Ca^{2+} Flux Through Promiscuous Cardiac Na^+ Channels: Slip-Mode Conductance

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The tetrodotoxin-sensitive sodium ion (Na^+) channel is opened by cellular depolarization and favors the passage of Na^+ over other ions. Activation of the β-adrenergic receptor or protein kinase A in rat heart cells transformed this Na^+ channel into one that is promiscuous with respect to ion selectivity, permitting calcium ions (Ca^{2+}) to permeate as readily as Na^+. Similarly, nanomolar concentrations of cardiotonic steroids such as ouabain and digoxin switched the ion selectivity of the Na^+ channel to this state of promiscuous permeability called slip-mode conductance. Slip-mode conductance of the Na^+ channel can contribute significantly to local and global cardiac Ca^{2+} signaling and may be a general signaling mechanism in excitable cells.

Contraction in heart muscle is activated by a transient increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that is triggered by the cardiac action potential (AP). During an AP, depolarization of the sarcolemmal (SL) and transverse tubule (TT) membranes activates voltage-gated L-type Ca^{2+} channels. Opening of these channels results in a small influx of Ca^{2+} that is amplified by Ca^{2+}-induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum (SR) (*1–3*). During excitation-contraction (EC) coupling, even brief openings of L-type Ca^{2+} channels can trigger openings of SR Ca^{2+}-release channels (ryanodine receptors, RyRs), because the Ca^{2+}-activated RyRs are located very close to (within 10 to 20 nm) the SL and TT membranes (*2, 4*) and thus are exposed to a high local $[Ca^{2+}]_i$ whenever neighboring L-type Ca^{2+} channels open (*5, 6*).

Individual Ca^{2+}-release events from the SR can be imaged as discrete events called "Ca^{2+} sparks" (*7*) if an intracellular Ca^{2+} indicator such as fluo-3 is used in conjunction with confocal microscopy and patch-clamp techniques. Ca^{2+} current across the plasma membrane (I_{Ca}) and the increase in $[Ca^{2+}]_i$ (and Ca^{2+} sparks) are activated in parallel by depolarization, both being detectable at about −50 mV, reaching a peak at about 0 mV, and falling to zero at +60 mV (*2, 7*). From these experiments, a case for I_{Ca} as the sole trigger for the voltage-gated SR Ca^{2+} release has been made. However, contraction of heart muscle and transitory increases in $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$ transients) can be observed even when Ca^{2+} flux through I_{Ca} has been eliminated by Ca^{2+} channel blockers or by positive membrane potentials (*8, 9*). Two explanations have been offered to account for these observations. (i) The Na^+/Ca^{2+} exchanger, activated by the depolarization, favors the entry of Ca^{2+} at increasingly positive potentials, and this entry of Ca^{2+} activates CICR (*8, 10, 11*). (ii) A voltage-gated release mechanism (perhaps similar to that in skeletal muscle) directly activates RyRs (*9, 12*). Our results suggest an alternative explanation: Under certain conditions Ca^{2+} flux through Na^+ channels can activate SR Ca^{2+} release.

We examined the $[Ca^{2+}]_i$ transient in isolated rat ventricular myocytes (Fig. 1) in the presence of agents that activate cyclic adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (protein kinase A, PKA). Activation of PKA is necessary to observe the hypothesized direct voltage-gated release of Ca^{2+} from the SR (*9*). Intracellular cAMP (50 μM) was used in Fig. 1A (Table 1). The Ca^{2+} influx that depends on

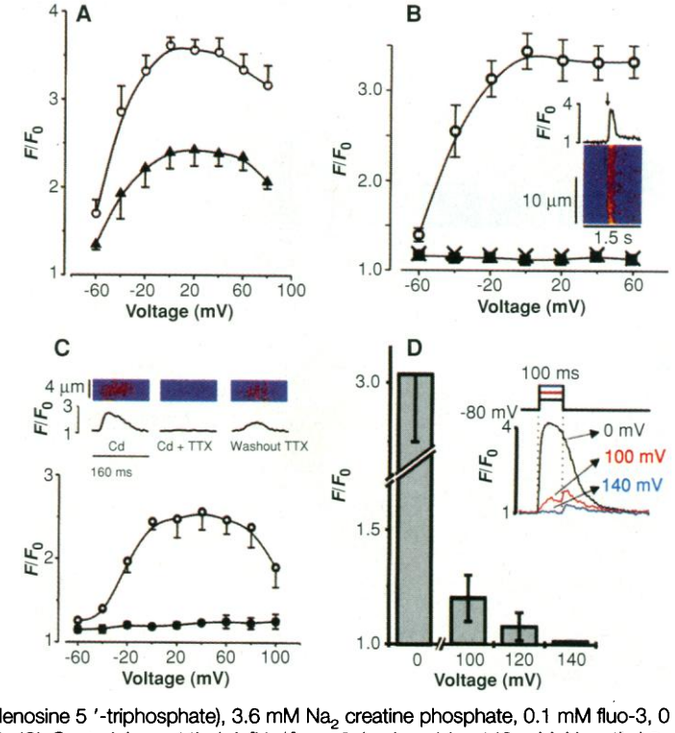

Fig. 1. TTX-sensitive $[Ca^{2+}]_i$ transients in isolated cardiac myocytes. (**A**) Activation of the $[Ca^{2+}]_i$ transient by depolarization. Single rat heart cells (*38*) were voltage-clamped with patch-clamp methods in whole-cell mode. Depolarizations were from −65 mV to test potentials ranging from −60 to +80 mV. Cells were held at −80 mV and then submitted to a slow (500 ms) ramp to −65 mV, were then held at −65 mV for 100 ms, and then were depolarized to the test potential. Extracellular solution contained 140 mM NaCl, 5.0 mM KCl, 0.33 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM Hepes (pH 7.4), 5.5 mM glucose. Internal solution contained 130 mM KCl, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 10 mM Hepes, 5 mM MgATP (adenosine 5 ′-triphosphate), 3.6 mM Na_2 creatine phosphate, 0.1 mM fluo-3, 0 mM NaCl, 50 μM cAMP. (○) Control (n = 11); (▲) $[Na^+]_o$ = 0 (replaced by 140 mM *N*-methyl-D-glucamine, NMDG), nifedipine = 5 μM (n = 8). T = 37°C. (**B**) Requirement of extracellular Ca^{2+} and blockade of $[Ca^{2+}]_i$ transient by ryanodine. $[Ca^{2+}]_i$ transients were elicited as in (A) and solutions were similar except that 100 μM $CdCl_2$ was used to block I_{Ca}. The $[Ca^{2+}]_i$ transient (○, n = 13) was abolished by 0 mM $[Ca^{2+}]_o$ (replaced by 2 mM Ba^{2+}) with 4 mM EGTA added (■). (**Inset**) A large $[Ca^{2+}]_i$ transient after caffeine application (10 mM at arrow) in 0 mM $[Ca^{2+}]_o$. The time course of the $[Ca^{2+}]_i$ transient is shown as fluorescence ratio F/F_o (top), and line-scan image (below). The confocal line-scan image of $[Ca^{2+}]_i$ is obtained by repeated sampling of the $[Ca^{2+}]_i$ signal along a line within the cell. Position within the cell is shown vertically and time is displayed horizontally. With 2 mM $[Ca^{2+}]_o$, the $[Ca^{2+}]_i$ transient was abolished by ryanodine (10 μM) (**X**). See Table 1. T = 37°C. (**C**) TTX-sensitive $[Ca^{2+}]_i$ transients. $[Ca^{2+}]_i$ transients were elicited by depolarizations from −65 mV with 100 μM Cd^{2+} to block I_{Ca} in the absence (○, n = 7) or presence of TTX (10 μM) (●). (Top) Individual line-scan images of $[Ca^{2+}]_i$ and corresponding $[Ca^{2+}]_i$ transients (as F/F_o) obtained on depolarization to +60 mV before (left), during (center), and after (right) the application of 10 μM TTX. (**D**) Elimination of the $[Ca^{2+}]_i$ transient at E_{Ca}. Conditions were as in (A). The bar graphs show mean values of the peak $[Ca^{2+}]_i$ transient (as F/F_o) (±SEM) during depolarization to 0 mV, +100 mV, +120 mV, and +140 mV from a holding potential of −65 mV (n = 6). E_{Ca} = 130 mV. (**Inset**) Sample $[Ca^{2+}]_i$ transient records along with the voltage protocol. T = 37°C.

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the Na^+/Ca^{2+} exchanger was blocked by removal of intracellular Na^+. A $[Ca^{2+}]_i$ transient was observed at +80 mV even though I_{Ca} is absent at this potential (see above and *13*). The addition of nifedipine (5 μM) to block any remaining Ca^{2+} influx via I_{Ca} and the removal of extracellular Na^+ (in the continued absence of intracellular Na^+) did not abolish the $[Ca^{2+}]_i$ transient at +80 mV. These results are consistent with a direct voltage-gated Ca^{2+}-release mechanism in the SR but incompatible with one that depends on Ca^{2+} influx mediated by the Na^+/Ca^{2+} exchanger. We repeated these experiments in the absence of extracellular Ca^{2+} (using Ba^{2+} to replace Ca^{2+}) to determine if Ca^{2+} influx played a role in triggering the $[Ca^{2+}]_i$ transient. Removal of extracellular Ca^{2+} blocked the $[Ca^{2+}]_i$ transient (Fig. 1B). The SR was apparently still replete with Ca^{2+} because caffeine still activated a robust $[Ca^{2+}]_i$ transient. This result demonstrates that Ca^{2+} influx is required for EC coupling and argues against the direct-coupling hypothesis. Furthermore, the observed voltage-gated $[Ca^{2+}]_i$ transient still depends on Ca^{2+} release from the SR via RyRs because the application of ryanodine (10 μM) in the presence of extracellular Ca^{2+} abolished the $[Ca^{2+}]_i$ transient (Fig. 1B).

Because no established pathway appeared to allow Ca^{2+} to enter and trigger the $[Ca^{2+}]_i$ transient, we tested unlikely routes of Ca^{2+} permeation. Sodium channels—voltage-gated proteins responsible for the Na^+ current, I_{Na}—are abundant in the SL and TT membranes of heart muscle and are highly selective for Na^+ permeation (*14*). We examined the role of I_{Na} in activating the $[Ca^{2+}]_i$ transient by applying the specific Na^+ channel blocker tetrodotoxin (TTX). Under conditions in which there was no Ca^{2+} influx through Ca^{2+} channels and no Ca^{2+} influx through the Na^+/Ca^{2+} exchanger, TTX (10 μM) abolished the $[Ca^{2+}]_i$ transients activated by depolarization (Fig. 1C). This result indicated that CICR might be activated by Ca^{2+} entering the cell through TTX-sensitive Na^+ channels. If so, the $[Ca^{2+}]_i$ transient should also be abolished at E_{Ca}, the membrane potential at which there should be no net entry of Ca^{2+} through any ion channel (*13*, *14*). The $[Ca^{2+}]_i$ transient declined to zero as the depolarization potential approached E_{Ca} (~130 mV under our experimental conditions) (Fig. 1D).

A quantitative examination of the role of I_{Na} in triggering the $[Ca^{2+}]_i$ transient (Fig. 2) made use of three complementary experimental approaches to measure I_{Na} (*15*). Under control conditions, depolarization to −65 mV activated I_{Na} but not a $[Ca^{2+}]_i$ transient (Fig. 2A). However, when cellular PKA was activated by the application of isoproterenol (1 μM), I_{Na} was increased and produced a clear $[Ca^{2+}]_i$ tran-

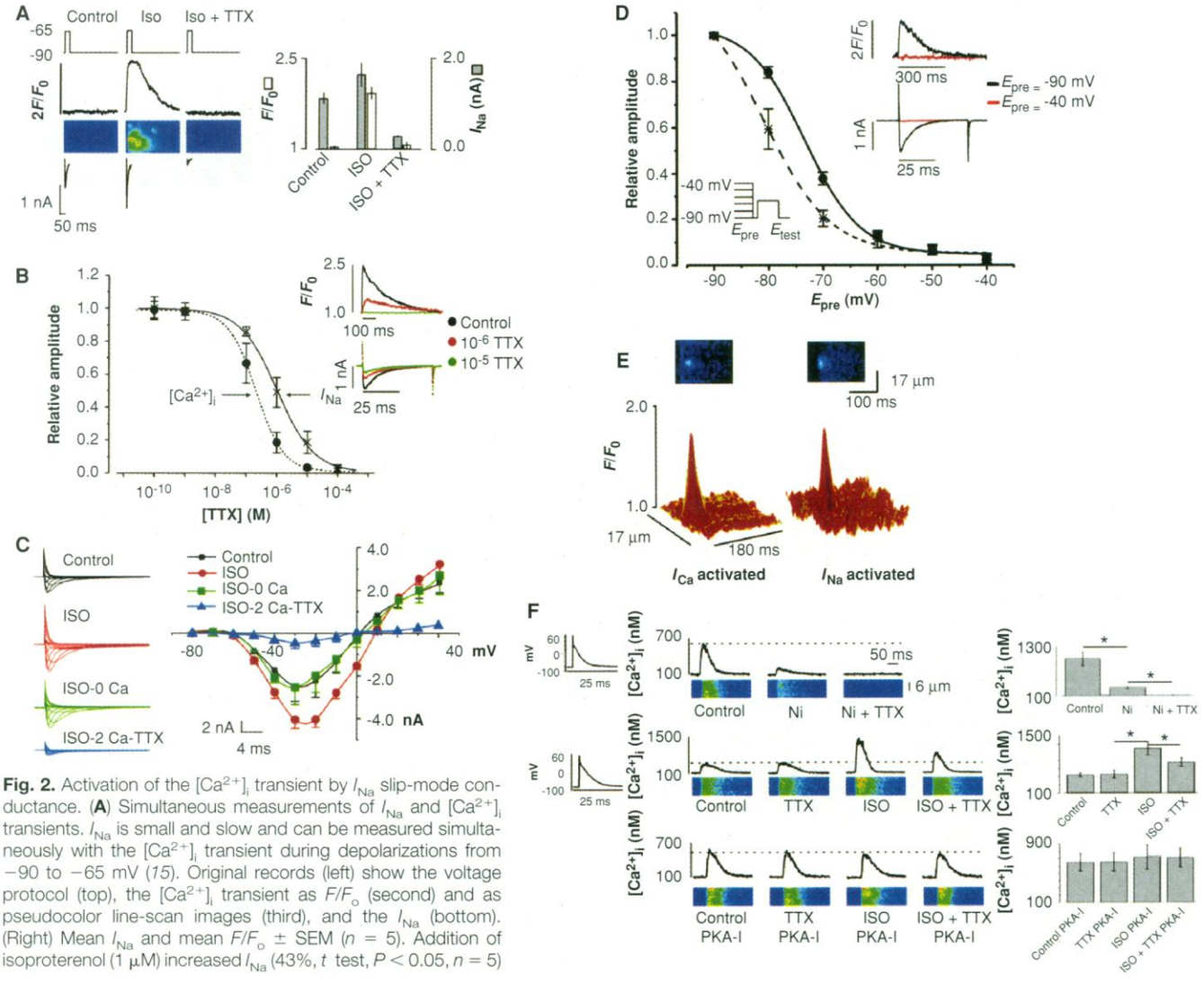

Fig. 2. Activation of the $[Ca^{2+}]_i$ transient by I_{Na} slip-mode conductance. (**A**) Simultaneous measurements of I_{Na} and $[Ca^{2+}]_i$ transients. I_{Na} is small and slow and can be measured simultaneously with the $[Ca^{2+}]_i$ transient during depolarizations from −90 to −65 mV (*15*). Original records (left) show the voltage protocol (top), the $[Ca^{2+}]_i$ transient as F/F_o (second) and as pseudocolor line-scan images (third), and the I_{Na} (bottom). (Right) Mean I_{Na} and mean F/F_o ± SEM (n = 5). Addition of isoproterenol (1 μM) increased I_{Na} (43%, t test, $P < 0.05$, n = 5)

sient (*16*). TTX (10 μM) blocked the $[Ca^{2+}]_i$ transient completely and about 80% of I_{Na}. Neither I_{Ca} nor the Na^+/Ca^{2+} exchanger could contribute to the activation of Ca^{2+} release from the SR because I_{Ca} is not activated by depolarizations to −65 mV and Ca^{2+} influx through the Na^+/Ca^{2+} exchanger was blocked by removal of intracellular Na^+.

This dynamically modulated Na^+ channel that is blocked by TTX and can conduct Ca^{2+} in the presence of physiological concentrations of Na^+ is without precedent. Indeed, Ca^{2+} is an established blocker of Na^+ channels (*17*) and does not "normally" permeate cardiac Na^+ channels (*18*). PKA, however, appears to be able to bring about a change in the selectivity of the Na^+ channel to permit Ca^{2+} permeation. Although PKA-dependent phosphorylation of the Na^+ channel is well established and occurs at multiple sites on the protein (*19*), the physiological role of this phosphorylation is uncertain. We identify the Na^+ channel as the pathway by which Ca^{2+} enters the cell to trigger CICR after PKA activation. This identification depends on the specific Na^+ channel–blocking toxin TTX (*14*, *20*). The ability of the Na^+ channel to be changed so that Ca^{2+} can permeate suggests that an unrecognized mode of operation of the Na^+ channel exists during which ions other than Na^+ are allowed to pass through the channel. We have called this behavior "slip-mode conductance."

To further examine how the $[Ca^{2+}]_i$ transient and I_{Na} depend on TTX, we performed step depolarizations to −65 mV in the presence of various concentrations of TTX (Fig. 2B). The TTX-dependent blockade of the $[Ca^{2+}]_i$ transient was 10-fold more sensitive to TTX than I_{Na} itself [dissociation constant (K_d) = 0.1 μM versus 1.0 μM, respectively]. This difference in K_d could be due to increased TTX sensitivity in the population of channels that were affected by PKA. Linkage between TTX affinity and ion selectivity may reflect colocalization of protein domains critical for both processes as suggested by investigations of mutated neuronal, skeletal, and cardiac Na^+ channels (*20*, *21*). However, the observed differences in TTX sensitivity may also be due to the mechanism by which Ca^{2+} flux through Na^+ channels activates Ca^{2+} release from the SR (see below).

To examine the PKA-induced slip-mode conductance of the Na^+ channel over a wider range of potentials, we reduced I_{Na} in magnitude and blocked cellular movement (to improve the clamp). Both intracellular Na^+ concentration ($[Na^+]_i$) and extracellular Na^+ concentration ($[Na^+]_o$) were made 10 mM, thereby establishing E_{Na} at 0 mV. The temperature was set at 24°C to reduce I_{Na} magnitude, and EGTA was added to the pipette solution to prevent cell movement. I_{Ca} was blocked with nifedipine (40 μM). Intracellular Cs^+ was used to block K^+ current. The current-voltage (*IV*)

Fig. 2. (*continued*) and produced a $[Ca^{2+}]_i$ transient (F/F_0 increased from 1.05 to 1.9, *t* test, $P < 0.05$, $n = 5$). The addition of TTX (10 μM) in the continued presence of isoproterenol blocked the $[Ca^{2+}]_i$ transient (reduction of 95%, $P < 0.05$, $n = 5$) and largely removed I_{Na} (reduction of 83 ± 6%, *t* test, $P < 0.05$, $n = 5$). Extracellular solutions contained 140 mM NaCl, 5 mM CsCl, 10 mM Hepes (pH 7.4), 2 mM $CaCl_2$, 4 mM 4-aminopyridine, 10 mM tetraethylammonium (TEA) chloride, 10 mM glucose, 1 mM $MgCl_2$, and 0.33 mM NaH_2PO_4. Intracellular (pipette) solution contained 130 mM KCl, 20 mM TEA, 10 mM Hepes (pH 7.2), 5 mM MgATP, 0.1 mM fluo-3. T = 24°C. (**B**) Blockade of I_{Na} and of the $[Ca^{2+}]_i$ transient by TTX. The sensitivities of I_{Na} (solid line) and of the $[Ca^{2+}]_i$ transient (dashed line) to [TTX] were examined under conditions like those in (A) except that the concentration of intracellular cAMP was 50 μM. The concentration of TTX that blocked half of I_{Na} was 1.0 μM and that for the $[Ca^{2+}]_i$ transient was 0.1 μM. Each set of data was fit by a least-squares method through use of a logistic dose-response equation, $y = [(A_1 - A_2)/(1 + (X/X_0)^p)] + A_2$, where A_1 is the initial amplitude value, A_2 is the final amplitude value, X_0 is the concentration of TTX that half-blocks I_{Na} or the $[Ca^{2+}]_i$ transient, and p equals the power of equation. For I_{Na}, $A_1 = 0.99$, $A_2 = 0.005$, $X_0 = 1.033$ μM, and $p = 0.724$; for the $[Ca^{2+}]_i$ transient, $A_1 = 0.988$, $A_2 = 0.009$, $X_0 = 0.109$ μM, and $p = 0.967$. (**Inset**) Sample records obtained simultaneously for $[Ca^{2+}]_i$ (top) and I_{Na} (bottom) indicating no TTX (black), 1 μM TTX (red), and 10 μM TTX (green). T = 24 °C. (**C**) The *IV* relation of I_{Na} in rat ventricular myocytes. Effect of PKA activation, Ca^{2+}, and TTX. I_{Na} measured as a function of potential in low $[Na^+]$ (*15*). Control conditions (crosses, black line, $n = 8$); isoproterenol (ISO, 1 μM) (filled circles, red line, $n = 8$); isoproterenol (1 μM) in 0 $[Ca^{2+}]_o$ (filled squares, green line, $n = 8$); TTX (10 μM) with isoproterenol (1 μM) and 2 mM $[Ca^{2+}]_o$ (filled triangles, blue line, $n = 8$). Sample I_{Na} records (left) for all conditions. Extracellular solutions contained 125 mM CsCl, 10 mM NaCl, 10 mM Hepes (pH 7.4), 5 mM glucose, 2 mM $CaCl_2$ and 1 mM $MgCl_2$. Extracellular $MgCl_2$ was increased to 3 mM when $[Ca^{2+}]_o$ was reduced to zero; intracellular (pipette) solutions contained 130 mM CsCl, 10 mM NaCl, 10 mM Hepes (pH 7.2), 5 mM MgATP, 5 mM EGTA. E_{Na} = 0 mV. T = 24°C. Error bars indicate SEM. (**D**) Inactivation of I_{Na} and the $[Ca^{2+}]_i$ transient. The reductions of I_{Na} (solid line) and of $[Ca^{2+}]_i$ transients (dashed line) by voltage were examined under conditions like those in (B). A 50-ms prepulse potential was used to inactivate I_{Na} and inactivate the associated $[Ca^{2+}]_i$ transient. The effects of inactivation were evaluated simultaneously by test depolarizations to −65 mV. The curves are the best fit line by a least-squares method with a Boltzmann equation, $y = [(A_1 - A_2)/(1 + e^{(V - V_0)/k})] + A_2$, where A_1, A_2, V_0, and k are the initial value, final value, the mid-point voltage, and the slope factor. (**Insets**) The voltage protocol (left) and sample records (right) obtained simultaneously during the evaluation depolarization to −65 mV after prepulse levels of −90 mV (black) and −40 mV (red). For I_{Na}, $A_1 = 1.03$, $A_2 = 0.046$, $V_0 = -73.28$ mV, and $k = 5.06$, and for the $[Ca^{2+}]_i$ transient $A_1 = 1.21$, $A_2 = 0.05$, $V_0 = -80.87$ mV, and $k = 6.08$. T = 24°C. Error bars are the SEM ($n = 4$). (**E**) Activation of Ca^{2+} sparks by Ca^{2+} influx through Na channels. Signal-averaged line-scan images (Fig. 1) of Ca^{2+} sparks are presented as surface plots. (Left) Ca^{2+} sparks activated by I_{Ca} ($n = 186$). Ca^{2+} sparks were activated by small depolarizations close to −50 mV or large depolarization with 1 μM nifedipine (*7*). A single sample Ca^{2+} spark as a line-scan image is shown above. Solutions were as in Fig. 1A except that $[Na^+]_i$ was 8 mM, $[Ca^{2+}]_o$ was 140 mM, and there was no cAMP. (Right) Ca^{2+} sparks activated by slip-mode conductance of I_{Na} ($n = 17$). I_{Ca} was blocked with 100 μM Cd^{2+} or nifedipine (5 μM). A sample Ca^{2+} spark as a line-scan image is shown above, and a signal-averaged Ca^{2+} spark is shown below ($n = 17$). Solutions were as in Fig. 1A with $[Na^+]_o$ at 140 mM. For both kinds of Ca^{2+} sparks, background and baseline fluorescence signals were subtracted. T = 35° to 37°C. Characteristics of the Ca^{2+} sparks activated by I_{Ca} or I_{Na} were similar: amplitude (F/F_o) = 1.69 ± 0.02 versus 1.74 ± 0.11 for $I_{Ca,L}$ versus I_{Na}, P = not significant (NS); time constants of decay = 16.75 ± 0.37 ms versus 17.58 ± 2.03 ms, P = NS; width (at half peak F/F_o) = 2.02 ± 0.13 mm versus 2.07 ± 0.13, P = NS. (**F**) Contribution of Ca^{2+} release activated by slip-mode conductance of the Na^+ channel to the normal $[Ca^{2+}]_i$ transient. AP clamps (*15*) were used to activate $[Ca^{2+}]_i$ transients with sample records shown on the left and statistics on right. (Top) $[Ca^{2+}]_i$ transients were largely blocked by Ni^{2+} (5 mM) with the remainder blocked by TTX (10 μM). Bar diagram on the right ($n = 4$). Ni^{2+} (5 mM) reduced the $[Ca^{2+}]_i$ transient by 78% and TTX blocked the remaining 22%. Intracellular (pipette) cAMP was 50 μM. (Middle) Effect of TTX under control or after treatment with isoproterenol (1 μM). Isoproterenol alone increased the $[Ca^{2+}]_i$ transient to 2.1 times the control value and TTX reduced this to 1.5 times the control value ($n = 12$). (Bottom) Protein kinase A inhibitor peptide (PKA-I) blocks the action of isoproterenol to increase the $[Ca^{2+}]_i$ transient. With 100 μM PKA-I added to the pipette filling solution, no change in the $[Ca^{2+}]_i$ transient was observed after TTX (10 μM), isoproterenol (1 μM), or both TTX and isoproterenol. Solutions were similar to those used in Fig. 2B except that no cAMP was added. The AP (top, left inset) applies to the top panel and was obtained during current clamp (zero current) in a zero $[Na^+]_i$ solution at 37°C. Resting potential = −81 mV, with a peak positive excursion at +63 mV and a duration for 90% repolarization of 7 ms. The AP (bottom, left inset) applies to the bottom two panels and was obtained with 8.2 mM $[Na^+]_i$ and no cAMP in the pipette. The AP 90% duration was 15 ms, holding potential (V_h) = −86 mV, peak voltage = +58 mV. The horizontal bars on the right show which data pair was compared for statistically significant differences. Those marked with an asterisk (*) were different ($P < 0.05$, Student's *t* test).

relations of I_{Na} were measured under four conditions: (i) control conditions, (ii) after the addition of isoproterenol (1 μM), (iii) in the maintained presence of isoproterenol but after removal of extracellular Ca^{2+}, and (iv) in the maintained presence of isoproterenol and 2 mM extracellular Ca^{2+} but with TTX (10 μM) added (Fig. 2C). The control *IV* relation shows a maximal inward current at around −30 mV, a zero-current potential (reversal potential) of 0 mV, and significant outward current at potentials positive to 0 mV. Treatment with isoproterenol activated additional inward current at potentials negative to +20 mV, shifted the reversal potential positive by 9 mV to +9 mV, and produced more outward current than seen in the control *IV* relation at potentials positive to +20 mV. Removing extracellular Ca^{2+} reversed all of these effects. Thus, in the absence of Ca^{2+}, the activation of slip-mode conductance by PKA does not change the apparent Na^+ permeability (P_{Na}) of the affected Na^+ channels. These results also indicate that a new population of Na^+ channels is not recruited by PKA activation. If new Na^+ channels were recruited, then I_{Na} in Ca^{2+}-free solutions should be larger than under control conditions. The measured shift in reversal potential from 0 mV (control) to +9 mV (isoproterenol) suggests that the relative permeability of Ca^{2+} through the Na^+ channel (P_{Ca}) compared to P_{Na} is significantly increased. The calculated P_{Ca}/P_{Na} increased from about 0.0 (control conditions) to 1.25 (isoproterenol) (*22*). However, if only a subpopulation of Na^+ channels were altered by PKA, then the P_{Ca}/P_{Na} per channel would be greater in the affected slip-mode channels and zero in the remaining normal-mode channels. TTX blocked virtually all of the current at all potentials, indicating that we were measuring I_{Na} during these experiments. There was, however, a residual current in 10 μM TTX, but the magnitude of this current is appropriate for TTX blockade of cardiac I_{Na}. Importantly, the residual current reverses at 0 mV not at +9 mV. This indicates that the PKA-modified I_{Na} is more sensitive to TTX than is the normal-mode I_{Na} (*22*), a finding consistent with Fig. 2B.

Another distinctive feature of the TTX-sensitive Na^+ channel is that it becomes inactivated at quite negative potentials. We therefore compared the voltage dependence of inactivation of I_{Na} to that of the TTX-sensitive $[Ca^{2+}]_i$ transient (Fig. 2D). The cells were held at −90 mV, and a 50-ms predepolarization step (E_{pre}) to various potentials was applied before a depolarization to −65 mV was used to evaluate the effect of prior depolarization (*15*). The half-inactivation potential ($V_{0.5}$) of I_{Na} was about −73 mV, whereas the $[Ca^{2+}]_i$ transient was half-inactivated at about −81 mV. The 8 mV more negative $V_{0.5}$ for the $[Ca^{2+}]_i$ transient relative to I_{Na} may reflect a real difference in the voltage dependence of the Na^+ channel subpopulation that exhibits slip-mode behavior after PKA activation as compared with the remainder of the population that still exhibits normal-mode conductance.

The negative shift in the voltage dependence of inactivation of the $[Ca^{2+}]_i$ transient versus I_{Na} may depend on coupling characteristics between the Ca^{2+}-flux characteristics (magnitude and kinetics) of the slip-mode Na^+ channels and triggering requirements of RyRs. The extremely brief open times of the Na^+ channels and the rapid inactivation of I_{Na} limits the Ca^{2+} influx through any one slip-mode Na^+ channel and may account for the absence of a measurable $[Ca^{2+}]_i$ increase when RyRs are blocked and slip-mode conductance is activated. These same features suggest that EC coupling gain (increase in $[Ca^{2+}]_i$ per unit Ca^{2+} influx) during slip-mode conductance should be very high and contrasts

Table 1. Experimental conditions.

Figure	T (°C)	$[Na^+]_i$ (mM)	$[Na^+]_o$ (mM)	V_h (mV)	Ramp to (mV)	Special voltage protocol	I_{Ca} blocker	TTX	Slip-mode conductance activator	Special condition	Intracellular cation
1A	37	0	140, 0	−80	−65		0, 5 μM nifedipine	0	50 μM cAMP		K^+
1B	37	0	140	−80	−65		100 μM Cd^{2+}	0	50 μM cAMP	Ba^{2+}, caffeine, ryanodine	K^+
1C	37	0	140	−80	−65		100 μM Cd^{2+}	0	50 μM cAMP		K^+
1D	37	0	140	−80	−65		100 μM Cd^{2+}	0	50 μM cAMP		Cs^+
2A	24	0	140	−90	NA	50-ms depol. to −65 mV	None	0, 10 μM	None, 1 μM ISO		K^+
2B	24	0	140	−90	NA	Step to −65 mV	None	10^{-4} to 10^2 μM	50 μM cAMP		K^+
2C	24	10	10	−90	None	*IV*: −80 to +40 mV	40 μM nifedipine	0, 10 μM	0, 1 μM ISO	0, 2 mM $[Ca]_o$; E_{Na} = 0 mV	Cs^+
2D	24	0	140	−90	NA	Step to −65 mV	None	0	50 μM cAMP	Inactivation *V* prepulse to −90 to −40 mV	K^+
2E (left)	37	0	140	−80	−45		None or 1 μM nifedipine	0	None		Cs^+
2E (right)	37	0	140	−80	−60		100 μM Cd^{2+} or 5 μM nifedipine	0	50 μM cAMP		K^+
2F (top)	37	0	140	−81	NA	AP clamp	0, 5 mM Ni^{2+}	0, 10 μM	50 μM cAMP		K^+
2F (middle)	37	0	140	−86	NA	AP clamp	None	0, 10 μM	0, 1 μM ISO		K^+
2F (bottom)	37	0	140	−86	NA	AP clamp	None	0, 10 μM	0, 1 μM ISO	PKA inhibitor peptide	K^+
3A (top)	37	0	140	−86	NA	AP clamp	None	0, 10 μM	0, 100 nM ouabain		K^+
3A (bottom)	37	0	140	−86	NA	AP clamp	None	0, 10 μM	PKA-I; 0, 100 nM ouabain		K^+
3B	37	0	140	−86	NA	AP clamp	None	0	10^{-12} to 10^{-6} nM ouabain or digoxin		K^+
3C	24	10	10	−90	NA	*IV*: −80 to +40 mV	40 μM nifedipine	0, 10 μM	100 nM ouabain	0, 2 mM $[Ca]_o$; E_{Na} = 0 mV	Cs^+

with the low gain seen during photolysis of caged Ca^{2+} (*3*, *23*). This difference in gain may account for the observation that slip-mode I_{Na} activates Ca^{2+} sparks (Fig. 2E), whereas photolysis of caged Ca^{2+} fails to do so (*23*). Because there are about 200 Na^+ channels per square micrometer (*24*) along the SL and TT membranes, about 20 times the density of L-type Ca^{2+} channels, it is likely that more than one slip-mode channel opens to activate a Ca^{2+} spark. Nevertheless, the closeness of $V_{0.5}$ values for inactivation of the $[Ca^{2+}]_i$ transient and I_{Na} further supports the identification of the Na^+ channels as the conductance pathway responsible for the new component of the $[Ca^{2+}]_i$ transient.

If slip-mode conductance of the Na^+ channel does indeed contribute to EC coupling, then the effects of its activation should be detectable during a normal AP. The addition of intracellular cAMP (50 μM) activated PKA and enhanced the $[Ca^{2+}]_i$ transients recorded during AP clamp experiments (*15*). Ni^{2+} (5 mM), which blocks both I_{Ca} and the Na^+/Ca^{2+} exchanger, partly inhibited the $[Ca^{2+}]_i$ transients. The remaining 22 ± 5% (n = 8) of the $[Ca^{2+}]_i$ transient was blocked by TTX (10 μM) (top row, Fig. 2F). However, when PKA was not activated, TTX (10 μM) did not block any of the $[Ca^{2+}]_i$ transient (middle row, Fig. 2F), a result consistent with Fig 2A. The $[Ca^{2+}]_i$ transient in the absence of PKA activation and with Na^+ channels blocked by TTX or voltage-dependent inactivation has been shown to be caused by CICR triggered by I_{Ca} (*7*). When PKA was activated by isoproterenol, then TTX reduced the AP triggered $[Ca^{2+}]_i$ transient by 29.4%. After TTX, the $[Ca^{2+}]_i$ transient was still elevated relative to that observed before PKA activation because PKA activation has multiple effects on cardiac myocytes. These effects include increases in I_{Ca}, increases in the sensitivity of RyRs to be triggered by $[Ca^{2+}]_i$, and increases in the SR Ca^{2+} content [by activating the SR Ca^{2+} adenosine triphosphatase (ATPase)] (*5*, *25*). To determine if PKA was involved or whether some other cAMP or isoproterenol-dependent process mediated these effects, we used the 20–amino acid peptide inhibitor of PKA, PKA-I (*26*). In the presence of PKA-I the $[Ca^{2+}]_i$ transient was not affected by TTX (10 μM); isoproterenol (1 μM) did not increase the $[Ca^{2+}]_i$ transient; and TTX did not alter the $[Ca^{2+}]_i$ transient after isoproterenol was added (bottom row, Fig. 2F). It would thus appear that isoproterenol activates slip-mode conductance by the signaling cascade initiated by its binding to β-adrenergic receptor (βAR). GTP-binding protein (G protein)–dependent activation of adenylate cyclase then leads to increased intracellular cAMP that activates PKA, which presumably phosphorylates the Na^+ channel to activate slip-mode conductance (Fig. 3D).

Cardiotonic steroids (CTSs) are digitalis-like agents used therapeutically to increase the strength of contraction of the heart. More recently, endogenous hormones almost identical to the CTS ouabain have been found at nanomolar concentrations in plasma (*27*). The distinguishing characteristic of the CTSs is their high affinity and specificity for the Na^+,K^+-ATPase ("Na^+ pump") to which they bind and inhibit. The mechanism by which CTS acts to strengthen the heartbeat is thought to depend on Na^+ pump inhibition (*28*). This inhibition reduces the extrusion of Na^+ from the cell and consequently leads to an increase in $[Na^+]_i$. The increase in $[Na^+]_i$ reduces the Na^+ gradient across the plasmalemmal membrane and thus reduces Ca^{2+} efflux through the Na^+/Ca^{2+} exchanger, increases $[Ca^{2+}]_i$, and thus increases the amount of Ca^{2+} within the SR. This sequence of events enables larger

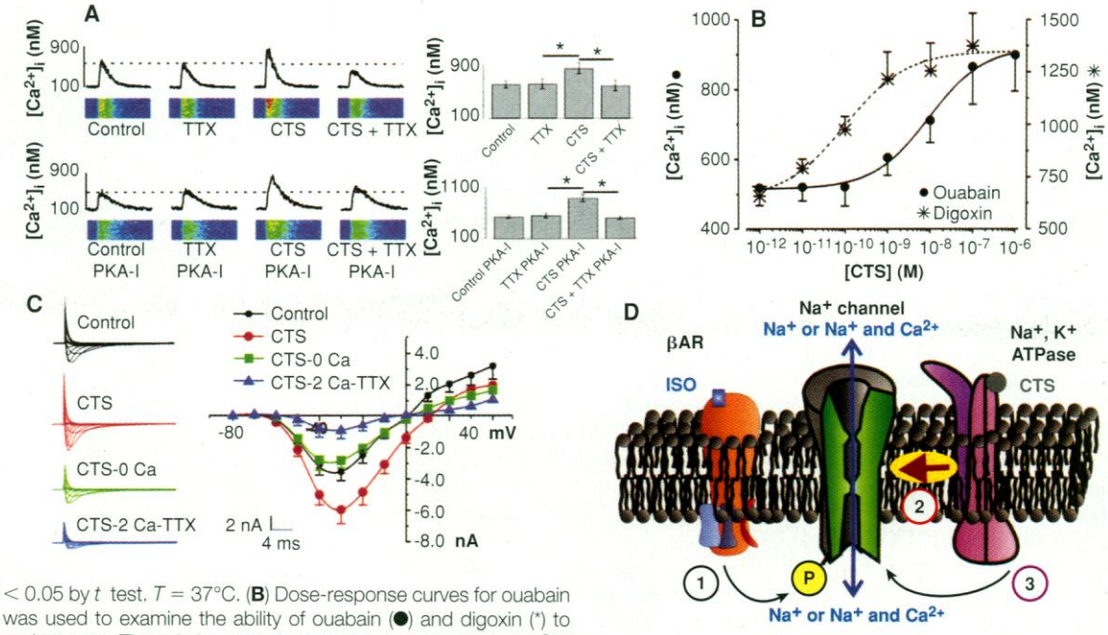

Fig. 3. Activation of the slip-mode conductance of the Na^+ channel by cardiotonic steroids (CTS). (**A**) An AP clamp was used to activate $[Ca^{2+}]_i$ transients (*15*). Sample records are shown on the left and statistics on the right. (Top) TTX (10 μM) alone did not affect the $[Ca^{2+}]_i$ transient, whereas the application of 100 nM ouabain (CTS) produced a 44% increase that was completely blocked by TTX (n = 12). (Bottom) Same as the top panel in the presence of 100 μM PKA-I (intracellular). PKA-I did not alter the actions of CTS on the $[Ca^{2+}]_i$ transient nor did it alter the ability of TTX to block the 64% increase (n = 12). Solutions were as in Fig. 2A. (*) $P < 0.05$ by t test. T = 37°C. (**B**) Dose-response curves for ouabain and digoxin. The AP clamp was used to examine the ability of ouabain (●) and digoxin (*) to activate the I_{Na} slip-mode conductance. The relative magnitudes of the increases in $[Ca^{2+}]_i$ transients produced by a CTS were plotted as a function of concentration over the range 10^{-12} to 10^{-6} M. Half-maximum activation occurred at a concentration of 0.1 nM for digoxin (n = 3) and 9.22 nM for ouabain (n = 8). The digoxin (dashed line) and ouabain (solid line), data were fit with a logistic dose-response equation by a least-squares routine (Fig. 2B). For digoxin, A_1 = 640, A_2 = 1350, X_0 = 0.1 nM, P = 0.59781; for ouabain, A_1 = 514.4, A_2 = 922.14, X_0 = 9.22 nM, P = 0.66. (**C**) The IV relation of I_{Na} in rat ventricular myocytes; effect of CTS, Ca^{2+}, and TTX. I_{Na} measured as a function of potential in low $[Na^+]$ (*15*). Control conditions (crosses, black line, n = 5); 100 nM ouabain (CTS) (red circles, red line, n = 5); 100 nM ouabain in 0 $[Ca^{2+}]_o$ (green squares, green line, n = 5); TTX (10 μM) with 100 nM ouabain and 2 mM $[Ca^{2+}]_o$ (filled triangles, blue line, n = 5). Solutions were as in Fig. 2C. T = 24 °C. (**D**) Model activation of slip-mode conductance of the Na^+ channel. β-Adrenergic receptor (βAR) binds isoproterenol, producing a G-protein–dependent activation of adenylate cyclase that activates PKA, which phosphorylates the Na^+ channel (1). This phosphorylation presumably activates slip-mode conductance. CTSs bind to the Na,K-ATPase, which may activate slip-mode conductance of the Na^+ channel by direct protein-protein interactions (2) or indirect factors (3).

$[Ca^{2+}]_i$ transients and stronger contractions of heart muscle to occur after CTS inhibition of the Na^+ pump. At high concentrations of CTSs, this explanation certainly holds. However, an increase in neither cell-wide nor local $[Na^+]_i$ has been observed after the application of therapeutic concentrations of CTSs (*29–32*). We therefore examined whether digitalis-like agents can increase the $[Ca^{2+}]_i$ transient under the conditions of our experiments and whether CTSs might activate slip-mode conductance of the Na^+ channel.

Treatment of cells with 100 nM ouabain increased the $[Ca^{2+}]_i$ transient when Ca^{2+} influx through the Na^+/Ca^{2+} exchanger was inhibited (Fig. 3A). TTX (10 μM) blocked the increase in the $[Ca^{2+}]_i$ transient activated by ouabain but did nothing in the absence of ouabain. Thus, it appears that slip-mode conductance of I_{Na} may contribute to the ouabain-induced increase in the cardiac $[Ca^{2+}]_i$ transient. Because the action of ouabain was not blocked by PKA-I peptide, ouabain does not appear to require PKA. Like isoproterenol or intracellular cAMP, ouabain can activate Na^+ channel slip-mode conductance but does so by a different mechanism.

The $K_{0.5}$ (dissociation constant for half-maximal activation) for ouabain to activate the $[Ca^{2+}]_i$ transient was about 9.2 nM (Fig. 3B). Ouabain or an isomer of ouabain is produced endogenously with circulating levels between 1 and 25 nM and a mean level around 1.4 nM for control adult humans and 3.4 nM for patients with essential hypertension (*27*). Thus, endogenous ouabain may modulate the cardiac $[Ca^{2+}]_i$ transient through activation of the slip-mode conductance of I_{Na}.

Patients in heart failure are frequently given the CTS digoxin because it increases the strength of contraction of the heart. We therefore examined the ability of digoxin to activate a $[Ca^{2+}]_i$ transient, under conditions identical to those used for ouabain. Digoxin augmented the $[Ca^{2+}]_i$ transient but with a $K_{0.5}$ of about 0.1 nM. This efficacy is consistent with digoxin's therapeutic concentration of about 1 nM.

The ability of TTX to block the CTS-dependent increase in the $[Ca^{2+}]_i$ transient suggests that CTSs activate slip-mode conductance of the Na^+ channel. We therefore used ouabain in place of isoproterenol to activate the Ca^{2+} flux through Na^+ channels in experiments like those reported in Fig. 2C. We measured I_{Na} from −80 mV to +40 mV. The *IV* relation reversed at E_{Na} (0 mV) as expected for I_{Na} under our experimental conditions. The addition of ouabain (100 nM) increased inward current at all potentials and shifted the reversal potential of I_{Na} from 0 to 10 mV. This shift in reversal potential suggests that CTS treatment increases P_{Ca}/P_{Na} from 0.0 (control) to 1.45 (in 100 nM ouabain) (*22*). In this regard, the current activated by CTS is similar to that activated by PKA. One difference was that the PKA-activated current was outward at potentials positive to +20 mV. Removal of extracellular Ca^{2+} in the continued presence of 100 nM ouabain decreased the inward current at potentials negative to E_{Na} to control levels, and the reversal potential returned to 0 mV. This supports the conclusion that CTSs activate Ca^{2+} flux through Na^+ channels and suggests that only a fraction of Na^+ channels is altered by the CTS treatment. Furthermore, these findings argue against the recruitment of a new population of Na^+ channels by CTSs. The increased outward current at positive potentials was not affected by the removal of extracellular Ca^{2+} and thus may be explained by an altered permeability of the Na^+ channel for other ions at positive potentials (for example, Cs^+ or K^+). In the continued presence of 100 nM ouabain and with 2 mM Ca^{2+} in the extracellular solution, TTX (10 μM) blocked virtually all of the inward and outward current. Thus, we conclude that Na^+ channels mediate the changes in conductance produced by CTS. The reversal potential of the small residual current was 0 mV, which indicates that TTX may preferentially block Na^+ channels exhibiting slip-mode conductance as noted above for PKA-activated slip-mode conductance (*22*).

Although slip-mode conductance increases P_{Ca}/P_{Na} of native cardiac Na^+ channels from about 0.0 to between 1.25 and 1.42, earlier studies have noted a finite, albeit small, Ca^{2+} permeation through native TTX-sensitive Na^+ channels from many cell types with $P_{Ca}/P_{Na} \sim 0.1$ (*14*, *33*). However, studies of single Na^+ channels in heart and nerve have suggested that Ca^{2+} is even less permeant and acts as a channel blocker (*17*, *18*), although some studies, with low $[Na^+]_o$ (1 mM or less) (*34*), have reported a small Ca^{2+} flux through Na^+ channels. In ventricular myocytes, atrial natriuretic factor (ANF) (*35*) was reported to activate Ca^{2+} flux through Na^+ channels, but these findings have not been reproduced, nor did we observe any effect of ANF in an experiment like that in Fig 2C (*36*).

Our results indicate that the βAR-mediated activation of PKA promotes a TTX-sensitive Ca^{2+} permeation pathway through the Na^+ channel. The Ca^{2+} flux through this pathway can increase subcellular Ca^{2+} and thereby activate SR Ca^{2+} release, Ca^{2+} sparks, and the $[Ca^{2+}]_i$ transient. We estimate that up to 30% of the $[Ca^{2+}]_i$ transient may be due to this pathway when PKA is activated and may depend on the phosphorylation of the Na^+ channel (Fig. 3D). Similarly, CTSs are activators of slip-mode conductance, local and global Ca^{2+} signaling, and cardiac contraction, but they use a unique signaling process. CTSs bind to the Na^+ pump, which may result in altered protein-protein interactions between the Na^+,K^+-ATPase and Na^+ channel to activate slip-mode conductance; alternatively, this binding may act on Na^+ channels by a less direct pathway (*37*). Because of the widespread availability and importance of the elements underlying slip-mode conductance (βAR, Na^+,K^+-ATPase, and TTX-sensitive Na^+ channels) and the similarity of Na^+ channels in diverse excitable cells, slip-mode conductance is probably a general signaling pathway.

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15. We use three methods to examine I_{Na} in these experiments. (i) Examination of I_{Na} and the $[Ca^{2+}]_i$ transient at negative potentials is possible when $[Na^+]$ inside and outside the cell is normal. At −65 mV, the time constant of inactivation of I_{Na} is about 10 ms and the inward current is about 1 to 2 nA. At this potential, $[Ca^{2+}]_i$ can be monitored, I_{Na} can be measured, and voltage can be controlled. (ii) Examination of I_{Na} over a wider voltage range was done at room temperature with $[Na^+]_i$ and $[Na^+]_o$ equal at 10 mM and with contraction and the $[Ca^{2+}]_i$ transient blocked by adding intracellular EGTA. This method reduces the magnitude of I_{Na} and slows it sufficiently to allow I_{Na} to be measured over a wide range of potentials. Because of the requirement to block contractions and the $[Ca^{2+}]_i$ transient, I_{Na} and the $[Ca^{2+}]_i$ transient cannot be measured simultaneously, but the actions on I_{Na} of PKA activation and $[Ca^{2+}]_o$ can be examined. (iii) To estimate the contributions of I_{Na} under nearly normal cellular conditions, we used a hybrid voltage-clamp protocol, the "action potential clamp." An AP clamp uses a stored action-potential waveform as the voltage command signal. The AP clamp permits the membrane potential to be controlled at all times during the procedure except for a brief period (less than a millisecond) when the AP clamp is settling. However, during this period the uncontrolled current that drives the membrane potential toward the Na^+ channel reversal potential, E_{Na}, assists the AP clamp because the initial depolarization potential of the AP clamp is close to E_{Na}. In a similar manner, some voltage-clamp depolarization test potentials are close to E_{Na}. Although the membrane currents cannot be measured accurately during the settling time of the clamp (less than 1 ms), the command potential and the uncontrolled current work to set the membrane potential at the same level.
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30. Comparison of the efficacy of CTS concentration on Na^+,K^+-ATPase activity is not simple. Reports on the assessment of concentrations of CTS for half-maximal inhibition do not always provide enough relevant information (for example, K^+ concentration). See T. Akera, in *Cardiac Glycosides, Part 1*, K. Greeff, Ed. (Springer-Verlag, New York, 1981), pp. 287–336. There are many additional uncertainties. K_d values for ouabain inhibition of heart Na^+,K^+-ATPase range from 1 nM to about 50 μM; this is due in part to methodological differences and to the existence of three α subunits that bind ouabain, with different affinities for ouabain and different species distributions (*31*).
31. In human heart there are three isoforms of the Na^+,K^+-ATPase, but in rat heart only α1 and α2 are present. (α1 and α2 have a similar affinity for Na^+, but the affinity of α3 is one-third as great) [E. A. Jewell, O. L. Shamraj, J. B. Lingrel, *Acta Physiol. Scand. Suppl.* **607**, 161 (1992)]. The K_d's for ouabain calculated by one method are, for example: α1, α2, and α3: 48 μM, 115 nM, and 1.6 nM, respectively [W. J. O'Brien, J. B. Lingrel, E. T. Wallick, *Arch. Biochem. Biophys.* **310**, 32 (1994)]. Nevertheless, nanomolar quantities of ouabain or other CTS-like digoxin provide both inotropic and toxic actions.
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37. The Na^+,K^+-ATPase is the only identified high-affinity ligand for CTSs. Thus, it is the likely site for the binding of CTSs to activate slip-mode conductance. Because the effects of CTSs were examined in the absence of intracellular Na^+, variation in Na^+ pump activity cannot account for the CTS-dependent Ca^{2+} signaling. Instead, the Na^+,K^+-ATPase may act as a CTS receptor with the Na^+ channel acting as the target protein. Signaling may depend on direct interactions between receptor and target proteins (Fig. 3D) but may involve other signaling components. Slip-mode conductance may thus depend on the local clustering of at least three proteins: βAR, Na^+ channel, and the Na^+,K^+-ATPase.
38. Single rat heart cells were prepared by standard enzymatic methods (*7*). Some of the experiments presented in this paper use conditions that are quite stressful for heart cells. Data for this and subsequent figures were taken only from cells that maintained normal morphology and showed no signs of SR Ca^{2+} overload (that is, spontaneous $[Ca^{2+}]_i$ waves) throughout the duration of experiments. In those experiments in which it was required to expose cells to 0 $[Na^+]_o$, cells were maintained in good condition by limiting their exposure to such solutions to the minimum (for example, 1 to 2 min).
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