Original Paper

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- β -S or anti- G_β 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 $\beta\gamma$ subunits of $\text{G}_{i/o}$ -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 $_{\theta}$ inhibitory peptide (PKC $_{\theta}$ -IP) was purchased from Santa Cruz Biotechnology (TX, USA). The selective PKC ϵ inhibitory peptide ϵ V1-2 (amino acids EAVSLKPT) was obtained from Merck Millipore (Hessen, Germany). The PKC $_{\delta}$ -selective inhibitor peptide (δ V1-1, 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 G66976 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_{CaL} .

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 CaCl_2 . 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 CaCl_2 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 CaCl_2 . 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 Ca^{2+} currents (I_{CaL}). Cells were placed in a recording dish and perfused with a bath solution containing (in mM): tetraethylammonium chloride (TEA-Cl) 140, CaCl_2 2, MgCl_2 0.5, glucose 5.5, CsCl 5, HEPES 10 (pH 7.35 with CsOH). Recording pipettes (World Precision Instruments, FL, USA) had 2–3 M Ω resistance when filled with internal solution containing (in mM): CsCl 100, tetraethylammonium 20, EGTA 5, HEPES 10, MgATP 5, Na_2GTP 0.4, and Na_2 -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: 1000, Cell Signaling Technology, MA, USA), antibody against PKC_δ (rabbit, 1: 500, Santa Cruz Biotechnology, TX, USA), antibody against PKC_ϵ (rabbit, 1: 700, Santa Cruz Biotechnology, TX, USA), antibody against PKC_θ (rabbit, 1: 500, Santa Cruz Biotechnology, TX, USA) and antibody against PKC_η (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 Chemiluminescent 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 $\times$ 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 μ l lysis buffer. The cell lysates were centrifuged for 15 min, and aliquots of the supernatants containing 0.2 μ g of protein were assayed for PKA activity. The activity is expressed as RLU⁻¹ (relative light units) per amount of protein.

Assay for PKC activity

PKC 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 supernatant 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 $\pm$ 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 $I/I_{\text{control}} = 1/(1 + 10^{(\log[IC_{50}] - X)n_h})$, where *X* is the decadic logarithm of the concentration used, 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: $G/G_{\text{max}} = 1/\{1 + \exp[-(V_{\text{half}} - V_m)/k]\}$, 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_{CaL} was fitted with the following negative Boltzmann equation: $I/I_{\text{max}} = 1/\{1 + \exp[-(V_{\text{half}} - V_m)/k]\}$, 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 Ca^{2+} currents (I_{CaL})

I_{CaL} 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 nifedipine (5 μ M), a specific L-type Ca^{2+} channel blocker (decrease $97.6 \pm 3.9\%$, *n* = 5). Addition of NF-1 (0.01 μ M) caused a significant decrease in peak I_{CaL} to 31.7% of the basal level (*n* = 11) (Fig. 1A and 1B). Following removal of NF-1, the amplitude of I_{CaL} partially recovered within 5 min (Fig. 1B and 1C). NF-1 affected I_{CaL} in a concentration-dependent manner (Fig. 1D). The relationship between the concentration of I_{CaL} and the degree of inhibition was described by a sigmoidal Hill equation, using which the concentration of I_{CaL} that produced half-maximal inhibition (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_{CaL} 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 μ M, 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, Fig. 2B). 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_{CaL} (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_{CaL} may be due to the retention of an increased proportion of channels in the inactivated state in adult rat ventricular myocytes.

Fig. 1. Nesfatin-1 (NF-1) decreases $I_{Ca,L}$ in adult rat ventricular myocytes. **A**, Time course of changes in $I_{Ca,L}$ amplitude mediated by 0.01 μ M NF-1. Numbers indicate the points used for sample traces. **B** and **C**, Representative traces (**B**) and summary data (**C**) of $I_{Ca,L}$ under control conditions, during exposure to 0.01 μ M NF-1, and during washout of NF-1 ($n = 11$). $I_{Ca,L}$ were elicited by a 200-ms long depolarizing step pulse from the holding potential of -55 mV to 0 mV. This protocol was used to calculate the peak decrease of $I_{Ca,L}$ induced by NF-1 in all the figures. **D**, Dose-response curve of NF-1-induced inhibition of $I_{Ca,L}$. The dotted line represents the best fit to the sigmoidal Hill equation. Numbers of cells tested at each concentration of NF-1 are indicated in brackets. * $p < 0.05$ vs. control.

Fig. 2. Effect of NF-1 on the biophysical properties of L-type Ca^{2+} channels. **A**, Current-voltage (I - V) plot showing the density of $I_{Ca,L}$ vs. test voltage, recorded before and after treatment with 0.01 μ M NF-1. **B**, Summary of results showing the density of $I_{Ca,L}$ at 0 mV as indicated in panel A ($n = 15$). **C** and **D**, NF-1 at 0.01 μ M did not significantly alter the steady-state activation curve of L-type Ca^{2+} channels (**C**, $n = 17$), but caused a leftward shift of the steady-state inactivation curve (**D**, $n = 12$). To determine the steady-state inactivation, $I_{Ca,L}$ was evoked by 80 ms test pulse to 0 mV after the 2 s conditioning pulses ranging from -80 to +30 mV with 10 mV increments. To determine the voltage-dependent activation, tail currents were elicited by repolarization to -55 mV after 60 ms test pulses from -50 to +40 mV in increments of 10 mV. * $p < 0.05$ vs. control.

The melanocortin 4 receptor (MC4-R) mediates NF-1-induced $I_{Ca,L}$ 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 $I_{Ca,L}$ 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 $I_{Ca,L}$ decrease, we examined the effects of NF-1 on $I_{Ca,L}$ in the presence of astressin-2B, a selective CRF-R2 antagonist, or anantin, an specific NRP-A antagonist. After pretreatment of cells with either astressin-2B (1 μ M) or anantin (0.1 μ M), NF-1 could still mediated a substantial decrease of the $I_{Ca,L}$ (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 $I_{Ca,L}$ (decrease 1.3 ± 0.7 %, $n = 10$) (Fig. 3D and E). These results suggest that NF-1 might act as a ligand of MC4-R to decrease $I_{Ca,L}$. As a complementary test of our hypothesis,

Fig. 3. MC4-R was involved in NF-1-induced response. A and B, Time course showing the effect of 0.01 μ M NF-1 on $I_{Ca,L}$ in the presence of astressin-2B (1 μ M for 30 min, A) or anantin (0.1 μ M for 30 min, B). C, Summary of results showing the effect of NF-1 at 0.01 μ M on $I_{Ca,L}$ in the presence of astressin-2B ($n = 9$) or anantin ($n = 11$). D and E, Time course (D) and summary of results (E) showing the pretreatment of ventricular myocytes with HS024 completely abolished the decrease in $I_{Ca,L}$ induced by 0.01 μ M NF-1 ($n = 10$). F, Western blot analysis showing the endogenous expression of MC4-R protein in adult rat ventricular myocytes; MC3-R was not detected. The blots shown are representative of three independent experiments. GAPDH was used as an internal control for the concentration of the proteins loaded. *** $p < 0.001$ vs. control.

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 $I_{Ca,L}$ requires the $\beta\gamma$ subunits of $G_{i/o}$ -protein

Having identified the cell surface receptor through which NF-1 decreases $I_{Ca,L}$, we sought to elucidate the intracellular signaling events linking MC4-R activation with $I_{Ca,L}$ 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 $I_{Ca,L}$, 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 $I_{Ca,L}$ (decrease $0.9 \pm 0.7\%$, $n = 7$, Fig. 4A and D), indicating that G-protein activation is required for NF-1 action. NF-1 increases intracellular cAMP level via $G\alpha_s$ in cultured neuronal cells isolated from rat hypothalamus [33]. To determine whether the MC4-R-mediated response also occurred via G_s , we examined the effects of NF-1 on $I_{Ca,L}$ in the presence of cholera toxin (CTX), which inactivates G_s by ADP-ribosylation. After pretreatment of cells with CTX (0.5 μ g/ml for 16 h), NF-1 still robustly inhibited $I_{Ca,L}$ (decrease $32.2 \pm 3.9\%$, $n = 11$, Fig. 4B and D). Conversely, inhibition of $G_{i/o}$ by pretreating ventricular myocytes with pertussis toxin (PTX, 0.2 μ g/ml for 16 h), which catalyzes the ADP ribosylation of the α -subunit of $G_{i/o}$ proteins, completely abolished the inhibitory effect of NF-1 (decrease $3.9 \pm 0.5\%$, $n = 8$, Fig. 4C and D). The NF-1-induced decrease of $I_{Ca,L}$ was sensitive to PTX but not to CTX, indicating that $G_{i/o}$, but not G_s , is involved. Next, to determine the role of the $\beta\gamma$ subunits of $G_{i/o}$ -protein ($G_{\beta\gamma}$) in NF-1 action, we tested a blocking antibody to the G_{β} subunit [34]. Dialysis of cells with anti- G_{β} antibody

Fig. 4. The $G_{\beta\gamma}$ subunit of $G_{i/o}$ -protein is involved in the NF-1-induced response. A through C, Time course showing the effect of NF-1 at 0.01 μ M on $I_{Ca,L}$ in the presence of GDP- β -S (1 mM, intracellularly applied, $n = 7$), cholera toxin (CTX, 0.5 μ g/ml pretreated for 16 h, $n = 11$), or pertussis toxin (PTX, 0.2 μ g/ml pretreated for 16 h, $n = 8$), respectively. D, Summary of results showing the effects of 0.01 μ M NF-1 on $I_{Ca,L}$ as indicated in panel A through C. E and F, Time course (E) and summary of results (F) showing the effect of NF-1 at 0.01 μ M on $I_{Ca,L}$ in the presence of anti- G_{β} (10 μ g/ml, intracellularly applied, $n = 9$) and denatured anti- G_{β} (10 μ g/ml, intracellularly applied, $n = 7$). *** $p < 0.001$ vs. control.

(10 μ g/ml) completely abolished the inhibitory effects of NF-1 (decrease $0.7 \pm 0.8\%$, $n = 9$, Fig. 4E and F), while dialysis with the boiled antibody did not (decrease $29.8 \pm 3.7\%$, $n = 7$, Fig. 4F).

NF-1-mediated inhibition of $I_{Ca,L}$ involves the novel protein kinase C (PKC)

Next, we investigated the mechanism underlying the NF-1-mediated inhibition of $I_{Ca,L}$. 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 μ M) or the PKG blocker KT-5823 (1 μ M) did not affect NF-1-mediated inhibition of $I_{Ca,L}$ (decrease $31.9 \pm 2.7\%$ for KT-5720, $n = 11$; decrease $30.6 \pm 3.9\%$ for KT-5823, $n = 9$; Fig. 5A-C). However, pretreatment of ventricular myocytes with 1 μ M KT-5720 abolished the ability of forskolin (20 μ M)-induced increase in PKA activity (Fig. 5D), indicating that KT-5720 was effective. PKC activation has been shown to modulate L-type Ca^{2+} channels [9, 35], therefore we investigated the role of PKC in NF-1-induced inhibition of $I_{Ca,L}$. NF-1 at 0.01 μ M significantly increased PKC activity (~ 2.1 fold) in adult ventricular myocytes (Fig. 5E). This response was abolished in cells pretreated with chelerythrine chloride (1 μ M) (Fig. 5E), an inhibitor of both novel and classical PKC isoforms. Pretreatment of cells with chelerythrine chloride (1 μ M) also abolished the ability of NF-1 to inhibit $I_{Ca,L}$ (decrease $2.2 \pm 2.5\%$, $n = 9$, Fig. 5F and H). Similar results were obtained when we used the classical and novel PKC antagonist calphostin C (50 nM) (decrease $2.5 \pm 0.3\%$, $n = 7$, Fig. 5H). Interestingly, Gö6976 (1 μ M), which blocks only classical PKC isoforms, did not block the effects of PKC on NF-1-induced decrease in $I_{Ca,L}$ (decrease $32.2 \pm 1.9\%$, $n = 12$, Fig. 5G and H).

Fig. 5. NF-1 induced I_{CaL} decrease requires novel PKC. A and B, Time course showing the inhibitory effects of 0.01 μ M NF-1 on I_{CaL} in the presence of KT-5720 (1 μ M for 30 min) or KT-5823 (1 μ M for 30 min), respectively. C, Summary of results showing that treatment of ventricular myocytes with either KT-5720 ($n = 11$) or KT-5823 ($n = 9$) did not affect the 0.01 μ M NF-1-induced I_{CaL} decrease. D, Bar graph showing the activity of PKA in control cells, ventricular myocytes treated with forskolin 20 μ M, and ventricular myocytes pre-incubated with KT-5720 (1 μ M for 30 min) and then treated with forskolin (20 μ M). Data are obtained from three separate experiments. E, NF-1 at 0.01 μ M induced a significant increase in PKC activity in adult rat ventricular myocytes. This response was blocked by the pretreatment of cells with chelerythrine chloride (1 μ M). Data are obtained from four separate experiments. F and G, Time course showing the inhibitory effects of 0.01 μ M NF-1 on I_{CaL} in the presence of chelerythrine chloride (Chel, 1 μ M for 30 min) or Gö6976 (1 μ M for 30 min), respectively. H, Summary of results showing that treatment of ventricular myocytes with either Chel ($n = 9$) or calphostin C (50 nM for 30 min, $n = 7$), but not Gö6976 ($n = 12$), completely abolished the 0.01 μ M NF-1-induced I_{CaL} decrease. # $p < 0.05$ vs. vehicle, ## $p < 0.01$ vs. vehicle, @ $p < 0.05$ vs. forskolin without KT5720, \$ $p < 0.05$ vs. NF-1 without Chel, *** $p < 0.001$ vs. control.

The novel PKC theta isoform (PKC_{θ}) is involved in NF-1-mediated I_{CaL} decrease

Unlike the classical PKC isoforms, which need both cytoplasmic Ca^{2+} and diacylglycerol (DAG) for activation, novel PKC isoforms do not require cytoplasmic Ca^{2+} for activation [36, 37]. Indeed, intracellular dialysis of the fast Ca^{2+} chelator BAPTA (20 mM) did not affect the NF-1-mediated decrease in I_{CaL} (decrease $31.9 \pm 4.2\%$, $n = 12$; Fig. 6A and B), further 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_{CaL} . 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_{δ}), epsilon (PKC_{ϵ}) and theta (PKC_{θ}), are expressed in rat ventricular myocytes; PKC eta (PKC_{η}) 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_{δ} (δ V1-1), PKC_{ϵ} (ϵ V1-2), and PKC_{θ} (PKC_{θ} -IP) revealed that PKC_{θ} -IP (10 μ M), but not its denatured form, could nearly completely abolish the inhibitory effects of NF-1 on I_{CaL} (decrease $3.8 \pm 0.5\%$ for PKC_{θ} -IP, $n = 10$, Fig. 6F and H; decrease $30.5 \pm 2.3\%$ for its denatured form, $n = 10$, Fig. 6G and H). After intracellular application of δ V1-1 (10 μ M), rottlerin (5 μ M), or ϵ V1-2 (200 μ M), the inhibitory effects of NF-1 on I_{CaL} remained unchanged (decrease $28.7 \pm 5.2\%$ for δ V1-1, $n = 9$, Fig. 6D and H; decrease $26.1 \pm 3.9\%$ for rottlerin, $n = 9$, Fig. 6H; decrease $29.2 \pm 2.6\%$ for ϵ V1-2, $n = 11$, Fig. 6E and H). The cellular activation of PKC is intimately

Fig. 6. NF-1 inhibits L-type Ca^{2+} channels through PKC θ pathway. A and B, Time course (A) and summary of results (B) showing the effect of NF-1 (0.01 μ M) on the I_{CaL} in the presence of BAPTA (20 mM, intracellularly applied, $n = 12$). C, Western blots were incubated with rabbit polyclonal antibodies (against PKC δ , PKC ϵ , PKC θ , and PKC η) to determine the protein levels of novel PKC isoforms in adult rat ventricular myocytes. GAPDH was used as an internal control for the concentration of the proteins loaded. Rat brain proteins were used as positive controls. The blots shown are representative of three independent experiments. D through G, Time course of 0.01 μ M NF-1 on I_{CaL} in the presence of δ V1-1 (10 μ M, intracellularly applied, D), ϵ V1-2 (200 μ M, intracellularly applied, E), PKC θ -IP (10 μ M, intracellularly applied, F), and denatured PKC θ -IP (10 μ M, intracellularly applied, G), respectively. H, Summary of results showing dialysis of cells with PKC θ -IP ($n = 10$), but not δ V1-1 ($n = 9$), ϵ V1-2 ($n = 11$), rottlerin (5 μ M for 30 min, $n = 9$), and denatured PKC θ -IP ($n = 10$), resulted in a near-complete elimination of the inhibitory effects of NF-1 on I_{CaL} . I, Effects of NF-1 on membrane PKC θ expression. Cells were treated with vehicle or NF-1 (0.01 μ M) for 15 min. Data are means $\pm$ SEM of triplicate samples and are representative of three independent experiments. ** $p < 0.01$ vs. control, *** $p < 0.001$ vs. control.

related to its translocation and binding to the plasma membrane and to further support our results, we examined the translocation of PKC θ 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 θ and a decrease in the cytosolic fraction when the cells were treated with NF-1 (0.01 μ 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 Ca^{2+} channels in adult ventricular myocytes. We propose that the marked decrease in I_{CaL} 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 θ 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 $G_{i/o}$ causing it to release the $G_{\beta\gamma}$ subunits, which causes an increase in PKC_{θ} 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_{CaL} . Whether PKC_{θ} directly phosphorylates L-type Ca^{2+} channels or acts on an intermediate protein remains unclear.

I_{CaL} 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 $G_{\beta\gamma}$ is involved in NF-1-mediated I_{CaL} inhibition because (1) the response was blocked by the nonselective G-protein inhibitor GDP- β -S; (2) pretreatment with PTX, but not with CTX, abolished the NF-1 response; and (3) dialysis of cells with G_{β} 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 $G_{i/o}$ -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 β_2 adrenergic receptor [41]. Moreover, cAMP/PKA-dependent stimulation of L-type Ca^{2+} channels by activation of the histamine receptors (H_2) receptors [42] has been described in normal mammalian heart. In this study, we found that NF-1 mediated inhibition of I_{CaL} 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_{CaL} response.

In this study, pretreatment of cells with classic and novel PKC antagonists completely abolished the NF-1-mediated inhibition of I_{CaL} in adult ventricular myocytes, whereas the classic PKC inhibitor Gö6976 exerted no such effects, suggesting that a novel PKC isoform was involved in MC4-R-mediated I_{CaL} response. These results are consistent with previous findings. In hippocampal neurons, inhibition of L-type Ca^{2+} channels by the neuromedin 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_{CaL} by hydrogen peroxide in arterial smooth muscle cells [45]. Similarly, in rat neonatal hippocampal neurons, enhancement of I_{CaL} by GABAB receptors via the PKC-dependent pathway has been reported [46]. Additionally, a biphasic effect on I_{CaL} 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 $\alpha 1C$ in *Xenopus* oocytes caused the enhancement of a I_{CaL} by a PKC activator, while the expression of short-NT $\alpha 1C$ 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_{CaL} 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 isozymes 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_{CaL} 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 $\alpha 1C$ [54] or β 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_{CaL} 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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