

Dendroaspis natriuretic peptide regulates the cardiac L-type Ca^{2+} channel activity by the phosphorylation of α_{1c} proteins

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Abbreviations: ANP, atrial natriuretic peptide; APD, action potential duration; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; d-DNP, C-terminal-deleted DNP; DNP, dendroaspis natriuretic peptide; IC_{50} , half maximal inhibitory concentration; $I_{\text{Ca,L}}$, L-type Ca^{2+} current; PKG, protein kinase G

Abstract

Dendroaspis natriuretic peptide (DNP), a new member of the natriuretic peptide family, is structurally similar to atrial, brain, and C-type natriuretic peptides. However, the effects of DNP on the cardiac function are poorly defined. In the present study, we examined the effect of DNP on the cardiac L-type Ca^{2+} channels in rabbit ventricular myocytes. DNP inhibited the L-type Ca^{2+} current ($I_{\text{Ca,L}}$) in a concentration dependent manner with a IC_{50} of 25.5 nM, which was blocked by an inhibitor of protein kinase G (PKG), KT5823 (1 μM). DNP did not affect the voltage dependence of activation and inactivation of $I_{\text{Ca,L}}$. The α_{1c} subunit of cardiac L-type Ca^{2+} channel proteins was phosphorylated by the treatment of DNP (1 μM), which was completely blocked by KT5823 (1 μM). Finally, DNP also caused the shortening of action potential duration in rabbit ventricular tissue by $22.3 \pm 4.2\%$ of the control ($n = 6$), which was completely blocked by KT5823 (1 μM). These results clearly

indicate that DNP inhibits the L-type Ca^{2+} channel activity by phosphorylating the Ca^{2+} channel protein via PKG activation.

Keywords: calcium channels, L-type; cyclic GMP-dependent protein kinases; dendroaspis natriuretic peptide; myocytes, cardiac; rabbits

Introduction

A new member of the natriuretic peptide family, dendroaspis natriuretic peptide (DNP), has been reported. DNP, originally isolated from the venom of the *Dendroaspis angusticeps* or green Mamba snake, is a peptide of 38 amino acids containing a 17 amino acid disulfide ring structure with a 15-residue C terminal extension (Schweitz *et al.*, 1992). This peptide shares structural similarity to atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) of myocardial cell origin, and C-type natriuretic peptide (CNP) of endothelial cell origin (Schweitz *et al.*, 1992). Reports indicate that DNP possesses biologic properties that are similar to the other natriuretic peptides (Schweitz *et al.*, 1992; Lisy *et al.*, 1999, 2001; Lee and Kim, 2002). Synthetic DNP potentially relaxes rodent aorta and isolated canine coronary arteries with potencies comparable to that of ANP (Schweitz *et al.*, 1992; Wennberg and Burnett, 1997; Collins *et al.*, 2000). Furthermore, DNP displaces ANP binding from the natriuretic peptide receptors (Schweitz *et al.*, 1992; Collins *et al.*, 2000). Interestingly, it has been reported that DNP immunoreactivity is present in human plasma and atrial myocardium (Schirger *et al.*, 1999; Lisy *et al.*, 2001) and is elevated in the plasma of humans with congestive heart failure (CHF) (Schirger *et al.*, 1999). More recently, Lisy *et al.* (2001) proposed that DNP has potential as a new intravenous agent for the treatment of decompensated CHF. Although synthetic DNP suggests its potential use in the cardiovascular disease states such as CHF, the effects of DNP on the cardiac function are poorly defined.

The voltage- and time-dependent slow (L-type) Ca^{2+} channels in the cardiac myocytes play a pivotal role in regulating the cardiac function, and can be

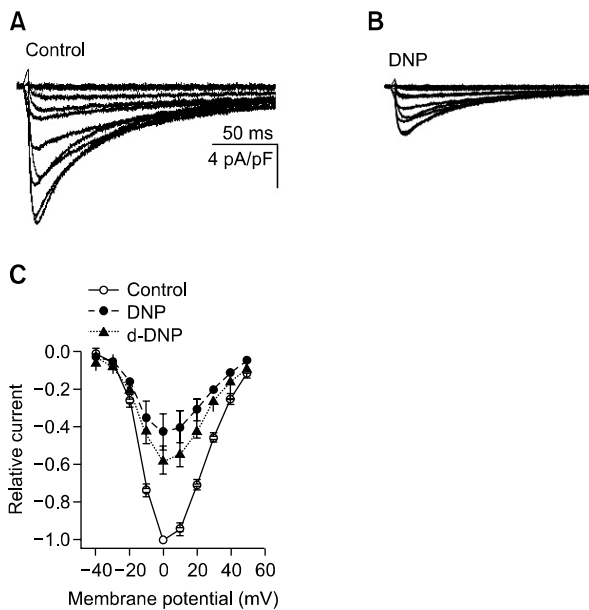


Figure 1. The inhibitory effect of DNP and d-DNP on the voltage-gated L-type Ca^{2+} currents, $I_{\text{Ca,L}}$ in adult rabbit ventricular myocytes. (A, B) Superimposed current traces were elicited by 300 ms pulses with 10 mV increments from a holding potential of -80 mV in the range of -40 to +50 mV in the absence (A) or presence (B) of DNP (0.1 μM). The interval between voltage steps was 5 seconds. (C) The current-voltage relationships of peak current in the absence or presence of DNP or C-terminal-deleted DNP (d-DNP; Des-Arg³⁰, Des-Pro³¹-DNP) (0.1 μM). The current values were normalized to the maximum level observed under control conditions for each experiment.

controlled by extrinsic factors such as hormones and by intrinsic factors such as cellular pH or ATP levels. L-type Ca^{2+} channel activity is increased by activation of cyclic AMP-dependent protein kinase (PKA), whereas is decreased by activation of cGMP-dependent protein kinase (PKG) (Sumii and Sperelakis, 1995). Additionally, DNP augments the formation of 3', 5' cyclic guanosine monophosphate (cGMP), a second messenger for the other natriuretic peptides, in aortic endothelial and smooth muscle cells (Schweitz *et al.*, 1992). Considering these, DNP could regulate the cardiac function by inhibiting the L-type Ca^{2+} channels through PKG activation. However, no investigations have been made into a casual relation between DNP, cyclic GMP or PKG, and the cardiac Ca^{2+} channels with cardiac action potential duration.

In the present study, we examined the effect of DNP on the cardiac L-type Ca^{2+} channels in rabbit ventricular myocytes. The present study clearly showed that DNP inhibits the cardiac L-type Ca^{2+} channel activity through PKG activation.

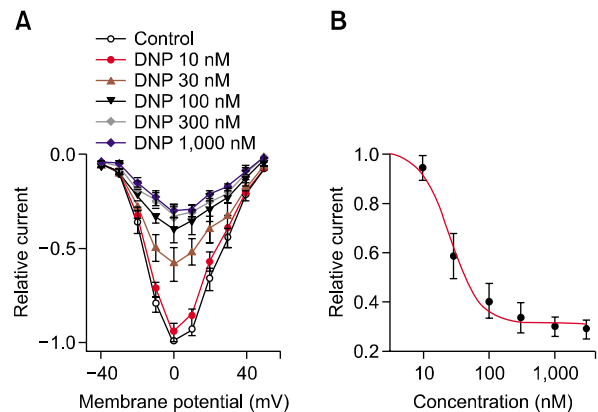


Figure 2. Concentration-dependence of DNP-induced $I_{\text{Ca,L}}$ inhibition in adult rabbit ventricular myocytes. (A) The current-voltage relationships of peak current in the absence or presence of DNP (10-1000 nM). The current values were normalized to the maximum level observed under control conditions for each experiment. (B) Concentration-dependent inhibition of the $I_{\text{Ca,L}}$ current evoked by a depolarizing pulse of 0 mV. The amplitudes of peak currents were normalized to the maximum current obtained in control, and plotted against DNP concentrations. Data were fitted with a Hill equation. Each point with vertical bars represents mean \pm SEM and each data was obtained from 5 myocytes.

Results

Voltage-gated L-type Ca^{2+} currents, $I_{\text{Ca,L}}$ are suppressed by DNP

The actions of DNP on $I_{\text{Ca,L}}$ were examined in adult rabbit ventricular myocytes. $I_{\text{Ca,L}}$ were elicited by depolarizing steps of 300 ms duration from a holding potential of -80 mV. DNP at a concentration of 0.1 μM decreased $I_{\text{Ca,L}}$ density to $40.2 \pm 7.0\%$ of the control at 0 mV ($P < 0.001$, $n = 6$; Figure 1). Figure 1C shows relations between the peak current and the potential of depolarizing pulse. The potential at which $I_{\text{Ca,L}}$ was greatest was somewhat variable (either 0 or +10 mV) from myocyte to myocyte. However, DNP did not shift the potentials for the threshold (-40 mV) and the maximum peak current (0 mV). In other words, DNP did not affect the voltage dependence of activation of $I_{\text{Ca,L}}$. Interestingly, C-terminal-deleted DNP (d-DNP; Des-Arg³⁰, Des-Pro³¹-DNP, 0.1 μM) also inhibited $I_{\text{Ca,L}}$ to $53.6 \pm 7.6\%$ of the control at 0 mV ($P < 0.001$, $n = 6$; Figure 1C). These results indicate that C-terminal of DNP (Arg³⁰ and Pro³¹ residues of DNP) is not essential in inhibiting L-type Ca^{2+} channels.

Figure 2 shows the concentration dependence of DNP-induced $I_{\text{Ca,L}}$ inhibition in rabbit ventricular myocytes. DNP inhibited $I_{\text{Ca,L}}$ at a concentration starting as low as 10 nM. Increasing the concentration of DNP led to progressively greater inhibition of $I_{\text{Ca,L}}$ (Figure 2), where IC_{50} of DNP was 25.5 ± 4.6 nM ($n = 5$).

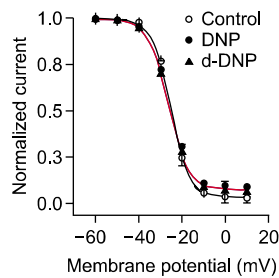


Figure 3. The effect of DNP on the inactivation curve of the voltage-gated L-type Ca^{2+} currents, $I_{\text{Ca,L}}$ in adult rabbit ventricular myocytes. The voltage protocol was elicited by 300 ms pulses with 10 mV increments from a holding potential of -80 mV in the range of -40 to +50 mV following a depolarizing pulse of 0 mV in the absence or presence of DNP (0.1 μM). The interval between voltage steps was 5 seconds. To build up an inactivation curve, we measured the peak current at the second depolarizing pulse of 0 mV. The current values were normalized to the maximum level observed under control conditions for each experiment.

To determine whether changes in the kinetics of the Ca^{2+} channels could account for the decrease of $I_{\text{Ca,L}}$, we analyzed the steady-state inactivation characteristics in the absence or presence of DNP (Figure 3). For these experiments, the following voltage protocol was applied. From a holding potential of -80 mV, 500 ms pulses with 10 mV increments in the range of -80 to +30 mV were applied, followed by a depolarizing pulse of 0 mV in the absence or presence of DNP (0.1 μM). DNP, although decreasing the peak $I_{\text{Ca,L}}$ density, had no effect on the steady state inactivation characteristics of $I_{\text{Ca,L}}$. Thus, the DNP-induced $I_{\text{Ca,L}}$ inhibition is most likely due to a decrease in open probability of the Ca^{2+} channels, rather than to a modification of their voltage dependence.

DNP is known to augment the formation of cGMP in aortic endothelial and smooth muscle cells and to be associated with marked increase of cGMP in its natriuretic and diuretic effects (Schweitz *et al.*, 1992; Collins *et al.*, 2000). Considering these results, we hypothesized that the DNP-induced $I_{\text{Ca,L}}$ inhibition could be mediated *via* PKG pathway. To confirm the involvement of PKG in the DNP-induced inhibition of $I_{\text{Ca,L}}$, we examined the effect of DNP on the $I_{\text{Ca,L}}$ in the presence of a PKG inhibitor, KT5823 (Figure 4). DNP at a concentration of 0.1 μM failed to suppress further the Ca^{2+} channel current in the rabbit ventricular myocytes pretreated with KT5823 (1 μM) for 30 min. This finding indicates that the DNP-induced inhibition of the Ca^{2+} channel current is likely to be mediated by PKG activation.

Cardiac L-type Ca^{2+} channel protein, α_{1c} is phosphorylated by DNP

Cardiac L-type Ca^{2+} channels are composed of α_{1c} ,

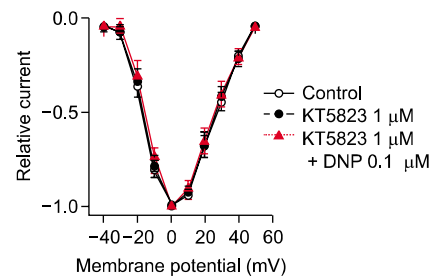


Figure 4. The influence of KT5823 (1 μM) on the inhibitory effect of DNP on the voltage-gated L-type Ca^{2+} currents, $I_{\text{Ca,L}}$ in adult rabbit ventricular myocytes. The ventricular myocytes were pretreated for 30 min with a protein kinase inhibitor KT5823 (1 μM) at room temperature. The currents were elicited by 300 ms pulses with 10 mV increments from a holding potential of -80 mV in the range of -40 to +50 mV in the absence (A) or presence (B) of DNP (0.1 μM). The interval between voltage steps was 5 seconds. The current-voltage relationships in the absence or presence of DNP (0.1 μM). The current values were normalized to the maximum level observed under control conditions for each experiment.

β_2 and $\alpha_{2\delta}$ subunits, and both the α_{1c} and β_2 have been demonstrated to be the direct targets of phosphorylation (Gao *et al.*, 1997; Puri *et al.*, 1997). In order to test whether or not the DNP-induced inhibition of the Ca^{2+} channel current was due to phosphorylation of the cardiac L-type Ca^{2+} channel proteins, we metabolically labeled rabbit ventricular myocytes with [^{32}P]orthophosphate and then incubated the cells with DNP and/or KT5823. α_{1c} subunits immunoprecipitates from the labeled cells treated with DNP (1 μM) for 10 min, contained [^{32}P] radioactivity (Figure 5), indicating that DNP can phosphorylate α_{1c} subunit of L-type Ca^{2+} channels. This DNP-induced phosphorylation of α_{1c} subunits proteins was completely blocked by KT5823 (1 μM). These α_{1c} subunit proteins had a similar molecular mass independent of drug treatments when analyzed on immunoblot using anti- α_{1c} antibody. This result indicates that DNP phosphorylates the cardiac L-type Ca^{2+} channel proteins *via* PKG activation.

Effect of DNP on the ventricular action potential

It is well known that the inhibition of cardiac L-type Ca^{2+} channels induces the shortening of cardiac action potential duration. To test whether DNP decreases the cardiac action potential, we examined the effect of DNP on the ventricular action potential in rabbit ventricular myocytes using conventional microelectrode technique (Figure 6). The resting membrane potentials of ventricular muscles was -80.5 ± 2.1 mV ($n=6$) and action potential durations (APD) at 100% repolarization was 193.9 ± 5.2 ms ($n=6$) in ventricular muscles stimulated at 2 Hz. The maximum effect of DNP on action potential

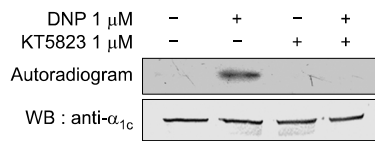


Figure 5. Phosphorylation of cardiac voltage-sensitive Ca^{2+} channel protein, α_{1c} , by protein kinase G in rabbit ventricular myocytes. Ventricular myocytes were incubated with [32 P]orthophosphate for 3h prior to treatment with DNP or KT5823. The labeled cells were treated with DNP for 10 min and/or with KT5823 for 1h. α_{1c} subunit of L-type Ca^{2+} channels was immunoprecipitated by anti- α_{1c} antibody. The immunoprecipitates were separated on SDS-polyacrylamide gels and the dried gels were exposed to XAR film (upper panel). After exposure to XAR film, the dried gel was swelled with distilled water and transferred into NC paper. The paper was blotted with anti- α_{1c} antibody (lower panel).

duration was observed about 30 min after exposure to the drug and remained relatively unchanged with further perfusion. Therefore, we exposed the tissue to the drugs for ~30 min or until the plateau effect of the drug was present. DNP (0.1 μ M) decreased the action potential duration at 90% repolarization (APD_{90}) by $22.4 \pm 4.2\%$ of APD_{90} before DNP ($n = 6$), which was completely blocked by KT5823 (1 μ M). These results suggest that DNP can cause the shortening of action potential duration through inhibition of L-type Ca^{2+} channel *via* PKG activation.

Discussion

In the present study, we have examined the effects of DNP on the cardiac L-type Ca^{2+} channels, and clarified its action mechanism at molecular level. Our results clearly showed that DNP inhibited the L-type Ca^{2+} channel activity by phosphorylating the L- Ca^{2+} channel α_{1c} protein *via* PKG activation. This is the first report showing the DNP-induced inhibition of L-type Ca^{2+} channels and its molecular mechanism.

In the present study, DNP inhibited the cardiac L-type Ca^{2+} channel activity through PKG activation in isolated rabbit ventricular myocytes. DNP is a 38-amino acid peptide that contains a 17-amino acid disulfide ring structure similar to ANP, BNP, and CNP (Schweitz *et al.*, 1992), all of which mediate biologic actions through particulate guanylyl cyclase receptors and generation of cGMP (Schweitz *et al.*, 1992). DNP substantially augments the formation of cGMP in aortic endothelial and smooth muscle cells (Schweitz *et al.*, 1992; Collins *et al.*, 2000). And, it has been proposed that cGMP play a role antagonistic to cAMP in regulating cardiac Ca^{2+} channels (Goldberg *et al.*, 1975). This is supported by several reports that the cardiac L-type Ca^{2+} channel activities were inhibited by 8-bromo-cyclic GMP, a potent activator of PKG, as well as PKG

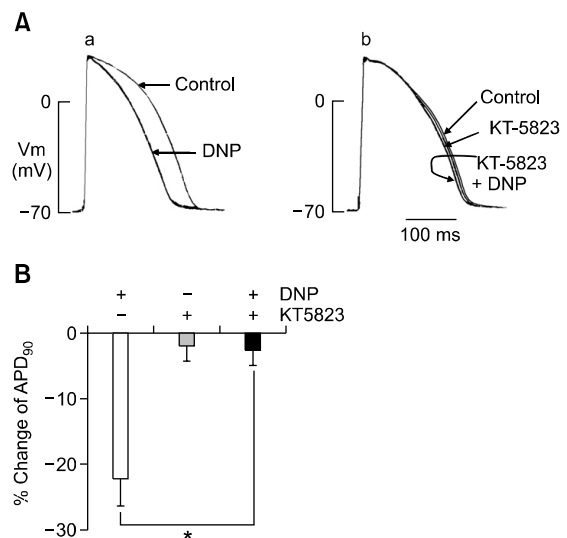


Figure 6. Effects of DNP (0.1 μ M) on action potentials in isolated rabbit ventricular muscles. (A) A typical representation of superimposed action potentials in the presence of DNP (a, $n = 6$) and in the combined presence of KT5823 (1 μ M, b). (B) Averaged effects of DNP (0.1 μ M) on APD_{90} of isolated rabbit ventricular muscles. Each column with vertical bar denotes the mean with S.E.M. from six observations. * $P < 0.05$.

(Sumii and Sperelakis, 1995). Furthermore, Tohse *et al.* (1995) found that ANP, structurally similar to DNP, inhibited the cardiac L-type Ca^{2+} channel activity through the intracellular production of cGMP and then activation of PKG. In the present study, we found that DNP inhibited the cardiac L-type Ca^{2+} channel activity through PKG activation and phosphorylated the pore-forming subunit of cardiac L-type Ca^{2+} channel protein, α_{1c} , which was completely blocked by PKG inhibitor. These results clearly indicate that DNP inhibits the cardiac L-type Ca^{2+} channel activity by phosphorylating the voltage-sensitive Ca^{2+} channel protein through PKG activation. However, further experiments are required to clarify which amino acids of cardiac Ca^{2+} channel α_{1c} should be phosphorylated by DNP to inhibit its activity.

During inhibiting the cardiac L-type Ca^{2+} channels, DNP did not affect the voltage dependence of activation and the inactivation characteristics of $I_{\text{Ca,L}}$, indicating that the DNP-induced $I_{\text{Ca,L}}$ inhibition is most likely due to a decrease in open probability of the Ca^{2+} channels, rather than to a modification of their voltage dependence. Additionally, there was no significant difference between the inhibitory effects of DNP and C-terminal-deleted DNP (Des-Arg³⁰, Des-Pro³¹-DNP) on $I_{\text{Ca,L}}$, indicating that C-terminal of DNP (Arg³⁰ and Pro³¹ residues of DNP) is not essential in inhibiting L-type Ca^{2+} channels.

Schirger *et al.* (1999) have reported that DNP-like immunoreactivity is present in human plasma and

atrial myocardium and is elevated in the plasma of patients with congestive heart failure. Additionally, Lainchbury *et al.* (2002) proposed that, although the molecular mechanism for the effects of DNP on the cardiac function is poorly defined, DNP may have a role as a cardiac unloading and natriuretic peptide in the dog. Based on our results, DNP inhibits the cardiac L-type Ca^{2+} channel, thereby regulating cardiac function including the cardiac contractility and action potential duration. This is supported by our additional findings that DNP (0.1 μM) caused shortening of the action potential duration in rabbit ventricular myocytes (Figure 6) and inhibited ventricular contractility in isolated rabbit ventricular tissues (data not shown). It is well known that the inhibition of cardiac Ca^{2+} channel results in shortening the action potential duration. Therefore, our study will be helpful when investigating the therapeutic uses or pathophysiological role of DNP in various cardiovascular diseases.

Methods

Action potential recording in rabbit ventricular tissues

All experiments were approved by the Experimental Animal Care and Ethics Committee of Chonbuk National University. Male New Zealand White rabbit (Han I, Jeonju, Korea) were housed under 12:12 light:dark cycles with free access to food and water. Male New Zealand White rabbits weighing about 1.8 to 2 kg were anesthetized by injection of pentobarbital into the marginal ear vein and were euthanized by cervical dislocation at between 10:00 and 12:00 h. Hearts were rapidly excised and transferred to a dissection bath filled with Tyrode's solution oxygenated with 97% O_2 and 3% CO_2 gas mixture. Ventricular muscles were carefully dissected free from the left ventricular wall. Each dissected tissue was mounted horizontally in a narrow channel of a tissue chamber and continuously superfused with oxygenated Tyrode's solution at 36–37°C. The size of dissected tissues ranged from 0.5 to 1 mm in width and was about 2 to 3 mm in length. The one end of each tissue was fixed by an insect pin to the bottom of the chamber coated with Sylgard. The tissue next to the insect pin was pressed against the floor by stimulating electrodes, which were used to elicit action potentials and contractions. The action potentials were elicited by stimulating the cardiac cells with square pulses (2 Hz, 1 ms duration, 20–30% above threshold voltage) by a stimulator *via* a stimulus isolation unit (WPI, Sarasota, FL). Action potentials were recorded with a 3 M KCl-filled microelectrode (10–20 megaohm) connected to an amplifier (KS-700, WPI), and were displayed on an oscilloscope (Dual beam storage 5113, Tektronix, Beaverton, OR). Tracings on the oscilloscope screen were photographed on a 35-mm film and also recorded on a chart recorder (RS 3400, Gould, Cleveland, OH). Rabbit cardiac tissue was superfused with a Tyrode's solution at a constant rate (5 ml/min). Tyrode's solution contained 137 mM NaCl, 5.4 mM KCl, 1.05 mM MgCl_2 , 0.45 mM

NaH_2PO_4 , 11.9 mM NaHCO_3 , 1.8 mM CaCl_2 and 5 mM dextrose. The solution was gassed with 97% O_2 + 3% CO_2 (pH 7.3–7.4).

Cell isolation

Single cardiac myocytes of male New Zealand White rabbits weighing 1.8 to 2 kg were prepared by enzymatic digestion as described previously (Kwak *et al.*, 1995). Rabbits were anesthetized by injection of pentobarbital into the marginal ear vein and were euthanized by cervical dislocation. After opening the chest cavity, the heart was excised and immersed in Krebs–Henseleit (KH) buffer solution (pH 7.35). Hearts were perfused retrogradely *via* the aorta in a Langendorff apparatus with KH solution for 5 min to clear visible blood. Hearts were then perfused with Ca^{2+} -free KH solution until hearts stopped beating, and then with Ca^{2+} -free KH solution containing 0.075% collagenase (CLS2, Worthington, Freehold, NJ) for 30 min. After enzymatic digestion, ventricular muscles were carefully dissected free from the left ventricular wall. The dissected tissues were placed in Ca^{2+} -free KH solution containing 1% bovine serum albumin, cut into small pieces and then mechanically dissociated into single cells. All cells used for experiments were rod-shaped with clear striations. The KH solution contained (in mM) 118 NaCl, 4.7 KCl, 1.2 MgSO_4 , 1.2 KH_2PO_4 , 10 HEPES, 25 NaHCO_3 , 10 pyruvate, 11 dextrose and 1 CaCl_2 .

Conventional whole cell patch clamp techniques

Conventional (ruptured patch) recording techniques were used to measure the voltage-gated L-type Ca^{2+} currents, $I_{\text{Ca,L}}$. The pipette solution contained (in mM) CsCl 125, tetraethylammonium chloride (TEA-Cl) 20, MgATP 5, creatine phosphate 3.6, EGTA 10, and HEPES 10 (pH 7.2) with CsOH. The bath solution contained (in mM) TEA-Cl 157, CaCl_2 1, MgCl_2 0.5, and HEPES 10 (pH 7.4) with CsOH. The junction potential with these solutions was 7.5 mV (calculated using Axoscope, version 1.1, Axon Instruments, Foster City, CA) and was not corrected. Data were acquired using a Pentium computer that controlled data acquisition hardware and software (pClamp 6.03, Axon Instruments) connected to Axopatch 200B amplifiers (Axon Instruments). Currents were filtered at 2 kHz and sampled at 4 to 10 kHz. A holding potential of -80 mV was used to inactivate Na^+ current. $I_{\text{Ca,L}}$ was elicited with step depolarization protocols, using test potentials in the range of -40 to +30 mV. $I_{\text{Ca,L}}$ densities were computed by dividing current amplitudes by the whole-cell capacitance. Peak Ca^{2+} current density refers to $I_{\text{Ca,L}}$ density at the peak of the current-voltage (I-V) curve.

Phosphorylation of cardiac voltage-sensitive Ca^{2+} channel protein, α_{1c} by DNP

Rabbit ventricular myocytes were washed twice with phosphate free Dulbecco's modified Eagle's medium (DMEM) containing 0.5% dialysed fetal calf serum. Cells were then incubated in 2 ml of phosphate free DMEM containing 1 mCi of [^{32}P]orthophosphate (Sigma-Aldrich, St. Louis, MO) for 4 h at 37°C. The cells were treated with DNP (Phoenix

Pharmaceuticals, Belmont, CA) for 10 min and/or with KT5823 for 1 h. Incubations were terminated by aspirating the medium, and the cells were washed repeatedly with ice-cold phosphate buffered saline to remove residual radioactive phosphate. The cells were lysed with a lysis buffer containing 20 mM HEPES, 1% Triton X-100, 0.1 M NaCl, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVO₄, and 10 µg/ml leupeptin (pH 7.2). After centrifugation (12,000 × *g*, 3 min), the soluble extract were precleared with 20 µl of protein A-agarose gel. The extracts were treated with a 10 µg of anti- α_{1c} antibody (Alomone labs, Jerusalem, Israel) for 4 h and then with 20 µl of protein A-agarose gel for 30 min. The immunoprecipitates were recovered by centrifugation and washed four times with a washing buffer containing 1% Triton X-100, 1% deoxycholate, 1% SDS, 150 mM NaCl, and 50 mM Tris (pH 8.5). The immunoprecipitates were separated on SDS-polyacrylamide gels. The dried gels were exposed to XAR film (Kodak, Rochester, NY). The dried gel was swelled with distilled water and transferred into NC paper. Protein bands were visualized with anti- α_{1c} antibody, alkaline phosphatase-labelled anti-rabbit antibody and a NBT/BCIP substrate kit (Bio-Rad, Hercules, CA).

Data analysis

For statistical analysis, Student's *t*-test was used to compare means, and a *P* value of less than 0.05 was considered significant. Data were presented as means ± SEM.

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