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Corticosteroids increase intracellular free sodium ion concentration via glucocorticoid receptor pathway in cultured neonatal rat cardiomyocytes[☆],[☆☆],[★]

Daisuke Katoh [*], Kenichi Hongo, Keiichi Ito, Takuya Yoshino, Yosuke Kayama, Makoto Kawai, Taro Date, Michihiro Yoshimura

Division of Cardiology, Department of Internal Medicine, The Jikei University School of Medicine, Tokyo 105-8461, Japan

A B S T R A C T

Background: Glucocorticoids as well as mineralocorticoid have been shown to play essential roles in the regulation of electrical and mechanical activities in cardiomyocytes. Excess of these hormones is an independent risk factor for cardiovascular disease. Intracellular sodium ($[Na^+]_i$) kinetics are involved in cardiac diseases, including ischemia, heart failure and hypertrophy. However, intrinsic mediators that regulate $[Na^+]_i$ in cardiomyocytes have not been widely discussed. Moreover, the quantitative estimation of altered $[Na^+]_i$ in cultured cardiomyocytes and the association between the level of $[Na^+]_i$ and the severity of pathological conditions, such as hypertrophy, have not been precisely reported.
Methods and results: We herein demonstrate the quantitative estimation of $[Na^+]_i$ in cultured neonatal rat cardiomyocytes following 24 h of treatment with corticosterone, aldosterone and dexamethasone. The physiological concentration of glucocorticoids increased $[Na^+]_i$ up to approximately 2.5 mM (an almost 1.5-fold increase compared to the control) in a dose-dependent manner; this effect was blocked by a glucocorticoid receptor (GR) antagonist but not a mineralocorticoid receptor antagonist. Furthermore, glucocorticoids induced cardiac hypertrophy, and the hypertrophic gene expression was positively and significantly correlated with the level of $[Na^+]_i$. Dexamethasone induced the upregulation of Na^+/Ca^{2+} exchanger 1 at the mRNA and protein levels.
Conclusions: The physiological concentration of glucocorticoids increases $[Na^+]_i$ via GR. The dexamethasone-induced upregulation of NCX1 is partly involved in the glucocorticoid-induced alteration of $[Na^+]_i$ in cardiomyocytes. These results provide new insight into the mechanisms by which glucocorticoid excess within a physiological concentration contributes to the development of cardiac pathology.

1. Introduction

The sodium ion (Na^+) is the primary determinant of the distribution of body fluids. While the extracellular Na^+ ($[Na^+]_o$) is ~140 mM, the concentration of free intracellular Na^+ ($[Na^+]_i$) is normally 4–16 mM, as exquisitely maintained by a series of ion channels and transporters [1–6]. This transsarcolemmal Na^+ gradient is a key regulator of various intracellular ions and metabolites. In the heart, $[Na^+]_i$ has been shown to increase in the presence of cardiac diseases, including ischemia, heart failure and hypertrophy [2–6]. Although the molecular mechanisms by which $[Na^+]_i$ increases in pathological conditions and the causal relationship between $[Na^+]_i$ and cardiac disease remain controversial, some reports have suggested that elevated $[Na^+]_i$ induces unfavorable effects in cardiomyocytes. For example, an ionophore monensin, which transports ions across the cell membrane and increases $[Na^+]_i$, has been reported to activate the hypertrophic gene expression via salt-inducible kinase 1 (SIK1), a kinase known to be critical for cardiac development, in a myocyte cell line [7]. On the other hand, elevated $[Na^+]_i$ increases the mitochondrial formation of reactive oxygen species in failing cardiac myocytes [8,9]. Moreover, a rise in $[Na^+]_i$ reduces Ca^{2+} extrusion via the Na^+/Ca^{2+} exchanger (NCX), which induces diastolic Ca^{2+} overload [5]. These reports suggest that elevated $[Na^+]_i$ in cardiomyocytes is a trigger for the development of pathological conditions in the heart.

Corticosteroids, including aldosterone and cortisol (in humans)/corticosterone (in rodents), regulate the absorption of sodium ions in

renal tubules via a member of the steroid receptor superfamily, mineralocorticoid receptor (MR). An excess of these hormones induces sodium retention, thereby causing hypertension [10,11]. Moreover, high levels of cortisol and aldosterone are known to be independent risk factors for cardiovascular events [11–13]. This may be partly due to the direct alteration of mineralocorticoid receptor (MR) signaling in the cardiovascular system in addition to secondary systemic activities of these hormones, such as the induction of hypertension, as MR antagonists are responsible for marked prognostic improvements in patients with heart failure [14–16]. In cardiomyocytes, MR is expressed, while 11beta-hydroxysteroid dehydrogenase type 2, which converts glucocorticoids to their inactive metabolites, is not [17,18]. Hence, under physiological conditions, most MRs are presumably occupied by cortisol/corticosterone. On the other hand, glucocorticoid receptor (GR), which displays high sequence homology with MR and binds glucocorticoids with higher affinity than the mineralocorticoid aldosterone, is also expressed in cardiomyocytes. However, the role of specific GR signaling in the cardiovascular system is poorly understood.

We and others have recently reported that aldosterone induces $[Na^+]_i$ elevation in cultured cardiomyocytes and that this effect is rapid and non-genomic and occurs in a mineralocorticoid receptor-independent fashion [19,20]. Although there is a previous report that 24-hour treatment with aldosterone activates Na^+/H^+ exchange (NHE) and increases $[Na^+]_i$ in cardiomyocytes using a sodium fluorescent indicator [21], the absolute value of the alteration in $[Na^+]_i$ is not available. To our knowledge, the effects of glucocorticoid/GR signaling on $[Na^+]_i$ handling in cardiomyocytes have not been previously reported. Moreover, the relationship between the severity of a pathological status, such as cardiac hypertrophy, and the level of $[Na^+]_i$ in cardiomyocytes has not been discussed.

The present study was conducted to identify the role of corticosteroids, including corticosterone, aldosterone and synthesized glucocorticoid dexamethasone, in the regulation of $[Na^+]_i$ in cardiomyocytes. The results suggest that NCX1 is involved, at least in part, in the pathogenesis of altered sodium ion handling under conditions of glucocorticoid excess via the GR pathway.

2. Materials and methods

2.1. Reagents

Corticosterone, dexamethasone, eplerenone and RU486 were purchased from Sigma-Aldrich. Aldosterone was purchased from Wako
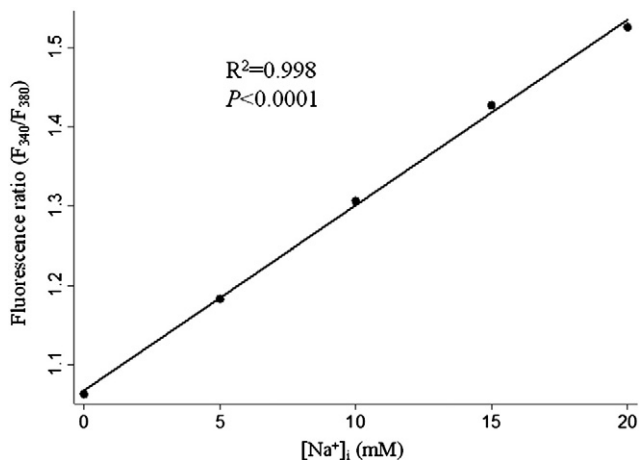
Fig. 1. *In vivo* calibration of SBFI. The *in vivo* calibration of SBFI was accomplished by exposing NRVM to various extracellular $[Na^+]$. Between 0 and 20 mM $[Na^+]_i$, the SBFI fluorescence ratio (F_{340}/F_{380}) exhibited a linear relationship with $[Na^+]_i$ ($R^2 = 0.998$, $P < 0.0001$).
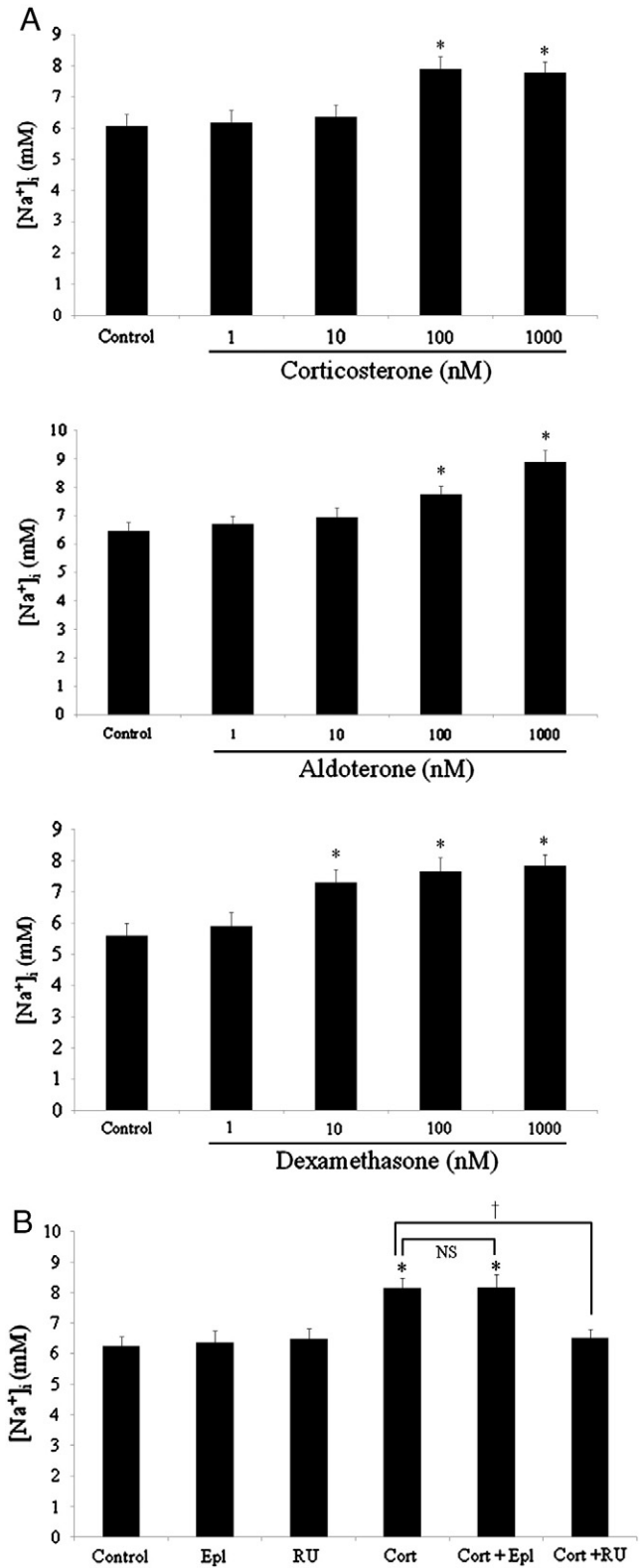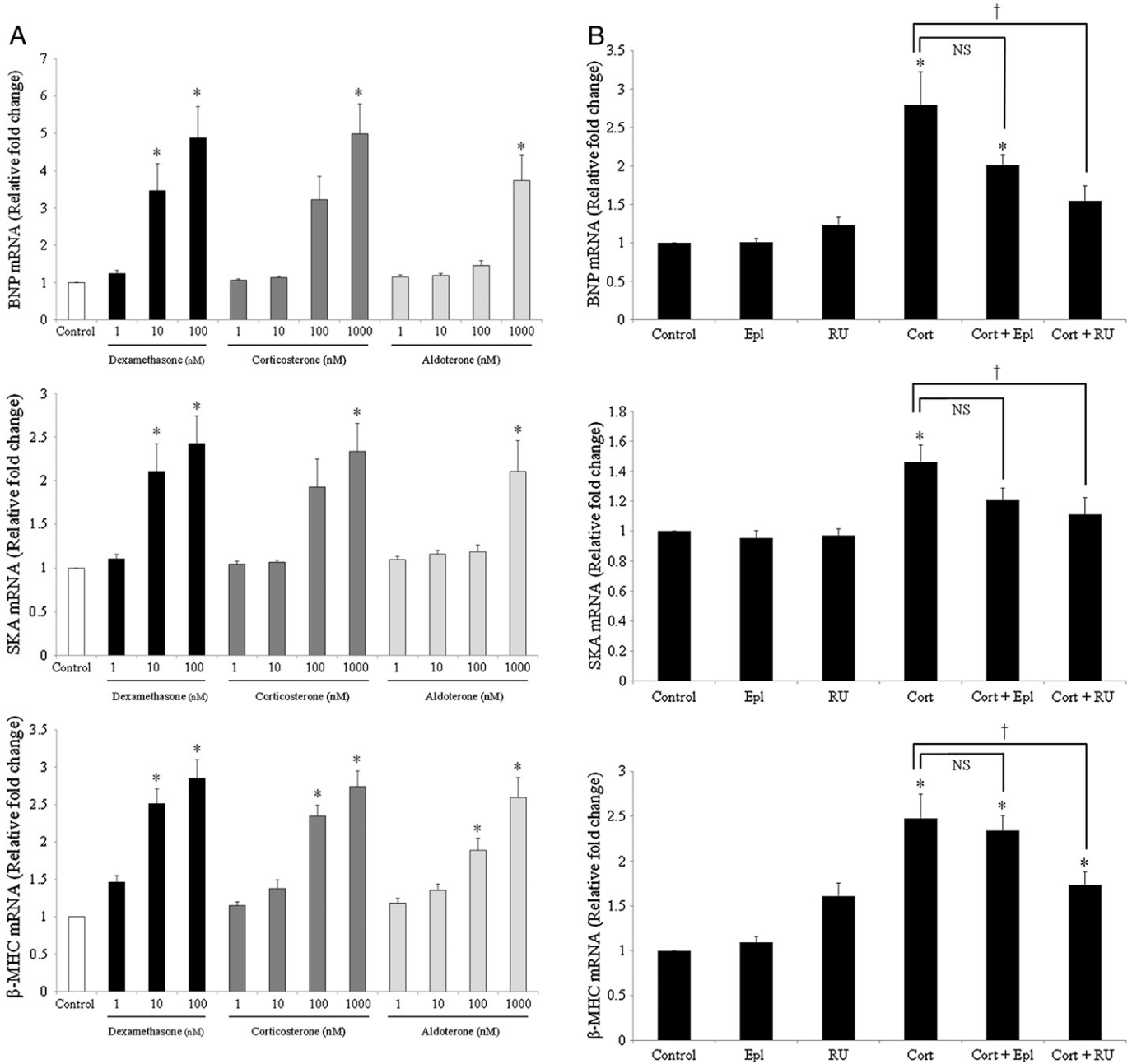

Fig. 2. Corticosteroids increase $[Na^+]_i$ via GR in NRVM. NRVM were treated with corticosterone, aldosterone and dexamethasone at concentrations of 1 to 1000 nM in the presence or absence of 1 μM of RU486 or 10 μM of eplerenone for 24 h. The quantitative estimation of $[Na^+]_i$ was carried out after treatment. A: corticosterone, aldosterone and dexamethasone increased $[Na^+]_i$ in a dose-dependent manner. The data represent the mean ± SEM of six to nine independent experiments. B: the corticosterone-induced $[Na^+]_i$ elevation was blocked by RU486 but not eplerenone. The data represent the mean ± SEM of seven independent experiments. Cort, 100 nM of corticosterone; Epl, 10 μM of eplerenone; RU, 1 μM of RU486. *$P < 0.05$ versus control group, †$P < 0.05$.

Pure Chemical Industries. Sodium-binding benzofuran isophthalate-acetoxymethylester (SBFI-AM) and Pluronic F-127 were purchased from Invitrogen. All primer sets for PCR were purchased from Applied Biosystems. Antibodies were purchased from the following companies: anti-NCX1 (Abcam); anti-NHE-1 (Santa Cruz Biotechnology); and anti-β-actin (Sigma-Aldrich).

2.2. Cell culture

All animal procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Research Committee of Jikei University. Neonatal rat ventricular myocytes (NRVM) were isolated from 1- to 3-day-old Sprague–Dawley rats according to the manufacturer's protocol from Worthington Biochemical. Purified NRVM were plated at a density of $1 * 10^5$ cells/well in 96-well clear bottom plates (Greiner) in low-glucose (1000 mg/l) DMEM (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen), 10% horse serum (Invitrogen), 20 mM HEPES, 200 μmol/l of bromodeoxyuridine, and antibiotics (100 U/ml of penicillin G and 100 μg/ml of streptomycin). The cells were allowed to attach at 37 °C in a 5% CO_2 atmosphere, and subconfluent myocyte monolayers were obtained after 48–72 h. Sixteen hours before treatment with the indicated agents, the medium was replaced with DMEM supplemented with 1% bovine serum albumin (Sigma-Aldrich).

Fig. 3. Corticosteroids induce hypertrophy in NRVM. The mRNA levels of the cardiac hypertrophic marker genes BNP, SKA and β-MHC in NRVM were determined using real-time PCR and normalized to GAPDH (A and B). A: NRVM were treated with the indicated dose of corticosterone, aldosterone and dexamethasone for 48 h. The data represent the mean ± SEM of six independent experiments. B: NRVM were treated with 100 nM of corticosterone in the presence or absence of 1 μM of RU486 or 10 μM of eplerenone for 48 h. The data represent the mean ± SEM of seven independent experiments. Cort, 100 nM of corticosterone; Epl, 10 μM of eplerenone; RU, 1 μM of RU486. *$P < 0.05$ versus control group, †$P < 0.05$. C: representative microscopic images of NRVM treated with 100 nM of dexamethasone in the presence or absence of 1 μM of RU486.
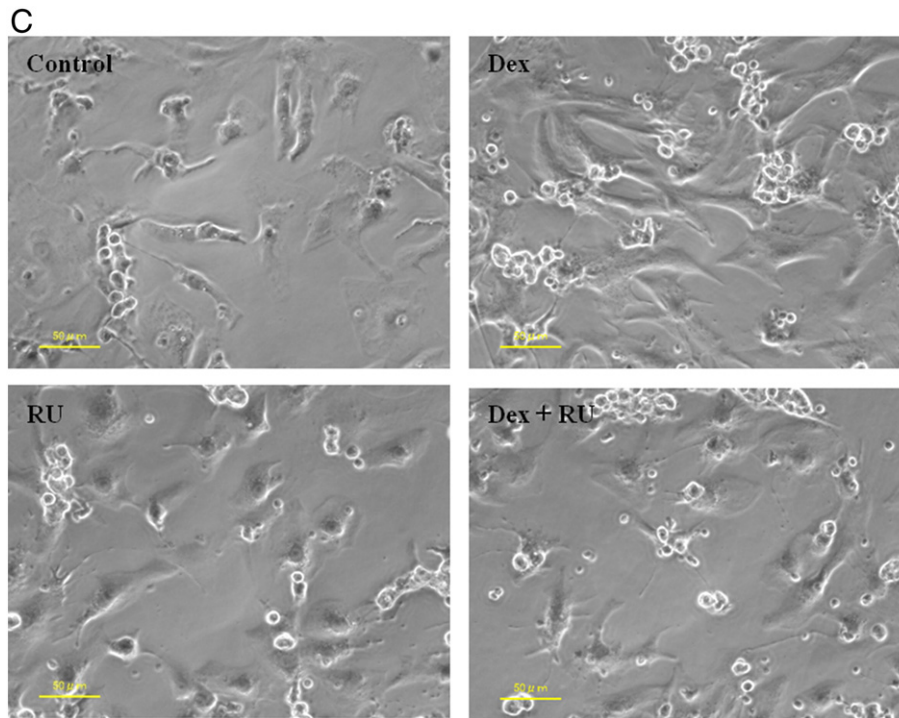
Fig. 3 (*continued*).

2.3. Measurement of $[Na^+]_i$ in NRVM

We measured $[Na^+]_i$ in NRVM using a microplate reader as previously reported with some modifications [22–25]. Briefly, NRVM were loaded with 5 μM of SBFI-AM dissolved in DMEM supplemented with 1% bovine serum albumin for 3 h at 37 °C in the presence of the nonionic surfactant, Pluronic F-127 (0.05% w/v). After washing out the external dye three times with Tyrode's solution (150 NaCl, 5.4 KCl, 1.2 MgCl$_2$, 0.4 NaH$_2$PO$_4$, 10 HEPES, 5 glucose and 1 CaCl$_2$, pH 7.4) in the presence of 1 mM of probenecid, leaving a final volume of 100 μl in each well, the fluorescence intensity was measured using an Infinite 200 PRO microplate reader (TECAN) at room temperature. Dual excitation measurements at 340 nm and 380 nm were obtained, and the emitted fluorescence was recorded at 510 ± 12.5 nm in the fluorescence bottom reading mode. The completion of hydrolysis was judged based on the attainment of a stable 340/380 nm ratio. The microplate reader is able to obtain measurements in each well of a plate within 90 s, and the fluorescence intensity was automatically recorded every 10 min. In each microplate, NRVM of the same preparation in 10 wells were prepared in the absence of SBFI to measure the background signals of the NRVM and microplates. The mean fluorescence signals from the 10 SBFI-unloaded wells at 340 nm and 380 nm were subtracted from the individual signals of the SBFI-loaded wells at each wavelength [22].

2.4. In vivo calibration of SBFI

The *in vivo* calibration of SBFI was accomplished, similar to that described in previous reports, by exposing the cardiomyocytes to various concentrations of extracellular $[Na^+]$ (0–20 mM) in the presence of 1 mg/l Gramicidin D (MP Biomedicals), 100 μM of strophanthidin (Sigma-Aldrich) and 2 mM EGTA, and the pH was adjusted to 7.1 with Tris base [22–25]. The solutions were prepared by mixing two solutions of equal ionic strength. One solution contained 145 mM Na$^+$ (30 mM NaCl, 115 mM sodium gluconate) without K$^+$, while the other contained 145 mM K$^+$ (30 mM KCl, 115 mM potassium gluconate) without Na$^+$. Calibration was performed at the end of each experiment.

2.5. Real-time PCR

Total RNA was extracted from cardiomyocytes cultured in 12-well plates using the PureLink RNeasy mini Kit (Applied Biosystems) according to the manufacturer's instructions. Two-step PCR was performed with TaqMan Reverse Transcription Reagents and a TaqMan Universal Master Mix Kit and quantified with the StepOne Real-time PCR system (Applied Biosystems). The expression of each gene was normalized to the level of GAPDH, which was stable under our experimental conditions.

2.6. Immunoblotting

Whole cell extracts were prepared in cell lysis buffer (Cell Signaling) and phenylmethylsulfonyl fluoride on ice and boiled LDS sample buffer (Novex). Equivalent amounts of protein were separated on 4–12% bis–Tris gel (Invitrogen) and electrically transferred to nitrocellulose or polyvinyl difluoride membranes (Invitrogen). The membranes were blocked with starting block (Thermo Fisher scientific) for 30 min at room temperature and incubated with primary antibodies overnight at 4 °C. The membranes were then washed with Tris-buffered saline with Tween 20, incubated with the appropriate secondary antibodies for 45 min at room temperature and developed using an enhanced chemiluminescence method according to the manufacturer's protocol (Thermo Fisher Scientific).

2.7. Statistical analysis

The data are expressed as the mean $\pm$ standard error of the indicated number of experiments. The statistical analyses were performed using Student's *t* test and one way ANOVA, followed by Tukey's test. Values of $P < 0.05$ were considered to be significant.

3. Results

3.1. Corticosteroids increase $[Na^+]_i$ in NRVM via GR

Fig. 1 shows a typical *in vivo* calibration experiment for SBFI in NRVM. Between 0 and 20 mM $[Na^+]_i$, the SBFI fluorescence ratio (340/380 nm) exhibited a linear relationship with $[Na^+]_i$ (coefficient correlation (R^2) of 0.998, $P < 0.0001$). The value of $[Na^+]_i$ in untreated NRVM calculated using our method was 6.1 ± 0.1 mM (n = 30). To examine the long-term effects of corticosteroids on $[Na^+]_i$, cardiomyocytes were treated with corticosterone, aldosterone and dexamethasone at a (patho)physiological concentration ranging from 1 to 1000 nM each. As shown in Fig. 2A, corticosterone, aldosterone and dexamethasone increase $[Na^+]_i$ in a dose-dependent manner, while dexamethasone has 10 times the potency of corticosterone and 100 times the potency of aldosterone. These data suggest that GR is involved in altered $[Na^+]_i$ handling, in accordance with the findings of previous reports showing the affinity of steroids to the GR [26–28]. The maximum increase in $[Na^+]_i$ in cardiomyocytes induced by these steroid agents was approximately 2.5 mM (an almost 1.5-fold increase compared to the control).

In order to examine the importance of GR signaling in the corticosteroid-induced $[Na^+]_i$ increase, we employed a GR antagonist, RU486, and an MR antagonist, eplerenone. Treatment with 1 μM of RU486 abolished the increase in $[Na^+]_i$ induced by 100 nM of corticosterone, which bound to both GR and MR in the cardiomyocytes, although 10 μM of eplerenone showed no effects (Fig. 2B). These results indicate that GR is required for altered $[Na^+]_i$ handling in cardiomyocytes treated with corticosteroids.

3.2. Corticosteroids induce cardiac hypertrophy via GR

As shown in Fig. 3A, corticosteroids induced the hypertrophic gene expression, including that of B-type natriuretic peptide (BNP), skeletal muscle alpha-actin (SKA) and beta-myosin heavy chain (β-MHC), in a dose-dependent manner. These effects were blocked by 1 μM of RU 486 but not 10 μM of eplerenone (Fig. 3B). Fig. 3C shows the changes in cell morphology of NRVM treated with 100 nM of dexamethasone in the presence or absence of 1 μM of RU486. Dexamethasone induced a marked increase in the cell surface area; this effect was abrogated by RU486, compatible with the results showing that corticosteroids induce the hypertrophic gene expression, and the findings of previous reports by others regarding the effects of glucocorticoids on morphological changes in cardiomyocytes [26,29].

3.3. Close association between $[Na^+]_i$ and the cardiac hypertrophic gene expression levels in NRVM treated with corticosteroids

As shown in Fig. 4, a close and significant positive association was observed between the level of $[Na^+]_i$ and the hypertrophic gene expression in NRVM treated with the indicated corticosteroids. This finding indicates that the extent of altered $[Na^+]_i$ is associated with the severity of pathophysiological conditions, such as hypertrophy, in cardiomyocytes.

3.4. NCX1 mRNA is induced by dexamethasone

In order to determine the mechanisms underlying the glucocorticoid-induced elevation of $[Na^+]_i$, we investigated the effects of dexamethasone on the expression of transporters that handle Na^+ influx in cardiomyocytes. Fig. 5A shows the mRNA expressions of NCX1, NHE-1 and $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1), the main transporters that influx Na^+ in cardiomyocytes [1]. Forty-eight hours of treatment with dexamethasone induced the NCX1 expression at the mRNA and protein levels in a dose-dependent manner; this effect was abrogated by 1 μM of RU-486 (Fig. 5A). A higher concentration of dexamethasone significantly
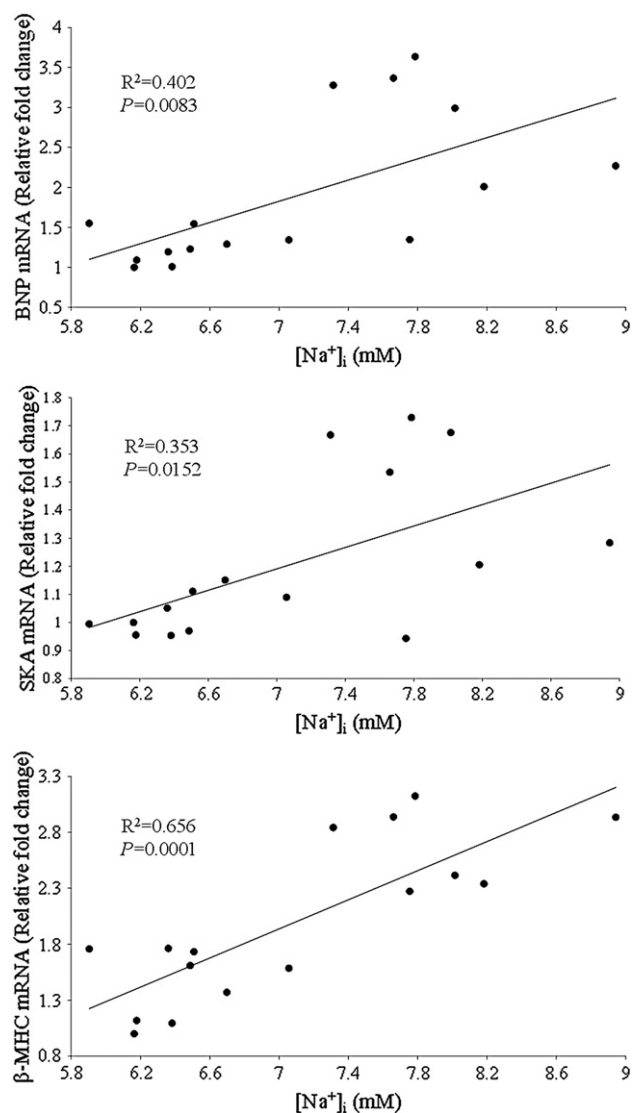

Fig. 4. Association between $[Na^+]_i$ and markers of cardiac hypertrophy. NRVM were treated with vehicle, corticosterone, aldosterone and dexamethasone at concentrations of 1 to 1000 nM in the presence or absence of 1 μM of RU486 or 10 μM of eplerenone for 48 h. The relationship between $[Na^+]_i$ and the hypertrophic gene expression was analyzed by using a linear regression analysis. The coefficient of determination (R^2) and P value are indicated in each panel.

reduced the NHE-1 mRNA expression. On the other hand, the NKCC1 mRNA expression was not changed by treatment with dexamethasone.

3.5. NCX1 proteins are induced by dexamethasone

The protein level of NCX1 was induced by treatment with 100 nM of dexamethasone; this effect was blocked by 1 μM of RU486, according to the mRNA expression. On the other hand, the NHE-1 protein level was not changed by treatment with dexamethasone (Fig. 5B). Dexamethasone induced the upregulation of NCX1 at the mRNA level after 12 h and at the protein levels after 18 h (Fig. 5C).

4. Discussion

In the present study, we found that chronic physiological concentrations of glucocorticoid treatment increased $[Na^+]_i$ in a dose-dependent manner in NRVM up to approximately 2.5 mM (an almost 1.5-fold

increase compared to the control) quantified using the sodium fluorescent indicator SBFI and *in vivo* calibration method. Supraphysiological concentrations (higher than 100 nM) of aldosterone also increased

$[Na^+]_i$. Furthermore, we confirmed that these effects were abrogated by a GR antagonist, but not an MR antagonist, suggesting that GR signaling is essential for $[Na^+]_i$ handling in cardiomyocytes.
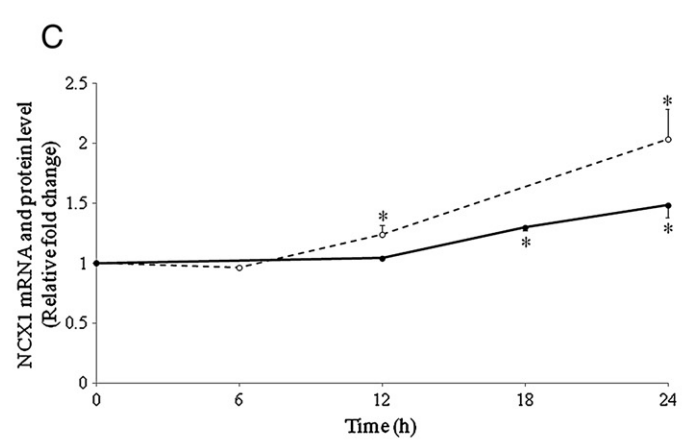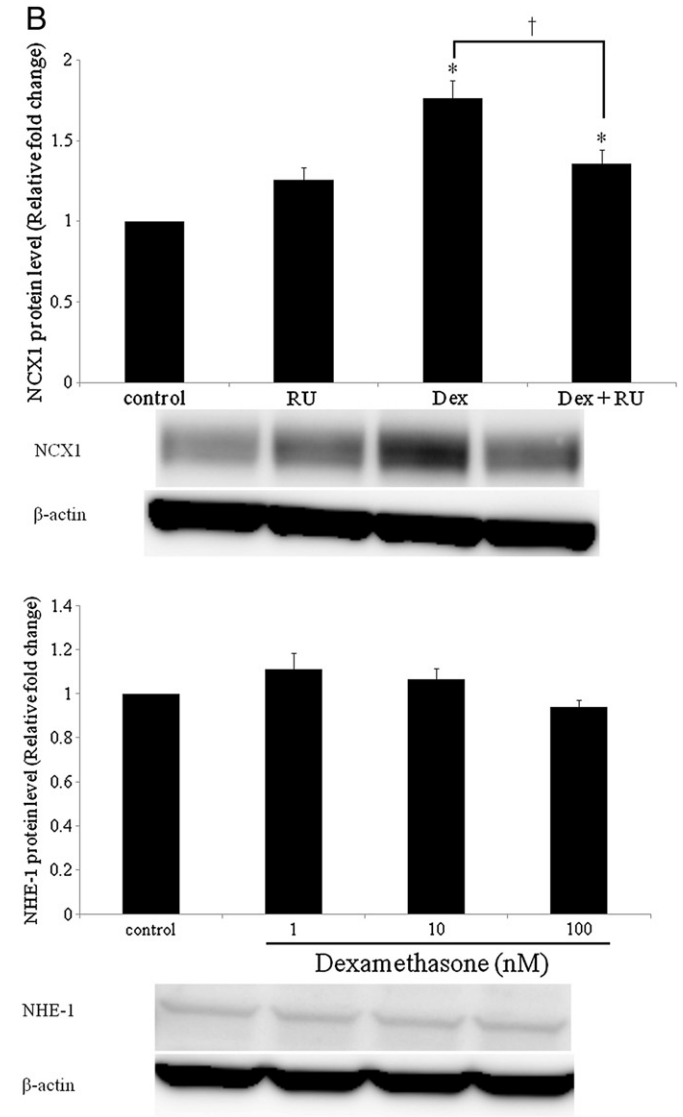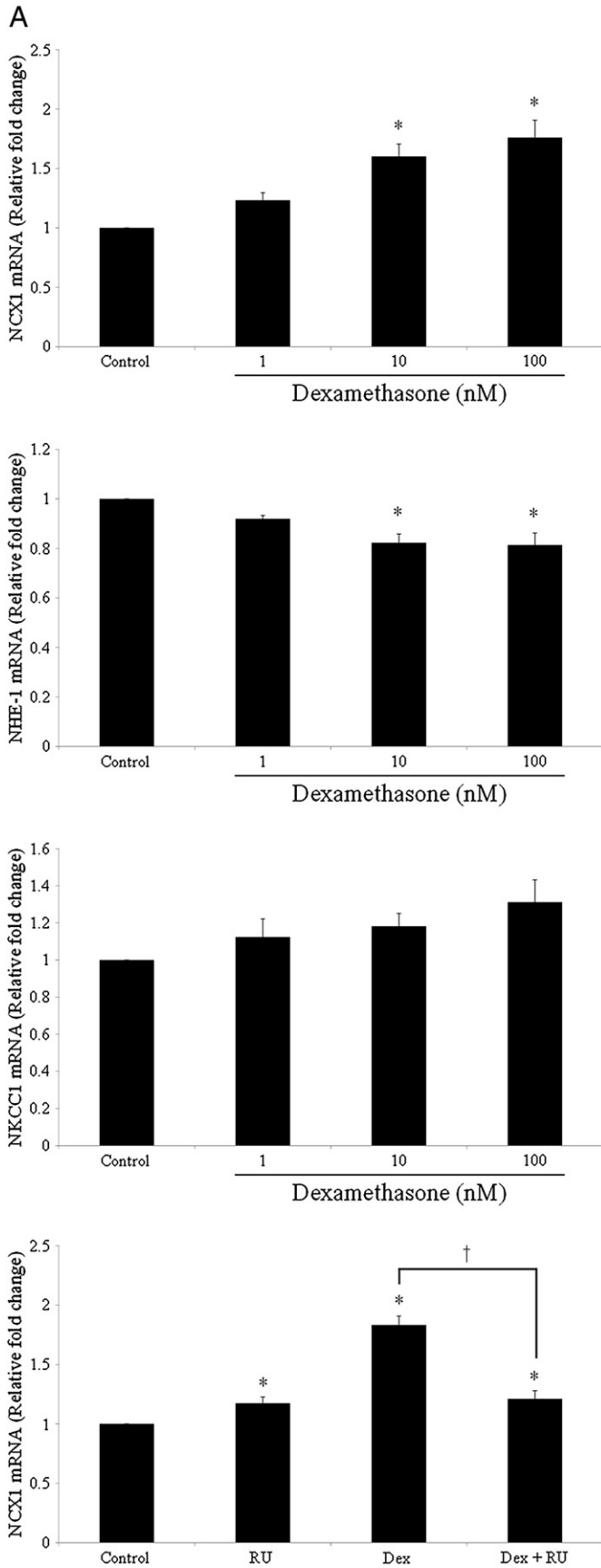
Few studies have tested the effects of intrinsic molecules (e.g. hormones) in altering $[Na^+]_i$ handling. For example, hyperthyroidism increases $[Na^+]_i$ and affects the expression of Na^+ channels, NCX and NHE [30]. On the other hand, $\alpha1$- or β-adrenoceptor stimulation reduces $[Na^+]_i$ [31–33]. We and others have previously reported that aldosterone rapidly induces $[Na^+]_i$ elevation in cultured cardiomyocytes in an MR-independent fashion [19,20]. Others have also reported that 24 h of treatment with 100 nM of aldosterone activates NHE-1 at the protein level in cardiomyocytes via MR and increases $[Na^+]_i$ measured according to the sodium indicator, sodium green [21]. However, the absolute value of the aldosterone-induced alteration in $[Na^+]_i$ remains unclear because no calibration methods were used in these studies. Furthermore, the variable dye concentrations in the cells and/or the cell size may affect the intensity of fluorescent indicator sodium green due to its single excitation measurements [20,21]. Even minor changes in global $[Na^+]_i$ have a large impact on the cell function, primarily because small increases in $[Na^+]_i$ affect Ca^{2+} fluxes through NCX, and the force of contraction has been reported to double with as little as a 1-mM increase in $[Na^+]_i$ [34]. Therefore, the accuracy of measurements of $[Na^+]_i$ is critical in studies of $[Na^+]_i$ in cardiomyocytes. In the present study, we measured $[Na^+]_i$ in NRVM using a microplate reader and employed *in vivo* calibration of SBFI, which exhibited adequate accuracy, comparable to that of the traditional microscopy-based method (Fig. 1). The value of $[Na^+]_i$ in untreated quiescent NRVM calculated using our method was 6.1 ± 0.1 mM, similar to the findings ranging from 5 to 13 mM in previous reports of neonatal cardiomyocytes [35, 36] and adult cells [23,24,37] measured using microscopy or spectrophotometers. The maximum increase in $[Na^+]_i$ in cardiomyocytes induced by corticosteroids was approximately 2.5 mM, which is a slightly smaller alteration than that previously reported in heart failure models [2–5]. This glucocorticoid-induced modest increase in $[Na^+]_i$ would have an impact on the subsequent development of cardiac pathology.

In the present study, we also confirmed that glucocorticoids induce hypertrophy in cardiomyocytes via GR. Chronic treatment with physiological concentrations of corticosteroids and supraphysiological concentrations of aldosterone upregulated the hypertrophic gene expression as well as the incidence of morphological changes, in agreement with the findings of previous reports [19,21,26,29,38–41]. Moreover, the corticosteroid-induced hypertrophic gene expression levels were positively and significantly associated with the level of $[Na^+]_i$, indicating that elevated $[Na^+]_i$ is involved in the pathogenesis of hypertrophy. Previously, an ionophore-induced small increase in $[Na^+]_i$ has been reported to activate the hypertrophic gene expression via SIK 1 in a myocyte cell line [7]. That study showed that $[Na^+]_i$ is an independent regulator of the transcription and activation of the gene expression in an atrial myocyte cell line. These results indicate that small increases in $[Na^+]_i$ induce cardiac hypertrophy, although further investigation is needed to clarify whether the glucocorticoid-induced elevation of $[Na^+]_i$ affects the hypertrophic gene expression via the SIK1 pathway and whether this phenomenon is applicable to ventricular cells.

$[Na^+]_i$ is determined by the balance between Na^+ influx and efflux. There are several pathways for Na^+ entry into cells, including the NCX, NHE, NKCC, and Na^+/HCO_3^- cotransporter and Na^+ channels [1,6]. In the present study, short-term exposure (<6 h) to dexamethasone did not increase $[Na^+]_i$ (data not shown), and the glucocorticoid-induced elevation in $[Na^+]_i$ was a GR-dependent genomic reaction. These results suggest that GR signaling modifies the expression of transporters that regulate $[Na^+]_i$. In this study, we found that chronic treatment with dexamethasone induced the NCX1 expression at both the mRNA and

protein levels in a dose-dependent manner; this effect was abrogated by a GR blocker. However, chronic dexamethasone treatment did not affect the NKCC1 mRNA expression or NHE-1 protein level (NHE-1 mRNA was slightly downregulated by 100 nM of dexamethasone). NCX, which transports three Na^+ ions in exchange for one Ca^{2+} ion, is the main route for Na^+ influx in resting cardiac cells, and NCX1 is the major isoform in cardiomyocytes [42]. Taken together, we speculate that NCX1 is involved in the glucocorticoid-induced elevation of $[Na^+]_i$. On the other hand, NCX has been reported to be highly expressed in late fetal and neonatal rat hearts, decreasing to an adult level for RNA by 20 days after birth [43]. Therefore, the involvement of NCX in $[Na^+]_i$ handling and hypertrophy in NRVM may be different from that observed in adult models. Further studies are needed to confirm the contribution of NCX1 in the glucocorticoid-induced alteration of $[Na^+]_i$ in cardiomyocytes.

5. Conclusions

We observed a significant role of corticosteroids in regulating $[Na^+]_i$ via the GR pathway in cardiomyocytes. We also confirmed that glucocorticoids induce hypertrophy in cardiomyocytes via GR. The corticosteroid-induced elevation of $[Na^+]_i$ was closely and significantly associated with the hypertrophic gene expression. The dexamethasone-induced upregulation of NCX1 is partly involved in the glucocorticoid-induced alteration of $[Na^+]_i$ in cardiomyocytes. These results provide new insight into the mechanisms by which glucocorticoid excess within a physiological concentration contributes to the development of cardiac pathology.

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Fig. 5. Effects of glucocorticoids on the expressions of NCX1, NHE-1, and NKCC1. NRVM were treated with the 100 nM of dexamethasone in the presence or absence of 1 μM of RU486 for 48 h. A: the mRNA levels of NCX1, NHE-1 and NKCC1 in the NRVM were determined using real-time PCR and normalized to GAPDH. The data represent the mean $\pm$ SEM of six independent experiments. Dex, 100 nM of dexamethasone; RU, 1 μM of RU486. *$P < 0.05$ versus control group, †$P < 0.05$. B: The NCX1 and NHE-1 protein levels in the NRVM were measured using immunoblotting. The data represent the mean $\pm$ SEM of six independent experiments. *$P < 0.05$ versus control group, †$P < 0.05$. C: time course of the NCX1 mRNA and protein levels in the dexamethasone-treated NRVM. NRVM were treated with 100 nM of dexamethasone for various durations. The relative fold changes in the mRNA (*open circles*) and protein levels (*closed circles*) of NCX1 at each time point are indicated. The data represent the mean $\pm$ SEM of four independent experiments. *$P < 0.05$ versus cells treated with vehicle.

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