Phorbol ester and endothelin-1 alter functional expression of Na\(^+\)/Ca\(^{2+}\) exchange, K\(^+\), and Ca\(^{2+}\) currents in cultured neonatal rat myocytes

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Submitted 19 April 2010; accepted in final form 23 November 2010

Puglisi JL, Yuan W, Timofeyev V, Myers RE, Chiamvimonvat N, Samarel AM, Bers DM. Phorbol ester and endothelin-1 alter functional expression of Na\(^+\)/Ca\(^{2+}\) exchange, K\(^+\), and Ca\(^{2+}\) currents in cultured neonatal rat myocytes. Am J Physiol Heart Circ Physiol 300: H617–H626, 2011. First published December 3, 2010; doi:10.1152/ajpheart.00388.2010.—Endothelin-1 (ET-1) and activation of protein kinase C (PKC) have been implicated in alterations of myocyte function in cardiac hypertrophy and heart failure. Changes in cellular Ca\(^{2+}\) handling and electrophysiological properties also occur in these states and may contribute to mechanical dysfunction and arrhythmias. While ET-1 or PKC stimulation induces cellular hypertrophy in cultured neonatal rat ventricular myocytes (NRVMs), a system widely used in studies of hypertrophic signaling, there is little data about electrophysiological changes. Here we studied the effects of ET-1 (100 nM) or the PKC activator phorbol 12-myristate 13-acetate (PMA, 1 μM) on ionic currents in NRVMs. The acute effects of PMA or ET-1 (≤30 min) were small or insignificant. However, PMA or ET-1 exposure for 48–72 h increased cell capacitance by 100 or 25%, respectively, indicating cellular hypertrophy. ET-1 also slightly increased Ca\(^{2+}\) current density (T and L type). Na\(^+\)/Ca\(^{2+}\) exchange current was increased by chronic pretreatment with either PMA or ET-1. In contrast, transient outward and delayed rectifier K\(^+\) currents were strongly downregulated by PMA or ET-1 pretreatment. Inward rectifier K\(^+\) current tended toward a decrease at larger negative potential, but time-independent outward K\(^+\) current was unaltered by either treatment. The enhanced inward and reduced outward currents also result in action potential prolongation after PMA or ET-1 pretreatment. We conclude that chronic PMA or ET-1 exposure in cultured NRVMs causes altered functional expression of cardiac ion currents, which mimic electrophysiological changes seen in whole animal and human hypertrophy and heart failure.

neonatal cardiomyocytes; phorbol 12-myristate 13-acetate; endothelin-1; hypertrophy; heart failure; neonatal rat ventricular myocytes

ENDOTHELIN-1 (ET-1) and protein kinase C (PKC) activation have been implicated in altered cell signaling and gene expression in cardiac hypertrophy and heart failure (2, 10, 11, 43, 45, 56). Neonatal rat ventricular myocytes (NRVMs) in primary culture have been used extensively as a model system to explore the molecular and cellular events responsible for hormonally induced cellular hypertrophy and contractile protein gene expression. In addition, many of the features of pressure overload-induced myocyte hypertrophy observed in vivo can be simulated using these cultured cells. For instance, the exposure of NRVMs to various neurohumoral agents (e.g., adrenergic agonists, ET-1, or angiotensin II) produces myocyte hypertrophy and changes in gene expression characteristic of hypertrophic myocardium in vivo (22, 31, 44, 59, 62). Several of these growth-promoting stimuli lead to the activation of one or more of the isoenzymes of PKC (19, 20, 33, 43, 52, 70). The direct activation of Ca\(^{2+}\)-dependent novel PKC isoenzymes by phorbol 12-myristate 13-acetate (PMA) in NRVMs also mimics aspects of the hypertrophic response to pressure overload in vivo. For example, PMA induces the expression of immediate early genes (15) and secondary response genes such as atrial natriuretic factor (58), β-myosin heavy chain (24, 54), and α-skeletal actin (29). PMA exposure also increases the overall cell protein expression (65) yet decreases the expression level and function of the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase 2a (SERCA2a) in NRVMs (8, 48, 50). The latter may be related to the downregulation of SERCA2a seen in many models of hypertrophy and heart failure (1, 51), contributing directly to mechanical cardiac dysfunction. Ca\(^{2+}\)-dependent PKC isoenzymes (PKC-α, -β, and -γ) do not appear necessary for the induction of cardiac hypertrophy in response to pressure overload in vivo, but the PKC-α isoenzyme may negatively regulate sarcoplasmic reticulum Ca\(^{2+}\) load and contractility, thereby affecting cardiac performance during the transition to heart failure (32). These data indicate that the PKC regulation of SERCA2 and Ca\(^{2+}\) handling is important for hypertrophy and heart failure in vivo, yet the effects of PKC activation on Ca\(^{2+}\) currents have not been examined in the NRVM model.

ET-1 expression is increased in animal models of cardiac hypertrophy, working in part through the activation of PKC (27, 45). ET-1 also potently stimulates NRVM hypertrophy (9, 61) and is partly dependent on the activation of a novel PKC-ε (21). Chronic ET-1 stimulation produces increased cell size and protein synthesis, increased transcription of myosin light chain-2, and atrial natriuretic factor, as well as enhanced sarcomeric assembly (14, 16, 36). Therefore, ET-1 and PKC activation are likely to be critical modulators of protein expression and phenotype in cardiac hypertrophy and heart failure.

Cardiac hypertrophy and failure are also characterized by alterations in electrophysiological properties, notably decreased transient outward K\(^+\) current (I\(_{\text{to}}\)), reduced inward rectifier K\(^+\) current (I\(_{\text{K1}}\)), modestly increased L-type and T-type Ca\(^{2+}\) currents (I\(_{\text{Ca,L}}\) and I\(_{\text{Ca,T}}\), respectively), and enhanced Na\(^+\)/Ca\(^{2+}\) exchange (Na\(_{\text{Ca}}\)X) (3, 6, 14, 39, 42, 46, 60, 64, 68). Indeed, these electrophysiological alterations may be important in triggered arrhythmias in heart failure. There is little information to date concerning how PKC activation alters ionic currents in NRVMs, although Gaughan et al. (17) found that

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chronic α-adrenergic activation in NRVMs altered the functional expression of Ca2+ current (ICa) and certain K+ currents. In addition, acute (<1 h) treatment of NRVMs with ET-1 was reported to activate reverse-mode NaCaX secondary to Na+/H+ exchange activation; however, the direct effects on NaCaX activity were not identified in response to ET-1 (14).

The aim of the present study is to characterize alterations in ionic current expression in NRVMs exposed to ET-1 and PMA. We measured the acute effects, which occur in 10–30 min (possibly because of the direct effects PKC-dependent phosphorylation), as well as the long-term effects induced by 48–72 h of exposure (referred to as “chronic treatment”) and then withdrawal during current measurements (which presumably reflect alterations in the functional expression of channel proteins or modulators). We measured ICa-L and ICa-T, NaCaX current (INaCaX), INa, IK1, and delayed rectifier K+ current (IKs). The acute effects were minimal, whereas chronic PMA and ET-1 exposure produced cellular hypertrophy and altered current densities in a manner consistent with reports in animal models of hypertrophy and heart failure.

METHODS

NRVM isolation and culture. All animal protocols were approved by the Animal Care and Use Committee at Loyola University Chicago and University of California Davis. Animals used in these experiments were handled in accordance with National Institutes of Health’s Guide for the Care and Use of Laboratory Animals (NIH Publication No 85-23, Revised 2010). NRVMs were isolated from the hearts of 1–3-day-old Sprague-Dawley rat pups via collagenase digestion as previously described (54). Dissociated cells were plated for 1 h in serum-free PC-1 medium (BioWhittaker, Walkersville, MD) to selectively remove nonmuscle cells. Myocytes were then plated in PC-1 medium at a density of 400 or 1,600 cells/mm² onto collagen-coated plastic 35- or 100-mm dishes or chamber slides and left undisturbed for 14–18 h. Unattached cells were removed by aspiration, and the attached cells were maintained in a solution of DMEM-medium 199 (4:1; GIBCO, Grand Island, NY) containing antibiotic/antimycotic solution. Ca2+-free and Mg2+-free Hanks’ balanced salt solution, acid soluble calf skin collagen, and antibiotic/antimycotic solution were obtained from Sigma Chemical (St. Louis, MO). PMA (1 μM) or ET-1 (100 nM) was added at this time, and for parallel control plates medium was refreshed without added PMA or ET-1. Medium was changed daily, and all electrophysiological studies were 48–72 h after the PMA/ET-1 addition time.

For studies of acute exposure to PMA or ET-1, control cells were used and recordings were made before and after 10–30 min exposure to 1 μM PMA or 100 nM ET-1. For chronic studies, the cells exposed for 48–72 h to PMA or ET-1 (or not for control) were studied after the removal of PMA or ET-1 from the medium for 1 to 2 h. This was done to minimize the potentially complicating direct acute effects of PMA or ET-1, allowing an assessment of altered current as a consequence of altered protein expression.

Electrophysiological measurements. All currents were recorded in whole cell ruptured patch voltage-clamp mode (Axopatch 200) at room temperature, except INaCaX (measured at 35°C) with pipettes of 1–4-MΩ resistance with partial series resistance compensation (71). Ca2+ channel currents (L and T type) were measured with Na+ and K+ absent. The bathing solution contained (in mM) 140 tetraethylammonium (TEA)-Cl, 6 CsCl, 2 MgCl2, 5 CaCl2, 10 HEPES, and 10 glucose (pH 7.4), adjusted with TEA-OH at room temperature. The pipette solution contained (in mM) 125 CsCl, 10 Mg2+-ATP, 20 HEPES, 10 EGTA, 0.3 GTP, and 10 TEA-Cl (pH 7.4), adjusted with CsOH at room temperature. ICa-L was measured from a holding potential (Eh) = −50 mV, with depolarizing steps of 10 up to +60 mV (200-ms duration). ICa,L was measured from Eh = −100 mV with 2 μM nifedipine to block ICa,L, with depolarizing steps of 10 up to +60 mV (200-ms duration).

INaCaX was measured as the current blocked by Ni (5 mM) under conditions where most other currents were blocked. The bath solution contained (in mM) 140 NaCl, 6 CsCl, 1 MgCl2, 5 HEPES, 10 glucose, 2 CaCl2, and 0.03 2,3-butanedione monoxime (pH 7.4) at 35°C, and the pipette solution contained (in mM) 14 NaCl, 55 Cs-methanesul-
K⁺ currents (Iₖo, Iₖs, and Iₖi) were measured using a bath solution containing (in mM) 138 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 0.3 CdCl₂, 10 HEPES, and 10 glucose (pH 7.4) with NaOH at room temperature. The pipette solution contained (in mM) 60 KCl, 70 K-aspartate, 10 NaH₂PO₄, 10 glucose, and 10 HEPES (pH 7.4) with NaOH at room temperature. For action potential (AP) recordings, patch pipettes were backfilled with a protease inhibitor cocktail (Roche). Myocytes were briefly homogenized with a tissue grinder, and protein concentrations were determined by BCA Protein Assay (ThermoScientific). Protein (30 μg/well) were run on a 4–20% gradient polyacrylamide gel at 80 V. Protein samples were transferred to nitrocellulose membranes overnight at 40 V. Membranes were blocked in 5% milk in TBS-Tween-20 for 1 h and incubated with primary antibodies overnight at 4°C with rocking. Primary antibodies for Na⁺/Ca²⁺ exchanger and GAPDH (Fitzgerald) were used at dilutions of 1:500 and 1:4,000, respectively. Horseradish peroxidase secondary antibodies (GE) were incubated at 1:4,000 for 1 h at 37°C. The membranes were washed 4 times in TBS-Tween-20, incubated with secondary antibodies at 1:4,000 for 1 h at 25°C, washed 4 times with TBS-Tween-20, and developed with 500 ml of ECL Western blotting detection reagent (GE). Primary antibodies for Na⁺/Ca²⁺ exchanger and GAPDH (Fitzgerald) were used at dilutions of 1:500 and 1:4,000, respectively. Horseradish peroxidase secondary antibodies (GE) were incubated at 1:4,000 for 1 h at room temperature. To record Iₖo, Eₒ was set at −70 mV with a 40-ms step to −60 mV, followed by depolarizing pulses from −30 to +120 mV for 300 ms (in 10-mV increments). The rapidly inactivating component was attributed to Iₖo (and was completely suppressed by 2 mM 4-aminopyridine), whereas the sustained component we refer to here is time-independent outward K⁺ current (Iₖs). To record Iₖs, Eₒ was −50 mV, followed by 10-mV depolarizing pulses from −20 to +80 mV for 3 s, returning to −25 mV for 4.5 s and finally to Eₒ. For Iₖi, Eₒ = −50 mV followed by 10 mV steps from −140 mV to 0 mV during 300 ms, returning to Eₒ afterward. Customized software was written to analyze the K⁺ currents.

Fig. 3. Chronic effects of ET-1 on IₖCa and T-type Ca²⁺ current (IₖCa,T). Representative traces from IₖCa,L (A) and IₖCa,T (B) during control and after pretreatment with 0.1 μM ET-1 for 48–72 h. C and D: current-voltage relationships for IₖCa,L and IₖCa,T, respectively, showing a significantly increased peak current at the highest values (3 preparations).

Fig. 4. Measurement of Na⁺/Ca²⁺ exchange current (IₐNa/Ca). A: voltage protocol. Starting at a holding potential of −90 mV follows a step to −45 mV to inactive Na⁺ current and then another step to 0 mV to inactive IₖCa,L. Finally, a ramp from +80 to −140 mV was used to assess the Ni-sensitive IₐNa/Ca. B: raw traces obtained during control conditions and after application of 5 mM Ni. C: current record obtained after subtraction of the 2 traces. IₖCa,L, Ca²⁺ current.
Membranes were visualized with Super Signal West Pico Chemiluminescent Substrate (ThermoScientific).

Compared values were judged different if unpaired Student’s two-sided \( t \)-test or one-way ANOVA resulted in \( P < 0.05 \). Values are expressed as means ± SE.

RESULTS

PMA and ET-1 treatment produce cellular hypertrophy. NRVMs have been used to model myocardial hypertrophy in response to neurohumoral agents, and optical methods indicated that ET-1 treatment for 24 h increases cell surface area (21). To further investigate the capacity of PKC activation and ET-1 treatment to induce hypertrophy in NRVMs, the cells were treated with PMA or ET-1 and the cell surface area was assessed by measuring cell \( C_{\text{m}} \). Figure 1 shows that 48–72 h exposure to 1 \( \mu \)M PMA or 0.1 \( \mu \)M ET-1 led to a significant increase in cell capacitance compared with control cells cultured for the same period of time. For PMA, the capac-

Fig. 5. Effect of PMA and ET-1 on \( I_{\text{Na/Ca}} \). A: acute effect of 10–30-min exposure to 1 \( \mu \)M PMA. No significant changes on \( I_{\text{Na/Ca}} \) can be noticed. B: similarly, no effects can be seen by 10–30-min exposure to 0.1 \( \mu \)M ET-1. C: PMA pretreatment for 48–72 h induces a significant increase on \( I_{\text{Na/Ca}} \). D: likewise, ET-1 pretreatment induces a significant (although smaller) increase on \( I_{\text{Na/Ca}} \). In all cases, pipette [Ca\(^{2+}\)] was 100 nM (4 preparations). Circles are control, triangles are PMA treatment, and squares are ET-1 treatment (white symbols, acute; and black symbols, chronic). * \( P < 0.05 \).

Fig. 6. Effects of PMA and ET-1 on transient outward K\(^{+}\) current (\( I_{\text{to}} \)). A: raw traces obtained after applying a voltage protocol in control conditions. Two components can be identified: a time-dependent component right after the stimulus and a steady-state component at the end of the pulse. B: raw traces obtained after pretreatment with PMA. C: transient component is severely reduced with PMA or ET-1 treatment. D: steady-state component, conversely, is unaffected by either PMA or ET-1 (3 preparations). \( I_{\text{SS}} \), time-dependent outward K\(^{+}\) current. * \( P < 0.05 \).
ittance increased from 66 ± 4 to 146 ± 8 pF (P < 0.05, n = 20 cells), and for ET-1, Cm increased from 69 ± 5 to 86 ± 5 pF (P < 0.05, n = 21 cells). This indicates that the cell surface area is increased by either treatment and is consistent with cellular hypertrophy caused by PKC activation in response to PMA exposure (59) or the hypertrophic effects of ET-1 (18).

**Effects of PMA and ET-1 on I_{Ca}.** Because Ca^{2+} currents are increased during hypertrophy and heart failure (64, 68), we examined the effects of acute versus chronic PKC activation on I_{Ca,L} and I_{Ca,T} in NRVMs. Figure 2, A and B, shows that in control cells, an incubation for 30 min with PMA or ET-1 did not alter I_{Ca,L} appreciably. While mean I_{Ca,L} was slightly decreased, this was not significant at any potential. The stimulation of PKC by phorbol esters has discordant effects on I_{Ca}. Dösemeci et al. (13) reported an increase in this current, whereas Tseng and Boyden (63) saw a decrease and Walsh and Kass (67) observed no change at all. Our experiments show that chronic PMA treatment increased I_{Ca}, but this increased current almost exactly matched the increase in cell capacitance, such that I_{Ca,L} density was not significantly changed by PMA (supplemental Fig. 1; note: all supplemental material may be found posted with the online version of this article). Therefore, I_{Ca,L} expression is not increased in response to PMA treatment in NRVMs. Figure 3 shows that a chronic pretreatment with ET-1 produced a slight increase in both I_{Ca,L} and I_{Ca,T}. Neither the reversal potential, activation voltage, nor the voltage for maximum I_{Ca} was altered by ET-1 for either channel type. The increase in I_{Ca,L} and I_{Ca,T} induced by ET-1 was only significant at potentials where the currents are near their largest values. While the blockade of I_{Ca,L} with nifedipine minimizes the potential contamination of I_{Ca,T} by I_{Ca,L} (and >95% of I_{Ca} activated from E_h = −50 was blocked), such a complication would be most apparent at positive potentials. This was not the case; moreover, the effect of ET-1 on I_{Ca,T} was significant at −30 mV where there is almost no detectable I_{Ca,L}. Thus chronic ET-1 pretreatment may slightly enhance both I_{Ca} types in these myocytes. Although the diverse effects of ET-1 on I_{Ca} have been reported (12, 28, 30), the experimental conditions of those reports were of acute treatment. The chronic effects of ET-1 reported here are more consistent with the pathophysiological condition of heart failure.

**Fig. 7.** Chronic effect of PMA and ET-1 on delayed rectifier K^{+} current (I_{Ks}). A: raw current traces obtained under control conditions. B: effect of pretreating neonatal cells for 48–72 h with 1 μM PMA. C: slowly activating component of I_{Ks} is significantly depressed by 0.1 μM ET-1. D: likewise, PMA decreased significantly I_{Ks}. E: tail currents are also reduced by ET-1. F: similar effect on tail currents is obtained by pretreatment with PMA (4 preparations). *P < 0.05.
PMA and ET-1 increase $I_{NaCa}$ expression but do not directly alter $I_{NaCa}$. We investigated the chronic versus acute effects of PKC activation on the NaCaX activity in NRVMs directly by electrophysiological recording. Figure 4A shows the protocol used to measure $I_{NaCa}$. Starting at $E_h = -90$, a step to $-45$ mV activates and inactivates Na$^+$ current. A subsequent step to 0 mV then activates and inactivates $I_{Ca}$. Finally, a ramp from $+80$ to $-140$ mV was used to assess the Ni-sensitive $I_{NaCa}$ (Fig. 4B). This protocol was repeated in the presence or absence of 5 mM Ni. Figure 4C shows a record of $I_{NaCa}$ after the subtraction of the two traces. An acute exposure of cells to either PMA (1 μM) or ET-1 (0.1 μM) for 10–30 min did not affect $I_{NaCa}$ (Fig. 5, A and B). In contrast, pretreatment (48–72-h exposure) with either PMA or ET-1 significantly increased $I_{NaCa}$ (Fig. 5, C and D). The reversal potential of $I_{NaCa}$ was not altered, assuring that the intracellular Na$^+$ and Ca$^{2+}$ concentrations dictated by the pipette solution were the same for control and experimental cells. The ratio of PMA to control or ET-1 to control for $I_{NaCa}$, indicating the fold change in NaCaX activation, was consistent at nearly all membrane potentials ($V_m$) values evaluated (2.16 ± 0.09 and 1.53 ± 0.07 for PMA (Fig. 5C) and ET-1 (Fig. 5D), respectively). However, this difference was significant only at $V_m$ values where the current was relatively large. To evaluate inward $I_{NaCa}$ after PMA pretreatment, an additional series of experiments was performed at elevated [Ca$^{2+}$]i (1 μM) to enhance $I_{NaCa}$ amplitude (supplemental Fig. 2). This procedure shifted the reversal potential to a more positive $V_m$ and confirmed the increased expression of inward $I_{NaCa}$. Furthermore, we measured the protein level expression for NaCaX and found a twofold increase for both ET-1 (2.26 ± 0.19, $P = 0.05$) and PMA (2.34 ± 0.27, $P = 0.05$) (supplemental Fig. 3). Therefore, the effects of PMA or ET-1 treatment on NRVMs mimic the protocol was repeated in the presence or absence of 5 mM Ni. Figure 4C shows a record of $I_{NaCa}$ after the subtraction of the two traces. An acute exposure of cells to either PMA (1 μM) or ET-1 (0.1 μM) for 10–30 min did not affect $I_{NaCa}$ (Fig. 5, A and B). In contrast, pretreatment (48–72-h exposure) with either PMA or ET-1 significantly increased $I_{NaCa}$ (Fig. 5, C and D). The reversal potential of $I_{NaCa}$ was not altered, assuring that the intracellular Na$^+$ and Ca$^{2+}$ concentrations dictated by the pipette solution were the same for control and experimental cells. 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Both the slowly developing outward current (Fig. 7, C and D) and tail currents at the end of the 4-s pulse (Fig. 7, E and F) were analyzed. An acute application of PMA or ET-1 did not produce substantive changes in $I_{Kr}$ (supplemental Fig. 5). In contrast, treatment for 48–72 h with PMA or ET-1 decreased $I_{Kr}$ (Figs. 7, C and D). Raw traces from PMA-treated cells are illustrated in Fig. 7B. Tail $K^+$ currents were also depressed by either PMA or ET-1 pretreatment. Figure 7, bottom, shows normalized tail currents from cells pretreated with ET-1 (Fig. 7E) and PMA (Fig. 7F). Consequently, our experiments with NRVMS suggest that there is $I_{Kr}$ functionally expressed, and chronic exposure to hypertrophic agonists re-
roduces its functional expression, consistent with prolonged APD seen in larger mammals during hypertrophy and heart failure. $I_{K1}$ is borderline reduced by PMA and ET-1 only at higher negative potentials. Inward rectifying K$^+$ current ($I_{K1}$) is diminished in adult heart failure (47) contributing to (among other mechanisms) the onset of arrhythmias. The strong $I_{K1}$ that is central in stabilizing the resting $V_m$ in ventricular myocytes was assessed in NRVMs using step pulses (duration, 300 ms) from $-140$ to $0$ mV. Raw control traces are shown in Fig. 8A. An acute application of PMA had no effect on $I_{K1}$ in NRVMs (supplemental Fig. 6). A chronic pretreatment with ET-1 (and PMA) produced a tendency for reduced NRVMs (supplemental Fig. 6). A chronic pretreatment with ET-1 (and PMA) produced a tendency for reduced $I_{K1}$ at the more negative $V_m$ (Fig. 8B), but this trend did not reach statistical significance; during the physiological voltage range ($0$ to $-100$ mV), no changes were observed.

APD is increased by PMA and ET-1. The above effects of 48–72-h PMA or ET-1 exposure to increase predominantly inward currents ($I_{Ca}$ and $I_{Na,Ca}$) and reduce outward currents ($I_{Na}$ and $I_{K}$) would be expected to prolong APD. Figure 9A shows AP traces with and without PMA and ET-1 pretreatment. Mean data (Fig. 9B) show that a significant prolongation on APD at 80% repolarization ($APD_{80}$) is observable for both treatments (control $APD_{80} = 252 \pm 11.7$, PMA $APD_{80} = 342 \pm 26.9$, and ET-1 $APD_{80} = 279 \pm 7.5$ ms; $P \leq 0.05$).

Noticeably, the resting $V_m$ did not change (control, $-64.7 \pm 2.6$; PMA, $-63.4 \pm 2.5$; and ET-1, $-61.3 \pm 1.4$ mV; not significant), which is consistent with the lack of significant changes in $I_{K1}$ within the physiological voltage range.

**DISCUSSION**

Here we characterized PMA and ET-1 effects (both acute and chronic) on the main ionic currents in NRVM APs and excitation-contraction coupling. PMA is a strong direct PKC activator, whereas ET-1 activates $G_q$-coupled receptors and activates both PKC and 1,4,5-inositol trisphosphate (IP3) production. Both are known to induce hypertrophy in NRVMs, and these cultured myocytes have been used in hundreds of studies to elucidate signaling pathways and alterations in the expression of key proteins in cardiac myocytes, typically upon chronic agonist activation. However, there has been relatively little characterization of ionic currents in NRVMs, especially in response to hypertrophic agonists. Acute exposures (30 min) sufficient to activate both PKC and IP3 production did not significantly alter $C_m$ or ion channel activity, suggesting that the acute regulation of these currents by PKC or IP3-dependent pathways is minimal. On the other hand, a 48–72-h exposure to PMA and ET-1 and washout before measurement resulted in substantial cellular hypertrophy and changes in the current density of many ionic currents. Because the acute effects were minimal, we infer that these chronic changes in current density are the result of the altered expression levels of either the channel proteins themselves or modulators (disproportionate to the degree of cellular hypertrophy). The cellular hypertrophy measured here as cell capacitance agrees with previous reports of the hypertrophic effects of PKC activators and is consistent with a PKC-dependent increase in cell surface area observed optically and by protein expression studies in response to ET-1 treatment of NRVMs (50).

$I_{Ca,L}$ density was not significantly altered by chronic PMA treatment, but it should be appreciated that the cell surface area was increased by $\sim 100\%$ (Fig. 1). This means that there was an upregulation of $I_{Ca,L}$ functional expression that kept pace almost perfectly with the cellular hypertrophy. In contrast, ET-1 pretreatment produced a modest increase in both $I_{Ca,L}$ and $I_{Ca,T}$. It is possible that the exaggerated functional upregulation of $I_{Ca,L}$ and $I_{Ca,T}$ is not PKC dependent (since PMA is such a strong PKC activator). ET-1 also causes IP3 production, which could induce Ca$^{2+}$-dependent changes in transcription. Indeed, IP3 and Ca$^{2+}$-calmodulin-dependent signaling can lead to an altered transcriptional regulation via calcineurin-NFAT and CaMKII-HDAC pathways (34, 69). In adult hearts, hypertrophy and heart failure have been associated with the enhanced density of $I_{Ca,T}$ (41) and either unaltered or slightly increased $I_{Ca,L}$ (7, 38, 46, 47).

It is well established that NaCaX plays a major role in myocyte Ca$^{2+}$ efflux (4). There are reports suggesting an acute PKC-dependent regulation of NaCaX (26, 57), but we did not detect the acute effects of PMA in NRVMs. However, a chronic treatment with ET-1 or PMA increased $I_{Na,Ca}$ density.
Again, this indicates that NaCaX is upregulated above and beyond the overall cellular hypertrophy. Such increases in NaCaX density are also seen in adult myocytes from hypertrophied (37) and failing hearts (46). Furthermore, computer models show how this increased NaCaX can be arrhythmogenic (49). It is possible that ET-1/PMA and hypertrophic signaling increase NaCaX expression, which is beneficial initially but becomes maladaptive in the transition from hypertrophy to heart failure (35).

\(I_\text{to} \) was dramatically reduced by chronic, but unaltered by acute, PMA and ET-1 exposure. Note that this reduction exceeds the extent of cellular hypertrophy (Fig. 6C), suggesting that there is a net decrease in the rate of \(I_\text{to} \) functional expression, not simply a dilution of the same number of \(I_\text{to} \) channels into a bigger cell. Notably \(I_\text{SS} \) density was unaltered by chronic PMA or ET-1 exposure, consistent with the idea that \(I_\text{SS} \) expression increases on pace with cellular hypertrophy (so something other than PKC or IP3 dominates control of this constancy of current density). \(I_\text{to} \) exhibits rapid activation and inactivation and is responsible for the early repolarization phase of the cardiac AP and, in adult rat and mouse myocytes, is the predominant repolarizing current. \(I_\text{to} \) reduction is a very common finding in both ventricular hypertrophy and heart failure (3, 6, 40, 47), and reduced \(I_\text{to} \) can contribute to AP prolongation (especially in rat and mouse). Notably, the early repolarization driven by \(I_\text{to} \) can also exert critical control over myocyte Ca\(^{2+}\) transients by influencing the driving force for \(I_{\text{Ca}} \) (53).

While \(I_{\text{KS}} \) is not usually demonstrable in adult rat or mouse hearts, we observed measurable \(I_{\text{KS}} \) in NRVMs, which is reduced by chronic exposure to hypertrophic agonists. Our findings that ET-1 or PMA treatment decrease \(I_{\text{KS}} \) are consistent with the findings of Volders et al. (66) of decreased \(I_{\text{KS}} \) in dogs with hypertrophy induced by atrioventricular blockade. Reduced \(I_{\text{KS}} \) enhances APD and favors the occurrence of early afterdepolarizations. In the theoretical model of ventricular cardiac myocytes implemented by LabHEART, a 50% decrease in \(I_{\text{KS}} \) increased the AP by 12% (APD\(_{90} = 202 \text{ ms control vs. } 181-\text{ms } I_{\text{KS}} \text{ reduced}) (49). Thus our results with NRVMs concur with theoretical and in vivo animal models of hypertrophy.

Notably, the net effects of PMA and ET-1 were to increase the currents that are predominantly inward currents during the AP (\(I_{\text{Ca}} \) and NaCaX) and also decrease repolarizing K\(^{+}\) currents (\(I_{\text{to}} \) and \(I_{\text{KS}} \)). These are qualitatively like many reports with respect to these currents in adult hypertrophy and heart failure and would all tend to prolong APD (also typical in adult hypertrophy and heart failure). Indeed, we measured AP prolongation in myocytes exposed to PMA or ET-1 for 48–72 h. By and large, PMA and ET-1 produced similar effects with the exception of \(I_{\text{Ca,L}} \), which would be consistent with PKC activation as the most likely candidate in driving most of the altered functional expression. Our results provide a useful survey of overall acute and chronic effects of ET-1 and PMA on NRVM currents, and this should be a valuable complement to the extensive biochemical and morphological work in this cellular model. However, this is only a starting point for more detailed mechanistic studies of each current to better understand the details of the signaling pathways, transcriptional regulation, and specific channel subunits involved in these changes.

NRVMs are a powerful cellular model that has been a workhorse for studies of cardiac cell signaling involved in hypertrophic remodeling of the heart. However, little was known about the fundamental changes in ion currents known to be associated with adult hypertrophy and heart failure in the setting of hypertrophic stimuli in NRVMs. We found little acute effect of PMA or ET-1 exposure on \(I_{\text{Ca}} \), NaCaX, or K\(^{+}\) currents in NRVMs. In contrast, chronic exposure to ET-1 (and for the most part PMA) produces changes in current levels that are comparable (at least in direction) with numerous reports from adult hypertrophy and heart failure models. Namely, there was a modest enhancement of \(I_{\text{Ca,L}} \) and \(I_{\text{Ca,T}} \), a greater increase in NaCaX, and a reduction in several K\(^{+}\) currents (\(I_{\text{to}} \) and \(I_{\text{KS}} \)) with a matching increase in APD. While this system and our data here are not a substitute for adult myocyte studies of hypertrophy, this characterization provides a valuable electrophysiological context for signaling studies in NRVMs.

**ACKNOWLEDGMENTS**

We thank Dr. Robert Rigor for critical reading of the manuscript and Dr. Kenneth Ginsburg for valuable suggestions.

**GRANTS**

This work was supported by National Heart, Lung, and Blood Institute Grants R37-HL-30077 and P01-HL-80101.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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