

Conventional protein kinase C isoforms and cross-activation of protein kinase A regulate cardiac Na⁺ current

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Abstract We tested the hypothesis that specific isoforms of protein kinase C (PKC) are responsible for modulation of Na⁺ current (I_{Na}) derived from the human cardiac Na⁺ channel using activators and inhibitors selective for specific PKCs. Experimental results demonstrated that I_{Na} suppression was mediated by activation of conventional PKCs (cPKCs) and possibly resulted from channel internalization. In the presence of cPKC inhibition, phorbol ester application unexpectedly increased Na⁺ current, an effect eliminated by inhibition of protein kinase A. These findings demonstrate complex modulation of cardiac I_{Na} by protein kinases and provide further evidence that PKC isoforms have distinct protein targets. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Sodium channel; Cardiac; Protein kinase C; Protein kinase A; Trafficking; *Xenopus* oocyte

1. Introduction

Alterations in Na⁺ channel behavior that modify Na⁺ current (I_{Na}) amplitude or channel gating can lead to the generation of life-threatening cardiac arrhythmias in humans [1,2]. Hence, the factors that regulate Na⁺ channel function are of considerable interest from both a pathophysiologic and therapeutic standpoint. As in brain and skeletal muscle, Na⁺ current in heart can be modulated by activation of protein kinase C (PKC), a family of kinases that modulate function and expression of many ion channels [3,4]. At least 11 isoforms of PKC have been identified [5] and grouped into three subfamilies based on structural considerations and requirements for activation: conventional (c) PKCs (α , β I, β II and γ) that require Ca²⁺, phospholipid, and diacylglycerol or phorbol esters for maximal activation; novel (n) PKCs (δ , ϵ , η , and θ) that also require phospholipid and diacylglycerol, but are insensitive to Ca²⁺; and atypical (a) PKCs (ζ and ι/λ), which do not bind Ca²⁺ or diacylglycerol, but are activated by phospholipids or other mediators such as free fatty acids. Along with this remarkable diversity, there is increasing evidence

that one or more PKC isoforms can mediate a specific physiological response [6].

We have previously demonstrated that Na⁺ current derived from expression of the human cardiac Na⁺ channel, hH1, is reduced by direct activation of PKC (using phorbol 12-myristate 13-acetate, or PMA), as well as α -adrenergic receptor stimulation, implicating a role for conventional PKCs (cPKCs) and/or nPKCs in this effect [7]. Subsequent studies have identified expression of at least nine PKC isoforms in human ventricular myocytes, including members of all three subfamilies [8]. The purpose of this investigation was to test the hypothesis that modulation of hH1 is mediated by a limited repertoire of PKCs using activators and inhibitors that selectively target PKC isoforms or subfamilies. Our results implicate a role for conventional isoforms, as well as potential activation of other kinases, in the effects of PKC on cardiac Na⁺ current.

2. Materials and methods

2.1. Materials

PMA, thymeleatoxin (Tx), bisindolylmaleimide I (Bis I), and ingenol 3,20-dibenzoate (IDB) were obtained from LC laboratories (Woburn, MA, USA), and the protein kinase A (PKA) inhibitor 5-24 from Calbiochem (La Jolla, CA, USA). The PKC- β inhibitor, LY379196, was a gift from Lilly Research Laboratories (Indianapolis, IN, USA). Peptide translocation inhibitors and activators of PKC isoforms, as well as peptides with the same amino acid composition in a random or 'scrambled' order, were kindly provided by Dr. Daria Mochly-Rosen. These peptides were synthesized at Stanford University (Stanford, CA, USA) and characterized previously [6].

2.2. Na⁺ channel expression

The hH1 DNA construct (in a modified pSP64T vector [9]) was linearized with *Xba*I and cRNA was transcribed using SP6 RNA polymerase (Boehringer Mannheim, Indianapolis, IN, USA). Defolliculated oocytes were obtained from *Xenopus laevis* frogs as previously described [10], and injected with cRNA diluted with RNase-free water to yield Na⁺ currents ≤ 6 μ A within 24–72 h after injection.

2.3. Electrophysiology and data analysis

Na⁺ currents were recorded using the two-electrode voltage clamp technique and cell membrane electrical capacitance was measured as previously described [7]. All experiments were conducted at room temperature (22 \pm 2°C).

Analysis of data was performed using custom programs designed to read and analyze pClamp data files. The reversal potential was estimated using a linear fit to the ascending limb of the current–voltage relationship. A paired *t* test was used to compare Na⁺ current properties before and after drug/peptide administration. Comparison of normalized I_{Na} density after different interventions was performed using one-way analysis of variance (ANOVA) with the Scheffé multiple comparison procedure, while the time-dependent effects of specific interventions were compared using ANOVA for repeated measures.

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Abbreviations: PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; aPKC, atypical PKC; PMA, phorbol 12-myristate 13-acetate; Tx, thymeleatoxin; Bis I, bisindolylmaleimide I; IDB, ingenol 3,20-dibenzoate; PKA, protein kinase A; PKI, protein kinase A inhibitor 5-24; ConA, concanavalin A

Results are presented as mean \pm S.E.M. or 95% confidence intervals where indicated.

2.4. Western blot analysis of PKC isoforms in *Xenopus oocytes*

Cells were homogenized and subjected to immunoblotting as described previously [8]. Yolk proteins were removed from the preparation by centrifugation at $12000\times g$ for 5 min. For most experiments, whole cell homogenate was used, with proteins separated using a 6% sodium dodecyl sulfate–polyacrylamide gel. To investigate PKC- β translocation, the homogenate was separated into soluble and particulate fractions by centrifugation at $100000\times g$ for 30 min and the particulate fraction used. An isoform was considered to be present if antibodies from at least two separate commercial sources (Transduction, 1:250; and Santa Cruz, dilutions reported previously [8]) recognized one or more identical bands of a size similar to that of the positive control (± 5 kDa).

3. Results and discussion

3.1. Activation of cPKCs reduces hH1 current

To investigate the role of cPKC isoforms in modulating hH1 current, we examined the effects of Tx, a compound that preferentially activates cPKC isoforms ($ED_{50} \approx 0.1$ μ M [11,12]). Bath application of Tx caused a significant reduction in Na^+ current at all test potentials examined ($-66 \pm 9\%$ at -10 mV in 40 min, Figs. 1A and 2A; $n=9$). The time course of this effect was slow (Fig. 1B), with a continual decrease in current for 40 min without saturation. Tx did not alter cell membrane capacitance ($+2 \pm 10\%$ in 40 min, Fig. 1B), indicating that measurable changes in cell membrane surface area were not involved. Na^+ currents were unchanged following bath superfusion of DMSO (CTRL, Fig. 2A), eliminating

the possibility of a non-specific effect due to either experimental time or vehicle.

To further characterize this effect, we investigated whether Tx altered the voltage dependence of channel gating. There was no significant change in the midpoint ($V_{1/2}$) of the I_{Na} activation or inactivation curve with Tx (Fig. 1C). Using a two-pulse protocol [7], the time course of Na^+ current recovery from inactivation was determined. The recovery process was best fit with a bi-exponential function, and Tx did not significantly alter either the fast or slow components ($\tau_{fast}/\tau_{slow} = 9.1 \pm 1.0/426 \pm 34$ ms before and $11.9 \pm 1.1/429 \pm 130$ ms after Tx). Taken together, these results with Tx are similar to those obtained previously with phorbol ester application and α -adrenergic receptor stimulation [7], suggesting that PKC modulates hH1 current by activating cPKC isoforms.

To further confirm this hypothesis, additional experiments were performed using inhibitors of PKC (Fig. 2A). Pre-incubation with the general PKC inhibitor Bis I (1 μ M) eliminated the reduction in Na^+ current seen with Tx, indicating that this effect was mediated by PKC activation. Receptors for activated C kinase, or RACKs, are anchoring proteins that target PKC isoforms to their substrates during translocation [6]. Based on the binding site of a RACK to the C2 or Ca^{2+} -binding region of PKC- β , a synthetic peptide termed β C2-4 was developed and found to specifically inhibit translocation and activation of cPKCs [6,13]. Pre-injection of β C2-4, but not the analogous peptide having a randomly scrambled amino acid sequence (β C2-4*), completely inhibited the effect of Tx to suppress hH1 current (Fig. 2A), demonstrating that this compound reduced hH1 currents by specific activation of

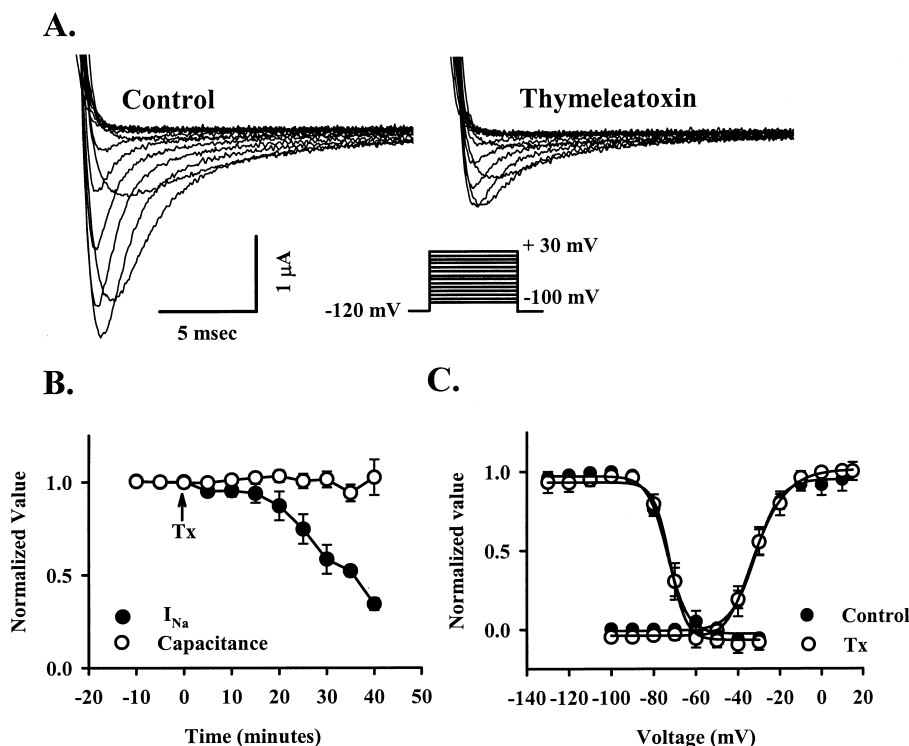


Fig. 1. Effect of Tx on hH1 current. A: Na^+ currents were recorded under control conditions (left) and after exposure to Tx 0.1 μ M for 40 min (right). The pulse protocol is shown in the inset. B: Normalized hH1 current amplitude (at -10 mV; \bullet) and cell membrane electrical capacitance (\circ) are shown as a function of time following bath application of Tx (arrow). C: Activation curves on the right (generated from data in A) and inactivation curves on the left (generated by two second pre-pulses from -140 to -30 mV, followed by a -10 mV test pulse) are shown under control conditions (\bullet) and after Tx (\circ). Data were normalized to peak current amplitude and the curves were fitted with a Boltzmann function. For activation, $V_{1/2}$ was -32 ± 1 mV before and -31 ± 1 mV after Tx; for inactivation, values were -73 ± 1 mV before and -73 ± 0.4 mV after Tx.

cPKC isoforms. β C2-4 itself caused a small increase in hH1 current size ($+9 \pm 7\%$ in 40 min, data not shown; $n = 5$), indicating that basal cPKC activity caused tonic suppression of Na^+ current amplitude.

3.2. PKC- β has a minor role in the effects of cPKC activation

While specific inhibitors of most individual PKC isoforms are currently not available, compounds that inhibit both PKC- β I and PKC- β II with high specificity (IC_{50} 0.03–0.05 μM vs. $\geq 0.3 \mu\text{M}$ for other PKC isoforms) have recently been described [14]. Following pre-incubation of cells with the PKC- β -specific inhibitor LY379196, Tx still caused marked suppression of hH1 current (reduction of I_{Na} in the presence of LY379196 0.1 mM did not differ significantly from Tx alone, Fig. 2A). Similar results were obtained following direct injection of LY379196, while exposure of cells to LY379196 alone had no effect on hH1 current (data not shown). Western analysis was performed to determine whether LY379196 could inhibit activation of PKC- β in *Xenopus* oocytes. As previously reported [15,16], immunoblotting revealed the presence of both PKC- α and PKC- β in these cells (as shown for PKC- β in Fig. 2B). In the presence of Tx, PKC- β expression in the particulate fraction increased, while pre-incubation with LY379196 reproducibly prevented this effect ($n = 3$), indicating that the PKC- β inhibitor could indeed inhibit translocation of the isoform in these cells. These data indicate that PKC- β plays a relatively minor role in the effect of cPKCs to modulate hH1 current.

With respect to other cPKC isoforms, expression of PKC- γ is almost exclusively limited to neuronal tissue [17,18], and we did not detect this isoform in *Xenopus* oocytes (although antibodies directed against mammalian PKCs may not recognize *Xenopus* protein). In light of the tissue-specific expression of PKC- γ , our data with LY379196 suggest that PKC- α is largely responsible for cPKC-mediated suppression of hH1 current.

3.3. Activation of nPKCs does not modulate hH1 current

While conventional and aPKC isoforms have been purified or cloned from *Xenopus* oocytes [15,16,19], the presence of nPKCs in these cells is also assured given the ubiquitous nature of some nPKCs (e.g. PKC- δ) [17], although we did not convincingly detect any nPKC isoforms by immunoblotting (data not shown). To determine whether nPKCs can also modulate hH1 current, we investigated the effects of IDB, a selective activator of nPKCs (at 0.01–0.05 μM) [20,21]. IDB (0.05 μM) had no effect on hH1 currents (Fig. 3A,C), although increasing concentrations caused a reduction in I_{Na} . While a specific inhibitor of nPKCs is currently not available, this reduction in I_{Na} was prevented by pre-injection of the cPKC inhibitor β C2-4 (Fig. 3B,C), indicating that it likely resulted from the non-specific activation of cPKCs known to occur at high concentrations of IDB [21].

Previous studies demonstrate that PKC- ϵ can regulate numerous cardiomyocyte properties including growth, response to ischemic preconditioning, and contraction rate, as well as the amplitude of cardiac L-type Ca^{2+} current [6,22,23]. To examine the role of PKC- ϵ in modulating hH1 current, oocytes were pre-injected with the isoform-specific peptide antagonist ϵ V1-2 prior to bath application of PMA 0.01 μM (at higher concentrations of PMA, the efficacy of the peptide inhibitor is reduced; Daria Mochly-Rosen, personal communication). Although the PKC- ϵ inhibitor blunted the effect of

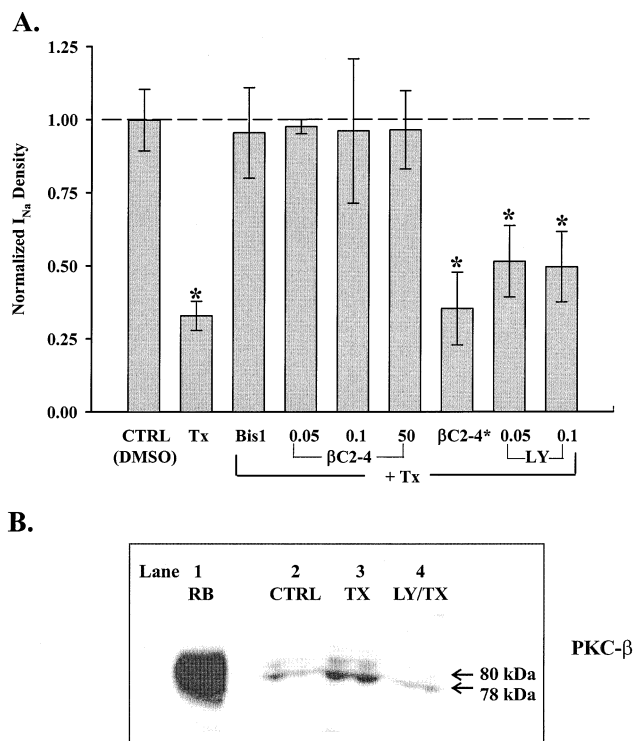


Fig. 2. Effect of PKC inhibitors. A: Bar graphs ($\pm 95\%$ confidence intervals; $n = 5-9$ each; $*P \leq 0.05$ compared to CTRL) display normalized I_{Na} density after a 40 min exposure to DMSO 0.01% (CTRL) or Tx (0.1 μM), and following a 40 min exposure to Tx after pre-incubation (20 min) or pre-injection (30 min) with either Bis 1 (1 μM); β C2-4 (final intracellular concentration 0.05, 0.1, or 50 μM); the scrambled peptide β C2-4* (50 μM); and LY379196 (0.05 or 0.1 μM). B: Rat brain (RB, positive control, lane 1), and the particulate fraction of oocytes exposed for 1 h to bath solution (lane 2), Tx (0.1 μM , lane 3), and pre-incubation (1 h) with LY379196 (0.1 μM) followed by Tx (lane 4), were subjected to Western analysis using an antibody to PKC- β (Santa Cruz, 1:1000).

PMA to reduce I_{Na} (Fig. 3C), this also occurred in cells injected with the scrambled inhibitor (ϵ V1-2*), indicating that it resulted from a non-specific effect of peptide injection. Moreover, injection of cells with the specific PKC- ϵ agonist ψ -RACK did not suppress Na^+ current (Fig. 3C). These results do not support a role for PKC- ϵ , or other nPKCs, in the modulation of hH1 current.

3.4. PMA increases hH1 current by cross-activation of PKA

Because PMA can activate both conventional and nPKC isoforms, we examined the effects of cPKC inhibition on modulation of hH1 current by PMA. Pre-injection of β C2-4 not only inhibited the PMA-induced reduction in I_{Na} , but also unmasked a transient increase in hH1 current by PMA (Fig. 4A). This finding resembled the transient initial increase in I_{Na} that we observed at low concentrations of Tx ($+5 \pm 2\%$ in 25 min at 50 nM; $n = 8$) and PMA ($+12 \pm 4\%$ in 25 min at 1 nM; $n = 4$). Previous work by us and others has shown that hH1 current is increased by activation of PKA [10,24]. In addition, it is well established that in some circumstances, stimulation of PKC can modulate the activity of PKA by multiple different mechanisms [25]. To test the hypothesis that the transient increase in I_{Na} was due to cross-activation of PKA, cells were injected with the peptide inhibitor of PKA 5-24 (PKI), as well as β C2-4. Pre-injection of PKI eliminated the effect of PMA to increase hH1 current in the presence of β C2-4 (Fig. 4A),

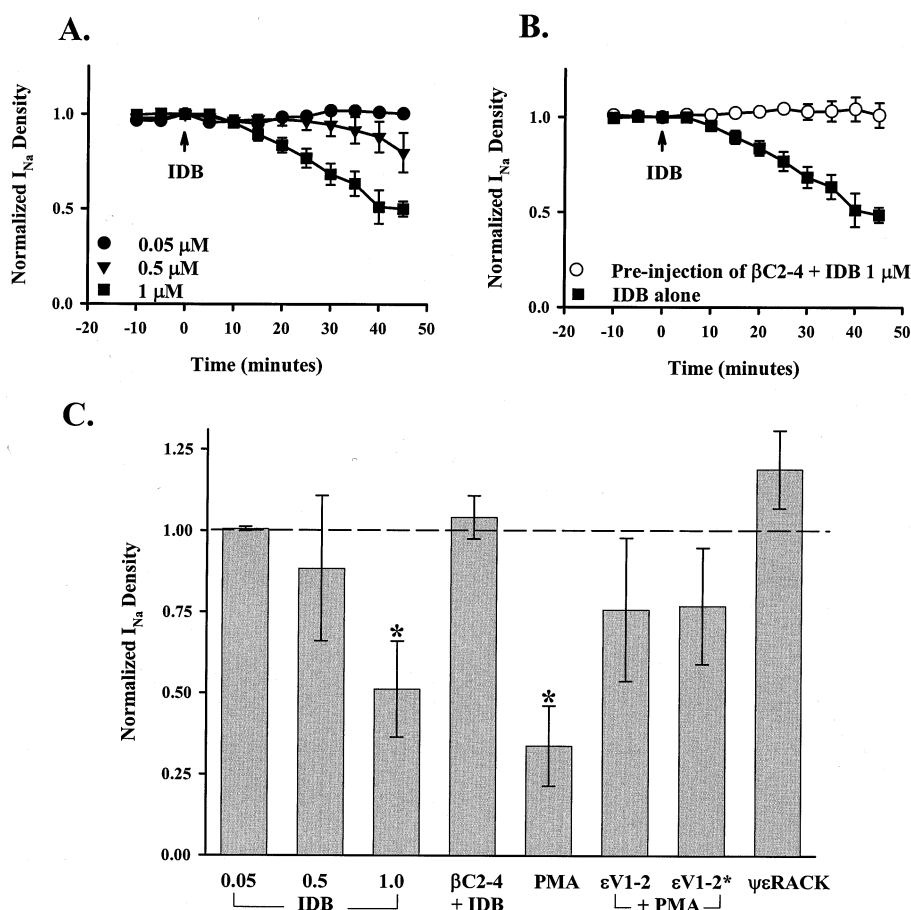


Fig. 3. Lack of effect of nPKC isoforms on hH1 current. A: Normalized I_{Na} density (at -10 mV) is shown over time following exposure to 0.05 μ M (●), 0.5 μ M (▼) and 1 μ M (■) IDB ($n=6$ each). B: In cells pre-injected with the cPKC inhibitor β C2-4, normalized I_{Na} density is shown following exposure to IDB (1 μ M, ○; $n=6$). Data obtained with IDB 1 μ M in the absence of β C2-4 pre-injection (■; also shown in A) are aligned for comparative purposes. C: Bar graphs ($\pm 95\%$ confidence intervals; $n=5-9$ each; $*P \leq 0.05$ compared to CTRL in Fig. 2A) of normalized I_{Na} density are shown after a 40 min exposure to: increasing concentrations (0.05, 0.5, 1.0 μ M) of IDB; IDB (1 μ M) after pre-injection (30 min) of β C2-4; PMA (0.01 μ M); PMA (0.01 μ M) after pre-injection (30 min) of ϵ V1-2 (0.1 μ M) or the scrambled peptide ϵ V1-2* (0.1 μ M); and injection of ψ RACK (0.1 μ M).

indicating that the enhancement of current was likely mediated by cross-activation of PKA.

The mechanism of PKA activation in these experiments remains uncertain. Phorbol esters themselves can activate

PKA, as recently described for modulation of K^+ current derived from the human ether-a-go-go-related gene product (hERG) [26]. In addition, physiologic activation of PKC can alter cAMP generation, with either increased or decreased

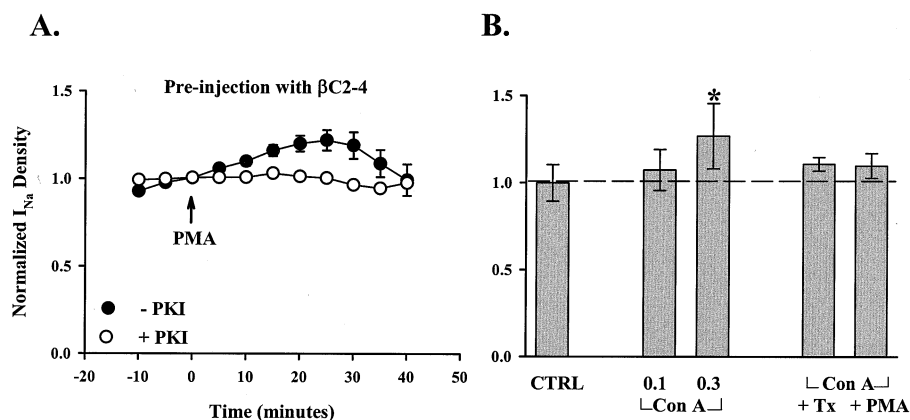


Fig. 4. Cross-activation of PKA and effects of ConA. A: The time course of normalized I_{Na} density is shown in oocytes pre-injected with the cPKC inhibitor β C2-4 alone (–PKI, ●, final intracellular concentration 50 μ M) or β C2-4 plus the PKA inhibitor (+PKI, ○, final intracellular concentration of PKI 10 μ M), followed by application of PMA 0.01 μ M. B: Bar graphs ($\pm 95\%$ confidence intervals; $n=4-6$ each; $*P \leq 0.05$ compared to CTRL) illustrate normalized I_{Na} density after a 40 min exposure to increasing concentrations (0.1, 0.3 mg/ml) of ConA, and to Tx (0.1 μ M) or PMA (0.01 μ M) after pre-incubation (30 min) with ConA (0.1 mg/ml).

production, to modulate PKA activity [25]. Thus, PMA could cause cross-activation of PKA either directly, or by activating a nPKC not stimulated by IDB. Previous studies have shown that the effects of PKC activation on cardiac Na^+ current derived from native myocytes and recombinant channels have been controversial, with both an increase and decrease reported [3,4,10,27,28]. In one study with ventricular myocytes, low concentrations of angiotensin II increased cardiac I_{Na} while high concentrations suppressed it [29], analogous to our results with Tx and PMA. Given our findings, a plausible explanation for this phenomenon is differential activation of PKA and PKC at different concentrations of agonist.

3.5. Concanavalin A (ConA) inhibits suppression of hH1 current

We have recently shown that the increase in hH1 current with PKA stimulation results from modulation of channel trafficking to increase the number of channels in the plasma membrane [10]. The slow, non-saturable reduction in I_{Na} that occurred with Tx (Fig. 1B) and PMA [7] suggested a similar mechanism. To begin to test the hypothesis that channel trafficking is involved in the effect of PKC activation, cells were initially exposed to ConA, a lectin that cross-links extracellular carbohydrate moieties and prevents internalization of membrane proteins [30]. Bath application of ConA caused a slow, concentration-dependent increase in Na^+ current (Fig. 4B), an expected effect if channel internalization was inhibited. Moreover, pre-incubation with ConA totally prevented suppression of hH1 current by either Tx or PMA (Fig. 4B). These results suggest that activation of PKC reduced cardiac Na^+ current by promoting channel internalization, rather than a direct effect to modulate channel function. It is unlikely that this reduction in I_{Na} results from non-specific internalization of oocyte plasma membrane, given that: (1) cell membrane electrical capacitance was unchanged despite a significant reduction in I_{Na} ; and (2) internalization of a large portion of oocyte membrane would have been required, while cells were unchanged microscopically.

Because ConA can cause other effects besides inhibition of membrane protein internalization [31], additional experiments are required to confirm the role of channel internalization in the effects of PKC activation. However, given the increasing recognition that the trafficking of membrane proteins into and out of the plasma membrane can be regulated by protein kinase activation, as for GLUT4 glucose transporters and the cystic fibrosis transmembrane conductance regulator CFTR [32], it is likely that ion channels are subjected to similar regulation. For example as noted above, we have shown that PKA activation increases trafficking of hH1 into the plasma membrane [10]. In addition in immature neurons, persistent activation of Na^+ channels can promote channel internalization [33].

In summary, our results demonstrate additional evidence that a specific physiological response can be mediated by a restricted number of PKC isoforms due to compartmentalization, or co-localization of substrate and enzyme at restricted locations within the cell. In addition, the potential to modify channel trafficking as well as function, and for agonist-mediated cross-activation of other kinases, illustrates that PKC modulation of cardiac Na^+ current is likely a complex process.

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