Nesfatin-1 Suppresses Cardiac L-type Ca\(^{2+}\) Channels Through Melanocortin Type 4 Receptor and the Novel Protein Kinase C Theta Isoform Pathway

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Key Words
Nesfatin-1 • L-type Ca\(^{2+}\) channels • Protein kinase C • Cardiomyocytes

Abstract
Background/Aims: Nesfatin-1 (NF-1), an anorexic nucleobindin-2 (NUCB2)-derived hypothalamic peptide, acts as a peripheral cardiac modulator and it can induce negative inotropic effects. However, the mechanisms underlying these effects in cardiomyocytes remain unclear. Methods: Using patch clamp, protein kinase assays, and western blot analysis, we studied the effect of NF-1 on L-type Ca\(^{2+}\) currents (\(I_{\text{Ca,L}}\)) and to explore the regulatory mechanisms of this effect in adult ventricular myocytes. Results: NF-1 reversibly decreased \(I_{\text{Ca,L}}\) in a dose-dependent manner. This effect was mediated by melanocortin 4 receptor (MC4-R) and was associated with a hyperpolarizing shift in the voltage-dependence of inactivation. Dialysis of cells with GDP-\(\beta\)-S or anti-\(G_{\beta}\) antibody as well as pertussis toxin pretreatment abolished the inhibitory effects of NF-1 on \(I_{\text{Ca,L}}\). Protein kinase C (PKC) antagonists abolished NF-1-induced responses, whereas inhibition of PKA activity or intracellular application of the fast Ca\(^{2+}\)-chelator BAPTA elicited no such effects. Application of NF-1 increased membrane abundance of PKC theta isoform (PKC\(_{\theta}\)), and PKC\(_{\theta}\) inhibition abolished the decrease in \(I_{\text{Ca,L}}\) induced by NF-1. Conclusion: These data suggest that NF-1 suppresses L-type Ca\(^{2+}\) channels via the MC4-R that couples sequentially to the \(\beta y\) subunits of \(G_{\beta\gamma}\)-protein and the novel PKC\(_{\theta}\) isoform in adult ventricular myocytes.

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Introduction

Nesfatin-1, an 82-amino-terminal fragment derived from the protein nucleobindin-2 (NUCB2), was first identified in hypothalamic regions and later in peripheral tissues [1]. NF-1 exerts a large array of behavioral effects and acts as an integral regulator of energy balance, circadian feeding rhythm, and related endocrine functions [2]. It also circulates in the bloodstream, with the plasma levels depending on nutritional state and other metabolic circumstances [3]. NF-1 reduces food and water intake and body weight gain in rodents [4], increases spontaneous physical activity and whole-body fat oxidation, and raises body temperature. In the cardiovascular system, central NF-1 activates the nervous circuits that are responsible for hypertension, an effect that is presumed to also occur via melanocortin-3/4 receptors [5]. Endogenously expressed NF-1 was recently identified in the heart of mammals [6, 7], suggesting that NF-1 may be an endogenous modulator of cardiac performance. Indeed, NF-1 has been shown to induce negative inotropism and lusitropism in vitro [8]. In rats, NF-1 attenuates cardiac performance as indicated by decreases in left ventricular maximum dP/dT (an index of myocardial contractility) and left ventricular fractional shortening in perfused hearts [6, 8]. However, the mechanisms underlying these effects of NF-1 in cardiomyocytes remain unknown.

L-type voltage-gated Ca\(^{2+}\) channels (VGCC) are voltage-dependent channels that open in response to membrane depolarization, permitting entry of Ca\(^{2+}\) into the cell [9, 10]. The depolarizing current through L-type VGCC contributes to the plateau phase of the cardiac action potential as well as to pacemaker activity in nodal cells [11]. The influx of Ca\(^{2+}\) subsequently triggers the release of intracellular Ca\(^{2+}\) stores from the sarcoplasmic reticulum, and the ensuing intracellular Ca\(^{2+}\) transient results in the activation of the myofilaments, allowing cell contraction [11-13]. Additionally, L-type VGCC can affect other cellular processes modulated by intracellular Ca\(^{2+}\), including gene expression and excitation-secretion coupling [9, 14-18]. Therefore, alterations in density or function of L-type VGCC have been implicated in a variety of cardiovascular diseases, including atrial fibrillation, heart failure, and ischemic heart disease [9, 19, 20]. Altering the properties of the VGCC could have detrimental effects on cardiac electrical and contractile functions [21]. Importantly, these channels are modulated by a variety of hormones, neurotransmitters, and cytokines, operating via G-protein coupled receptors and second messengers [22], and thereby profoundly affecting the functions of target tissue.

Nonetheless, there is little information available on the underlying mechanism of the effect of NF-1 on cardiac L-type Ca\(^{2+}\) channels. Therefore, the present study was undertaken to elucidate the signaling pathways involved in L-type Ca\(^{2+}\) channel modulation by NF-1 in adult rat ventricular myocytes.

Materials and Methods

Chemicals and drugs

All chemicals were obtained from Sigma-Aldrich (MO, USA), unless otherwise stated. The PKC\(_\text{\theta}\) inhibitory peptide (PKC\(_\text{\theta}\)-IP) was purchased from Santa Cruz Biotechnology (TX, USA). The selective PKC\(_\varepsilon\)-selective inhibitor peptide εV1-2 (amino acids EAVSLKPT) was obtained from Merck Millipore (Hessen, Germany). The PKC\(_\varepsilon\)-selective inhibitor peptide (6V1-1, amino acids SFNSYELGSL) was synthesized by GenScript Corporation (NJ, USA). Stock solutions of nesfatin-1 (NF-1), GDP-β-S, pertussis toxin (PTX), cholera toxin (CTX), anantin, and BAPTA were prepared in distilled deionized water. Stock solutions of calphostin C, KT-5720, KT-5823, forskolin, rottlerin, chelerythrine chloride, and Gö6976 were prepared in dimethyl sulfoxide (DMSO). The concentration of DMSO in the bath solution is expected to be less than 0.01%. Preliminary experiments showed that the presence of equivalent amounts of DMSO in the bath solution in the absence of any drug did not modify the basal cardiac I\(_{\text{Ca,L}}\).

Isolation of ventricular myocytes

All experiments were performed in accordance with the animal care guidelines of the local institutional ethical committee, and this study conforms to the Guide for the Care and Use of Laboratory Animals published
by the US National Institute of Health (NIH). Ventricular myocytes were isolated from the hearts of adult male Wistar rats (250-300 g of weight), as described previously [23]. Rats were heparinized (4 IU/g ip) and anesthetized with pentobarbital sodium (50 mg/kg). The heart was dissected out and transferred to ice-cold Tyrode’s solution. The aorta was cannulated, and the heart was mounted on a Langendorff perfusion apparatus and perfused at 36-37 °C with a standard calcium-free Tyrode solution and supplemented with 0.2 mM EGTA (3 min) and then with the same Tyrode solution containing 251 IU/ml of collagenase type II (Worthington, NJ, USA) and 0.1 mM CaCl₂. The left ventricles were dissected out, cut into small pieces, and gently shaken for 3 min in a standard Tyrode solution containing 0.1 mM of CaCl₂ to disperse the isolated cells. The resulting cell suspensions were filtered through a 250-μm nylon mesh, centrifuged for 3 min, and resuspended in the Tyrode’s solution containing 0.5 mM CaCl₂. Cells with a rod shape and clear cross striation were used for experiments.

**Patch-clamp experiments**

Experiments were carried out at room temperature (24-26 °C) on rat ventricular myocytes using a MultiClamp 700B amplifier (Molecular Devices, CA, USA). Conventional whole-cell patch-clamp recordings were performed to measure L-type Ca²⁺ currents (I_{Ca,L}). Cells were placed in a recording dish and perfused with a bath solution containing (in mM): tetraethylammonium chloride (TEA-Cl) 140, CaCl₂ 2, MgCl₂ 0.5, glucose 5.5, CsCl 5, HEPES 10 (pH 7.35 with CsOH). Recording pipettes (World Precision Instruments, FL, USA) had 2-3 MΩ resistance when filled with internal solution containing (in mM): CsCl 100, tetraethylammonium 20, EGTA 5, HEPES 10, MgATP 5, Na₂GTP 0.4, and Na₂-creatine phosphate 5 (pH adjusted to 7.2 with CsOH). The data were acquired and analyzed using the pCLAMP 10.3 of programs (Molecular Devices, CA, USA). Current traces were corrected using on-line P/6 trace subtraction. Series resistance was compensated to the maximal extent (at least 75%). In experiments in which cells were dialyzed with compounds or peptides, current measurements were started at least 5 min after breaking the patch.

**Western blotting**

Western blot analysis was performed as previously described [17, 24, 25]. In brief, cells were homogenized by sonication at 4 °C in homogenization buffer containing protease inhibitors. The protein content was determined according to the Bradford method. Equivalent amounts of proteins (20 μg) were separated by 7.5% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, PA, USA). The membranes were blocked with 5% skim milk in phosphate-buffered saline (PBS) for 1 hour at room temperature. Blotted proteins were probed with the following primary antibodies: antibody against MC3-R (rabbit, 1: 500, Cell Signaling Technology, MA, USA), antibody against MC4-R (rabbit, 1: 100, Cell Signaling Technology, MA, USA), antibody against PKCδ (rabbit, 1: 500, Santa Cruz Biotechnology, TX, USA), antibody against PKCε (rabbit, 1: 700, Santa Cruz Biotechnology, TX, USA), antibody against PKCθ (rabbit, 1: 500, Santa Cruz Biotechnology, TX, USA) and antibody against PKCη (rabbit, 1: 1000, Santa Cruz Biotechnology, TX, USA). An antibody against GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (1: 3000, Cell Signaling Technology, MA, USA) was used as an internal control for the concentration of proteins loaded. A secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (immunoglobulin G) (GE Healthcare, PA, USA) was used in combination with the enhanced chemiluminescence detection system (SuperSignal West Pico Chemiluminiscent Substrate, Pierce, Life Technologies, CA, USA) to visualize the primary antibodies.

**Subcellular fractionation**

Cells were treated with 0.01 μM NF-1 for 15 min. The cells were extracted with lysis buffer and sonicated on ice (3 × 10 s cycles). The mixture was centrifuged for 10 min at 800 g and the supernatant was saved and centrifuged at 100,000 g for 45 min. The supernatant was taken as the cytosol fraction. The pellet was resuspended in lysis buffer plus 1% Triton X-100 and centrifuged as before. The supernatant was collected as the membrane fraction. Protein concentrations of the fractions were determined using Bio-Rad assay (Bio-Rad Laboratories, CA, USA). Cells incubated with medium only were used as controls.

**Determination of PKA activity**

PKA activity was determined by enzyme-linked immuno sorbent assay (ELISA, Promega, WI, USA), according to the manufacturer’s instructions and as described previously [18, 24, 26]. In brief, cells were
pretreated with either vehicle or KT-5720 for 30 min, followed by treatment with either vehicle or forskolin for 10 min. The cells were washed with ice-cold PBS and incubated with 200 μl lysis buffer. The cell lysates were centrifuged for 15 min, and aliquots of the supernatants containing 0.2 μg of protein were assayed for PKA activity. The activity is expressed as RLU⁻¹ (relative light units) per amount of protein.

**Assay for PKA activity**

PKA activity was measured as described previously [25, 27-29]. The cells were pretreated with either vehicle or chelerythrine chloride for 30 min, followed by treatment with NF-1 for 15 min. The cells were homogenized in PKC extraction buffer. The lysates were centrifuged at 14,000 g for 15 min and the supernatants were collected for detecting the activity of kinase. Detection of PKC activity was performed with PepTag Non-Radioactive PKC assay kit (Promega, WI, USA), and the activity of PKC was calculated according to manufacturer’s instructions.

**Statistical analysis**

All data are presented as mean ± S.E.M. When the comparison was made between only two treatment groups, unpaired Student’s t-tests were used. One-way analysis of variance (ANOVA) with a post hoc Bonferroni’s multiple-comparison test was used when three or more groups were compared. Probability values (p) < 0.05 were accepted as statistically significant. GraphPad Prism 5.0 (GraphPad Software, CA, USA) was used to analyze the data. Concentration-response curves were fitted by the sigmoidal Hill equation 1/I/I

\[ \frac{I}{I_{max}} = \frac{1}{1 + 10^{(\log IC_{50} - \log X)}} \]

where \( X \) is the decadic logarithm of the concentration used, IC

\[ IC_{50} \]

is the concentration at which the half-maximum effect occurs, and \( n_h \) is the Hill coefficient. Activation data were fitted by the following modified Boltzmann equation: \( G/I_{max} = 1/(1 + \exp[-(V_{half} - V_{m})/k]) \), where \( G_{max} \) is the fitted maximal conductance, \( V_{half} \) is the membrane potential for half-activation, and \( k \) is the slope factor. Steady-state inactivation of \( I_{Ca,L} \) was fitted with the following negative Boltzmann equation: \( I/I_{max} = 1/(1 + \exp[-(V_{half} - V_{m})/k]) \), where \( I_{max} \) is maximal current, \( V_{half} \) is the membrane potential for half-inactivation, and \( k \) is the slope factor.

**Results**

**Nesfatin-1 (NF-1) decreases L-type Ca²⁺ currents (I_{Ca,L})**

\( I_{Ca,L} \) were recorded in isolated rat ventricular myocytes using whole-cell voltage clamping. Currents were elicited by stepping voltage from a holding potential of -55 to 0 mV for 200 ms at 10-second intervals. These currents were blocked by nicardipine (5 μM), a specific L-type Ca²⁺ channel blocker (decrease 97.6 ± 3.9 %, \( n = 15 \)). Addition of NF-1 (0.01 μM) caused a significant decrease in peak \( I_{Ca,L} \) to 31.7% of the basal level (\( n = 11 \)) (Fig. 1A and 1B). Following removal of NF-1, the amplitude of \( I_{Ca,L} \) partially recovered within 5 min (Fig. 1B and 1C). NF-1 affected \( I_{Ca,L} \) in a concentration-dependent manner (Fig. 1D). The relationship between the concentration of \( I_{Ca,L} \) and the degree of inhibition was described by a sigmoidal Hill equation, using which the concentration of \( I_{Ca,L} \) that produced half-maximal inhibition (IC

\[ IC_{50} \]

was found to be 17.9 nM with the apparent Hill coefficient of 0.87 (Fig. 1D).

**NF-1 induces hyperpolarizing shifts in the steady-state inactivation curve**

Next, we determined whether the biophysical properties of \( I_{Ca,L} \) were affected by NF-1. A current–voltage (I–V) curve was evoked by a series of depolarizing pulses ranging from a holding potential of -55 mV to test potentials between -50 and +50 mV (10 mV intervals). At an NF-1 concentration of 0.01 μM, the I–V curve significantly up-shifted (\( n = 15 \), Fig. 2A), and at 0 mV, the current density decreased from 6.3 ± 0.5 pA/pF to 4.5 ± 0.8 pA/pF (\( n = 15 \)). We also looked for NF-1-induced changes in the voltage dependences of activation and inactivation. We observed a significant hyperpolarizing shift of ~8.5 mV in the steady-state inactivation profile of \( I_{Ca,L} \) (\( V_{50} \) from -24.2 ± 1.9 mV to -32.7 ± 2.7 mV; \( n = 12 \)) (Fig. 2D), whereas we found no significant changes in the activation properties (\( V_{50} \) from -11.3 ± 0.8 mV to -12.1 ± 0.7 mV; \( n = 17 \)) (Fig. 2C). These results together suggest that the NF-1-induced decrease in \( I_{Ca,L} \) may be due to the retention of an increased proportion of channels in the inactivated state in adult rat ventricular myocytes.
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The melanocortin 4 receptor (MC4-R) mediates NF-1–induced ICa,L decrease

Previous studies have shown that the corticotropin-releasing factor type 2 receptor (CRF-R2), the natriuretic peptide receptor A (NRP-A) and the MC4-R subtypes are involved in NF-1 mediated effects [5,8,30]. We tested the extent to which CRF-R2, NRP-A and MC4-R might participate in ICa,L responses to NF-1. Both CRF-R2 and NRP-A are endogenously expressed in adult rat cardiomyocytes [8,31]. To determine whether these receptors are involved in the NF-1-induced ICa,L decrease, we examined the effects of NF-1 on ICa,L in the presence of astressin-2B, a selective CRF-R2 antagonist, or anantin, an specific NPR-A antagonist. After pretreatment of cells with either astressin-2B (1 µM) or anantin (0.1 µM), NF-1 could still mediated a substantial decrease of ICa,L (decrease 32.8 ± 2.5 % for astressin-2B, n = 9; decrease 31.7 ± 3.2 % for anantin, n = 11; Fig. 3A-C). However, pretreatment of the cells with HS024 (2 nM), a specific MC4-R antagonist, completely abolished the NF-1-induced decrease in ICa,L (decrease 1.3 ± 0.7 %, n = 11) (Fig. 3D and E). These results suggest that NF-1 might act as a ligand of MC4-R to decrease ICa,L. As a complementary test of our hypothesis,
we further examined the protein expression profiles of MC4-R in rat ventricular myocytes by immunoblotting with subunit-specific antibodies. Immunoblot analysis revealed that MC4-R is endogenously expressed in adult rat ventricular myocytes, whereas MC3-R was not detected (Fig. 3F).

The MC4-R-mediated inhibition of $I_{\text{Ca,L}}$ requires the $\beta\gamma$ subunits of $G_{\text{i/o}}$-protein

Having identified the cell surface receptor through which NF-1 decreases $I_{\text{Ca,L}}$, we sought to elucidate the intracellular signaling events linking MC4-R activation with $I_{\text{Ca,L}}$ inhibition. MC4-R is a G-protein coupled receptor that acts through heterotrimeric G-proteins [32]. To determine whether G-proteins are involved in NF-1-mediated inhibition of $I_{\text{Ca,L}}$, we dialyzed the non-hydrolysable GDP analogue guanosine-5'-O-(2-thiodiphosphate) (GDP-β-S) into cells. GDP-β-S (1 mM) completely abolished the NF-1-induced decrease in $I_{\text{Ca,L}}$ (decrease 0.9 ± 0.7%, $n$ = 7, Fig. 4A and D), indicating that G-protein activation is required for NF-1 action.

NF-1 increases intracellular cAMP level via $G_{\text{aS}}$ in cultured neuronal cells isolated from rat hypothalamus [33]. To determine whether the MC4-R-mediated response also occurred via $G_{\text{aS}}$, we examined the effects of NF-1 on $I_{\text{Ca,L}}$ in the presence of cholera toxin (CTX), which inactivates $G_{\text{aS}}$ by ADP-ribosylation. After pretreatment of cells with CTX (0.5 µg/ml for 16 h), NF-1 still robustly inhibited $I_{\text{Ca,L}}$ (decrease 32.2 ± 3.9%, $n$ = 11, Fig. 4B and D). Conversely, inhibition of $G_{\text{aS}}$ by pretreating ventricular myocytes with pertussis toxin (PTX, 0.2 µg/ml for 16 h), which catalyzes the ADP ribosylation of the α-subunit of $G_{\text{i/o}}$ proteins, completely abolished the inhibitory effect of NF-1 (decrease 3.9 ± 0.5%, $n$ = 8, Fig. 4C and D). The NF-1-induced decrease of $I_{\text{Ca,L}}$ was sensitive to PTX but not to CTX, indicating that $G_{\text{aS}}$ but not $G_{\text{aS}}$ is involved. Next, to determine the role of the $\beta\gamma$ subunits of $G_{\text{aS}}$-protein ($G_{\beta\gamma}$) in NF-1 action, we tested a blocking antibody to the $G_{\beta\gamma}$ subunit [34]. Dialysis of cells with anti-$G_{\beta\gamma}$ antibody
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(10 μg/ml) completely abolished the inhibitory effects of NF-1 (decrease 0.7 ± 0.8%, n = 9, Fig. 4E and F), while dialysis with the boiled antibody did not (decrease 29.8 ± 3.7 %, n = 7, Fig. 4F).

**NF-1-mediated inhibition of ICa,L involves the novel protein kinase C (PKC)**

Next, we investigated the mechanism underlying the NF-1-mediated inhibition of ICa,L. We found no evidence for the involvement of protein kinase A (PKA) or protein kinase G (PKG) in this process, as pretreatment of cells with the PKA inhibitor KT-5720 (1 μM) or the PKG blocker KT-5823 (1 μM) did not affect NF-1-mediated inhibition of ICa,L (decrease 31.9 ± 2.7 % for KT-5720, n = 11; decrease 30.6 ± 3.9 % for KT-5823, n = 9; Fig. 5A-C). However, pretreatment of ventricular myocytes with 1 μM KT-5720 abolished the ability of forskolin (20 μM)-induced increase in PKA activity (Fig. 5D), indicating that KT-5720 was effective. PKC activation has been shown to modulate L-type Ca2+ channels [9, 35], therefore we investigated the role of PKC in NF-1-induced inhibition of ICa,L. NF-1 at 0.01 μM significantly increased PKC activity (~2.1 fold) in adult ventricular myocytes (Fig. 5E). This response was abolished in cells pretreated with chelerythrine chloride (1 μM) (Fig. 5E), an inhibitor of both novel and classical PKC isoforms. Pretreatment of cells with chelerythrine chloride (1 μM) also abolished the ability of NF-1 to inhibit ICa,L (decrease 2.2 ± 2.5%, n = 9, Fig. 5F and H). Similar results were obtained when we used the classical and novel PKC antagonist calphostin C (50 nM) (decrease 2.5 ± 0.3%, n = 7, Fig. 5H). Interestingly, Gö6976 (1 μM), which blocks only classical PKC isoforms, did not block the effects of PKC on NF-1-induced decrease in ICa,L (decrease 32.2 ± 1.9%, n = 12, Fig. 5G and H).
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The novel PKC theta isoform (PKC\( \theta \)) is involved in NF-1-mediated \( I_{\text{Ca,L}} \) decrease

Unlike the classical PKC isoforms, which need both cytoplasmic \( \text{Ca}^{2+} \) and diacylglycerol (DAG) for activation [36, 37], intracellular dialysis of the fast \( \text{Ca}^{2+} \) chelator BAPTA (20 mM) did not affect the NF-1-mediated decrease in \( I_{\text{Ca,L}} \) (decrease 31.9 ± 4.2%, \( n = 12 \); Fig. 6A and B), supporting the involvement of novel PKC isoforms in this process. Next, we aimed to identify the PKC isoform involved in the NF-1-mediated inhibition of \( I_{\text{Ca,L}} \). For this, we first investigated the protein expression profiles of novel PKC isoforms in adult rat ventricular myocytes. Western blot analysis revealed that PKC delta (PKC\( \delta \)), epsilon (PKC\( \varepsilon \)) and theta (PKC\( \theta \)) are expressed in rat ventricular myocytes; PKC eta (PKC\( \eta \)) could not be detected (Fig. 6C). Rat brain expresses all four novel PKC isoforms and was used as a positive control for detecting each PKC isoform (Fig. 6C). Dialysis of cells with inhibitory peptides of PKC\( \delta \) (\( \delta V1-1 \)), PKC\( \varepsilon \) (\( \varepsilon V1-2 \)), and PKC\( \theta \) (PKC\( \theta \)-IP) revealed that PKC\( \theta \)-IP (10 \( \mu \)M), but not its denatured form, could nearly completely abolish the inhibitory effects of NF-1 on \( I_{\text{Ca,L}} \) (decrease 3.8 ± 0.5% for PKC\( \theta \)-IP, \( n = 10 \), Fig. 6F and H; decrease 30.5 ± 2.3% for its denatured form, \( n = 10 \), Fig. 6G and H). After intracellular application of \( \delta V1-1 \) (10 \( \mu \)M), rottlerin (5 \( \mu \)M), or \( \varepsilon V1-2 \) (200 \( \mu \)M), the inhibitory effects of NF-1 on \( I_{\text{Ca,L}} \) remained unchanged (decrease 28.7 ± 5.2% for \( \delta V1-1 \), \( n = 9 \), Fig. 6D and H; decrease 26.1 ± 3.9% for rottlerin, \( n = 9 \), Fig. 6H; decrease 29.2 ± 2.6% for \( \varepsilon V1-2 \), \( n = 11 \), Fig. 6E and H). The cellular activation of PKC is intimately...
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related to its translocation and binding to the plasma membrane and to further support our results, we examined the translocation of PKC\( \theta \) from the cytosolic to the membrane fractions in ventricular myocytes after treatment with NF-1. Western blot analysis revealed an increase in membrane-bound PKC\( \theta \) and a decrease in the cytosolic fraction when the cells were treated with NF-1 (0.01 \( \mu \)M) (Fig. 6I).

**Discussion**

The present study identified a novel functional role of NF-1 and the findings provide evidence of new mechanisms for modulating L-type \( \text{Ca}^{2+} \) channels in adult ventricular myocytes. We propose that the marked decrease in \( I_{\text{Ca,L}} \) induced by NF-1 is mediated through MC4-R, which is coupled to the \( G_{\beta\gamma} \) subunits of \( G_{i/o} \), leading to the subsequent activation of the novel PKC\( \theta \) isoform (see Fig. 7).

In addition to its anorexigenic effects, recent studies have shown that the activity of NF-1 in the autonomic nervous system involves the central melanocortin system and corticotropin-releasing factor (CRF) receptors [3, 5, 8, 30]. For example, pretreatment of rats with either the CRF-R2 antagonist astressin-2B [30] or the MC4-R antagonist SHU9119 [5] abolished both the anorexigenic action and the negative inotropic action of NF-1. In the present study, we found that MC4-R, but not CRF-R2, was involved in NF-1-induced cardiac
Fig. 7. Proposed signaling pathway involved in the effect of NF-1 on L-type Ca\(^{2+}\) channels. NF-1 binds to MC4-R, which is coupled to the G-protein G\(_{\beta\gamma}\), causing it to release the G\(_{\beta}\) subunits, which causes an increase in PKC activity, and subsequent inhibition of the L-type Ca\(^{2+}\) channels. Neither PKA or classic PKC isoforms are necessary for MC4-R-mediated decrease in I\(_{\text{Ca,L}}\). Whether PKC\(_{\beta}\) directly phosphorylates L-type Ca\(^{2+}\) channels or acts on an intermediate protein remains unclear.

I\(_{\text{Ca,L}}\) decrease, as pretreatment of cells with HS024 completely abolished responses to NF-1, whereas pretreatment with the CRF antagonist astressin-2B did not. MC4-R activation occurs through both PTX-sensitive and PTX-insensitive G-protein coupled signaling [8, 33, 38]. Interestingly, in adult ventricular myocytes, we found that G\(_{\beta}\) is involved in NF-1-mediated I\(_{\text{Ca,L}}\) inhibition because (1) the response was blocked by the nonselective G-protein inhibitor GDP-\(\beta\)-S; (2) pretreatment with PTX, but not with CTX, abolished the NF-1 response; and (3) dialysis of cells with G\(_{\beta}\) antibody, but not the boiled, denatured antibody, completely blocked the inhibitory effects of NF-1. Together these data support the involvement of the G\(_{\beta\gamma}\) subunit of G-protein in rat ventricular myocytes.

There are several examples of the modulation of L-type Ca\(^{2+}\) channels by PKA activation. In GT1-7 hypothalamic neurons, for example, the inhibition of L-type Ca\(^{2+}\) channels by the CB1 cannabinoid receptor was prevented by application of PKA inhibitors [39]. Similarly, inhibition of the Cav1.2 channel by activation of the integrin receptor was abolished by PKA antagonists [40]. In contrast, PKA-induced L-type Ca\(^{2+}\) channel stimulation has also been described [9]. In rat ventricular myocytes, PKA enhanced the activation of L-type Ca\(^{2+}\) channels by the \(\beta2\) adrenergic receptor [41]. Moreover, cAMP/PKA-dependent stimulation of L-type Ca\(^{2+}\) channels by activation of the histamine receptors (H\(_1\)) receptors [42] has been described in normal mammalian heart. In this study, we found that NF-1-mediated inhibition of I\(_{\text{Ca,L}}\) was not affected by the PKA antagonist KT-5720, which likely acts effectively by blocking forskolin-induced increase of cAMP concentration. This suggests that some mechanism other than the cAMP/PKA pathway is involved in the NF-1-induced I\(_{\text{Ca,L}}\) response.

In this study, pretreatment of cells with classic and novel PKC antagonists completely abolished the NF-1-mediated inhibition of I\(_{\text{Ca,L}}\) in adult ventricular myocytes, whereas the classic PKC inhibitor G66976 exerted no such effects, suggesting that a novel PKC isoform was involved in MC4-R-mediated I\(_{\text{Ca,L}}\) response. These results are consistent with previous findings. In hippocampal neurons, inhibition of L-type Ca\(^{2+}\) channels by the neuropeptide U type 1 receptor was prevented by application of PKC inhibitors [43]. Similarly, in neostriatal neurons, PKC was involved in muscarinic M1 receptor-induced inhibition of L-type Ca\(^{2+}\) channels [44]. In contrast, PKC has been implicated in the enhancement of I\(_{\text{Ca,L}}\) by hydrogen peroxide in arterial smooth muscle cells [45]. Similarly, in rat neonatal hippocampal neurons, enhancement of I\(_{\text{Ca,L}}\) by GABAB receptors via the PKC-dependent pathway has been reported [46]. Additionally, a biphasic effect on I\(_{\text{Ca,L}}\) by the PKC activator phorbol esters in studies of neonatal rat ventricular myocytes and adult canine ventricular myocytes was shown with an initial stimulation followed by an inhibition [9, 42]. Moreover, in heterologous systems, expression of human long-NT \(\alpha1C\) in Xenopus oocytes caused the enhancement of a I\(_{\text{Ca,L}}\) by a PKC activator, while the expression of short-NT \(\alpha1C\) was inhibited [11, 47]. These results suggest that the regulatory effects of PKC on L-type Ca\(^{2+}\) channels can be variable in different tissues expressing different L-type Ca\(^{2+}\) channel subtypes (Cav1.2 or Cav1.3). An alternative hypothesis is that PKC phosphorylates an intermediate protein that, in turn, down-regulates the L-type Ca\(^{2+}\) channels. In addition, the expression and/or activation of endogenous PKC isoforms is tissue/cell specific; therefore, the modulatory effects of PKC on I\(_{\text{Ca,L}}\) may be closely related to the isoform(s) of PKC that are activated by a particular signaling pathway or
chemical [48, 49]. First, expression of PKC isoforms in the heart is developmentally regulated, species-dependent, and disease-specific [42]. Activation of PKC involves translocation of the enzyme to specific targets, and different isoforms show different patterns of subcellular localization upon activation, corresponding to the subcellular localization of the specific substrates [27, 48]. In the present study, we suggest that the PKC\(\theta\) isoform is involved in the NF-1-induced response, as NF-1-mediated inhibition of \(I_{\text{Ca,L}}\) was blocked by intracellular application of PKC\(\theta\) whereas application of the denatured form of PKC\(\theta\) had no effect. Indeed, PKC\(\theta\) translocates to cross-striated regions in ventricular myocytes, near T-tubules where L-type Ca\(^{2+}\) channels are localized [50]. Second, PKC modulation of L-type Ca\(^{2+}\) channels may involve PKC-interacting proteins, which is the case for the Cav2.2 N-type channel [51]. PKC-interacting proteins confer specificity on individual PKC isoforms by regulating their activity and cellular location, endowing isoforms with the ability to mediate specific cellular functions [52, 53]. Finally, cell-specific splice variants of Cav1.2 \(\alpha1C\) [54] or \(\beta\) subunits [55] might modulate the pharmacological properties of L-type Ca\(^{2+}\) channels in different ways. In addition, previous studies have shown that \(G_{\beta\gamma}\) subunits can activate PKC via PLC\(\beta\) [56]. In this study, an intermediate protein could also be involved in the observed \(G_{\beta\gamma}\)-mediated PKC\(\theta\) activation. For example, studies by others suggest that the immediate downstream mediator of \(G_{\beta\gamma}\) is PI3K [57-59], which is upstream of the novel PKC [60]. Although further investigation is required to more fully understand the underlying mechanisms, we cannot exclude the possibility that an intermediate effector, such as PI3K or PLC\(\beta\), may be involved in the \(G_{\beta\gamma}\)-induced PKC\(\theta\) activation.

In summary, our data provide evidence of new mechanisms involved in the modulation of L-type Ca\(^{2+}\) channels by NF-1 in adult rat ventricular myocytes. We propose that the significant decrease in \(I_{\text{Ca,L}}\) induced by NF-1 is mediated through MC4-R and involves the \(G_{\beta\gamma}\) subunit of \(G_{i/o}\)-protein and the downstream PKC\(\theta\) pathway. We found no evidence of a role for PKA or PKG signaling. This novel mechanism may contribute directly to the influence of NF-1 on cardiac performance, including ventricular contraction, in the mammalian cardiovascular system.

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Disclosure Statement

The authors declare that they have no conflict of interests.

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