

# Evidence for functional role of $\epsilon$ PKC isozyme in the regulation of cardiac $\text{Na}^+$ channels

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**Xiao, Guang-Qian, Yongxia Qu, Zhou-Qian Sun, Daria Mochly-Rosen, and Mohamed Boutjdir.** Evidence for functional role of  $\epsilon$ PKC isozyme in regulation of cardiac  $\text{Na}^+$  channels. *Am J Physiol Cell Physiol* 281: C1477–C1486, 2001.—Investigation of the role of individual protein kinase C (PKC) isozymes in the regulation of  $\text{Na}^+$  channels has been largely limited by the lack of isozyme-selective modulators. Here we used a novel peptide-specific activator ( $\epsilon$ V1–7) of  $\epsilon$ PKC and other peptide isozyme-specific inhibitors in addition to the general PKC activator phorbol 12-myristate 13-acetate (PMA) to dissect the role of individual PKCs in the regulation of the human cardiac  $\text{Na}^+$  channel hH1, heterologously expressed in *Xenopus* oocytes. Peptides were injected individually or in combination into the oocyte. Whole cell  $\text{Na}^+$  current ( $I_{\text{Na}}$ ) was recorded using two-electrode voltage clamp.  $\epsilon$ V1–7 (100 nM) and PMA (100 nM) inhibited  $I_{\text{Na}}$  by  $31 \pm 5\%$  and  $44 \pm 8\%$  (at  $-20$  mV), respectively. These effects were not seen with the scrambled peptide for  $\epsilon$ V1–7 (100 nM) or the PMA analog  $4\alpha$ -phorbol 12,13-didecanoate (100 nM). However,  $\epsilon$ V1–7- and PMA-induced  $I_{\text{Na}}$  inhibition was abolished by  $\epsilon$ V1–2, a peptide-specific antagonist of  $\epsilon$ PKC. Furthermore, PMA-induced  $I_{\text{Na}}$  inhibition was not altered by 100 nM peptide-specific inhibitors for  $\alpha$ -,  $\beta$ -,  $\delta$ -, or  $\eta$ PKC. PMA and  $\epsilon$ V1–7 induced translocation of  $\epsilon$ PKC from soluble to particulate fraction in *Xenopus* oocytes. This translocation was antagonized by  $\epsilon$ V1–2. In native rat ventricular myocytes, PMA and  $\epsilon$ V1–7 also inhibited  $I_{\text{Na}}$ ; this inhibition was antagonized by  $\epsilon$ V1–2. In conclusion, the results provide evidence for selective regulation of cardiac  $\text{Na}^+$  channels by  $\epsilon$ PKC isozyme.

protein kinase C; two-electrode voltage clamp; peptides; *Xenopus* oocyte; electrophysiology

CARDIAC  $\text{Na}^+$  channels determine cell excitability and are responsible for conduction velocity of the action potential. They are the targets of several anti-arrhythmic drugs (13) and kinases (12, 51). Both protein kinase A and protein kinase C (PKC) have been implicated in the modulation of  $\text{Na}^+$  channels (12, 51). Two subfamilies of PKC isozymes can be stimulated by the tumor-promoting drug  $4\beta$ -phorbol 12-myristate 13-acetate (PMA): the conventional PKC (cPKC) isozymes

$\alpha$ -,  $\beta$ I-,  $\beta$ II-, and  $\gamma$ PKC, which contain the  $\text{Ca}^{2+}$  binding domain (C2-containing), and the novel PKC (nPKC) isozymes  $\delta$ -,  $\theta$ -,  $\epsilon$ -, and  $\eta$ PKC, or C2-less isozymes (8).

The regulation of  $\text{Na}^+$  channels by PKC has been studied using general PKC activators such as PMA (7) and 1-oleoyl-2-acetyl-*sn*-glycerol (34). In general, activation of PKC by these non-isozyme-specific activators leads to a reduction in  $\text{Na}^+$  current ( $I_{\text{Na}}$ ) in both brain and heart (25, 31, 32). The characterization of the role of individual PKC isozymes in the regulation of ion channels in general and  $\text{Na}^+$  channels in particular has been largely limited by the lack of isozyme selective activators and inhibitors. Identification of the particular isozyme(s) that mediates the regulation of  $\text{Na}^+$  channels is essential for our better understanding of the regulation of  $I_{\text{Na}}$  in physiological and pathological settings. Recently, we have demonstrated, using novel peptide activators and inhibitors of individual isozymes (16, 54), that C2-containing isozymes and  $\epsilon$ PKC play an important role in mediating PMA-induced inhibition of L-type  $\text{Ca}^{2+}$  channels. PKC activation has been associated with the translocation of PKC isozymes from one intracellular compartment to another (10, 27). This translocation event is required for the functional PKC isozymes (40) and is mediated, at least in part, by the binding of activated PKC isozymes to the selective anchoring proteins (RACKs, or receptors for activated C-kinase) that anchor them to different subcellular sites and consequently activate them (28). Anchoring is required for the proper function of individual PKC isozymes. Inhibition or activation of anchoring will alter function. Peptides that mimic either the PKC binding site on RACKs or the RACK binding site on PKC are translocation inhibitors of PKC that inhibit the function of the enzyme (41). On the other hand, a peptide that binds PKC, opens up PKC structure, exposes the catalytic site, and enables anchoring to RACKs will be a PKC agonist (41). On the basis of this rationale, peptide inhibitors and activators of particular PKC isozymes have been developed to inhibit and activate interaction of individual PKC

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isozymes with their respective RACKs, thus altering their translocation and function as well (19, 41). Using these peptides, we examined the potential role of individual PKC isozymes in the regulation of cloned human Na<sup>+</sup> channels expressed in *Xenopus* oocytes and of Na<sup>+</sup> channels in rat ventricular myocytes.

## MATERIALS AND METHODS

**Preparation of *Xenopus* oocyte and cRNA injection.** Mature female *Xenopus* frogs, purchased from Xenopus I (Ann Arbor, MI), were anesthetized with 1.5 mg/ml tricaine. Surgically removed ovarian lobes were dissected and treated for 1.5 h with 1.5 mg/ml collagenase type IA dissolved in Ca<sup>2+</sup>-free ND96 medium (in mM: 96 NaCl, 2 KCl, 2 MgCl<sub>2</sub>, and 5 HEPES, pH 7.4). Stage IV and V oocytes were selected. Plasmids encoding human cardiac Na<sup>+</sup> channel hH1 subunit, pCDNA3.1<sup>+</sup>-SCN5A, were generously given by Dr. Robert S. Kass (Columbia University, New York, NY). Plasmids were first linearized with restriction enzymes, and *in vitro* transcription was carried out using the mMSSAGE mMACHINE (Ambion, Austin, TX). Each oocyte was injected with 50 nl of hH1 cRNA. The injected oocytes were stored at 18°C in Leibovitz's L-15 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 50 U/ml penicillin/streptomycin. Currents were recorded from the third to the fourth day.

**Isolation of cardiac myocytes.** Cardiac myocytes were obtained from hearts of Wistar rats (200–250 g) by enzymatic dissociation as previously described (16, 54). Briefly, hearts were perfused with HEPES-buffered solution containing (in mM) 117 NaCl, 5.4 KCl, 4.4 NaHCO<sub>3</sub>, 1.5 NaH<sub>2</sub>P0<sub>4</sub>, 1.7 MgCl<sub>2</sub>, 20 HEPES, 11 glucose, 10 creatine, and 20 taurine. Hearts were then perfused with the same solution containing collagenase type B (1.0–2.0 mg/ml; Boehringer Mannheim, Indianapolis, IN) for 25–30 min. The softened ventricular tissues were removed, cut into small pieces, and mechanically dissociated by trituration. Cells were suspended in petri dishes containing HEPES buffer with 1 mM CaCl<sub>2</sub> and 0.5% BSA (pH 7.4). All solutions used for perfusion were gassed with 100% O<sub>2</sub> and warmed to 37°C. After incubation for 30 min, a small aliquot of the medium containing single cells was transferred to a chamber mounted on the stage of an inverted microscope (Nikon, Tokyo, Japan). Rod-shaped, non-contracting cells with clear striations were used for the whole cell voltage-clamp studies. All experiments were carried out at room temperatures (22–24°C).

**Solutions and drugs for oocytes.** The composition of external solution for *I*<sub>Na</sub> recording is ND96 (20). V1- or C2-region-derived peptides (100 nM) were injected individually or in combination, as indicated, in a total volume of 50 nl (1/20 of oocyte volume). Proper diffusion of the peptides into the cytoplasm is reached within 10–15 min as previously reported (48). Ten to fifteen minutes after injection of the antagonist peptide, oocytes were superfused with PMA or 4α-phorbol 12,13-didecanoate (4αPDD). For εV1–7 (εPKC agonist peptide), the time course of *I*<sub>Na</sub> was recorded immediately after injection. The peptides εV1–7 [HDAPIGYD; εPKC agonist, also termed pseudo-εRACK (Ψ-εRACK)] (10), εV1–2 (EAVSLKPT; εPKC antagonist), αC2–4 (SLNPQWNET; αPKC antagonist), βC2–4 (SLNPEWNET; βPKC antagonist), and ηV1–2 (EAVGLQPT; ηPKC antagonist) were synthesized at Genemed Synthesis (South San Francisco, CA). All peptides used were >90% pure. All chemicals were purchased from Sigma or otherwise indicated.

**Solutions and drugs for rat ventricular myocytes.** The composition of external solution for *I*<sub>Na</sub> recordings was (in mM) 100 tetraethylammonium (TEA)-Cl, 15 NaCl, 5 CsCl, 0.1

CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, and 10 glucose (pH 7.4 adjusted with CsOH). L- and T-type Ca<sup>2+</sup> currents were blocked by CoCl<sub>2</sub> (5 mM) and NiCl<sub>2</sub> (1 mM), respectively. The internal solution contained (in mM) 135 CsOH, 135 L-aspartic acid, 1 MgCl<sub>2</sub>, 10 EGTA, 10 HEPES, 4 Mg-ATP, and 0.1 Na-GTP (pH 7.1–7.2 adjusted with CsOH). Peptides were added to the peptide solution at a concentration of 100 nM as previously described (16, 54).

**Oocyte *I*<sub>Na</sub> recordings.** The expressed *I*<sub>Na</sub> was recorded with a two-electrode voltage-clamp technique using a GeneCLAMP 500 amplifier (Axon Instrument, Foster City, CA). The volume of the recording chamber was 0.3 ml, and the rate of perfusion was 0.3 ml/min. Oocytes were impaled with electrodes filled with 3 M KCl in ND96 external solution. Oocytes with membrane potential more negative than –40 mV were used for current recording. For *I*<sub>Na</sub> (20) current-voltage (*I*-*V*) relations, oocytes were depolarized from a holding potential of –130 mV to tests ranging from –100 to 70 mV with increments of 5 mV. A depolarization pulse to –20 mV from a holding potential of –130 mV recorded the time course for *I*<sub>Na</sub>. Oocyte membrane capacitance was calculated from the capacitance transient during a voltage step from –130 to –120 mV. The steady-state inactivation of *I*<sub>Na</sub> was obtained by using the double-pulse protocol. Prepulse potentials ranging from –130 to 70 mV were applied and then followed by a 5-ms interpulse interval at a potential of –130 mV. The membrane was then depolarized for 200 ms to test potentials of –20 mV. Steady-state inactivation was measured as the ratio of *I* to *I*<sub>max</sub> (*I*/*I*<sub>max</sub>), where *I*<sub>max</sub> is the maximum current amplitude elicited during the test pulse to –20 mV after the most hyperpolarizing prepulse. The current ratio was plotted as a function of the prepulse potential. The curves were obtained by fitting the data points with Boltzmann distribution of the form  $f_{\text{inf}}(V) = 1/[1 + \exp\{(V_m - V_{0.5})/k\}]$ , where  $f_{\text{inf}}(V)$  is the steady-state inactivation parameter,  $V_m$  is membrane voltage,  $V_{0.5}$  is the half-maximum inactivation potential, and  $k$  is the slope factor. Current recording was done at room temperature (22 ± 2°C).

**Myocyte *I*<sub>Na</sub> recordings.** Whole cell *I*<sub>Na</sub> recording was performed using an Axopatch 200B amplifier with a CV-203BU head stage and pCLAMP software (Axon Instruments). Suction pipettes were made from borosilicate glass capillaries using a horizontal puller (Sutter Instrument, Novato, CA). When filled with pipette solution, tips had resistances ranging between 0.8 and 1.2 MΩ. The tip potential was compensated before the formation of membrane seals. After a seal formed, transient application of negative pressure ruptured the membrane. Hyperpolarizing voltage-clamp steps (to –10 mV from a holding potential of 0 mV) were used to record cell capacitance, which was calculated by integrating the area under the uncompensated capacitance transient and dividing this area by the voltage step. Cell capacitance and pipette series resistance were both compensated before the onset of the experiment. To record *I*<sub>Na</sub> time course, cells were depolarized to –25 mV for 50 ms from a holding potential of –90 mV (24). All experiments were performed at room temperature (22–24°C). Data acquisition, voltage protocols, and analysis were performed using the pCLAMP suite of software (Axon Instruments). We allowed 5–8 min for *I*<sub>Na</sub> to reach steady state and also for peptides to properly enter the cell (16, 54). Therefore, the *time 0* shown in Fig. 8 represents about 5–8 min after formation of whole cell configuration.

**Immunoprecipitation and Western blot.** Stage IV–V oocytes were treated with either 1) PMA (100 nM) or εV1–7 (100 nM) or 2) PMA or εV1–7 plus εV1–2 (100 nM). Membranes were obtained from these oocytes 30 min after the above treatment and purified as previously described (52).

Briefly, 50–90 treated oocytes were homogenized in 10% sucrose, 15 mM NaCl, 5 mM KCl, and 20 mM HEPES, pH 7.5, supplemented with proteinase inhibitor cocktail (37). After centrifugation, membrane fractions from 20–50% sucrose gradient interface were collected as particulate fractions, and pellet fractions from 10% and 10–20% sucrose gradient interface were collected as cytosolic fractions. Each fraction was homogenized and solubilized in 2.5 ml of buffer (75 mM KCl, 75 mM NaCl, and 50 mM Na-phosphate, pH 7.2, plus 2 mg/ml soybean lipids and 1% Triton X-100) and centrifuged for supernatant collection.

Mouse-raised antibodies against εPKC (DB Transduction, Piscataway, NJ) were used to immunoprecipitate εPKC. Briefly, anti-εPKC antibody was added to the supernatant, which was precleared with protein A-Sepharose and shaken at 4°C for 4 h. We added 25 μl of 50% protein A-Sepharose beads for every 1 ml of sample and incubated overnight. Protein A-Sepharose antibody/antigen complex was collected by centrifugation, washed, and eluted in reducing SDS sample buffer by boiling for 5 min. For Western blot assay, 35 μl/lane of the above immunoprecipitated proteins were subjected to 8% SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane by electrophoresis. The blot was blocked for 2 h in blocking buffer [5% nonfat dry milk in wash buffer (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween 20)] and washed twice in wash buffer. For immunoreaction, the blot was incubated with anti-εPKC antibody at room temperature for 1.5 h. Blots were washed completely with wash buffer. Immunodetection was carried out with a 1:1,500-diluted horseradish peroxidase conjugated anti-mouse IgG (Amersham Pharmacia Biotech, Piscataway, NJ) secondary antibody for 1 h at room temperature. Blots were washed again and then incubated with the enhanced chemiluminescence detection reagent (Amersham Pharmacia Biotech) for 1 min and exposed to X-ray film.

**Data analysis.** Data acquired were stored and then analyzed off-line with pCLAMP 6 software (Axon Instruments). All values were measured as the difference between zero and the peak current. All measurements of  $I_{Na}$  changes were performed at 30 min to avoid potential time-dependent internalization of plasma membrane in oocytes reported after 30 min of exposure to phorbol esters (46). Microcal Origin v5.0 (Microcal Software) was used to generate figures and perform statistical analysis. Data are presented as means ± SE. Percent inhibition was calculated as the difference in the current amplitude caused by the intervention(s), divided by the control value. Student's paired *t*-test was used to compare the data before and after interventions. Unpaired *t*-test or ANOVA was used to compare the data between groups. A value of  $P < 0.05$  was considered statistically significant.

**RESULTS**

**PMA inhibited  $I_{Na}$  expressed in *Xenopus* oocytes.** To investigate whether, under our experimental conditions, PKC is involved in the modulation of  $I_{Na}$ , we first used a general PKC activator, PMA, and a general PKC inhibitor, calphostin C. Figure 1 shows the effect of PMA in the absence (Fig. 1, A and B) and presence of calphostin C (Fig. 1D). Exposure of oocytes to PMA (100 nM) resulted in a slow and time-dependent inhibition of peak  $I_{Na}$  (Fig. 1A). The *I-V* relations during control and PMA at 30 min are shown in Fig. 1B. PMA inhibited  $I_{Na}$  by  $44.3 \pm 8.2\%$  at  $-20$  mV ( $n = 8, P < 0.05$  compared with control). The specificity of PMA effects on  $I_{Na}$  was confirmed by comparing its effects to another phorbol ester, 4αPDD, which does not activate PKC (11, 44). PMA effects were not seen [ $2.9 \pm 2.2\%$ ,  $n = 5, P =$  not significant (NS) compared with control]

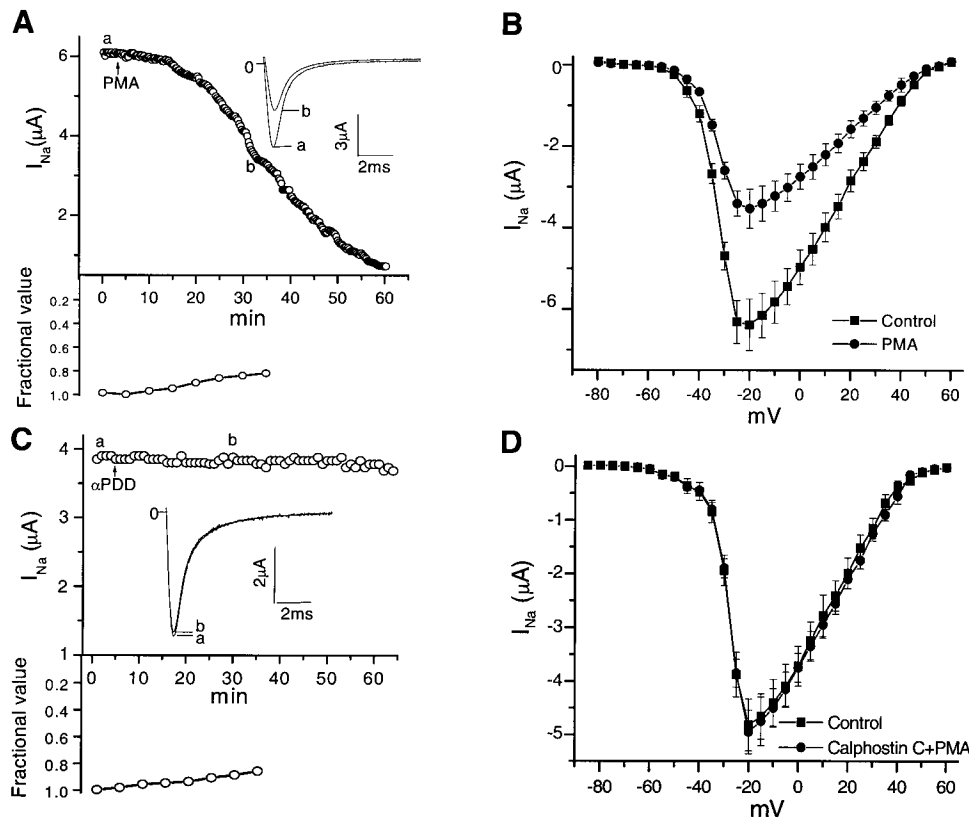


Fig. 1. Inhibition of Na<sup>+</sup> current ( $I_{Na}$ ) by phorbol 12-myristate 13-acetate (PMA). A: time course of peak  $I_{Na}$  inhibition by PMA (100 nM). B: current-voltage (*I-V*) relations of  $I_{Na}$  during control and 30 min after superfusion of PMA (100 nM) in 6 oocytes. C: specificity of PMA effects on  $I_{Na}$  as confirmed by comparing its effects to another phorbol ester, 4α-phorbol 12,13-didecanoate (αPDD; 100 nM), which does not activate protein kinase C (PKC). Oocyte capacitance expressed in fractional values is shown in A and C (bottom). D: *I-V* relations of  $I_{Na}$  during control ( $n = 4$ ) and 30 min after PMA superfusion of 4 oocytes pretreated with calphostin C, a general PKC inhibitor. Selected  $I_{Na}$  tracings ( $-20$  mV) at the times indicated by a and b are shown in insets in A and C. Time 0 corresponds to the time of oocyte impalement; arrows refer to the onset of drug superfusion.

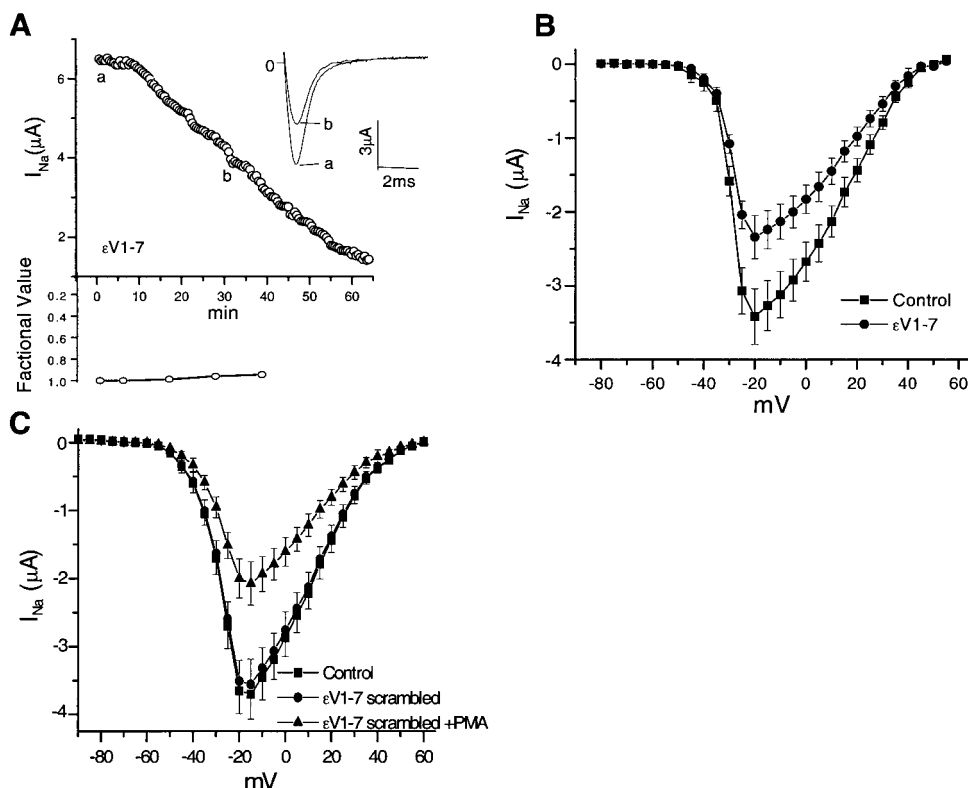
with its inactive analog, 4αPDD, at the same concentration of 100 nM (Fig. 1C). PMA and 4αPDD effects at 30 min on the cell capacitance of oocytes are shown in the lower part of Fig. 1, A and C, respectively. PMA and 4αPDD reduced oocyte cell capacitance by  $17 \pm 3.7\%$  ( $n = 6$ ,  $P < 0.05$ ) and  $15 \pm 3.6\%$  ( $n = 5$ ,  $P < 0.05$ ), respectively. Calphostin C superfusion for 10–15 min before the onset of PMA application completely blocked PMA inhibition of  $I_{\text{Na}}$  (only  $4.6 \pm 3.3\%$ ,  $n = 4$ ,  $P = \text{NS}$  compared with control). These experiments indicate that PMA inhibition of  $I_{\text{Na}}$  is mediated through PKC.

**Peptide activator of εPKC, εV1-7, inhibited  $I_{\text{Na}}$  expressed in *Xenopus* oocytes.** To selectively activate εPKC, we used a novel peptide, εV1-7, also termed pseudo-εRACK (Ψ-εRACK), which is derived from the regulatory V1 region of εPKC and was previously shown to selectively activate the translocation of εPKC (10). This is the first and only available agonist peptide activator of one single PKC isozyme. Figure 2 shows the effect of this εPKC agonist, εV1-7, and its scrambled peptide (negative control) on  $I_{\text{Na}}$ . Recording of  $I_{\text{Na}}$  began immediately after injection of εV1-7 (100 nM) or its scrambled peptide (100 nM). Figure 2A illustrates the time course of εV1-7 inhibition of  $I_{\text{Na}}$  from one oocyte. εV1-7 at 30 min reduced oocyte cell capacitance by  $3.9 \pm 3.0\%$  (Fig. 2A, bottom;  $n = 5$ ,  $P = \text{NS}$ ). Figure 2B shows the  $I$ - $V$  relations of  $I_{\text{Na}}$  during control and 30 min after the injection of εV1-7. εV1-7 inhibited peak  $I_{\text{Na}}$  by  $30.5 \pm 4.5\%$  ( $n = 15$ ,  $P < 0.05$  compared with control). Figure 2C shows the lack of effect of εV1-7 scrambled peptide on  $I_{\text{Na}}$   $I$ - $V$  relations ( $3.2 \pm 2\%$ ,  $n = 5$ ,  $P = \text{NS}$  compared with control). However, PMA

superfusion of seven other oocytes preinjected with εV1-7 scrambled peptide resulted in  $I_{\text{Na}}$  inhibition by  $40.1 \pm 6.3\%$  ( $n = 7$ ,  $P < 0.05$  compared with εV1-7 scrambled peptide).

To further evaluate the selectivity of the peptide εV1-7 on  $I_{\text{Na}}$  and its mechanism in the regulation of  $I_{\text{Na}}$ , we studied the effect of εV1-7 on  $I_{\text{Na}}$  in the presence of a peptide-specific inhibitor of εPKC, εV1-2, which has been shown to selectively inhibit the translocation of εPKC (10). Figure 3 shows the effect of PMA (100 nM) and εV1-7 (100 nM) on  $I_{\text{Na}}$  in the presence of εV1-2 peptide (100 nM). Figure 3A shows the time course of the PMA effect on peak  $I_{\text{Na}}$  from one oocyte injected with εV1-2. Figure 3B shows the  $I$ - $V$  relations during control and 30 min after PMA application in oocytes injected with εV1-2 peptide. εV1-2 peptide antagonized the PMA inhibitory effect on  $I_{\text{Na}}$  (only  $8.1 \pm 3.5\%$ ,  $n = 5$ ,  $P < 0.05$  compared with PMA alone). Figure 3C shows the time course of peak  $I_{\text{Na}}$  from one oocyte after the coinjection of both εV1-7 and εV1-2 peptides. εV1-7 failed to significantly inhibit  $I_{\text{Na}}$  in the presence of εV1-2, indicating that εV1-7 inhibited  $I_{\text{Na}}$  by functionally activating the translocation of εPKC. Figure 3D shows the  $I$ - $V$  relations during control and 30 min after the coinjection of εV1-2 plus εV1-7. The effect of εV1-7 on  $I_{\text{Na}}$  was completely blocked by εV1-2 peptide ( $4.4 \pm 3.1\%$ ,  $n = 6$ ,  $P = \text{NS}$  compared with 4αPDD). Together, these results demonstrate the ability of the novel peptide εV1-7 to activate one single PKC isozyme, εPKC, and the ability of peptide εV1-2 to block these effects, thus altering  $I_{\text{Na}}$  channel function.

Fig. 2. Inhibition of  $I_{\text{Na}}$  by peptide εV1-7. **A:** time course of peak  $I_{\text{Na}}$  recorded in an oocyte injected with the peptide εV1-7 (100 nM), a specific εPKC activator. **Bottom:** oocyte capacitance expressed in fractional values. **Inset:** selected  $I_{\text{Na}}$  tracings ( $-20$  mV) at the times indicated by *a* (control) and *b* (30-min perfusion of PMA). **B:**  $I$ - $V$  relations of  $I_{\text{Na}}$  during control and 30 min after injection of εV1-7 in 8 oocytes. **C:**  $I$ - $V$  relations of  $I_{\text{Na}}$  during control ( $n = 5$ ) and 30 min after injection of εV1-7 scrambled peptide, followed by 30 min of superfusion of PMA (100 nM) in the same oocytes injected with εV1-7 scrambled peptide ( $n = 5$ ).



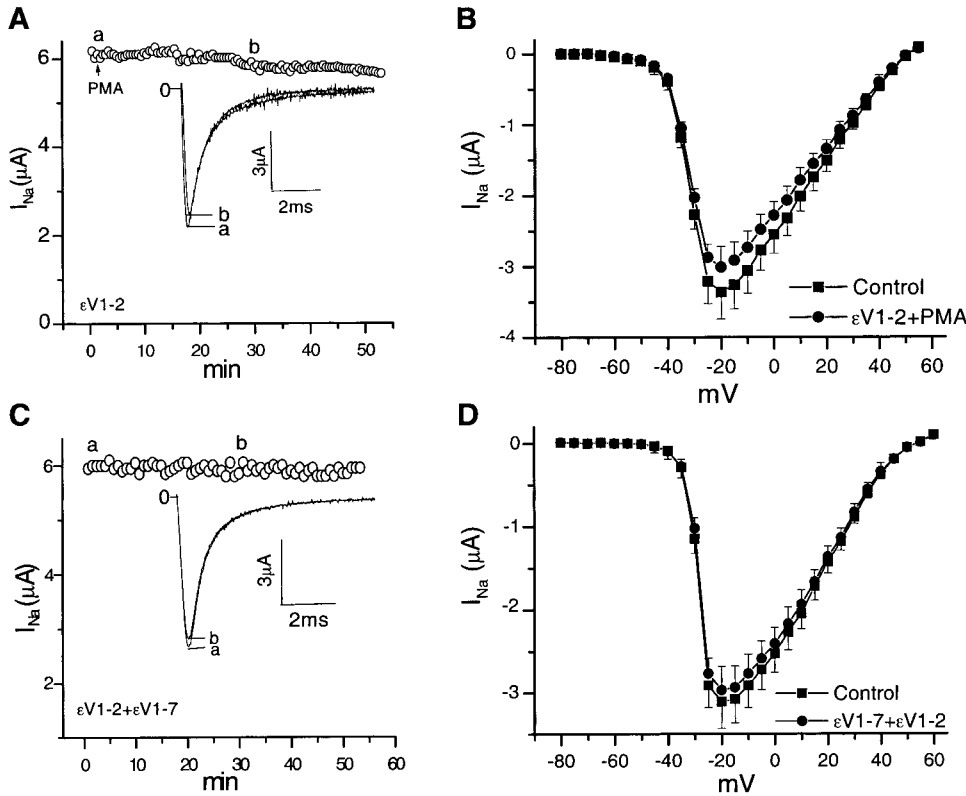


Fig. 3. Effect of PMA and εV1-7 peptide on  $I_{Na}$  in the presence of the peptide inhibitor of εPKC, εV1-2. **A:** time course of peak  $I_{Na}$  recorded from an oocyte injected with εV1-2 (100 nM) and superfused with PMA (100 nM). **B:**  $I$ - $V$  relations of  $I_{Na}$  during control and 30 min after superfusion of PMA in 5 oocytes injected with εV1-2 peptide. **C:** time course of peak  $I_{Na}$  recorded from an oocyte injected with both εV1-2 (100 nM) and εV1-7 (100 nM) peptides. **D:**  $I$ - $V$  relations during control and 30 min after injection of both εV1-7 (100 nM) and εV1-2 (100 nM) peptides in 5 oocytes. Selected  $I_{Na}$  tracings (-20 mV) at the times indicated are shown in insets in **A** and **C**. Time 0 corresponds to the time of oocyte impalement; arrows refer to the onset of drug superfusion.

PMA inhibition of  $I_{Na}$  was not altered by peptide-specific antagonists of α-, β-, δ-, and ηPKC isozymes. Figure 4A shows the  $I$ - $V$  relations of  $I_{Na}$  during control and 30 min after PMA superfusion of oocytes injected

with αC2-4 peptide;  $I_{Na}$  was decreased by  $44.9 \pm 7.9\%$  ( $n = 6$ ,  $P = NS$  compared with PMA alone). Figure 4B shows the  $I$ - $V$  relations of  $I_{Na}$  during control and 30 min after PMA superfusion of oocytes injected with

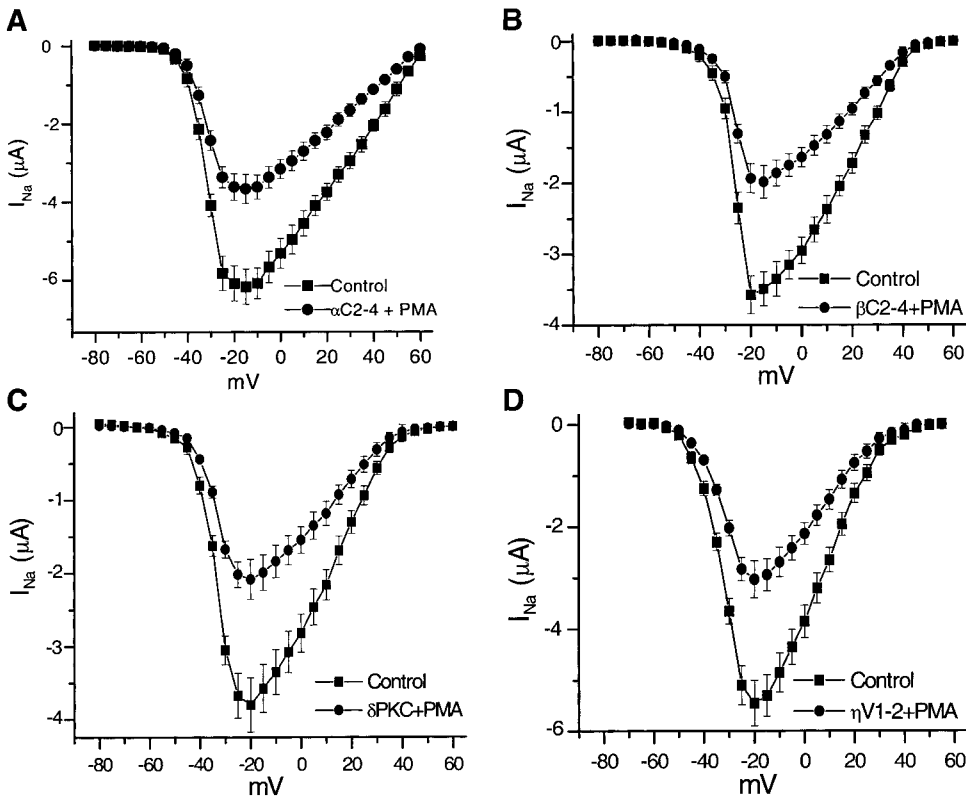
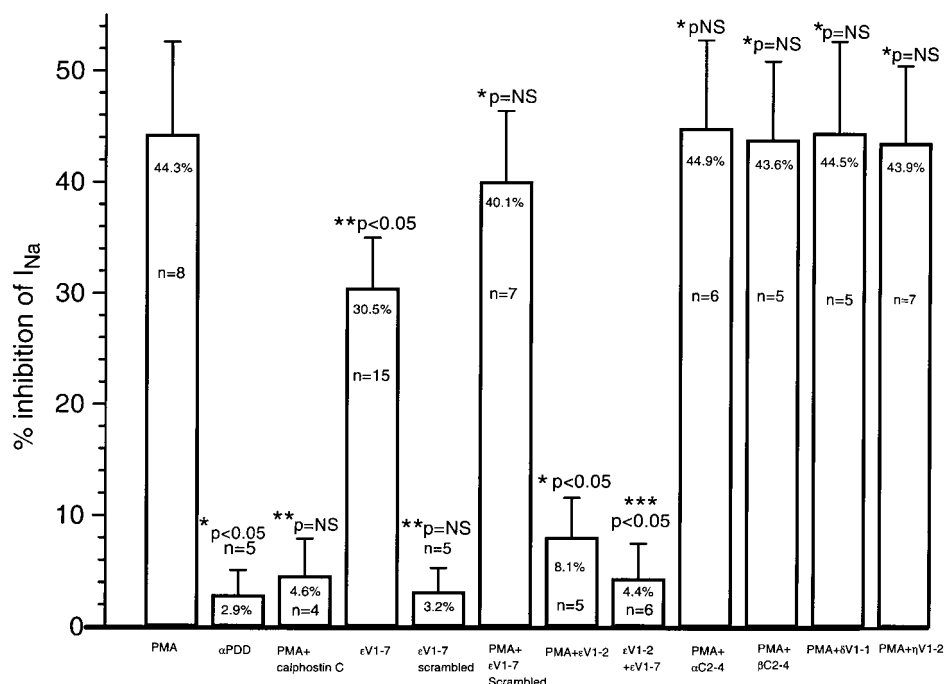


Fig. 4. Effect of PMA on  $I_{Na}$  in the presence of peptide inhibitors of α-, β-, δ-, and ηPKC isozymes. **A-D:**  $I$ - $V$  relations of  $I_{Na}$  during control and 30 min after superfusion of PMA (100 nM) in the presence of αC2-4 (100 nM,  $n = 6$ ), βC2-4 (100 nM,  $n = 5$ ), δV1-1 (100 nM,  $n = 5$ ), and ηV1-2 (100 nM,  $n = 7$ ) peptides, respectively.

Fig. 5. Summary of percent inhibition of  $I_{\text{Na}}$  by PMA,  $\alpha$ PDD, calphostin C, and currently available PKC isozyme-specific peptide agonist and antagonists. \* $P$  value indicates comparison with PMA alone. \*\* $P$  value indicates comparison with  $\alpha$ PDD superfusion. \*\*\* $P$  value indicates comparison with  $\epsilon$ V1–7 alone. NS, not significant.



$\beta$ C2–4 peptide;  $I_{\text{Na}}$  was decreased by  $43.6 \pm 6.9\%$  ( $n = 5$ ,  $P = \text{NS}$  compared with PMA alone). Figure 4C shows the  $I$ - $V$  relations of  $I_{\text{Na}}$  during control and 30 min after PMA superfusion of oocytes injected with  $\delta$ V1–1 peptide;  $I_{\text{Na}}$  was decreased by  $44.5 \pm 8.2\%$  ( $n = 5$ ,  $P = \text{NS}$  compared with PMA alone). Figure 4D shows the  $I$ - $V$  relations of  $I_{\text{Na}}$  during control and 30 min after PMA superfusion of oocytes injected with  $\eta$ V1–2 peptide;  $I_{\text{Na}}$  was decreased by  $43.9 \pm 7.0\%$  ( $n = 7$ ,  $P = \text{NS}$ , compared with PMA alone). These results demonstrate that  $\alpha$ -,  $\beta$ -,  $\delta$ -, and  $\eta$ PKC isozymes did not alter PMA-induced  $I_{\text{Na}}$  inhibition. A summary of all the above results is shown in Fig. 5.

PMA and  $\epsilon$ V1–7 did not alter steady-state inactivation of  $I_{\text{Na}}$  expressed in *Xenopus* oocytes. The effects of PMA and  $\epsilon$ V1–7 on steady-state inactivation of  $I_{\text{Na}}$  were also investigated using the double-pulse protocol as indicated in MATERIALS AND METHODS. Figure 6 shows the averaged normalized data plotted against the prepulse potentials for PMA (A) and  $\epsilon$ V1–7 (B). The curves in Fig. 6 were obtained by fitting the data points with the Boltzmann distribution described in MATERIALS AND METHODS. The inactivation curves were nearly identical between control and either PMA or  $\epsilon$ V1–7 in Fig. 6, A or B, respectively, suggesting that PMA or peptide  $\epsilon$ V1–7 did not change the kinetics of voltage-dependent inactivation of  $I_{\text{Na}}$ . For the control group ( $n = 6$ ),  $V_{0.5}$  was  $-80 \pm 4.3$  mV and  $k$  was  $4.4 \pm 0.7$  mV, whereas for the PMA group ( $n = 6$ ),  $V_{0.5}$  was  $-84 \pm 6.8$  mV and  $k$  was  $4.3 \pm 0.9$  mV. Similarly, for the control group ( $n = 5$ ),  $V_{0.5}$  was  $-79.7 \pm 3.8$  mV and  $k$  was  $4.5 \pm 0.5$  mV, whereas for the  $\epsilon$ V1–7 group ( $n = 5$ ),  $V_{0.5}$  was  $-82.6 \pm 6.4$  mV and  $k$  was  $4.4 \pm 0.8$  mV.

PMA and  $\epsilon$ V1–7 induced translocation of  $\epsilon$ PKC in *Xenopus* oocytes. To demonstrate that the functional inhibition of  $I_{\text{Na}}$  is associated with biochemical trans-

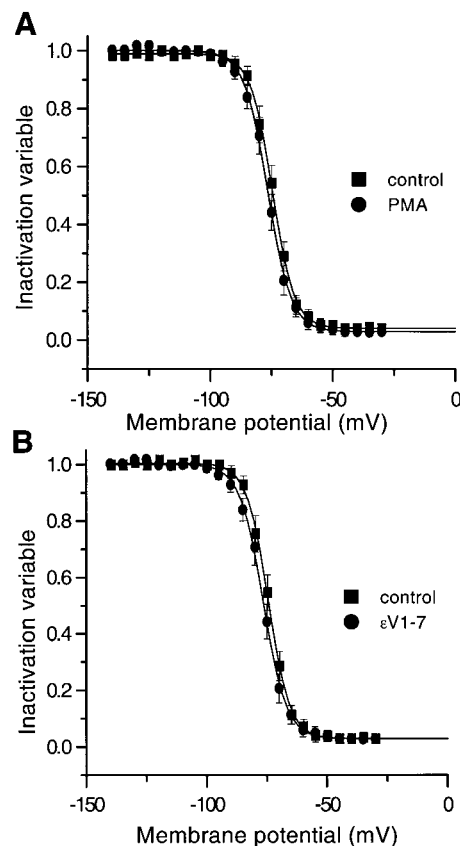


Fig. 6. Effect of PMA and  $\epsilon$ V1–7 on  $I_{\text{Na}}$  steady-state inactivation curves. Steady-state inactivation curves were fit through mean data points using the Boltzmann equation  $f_{\text{inf}}(V) = 1/[1 + \exp\{(V_m - V_{0.5})/k\}]$ , where  $V_m$  is membrane voltage,  $V_{0.5}$  is the half-maximum inactivation potential, and  $k$  is the slope factor. A:  $V_{0.5}$  was  $-80 \pm 4.3$  mV and  $k$  was  $4.4 \pm 0.7$  mV for control ( $n = 6$ ), and  $V_{0.5}$  was  $-84 \pm 6.8$  mV and  $k$  was  $4.3 \pm 0.9$  mV for PMA ( $n = 6$ ). B:  $V_{0.5}$  was  $-79.7 \pm 3.8$  mV and  $k$  was  $4.5 \pm 0.5$  mV for control ( $n = 5$ ), and  $V_{0.5}$  was  $-82.6 \pm 6.4$  mV and  $k$  was  $4.4 \pm 0.8$  mV for  $\epsilon$ V1–7 ( $n = 5$ ).

location of εPKC from the cytosol to the membrane, we performed Western blot assays on oocytes treated with εPKC activators and/or inhibitors. Figure 7 shows the translocation of εPKC in oocytes by PMA (100 nM) and εV1-7 (100 nM) and its inhibition by εV1-2 (100 nM). Figure 7, lanes 2 and 4, show that εPKC was translocated from the soluble to the particulate fraction by PMA and εV1-7, respectively. Figure 7, lanes 6 and 8, show that εV1-2 antagonized PMA- and εV1-7-induced εPKC translocation from the soluble to the particulate fraction, respectively. Similar results were obtained in a total of four independent experiments.

*PMA and εV1-7 inhibited I<sub>Na</sub> in rat ventricular myocytes.* We next tested whether εPKC also modulates I<sub>Na</sub> in native cardiac myocytes. I<sub>Na</sub> was recorded from rat ventricular cells, and the effects of εPKC activation were investigated. Figure 8A shows the time course of the effect of 100 nM PMA on I<sub>Na</sub> in the absence or presence of intrapipette εV1-2 (100 nM). PMA inhibited I<sub>Na</sub> by 53 ± 5.9% (n = 5, P < 0.05). However, in the presence of εV1-2, PMA-induced I<sub>Na</sub> inhibition was reduced to 22.4 ± 7% (n = 4, P < 0.05 compared with PMA alone). Figure 8B shows the time course of the effect of εV1-7 (100 nM) on I<sub>Na</sub> in the absence and presence of intrapipette εV1-2 (100 nM). εV1-7 inhibited I<sub>Na</sub> by 34.3 ± 5.4% (n = 6, P < 0.05). This inhibition of I<sub>Na</sub> by εV1-7 was completely abolished by εV1-2 (5.4 ± 1.4%, n = 4, P > 0.05). All together, the data obtained in native cardiac myocytes indicate that selective εPKC activation also inhibited I<sub>Na</sub>. These results are similar to those obtained in *Xenopus* oocytes.

**DISCUSSION**

The present study is the first to show that εPKC is involved in PMA-induced inhibition of the cloned human I<sub>Na</sub> expressed in *Xenopus* oocyte and I<sub>Na</sub> recorded from rat ventricular myocytes. It is evident that identification of the particular isozyme(s) that mediates the regulation of Na<sup>+</sup> channels is of important therapeutic implications.

*Regulation of I<sub>Na</sub> by PKC.* The characterization of the role of individual PKC isoforms in the regulation of ion channels in general and Na<sup>+</sup> channels in particular

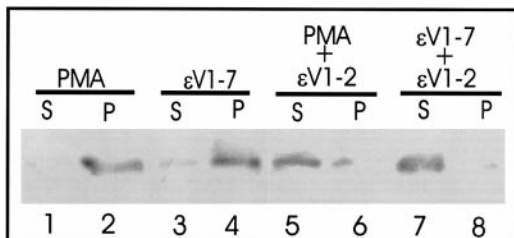


Fig. 7. Translocation of εPKC by PMA and εV1-7: Western blot analysis was performed in oocytes treated with PMA (100 nM) and/or εPKC peptide modulators (100 nM). Lanes 2 and 4 show that εPKC translocated from the soluble (S) to the particulate (P) fractions by PMA and εV1-7, respectively. Lanes 6 and 8 show that εV1-2 antagonized PMA- and εV1-7-induced εPKC translocation from soluble to particulate fractions, respectively.

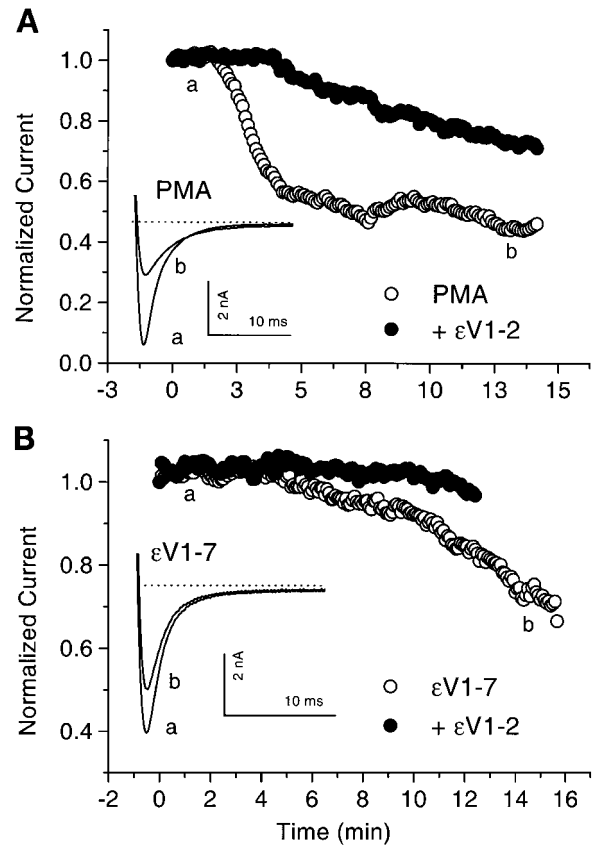


Fig. 8. Effects of PMA and εV1-7 on I<sub>Na</sub> in rat ventricular myocytes. A: time course of peak I<sub>Na</sub> from 2 different myocytes superfused with PMA (100 nM, ○) and PMA + εV1-2 (100 nM, ●). B: time course of peak I<sub>Na</sub> from 2 different myocytes dialyzed with εV1-7 (100 nM, ○) and εV1-2 (100 nM) + εV1-7 (●). Insets: selected current traces at the times indicated by a and b.

has been largely limited by the lack of isozyme selective activators and inhibitors. While several previous studies implicated PKC in the regulation of Na<sup>+</sup> channels, the role and the identity of the isozyme(s) responsible for this regulation remain largely unexplored. Heterologously expressed rat brain (rBIIA) (7, 32) and human cardiac Na<sup>+</sup> channel currents (hH1) (31) were reduced upon PKC activation. Although both rBIIA and hH1 contain consensus sites for phosphorylation by PKC, most of the sites are not conserved between these two isoforms. In one study (31), elimination of conserved consensus PKC sites in the hH1 interdomain III-IV linker, which contains the putative PKC site (Ser-1503), does not completely eliminate the PMA-induced I<sub>Na</sub> inhibition, implying that other phosphorylation site(s) may exist.

Our present findings in *Xenopus* oocytes showing that I<sub>Na</sub> is inhibited by PMA, a general activator of PKC, are consistent with previous studies using a heterologous expression system (7, 31, 34, 35). In addition, the use of a novel peptide-specific activator of εPKC, εV1-7, mimicked PMA effects on I<sub>Na</sub>, and the use of a peptide-specific inhibitor of εPKC, εV1-2, prevented these effects, thus establishing the involvement of εPKC in the regulation of Na<sup>+</sup> channels. Al-

though PMA inhibition of  $I_{Na}$  (44.3%) is slightly higher than εV1-7 inhibition (30.5%), it appears that activation of εPKC alone by the peptide εV1-7 is sufficient to inhibit  $I_{Na}$ . Because of the unavailability of peptide-specific activators of other PKC isozymes, we do not exclude the possibility that other isoforms may be involved in the regulation of Na<sup>+</sup> channels. PMA has been reported to cause time-dependent internalization of plasma membrane in oocytes (46) after 30 min of exposure. In the present study, whereas PMA (100 nM) and 4αPDD (100 nM) at 30 min significantly reduced oocyte cell capacitance by about 17% and 15%, respectively, εV1-7 did not significantly alter the oocyte cell capacitance (4%). This finding indicates that PMA effects on oocyte cell capacitance are likely due to a nonspecific effect. Phorbol esters have been reported to mediate some of their responses through β-chimerin, a member of the GTPase-activating proteins that lacks the functional kinase domain (3, 4). It is therefore possible that some of PMA-nonspecific effects may be mediated through the chimerin family via a yet unknown mechanism. However, PMA inhibition of  $I_{Na}$  (44.3%) far exceeds the nonspecific effect, suggesting that PMA regulates Na<sup>+</sup> channels through a PKC pathway. This is further supported by the fact that part of the PMA effects on  $I_{Na}$  were reversed by the general PKC antagonist calphostin C and by the εPKC-specific peptide antagonist εV1-2. In addition, biochemical data showed that both PMA and εV1-7 induced the translocation of εPKC from the cytosol to the membrane. This translocation was inhibited by the εPKC-specific peptide inhibitor εV1-2. In the present study, we showed by Western blot that, in *Xenopus* oocytes, εPKC can be detected, activated, and translocated. This biochemical finding is consistent with our functional data demonstrating that εPKC activation leads to  $I_{Na}$  inhibition. It is noteworthy that previous studies using *Xenopus* oocytes have demonstrated the existence of at least six other PKC isozymes including α-, βI-, βII-, γ-, δ-, and ζPKC (9, 18). Similarly, in native rat cardiac myocytes, we showed that εPKC activation results in  $I_{Na}$  inhibition, consistent with the results obtained in *Xenopus* and consistent with previously published reports in native cardiac myocytes (34, 49, 50). However, in one study (29), activation of PKC increased  $I_{Na}$ .

*Proposed mechanism of peptide εV1-7 action.* Peptide εV1-7 is the first isozyme-selective PKC activator that induces εPKC translocation from the cytosol to the particulate fraction (10). The molecular basis underlying the action of the peptide εV1-7 on εPKC has not been fully explored. It has been suggested that this peptide acts by interfering with the intramolecular interaction within εPKC between the RACK-binding site and the pseudo-RACK site, thereby mimicking the conformational change and dissociation of this intramolecular interaction that occurs upon activation of εPKC, rendering PKC more accessible to its anchoring protein (10). The evidence that a peptide translocation activator for εPKC, εV1-7, functionally inhibited  $I_{Na}$  suggests that translocation activators should be ago-

nists of PKC function, independent of the amount of second messengers that normally activate PKC. This finding further suggests that the translocation of PKC isozymes is essential for the full function of endogenous PKC activation. Phosphorylation of ion channel proteins is the key mechanism in signal transduction pathways that alter channel properties and influence excitability, and thus the physiological function, of excitable cells (22). The molecular mechanisms by which PKC regulates cardiac Na<sup>+</sup> channels are not completely defined.

*Physiological and pathophysiological significance of the regulation of  $I_{Na}$  channels by PKC.* In the last few years, research in the general area of signal transduction has advanced significantly. As a result, PKC has emerged as a key component along signal transduction pathways. PKC has been involved in the modulation of ion channels (11, 21, 33, 45, 47, 54), inotropic and chronotropic effects (5, 11, 23, 26, 53), gene expression (6, 38), secretion of cardiac factors (17, 36), hypertrophy (14, 39), ischemia, and infarction (43). It therefore becomes critical to characterize and gain insight on how PKC and, most importantly, its multiple isozymes regulate cardiac ion channels, in both physiological and pathological settings. In the heart, Na<sup>+</sup> channels determine excitability and conduction velocity of the action potential (1, 2, 15, 30, 42) and, thus, constitute the key elements in the genesis of arrhythmias. The ability to dissect the individual role of PKC isozymes in the regulation of Na<sup>+</sup> channels may provide functional information that will help in the design of isozyme-targeted therapeutics.

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