltage clamp errors or methodological differ-

TABLE I
τ_c AND τ_h IN PLYMOUTH *MYXICOLA INFUNDIBULUM*

Axon	Potential	τ_c	τ_h	τ_c/τ_h
	mV	*ms*	*ms*	
84MP28	−34	13.66	11.65	1.17
84MP27	−27	13.13	11.25	1.17
84MP26	−23	12.45	11.46	1.09
84MP27	−17	12.15	11.66	1.04
84MP31	−15.5	5.71	5.31	1.08
84MP10	−14	8.50	6.45	1.32

Internally perfused preparations. K-glutamate/KF perfusate (see methods). ½ Na ASW. 2 mM 3,4-diaminopyridine. Temperature was 5°C.

TABLE II
τ_c AND τ_h IN NORTH AMERICAN *MYXICOLA INFUNDIBULUM*

Axon	Potential	τ_c	τ_h	τ_c/τ_h
	mV	*ms*	*ms*	
82M31	−34.5	9.83	1.11	8.86
82MNH2	−30	9.42	2.51	3.75
82M29	−17.5	6.07	1.67	3.63

All experimental conditions as for Table I.

ences. It has been seen in studies done with an axial wire clamp, both on inact and perfused axons, with the double sucrose gap clamp (Oxford and Pooler, 1975), and with currents recorded from single Na channels with the patch clamp (Nagy and Hof, 1984). Here we have shown that in two geographically distinct populations of the same species one population does and the other does not display this property. Again, this difference cannot be attributed to any obvious methodological issues, and seems, then, to reflect a genuine difference in kinetic behavior between the two populations. As the two populations are of the same species these results raise the possibility that dramatic differences in Na channel kinetics may not necessarily reflect basic differences in the organization of the Na channel gating machinery. Rather, they might reflect only quantitative differences, for example in the values or relative values of some of the rate constants in the same kinetic scheme.

These experiments provide no definite information as to the origin of the different behavior in the two populations. They have been both exposed to different environmental and hence feeding conditions and are, presumably, not interbreeding. One possibility, then, is that feeding or other environmental conditions can have a regulatory effect on ionic channel function. Such effects could be the origin of the different pattern of electrical activity on exposure to glucose seen in pancreatic B-cells from mice of the same strain reared under different feeding and other environmental conditions (Lebrun and Atwater, 1985). The differences in electrical response are lost when animals from the two sources are held under identical laboratory conditions. Alternatively, there could be some small genetically based difference in Na channel gate structure between the two populations. Even a single side chain difference at a critical site could presumably produce a large difference in the energy barrier between conformational states of the Na channel gate. In fact, different larval development characteristics are known to exist in geographically separate populations of the same species of another tube-dwelling polychaete, *Streblospio benedicti* (Levin, 1984). These developmental differences persist when animals from the separate populations are cultured in the laboratory under identical conditions, and individuals from these separate populations are interfertile.

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