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Characterization of Noradrenaline-Induced Increases in Intracellular Ca^{2+} Levels in Chinese Hamster Ovary Cells Stably Expressing Human α_{1A}-Adrenoceptor

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Abstract. The mechanism for noradrenaline (NA)-induced increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and physiological significance of Na^+ influx through receptor-operated channels (ROCs) and store-operated channels (SOCs) were studied in Chinese hamster ovary (CHO) cells stably expressing human α_{1A}-adrenoceptor (α_{1A}-AR). $[Ca^{2+}]_i$ was measured using the Ca^{2+} indicator fura-2. NA (1 μM) elicited transient and subsequent sustained $[Ca^{2+}]_i$ increases, which were inhibited by YM-254890 ($G_{\alpha q/11}$ inhibitor), U-73122 (phospholipase C (PLC) inhibitor), and bisindolylmaleimide I (protein kinase C (PKC) inhibitor), suggesting their dependence on $G_{\alpha q/11}$/PLC/PKC. Both phases were suppressed by extracellular Ca^{2+} removal, SK&F 96365 (inhibitor of SOC and nonselective cation channel type-2 (NSCC-2)), LOE 908 (inhibitor of NSCC-1 and NSCC-2), and La^{3+} (inhibitor of transient receptor potential canonical (TRPC) channel). Reduction of extracellular Na^+ and pretreatment with KB-R7943, a Na^+/Ca^{2+} exchanger (NCX) inhibitor, inhibited both phases of $[Ca^{2+}]_i$ increases. These results suggest that 1) stimulation of α_{1A}-AR with NA elicits the transient and sustained increases in $[Ca^{2+}]_i$ mediated through NSCC-2 that belongs to a TRPC family; 2) Na^+ influx through these channels drives NCX in the reverse mode, causing Ca^{2+} influx in exchange for Na^+ efflux; and 3) the $G_{\alpha q/11}$/PLC/PKC-dependent pathway plays an important role in the increases in $[Ca^{2+}]_i$.

Keywords: α_{1A}-adrenoceptor, store-operated channel, nonselective cation channel, Na^+/Ca^{2+} exchanger, intracellular free Ca^{2+} concentration

Introduction

The α_{1A}-adrenoceptor (α_{1A}-AR) belongs to the superfamily of G protein-coupled receptors (GPCRs) that transduce the binding of their agonists such as the neurotransmitter noradrenaline (NA) into activation of G protein-regulated effectors and elevation of corresponding second messengers. In general, stimulation of α_{1A}-ARs with NA activates $G_{q/11}$ protein-coupled phospholipase Cβ (PLCβ) to increase the second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), thereby causing an initial transient increase and a subsequent sustained increase in $[Ca^{2+}]_i$ (1). In most nonexcitable cells including Chinese hamster ovary (CHO) cells, this interdependent mobilization of Ca^{2+} is considered to be attributed to rapid, transient release of Ca^{2+} stored in the endoplasmic reticulum (ER), followed by slowly developing Ca^{2+} entry through store-operated channels (SOCs) and/or receptor-operated channels (ROCs) such as Ca^{2+}-permeable nonselective cation channels (NSCCs) (2 – 5). Our previous studies have demonstrated that NA-induced sustained Ca^{2+} influx through NSCCs plays an essential role in cell proliferation and arachidonic acid release following stimulation of α_{1A}-AR expressed in CHO cells (1, 6). Interestingly, Mori and colleagues (7) have demonstrated that the essential component of NA-activated NSCC is the transient receptor potential canonical (TRPC) channel homologue TRPC6 since heterologous expression of murine TRPC6 in human embryonic

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kidney (HEK293) cells reproduced the biophysical and pharmacological properties of NA-activated NSCC. These findings suggest that voltage-independent (non-voltage-gated) cation channels (SOCs, ROCs, NSCCs, and TRPC channels) are involved in NA-induced Ca^{2+} influx.

Ca^{2+} signal plays a key role in controlling diverse cellular functions such as contraction, proliferation, and transcription (8). GPCR mediating Ca^{2+} influx across the plasma membrane, therefore, is strictly regulated by complicated mechanisms involving cation channels such as SOCs and ROCs (2, 9). On the other hand, many studies have shown that most of these channels allow the entry of Na^+ in addition to Ca^{2+} and the permeability to Na^+ varies from one channel to another (9 – 11). This implies that stimulation of GPCR can increase intracellular Na^+ concentration ($[Na^+]_i$) via cation channels possessing properties of NSCCs. In this regard, recent evidence suggests that Na^+ influx plays an important role in GPCR-mediated increase in $[Ca^{2+}]_i$. In isolated cardiac cells, stimulation of $G_{q/11}$ protein-coupled endothelin type A receptor (ET_AR) with its agonist endothelin-1 (ET-1) activates the Na^+/H^+ exchanger (NHE) via a PKC-dependent pathway, causing an increase in $[Na^+]_i$ that in turn drives the Na^+/Ca^{2+} exchanger (NCX) operating in the reverse mode to transport Ca^{2+} into cells in exchange for Na^+ efflux, leading to an increase in $[Ca^{2+}]_i$ (12 – 14). Moreover, activation of NHE by NA was observed in CHO cells expressing α_{1A}-AR (15), where mRNA expression for three members (NCX1, NCX2, and NCX3) of NCX was detected by RT-PCR (16). These raise the possibility that α_{1A}-AR modulates Na^+/Ca^{2+} exchange via increased Na^+ influx through NSCCs or NHE, resulting in an increase in $[Ca^{2+}]_i$.

The present study attempted to elucidate the mechanisms for NA-induced increase of $[Ca^{2+}]_i$ in CHO cells expressing human α_{1A}-AR using inhibitors for $G_{q/11}$ protein-regulated signaling molecules, cation channels, and transporters. In addition, the functional significance of Na^+ entry and NCX in the NA-induced $[Ca^{2+}]_i$ elevation were also examined using reduction of extracellular Na^+ concentrations and KB-R7943, an inhibitor of NCX, respectively.

Materials and Methods

Materials

The pCR3 mammalian expression vector containing human α_{1A}-AR cDNA was kindly provided by Dr. Ikunobu Muramatsu (Fukui University, Japan).

YM-254890 and LOE 908 ((*R,S*)-(3,4-dihydro-6,7-dimethoxy-isoquinoline-1-yl)-2-phenyl-*N,N*-di-[2-(2,3,4-trimethoxyphenyl)ethyl]-acetamide) were kindly provided by Astellas Pharma, Inc. (Tokyo) and Nippon Boehringer Ingelheim Co., Ltd. (Hyogo), respectively.

(−)-Noradrenaline (+)-bitartrate, 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), amiloride, lanthanum (III) chloride, G418, and probenecid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bisindolylmaleimide I (BIS I, 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide), U-73122 (1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino) hexyl]-1*H*-pyrrole-2,5-dione), SK&F 96365 (1-[β-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl]-1*H*-imidazole), and KB-R7943 (2-(2-(4-(4-nitrobenzyloxy) phenyl)ethyl)isothiourea) were from Calbiochem (San Diego, CA, USA). Fura-2/acetoxymethyl ester (fura-2/AM), fluo-3/AM, and Pluronic F-127 were from Dojindo Laboratories (Kumamoto). The other regents used were of the highest grade in purity.

Cell culture

CHO cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin and maintained at 37°C in humidified air with 5% CO_2.

Stable expression of human α_{1A}-AR in CHO cells

The pCR3 mammalian expression vector containing human α_{1A}-AR cDNA was transfected into CHO cells using a TransIT™-CHO transfection kit (Mirus Bio Corporation, Madison, WI, USA) according to the manufacturer's instructions. Transfected cells were selected using resistance to 800 $\mu g \cdot ml^{-1}$ G418.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured as described previously (16). Briefly, CHO cells were incubated with 4 μM fura-2/AM or 10 μM fluo-3/AM admixed with 2.5 mM probenecid and 0.04% Pluronic F-127 at 37°C for 45 min under reduced light. After collecting and washing cells, the cells were suspended in Ca^{2+}-free Krebs-HEPES solution (140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 11 mM D-(+)-glucose, 10 mM HEPES; adjusted to pH 7.3 with LiOH) at 4×10^5 cells/ml. $CaCl_2$ was added to 0.5-ml aliquot of the cell suspension at the final concentration of 1 mM, when necessary. Changes of $[Ca^{2+}]_i$ in cells were measured at 25°C using a CAF-110 spectrophotometer (JASCO, Tokyo) with the excitation wavelengths of 340 and 380 nm and emission wavelength of 500 nm for fura-2 and the emission wavelength of 540 nm and the excitation wavelength of 490 nm for fluo-3.

In the experiments with EIPA and amiloride, $[Ca^{2+}]_i$ was measured using fluo-3 instead of fura-2 because these drugs at the concentrations used in the present

study was found to interfere with the fluorescence signals of fura-2 (data not shown).

Data analyses

Data were collected and analyzed using a MacLab/8s and Chart (v. 3.5) software (ADInstruments Japan, Tokyo). The concentration-response curves for NA were constructed to evaluate its EC_{50} value, which is the effective NA concentration eliciting a half-maximal response, using GraphPad PRISM™ (version 3.00; GraphPad Software, San Diego, CA, USA). The concentration-inhibition curves were also obtained with GraphPad PRISM™ in order to estimate the IC_{50} values, which are the concentrations producing 50% inhibition of the control response. The EC_{50} and IC_{50} values were converted to logarithmic values (pEC_{50} and pIC_{50}) for analysis. Data are presented as means ± S.E.M., where n refers to the number of experiments. The significance of the difference between mean values was evaluated with GraphPad PRISM™ by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A *P* value less than 0.05 was considered to indicate significant differences.

Results

Characterization of NA-induced transient and sustained increases in $[Ca^{2+}]_i$

Figure 1A shows a representative trace illustrating NA (1 μM)-induced increase in $[Ca^{2+}]_i$ in CHO cells stably expressing human α_{1A}-AR. NA elicited concentration-dependent increases in $[Ca^{2+}]_i$ that consist of an initial transient phase and a subsequent sustained phase (Fig. 1B). The maximum increases in $[Ca^{2+}]_i$ by NA and its pEC_{50} values were 149.1 ± 5.1 nM and 7.56 ± 0.05 for the transient phase and 108.1 ± 1.5 nM and 7.39 ± 0.05 for the sustained phase, respectively (n = 6 for each). The NA (1 μM)-induced transient and sustained $[Ca^{2+}]_i$ increases were markedly reduced by removal of Ca^{2+} from the medium (the residual component of transient phase, 27.3 ± 3.4%; sustained phase, 17.0 ± 1.3%, n = 6 for each; Fig. 1C).

Pharmacological identification of Ca^{2+} channels involved in the transient and sustained increases in $[Ca^{2+}]_i$ in response to NA

To pharmacologically identify Ca^{2+} channels involved in the NA-induced increases in $[Ca^{2+}]_i$, we examined the effects of several Ca^{2+} channel blockers on the NA-induced transient and sustained increases in $[Ca^{2+}]_i$. Nifedipine, an inhibitor of L-type voltage-operated Ca^{2+} channel (VOCC), at concentrations up to 30 μM had no effect on the NA-induced Ca^{2+} responses (Fig. 2A). However, the NA-induced transient and sustained increases in $[Ca^{2+}]_i$ were concentration-dependently inhibited by SK&F 96365, an inhibitor of SOC and NSCC-2 (17), and LOE 908, an inhibitor of NSCC-1 and NSCC-2 (17) (Fig. 2: B and C). The inhibitory effects of these compounds at 30 μM on the transient and sustained phases were 79.4 ± 4.5% and 100.2 ± 1.7% for SK&F 96365 (n = 5) and 92.0 ± 1.1% and 91.3 ± 0.9% for LOE 908 (n = 5), respectively. Moreover, the transient and sustained increases in $[Ca^{2+}]_i$ triggered by NA were sensitive to La^{3+} (Fig. 2D), which is reported to inhibit certain members of the TRPC subfamily (3); and the inhibitory effects at 100 μM were 61.9 ± 4.4%

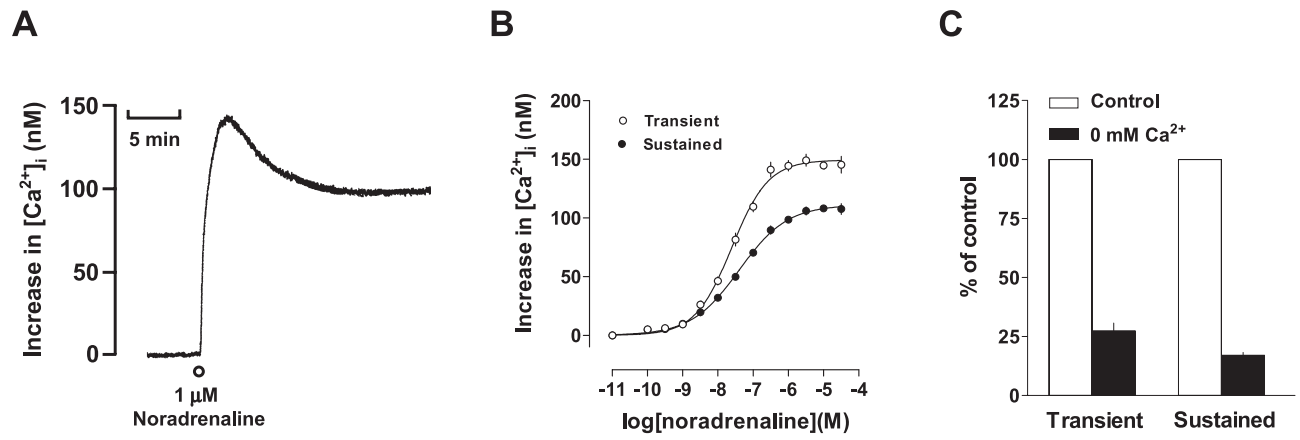

Fig. 1. Characterization of the NA-induced increase in $[Ca^{2+}]_i$ in the CHO cells stably expressing human α_{1A}-AR. $[Ca^{2+}]_i$ was measured using the Ca^{2+} indicator fura-2. A: A typical trace illustrating the $[Ca^{2+}]_i$ increases induced by NA (1 μM) in the presence of 1 mM Ca^{2+}. B: Concentration-response curves for the NA-induced transient and sustained $[Ca^{2+}]_i$ increases (n = 6). C: Effects of removal of extracellular Ca^{2+} on the transient and sustained phases generated by 1 μM NA (n = 6). The maximum $[Ca^{2+}]_i$ level induced by 1 μM NA for each phase in the presence of 1 mM Ca^{2+} was set to 100% as a control. Data are presented as means ± S.E.M. of the results obtained from n separate experiments.

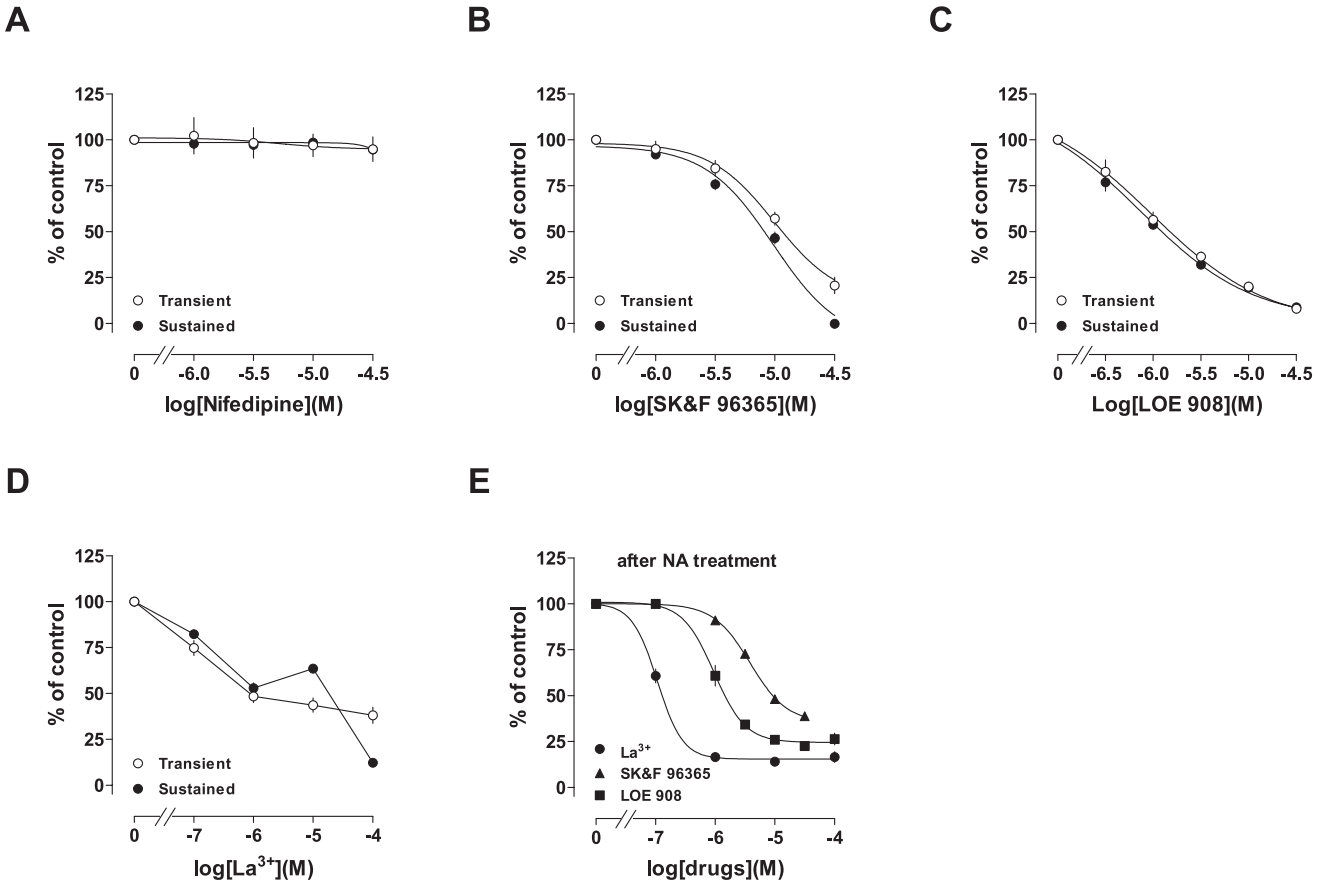

Fig. 2. Effects of pretreatment or post-treatment with cation channel blockers (nifedipine (A), SK&F 96365 (B, E), LOE 908 (C, E) and La^{3+} (D, E)) on the transient and sustained increases in $[Ca^{2+}]_i$ induced by 1 μM NA. These drugs were added 5 min before stimulation with NA (A – D) or during the sustained phase after stimulation with NA (E). The maximum $[Ca^{2+}]_i$ level induced by 1 μM NA for each phase in the presence of 1 mM Ca^{2+} was set to 100% as a control. Data are presented as means ± S.E.M. of the results obtained from 5 separate experiments.

and 87.8 ± 1.9%, respectively (n = 5). To further characterize the NA-induced sustained $[Ca^{2+}]_i$ increase, SK&F 96365, LOE 908, and La^{3+} were added after NA treatment. Figure 2E shows that the sustained phase was concentration-dependently suppressed by these inhibitors after NA treatment.

Importance of Na^+ influx in the NA-induced increases in $[Ca^{2+}]_i$

It is generally accepted that ROCs and SOCs allow the entry of Na^+ in addition to Ca^{2+}, resulting in an increase in $[Na^+]_i$ (9). However, the functional significance of Na^+ influx through these channels in the NA-induced increase in $[Ca^{2+}]_i$ is not well-known. To determine the role of Na^+ influx in the Ca^{2+} response to NA, the effects of reduction of extracellular Na^+ concentrations ($[Na^+]_e$) were tested. For this purpose, the $[Na^+]_e$ (140 mM) in normal Krebs-HEPES solution was reduced by replacing NaCl with equimolar LiCl. As shown in Fig. 3: A and B, the NA-induced transient and sustained increases in $[Ca^{2+}]_i$ in normal Krebs-HEPES solution was unaffected by reducing $[Na^+]_e$ to 70 mM, but significantly suppressed by further reduction to either 10 or 0 mM. The inhibitory effects of reducing $[Na^+]_e$ to 0 mM on the transient and sustained phases were about 40% and 60%, respectively (n = 5), when compared with the responses in normal Krebs-HEPES solution containing 140 mM Na^+. These results suggest that Na^+ influx plays an important role in the transient and sustained $[Ca^{2+}]_i$ increases in response to NA.

In cardiac cells, stimulation of ET_AR with ET-1 activates the Na^+/H^+ exchanger (NHE), causing an increase in $[Na^+]_i$ as well as intracellular alkalinization (18). In addition, amiloride-sensitive Na^+ channel is reported to participate in extracellular Na^+ influx (19). To determine whether these Na^+ entry pathways are involved in the NA-induced increases in $[Ca^{2+}]_i$, the effects of EIPA, an inhibitor of NHE (13), and amiloride, an inhibitor of amiloride-sensitive Na^+ channel (20), were examined. However, the transient and sustained $[Ca^{2+}]_i$ increases in

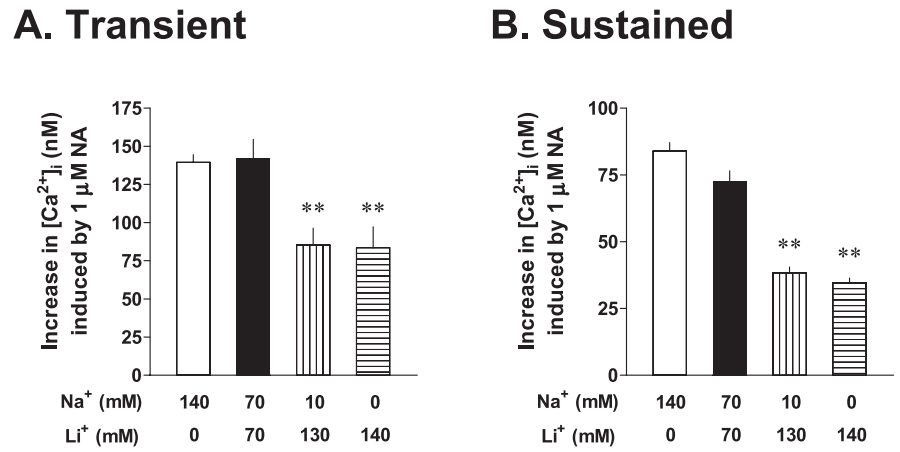

Fig. 3. Effects of reducing extracellular concentrations of Na^+ on the transient (A) and sustained increases (B) in $[Ca^{2+}]_i$ induced by 1 μM NA. The extracellular concentration of Na^+ was reduced by replacing Na^+ with Li^+. Data are presented as means ± S.E.M. of the results obtained from 5 separate experiments. ***P*<0.01: significantly different from the value at 140 mM Na^+.

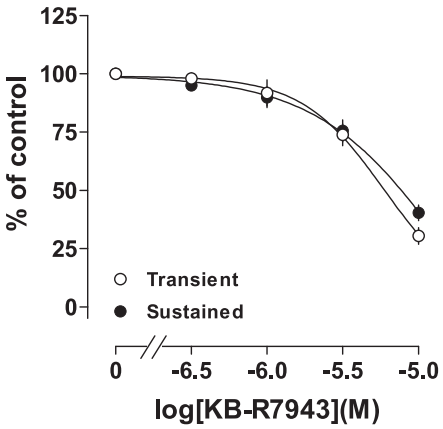

Fig. 4. Inhibitory effects of pretreatment with KB-R7943, an inhibitor of NCX, on the transient and sustained increases in $[Ca^{2+}]_i$ induced by 1 μM NA. The maximum $[Ca^{2+}]_i$ level induced by 1 μM NA for each phase in the presence of 1 mM Ca^{2+} was set to 100% as a control. Data are presented as means ± S.E.M. of the results obtained from 5 separate experiments.

response to NA were insensitive to both inhibitors at concentrations up to 10 μM (data not shown). Therefore, NHE and amiloride-sensitive Na^+ channels do not seem to be major Na^+ entry pathways that contribute to the increase in $[Na^+]_i$.

Involvement of NCX in the NA-induced increases in $[Ca^{2+}]_i$

How is the Na^+ influx associated with the $[Ca^{2+}]_i$ elevation? A potential candidate for such a link is the NCX that mediates Na^+/Ca^{2+} exchange with a ratio of 3 Na^+ : 1 Ca^{2+}, depending on the electrochemical gradient of Na^+ and Ca^{2+} across the plasma membrane. Recently, we have demonstrated that mRNAs for three members of NCX (NCX-1, NCX-2, and NCX-3) were expressed in CHO cells stably expressing ET_AR (16). To determine whether NCX is responsible for the NA-induced $[Ca^{2+}]_i$ increases, therefore, the effect of KB-R7943, an NCX inhibitor (21), was examined. KB-R7943 induced a concentration-dependent inhibition of the transient and sustained $[Ca^{2+}]_i$ increases in response to NA (Fig. 4). The inhibitory effects on the transient and sustained Ca^{2+} responses were 69.6 ± 3.5% and 59.7 ± 3.4%, respectively (n = 5). This result suggests that NCX operating in the reverse mode plays a major role in the NA-induced transient and sustained Ca^{2+} responses.

Pharmacological identification of signaling molecules involved in the transient and sustained increases in $[Ca^{2+}]_i$ induced by NA

To pharmacologically confirm the member(s) of the G proteins involved in the NA-induced transient and sustained increases in $[Ca^{2+}]_i$, the effects of YM-254890, a selective $G_{\alpha q/11}$ inhibitor (22), was examined. In general, α_1-AR is thought to couple with G_q protein in CHO cells (6, 23). As shown in Fig. 5A, YM-254890 suppressed the transient and sustained increases in $[Ca^{2+}]_i$ with different pIC_{50} values (for the transient phase, 6.33 ± 0.08; for the sustained phase, 6.99 ± 0.05, n = 5 for each). The sustained $[Ca^{2+}]_i$ increase was abolished by 0.1 μM YM-254890, which did not affect the transient $[Ca^{2+}]_i$ increase. The inhibitory effect of YM-254890 was about 3-fold more potent for the sustained phase than for the transient phase. These results suggest that NA induces the transient and sustained $[Ca^{2+}]_i$ increases via $G_{\alpha q/11}$.

The principal downstream effector for $G_{\alpha q/11}$ is PLCβ

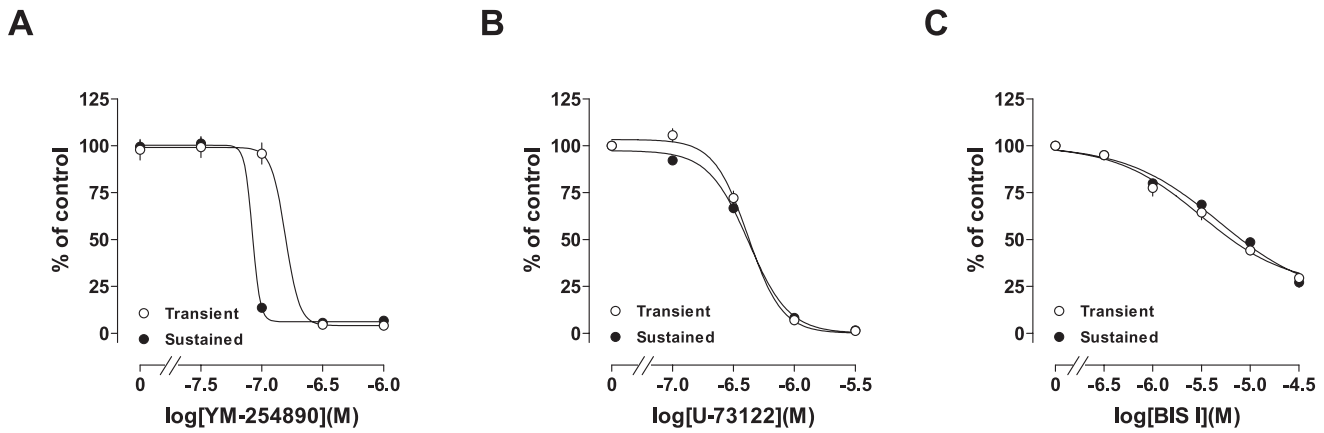

Fig. 5. Inhibitory effects of pretreatment with (A) YM-254890 as a $G_{\alpha q/11}$-selective inhibitor, (B) U-73122 as a PLC inhibitor, and (C) BIS I as a PKC inhibitor on the transient and sustained increases in $[Ca^{2+}]_i$ induced by 1 μM NA. The maximum $[Ca^{2+}]_i$ level induced by 1 μM NA for each phase in the presence of 1 mM Ca^{2+} was set to 100% as a control. Data are presented as means ± S.E.M. of the results obtained from 5 separate experiments.

that cleaves the membrane lipid phosphatidylinositiol-4,5-bisphosphate (PIP_2) into the second messengers IP_3 and DAG, both of which modulate $[Ca^{2+}]_i$ (24). To clarify whether PLCβ is involved in the NA-induced increase in $[Ca^{2+}]_i$, the effects of U-73122, a PLC inhibitor, on the NA-induced $[Ca^{2+}]_i$ responses were examined. As shown in Fig. 3B, U-73122 inhibited the transient and sustained increases in $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 5B). These results unequivocally indicate that the Ca^{2+} mobilization activated by NA depends on PLC activation.

DAG, which is the product of PLC activation, can activate the major regulatory kinase PKC. To determine the role of PKC in the NA-induced $[Ca^{2+}]_i$ increase, the effect of BIS I, a PKC inhibitor (25), was examined. Pretreatment with BIS I induced the concentration-dependent inhibition of the transient and sustained increases in $[Ca^{2+}]_i$ (Fig. 5C), indicating that PKC plays an important role in the transient and sustained increases in $[Ca^{2+}]_i$ induced by NA.

Discussion

In the present study, we attempted to determine the mechanisms underlying the NA-induced increases in $[Ca^{2+}]_i$ in CHO cells stably expressing human α_{1A}-AR, focusing on Na^+ influx through ROCs and SOCs. As shown in Fig. 1A, the activation of α_{1A}-AR with NA induced transient and subsequent sustained increases in $[Ca^{2+}]_i$. It is generally thought that the initial Ca^{2+} transient phase produced by $G_{q/11}$ protein-coupled receptors is generated by IP_3-dependent Ca^{2+} release from the intracellular Ca^{2+} store, and the resulting depletion of the Ca^{2+} store triggers the activation of several types of cation channels such as SOC, leading to the sustained $[Ca^{2+}]_i$ increase by initiating extracellular Ca^{2+} entry (26). However, the transient and subsequent sustained Ca^{2+} responses were suppressed following removal of extracellular Ca^{2+}, indicating that Ca^{2+} influx is critical for both of them. Thus, the classic theory is insufficient to account for the initial Ca^{2+} transient increase presented in this study.

We therefore explored the route for the Ca^{2+} entry upon stimulation of α_{1A}-AR expressed in CHO cells. The potential candidates are voltage-independent Ca^{2+} channels such as SOCs and ROCs (designated as non-selective cation channel type 1 and type 2; abbreviated to NSCC-1 and NSCC-2, respectively) rather than VOCCs, because nonexcitable cells including CHO cells ordinarily lack VOCCs (27). In fact, nifedipine, a blocker of L-type VOCC, had no effect on the NA-induced increases in $[Ca^{2+}]_i$ in CHO cells expressing α_{1A}-AR.

To characterize the voltage-independent Ca^{2+} channels involved in the Ca^{2+} responses to NA, we utilized voltage-independent Ca^{2+} channel blockers, SK&F 96365 and LOE 908, which are very useful pharmacological tools for differentiating the three types of Ca^{2+}-permeable channels, SOCs, NSCC-1, and NSCC-2 (17, 28). We have previously demonstrated that NSCC-1 is sensitive to LOE 908, but resistant to SK&F 96365; NSCC-2 is sensitive to both LOE 908 and SK&F 96365; and SOC is resistant to LOE 908, but sensitive to SK&F 96365 (17, 28). In the present study, the NA-induced transient and sustained increases in $[Ca^{2+}]_i$ were inhibited by LOE 908 and SK&F 96365, indicating the involvement of NSCC-2.

Although the contribution of ROCs including NSCC-

2 to the Ca^{2+} entry induced by stimulation of α_{1A}-AR with NA is predictable from a variety of pharmacological and physiological data, the molecular entity of ROCs is unknown. Recently, some studies with molecular and electrophysiological techniques have identified TRPC channels as potential candidates for Ca^{2+}-permeable cation channels, which are operated by the emptying of the intracellular Ca^{2+} store and the second messenger DAG resulting from PLC activation (26, 29). The TRPC family is divided phylogenetically into four distinct subfamilies (TRPC1; TRPC2; TRPC3, 6, and 7; and TRPC4 and 5). Most of these channels are expressed in many types of cells and have been proposed to operate as SOCs and/or ROCs (3). In addition, several reports have shown that a subfamily consisting of TRPC3, 6, and 7 can be blocked by La^{3+} (3). In the present study, La^{3+} inhibited both transient and sustained $[Ca^{2+}]_i$ increases induced by NA, suggesting that the TRPC channel member(s) would contribute to the Ca^{2+} response to NA.

It is well-known that Ca^{2+}-permeable cation channels allow passage of Na^+ as well as Ca^{2+}. An increase in $[Na^+]_i$ is suggested to functionally modify the NCX that couples Na^+ transport to Ca^{2+} transport (2). Indeed, the NA-induced $[Ca^{2+}]_i$ increase was inhibited by reduction of $[Na^+]_e$, as shown in Fig. 3. In addition to cation channels, NHE and amiloride-sensitive Na^+ channels are reported to function as Na^+ influx pathways. However, EIPA, an inhibitor of NHE (13), and amiloride, an inhibitor of the amiloride-sensitive Na^+ channel (20), had no effect on the NA-induced Ca^{2+} response (data not shown). These data indicate that the Na^+ influx following stimulation of α_{1A}-AR with NA is not mediated by NHE or amiloride-sensitive Na^+ channels but probably by Ca^{2+}-permeable cation channels.

To clarify whether the NA-induced $[Ca^{2+}]_i$ increases result from the activation of the NCX reverse mode driven by the increase in $[Na^+]_i$ through NSCC-2, we employed KB-R7943, a NCX inhibitor (30 – 32). KB-R7943 partially inhibited the NA-induced transient and sustained $[Ca^{2+}]_i$ increases. Moreover, we have recently shown that mRNAs for all members of NCX (NCX-1, NCX-2, and NCX-3) are expressed in CHO cells stably expressing ET_AR and that the NCX can operate following stimulation of ET_AR with ET-1 (16). These findings suggest that NCX plays a functional role in the NA-induced transient and sustained increases in $[Ca^{2+}]_i$.

Interestingly, such physiological and functional coupling between NSCC and NCX presented in this study was also observed in HEK293 cells overexpressing TRPC3 where NCX1 can physically associate with TRPC3 to form a Ca^{2+}-signaling complex (33). TRPC3 is suggested to convert PLC-derived signals into local accumulation of Na^+ but not Ca^{2+}, while NCX would function as a Na^+ sensor to convert $[Na^+]_i$ increase into Ca^{2+} signaling (33). Moreover, in rat cardiac myocytes, activation of PLC via G_q protein-coupled angiotensin receptor recruits the TRPC3-NCX1 complex to the plasma membrane to trigger Ca^{2+} influx (34). Taken together, the physiological association of NSCC with NCX in response to the stimulation of $G_{q/11}$ protein-coupled receptors may serve as the regulatory mechanism to maintain Ca^{2+} homeostasis.

Finally, the pharmacological properties of NA-induced $[Ca^{2+}]_i$ increases were characterized using YM-254890 (an inhibitor of $G_{\alpha q/11}$) and U-73122 (an inhibitor of PLC). YM-254890 completely suppressed the NA-induced sustained and transient phases of $[Ca^{2+}]_i$ increases with different potencies. These results indicate that both phases totally depend on $G_{\alpha q/11}$, but the different sensitivity of both phases might be explained as follows: (1) amounts of $G_{q/11}$ protein required for triggering the Ca^{2+} responses differ between both phases, and (2) different members of the $G_{\alpha q/11}$ subfamily are involved in both phases of Ca^{2+} responses.

Furthermore, abolition of both phases of the NA-induced $[Ca^{2+}]_i$ increases by U-73122, an inhibitor of PLC, provides direct evidence that the Ca^{2+} responses to NA in CHO cells expressing α_{1A}-AR are entirely dependent on the $G_{\alpha q/11}$/PLC pathway. On the other hand, a recent report suggested that another G protein, G_{13}, couples with α_{1A}-AR and plays a major role for NA-induced NSCC activation and arachidonic release in CHO cells (35). Taken together, the activation of both $G_{q/11}$ and G_{13} may be required for generation of the Ca^{2+} responses to NA, like ET_AR expressed in CHO cells where both G_q and G_{12} are needed to activate NSCC-2 (17, 28, 36).

In general, activation of $G_{\alpha q/11}$/PLC results in generation of the second messenger DAG that can activate the major regulatory kinase PKC. PKC is reported to modulate the activity of signaling molecules including cation channels and ion transporters. In cardiac cells, PKC activated by ET-1 induces the phosphorylation of the NCX protein (37) and an increase in NCX-mediated outward ionic current as an indicator of reverse mode NCX (38). On the other hand, certain members of the TRPC family are partially inhibited by PKC (2). In the present study, pretreatment with BIS I, a PKC inhibitor (25), partially inhibited the action of NA, suggesting that both phases of the NA-induced $[Ca^{2+}]_i$ increases are mediated by PKC-dependent pathway(s). The action of PKC may be either activation of NCX, inhibition of TRPC channels, or both.

In summary, the present study demonstrated the physiological mechanisms underlying the Ca^{2+} mobiliza-

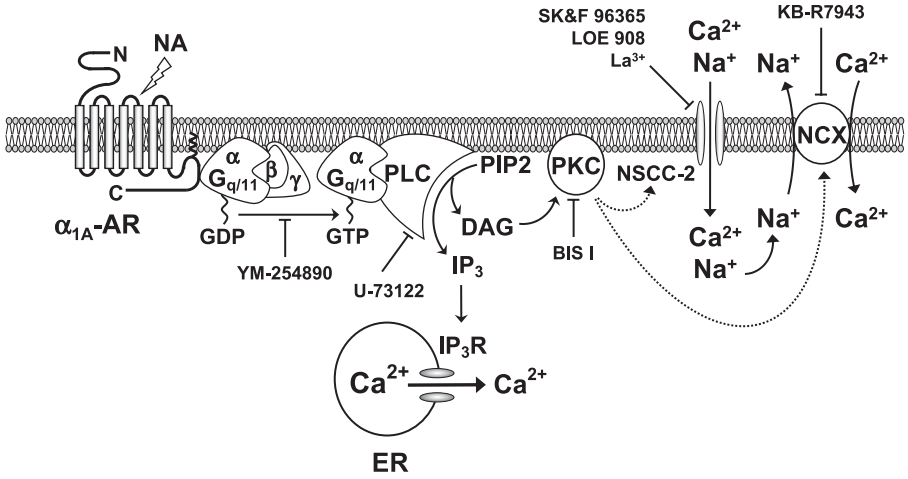

Fig. 6. Possible mechanism involving Ca^{2+} signaling activated by NA in the CHO cells expressing α_{1A}-AR. The application of NA causes $G_{q/11}$ protein-mediated activation of PLC that forms IP_3 and DAG. IP_3R mediating Ca^{2+} release from ER is mainly involved in the transient Ca^{2+} response to NA. In addition, activation of the $G_{q/11}$/PLC pathway elicits facilitation of Ca^{2+} and Na^+ entry via NSCC-2 which belongs to TRPC. PKC activated by DAG is involved in the increase in $[Ca^{2+}]_i$. Speculative mechanisms responsible for the Ca^{2+} responses via PKC are indicated with dotted lines. The increase in $[Na^+]_i$ resulting from Na^+ influx via NSCC-2 eventually triggers Ca^{2+} influx via the NCX operating in the reverse mode.

tion activated by the stimulation of α_{1A}-AR with NA (Fig. 6). The increase in $[Ca^{2+}]_i$ in response to NA in CHO cells expressing α_{1A}-AR results from Ca^{2+} influx via the reverse mode of NCX in exchange for outward Na^+ transport. The operation of NCX in the reverse mode is caused by an increase in $[Na^+]_i$ as a driving force, which is provided by Na^+ influx through NSCC-2 as ROC. NSCC-2 and NCX are activated by α_{1A}-AR through the $G_{\alpha q/11}$/PLC pathway.

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