Increased expression of the Na^+/Ca^{2+} exchanger in the rat heart after immobilization stress is not induced by cortisol

Lubomira Zacikova[a], Richard Kvetnansky[b], Olga Krizanova[a,*]

[a] *Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlarska 5, 833 34 Bratislava, Slovak Republic*
[b] *Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovak Republic*

Received 1 July 1999; received in revised form 19 July 1999

Abstract Calcium homeostasis is crucial for the proper function of cardiac cells. Since the Na^+/Ca^{2+} exchanger is an important modulator of calcium homeostasis especially in the heart, the objective of this study was to investigate the effect of immobilization stress on the high capacity Na^+/Ca^{2+} exchanger in rat heart ventricles and atria. Repeated immobilization stress increased both the mRNA and the protein level and the activity of the Na^+/Ca^{2+} exchanger in the left, but not the right ventricle of rat heart. Since corticosterone is rapidly increased during the stress stimulus, it might be assumed that mRNA of the Na^+/Ca^{2+} exchanger is increased through a glucocorticoid responsive element. However, we have found that cortisol did not change the Na^+/Ca^{2+} exchanger at the mRNA or protein levels. These results clearly show that this effect of stress is not mediated via cortisol.

Key words: Na^+/Ca^{2+} exchanger; Stress; Cortisol; Gene expression

1. Introduction

Stress is a stimulus which is known to be deeply involved in the development of some cardiovascular complications, mainly hypertension, myocardial infarction, heart failure, etc. [1]. Immobilization has been proved to be one of the most potent stress models, since it activates both components of the sympathoadrenal system, i.e. adreno-medullary and sympathoadrenal [2]. Immobilization stress affects several metabolic pathways. Alterations in the activity of several enzymes and/or receptors due to the immobilization stress result from an increase in the amount and expression of these proteins. Activation of the sympathoadrenal system leads to the outflow of catecholamines from the adrenal medulla and the sympathetic nerve endings. Glucocorticoids are also increased during the stress and influence the synthesis of catecholamines. Munck et al. [3] proposed that stress-induced increase in glucocorticoid levels protects the organism by restraining defence reactions activated by the stressor. Glucocorticoids (e.g. cortisol) are hormones which affect several metabolic pathways, e.g. of carbohydrates, lipids and proteins. They reduce inflammation, and enhance immunity and thus resistance to infection. Cortisol also has a positive inotropic effect on the heart. It is proposed that this effect is mediated, at least partially, by a direct potentiating effect on the calcium current I_{Ca} [4]. It has been shown that high levels of glucocorticoids are neurotoxic, because they alter calcium homeostasis of hypothalamic neurons by increasing calcium voltage-dependent influx, especially in aged neurons [5].

The Na^+/Ca^{2+} exchanger serves as the principal calcium extrusion mechanism in the heart muscle [6,7]. The extrusion of calcium by the Na^+/Ca^{2+} exchanger increases as intracellular calcium is elevated. Two explicit roles of the Na^+/Ca^{2+} exchanger in excitation-contraction coupling in the heart have been described: (i) the Na^+/Ca^{2+} exchanger regulates calcium content of the sarcoplasmic reticulum by regulating the resting $[Ca^{2+}]_i$ level, and (ii) the Na^+/Ca^{2+} exchanger can directly contribute to the calcium that enters the myocardial cells during the upstroke and plateau phases of the action potential [8]. The Na^+/Ca^{2+} exchanger can extrude calcium at different rates during the plateau phase of the action potential [9]. It can also, at least partially, contribute to calcium-induced calcium release via the sarcoplasmic reticulum calcium release channels [10–13].

The Na^+/Ca^{2+} exchanger has already been cloned from dog heart [14], human heart [15] and cow heart [16]. From that time, modulation of its expression and structure-function studies have been performed extensively. Modulation of the Na^+/Ca^{2+} exchanger by protons, sodium, calcium, ATP, etc. involves the interactions of these agents with the intracellular loop of the exchanger [8].

The objective of this study was to determine the effect of single and repeated immobilization stress on the mRNA and protein levels of the Na^+/Ca^{2+} exchanger in the left and right ventricles of the rat heart. In order to understand the mechanism of the effect of stress on the Na^+/Ca^{2+} exchanger in the rat heart, it was worth investigating also the involvement of cortisol in this mechanism.

2. Materials and methods

2.1. Animals

The protocol used was approved by the Animal Care Committees of the Slovak Academy of Sciences and the Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovak Republic. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health. Male Sprague-Dawley rats (weighing 300–450 g) were used for all experiments. Before initiation of the experimental procedures, animals were housed 3–4 per cage for at least 7 days. Room temperature was held at 23 ± 2°C and periodic 12-h alteration of the light and dark was performed during the whole experiment. Food and water were available ad libitum.

2.2. Immobilization stress

The immobilization protocol described by Kvetnansky and Mikulaj [2] was used. Rats were immobilized one (IMO1), six (ADC) or seven (IMO7) times for 2 h, except for the control group. Animals were killed immediately after the last immobilization, except for the 'adapted control' group (ADC), in which animals were immobilized

*Corresponding author. Fax: (421) (7) 54773666.
E-mail: umfgkriz@kramare.savba.sk

PII: S0014-5793(99)01078-9

for six times and subsequently kept in their cages for 24 h. Hearts were dissected, dried of blood, washed in ice-cold physiological solution, and frozen in liquid nitrogen.

2.3. Implantation of osmotic cortisol minipumps

In spite of the fact that the endogenous glucocorticoid in rats is corticosterone and not cortisol, we applied cortisol because of its better solubility. After skin incision, in the dorsoventral direction in the dorsal area of the neck between the shoulder blades, we slotted the osmotic minipump with cortisol or saline into the skin in the rostral direction. Surgery was completed under pentobarbital anesthesia (50 mg/kg body weight). Cortisol was applied continuously at a concentration of 25 mg/kg/day for either 24 h or 7 days at a rate of about 1 μl/h. This concentration of cortisol was shown to be sufficient to increase the endogenous glucocorticoid levels to the same amounts as were found in stressed rats [17].

2.4. Calcium transport into the membrane proteoliposomes from rat hearts

Rat heart plasma membranes were prepared by ultracentrifugation as described previously by Krizanova et al. [18]. Ca^{2+} transport was measured according to Reeves and Sutko [19] with some modifications. Briefly, the reconstitution was done with an asolectin-protein ratio of 40:1. The mixture was solubilized in 1% 3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS). After solubilization, CHAPS was removed on a Sephadex G-50 column (Pharmacia, Sweden). Multilamellar vesicles were eluted with 20 mM Tris-HCl, 160 mM NaCl, pH 7.4. The proteoliposomes were sonicated for 2×30 s to obtain unilamellar particles. Proteoliposomes were then incubated with 40 μM $^{45}Ca^{2+}$, 20 mM Tris-HCl, pH 7.4, 160 mM KCl and/or NaCl solution for 10 min. Afterwards, free calcium was removed on CM-Sepharose (Pharmacia, Sweden). Radioactivity was measured after addition of Bray's scintillation cocktail in a Beta counter (Rackbeta, LKB).

2.5. RNA preparation

RNA was isolated from the hearts according to the procedure of Chomczynski and Sacchi [20], using guanidine isothiocyanate (Fisher Scientific, USA) and phenol-chloroform extraction. Concentration and purity of RNA were determined in triplicate spectrophotometrically on a UV 3000 (Shimadzu).

2.6. Relative quantification of mRNA levels by RT-PCR

Reverse transcription was done from 5 μg of total RNA using a First-Strand cDNA Synthesis kit (Pharmacia Biotech, Sweden). We used primer pd$(N)_6$ (Pharmacia, Sweden). Specific PCR for the Na^+/Ca^{2+} exchanger was done afterwards using primers NCX1 (5′-GGG CAG CTT CTC CTC CCC ACA TTC-3′) and NCX2 (5′-AGG CGG CTT CTC TTA TAC AAG TAC-3′). The NCX primers were designed according to cDNA. After the initial denaturation, 35 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min and polymerization at 72°C for 1 min were performed. PCR products were analyzed on 2% agarose gels. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is commonly used as an internal control in many cell systems [21–23]. Therefore, as a control for semi-quantitative evaluation of PCR, GAPDH primers (GAPDH1: 5′-AGA TCC ACA ACG GAT ACA TT-3′; GAPDH2: 5′-TCC CTC AAG ATT GTC AGC AA-3′) were used to amplify a 309 bp fragment from each first strand sample [24]. After the initial denaturation at 94°C for 5 min, 30 cycles of PCR at 94°C for 1 min, 60°C for 1 min and 72°C for 3 min were performed. Since it is known that the fragment amplification by PCR is linear only up to a limited number of cycles, prior to the experiment we tested whether under these conditions PCR is working in the linear range for both the Na^+/Ca^{2+} exchanger and GAPDH. For the verification, 15, 20, 25, 30, 35, 37 and 40 cycles were chosen. We found that in our experiments, 35 cycles for the Na^+/Ca^{2+} exchanger and 30 cycles for the GAPDH is still in the linear range of PCR amplification.

2.7. Western blot analysis

Protein assays were done by the method of Lowry et al. [25]. 20 μg of each protein extract was separated by electrophoresis on a 6.5% sodium dodecyl sulfate-polyacrylamide gel and then transferred to a nitrocellulose membrane, using semi-dry blotting. The blot was blocked in 5% non-fat dry milk diluted in TBST and then incubated with the mouse monoclonal antibody against Na^+/Ca^{2+} exchanger (Affinity Bioreagents, USA). Primary antibody reacts with the Na^+/Ca^{2+} exchanger in kidney and cardiac tissues of human, canine, rabbit, guinea pig and rat. The epitope for this antibody is in the region of amino acids 371–525, which is on the intracellular side of the plasma membrane. We used a secondary anti-mouse antibody conjugated to horseradish peroxidase (Amersham, UK). The protein bands were detected with a chemiluminescent detection system, ECL analysis (Amersham, UK). The signal for the Na^+/Ca^{2+} exchanger was expected to be at 120 kDa.

2.8. Statistical analysis

Each value represents the average for six animals. Results are presented as means ± S.E.M. Statistical differences among groups were determined by one way analysis of variance (ANOVA). Statistical significance was defined as $P < 0.05$. For multiple comparisons, an adjusted *t*-test with *P* values corrected by the Bonferroni method was used (Instat, GraphPad Software, USA).

3. Results

As determined by Western blot analysis with a monoclonal antibody against rat Na^+/Ca^{2+} exchanger, the highest density of this transport system was observed in the left ventricle (LV; 96 ± 10 arbitrary units) (Fig. 1). Both atria (LA, RA) had much lower amounts of the Na^+/Ca^{2+} exchanger protein (33 ± 1.2 and 33 ± 2 arbitrary units) compared to LV. Therefore, in further experiments we compared the effect of immobilization stress separately on the left and right ventricles. Single immobilization (IMO1) stress for 2 h did not affect the gene expression of the Na^+/Ca^{2+} exchanger either in the left or in the right ventricle (Fig. 2). However, expression of the gene for the Na^+/Ca^{2+} exchanger was significantly increase in the left ventricle after six (ADC) and/or seven (IMO7) immobilizations (Fig. 2). In the right ventricle, no significant increase was observed even after the repeated immobilization (Fig. 2). This observation correlates with results obtained by Western blot analysis with mouse monoclonal antibody against rat Na^+/Ca^{2+} exchanger (Fig. 3). In the left ventricle, the protein level of the Na^+/Ca^{2+} exchanger was also increased 11–13 times after the repeated immobiliza-

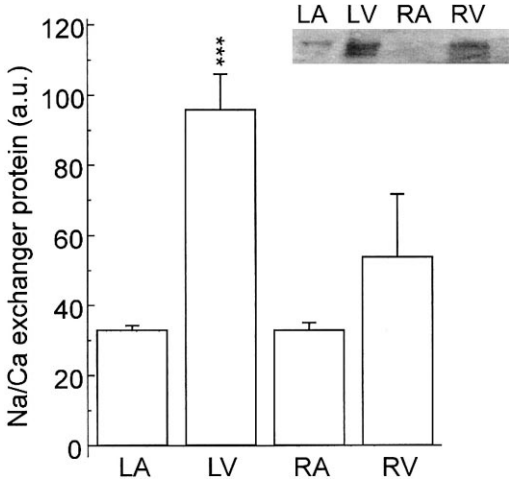

Fig. 1. Distribution of the Na^+/Ca^{2+} exchanger protein in individual parts of the rat heart. The highest level of the Na^+/Ca^{2+} exchanger as determined by Western blot analysis with the monoclonal antibody against rat Na^+/Ca^{2+} exchanger was found in the left ventricle (LV). The smallest amounts were found in the left and right atria (LA, RA). Results are mean ± S.E.M. and are the average from at least three animals. ***$P < 0.001$, compared to atria.

tion (ADC, IMO7, Fig. 3), compared to controls (C) and/or the group of animals immobilized only once (IMO1). In the right ventricle, no increase was observed in the Na^+/Ca^{2+} exchanger protein after repeated immobilization compared to the controls (Fig. 3). Activity of the Na^+/Ca^{2+} exchanger was also increased after repeated immobilization in the left, but not in the right ventricle (Fig. 4).

Immobilization stress is known to rapidly elevate the concentration of glucocorticoids [17]. Therefore, one possible explanation for the increased gene expression of the Na^+/Ca^{2+} exchanger might be mediated via the glucocorticoid responsive element. We examined whether continuous administration of cortisol would mediate the same effect. However, no differences were observed in Na^+/Ca^{2+} exchanger gene expression in cortisol treated (65 ± 6 arbitrary units) or control animals (62 ± 10 arbitrary units; Fig. 5A), compared to the housekeeper GAPDH gene. Western blot analysis with the monoclonal antibody against rat Na^+/Ca^{2+} exchanger did not reveal any differences in Na^+/Ca^{2+} exchanger protein level after cortisol treatment compared to controls (Fig. 5B) either. Interestingly, we found that activity of the Na^+/Ca^{2+} exchanger in rat heart (Fig. 5C) decreased rapidly (from 48 ± 11 nmol Ca^{2+}/h to 13.5 ± 2.9 nmol Ca^{2+}/h) after continuous administration of cortisol for 24 h. After administration of cortisol for 7 days, reduction in the Na^+/Ca^{2+} exchanger's activity was even more pronounced (to 4.8 ± 1.4 nmol Ca/h).

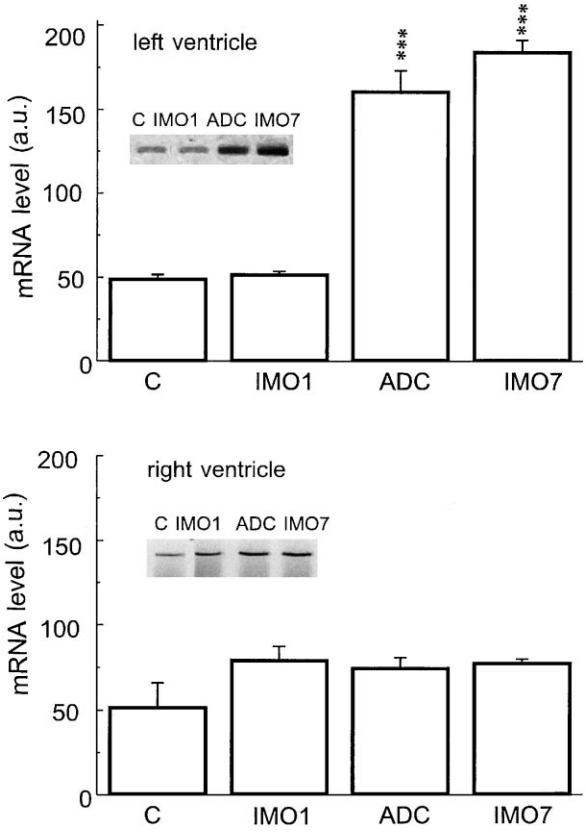

Fig. 2. mRNA levels of the Na^+/Ca^{2+} exchanger in left and right ventricles after a single (IMO1) and repeated (IMO7) immobilization. C: control group; IMO1: group of rats immobilized once for 2 h; ADC: adapted control, where animals were immobilized six times and left in home cages for 24 h before decapitation; IMO7: group of rats immobilized seven times for 2 h. Results are mean ± S.E.M. and are the average from at least six animals. $***P < 0.001$, compared to controls (C).

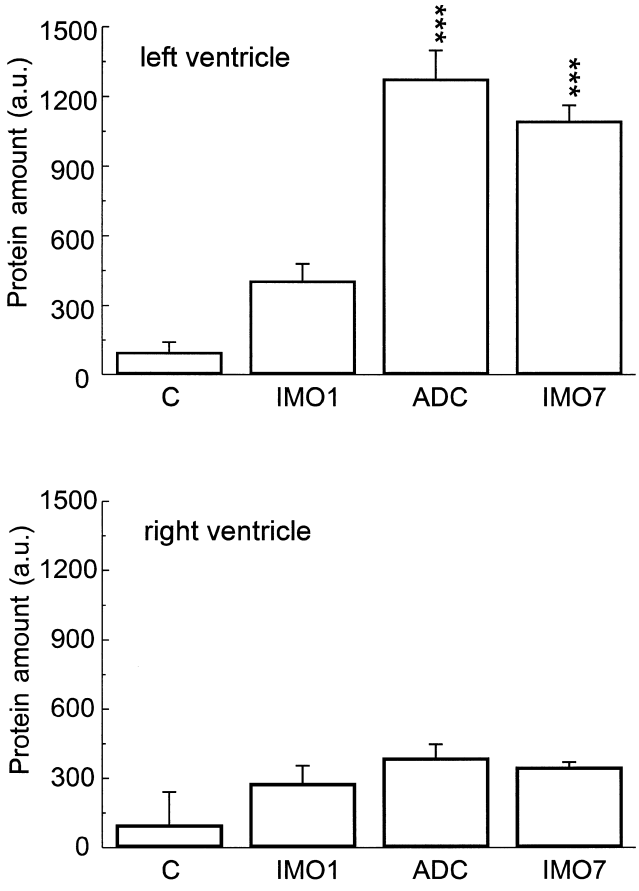

Fig. 3. Western blot analysis of the Na^+/Ca^{2+} exchanger in left and right ventricles as determined by monoclonal antibody against rat Na^+/Ca^{2+} exchanger. C: control group; IMO1: group of rats immobilized once for 2 h; ADC: adapted control, where animals were immobilized for six times and left in home cages for 24 h before decapitation; IMO7: group of rats immobilized seven times for 2 h. Results are mean ± S.E.M. and are the average from at least six animals. $***P < 0.001$, compared to controls (C).

4. Discussion

Stress is deeply involved in the development of serious cardiovascular complications, mainly hypertension, myocardial infarction, heart failure, etc. Although the primary mechanism of this effect is still not known, several parameters have been reported to be altered. It was shown that immobilization stress, a well-defined model of stress, affects the expression of several systems, e.g. Ca^{2+} channels in the rat kidney [26], catecholamine biosynthetic enzymes [27,28], etc. Since the Na^+/Ca^{2+} exchanger is known to play an important role in calcium homeostasis in the heart [6,7], we compared mRNA and protein levels, as well as the activity of the Na^+/Ca^{2+} exchanger in the rat heart after a single and repeated immobilization. We focused on cardiac ventricles, since we found that Na^+/Ca^{2+} exchanger is much more abundant in ventricles than in atria (Fig. 1). Our results are consistent with the report of Wang and coworkers [29] on human heart, who reported that levels of the Na^+/Ca^{2+} exchanger were 30–50% lower in atria compared to ventricles. After the single immo-

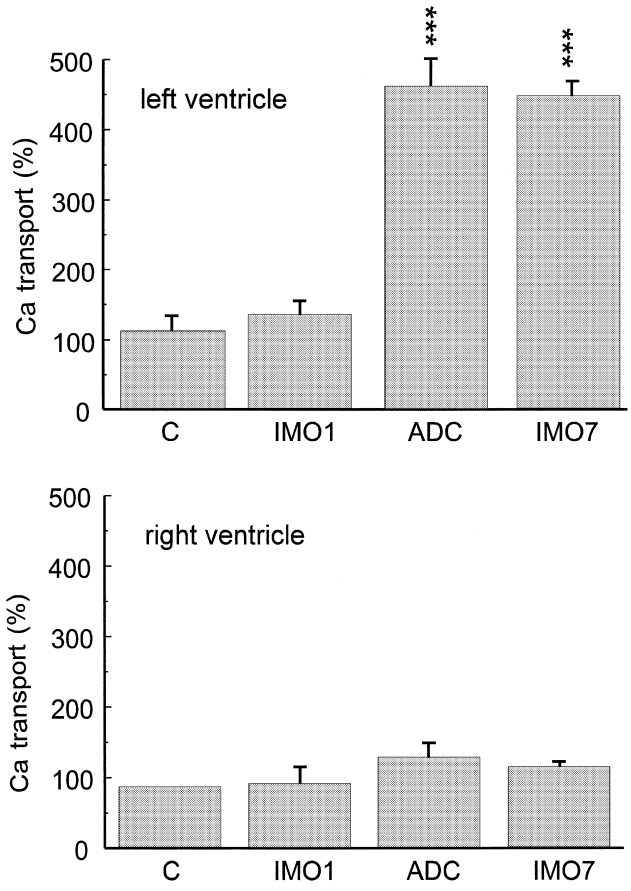

Fig. 4. Activity of the Na^+/Ca^{2+} exchanger in the left and right ventricles as determined by $^{45}Ca^{2+}$ transport into the proteoliposomes. C: control group; IMO1: group of rats immobilized once for 2 h; ADC: adapted control, where animals were immobilized for six times and left in home cages for 24 h before decapitation; IMO7: group of rats immobilized seven times for 2 h. Each measurement is the average from at least six animals. Results are displayed as mean ± S.E.M. $***P < 0.001$.

bilization we did not observe any change either in expression or in protein level, and the activity of this system in rat heart ventricles. This observation was not surprising, because of the short duration of the stimulus. In cardiac left ventricles of the repeatedly immobilized rats we observed changes in Na^+/Ca^{2+} exchanger mRNA levels, which correlated with the elevated amount of the Na^+/Ca^{2+} exchanger's protein and finally, with the activity of this transport system. The mechanism by which stress increases gene expression of the Na^+/Ca^{2+} exchanger is not clear yet. Since the work of Kvetnansky et al. [17] shows that the level of glucocorticoids during stress is rapidly elevated, we performed further experiments to determine whether this effect might be caused by a glucocorticoid responsive element. Therefore, cortisol was applied to the animals continuously either for 24 h of for 7 days and the level of Na^+/Ca^{2+} exchanger expression was quantified. Our results show that gene expression and Na^+/Ca^{2+} exchanger protein were not changed after cortisol administration and therefore, immobilization cannot modulate the Na^+/Ca^{2+} exchanger via glucocorticoids. The continuous cortisol treatment suppressed, and did not enhance, activity of the Na^+/Ca^{2+} exchanger. These surprising results point to an additional and completely different mechanism of modulation of the Na^+/Ca^{2+} exchanger's activity through the glucocorticoids. Down-regulation of the activity of the Na^+/Ca^{2+} exchanger by glucocorticoids, although not in the heart, but in rat aorta was described by Smith and Smith [30]. These authors found that the activity of the Na^+/Ca^{2+} exchanger was suppressed by cortisol treatment for 24 h by approximately 55%. Based on their observation that the gene expression of the Na^+/Ca^{2+} exchanger in rat aorta is also decreased by

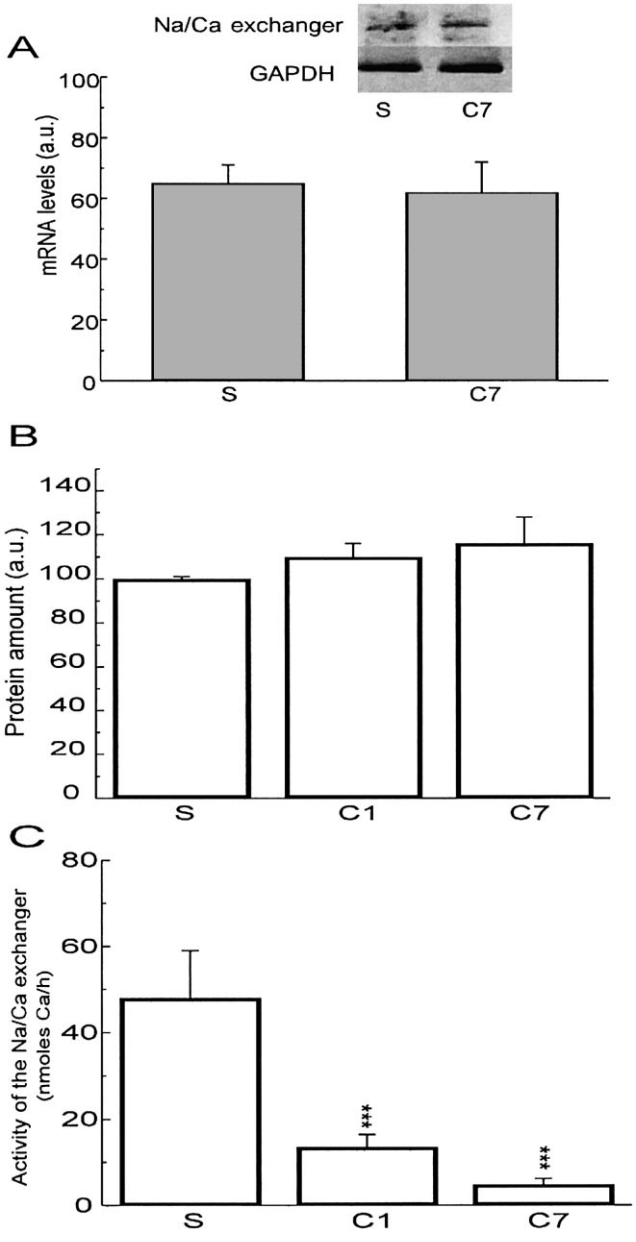

Fig. 5. mRNA levels, protein amount and the activity of the Na^+/Ca^{2+} exchanger in the hearts of saline treated control rats (S) and rats treated with cortisol for 1 (C1) or 7 (C7) days. mRNA levels are compared relatively with the housekeeper GAPDH. No changes relative to GAPDH were observed between the S and C7 groups. Protein level was also not affected by the cortisol treatment. In contrast, the activity of the Na^+/Ca^{2+} exchanger decreased significantly after 1 (C1) and/or 7 (C7) days of cortisol treatment. Results are displayed as mean ± S.E.M. $*P < 0.05$; $***P < 0.001$, compared to saline treated controls. Each measurement is the average from at least six animals.

cortisol, Smith and Smith [30] proposed that cortisol suppressed directly Na^+/Ca^{2+} exchanger expression, probably via a glucocorticoid responsive element. Based on our results, we propose rather an indirect effect of glucocorticoids with subsequent down-regulation of the Na^+/Ca^{2+} exchanger's activity.

We can only speculate about the physiological relevance of our observation, based on the role of calcium in the normal and failing heart. The diastolic and systolic dysfunction in the failing heart appears to be related to altered Ca^{2+} handling of the cardiac myocytes [31]. In chronic heart failure the myocardium undergoes a phenotypic change which includes alterations of the activity of enzymes regulating calcium homeostasis. The sarcoplasmic reticulum calcium ATPase is depressed in both function and expression. At the same time the sarcolemmal Na^+/Ca^{2+} exchanger is increased in both function and expression [32]. The Na^+/Ca^{2+} exchanger mRNA levels and thus its activity might be a powerful compensatory mechanism for retaining cardiac contractility in chronic heart failure [33] and end stage heart failure [34]. All these changes may significantly contribute to the altered calcium homeostasis in the cell and thus to the pathophysiological consequences.

It was already shown that Na^+/Ca^{2+} exchanger can be modulated by various stimuli: glycerophosphoinositols [35], reduced by cocaine [36], halothane [37]. All results described above point to the vulnerability of the Na^+/Ca^{2+} exchanger to various stimuli.

Another mechanism by which the Na^+/Ca^{2+} exchanger can be elevated by stress is through α-adrenergic receptors. This proposal is highly supported by the data of Reinecke et al. [38], who demonstrated that activation of the α_1-adrenoceptor up-regulates the expression of the Na^+/Ca^{2+} exchanger. Stress rapidly increases catecholamines [39–41], which leads to the increased activation of the adrenergic receptors.

In conclusion, our results clearly show that during stress stimuli, two independent effects participate in the modulation of the Na^+/Ca^{2+} exchanger: (i) up-regulation of the levels of mRNA of the Na^+/Ca^{2+} exchanger affecting its protein and the activity. This effect was observed only after the repeated immobilization (7×). This increase is not mediated via the glucocorticoid responsive element. (ii) An additional effect on the Na^+/Ca^{2+} exchanger may be due to the increased levels of glucocorticoids during stress. As we show in this paper, the effect caused by the cortisol treatment is a down-regulation of the activity of the Na^+/Ca^{2+} exchanger in the rat heart without affecting the mRNA or the protein levels. This effect was observed even after the single immobilization, but obviously was abolished by the stronger effect mediated by the unknown pathway of stress response. However, the physiological relevance of the dual regulation of the Na^+/Ca^{2+} exchanger during stress remains to be elucidated.

Acknowledgements: This work was supported from Grant VEGA (SK) 2/4128/99. The authors wish to thank Olga Rantova and Silvia Zakova for their technical assistance. The authors are grateful to Drs. Juhaszova (University of Maryland, Baltimore, MD, USA) and Stankovicova (Faculty of Pharmacy, UK, Bratislava) for valuable comments on the manuscript and to Dr. Rusnak for help with the implantation of the minipumps.

References

[1] Goldstein, D.S. (1996) in: Stress: Molecular Genetic and Neurobiological Advances (McCarty, R., Aguilera, G., Sabban, E.L. and Kvetnansky, R., Eds.), Vol. 2, pp. 833–862, Gordon and Breach, New York.
[2] Kvetnansky, R. and Mikulaj, L. (1970) Endocrinology 87, 738–743.
[3] Munck, A. and Naray-Fejes-Toth, A. (1992) Mol. Cell. Endocrinol. 90, C1–4.
[4] Yano, K., Tsuda, Y., Kaji, Y., Kanaya, S., Fujino, T. and Niho, Y. (1994) Jpn. Circ. J. 58, 836–843.
[5] Gareri, P., Mattace, R., Nava, F. and De Sarro, G. (1995) 26, 1651–1657.
[6] Bridge, J.H.B., Smolley, J.R. and Spitzer, K.W. (1990) Science 248, 376–378.
[7] Crespo, L.M., Grantham, C.J. and Cannell, M.B. (1990) Nature 345, 618.
[8] Schulze, D., Kofuji, P., Hadley, R., Kirby, M.S., Kieval, R.S., Doering, A., Niggli, E. and Lederer, W.J. (1993) Cardiovasc. Res. 27, 1726–1734.
[9] Mullins, L.J. (1989) in: Physiology and Pathophysiology of the Heart (Sperelakis, N., Ed.), pp. 241–251, Academic Press, Boston, MA.
[10] Lederer, W.J., Niggli, E. and Hadley, R.W. (1990) Science 248, 283.
[11] Leblanc, N. and Hume, J.R. (1990) Science 248, 372–376.
[12] Wier, W.G. (1991) Ann. NY Acad. Sci. 639, 366–374.
[13] Sipido, K.R., Maes, M. and Van de Werf, F. (1997) Circ. Res. 81, 1034–1044.
[14] Nicoll, D.A., Longoni, S. and Philipson, K.D. (1990) Science 250, 562–565.
[15] Kofuji, P., Hadley, R.W., Kieval, R.S., Lederer, W.J. and Schulze, D.H. (1992) Am. J. Physiol. 263, 1241–1249.
[16] Aceto, L.F., Condrescu, M. and Kroupis, C. et al. (1992) Arch. Biochem. Biophys. 298, 553–560.
[17] Kvetnansky, R., Fukuhara, K., Pacák, K., Cizza, G., Goldstein, D.S. and Kopin, I.J. (1993) Endocrinology 133, 1411–1419.
[18] Krizanova, O., Novotova, M. and Zachar, J. (1990) FEBS Lett. 267, 311–315.
[19] Reeves, J.P. and Sutko, J.L. (1979) Proc. Natl. Acad. Sci. USA 76, 590–594.
[20] Chomczynski, P. and Sacchi, R. (1987) Anal. Biochem. 162, 156–159.
[21] Diamond, S.L., Sharefkin, J.B., Dieffenbach, C.W., McIntire, L.V., Frasier-Scott, K. and Eskin, S. (1990) J. Cell. Physiol. 143, 364–371.
[22] Sharefkin, J.B., Diamond, S.L., Eskin, S.G., McIntire, L.V. and Dieffenbach, C.W. (1991) J. Vasc. Surg. 14, 1–9.
[23] Dveksler, G.S., Basile, A.A. and Dieffenbach, C.W. (1992) PCR Methods Appl. 1, 283–285.
[24] Terada, Y., Tomita, K., Nonoguchi, H. and Marumo, F. (1993) Kidney Int. 43, 1251–1259.
[25] Lowry, O.H., Rosebrough, N.J., Farr, A.I. and Randal, R.J. (1951) J. Biol. Chem. 193, 256–272.
[26] Hudecova, S., Kvetnansky, R. and Krizanova, O. (1996) in: Stress: Molecular Genetic and Neurobiological Advances (McCarty, R., Aguilera, G., Sabban, E.L. and Kvetnansky, R., Eds.), Vol. 2, pp. 751–760, Gordon and Breach, New York.
[27] Sabban, E.L., Hiremagalur, B., Nankova, B. and Kvetnansky, R. (1995) Ann. NY Acad. Sci. 771, 327–338.
[28] Viskupic, E., Kvetnansky, R., Sabban, E.L., Fukuhara, K., Weise, V.K., Kopin, I.J. and Schwartz, J.P. (1994) J. Neurochem. 63, 808–814.
[29] Wang, J., Schwinger, R.H., Frank, K., Muller-Ehmsen, J., Martin-Vasallo, P., Pressley, T.A., Xiang, A., Erdmann, E. and McDonough, A.A. (1996) J. Clin. Invest. 98, 1650–1658.
[30] Smith, L. and Smith, J.B. (1994) J. Biol. Chem. 269, 27527–27531.

[31] Studer, R., Reinecke, H., Vetter, R., Holtz, J. and Drexler, H. (1997) Basic Res. Cardiol. 92, 53–58.
[32] Just, H. (1996) Eur. Heart J. 17, 2–7.
[33] Flesch, M., Schwinger, R.H., Schiffer, F., Frank, K., Sudkamp, M. and Kuhn-Regnier, F. (1996) Circulation 94, 992–1002.
[34] Drexler, H. (1994) Arch. Mal. Coeur Vaiss. 87, 13–16.
[35] Luciani, S., Antolini, M., Bova, S., Cargnelli, G., Cusinato, F., Debetto, P., Trevisi, L. and Varotto, R. (1995) Biochem. Biophys. Res. Commun. 206, 674–680.
[36] Yuan, C. and Acosta, D. (1994) J. Mol. Cell. Cardiol. 26, 1415–1419.
[37] Baum, V.C. and Wetzel, G.T. (1994) Anesth. Analg. 78, 1105–1109.
[38] Reinecke, H., Vetter, R. and Drexler, H. (1997) Cardiovasc. Res. 36, 216–222.
[39] Kvetnansky, R., Palkovits, M., Mitro, A., Torda, T. and Mikulaj, L. (1997) Neuroendocrinology 23, 257–267.
[40] Kvetnansky, R. and Sabban, E.L. (1993) in: Tyrosine Hydroxylase: From Discovery to Cloning (Naoi, M. and Parvez, S.H., Eds.), pp. 253–281, VSP, Utrecht.
[41] Nagatsu, T., Kobayashi, K. and Ichinose, H. (1995) Ann. NY Acad. Sci. 771, 159–165.