Role of Nitric Oxide in the Regulation of Mechanosensitive Ionic Channels in Cardiomyocytes: Contribution of NO-Synthases

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The role of NO in the regulation of currents passing through ion channels activated by cell stretching (mechanically gated channels, MGC), particularly through cation-selective K^+-channels TRPC6, TREK1 (K_{2P}2.1), and TREK2 (K_{2P}10.1), was studied on isolated mouse, rat, and guinea pig cardiomyocytes using whole-cell patch-clamp technique. In non-deformed cells, binding of endogenous NO with PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-1-oxy-3-oxide) irreversibly shifted the diastolic membrane potential towards negative values, modulates K_{ir}-channels by reducing I_{K1}, and blocks MGC. Perfusion of stretched cells with PTIO solution completely blocked MG-currents. NO-synthase inhibitors L-NAME and L-NMMA completely blocked MGC. Stretching of cardiomyocytes isolated from wild type mice and from NOS1$^{-/-}$- and NOS2$^{-/-}$- knockout mice led to the appearance in MG-currents typical for the specified magnitude of stretching, while stretching of cardiomyocytes from NOS3$^{-/-}$- knockout mice did not produce in MG-current. These findings suggest that NO plays a role in the regulation of MGC activity and that endothelial NO-synthase predominates as NO source in cardiomyocyte response to stretching.

Key Words: *mechanically gated channels; PTIO; L-NAME; LNMMA; NOS3*

Ionic channels activated by cell stretching (mechanically gated channels: MGC), particularly cation-non-selective [3-5] and, as it was demonstrated later, K^+-channels TRPC6, TREK1 (K_{2P}2.1), and TREK2 (K_{2P}10.1) [2] play the key role in mechanoelectrical feedback in the heart. However, little is known about direct pharmacological regulation of MGC conductivity. At the same time, some experimental studies on the whole heart [6,8,9] suggest that MGC can be directly regulated by NO-synthases (NOS) via NO production [7].

Here we studied the role of NO as a possible regulator of MGC activity in isolated cardiomyocytes and elucidated functional role of different types of NO-synthases using NO-synthase inhibitors and NOS-knockout animals.

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MATERIALS AND METHODS

Experiments were carried out on cardiomyocytes isolated from the left ventricle of male Wistar rats, guinea pigs, and wild type and NOS1$^{-/-}$-, NOS2$^{-/-}$-, NOS3$^{-/-}$-knockout mice. Standard Langendorf method was used for cardiomyocyte isolation [3,4]. Isolated myocytes were placed into an experimental chamber (0.5 ml, 37°C, solution change over 15 sec) and perfused with a solution contained (mmol/liter): 150 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 20 glucose, and 5 HEPES/NaOH (pH 7.4). Patch-pipette solution contained (mmol/liter): 140 KCl, 5.5 $MgCl_2$, 5 Na_2ATP, 0.05 EGTA, and 10 HEPES/KOH (pH 7.2). After patch-pipette suc-

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tion to cell membrane and recording of whole-cell configuration, another fire-polished patch-pipette was placed at a distance of 40 μ to adhere to the cell. Standardized cell stretching by 10 μ was performed using fire-polished patch-pipette and digital micromanipulator with a stepping motor (MP 285, Sutter, "Novato", motor step 0.2 μ) [3].

Current was registered using a patch-clamp amplifier RK-300 (Biologic), and digitized using PowerCED system (Cambridge Instruments). The relationship between membrane currents and the potential was assessed by volt-ampere characteristics (I-V curves). Holding potential (Vhp) for Na_V-channel inactivation was -45 mV. To exclude the contribution of Ca_V-channels, the membrane potential first was fixed at 50 mV for 50 msec and then the cell was repolarized from 50 mV to -100 mV at a rate of -100 mV/sec (ramp-like repolarization) [1,2]. At the intersection point of I-V curve with the axis of potential, the current is null and this point is equivalent to diastolic membrane potential (V_0), which is commonly estimated using current-clamp approach. The peak of I-V curve at -60 mV is attributed to I_{K1}-current through K_{ir}-channels. When the potential was lower -20 mV, conductance of two types of channels had an impact on I-V curve: ΔG_{K1} and cation-non-selective ΔG_{ns}, determined by MGC activity. If the potential is higher than -20 mV, MG-currents are determined by K^+ -channels TRPC6, TREK1 (K_{2P}2.1) and TREK2 (K_{2P}10.1) [1].

The results were statistically processed using ANOVA and Bonferroni tests. Data is presented as mean±error of the mean.

RESULTS

For measuring activity of mechanosensitive ion channels under conditions of NO removal, we used a compound acting as a scavenger of endogenous NO, PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-1-oxy-3-oxide, 500 μmol/liter). In experimental series I we demonstrated the reaction of non-deformed cell to perfusion with PTIO-containing solution. In the control, I-V curve (I_C) for guinea pig cardiomyocytes (Fig. 1, *a*) has a pronounced N-like shape and V_0= -84±5 mV (*n*=10). In the presence of PTIO, V_0 was shifted towards negative values to -94±2 mV (*n*=10) as soon as after 3 min. Moreover, I_{K1} forming the peak on I-V curve decreased and the peak is shifted from -60 mV to -80 mV. On the whole, all currents determining I-V curve (I_{PTIO}) decreased at both negative and positive potentials. Cell stretching by 10 and 12 μ against the background of PTIO perfusion did not affect the currents. We suppose that presence of NO is critical for MGC activity and elimination of endogenous NO leave no possibility for channels to open. PTIO was not washed out for 5 min ($^{W}I_{PTIO}$ curve; Fig.1, *b*). Moreover, even long-term washout from PTIO (up to 15 min) did not restore the I-V curve to the initial level.

In experimental series II, stretched cell was perfused with PTIO. Current in control (I_C: has a N-lake shape and V_0=-86±5 mV, *n*=10) and its changes during cell stretching were demonstrated (I_S; Fig. 2, *a*). Cell stretching shifted the curve towards negative voltage values and shifted V_0 to -39±4 мВ (*n*=10). Differential current I_{MGC} (I_{S-C}; Fig. 2, *b*) was -0.41 nA ($I_{MGC(-45\ mV)}$=

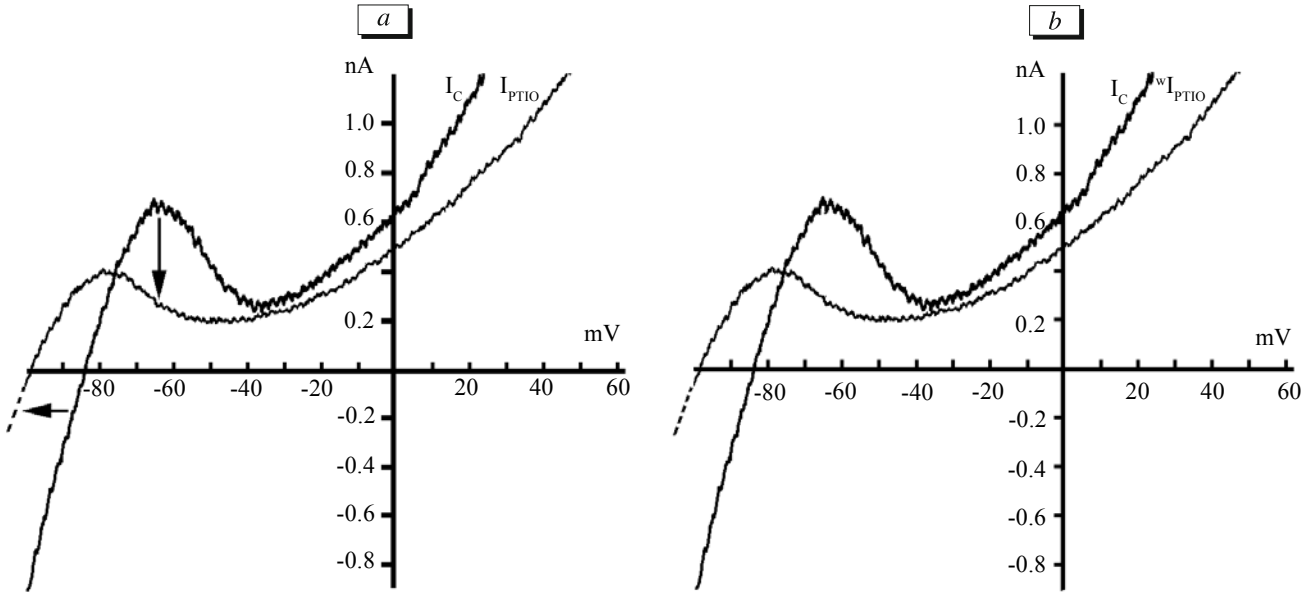

Fig. 1. I-V curves for guinea pig cardiomyocyte membranes during the perfusion of non-stretched cell with PTIO solution (500 μmol). *a*) I-V curve in control (I_C) and after 3-min perfusion with PTIO (I_{PTIO}). Arrows show direction of the curve shift during perfusion with PTIO. *b*) after 5 min of PTIO washout ($^{W}I_{PTIO}$). Control: I_C.

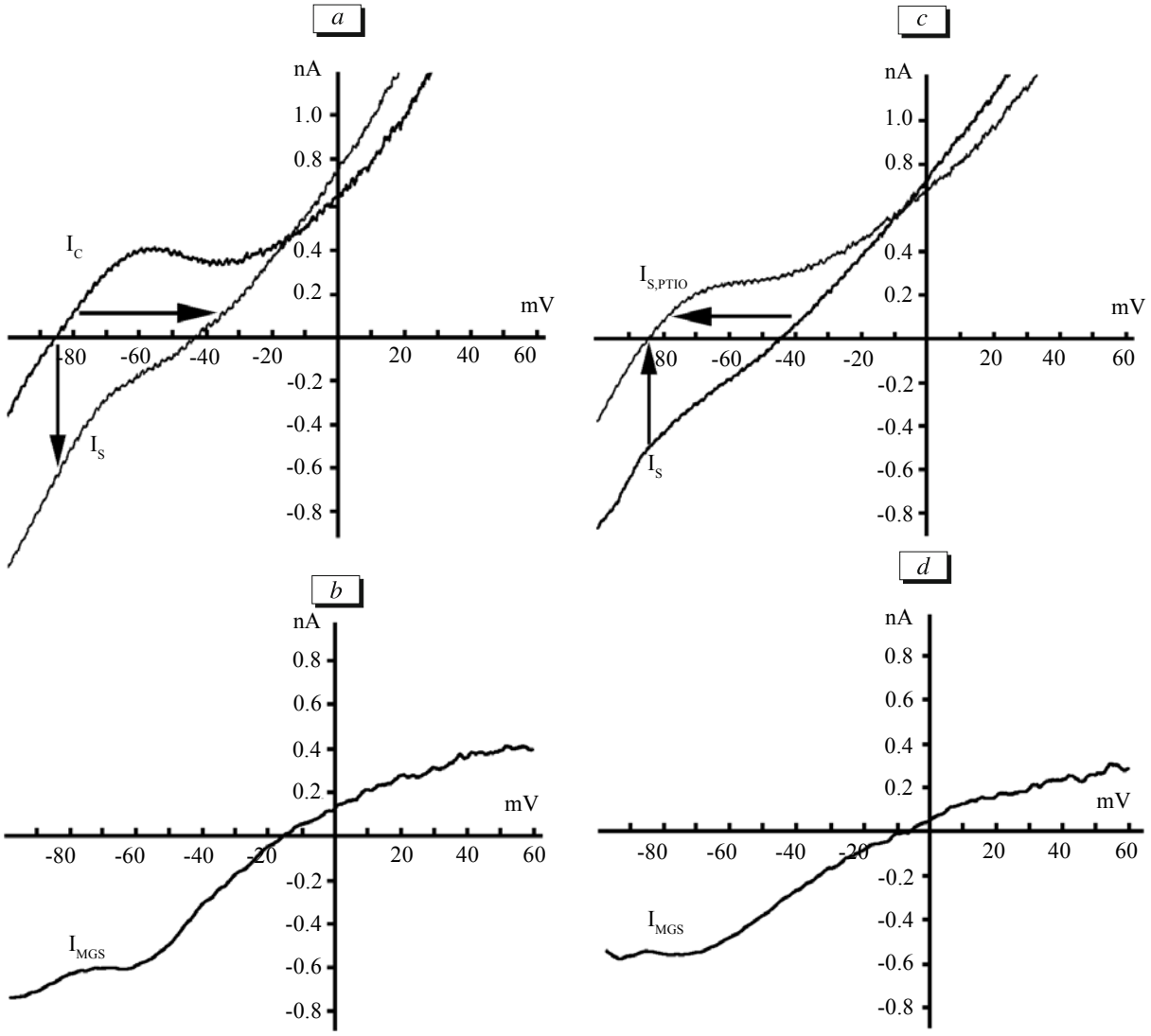

Fig. 2. I-V curves demonstrating relationship between the current through MGC and stretching (10 μ) of guinea pig cardiomyocytes during perfusion with PTIO (500 μmol) against the background of stretching. *a*) I-V curve in the control (I_C) and against the background of cell stretching (I_S). Arrows show direction of the curve shift during cell stretching; *b*) differential current I_{MGC} activated by cell stretching (I_{S-C}); *c*) I-V curve for stretched cell (I_S) against the background of 8 min perfusion with PTIO ($I_{S,\ PTIO}$). Arrows show direction of I_S shift during cell stretching against the background of PTIO; *d*) differential current I_{MGC}, activated by cell stretching after 8-min perfusion with PTIO.

-0.40±0.02 nA, E_{rev}=-12±3 mV, *n*=10). These results are in line with the data indicting that cardiomyocyte stretching results in the appearance of $I_{MGC(-45\ мВ)}$=-0.42±0.04 nA (*n*=8) in K_i/K_o medium [4]. Thus, cell stretching increased currents through MGC and depolarized the membrane.

Changes in I-V curve for stretched cardiomyocyte (I_S) after 8-min cell perfusion with PTIO were demonstrated ($I_{S,\ PTIO}$; Fig. 2, *c*). Despite cell stretching, PTIO restored the curve to the initial state, the I-V curve again acquired the N-like shape and crossed X-axis at V_0=-86±4 mV (*n*=10). Differential current I_{MGC} ($I_{S-S,\ PTIO}$; Fig. 2, *d*) was 0.33 nA ($I_{MGC\ (-45mV)}$=0.35±0.03 nA, E_{rev}=-2±2 mV, *n*=10). Thus, perfusion with PTIO against the background of cell stretching led to inhibition of MG-currents due to MGC blockage. Therefore, NO is essential for MGC functioning with both ΔG_{ns} and ΔG determined by K^+-ions. The curve obtained under these conditions virtually coincided with the control one except in none experiments the peak at -60 mV formed by I_{K1} did not completely return to the baseline state. It can be hypothesized that PTIO merely modulates activity of K_{ir}-channels decreasing I_{K1} without blockage and I_{K1} remained under the influence of cell stretching. These data were obtained on guinea pig, rat and mouse cardiomyocytes.

Differences in the effects of PTIO on non-deformed and stretched cells are to be specially discussed. The mechanism of these effects can be as follows. In non-stretched cell, PTIO blocks baseline MG-currents, which shifts V_0 towards more negative values. In stretched cell, PTIO also blocks MG-currents, including baseline ones,

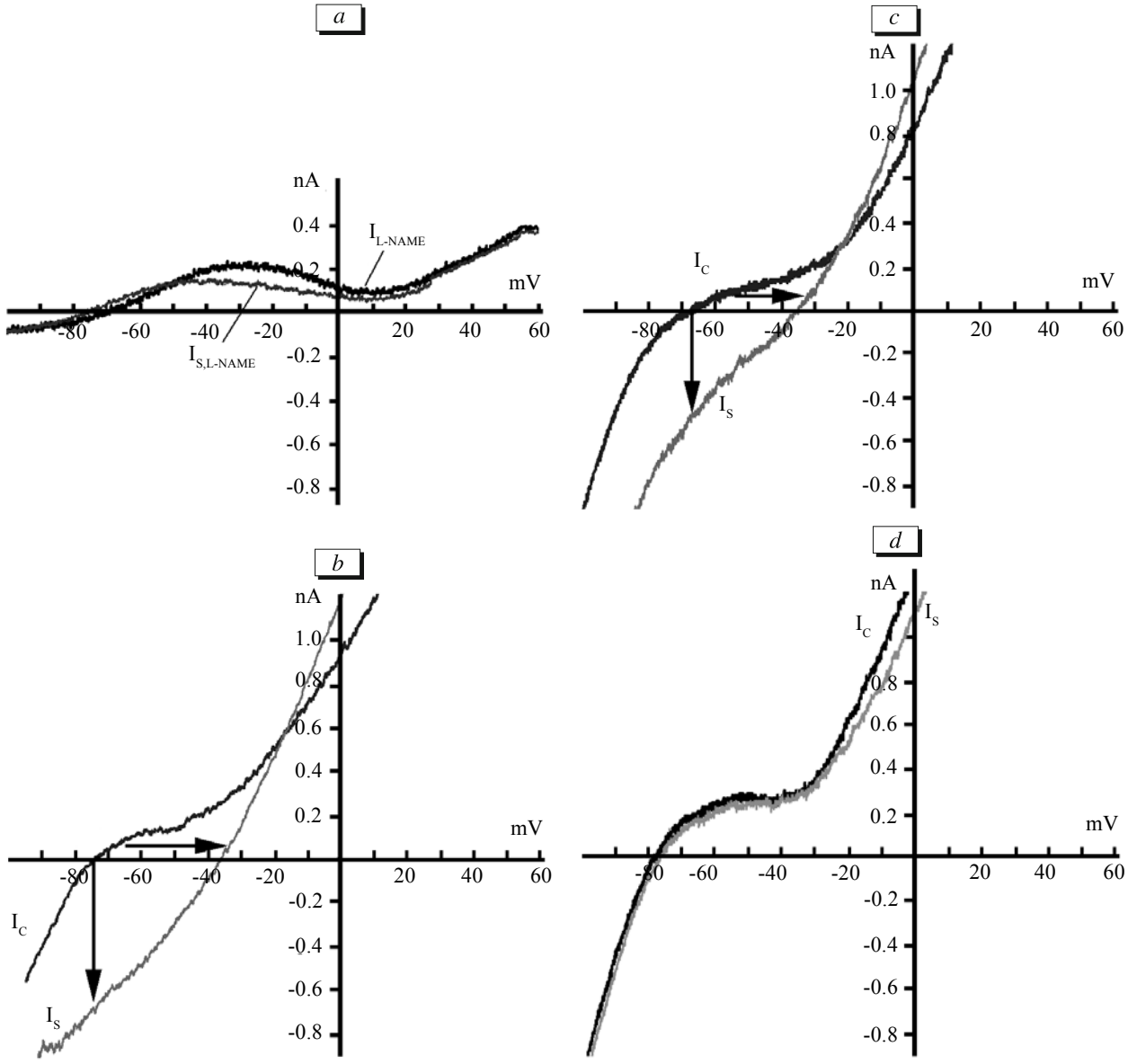

Fig. 3. Effects of NOS inhibition or elimination on MG-currents in cardiomyocytes. *a*) I-V curve for guinea pig cardiomyocytes after preincubation in L-NAME solution ($I_{L\text{-}NAME}$; 20 μmol) against the background of cell stretching by 10 μ ($I_{S,\,L\text{-}NAME}$); *b*) I-V curve for wild type mouse cardiomyocyte in control (I_C) and during stretching (I_S); *c*) currents in cardiomyocytes from NOS1$^{-/-}$ mice in the control (I_C) and during stretching (I_S). Arrows show direction of curve shift during cell stretching; *d*) currents in NOS3$^{-/-}$-mouse cardiomyocytes in the control (I_C) and during stretching (I_S).

but the cell is in stretched state and mechanisms typical for stretching are still active and contribute to membrane potential in such a way that V_0 fluctuate around the baseline values under the influence of PTIO.

For evaluation of the role NO-syntases in realization of this effect we used nonselective NO-synthase inhibitors. In experimental series I we used L-NAME (L-N^G-nitroarginine methyl ester (hydrochloride), 2 h preincubation). Stretching of the cell preincubated with L-NAME in concentrations 20 μmol/liter (Fig. 3, *a*) and 100 μmol/liter did not affect I-V curve ($I_{S,L\text{-}NAME}$), hence, MGC were completely blocked. MGC are not blocked when L-NAME concentration decreases to 2 μmol/liter. The reaction to stretching is preserved, and relaxation returns I-V curve to baseline parameters. Application of L-NMMA (N_ω-methyl-L-arginine acetate, 200 μmol/liter, 2 h preincubation) produced similar results: the cells exposed to the inhibitor did not respond to stretching. Thus, NO-synthase inhibitors L-NAME and L-NMMA completely blocked MG-current due to lack of endogenous NO.

From pharmacological point of view, the effects of different NO-synthases are virtually undistinguishable. This problem can be solved by using of knockout animals. Typical examples represent MG-currents in response to cell stretching in wild type and NOS1$^{-/-}$

and NOS3$^{-/-}$-knockout mice (Fig. 3, *b*-*d*). In cardiomyocytes from wild type mice and from *NOS1*- and *NOS2*-knockout mice, MG-currents in response to cell stretching were equal and characteristic for the corresponding magnitude of stretching (Fig. 3, *b*, *c*). Alternatively, in cardiomyocytes from NOS3$^{-/-}$-mice deformation did not induce MG-current (Fig. 3, *d*), therefore, NOS3 is more essential source of NO for myocyte response to stretching.

All results indicate that NO is involved in the functioning of mechanosensitive ionic channels, moreover, it is able to open MGC without cell stretching, but without NO MGC conductance is impossible even in case of effective stretching. Endothelial NOS is the most essential one for realization of heart cell response to stretching.

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