Letters to the Editor

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Gap Junction Shape and Orientation at the Cardiac Intercalated Disk

In a recent study on canine myocardium, Hoyt et al present some fascinating new insights into the form and distribution of gap junctions in relation to the complex three-dimensional nature of the intercalated disk of working ventricular myocytes. Their conclusions, based on morphometric analysis of data from thin sections and on serial sectioning and scanning electron microscopy, include the following: 1) small gap junctions, accounting for ~20% of gap junction membrane, are present in the plicate (i.e., fascia adherens-containing) regions of the intercalated disk; 2) gap junctions do not, as some had thought, occur at the true lateral surfaces of the myocyte body (i.e., in "non-intercalated disk regions"), and 3) each myocyte is connected via intercalated disks to an average of ~9 other myocytes. These findings add substantially to the debate on the structural basis for coupling between myocytes, and draw attention to aspects of junction organization not adequately taken account of previously. However, one central conclusion from this study in particular merits further discussion. This concerns the shape and orientation of the large gap junctions located in the intercalate zones of the disk. According to Hoyt et al., these junctions (which account for ~80% of total gap junction membrane) occur in the form of ribbon-like structures (averaging 1.3 x 5.1 μm), oriented with their long axes running transversely with respect to the longitudinal axis of the cell, that is, partially circumscribing, in discontinuous fashion, the "finger-like" processes making up the disk. This conclusion, summarized in the drawing in Figure 8 of their paper, is derived principally from measurements of gap junction profile length in sections cut in three orthogonal planes. One approach that overcomes some of the limitations inherent in using sections to reconstruct the shape and distribution of membrane domains is provided by the technique of freeze-fracture electron microscopy. Because this technique involves splitting membranes along an internal hydrophobic plane, it can produce large expanses of membranes, in which junctions (and other specializations) are displayed en face. Apart from the advantages of directly visualizing junction shape and topographical distribution, freeze-fracture can provide much larger samples of individual junctions for inspection than it would realistically be feasible to reconstruct from serial thin sections. The freeze-fracture technique has been widely applied in numerous studies on myocardial membrane structure in the past, including those described as elongated, no consistent pattern of orientation is apparent. From the junctional pleomorphism apparent in Figures 1 and 2, it is clear that larger samples of junctions are needed to gain a truly representative impression of junction shape and orientation. Accordingly, Figure 3 shows tracings of a random sample of 30 gap junctions, taken from replicas in which the myocyte’s longitudinal axis could be determined. This confirms that although a proportion of the junctions do appear elongated, such junctions are neither long extended ribbon-like structures nor are they preferentially arranged with their longer axis transverse to that of the cell. It should be noted that gap junctions in unfixed rabbit myocardium frozen directly (without any chemical pretreatment) tend to have more irregular borders than those illustrated here in fixed and glycerinated samples, but

To explore this apparent discrepancy I have reexamined shape and orientation of gap junctions in freeze-fracture replicas of glutaraldehyde-fixed and glycerinated rabbit left ventricle. Two complete gap junctions (GJ) and portions of a further two (GJ*) are visible. The expanse of intercalated membrane is derived from a myocyte that has been fractured away, leaving only the outermost half-membrane leaflet of its plasma membrane (seen in E-face view). The adjacent myocyte, with which its gap junctions are shared, is shown in cross-fracture view at the top of the picture. Longitudinally arranged spaces occupied by myofibrils (MF), interspersed with rows of elongated mitochondria (Mi) permit determination of the cell's longitudinal axis (LA). (This orientation is confirmed by many additional morphological markers in the montage from which this example is taken.) A plicate membrane zone (P1), which has been cross-fractured, rises vertically from the disk membrane at the site marked between the pair of filled arrows. T, Transverse tubule; To, transverse tubule opening; XP, cross-fractured plasma membrane; ES, extracellular space. Magnification, ×32,500.

FIGURE 1. Freeze-fracture replica showing face-on view of part of the intercalate membrane of an intercalated disk from glutaraldehyde-fixed and glycerinated rabbit left ventricle. Two complete gap junctions (GJ) and portions of a further two (GJ*) are visible. The expanse of intercalated membrane is derived from a myocyte that has been fractured away, leaving only the outermost half-membrane leaflet of its plasma membrane (seen in E-face view). The adjacent myocyte, with which its gap junctions are shared, is shown in cross-fracture view at the top of the picture. Longitudinally arranged spaces occupied by myofibrils (MF), interspersed with rows of elongated mitochondria (Mi) permit determination of the cell's longitudinal axis (LA). (This orientation is confirmed by many additional morphological markers in the montage from which this example is taken.) A plicate membrane zone (P1), which has been cross-fractured, rises vertically from the disk membrane at the site marked between the pair of filled arrows. T, Transverse tubule; To, transverse tubule opening; XP, cross-fractured plasma membrane; ES, extracellular space. Magnification, ×32,500.
again no obvious relation between junction shape and orientation with respect to the cell's long axis is apparent. The same general conclusions hold for rat ventricular myocytes. To follow up these findings, we have initiated further studies to map in detail the shape and distribution of gap junctions in low magnification survey freeze-fracture views of complete interplicate zones and entire intercalated disks.

It is, of course, important to bear in mind that as with any investigative tool, freeze-fracture is not without limitations. The larger a domain becomes, for example, the more likely is part of it to lie outside the field of membrane exposed, and undulating topology such as that found in parts of the intercalate zones of the disk will tend to encourage departure of the fracture plane from the plasma membrane. Such factors, it could plausibly be argued, would reduce the probability of disclosing, in their entirety, large transversely oriented ribbon-like junctions. Moreover, when images of membrane areas that are highly curved in three dimensions in the native cell are projected onto two dimensions (as in a standard micrograph), some distortion of the observed junctional shape will occur. However, many of the views we have examined involve extensive, relatively flat areas of membrane in which a whole series of complete, individual large junctions is visualized (Figure 1), and distortion in perception of junction shape can be avoided by tilting the specimen and by taking advantage of the three-dimensional perspective provided by stereo imaging (Figure 2). But even without the benefit of such views, if ribbon-like gap junctions were common, clear evidence for their presence should still be apparent in the more limited views that have repeatedly been provided by standard freeze-fracture in the past.

How, then, may these observations be reconciled with the findings of Hoyt et al. At first sight there seems no obvious reason why thin section profile measurements taken in different planes should fail to give accurate averages of junctional domains, but the implications of a paper by Chen et al published in the same issue of Circulation Research, suggest that it may not be safe to take this premise for granted. By applying lanthanum as an in situ negative stain before thin sectioning, these authors discovered that tangentially cut gap-junctional membrane is markedly more prevalent than one is led to believe from routine examination of standard positively stained sections. This means that in morphometric analysis of standard sectioned material, substantial amounts of junctional membrane are likely to escape detection, leading to serious underestimation of junc-

![Figure 2](http://circres.ahajournals.org/)

*Figure 2.* Stereo electron micrographs showing part of a freeze-fractured intercalated disk from glutaraldehyde-fixed and glycinated rabbit interventricular septum. Viewing in stereo brings out details of the disk structure in full three-dimensional perspective, and allows junctions to be studied on highly curved areas of membrane, where an accurate view of their shape is otherwise difficult to obtain. Extended views of the interplicate membranes of two adjacent cells have been exposed. These face out laterally from the myocyte body, undulating as they wrap around the sides of the myofibril ends. The plicate membrane zones, which lie as discrete "islands" in a plane perpendicular to this "lateral" membrane, have been mainly cross-fractured to reveal the underlying cytoplasm (XC); the interdigitating cell processes at the borders of the plicate zones are nevertheless clearly seen (PI). Five gap junctional areas (gj) are present in the interplicate membrane. Three junctions in the lower part of the field (gj1,2,3) are viewed as plateaux, face-on. One of these (gj1) is elongated both in the transverse and in the longitudinal axis (LA) of the cell. Another (gj2) lies at an angle of −25° from the longitudinal axis. A large, irregular gap junction (gj3) at the top of the field can be followed as it curves around the side of the cell; the transverse dimension of this junction is −1.3 fold greater than its maximal longitudinal dimension. (Part of this gap junction is seen in E-face view [ ], and a desmosome [small arrow] lies in its interior.) Note that the ribbon-like patch of gap junctional membrane marked gj4 (seen in both P-face and E-face view) represents only a small portion of a junction that extends beneath the cross-fractured cytoplasm alongside. (When junctions are fractured in this way, their overall shape cannot be determined with certainty.) Magnification, ×14,600.
sectioning could not have been foreseen without the benefit of the Chen et al study, the possibility of some shortfall in accuracy from this source is difficult to exclude completely. If such inaccuracy were to arise, it would not be expected necessarily to affect all section planes equally; those taken parallel with the long axis of the cell would, because of the lateral-facing undulations of the intercalate membrane, tend to contain a particularly high proportion of tangentially sectioned junctional (and other) membrane. Sections taken at right angles to the myocyte's long axis would, on the other hand, generally give clear transversely sectioned intercalate membrane, with well-defined cross-sectional gap junction profiles. If the tangential-section effect were to lead to undermeasurement of gap junction profile length, it would do so primarily with the former types of section, rather than the latter, thereby creating the false impression of transverse elongation in a domain that is, in reality, rounded. Failure to detect a proportion of the tangentially sectioned membrane could also introduce errors in the perception of junctional shape from serial sections as well as in the estimation of total quantity of junctional membrane and of the proportion of junctional membrane located in the plicate zone.

It should, however, be emphasized that all these possibilities are entirely conjectural at present, and it is difficult to be certain of the precise impact of the tangential section effect on the interpretation of the full range of morphometric data published by Hoyt et al1 and others. There are, moreover, other plausible explanations for the unexpected junction shape and orientation these authors propose. As their study was confined to dog myocardium, for example, the possibility that transversely oriented gap junction "ribbons" might be a feature peculiar to this species, not present in rabbit and rat, must still remain open. Further quantitative thin section studies aided by lanthanum staining, and extension of freeze-fracture examination of gap junctions to canine myocardium, may shed further light on these questions.

References

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Reply to the Preceding Letter
We thank Dr. N. J. Severs for his interest in our work on the three dimensional structure and distribution of gap junctions in canine myocardium. Analysis of Severs' elegant freeze-fracture images as well as review of our experience with the technique indicates that small, ovoid gap junctions are readily apparent in freeze-fracture replicas while long, ribbon-shaped junctions described in our analysis of ultrathin sections are apparently not seen in freeze-fracture preparations. In our studies of canine

Figure 3. The typical variation in gap junction shape, size, and orientation in relation to the cell's longitudinal axis is illustrated in this random selection of 30 gap junctions traced from the intercalate zones of intercalated disks of glutaraldehyde-fixed and glycerinated rabbit left ventricular myocytes. Unfixed, directly frozen samples show junctions of similar overall shape, though with more irregular borders than those illustrated here; whether this difference is due to fixation or the freezing process itself is uncertain. However, with neither preparative procedure do the junctions appear consistently elongated across the longitudinal cell axis. Of the two procedures, the junctions from the fixed samples shown here are the more appropriate to compare with the data on thin sectioned (i.e., fixed) junctions of Hoyt et al. Scale bar represents 1 µm.
myocardium, we consistently observed long (up to 8 μm), continuous gap junction profiles located in offset intercalate segments of intercalated disks. These junctions are oriented transverse to the long fiber axis such that their full “length” is apparent only in transverse planes of section. We believe their existence to be indisputable. We also described more numerous, roughly circular or ovoid gap junctions.

A plausible explanation for the discrepancy raised by Severs may relate to mechanical characteristics of the freeze-fracture process and inability of fracture cleavage planes to follow the tortuous three-dimensional course of the long gap junctions. As shown in Figures 2 and 4 of our paper, the long gap junctions invariably exhibit a highly convoluted and folded surface configuration, frequently bending at acute angles around mitochondria or other structures. Freeze-fracture planes generally reveal relatively flat expanses of membrane in which only limited three-dimensional topography is apparent. We believe it unlikely that a cleavage plane would follow a contour similar to that taken by a long gap junction, especially when these junctions are located in intercalate segments that are separated from broad expanses of sarcolemma by steps of the intercalated disk. In any event, we have not observed a replica to follow such a contour and would suggest that folding of the replica to this degree may result in collapse of the preparation. Thus, we would suggest that freeze fracturing preferentially reveals small, ovoid gap junctions situated in relatively smooth, unfolded expanses of the sarcolemma.

We reported that up to 16.8% of intercalated disk profile length was composed of gap junctions, a value comparable to that reported by Chen et al.2 and Page and McCallister.3 Our measured gap junction surface density values are two to four times greater than those reported previously in studies cited by Chen et al.2 Thus, we believe that we have not significantly underestimated gap junction profile length in positively stained sections.

We believe it unlikely that long, ribbon-shaped, transversely situated gap junctions are unique to canine myocardium. Similar gap junction configurations have been described in rat left ventricular myocardium by Page and McCallister,3 and we have observed long, ribbon-like gap junctions in human myocardium (unpublished observations).

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Comments on “Nonlinear Relation Between V^ and IN, in Canine Cardiac Purkinje Cells” (Circ Res 1988;63:386–398)

The authors have used an elegant technique to obtain data on sodium current and action potential upstroke in the Purkinje cell membrane. They compared, for the same membrane and the same experimental conditions, relative changes in the peak sodium current under voltage clamp to relative changes in maximal upstroke velocity (V_{peak}) of the free-running action potential. Voltage-dependent inactivation, tetrodotoxin block, and quinidine block were used to alter sodium channel availability. They found a nonlinear relationship between V_{peak} and peak sodium current for all three conditions and for different temperatures.

These data can be analyzed slightly differently by considering the sodium membrane conductance (g_{Na}) relating the transmembrane potential (V) to the sodium current (I_{Na}), i.e.,

\[ I_{Na} = g_{Na}(V - E_{Na}) \]  

(1)

where \( E_{Na} \) is the sodium equilibrium potential. Under the same level of voltage clamp (as in the protocol of Figure 1A) the sodium driving force is constant and relative changes in I_{Na} and g_{Na} are equal. If \( I_{p} \) and \( g_{p} \) represent peak I_{Na} and peak g_{Na}, respectively, with \( g_{p} \), and \( g_{Na} \) corresponding to the reference state, we have

\[ \frac{g_{p}}{g_{Na}} = \frac{I_{p}}{I_{Na}} \]  

(2)

At the time of V_{max}, the following relation holds if we assume that the non-sodium current is negligible

\[ C_{m}(\sqrt{V_{max}} - V_{Na} - E_{Na}) \]  

(3)

Here \( C_{m} \) is the membrane capacitance, \( V \) is the membrane potential at V_{max}, and \( g_{Na} \) is an estimate of peak g_{Na}. Model studies have shown that peak g_{Na} is slightly delayed relative to peak I_{Na} during a membrane action potential such that \( \frac{g_{p}}{g_{Na}} \) may be expected to be slightly lower but quite close to \( I_{Na} \). The sodium driving force is not constant since \( V \) assumes different values as V_{max} changes (as in the protocol of Figure 1B). Therefore, if V_{max}, g_{p}, and I_{Na} correspond to the reference state, we have

\[ \frac{g_{p}}{g_{Na}} = \frac{V_{max} - E_{Na}}{(V_{max} - V_{Na})} \]  

(4)

This equation indicates that relative changes in V_{max} give an estimate of changes in peak g_{Na} provided that corrections for variations in the sodium driving force are made. Without this correction, there is no sound theoretical basis to compare directly relative changes in V_{max} with changes in peak I_{Na} measured under voltage clamp.

The above observations were reported in a previous model study.2 The same model was used here with the protocols proposed by the authors, and the results are summarized in Figures 1A and 1B which reproduce, respectively, Figures 5A and 6A of the paper, while panels C and D correspond to Figure 7. Not surprisingly, the relation between the fractional values of V_{max} and I_{Na} is nonlinear since V (the voltage at which V_{max} occurs) is not constant. The linearity of the relation becomes quite good when a valid comparison is made, that is between the fractional change in peak g_{Na} estimated from V_{max} (i.e., \( g_{p} \), Equation 4) and the fractional change in peak g_{Na} itself (i.e., \( g_{p} \), Equation 2).

Both peak I_{Na} under voltage clamp and V_{max} of the free-running action potential provide estimates of relative changes in peak g_{Na}. Because the potential at which V_{max} occurs changes with g_{Na}, the sodium driving force at the time of V_{max} is not constant and the current is affected. Therefore, a correction must be applied to fractional changes in V_{max} before they are compared with fractional changes in peak I_{Na}. It would be interesting to see how much of the nonlinearity reported by the authors can be removed by this correction. The model study suggests that the relation
FIGURE 1. Using a model of the cardiac cell membrane to illustrate the estimation of relative changes in peak ($I_p$) sodium current ($I_{Na}$) from maximal upstroke velocity ($V_{max}$) of the action potential. A: $I_{Na}$ responses under voltage clamp at 0 mV following 500-msec conditioning steps to various voltage levels, as indicated. B: Action potential upstrokes on release from a 50-msec clamp at −45 mV, and following previous 500-msec conditioning steps at the values indicated. C: Fractional $I_p$ (from panel A) and $V_{max}$ (from panel B) values plotted against the conditioning potential level. The full line is a fit to measure values using the equation proposed by the authors (their Equation 2). D: Fractional $V_{max}$ (**) and fractional peak membrane conductance ($g_p$) of Equation 4 (*) against fractional $I_p$ (or $g_p$ since both are equal, Equation 2).

becomes nearly linear, thereby indicating that the proper use of $V_{max}$ measurements can provide a reasonably reliable estimate of peak $g_p$, changes in cardiac cells.

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References

Reply to the Preceding Letter

We appreciate your comments on our article, “Nonlinear Relation Between $V_{max}$ and $I_{Na}$ in Cardiac Purkinje Cells,” in which we experimentally determined the relation between two different measurements of $I_{Na}$ availability (maximal upstroke velocity [$V_{max}$] and fraction sodium current [$I_{Na}$]). The problems and potential sources of error with $V_{max}$ have been amply described in our article and in your letter. You point out correctly that $V_{max}$ should be adjusted for any significant change in Na⁺ driving force that would occur if the membrane potential at $V_{max}$ changes. Your Hodgkin-Huxley model simulations show that the discrepancy between $V_{max}$ and $I_{Na}$ becomes minimal after correction for the Na⁺ driving force. Since our experiments failed to demonstrate such an important role for Na⁺ driving force, we need to consider whether either 1) our experiments are in error or we misinterpreted them, or 2) your model simulations do not apply to our experimental conditions.

Data from our single Purkinje cell experiments showed that changes in Na⁺ electrochemical driving force did not appear to make a large contribution to the nonlinear relation between $V_{max}$ and $I_{Na}$. As shown in Figure 8 (page 392) of our article, the nonlinear behavior was largely independent of the Na⁺ electrochemical driving force when extracellular sodium was decreased from 120 to 45 mM. Furthermore, Figure 10 (page 393) showed that the largest change in membrane potential at $V_{max}$ (and consequently the Na⁺ electrochemical driving force) occurred at an experimental temperature of 26°C, but it was at this temperature that $V_{max}$ best approximated a linear relation to $I_{Na}$ (see Figure 9, page 393).

To demonstrate more clearly the role of electrochemical driving force on the nonlinearity of $V_{max}$ to $I_{Na}$, we include here...
additional data comparing the membrane potential at $V_{\text{max}}$ when sodium channel availability was 100% (holding potential $V_{\text{hp}}$ of -150 mV) with the membrane potential at $V_{\text{max}}$ when sodium channel availability was reduced to 57% ($V_{\text{hp}}$ of -120 mV). The availability of $I_N$ from different $V_{\text{hp}}$ was determined by measurement of peak $I_N$ during voltage clamp. Voltage inactivation reduced the number of available sodium channels and thereby reduced the upstroke velocity and established conditions for which correction for Na$^+$ driving force might be required. Action potential upstrokes were elicited as described in the article. Briefly, the cell was released from voltage clamp after prerelease steps of 50 μsec to release potentials from -130 to -40 mV. We have already shown that the latency to $V_{\text{max}}$ did not alter $V_{\text{max}}$ under our experimental conditions (see Figure 3, page 389). The magnitudes of $V_{\text{max}}$ were 243±0.6 V/sec (SEM, n=9) when $V_{\text{hp}}$ was -150 mV and 144±0.7 V/sec (n=9) when $V_{\text{hp}}$ was -120 mV.

The membrane potentials at $V_{\text{max}}$ are shown in Figure 1A. When $V_{\text{hp}}$ was -150 mV, the membrane potential at $V_{\text{max}}$ was constant and averaged -21.4±0.4 mV (n=9) over the large range of prerelease potentials of -150 to -40 mV. When $V_{\text{hp}}$ was -120 mV, the membrane potential at $V_{\text{max}}$ was also constant at -23.1±0.4 mV (n=9). Note that the membrane potentials at $V_{\text{max}}$ were similar even though the availability of sodium channels (as assessed by $I_N$) had decreased to 57% and $V_{\text{max}}$ had decreased to 144 from 243 V/sec. The relation between fractional $I_N$ and fractional $V_{\text{max}}$ is shown in Figure 1B. The straight line is the expected relation if fractional $V_{\text{max}}$ were a linear estimate of fractional $I_N$. The data show that $V_{\text{max}}$ consistently overestimated availability of Na$^+$ channels as measured by peak $I_N$ during voltage clamp. At a fractional $I_N$ of 0.57, $V_{\text{max}}$ overestimated $I_N$ by nearly 20% even though the membrane potential at $V_{\text{max}}$ had not changed. Similar conclusions can be made from examination of both Figures 9A and 10 in the original article. Figure 10 showed that the membrane potential at $V_{\text{max}}$ did not change until $V_{\text{max}}$ had decreased by more than one-half of its peak value while Figure 9A showed that $V_{\text{max}}$ significantly overestimated $I_N$ before fractional $V_{\text{max}}$ had decreased to one-half its peak value. Consequently, we find in our experiments that changes in the Na$^+$ driving force were not a major factor determining the nonlinear relation of $V_{\text{max}}$ to $I_N$.

If our experiments are correct in showing that changes in Na$^+$ driving force do not account for the discrepancy between the two measures of estimating sodium channel availability, why do your model simulations of the Hodgkin-Huxley equations predict otherwise? In your article, you state that the Hodgkin-Huxley model parameters allowed for a larger peak sodium membrane conductance ($g_{Na}$) and a greater time duration of $g_{Na}$, causing $I_N$ during an action potential upstroke to be largely determined by the electrochemical gradient (see page 346). The longer duration of $g_{Na}$ resulted from the choice of a relatively fast $r_m$ and a relatively slow $n$ (e.g., at -40 mV you chose $r_m$ to be about 0.16 msec and $n$ to be about 5.0 msec for a ratio of $r_m$ to $n$ of 1:31). This large ratio allowed for rapid activation and relatively slow inactivation and caused $g_{Na}$ to approach maximal $g_{Na}$. Because activation was almost complete during the action potential upstroke, Na$^+$ driving force could predominate.

Although it should be noted that the Hodgkin-Huxley equations do not accurately model $I_N$, kinetics and that ample support for coupled kinetic models of the sodium channel are in the literature, $I_N$ can be fitted to that model to estimate a $r_m$:$n$ ratio. For example, at 12°C, where the nonlinearity between $V_{\text{max}}$ and $I_N$ was the greatest in our experiments, we fitted $I_N$ to the Hodgkin-Huxley model. At -40 mV, $r_m$ was in the range of 0.5 msec and $n$ was about 2.5 msec, a ratio of only 1:5. This much smaller ratio produces peak $g_{Na}$ that is much less than maximal $g_{Na}$. At the single channel level, the probability that a channel will open decreases as $n$ (inactivation) approaches $r_m$ (activation).

**FIGURE 1. A: Membrane potentials at maximal upstroke velocity ($V_{\text{max}}$) when release potentials were varied.** From a holding potential ($V_{\text{hp}}$) of -150 mV (C), potentials at $V_{\text{max}}$ averaged -21.4±0.4 mV (n=9). From a $V_{\text{hp}}$ of -120 mV (X), potentials at $V_{\text{max}}$ averaged -23.1±0.4 V/sec (n=8). Note that the membrane potential at $V_{\text{max}}$ was nearly identical even though $V_{\text{max}}$ decreased from 243 to 144 V/sec. B: Fractional $V_{\text{max}}$ compared with fractional peak $I_N$, obtained from sodium channel availability plots as previously described. Also plotted is the line of identity. The experimental points deviate from the line of identity, showing that fractional $V_{\text{max}}$ overestimates $I_N$. Even though fractional $V_{\text{max}}$ decreased to 0.60 of its maximum value, the membrane potential at $V_{\text{max}}$ was not significantly changed. Cell 33.03.26, 27, 28, 19°C.

**In summary,** our experimental data did not show a major role for driving force in causing nonlinearity in the $V_{\text{max}}$-$I_N$ relation. We conclude that the nonlinearity of $V_{\text{max}}$ to $I_N$ under our experimental conditions arose from recruitment of a proportionally larger number of sodium channels during slower action potential upstrokes (see Figure 10). This suggests that at the single channel level, the fraction of channels that inactivate
without ever opening and the temporal dispersion of channel opening may be important in the nonlinearity of $V_{\text{max}}$ to $I_{\text{Na}}$.  

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