Whether ‘‘Slip-Mode Conductance’’ Occurs

Excitable cells rely on selective ionic conductances for electrical signaling. The work of Hodgkin and Huxley (1) propelled the modern dissection of the mechanisms of selectivity. Their formulation postulated two independent sets of ionic conductances, Na⁺ and K⁺, whose relative permeabilities changed during the course of excitation. Mulkins subsequently proposed that Na⁺ and K⁺ traverse the membrane through a single set of pores that changed from being Na⁺-selective to K⁺-selective (2). That view is no longer held as tenable. Several lines of experimental evidence, notably single-channel recordings, established convincingly that the membrane contains distinct sets of pores, each with its own distinctive selectivity properties (3, 4). Nevertheless, the prevailing view that ion channel selectivity is preserved during normal electrical activity was recently challenged in a report by L. F. Santana et al. (5).

Voltage clamp experiments in rat ventricular cells led to the proposal that voltage-dependent Na⁺ channels change their selectivity in response to cyclic AMP–dependent phosphorylation. Unexpectedly, such channels conduct Ca²⁺ well; similar ‘‘slip-mode conductance’’ responses were seen during exposure to cardiac glycosides. The extensive evidence against flexible selectivity, as well as major technical concerns over that report (5), prompted us to question whether or not this idea has a genuine biological basis. Ventricular myocytes are large cells in which voltage control is notoriously difficult to achieve (6); the large number of Ca²⁺-sensitive ionic currents (7) further complicates the interpretation of the results. To test for the existence of slip-mode conductance, we expressed human hH1 (8) or rat rH1 (9) cardiac Na⁺ channels heterologously in Chinese hamster ovary (CHO) cells. These small cells are readily voltage-clamped, have few endogenous channels, and support cyclic AMP-mediated responses (10).

We performed whole-cell patch clamp recordings of membrane current (11). In 20 mM external Na⁺, a family of typical Na⁺ currents was elicited (Fig. 1A) by depolarizing voltage pulses from −100 mV to potentials from −80 mV to +60 mV. As shown in the I-V relation (panel B), the currents reach a maximum amplitude at −20 mV and demonstrate a reversal potential near +60 mV. Such currents increased in amplitude in response to 1 μM isoproterenol or 10 μM dibutyl cyclic AMP (32 ± 5%, n = 9; panel C, ▲), confirming the well-established response to phosphorylation (10). However, no ionic current was measurable when the external solution was switched to 0 Na⁺/10 mM Ca²⁺ (1 ± 2% of basal current, n = 7; panel C, ▼). That Na⁺ channels were still present and functional was verified by restoring Na⁺ to the external solution (panel C, ▲). Figure 1D shows the time course of the phosphorylation-mediated increase in peak I_{Na} and flux of Na⁺ (▼) versus Ca²⁺ (▲) through the modified rH1 Na⁺ channels. Similarly, there was no measureable Ca²⁺ flux through phosphorylated hH1 channels in 0 Na⁺/5 Ca²⁺ solution (Fig. 1E). Results were comparable with either the human or rat channels, excluding a possible species-specific response.

These experiments relied on expression of the α subunit alone. Such a test was motivated by the fact that, in rat myocardium, Na⁺ channels consist only of α subunits (12), meaning it unlikely that β subunits somehow contributed to the original observations of slip mode conductance in rat heart cells. Nevertheless, to exclude the possibility that β subunit coexpression is required, we performed further experiments in CHO cells that coexpress the α and β1 subunits (13, 14). In these experiments we quantified both major manifestations of ion selectivity: reversal potential (E_{rev}), and ion flux. These two reflections of selectivity are complementary. The reversal potential in solutions of mixed ion composition yields relative ion permeabilities (4), but E_{rev} measurements can be problematic methodologically because small changes, resulting from junction potential drift for example, are difficult to exclude. For Na⁺ currents, the very nature of the measurements necessitates that small inward and outward currents be quantified reliably, which is difficult to do in large cells (for example, cardiac myocytes) with a variety of ionic conductances. In principle, the use of TTX subtraction may help in distinguishing among ions.

We took membrane current recordings from a representative CHO cell that coexpressed α + β1 subunits (Fig. 2A). The Na⁺ equilibrium potential was set to 0 mV by including 20 mM [Na⁺] in both the internal and external solutions (15). Membrane current was first recorded at baseline in the absence of cyclic AMP, but with 2 mM external [Ca²⁺]. The currents reversed at 0 mV (▲). The addition of 50 μM dibutyryl cAMP increased both inward and outward Na⁺ current, as expected from a simple increase in Na⁺ channel open probability. E_{rev} did not change despite the continuing presence of Ca²⁺ in the external solution (16) (●). Subsequent removal of external Ca²⁺ increased the amplitude of the Na⁺ currents at negative potentials; this effect is expected from the known voltage-dependent block of Na⁺ channels by external Ca²⁺ (17), but is in the opposite direction to the change that would have been expected had the channels been permeable by Ca²⁺. Once again, the current reversed at 0 mV (▲). The single experiment shown in Fig. 2A to C was representative of five cells, whose mean current–voltage relations are shown (B). On removal of Ca²⁺ in the continued presence of external Na⁺, E_{rev} did not change (16). This stability differs from the shift of 5.1 mV (18), which would have been seen had the relative Ca²⁺/Na⁺ permeabilities equaled 1.25, as stated in the report (5).

Inward currents disappeared (Fig. 2B) when the cells were bathed with an external solution containing 2 mM Ca²⁺ but no Na⁺ (▼). This observation further confirms the absence of an appreciable calcium conductance through Na⁺ channels. Results were indistinguishable between hH1 channels coassembled with human heart (hβ1) or rat brain (rβ1) β1 subunits. Confirmation that β1 in fact expresses functional subunits was derived from parallel experiments in which the same β1 cDNA increased current amplitude and shifted inactivation when coexpressed with Na⁺ channel α subunits (19). Because no E_{rev} shift was observed in mixed Na⁺/Ca²⁺ solutions, there is no basis for the notion that ‘‘slip mode’’ requires the modulatory presence of external Na⁺ ions.

Our results contradict findings in rat ventricular myocytes (5) and in HEK cells (20). We have used CHO cells, which are known to support cyclic AMP–mediated responses and which contain few endogenous conductances. The first reports of cyclic AMP–dependent upregulation of Na⁺ currents were from expression studies in CHO cells (10). HEK cells have the virtue of being readily transfected (21), but they do not consistently support cyclic AMP–dependent responses; several lines of evidence indicate that protein kinase activity is so high in the basal condition that kinase inhibitors must be added to reveal directionally appropriate responses (22).
Fig. 1. Testing for Ca\(^{2+}\) flux through phosphorylated Na\(^{+}\) channels. (A) Membrane currents recorded in CHO-K1 cells transfected with the rat cardiac Na\(^{+}\) channel (h1) \(\alpha\) subunit cDNA (3). (B) I-V relation peaks at \(-20\) mV and reverses near +60 mV. (C) Basal Na\(^{+}\) currents recorded with the use of 20 mM external Na\(^{+}\) as the permeant ion are increased in magnitude when phosphorylated (▲) during exposure to dibutyl cyclic AMP (10 \(\mu\)M). Complete exchange of the extracellular solution from 20 mM Na\(^{+}\)/0 Ca\(^{2+}\) to 0 Na\(^{+}\)/10 mM Ca\(^{2+}\) abolishes all inward currents (▼). Na\(^{+}\) current is restored on washout of Ca\(^{2+}\) and return of Na\(^{+}\) (20 mM) to the external solution (▲). (D) Peak inward current at \(-20\) mV plotted over the course of the above experiment illustrates the time course for the phosphorylation-mediated increase in peak Na\(^{+}\) current (▲) and the complete exchange of Ca\(^{2+}\) for Na\(^{+}\) in the recording solution. There was no measurable inward current in the absence of external Na\(^{+}\) with 10 mM Ca\(^{2+}\) as the putative charge carrier (▼). Washout of Ca\(^{2+}\) with the 20 mM Na\(^{+}\)/0 Ca\(^{2+}\) solution fully restored the current. Solution changes were repeated with similar results. (E) Human heart Na\(^{+}\) channel \(\alpha\) subunit expressed in CHO-K1 cells (2) also provided no evidence for Ca\(^{2+}\) flux in the absence of external Na\(^{+}\). Exposure to isoproterenol (1 \(\mu\)M) produced the phosphorylation-mediated increase in peak current (▲). Replacement of external Na\(^{+}\) (10 mM) with Ca\(^{2+}\) (5 mM) did not produce inward current (▼). Washout of Ca\(^{2+}\) with Na\(^{+}\) (10 mM) confirmed that there was no loss of functional channels during this manipulation (▲).
HEK cells also contain a variety of endogenous conductances that may interfere with the quantification of Na⁺ current reversal potentials. These include an endogenous TTX-sensitive Na⁺ channel (21) and a dihydropyridine-sensitive Ca⁺ channel (23). The Ca⁺ channel literature raises the caution that endogenous channels may interact with exogenously expressed β subunits in an unanticipated manner, clouding the interpretation of multisubunit expression studies (24). HEK cells also possess a variety of endogenous K⁺ and Cl⁻ conductances (25), which may complicate attempts to measure small differences in Na⁺ current reversal potential.

We have not observed Na⁺ currents in nontransfected CHO cells, but such endogenous TTX-sensitive Na⁺ currents (26, 27). To verify that our findings reflect the behavior of TTX-resistant cardiac Na⁺ channels, we measured I Na in hH1 + β1 + GFP transfected CHO cells under drug-free conditions and in the presence of low- and high-dose TTX (n = 7). The lower concentration of 100 nM would be expected to block >95% of TTX-sensitive channels, but only suppressed the current by 10.7 ± 1.4% (n = 7). In contrast, a higher concentration appropriate to inhibit cardiac channels (5 μM TTX) blocked 62.1 ± 2.9% (n = 7) of the current in agreement with Krafte et al. (27). Our results indicate that at least 90% of current in transfected cells arises from the TTX-resistant hH1 (cardiac) Na⁺ current. This conclusion is further bolstered by the observation that nongreen cells in the same dishes (n = 2) and CHO cells transfected with GFP alone (n = 4) had no observable Na⁺ currents under identical recording conditions.

In summary, our experiments show that cardiac Na⁺ channels are up-regulated by cyclic AMP, but that such up-regulation is not accompanied by changes in their Na⁺/Ca⁺ selectivity. Na⁺ channels are not measurably permeant to Ca⁺ either in the basal state or after cyclic AMP–dependent stimulation. Our experiments were designed to investigate the molecular basis of the reported “slip mode”: neither the cardiac α subunit alone nor α + β1 subunits suffice to confer such a phenomenon. Furthermore, cyclic AMP–dependent upregulation occurs without an associated change in selectivity, which implies that “slip mode” does not reflect a direct consequence of phosphorylation of either subunit. For these reasons, we conclude that the reports of “slip-mode conductance” represent a technical artifact, possibly arising from suboptimal voltage control.
with an intracellular (pipette) solution containing (in mM): 135 CsCl, 10 TEA-Cl, 10 Hepes, 5 EGTA, 4 MgATP, 2 CaCl2, pH 7.3 with CsOH.


13. The accessory subunit expressed in cardiac muscle is β1 (14).


15. Human and rat Na+ channel β1 subunit cDNA were subcloned into a mammalian expression vector (pGFPs) for bicistronic expression of the β1 subunit and GFP reporter [L. L. Isum et al., Science 256, 839 (1992); Y. H. Chu, B. D. O. Weggler, H. H. Ropers, A. L. George, Am. J. Physiol. 263, 629 (1992)].

16. D. J. Costanzo, M. T. Perez-Garcia, G. F. Tomaselli, and E. Marban, J. Biol. Chem. 272, 31598 (1997). Coexpression of α1 and β1 Na+ channel subunits was accomplished by cotransfecting equal masses of each plasmid into CHO-K1 cells, which provided a molar excess of β1 subunit cDNA due to the differences in length of coding sequences. The control extracellular recording solution was composed of (in mM): 125 CsCl, 20 NaCl, 2 CaCl2, 1 MgCl2, 5 dextrose, 10 Hepes, pH 7.3 with CsOH (except for the experiments described in Fig. 3, in which CsCl is replaced with NaCl). Dibutyryl-cAMP (50 μM) was present in all solutions except the initial control solution (Figs. 1, A and B, and 2, A and B, all figures). MgCl2 was increased to 3 mM when CaCl2 was removed from the bath (A) CsCl was increased to 145 mM to maintain the osmolarity of the 0 Na+ solution. Cells were dialyzed with an intracellular (pipette) solution containing (in mM): 125 CsCl, 20 NaCl, 10 Hepes, 5 EGTA, 4 MgATP, 0.33 MgCl2, pH 7.3 with CsOH.

17. Reversal potentials were determined by performing a linear regression of the ascending (linear) portion of each individual i-V and averaging the individual values of Erev for each solution. The experimentally determined Erev values equal 1.3 ± 1.0 mV, n = 5 in 20 Na+; 2 CaCl2; −0.1 ± 0.6 mV, n = 5 in 20 Na+; 2 CaCl2; 50 μM dB-cAMP; −0.6 ± 0.5 mV, n = 5 in 20 Na+; 2 CaCl2; 50 μM dB-cAMP; −37.8 ± 2.0 mV in 0 Na+; 2 CaCl2; 50 μM dB-cAMP (v). Junction potential offsets for the internal recording solutions measured with respect to the internal pipette solution were consistently between 0.8 to 1.2 mV. The calculated junction potentials for all four solutions at 22°C equals ±0.7 mv with respect to the pipette solution (AxoScope, Axon Instruments, city state). When the experimentally determined Erev values (Fig. 2) were corrected for the measured junction potential offsets, the results equaled: +0.1 mV for 20 Na+, 2 CaCl2; −1.3 mV for 20 Na+, 2 CaCl2; 50 μM dB-cAMP; −1.6 mV for 20 Na+, 0 CaCl2; 50 μM dB-cAMP; and −37.0 mV for 0 Na+, 2 CaCl2; 50 μM dB-cAMP (v).


19. A solution with a potential of 5.1 mV was calculated with the use of an explicit solution that Erev for a channel conducting both divalent and monovalent ions using Pm, 1 and Pr, 2. 25. The equation was derived from the Goldman-Hodgkin- Katz constant field equation by D. L. Campbell, W. R. Giles, J. R. Hume, D. Noble, and E. F. Shibata J. Physiol. 403, 267 (1988).

20. B1 cDNA was cotransfected with the rat skeletal muscle μ1 subunit cDNA in HEK cells. The midpoint of steady-state inactivation measured per J. R. Barfield, H. B. Nuss, D. Romashko, E. Marban, and G. F. Tomaselli [J. Gen. Physiol. 107, 643 (1996)] shifted from −66.9 ± 1.3 mV (n = 3) to −58.9 ± 0.2 mV (n = 3, p < 0.01), in agreement with S. N. Wright, S.-Y. Wang, H. F. Xiao, and G. K. Wang [Biophys. J. 76, 233 (1999)]. Current density also increased with β1 subunit expression consistent with H. B. Nuss, N. Chiamvontat, M. T. Perez-Garcia, G. F. Tomaselli, and E. Marban, J. Gen. Physiol. 106, 1171 (1995); and T. Yamagishi, G. F. Tomaselli, and E. Marban, unpublished data.


28. Supported by National Institutes of Health grant RO1-HL-52768 (EM) and an American Heart Association Scientist Development Grant (HNB). We thank W. Catterall for providing the rat cardiac Na+ channel α subunit cDNA.

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Santana et al. (I) observed a tetrodotoxin (TTX)-blockable Ca2+ current in rat ventricular myocytes after several pharmacological treatments [cyclic AMP, [3H]propranolol (ISO), or the cardiotonic steroids] that was distinct from those generating the classical Na+ current seen in the presence of ISO (a). Sodium channels (or whatever the native channel is) are phosphorylated in response to various stimuli, including PKA-dependent conversion of classical Na+ channels. The en-...
The increase in TTX-blockable Ca\(^{2+}\) current in the presence to that in the absence of 1 mM Ca\(^{2+}\) errors. Scale: 500 pA, 20 ms. (Fig. 1A, upper traces). Thus, if ISO induces any appreciable Ca\(^{2+}\) permeability in classical Na\(^{+}\) channels, this slippage should be evidenced by the appearance of a faster inactivating component in the TTX-blockable Ca\(^{2+}\) current, but none was detected (Fig. 1A, lower traces). Therefore, our experiments are not consistent with a change in selectivity of classical Na\(^{+}\) channels induced by conditions that promote channel phosphorylation, but are in agreement with findings in guinea pig, rabbit, and canine ventricular myocytes, as well as in rat cardiac Na\(^{+}\) (SKM2) channels expressed heterologously in frog oocytes (4).

**References and Notes**


3. Isolated rat ventricular myocytes were obtained by a standard enzymatic dispersion technique described previously [C. W. Balke and W. G. Wier, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4417 (1992)]. Membrane currents were recorded using the whole-cell configuration of the patch clamp technique. Patch pipettes were as described previously (2). Series resistance compensation was used in all experiments. Currents were filtered at 2 kHz and digitized at 20 kHz. The external solution was composed of 140 mM tetraethylammonium chloride (TEA-Cl), 10 mM glucose, 10 mM Hepes, 10 mM CsCl, 1 mM MgCl\(_2\) (pH adjusted to 7.3 with CsOH) and either 1 mM Na\(^{+}\) plus 0.5 mM Ca\(^{2+}\) or 1 mM Ca\(^{2+}\) only as noted. La\(^{3+}\) (10 mM) was included in all external solutions to suppress L-type Ca\(^{2+}\) currents. When indicated, isoproterenol (1 \(\mu\)M plus 10 \(\mu\)M EDTA to prevent oxidation) was added to this solution. The electrode filling solution contained 120 mM glutamic acid, 120 mM CsOH, 10 mM Hepes, 0.33 mM MgCl\(_2\), 20 mM TEA-Cl, 4 mM adenosine 5'-triphosphate (Mg salt), and 5 mM EGTA (pH adjusted to 7.3 with CsOH). Temperature was 21 to 23°C. The holding potential was -100 mV. Depolarizing pulses varied between -60 and -25 mV, in increments of 5 mV. Analyses were performed on records subtracted with the use of 10 \(\mu\)M TTX.


5. Animals used in the present study were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine and the Guide for the Care and Use of Laboratory Animals (National Institute of Health and Human Services publication No. (NIH) 85-23, revised 1985).

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**Response:** The TTX-sensitive Na⁺ channel is selective for Na⁺ over other monovalent and divalent cations (1–5). Recently, however, we reported that the ion selectivity of cardiac Na⁺ channels could be dynamically modulated (6). Following phosphorylation by protein kinase A (PKA), the Ca²⁺ permeability of the Na⁺ channel (P_{Ca}) could increase relative to Na⁺ permeability (P_{Na}) so that the permeability ratio (P_{Ca}/P_{Na}) was greater than 1. Called “slip-mode conductance” of the Na⁺ channel, this behavior was shown to be functionally important for heart cells when it was activated. First, it was shown that Ca²⁺ influx through Na⁺ channels alone could trigger Ca²⁺-induced Ca²⁺-release (CICR); activating Ca²⁺ sparks and small [Ca²⁺], transients. Second, it was demonstrated that Ca²⁺ influx through Na⁺ channels under near-physiological conditions using action potential “clamp” experiments. These results demonstrated that I_{Na} could activate measurable Ca²⁺ influx, Ca²⁺ sparks or [Ca²⁺], transients only when slip-mode conductance was activated. We also carried out quantitative examination of I_{Na} in heart cells under conditions that made it possible to measure this current. Low [Na⁺] at cool temperatures kept I_{Na} to values that could be reliably measured (1 to 4 nA). With the use of this approach, we measured large and unambiguous positive shifts in the I_{Na} reversal potential, E_{rev}, of 9 mV to 10 mV, when slip-mode conductance was activated. This shift in E_{rev} suggested that an increase of P_{Ca}/P_{Na} from a very low value (0.0 to 0.1) to large values (1.2 to 1.4) occurred when slip-mode conductance was activated. In their comments, neither Nuss and Marban nor Balke et al. have repeated these experiments in heart cells under conditions identical to those of our report (6). Consequently their comments do not directly address our report (6) and must be interpreted in light of the assumptions they have made. Balke et al. discuss a phenomenon that appears to be unrelated to slip-mode conductance of the Na⁺ channel. Nuss and Marban, however, did raise a question that we too have been examining for the past year: Can slip-mode conductance of the cardiac Na⁺ channel. Voltage-dependence of Na⁺ channel current (I_{Na}) is compared under four conditions: control (n = 8), following the addition of 500 μM Na⁺-dibutyryl cAMP (dbcAMP) (2 μM Ca²⁺) and 10 μM TTX (n = 4). The measured mean E_{rev} was close to E_{Na} for each curve: E_{Na} = –0.23 ± 0.72 mV (n = 8) (control), +0.82 ± 0.86 mV (n = 7) (dbcAMP, 2 mM Ca²⁺), +0.63 ± 0.77 mV (n = 7) (dbcAMP, 0 mM Ca²⁺), –2.04 ± 0.16 mV (n = 4) (dbcAMP, 2 mM Ca²⁺, TTX). When compared to the control, the addition of dbcAMP led to a significant increase in peak I_{Na} (21 ± 6% (p < 0.05, n = 8), with a significant shift in E_{rev} from 0.18 ± 0.65 mV to +4.00 ± 0.36 mV (p < 0.0001, n = 8). E_{rev} = 0.63 ± 0.76 mV (dbcAMP, 0 mM Ca²⁺); E_{rev} = –0.80 ± 1.25 mV (dbcAMP, 2 mM Ca²⁺, TTX). (*) indicates a significant difference between dbcAMP average I_{Na} and control I_{Na} (p < 0.05). An enlargement of the region of the reversal potentials shown as an inset. P_{Ca}/P_{Na}, for the measured ΔE_{rev} indicates an increase from about 0.04 (control) to 0.79 (dbcAMP), calculated from Campbell et al. (10). Addition of TTX (10 μM) reduced I_{Na} at 30 mV by 78.8 ± 4.7% (p < 0.05, n = 8) with a significant shift in E_{rev} back towards the control –0.59 ± 1.25 mV (p < 0.05, n = 8) when compared to the E_{rev} for I_{Na} in dbcAMP alone. (C) PKA-inhibitory peptide. Averaged I_{Na} IV relationships (n = 5) for HEK293 cells expressing α and β subunits of the human cardiac Na⁺ channel observed when PKA-inhibitory peptide (PKA-I) was added (100 μM) to the pipette filling solution (2). Control solutions were otherwise similar to those in A. IV relationships of I_{Na} were obtained under control conditions, following the addition of 500 μM Na⁺-dbcAMP (2 Ca²⁺) and following the addition of 500 μM Na⁺-dbcAMP (2 Ca²⁺) with 10 μM TTX. E_{rev} = –0.02 ± 0.44 mV (Control), E_{rev} = –0.05 ± 0.41 mV (dbcAMP, 2 Ca²⁺), E_{rev} = –0.2 ± 0.84 mV (dbcAMP, 2 mM Ca²⁺, TTX). Thus E_{rev} was similar in all conditions (control, dbcAMP, dbcAMP + TTX) and close to E_{Na} (0 mV, 0.63 mV and 0.63 mV respectively). (D) β only. Averaged I_{Na} IV relationships (n = 8) for HEK293 cells expressing only β subunits only under control conditions (left) and following control conditions (left) with I_{Na} records from a single cell (right). No significant measured I_{Na} is observed. (E) Sham transfection. Averaged I_{Na} IV relationships (n = 6) for HEK293 cells exposed to all aspects of the transfection process but with no added vector (left) with I_{Na} records from a single cell (right). No significant I_{Na} is observed. E_{Na} = 0 under control conditions (20 mM Na⁺), E_{Na} = 0.63 mV in dbcAMP (20.5 Na⁺, 2 Ca²⁺), E_{Na} = 0.63 mV in dbcAMP (20.5 Na⁺, 0 Ca²⁺).
Na⁺ channel be observed in an heterologous expression system? The experiments presented below, in contrast to those of Nuss and Marbán, show that Ca²⁺ can permeate Na⁺ channels in an heterologous expression system and thus provide strong evidence in support of our original findings and hypotheses (6).

If slip-mode conductance of the cardiac Na⁺ channel only relies on appropriate phosphorylation of the cardiac Na⁺ channel and the presence of Na⁺ channels, then it should be possible to reproduce the changes in I_{Na} and in Ca²⁺ flux that we have seen in heart using an heterologous expression system. A key factor in any such experiment is setting up the conditions in the correct manner. We chose to use HEK293 cells because of the low level of voltage-gated channels expressed in these cells (7). However, we did not know a priori what part of the heterotrophic Na⁺ channel was responsible for slip-mode conductance. Although virtually all features of cardiac Na⁺ channels have been attributed to the α subunit and can be observed when this subunit of the Na⁺ channel is expressed in an heterologous system (2, 8), there are two other Na⁺ channel subunits, β₁ and β₂. Because of the importance of the α subunit, we examined it in HEK293 cells first. I_{Na} occurred when only the α subunit of the human isoform of the cardiac Na⁺ channel (hH1α) was expressed in HEK293 cells (Fig. 1A). If slip-mode conductance of the Na⁺ channel could be produced when only the α subunit was expressed, then a shift of E_{rev} would be expected when PKA is activated. However under all conditions tested, E_{rev} remained at the Na⁺ equilibrium potential (E_{Na}), which was 0 mV under control conditions and 0.63 mV in Na⁺-dbcAMP (10). A small increase in I_{Na} magnitude following the addition of dbcAMP is consistent with published results (9), as is the block of I_{Na} by TTX (10 μM).

Under the ionic conditions of these experiments, an increase in P_{Ca/PNa} would have led to inward current at E_{Na} and a positive shift in E_{rev} (10). Because neither was observed, we deduced that there was no significant change in P_{Ca/PNa} (10), a finding similar to that observed by Grant et al. (11). How can these findings (Fig. 1A) be reconciled with the finding that Na⁺ channels in rat heart became permeable to Ca²⁺ following the activation of PKA (2)? Could our earlier results with rat heart cells (6) simply be wrong? That conclusion seemed unlikely, because multiple investigative methods were used to demonstrate slip-mode conductance of the cardiac Na⁺ channel in rat myocardyocytes. We thus examined the possibility that a missing factor or channel subunit was responsible for the absence of any PKA-activated increase in P_{Ca/PNa} when only the α subunit was expressed (see also Table 1). Because intact heart cells express α and both β subunits (12) and recent evidence suggests that both α and β subunits are associated with each other in heart (13) and in heterologous expression systems (14), we examined HEK293 cells that co-express α and β₁ subunits.

We measured the current-voltage (IV) relationships for I_{Na} in HEK293 cells that co-express α and β₁ subunits of the Na⁺ channels under several experimental conditions (Fig. 1B). Under control conditions, E_{rev} = −0.23 mV, a value statistically indistinguishable from E_{Na} of 0 mV. After the addition of 500 μM Na⁺-dbcAMP, a significant increase of E_{rev} was observed (E_{rev} = 4.00 mV, P < 0.0001, n = 8). This increase in E_{rev} suggests that P_{Ca/PNa} has increased from close to zero to 0.79 (10), indicating that Ca²⁺ was almost as permeable through Na⁺ channels as was Na⁺. The altered E_{rev} was returned to the control level by the removal of extracellular Ca²⁺ (replaced by Mg²⁺), a result that also supports the conclusion that Ca²⁺ permeation underlies the shift of E_{rev} following the addition of dbcAMP. In the maintained presence of dbcAMP and with 2 mM [Ca²⁺]o, TTX significantly reduced I_{Na} and also produced a significant negative shift in E_{rev} back to control conditions. We thus concluded that first, the observed TTX-sensitivity confirms the involvement of cardiac Na⁺ channels in the measured currents. Second, Na⁺ channels altered by PKA may be more sensitive to TTX when in slip-mode conductance than when they are not. If Na⁺ channels in slip-mode and those not in slip-mode were equally sensitive to TTX, then TTX would not have shifted E_{rev} back to E_{Na}. [This finding is identical to that observed in intact rat ventricular myocytes (6).] Third, protein-protein interactions between the α and β₃ subunits are.

### Table 1. Modulation of P_{Ca/PNa}

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Control</th>
<th>PKA activation</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 cardiac cells: Na⁺ = Naᵢ = 10 mM; Caᵢ = 2 mM; Caᵦ = 0; Monovalent replacement: Cs⁺</td>
<td>0</td>
<td>0</td>
<td>+9</td>
</tr>
<tr>
<td>2 α subunit only in HEK293 cells: Naᵢ = 20 mM; Naᵦ = 20 mM (control); Naᵦ = 20.5 mM (dbcAMP); Caᵢ = 2 mM; Caᵦ = 100 nM; Monovalent replacement: Cs⁺</td>
<td>0</td>
<td>−0.23</td>
<td>+0.63</td>
</tr>
<tr>
<td>3 α₁β₁ subunits in HEK293 cells: Naᵢ = Naᵦ = 20 mM (control); Naᵦ = 20.5 mM (dbcAMP); Cₐᵦ = 2 mM; Caᵦ = 100 nM; Monovalent replacement: Cs⁺</td>
<td>0.18</td>
<td>0.04</td>
<td>+0.63</td>
</tr>
<tr>
<td>4 PKA-I peptide and α₁β₁ subunits in HEK293 cells: Naᵢ = Naᵦ = 20 mM (control); Naᵦ = 20.5 mM (dbcAMP); Caᵦ = 2 mM; Caᵦ = 100 nM; Monovalent replacement: Cs⁺</td>
<td>0</td>
<td>−0.02</td>
<td>+0.63</td>
</tr>
<tr>
<td>5 α₁β₁ subunits in HEK293 cells: altered ionic conditions; Naᵢ = Naᵦ = 10 mM; Caᵦ = 5 mM; Caᵦ = 0 nM; Monovalent replacement: Cs⁺; 10 mM TEA</td>
<td>0.94</td>
<td>0.04</td>
<td>1.2</td>
</tr>
<tr>
<td>6 α₁β₁ subunits in HEK293 cells: very low Na⁺; Naᵢ = 10 mM; Naᵦ = 0.5 mM (dbcAMP); Caᵦ = 5 mM; Caᵦ = 0 nM; Monovalent replacement: Cs⁺; 10 mM TEA</td>
<td>−79</td>
<td>n.d.</td>
<td>−76.2</td>
</tr>
<tr>
<td>7 α₁β₁, β₂ subunits in HEK293 cells: Naᵦ = 20 mM (control); Naᵦ = 20.5 mM (dbcAMP); Caᵦ = 2 mM; Caᵦ = 100 nM; Monovalent replacement: Cs⁺; 10 mM TEA</td>
<td>0.01</td>
<td>0.01</td>
<td>0.63</td>
</tr>
<tr>
<td>8 α₁β₁ subunits in HEK293 cells: altered monovalent cation: Naᵦ = Naᵦ = 20 mM (control); Naᵦ = 20.5 mM (dbcAMP); Caᵦ = 2 mM; Caᵦ = 100 nM; Monovalent replacement: NMG⁺</td>
<td>0.53</td>
<td>0.11</td>
<td>0.63</td>
</tr>
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</table>

n.d., experiment not done.
important for proper Na⁺ channel function and provides a functional reason role for the β₃ subunit of the Na⁺ channel in heart. PKA-dependent phosphorylation underlies the activation of slip-mode conductance (Fig. 1C), which can be prevented by adding intracellular PKA inhibitory peptide (PKA-I, 100 μM).

Two control experiments (Fig. 1, D and E) indicated that neither the expression of the β₃ subunit alone nor a sham transfection of HEK293 cells altered the HEK293 cells to produce I_{Na} under control conditions or following exposure to dbcAMP. We thus conclude that there are no interfering voltage-gated currents in these cells to confound interpretation.

Ca²⁺ permeation through the cardiac Na⁺ channel is thus confirmed (Fig. 1) in support of our report (6). The α subunit alone is not enough (Fig. 1) to support this change in selectivity. Instead, it was found that the co-expression of an α Na⁺ channel subunit along with a β subunit was required for us to observe slip-mode conductance of the Na⁺ channel in an heterologous expression system. The α subunit is central to our understanding of ion permeation through Na⁺ channels because it contains the ion channel, the selectivity filter, the TTX binding site, and the PKA consensus phosphorylation sites. However, we have no structural information on how the α subunit is altered to produce slip-mode conductance. Also we do not know what the β subunit does and whether or not its action requires other factors. Intuition from nonselective channels, including mutated Na⁺ channels (15), suggests that larger changes in E_{rev} might be observed in bi-ionic conditions (for example, high Ca²⁺ outside and high Na⁺ inside with low Ca²⁺ inside and low Na⁺ outside). One finding (Fig. 2, A and B) was counterintuitive. HEK293 cells were made to express α and β subunits (as was shown in Fig. 1), but with somewhat reduced intracellular and extracellular [Na⁺] (10 mM) and increased extracellular [Ca²⁺] (5 mM).

Slip-mode conductance was readily activated by dbcAMP, leading to a positive shift of E_{rev} of more than 7 mV, more than was observed in Fig. 1. Unexpectedly, however, P_{Ca/PNa} was only 0.32. Experiments (16) similar to those of Fig. 2, A and B, but with extracellular [Ca²⁺] at 2 mM led to a larger calculated P_{Ca/PNa} of 0.95. Thus, although extracellular Ca²⁺ permeates the Na⁺ channel during slip-mode conductance, it also tends to block the conductance.

To further examine the role of extracellular Na⁺ in producing slip-mode conductance, we reduced extracellular [Na⁺] from 10.5 to 0.5 after slip-mode had been activated by dbcAMP, and we recorded I_{Na} at four select potentials from one cell (Fig. 2A). Switching to an extracellular Na⁺ of 0.5 mM with continued exposure to dbcAMP led to the virtual abolition of measured Ca²⁺ flux through Na⁺ channels. Fig. 2B shows the three IV plots (n = 7) that correspond to the conditions noted above. Reduction of extracellular Na⁺ to 0.5 mM led to the apparent abolition of inward I_{Na}, including any component carried by Ca²⁺. This observation was the second unexpected finding in these experiments (Figs. 2A and B). The I_{Na} IV plot shows that outward currents began to appear at potentials positive to −37 mV, which suggested that the "reversal potential" for I_{Na} was −37 mV or more negative. This suggests that under these conditions P_{Ca/PNa} is 0.11 or less (10). Taken together, these data (Fig. 1 and Fig. 2, A and 2B) suggest that even when slip-mode conductance is activated by PKA, as [Na⁺] increases, does P_{Ca/PNa}. These experiments, as noted above, also support a blocking action of high extracellular [Ca²⁺]. Dual actions of extracellular Ca²⁺ are consistent with the hypothesis that two independent processes are involved, one involving Ca²⁺ that blocks Na⁺ channels (previously established) and another that permits it to permeate.

We do not find (Fig. 2, A and B) support for the inward current described by Balke et al. In HEK293 cells that co-express α and β subunits of the cardiac Na⁺ channel, the absence of inward current in 0.5 mM [Na⁺] in 5 mM [Ca²⁺] suggests that the current they describe is not the result of H₁α and β₂ subunits. Furthermore, our experiments (Fig. 2) rule out their suggestion that slip-mode conductance of the cardiac Na⁺
A  &  NMG &  0.25 &  mV &  -100 & -20 &  20 &  80 &  \( \text{Control} \) &  \( \text{dbcAMP} \) \\
C  &  K\(^+\) &  0.4 &  20 &  40 mV &  \( \text{Control} \) &  \( \text{dbcAMP} \) \\
E  &  Ba\(^{2+}\) &  1.2 &  -80 &  0 &  20 mV &  \( \text{Control} \) &  \( \text{dbcAMP} \)

Fig. 3. Permeation by other ions during slip-mode conductance in HEK293 cells expressing \( \alpha \) and \( \beta_1 \) subunits. N-methyl-d-glucamine (NMG\(^-\)) was used in these experiments. (A) Selectivity of the Na\(^+\) channel for NMG\(^-\) (compared to Na\(^+\)) in the absence of Ca\(^{2+}\) before and after PKA activation. Solutions had the following ion composition: [Na\(^+\)]\(_i\) = 120 mM; [Na\(^+\)]\(_o\) = 10 mM; [Ca\(^{2+}\)]\(_i\) = 0 mM; [Ca\(^{2+}\)]\(_o\) = 0 mM; [NMG\(^-\)]\(_i\) = 120 mM; [NMG\(^-\)]\(_o\) = 10 mM; [Mg\(^{2+}\)]\(_i\) = 1 mM; [Mg\(^{2+}\)]\(_o\) = 4 mM. Depolarizations from a holding potential of −110 mV to a potential over the range −100 to −80 mV in 5 mV steps were applied. IV relationships before and after 500 \( \mu \)M Na\(^+-\)dbcAMP are shown. A statistically insignificant reduction of \( I_{Na} \) was observed following dbcAMP, 24 ± 5.9%, \( n = 6, p = n.s. \). No significant change in \( E_{rev} \) was observed (control: 55.4 ± 2.1 mV; dbcAMP: 56.7 ± 1.5 mV, \( n = 7, p = n.s. \)). A \( P_{\text{NMG}}/P_{\text{Na}} \) ratio < 0.03 can account for the measured \( E_{rev} \) under both conditions. (B) Selectivity of the Na\(^+\) channel for Cs\(^+\) (compared to Na\(^+\)) in the absence of Ca\(^{2+}\) before and after PKA activation. Ion composition: [Na\(^+\)]\(_i\) = 100 mM; [Na\(^+\)]\(_o\) = 10 mM; [Ca\(^{2+}\)]\(_i\) = 0 mM; [Ca\(^{2+}\)]\(_o\) = 0 mM; [Cs\(^+\)]\(_i\) = 60 mM; [Cs\(^+\)]\(_o\) = 27 mM; [NMG\(^-\)]\(_i\) = 75 mM; [NMG\(^-\)]\(_o\) = 18 mM; [Mg\(^{2+}\)]\(_i\) = 1 mM; [Mg\(^{2+}\)]\(_o\) = 4 mM. Depolarizations from a holding potential of −110 mV to a potential over the range −80 mV to +70 in 10 mV steps were applied. IV relationships before and after dbcAMP are shown. A statistically insignificant reduction of \( I_{Na} \) was observed following dbcAMP, 28.5 ± 21.2% reduction, \( n = 4, p = n.s. \). A statistically insignificant reduction of \( E_{rev} \) was observed (control: 47.2 ± 1.9 mV under control conditions and 45.2 ± 1.8 mV in dbcAMP, \( n = 4, p = n.s. \)) corresponding to similar ratios \( P_{\text{Cs}}/P_{\text{Na}} \). (C) Selectivity of Na\(^+\) channel for K\(^+\) compared to Na\(^+\) in the absence of Ca\(^{2+}\) before and after PKA activation. Ion composition: [Na\(^+\)]\(_i\) = 100 mM; [Na\(^+\)]\(_o\) = 10 mM; [Ca\(^{2+}\)]\(_i\) = 0 mM; [Ca\(^{2+}\)]\(_o\) = 0 mM; [K\(^+\)]\(_i\) = 60 mM; [K\(^+\)]\(_o\) = 27 mM; [NMG\(^-\)]\(_i\) = 75 mM; [NMG\(^-\)]\(_o\) = 18 mM; [Mg\(^{2+}\)]\(_i\) = 1 mM; [Mg\(^{2+}\)]\(_o\) = 4 mM. Depolarizations from a holding potential of −110 mV to a potential over the range −100 mV to +17 mV in 5 mV steps were applied. IV relationships before and afterdbcAMP are shown. A statistically significant reduction of \( I_{Na} \) was observed following dbcAMP, 16.1% ± 8.6% reduction, \( n = 4, p = 0.05 \). A 28.5% ± 4.3% reduction was observed following dbcAMP, \( n = 4, p = 0.05 \). A statistically significant reduction of \( E_{rev} \) was observed following dbcAMP, 4.8% ± 1.5 mV, \( n = 4, p = 0.05 \). (D) Selectivity for Ca\(^{2+}\) over Na\(^+\) in the presence of NMG\(^-\). Depolarizations from a holding potential of −108 mV to potential in the range −78 to +17 mV in 5 mV steps were applied. IV relationships for \( I_{Na} \) in control conditions and after the application of 500 \( \mu \)M Na\(^+-\)dbcAMP for 5 cells are shown. Control conditions include [Na\(^+\)]\(_i\) = 20 mM; [Na\(^+\)]\(_o\) = 20 mM; [Ca\(^{2+}\)]\(_i\) = 2 mM; [Ca\(^{2+}\)]\(_o\) = 100 mM; [K\(^+\)]\(_i\) = 0 mM; [K\(^+\)]\(_o\) = 0 mM; [NMG\(^-\)]\(_i\) = 125 mM; [NMG\(^-\)]\(_o\) = 25 mM; [Mg\(^{2+}\)]\(_i\) = 0 mM; [Mg\(^{2+}\)]\(_o\) = 4 mM. A 16.1% ± 8.6% reduction of \( I_{Na} \) was observed following dbcAMP, \( E_{rev} \) increased from 0.53 ± 1.5 mV to 6.35 ± 1.76 mV, \( n = 4, p < 0.05 \), which suggests that \( P_{\text{Ca}}/P_{\text{Na}} \) was increased from 0.11 to 1.48. (E) Selectivity for Ba\(^{2+}\) through Na\(^+\) channels. Depolarizations from a holding potential of −110 mV to a range of potentials from −80 to +15 mV in 5 mV steps were applied. IV relationships in control conditions and after the application of 500 \( \mu \)M Na\(^+-\)dbcAMP are shown for six cells. Control conditions include [Na\(^+\)]\(_i\) = 20 mM; [Na\(^+\)]\(_o\) = 20 mM; [Ba\(^{2+}\)]\(_i\) = 2 mM; [Ba\(^{2+}\)]\(_o\) = 100 mM; [K\(^+\)]\(_i\) = 0 mM; [K\(^+\)]\(_o\) = 0 mM; [NMG\(^-\)]\(_i\) = 125 mM; [NMG\(^-\)]\(_o\) = 125 mM; [Mg\(^{2+}\)]\(_i\) = 0 mM; [Mg\(^{2+}\)]\(_o\) = 4 mM. A statistically insignificant reduction of peak \( I_{Na} \) was observed following dbcAMP. No change in \( E_{rev} \) was observed; it remained nearly constant and close to \( E_{Na} \). Control: −0.83 ± 0.27 mV; after dbcAMP: −0.65 ± 0.4 mV, \( n = 6, p = n.s. \). \( P_{\text{Ba}}/P_{\text{Na}} \) could not be distinguished from zero.
channel is the result of the same putatively novel channel protein that Balke et al. state is responsible for the current they observe.

Just as the increase in $P_{Ca}/P_{Na}$ during slip-mode conductance should lead to a measured shift in $E_{rev}$ (Figs. 1 and Fig. 2, A and B), it should also produce measurable $Ca^{2+}$ influx in HEK293 cells. With the use of an amphotericin perforated patch-clamp method (17) with cells loaded with the $Ca^{2+}$-sensitive indicator fluo-3, we measured $[Ca^{2+}]_{i}$ during $Na^{+}$ channel activation. This method permitted patch-clamp control while measuring $[Ca^{2+}]_{i}$, and avoided the loss of $Ca^{2+}$ into the pipette which can severely distort $[Ca^{2+}]_{i}$ measurements (18). Because this approach uses an entirely different method to investigate $Ca^{2+}$ entry through cardiac $Na^{+}$ channels, it provided an independent check on the earlier measured changes in $E_{rev}$. In particular, this method of measuring $Ca^{2+}$ influx through $Na^{+}$ channels does not depend on tip-potential measurements. For these experiments, we transfected the HEK293 cells with all three relevant cardiac $Na^{+}$ channel subunits: hH1, the universal $\beta$ subunit, and a $\beta_{1}$ subunit cloned from human heart (13). We did this after finding that slip-mode conductance of cardiac $Na^{+}$ channels could also be measured when only $\alpha$ and $\beta_{2}$ were co-expressed (16). Our overall experience with cardiac $Na^{+}$ channels suggests that the triple transection ($\alpha$, $\beta_{1}$, $\beta_{2}$) provides the most reliable expression of $I_{Na}$, and the most robust slip-mode conductance. We took fluorescence images (Fig. 2C) of HEK293 cells containing the $Ca^{2+}$ indicator fluo-3 under control conditions (that is, no PKA activation) following 100 depolarizing pulses to activate $I_{Na}$ (left), and then following 100 depolarizing pulses in the presence of 500 $\mu$M dbcAMP (middle), and following the removal of extracellular $Ca^{2+}$ (right). External $[Na^{+}]$ and pipette $[Na^{+}]$ concentrations were 20 $mM$ and extracellular $[Ca^{2+}]$ was 2 $mM$ (as in Fig. 1). The top set of images in Fig. 2C shows triply transfected cells, the middle set shows triply transfected cells in the presence of 10 $\mu$M TTX, and the bottom set shows cells transfected with only the $\alpha$ subunit. Supporting our interpretation of Figs. 1B and 2B, we found (Fig. 2C) that the addition of dbcAMP enabled $Na^{+}$ channels in HEK293 cells to become permeable to $Ca^{2+}$ if they were composed of $\alpha$, $\beta_{1}$, and $\beta_{2}$ subunits but not if they expressed the $\alpha$ subunit only. The increase of $[Ca^{2+}]_{i}$, arising from the flux of $Ca^{2+}$ through $Na^{+}$ channels depended on the number of depolarizing pulses and thus on the number of times that $I_{Na}$ was activated (Fig. 2D) and was blocked by TTX. An increase of $[Ca^{2+}]_{i}$, from 100 nM to about 325 nM after 100 pulses is consistent with a $Na^{+}$ current having a magnitude of 300 pA with an inactivation time constant of 2 ms if 10% of the current is carried by $Ca^{2+}$ and this $Ca^{2+}$ flux enters a 16-$\mu$m-diameter cell with a buffering power of 60. The $\Delta[Ca^{2+}]_{i}$ achieved after 100 pulses was proportional to the measured peak $I_{Na}$ (Fig. 2E), a finding also consistent with the hypothesis that $Ca^{2+}$ can permeate $Na^{+}$ channels that exhibit slip-mode conductance. The elevated $[Ca^{2+}]_{i}$ fell towards the prestimulation level ($\tau = 36 \text{ s}$) when extracellular $Ca^{2+}$ was removed (Fig. 2F). When HEK293 cells were triply transfected, $I_{Na}$ had a reversal potential at $E_{Na}$ before slip-mode was activated by PKA (Fig. 2G). After slip-mode had been activated, $E_{rev}$ moved towards $E_{Ca}$ by 5.0 $mV$, which was consistent with an increase in $P_{Ca}/P_{Na}$ from 0 to 1.1, a change comparable to the increase in $P_{Ca}/P_{Na}$ seen in rat heart cells ($P_{Ca}/P_{Na} = 1.2$) following PKA activation. Finally, protein immunoblots (Fig. 2H) indicated that both $\beta_{1}$ and $\beta_{2}$ subunits were expressed in these HEK293 cells following the triple transfection. $\beta_{2}$ associated with the $\alpha$ subunit following the triple transfection (Fig. 2H (ii)) (19). The $\beta_{2}$ dissociates from $\alpha$ under these preparative conditions because, unlike $\beta_{1}$, it is not attached by disulfide bonds. These results and others (13, 14) provide direct evidence that the three cardiac $Na^{+}$ channel subunits are associated to form the normal cardiac $Na^{+}$ channel. All three subunits also contribute functionally to $Na^{+}$ channel behavior. The triply transfected HEK293 cells expressed $I_{Na}$ better and more reliably than the other subunit combinations we tested. Following PKA activation by dbcAMP, there was a decrease in the peak $I_{Na}$ at $-30 mV$ in the triply transfected HEK293 cells, a finding different to that observed in rat heart cells and in Figs. 1B and 2B. Additional studies will be needed to better understand this property of $Na^{+}$ channels.

A question raised by these findings (Figs. 1 and 2) is whether the $Na^{+}$ channel may also become permeable to other cations under conditions that activate slip-mode conductance. This topic was investigated in HEK293 cells expressing $\alpha$ and $\beta_{1}$ subunits. In the absence of extracellular $Ca^{2+}$, $NMG^{+}$ does not readily pass through the $Na^{+}$ channel before or after activation of slip-mode conductance ($P_{NMG^{+}}/P_{Na} < 0.03$); there is no significant change in $E_{rev}$ (Fig. 3A). Under control conditions $P_{Ca}/P_{Na}$ was about 0.10 and, following PKA activation, increased insignificantly to 0.12 (Fig. 3B). Thus, the $Na^{+}$ channel permitted a very small amount of $Cs^{+}$ and almost no $NMG^{+}$ to permeate; the permeability of neither cation was significantly modulated by PKA activation. Consistent with these observations was the insignificant reduction of peak $I_{Na}$ that followed PKA activation in $Cs^{+}$ (decreasing 28.5 $\%$ 21.2 $\%$, $p = n.s., n = 6$). In $NMG^{+}$, the reduction of 24.6 $\%$ 5.9 $\%$ ($p = n.s., n = 6$) was also insignificant. In contrast, $K^{+}$ ions were more readily conducted by $Na^{+}$ channels (Fig. 3C) under control conditions than either $Cs^{+}$ or $NMG^{+}$ ions. We examined, in the absence of $Ca^{2+}$, the relative changes in $I_{Na}$ when $K^{+}$ and $Na^{+}$ were present and when $NMG^{+}$ was the impermeant cation (chosen because it is the least permeant) (Fig. 3A). In the presence of $K^{+}$, the maximum $I_{Na}$ was reduced by 58.2 $\pm$ 16 $\%$ ($p < 0.05, n = 4$) following the application of dbcAMP. In these experiments $E_{rev}$, the expected $E_{rev}$ for $I_{Na}$ was 59 $mV$ if there is no permeability by $K^{+}$ through $Na^{+}$ channels. We found that under control conditions $E_{rev}$ was 36.75 $\pm$ 0.78 $mV$ ($n = 4$) indicating $P_{Ca}/P_{Na}$ was about 0.25 under these ionic conditions. Following PKA activation $E_{rev}$ shifted to 27.73 $\pm$ 4.31 $mV$ ($p < 0.05, n = 4$), which indicated that $P_{Ca}/P_{Na}$ almost doubled to 0.47. We concluded that $K^{+}$ permeation of the cardiac $Na^{+}$ channel (in the absence of $Ca^{2+}$) is also modulated by PKA-dependent phosphorylation. Nevertheless, compared to the single amino acid mutations of the selectivity filter (15), the changes in ion selectivity that we observed were subtle and specific following PKA-dependent phosphorylation.

Because $Ca^{2+}$ permeation arises as PKA-activation occurs, and because $Ba^{2+}$ permeates all known $Ca^{2+}$ channels (I), we examined the extent to which $Ba^{2+}$ could permeate $Na^{+}$ channels in slip-mode conductance. Because $NMG^{+}$ was the least permeant monovalent cation tested, it was used as the impermeant monovalent cation during these experiments. We examined $Ca^{2+}$ flux through $Na^{+}$ channels (Fig. 3D) under conditions similar to those used in the $Ba^{2+}$ permeation experiments (below). First, with 2 $mM$ extracellular $Ca^{2+}$, $E_{rev}$ shifted from 0.53 $\pm$ 1.5 $mV$ (control) to 6.35 $\pm$ 1.76 $mV$ (dbcAMP) ($p < 0.05, n = 4$), which suggested that following PKA activation $P_{Ca}/P_{Na}$ was 1.48. (inset in Fig. 3D). Although a large increase in $P_{Ca}/P_{Na}$ was observed, there was a reduction of peak $I_{Na}$ produced by dbcAMP, similar to that seen in Fig. 2G. These results provide additional evidence that specific ionic conditions influence $I_{Na}$ and PKA-induced changes in $P_{Ca}/P_{Na}$ of cardiac $Na^{+}$ channels. In contrast, when $Ba^{2+}$ was used to replace $Ca^{2+}$ in the extracellular solution, no change in $E_{rev}$ was observed following activation of PKA. $E_{rev}$ shifts slightly from $-0.83 \pm 0.27 mV$ to $0.65 \pm 0.4 mV$ ($p = n.s., n = 6$). This result suggested that $P_{Ba^{2+}}/P_{Na^{+}}$ was about zero whether PKA was activated or not. Although $Ba^{2+}$ normally can permeate $Ca^{2+}$ channels, in other biological processes $Ba^{2+}$ is readily distinguished from $Ca^{2+}$. For example, although $Ca^{2+}$ flux through L-type $Ca^{2+}$ channels augments $I_{Na}$ inactivation, $Ba^{2+}$ flux through L-type $Ca^{2+}$ channels does not. A second example is found in the
“Ca\(^{2+}\)”-induced Ca\(^{2+}\)”-release phenomenon in heart. Normally Ca\(^{2+}\)”-release channels in the sarcoplasmic reticulum cryanodin receptors are rapidly activated by Ca\(^{2+}\”), but not by Ba\(^{2+}\). The absence of Ba\(^{2+}\) permeation through Na\(^{+}\) channels activated by PKA adds to the evidence that the permeation process during slip-mode conduction of the Na\(^{+}\) channel is distinctive and involves regulation by the permeant ions themselves.

We thus confirm the ability of PKA phosphorylation of cardiac Na\(^{+}\) channels to enable Ca\(^{2+}\”\) flux as we originally proposed in our report (6). The experiments presented here were carried out in an heterologous expression system using HEK293 cells. These cells do not have the numerous voltage-gated currents (Fig. 1) seen in heart muscle cells. The human heart Na\(^{+}\) channel \(\alpha\) subunit (hH1α) was expressed along with either the \(\beta_1\) subunit or the \(\beta_2\) subunit, or both. All three combinations of \(\alpha\) subunit plus \(\beta\) subunits could produce slip-mode conductance. However, neither \(\beta_1\) nor \(\beta_2\) subunit alone was seen to produce PKA-dependent Ca\(^{2+}\”\) permeation of the Na\(^{+}\) channel. We speculate that it is the \(\alpha\) subunit that has the ability to produce slip-mode conductance, but only under specific conditions. One condition that is necessary is the co-expression of either \(\beta_1\) or \(\beta_2\) subunits (or both subunits). We surmise that the \(\beta\) subunits enable the \(\alpha\) subunit to function properly by one or more of the following means. They may help it fold properly in the SL membrane and stabilize important conformations of the protein, or directly modulate \(\alpha\) subunit function, or help the \(\alpha\) subunit associate with other important proteins. Co-transfection of \(\alpha\) and one of the \(\beta\) subunits did not always enable measurable slip-mode conductance of \(I_{Na}\). Why such variability occurred and how it depended on conditions known to affect subunit assembly (that is stoichiometry of expression, expression conditions, unidentified co-factors, the cell line used for expression, timing and conditions of the transfection, or other factors) remains unknown at this time. Presumably one or more of these conditions was not met in the experiments carried out by Nuss and Marbán and “different conditions” accounted for their negative results. We have directly addressed each of the concerns expressed in their comment. Also we have more completely characterized the molecular requirement for slip-mode conductance of cardiac Na\(^{+}\) channels.

We have identified and characterized important new features of TTX-sensitive Na\(^{+}\) channels from human heart. Most significantly, we demonstrate that Ca\(^{2+}\”\) can permeate cardiac Na\(^{+}\) channels following PKA activation during “slip-mode” conductance in an heterologous expression system. This permeation by Ca\(^{2+}\”\) of Na\(^{+}\) channels is evidenced by shifts in \(E_{rev}\) and by measured Ca\(^{2+}\”\) influx seen as increases in [Ca\(^{2+}\”\)]. That the \(\beta\) subunits, with no previously identified function in heart, are necessary to support this conductance mode of the \(\alpha\) subunit was unexpected. Additional unexpected features of slip-mode conductance of the cardiac Na\(^{+}\”\) channel have been identified and include facilitation by Na\(^{+}\”), block and permeation by Ca\(^{2+}\”\), and permeation by K\(^{+}\”\).

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\[ \text{References and Notes} \]

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8. \(I_{Na}\) attributed to the \(\alpha\) subunit alone did, however, appear to be sloved kinetically compared to the native \(I_{Na}\) (7), but it still had a different behavior than the \(\beta\) subunit does. For example, although slow inactivation of \(I_{Na}\) is observed when skeletal muscle \(\alpha\) subunits...
were expressed in Xenopus oocytes [H. B. Nuss, N. Chimavimov, M. T. Perez-Garcia, G. F. Tomaselli, E. Marbán, J. Gen. Physiol. 106, 1171 (1995)], rapid inactivation of $I_{Na}$ was observed when the α subunits were expressed in HEK293 cells [C. Ukonomu, J. Zhou, F. J. Sigworth, W. S. Agnew, Neuron 8, 663 (1992)]. This raises the question of the mechanism of acceleration of inactivation of the brain and skeletal muscle $I_{Na}$ following the co-expression of the β subunit with the α subunit in Xenopus oocytes [A. Toib, V. Lyakhov, S. Marom, J. Neurosci. 18, 1893 (1998); H. B. Nuss, N. Chimavimov, M. T. Perez-Garcia, G. F. Tomaselli, E. Marbán, J. Gen. Physiol. 106, 1171 (1995)]. Nevertheless, it is widely suggested that the β subunit does appear to significantly influence $I_{Na}$ kinetics when co-expressed with non-cardiac α subunits. In heart, however, the question is murky. Marbán and co-workers have examined the issue and note that there are clear, albeit much smaller, kinetic effects of β on H1I α subunit dependent $I_{Na}$ [H. B. Nuss, N. Chimavimov, M. T. Perez-Garcia, G. F. Tomaselli, E. Marbán, J. Gen. Physiol. 106, 1171 (1995)]. Nevertheless, their overall-assessment is that “… the functional role of this subunit in heart is uncertain …” Perhaps they were influenced by the absence of demonstrable association between α and β1 in heart [N. Makita, P. B. J. Bennett, A. L. J. George, J. Biol. Chem. 269, 7571 (1994)] with the small effects observed. More recent experiments now suggest there is an association between cardiac α and β1 subunits (12–14).

9. $I_{Na}$ was reported to increase when the α subunit was expressed in Xenopus oocytes and activated by CAMP [B. Frohnwieser, L. Q. Chen, W. Schreibmayer, R. G. Kallen, J. Physiol. 498, 309 (1997)] or when intact rabbit heart cells were exposed to isoproterenol [J. J. Matsuda, H. Lee, E. F. Shibata, Circ. Res. 70, 199 (1992)] but $I_{Na}$ was found to decrease in neonatal rat heart cells [B. Schubert, A. M. J. VanDongen, G. E. Kirsch, A. M. Brown, Scienc 245, 516 (1988); B. Schubert, A. M. VanDongen, G. E. Kirsch, A. M. Brown, Am. J. Physiol. 258, H977 (1990)].

10. To calculate relative permeability of ions through channels, reversal potential ($E_{rev}$) (i.e. the zero-current potential) measurement are made under different ionic conditions. Our experiments presented here use $E_{rev}$ to calculate the relative permeability of Ca$^{2+}$ ($P_{Ca}$) to Na$^{+}$ ($P_{Na}$) through the Na$^{+}$ channel. We used a solution of the Nerst-Planck equation with constant field assumption presented by Campbell et al. in 1988 [D. L. Campbell, W. R. Giles, J. R. Hume, D. Noble, E. F. Shibata, J. Physiol. 403, 267 (1988); this solution is equivalent to that of C. A. Lewis [Biol. 286, 407 (1979)]. The reversal potential ($E_{rev}$) for a channel with two ions that are permant must lie at or between the equilibrium potentials for the two ions (here $E_{Na}$ and $E_{Ca}$). Since we were examining possible changes in $P_{Ca}/P_{Na}$ for the Na$^{+}$ channel, an accurate assessment of measured potential was needed relative to $E_{rev}$. We opted to use one of two methods to correct for small (~3 mV) systematic errors in the measured potential compared to $E_{rev}$: 1. The zero-current potential for $I_{Na}$ was measured in Na$^{+}$-containing solutions (and known [Na$^{+}$]i and [Na$^{+}$]o) but with 0 mM [Ca$^{2+}$]i, and 0 mM [Ca$^{2+}$]o. It was assumed that the potential should be equal to $E_{rev}$ as calculated by the known Na$^{+}$ concentrations and all potentials were adjusted by the small measured error. 2. A steady-state tip potential was measured using the relevant pipette solution. All potentials were then adjusted by this estimated error. The adjustments were less than 3 mV and only a single adjustment was applied to a complete data set. Since the first method is preferred, it was applied whenever possible (Fig. 1, A and B, and Fig. 3, A, B, C, and E). The second method was applied elsewhere. The first method assumes that any monovalent cations present in the intracellular or extracellular solutions other than Na$^{+}$ do not contribute significantly to measured potentials. We determined that Cs$^{+}$ and NMG$^{+}$ could be used because they have two important properties—1. $P_{Cs}/P_{Na}$ and $P_{NMG}/P_{Na}$ are close to zero; 2. $P_{Ca}/P_{Na}$ and $P_{Ca}/P_{Ca}$ do not change following application of PKA activators such as dibutyryl CAMP (dbcAMP) (see Fig. 3).


12. Two β subunits have been identified in heart, β1 and β2. There is only one gene for β1, which is responsible for expression of this subunit in all tissues. β1 is identified in brain [L. L. Isom, D. S. Ragsdale, K. S. DeJongh, R. E. Westenbroek, B. F. X. Reber, W. A. Catterall, Cell 83, 433 (1995)] was recently characterized by L. N. Mattei and L. Isom (in heart), clone 149022 was subcloned into the EcoRI and Xhol sites of pcDNA3.1 (Zero+) (Invitrogen) and resequenced to confirm the orientation as well as the lack of mutations. Evidence by western of subunit association is shown in Fig. 2.


19. L. L. Isom, T. Scheuer, A. B. Brownstein, D. S. Ragsdale, B. J. Murphy, W. A. Catterall, J. Biol. Chem. 270, 3306 (1995). The antibodies used here were rabbit polyclonal antibodies generated against multiple-antigenic peptides (MAP) by Research Genetics (Huntsville, AL). MAPs specific to the cytoplasmic domains of β1 (LAIT-SEKENCEITovoVEI) or β2 (KCVRRKKEQKLSTD) were synthesized by the Protein and Carbohydrate Structure Core facility at the University of Michigan. The doublet observed for the brain β1, subunit in Fig. 2H(i) was first noted by E. M. Sulkowski and W. A. Catterall [J. Biol. Chem. 265, 12393 (1990)]. Additional investigations of α-β1 and α-β2 interactions by L. L. Isom and co-workers supports our findings (work in progress).

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