Korean J Physiol Pharmacol
Vol 12: 259-265, October, 2008

PKC-Independent Stimulation of Cardiac Na^+/Ca^{2+} Exchanger by Staurosporine

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$[Ca^{2+}]_i$ transients by reverse mode of cardiac Na^+/Ca^{2+} exchanger (NCX1) were recorded in fura-2 loaded BHK cells with stable expression of NCX1. Repeated stimulation of reverse NCX1 produced a long-lasting decrease of Ca^{2+} transients ('rundown'). Rundown of NCX1 was independent of membrane PIP_2 depletion. Although the activation of protein kinase C (PKC) was observed during the Ca^{2+} transients, neither a selective PKC inhibitor (calphostin C) nor a PKC activator (PMA) changed the degrees of rundown. By comparison, a non-specific PKC inhibitor, staurosporine (STS), reversed rundown in a dose-dependent and reversible manner. The action of STS was unaffected by pretreatment of the cells with calphostin C, PMA, or forskolin. Taken together, the results suggest that the stimulation of reverse NCX1 by STS is independent of PKC and/or PKA inhibition.

Key Words: Na^+/Ca^{2+} exchange, Rundown, Staurosporine, Protein kinase C, BHK cell

INTRODUCTION

Cardiac Na^+/Ca^{2+} exchanger (NCX1) is a bi-directional electrogenic Ca^{2+} transporter and contributes to the Ca^{2+} extrusion during the cardiac action potential (Blaustein and Lederer, 1999). It has been recognized that NCX1 operates with the fixed exchange ratio of $3Na^+ : 1Ca^{2+}$ (Reeves and Hale, 1984), however, recent work suggested multiple transporting mechanisms (Kang and Hilgemann, 2004). The forward mode of NCX1 produces an inward exchange current and removes Ca^{2+} out of the cells, while the reverse mode generates an outward exchange current and brings Ca^{2+} into the cells. Regulations of NCX1 by cytoplasmic Ca^{2+} and membrane phosphatidylionositol-4,5-bis-phosphate (PIP_2) have been well described in excised giant patch membranes (Hilgemann, 1990; Hilgemann et al, 1992a; Hilgemann et al, 1992b; Hilgemann and Ball, 1996). The action of PIP_2 on NCX1 can be determined by phospholipase C (PLC) activity. The effects of cytoplasmic Ca^{2+} on NCX1 can be mediated by either direct allosteric Ca^{2+} activation (Hilgemann et al, 1992b; Reeves and Condrescu, 2003) or multiple Ca^{2+} dependent signaling pathways (Iwamoto et al, 1998; Opuni and Reeves, 2000; Katanosaka et al, 2005; Zhang et al, 2006; Yaradanakul et al, 2007).

Recent studies with Baby Hamster Kidney cells stably expressing NCX1 (BHK-NCX1) demonstrated that a repeated activation of reverse NCX1 produces a long-term inactivation or rundown of the exchanger activity (Lee and Kang, 2007; Yaradanakul et al, 2008), and we suggested that, at least in part, the Ca^{2+} entered the cells through the reverse exchanger produces rundown (Lee and Kang, 2007). Recently, Hilgemann and colleagues (2008) demonstrated that a huge Ca^{2+}-dependent membrane fusion is triggered by massive Ca^{2+} influx via reverse NCX1. In their study, concentrations of triggering Ca^{2+} are far more than physiological cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) ranges, and the contributions of phospholipases and the cleavage of PIP_2 are independent of the fusion. They also demonstrated a long-lasting, irreversible rundown of NCX1 current in response to a repeated Ca^{2+} influx, albeit the mechanisms are not clear (Yaradanakul et al, 2008). Therefore, cellular mechanisms that cause the Ca^{2+}-induced rundown of NCX1 are yet to be defined.

In the present study, a long-lasting rundown of $[Ca^{2+}]_i$ transients triggered by reverse NCX1 was recorded from intact BHK-NCX1 cells. With confocal imaging technique, a strong activation of protein kinase C (PKC) was detected during the Ca^{2+} transients. Therefore, a question arises whether rundown of reverse NCX1 and consequent Ca^{2+} transient are mediated by PKC activation. By a series of experiments with several PKC modulators, the present work found that a non-specific PKC inhibitor staurosporine (STS) can reverse rundown of reverse NCX1, independent of PKC and/or PKA inhibition.

METHODS

NCX1-expressing BHK cells and cDNA

BHK fibroblasts permanently expressing NCX1 (BHK-NCX1) were provided by Dr. K.D. Philipson (UCLA, USA). BHK-NCX1 cells were cultured as described previously (Linck et al, 1998). For the measurement of $[Ca^{2+}]_i$, confocal imaging, and whole-cell currents, cells were detached with

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ABBREVIATIONS: PH domain, pleckstrin homology domain.

trypsin (0.25%). cDNAs for PKC β-II fused with green fluorescence protein (PKC β-II-GFP) and PLC δ 1-PH fused with GFP (PLC δ 1-PH-GFP) were kindly provided by Dr. D. M. Shin (Yonsei University, Korea). The cDNAs were transiently transfected to BHK-NCX1 cells in 35-mm dishes using *Lipofectamine 2000* (Invitrogen, USA) according to the manufacturer's manual, and the cells were further incubated for 24~48 h.

Confocal imaging

BHK-NCX1 cells were transiently transfected with PLC δ 1-PH-GFP or PKC β-II-GFP. The cells were visulalized with a Zeiss (Oberkochen, Germany) 510 confocal laser scanning microscope (excitation 488 nm) in normal Tyrode solution (room temperature). The time lapse images obtained were analysed using Zeiss LSM image examiner and quantified with Origin 6.1 software (OriginLab, USA). Fluorescence intensity along the horizontal axis was plotted as a histogram, and time-dependent changes in fluorescence intensity from the cytosol (F_c) was normalized to that of the plasma membrane (F_m).

$[Ca^{2+}]_i$ measurements

BHK-NCX1 cells were incubated in normal Tyrode solution containing 4 μM fura 2-AM for 30 min at room temperature. Cells were washed twice with Tyrode solution (in mM/L, 140 NaCl, 5 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 3 glucose, and 10 HEPES, pH 7.4 with NaOH) and placed in dark (30 min) for permitting the cells to hydrolyze the ester form. Microfluorometer (Deltascan, PTI, USA) was attached to an inverted fluorescence microscope (Diaphot 300, Nikon, Japan) with a 40X (0.75 NA) objective. Cells were placed into a recording chamber and alternatively excited (340 and 380 nm) at 5 Hz frequency, and the fluorescence emitted at 510 nm was collected via a photomultiplier tube. A mechanical diaphragm situated at an image plane in the emission path limited the measurement to the cells. The measurement was carried out at room temperature (25°C). To activate reverse NCX1, the cells were alternatively switched from normal Tyrode solution (140 mM Na^+ with 1 mM Ca^{2+}) to Na^+-free solution with 1 mM Ca^{2+} ('0-Na, 1-Ca pulse'). The fluorescence ratios (F_{340}/F_{380}) were corrected for background fluorescence and recorded using Felix 1.1 (PTI). The data were analyzed using Origin 6.1 software.

Whole-cell patch clamp

As described previously (Lee and Kang, 2007), conventional whole-cell recordings of reverse mode of NCX1 currents were recorded at room temperature, using Axopatch-1D patch clamp amplifier (Axon Instruments, U.S.A.). The currents were filtered at 1 kHz (−3dB frequency) and sampled at 5 kHz. NCX1 currents were recorded with the pipette solution containing (in mM): NaOH 50, N-methyl-D-glucamine (NMDG) 70, CsOH 15, EGTA 10, $CaCl_2$ 3.5, HEPES 50, $MgCl_2$ 0.7, Mg-ATP 2, and pH 7.2 adjusted with aspartic acid (100 nM free $[Ca^{2+}]$). Bath solution contained (in mM): NaOH 30, NMDG 120, EGTA 0.5, $CaCl_2$ 3.5 (or 0 for Ca^{2+}- free), HEPES 15, $MgCl_2$ 2, glucose 10, and pH 7.4 adjusted with aspartic acid. At a holding potential of 0 mV, an alternative switching from the Ca^{2+}-free solution to the 3 mM Ca^{2+}-containing bath solution (i.e. Ca^{2+} pulse) activated an outward NCX1 current.

Chemicals and statistics

Fura 2-AM was purchased from Molecular Probes, and all other chemicals were from Sigma (USA). The values given in the text are mean±S.E.M. with n, the sample size.

RESULTS

Rundown of reverse NCX1

BHK-NCX1 cells were loaded with a Ca^{2+} indicator dye (fura-2), and were repetitively stimulated with Na^+-free, 1 mM Ca^{2+}-containing solution ('0-Na, 1-Ca pulses') to activate reverse NCX1. When reverse exchanger was repeatedly activated, amplitudes of the $[Ca^{2+}]_i$ transient were gradually decreased ('rundown'). Rundown of exchanger activity was irreversible and lasted for a long period of recordings (>1 hr, data not shown). The degrees of rundown were not greatly changed by varying intervals between the pulses (data not shown). The most prominent rundown occurred between the initial Ca^{2+} transient and the second Ca^{2+} transient. The amplitudes of the 2nd and the 3rd Ca^{2+} transients were reduced to 48±12 and 35±9.7% of the initial transients, respectively (n=13, Fig. 1A,D). Kinetic changes of the Ca^{2+} transient were also observed during rundown. The rising times of each Ca^{2+} transient were lengthened as the transients were repeated. The time-to-peaks were 40±2.7, 63±4.8, and 70±6.3 sec for the initial, the 2nd, and the 3rd Ca^{2+} transient, respectively (Fig. 1B).

If intracellular Na^+ concentration ($[Na^+]_i$) was decreased during the recordings, it could reduce the driving force for Na^+ efflux and produce rundown. To address this issue, $[Na^+]_i$ was monitored with a Na^+ dye (SBFI). Although $[Na^+]_i$ was transiently decreased during the Ca^{2+} influx period (i.e. in Na^+-free solution), it was rapidly recovered by switching to 140 mM Na^+-containing Tyrode solution (i.e. turn off the exchanger). More importantly, no depletion of basal $[Na^+]_i$ level was observed during the entire period of recording (>1 hr) (data not shown). Furthermore, inhibition of Na-pump with ouabain (0.5 mM) that can increase $[Na^+]_i$ and enhance the driving force for reverse exchange, did not prevent rundown (Fig. 1A).

If the Ca^{2+} which entered during the initial activation of reverse NCX1 depletes stored Ca^{2+} by a Ca^{2+}-induced Ca^{2+}-release (CICR) mechanism, amplitudes of the initial Ca^{2+} transients would always be higher than the next transients. To address this issue, sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitors (20 μM cyclopiazonic acid or 1 μM thapsigargin) were applied for>10 min before the initial pulse. The SERCA inhibitors weakly increased $[Ca^{2+}]$i, however, did not change the degrees of rundown (n=>10, data not shown). Since the results excluded the possibilities of contribution of CICR and Ca^{2+} stores, the rest of experiments were carried out without SERCA inhibitors.

As described previously (Lee and Kang, 2007), a long-lasting rundown of whole-cell NCX1 current was duplicated, as shown in Fig. 1C. Time courses of rundown between the Ca^{2+} transients and the current were not greatly different. In both cases, a prominent rundown occurred after the initial stimulation. Amplitudes of the 2nd NCX1 were 48±12% (n=13) and 20±3.2% (n=7) of the initial ones, for Ca^{2+} transient and whole-cell current, respectively (Fig. 1D).

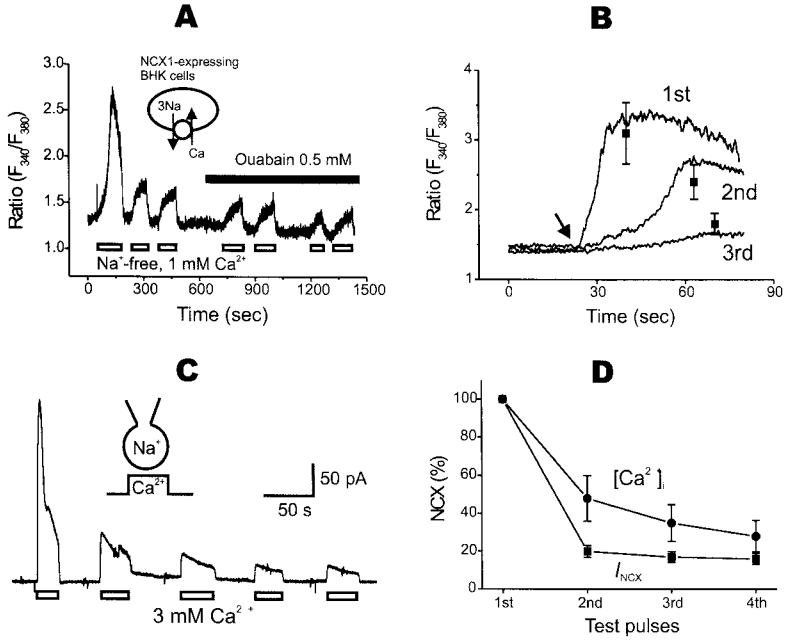

Fig. 1. Rundown of reverse mode of Na^+/Ca^{2+} exchanger (NCX1). (A) When NCX1 was repeatedly stimulated with Na^+-free, 1 mM Ca^{2+}-containing solution, a progressive decrease of the Ca^{2+} transients was observed. A Na^+-pump inhibitor, ouabain, did not enhance the transients. (B) Increased rising time of the Ca^{2+} transients was observed during rundown. The representative traces recorded from the same cell were drawn for comparison. An arrow indicates the starting time of stimulation. The mean values of the time-to-peak values are described in text. The peak ratio values of the 1st, 2nd, and 3rd Ca^{2+} transient were 3.1±0.44, 2.4±0.25, and 1.8±0.15, respectively (n=4). (C) Rundown of the outward whole-cell NCX1 current. (D) The degrees and the time courses of rundown obtained from the Ca^{2+} transients (n=13) and the whole-cell current (n=7) were compared. Amplitudes of the 2nd, 3rd, and 4th Ca^{2+} transient were reduced to 48±12, 35±9.7, and 28±8.4% of the initial ('1st') transient, respectively. Amplitudes of the 2nd, 3rd, and 4th current were reduced to 20±3.2, 17±2.8, and 16±2.9% of the initial one, respectively.

PKC activation during the Ca^{2+} transients

It was questioned whether PKC activation and PIP_2 depletion mediate rundown. To find out the activity of PKC and the changes in membrane PIP_2 concentrations, the translocation of PKCβ-II-GFP or PLCδ1-PH-GFP was monitored by a confocal imaging. As shown in Fig. 2 (upper panel), a reversible translocation of PKCβ-II-GFP between the cytoplasm and the plasma membrane was observed when reverse exchanger was activated by 0-Na, 3-Ca^{2+} pulse. Although data are not shown, a reversible translocation of GFP-fused MARCKS (myristoylated alanine-rich protein kinase C substrate) protein, which has been employed as an indicator for activation of PKC, was observed during the Ca^{2+} transients. These results strongly indicated that PKCs are activated by $[Ca^{2+}]_i$ raised.

On the contrary to PKC activation, membrane PIP_2 concentrations were not changed by the Ca^{2+} transients. Only a spontaneous quenching of the fluorescence, which can also be seen in the absence of the Ca^{2+} transients, was detected during the imaging period (Fig. 2, lower panel). On the other hand, a reversible translocation of the PH-GFP was easily detected when PLC-coupled purinoceptors were stimulated with 0.1 mM ATP (data not shown). Therefore, the results excluded the possibility of contributions of Ca^{2+}-activated PLCs and consequent depletion of PIP_2 to rundown. This was further supported by ineffectiveness of a PLC inhibitor U73122 (1 μM) on rundown of NCX1 (n=4, data not shown).

PKC-independent stimulation of NCX1 by STS

As PKC activation was observed during the Ca^{2+} transients (Fig. 2), a question of whether PKC modulators affect the degrees of rundown was examined. However, as presented in Fig. 3, neither a selective PKC inhibitor (1 μM calphostin C) nor a PKC activator (phorbol 12-myristate 13-acetate; PMA, 0.1 μM) changed rundown. Another PKC inhibitor, chelerythrine, was impossible to test because of its fluorescence. In Fig. 3D, the degrees and the time courses of rundown in the presence of calphostin C or PMA were compared with the control.

On the contrary to the above PKC modulators, a non-specific PKC inhibitor, staurosporine (STS, 0.1 μM), effectively reversed rundown. Before the treatment with STS, the degrees of rundown by repeated stimulation were similar to the control group. When STS (0.1 μM) was applied in the middle of the stimuli, however, the Ca^{2+} transients gradually increased and became higher than the initial level (Fig. 3C, D). The action of STS was reversible and dose-dependent (Fig. 4). As shown in Fig. 4A, a marked rundown, which occurred during the repeated stimulation, was rapidly restored by a brief treatment of STS (0.1 μM), and then a next rundown was processed after the washing of STS. When STS (50~100 nM for>5 min) was applied prior to the initial stimulation, the development of rundown was markedly inhibited (Fig. 4B). As the concentrations of STS increased (50 nM~0.3 μM), the Ca^{2+} transients were increased dose-dependently (Fig. 4B).

As STS has been known to inhibit PKA as well as PKC (Ruëgg and Burgess, 1989), we tested whether the STS-induced recovery of rundown is affected by pretreatment of the cells with calphostin C (1 μM), PMA (0.1 μM), or a PKA activator forskolin (1 μM) (Fig. 5). As expected, pretreatment of the cells with each chemical (>15 min) did not change the initial rundown (compare the 1st and the 2nd Ca^{2+} transients in each panel of Fig. 5A~C). When STS (0.1 μM) was applied after the 2nd Ca^{2+} transients, it progressively recovered the rundown even in the presence of each chemical. The summarized data with time courses of rundown and recovery are illustrated in Fig. 5D. Taken together, the data presented in Figs. 3~5 suggest that STS- induced recovery is independent of inhibition of PKC and/or PKA.

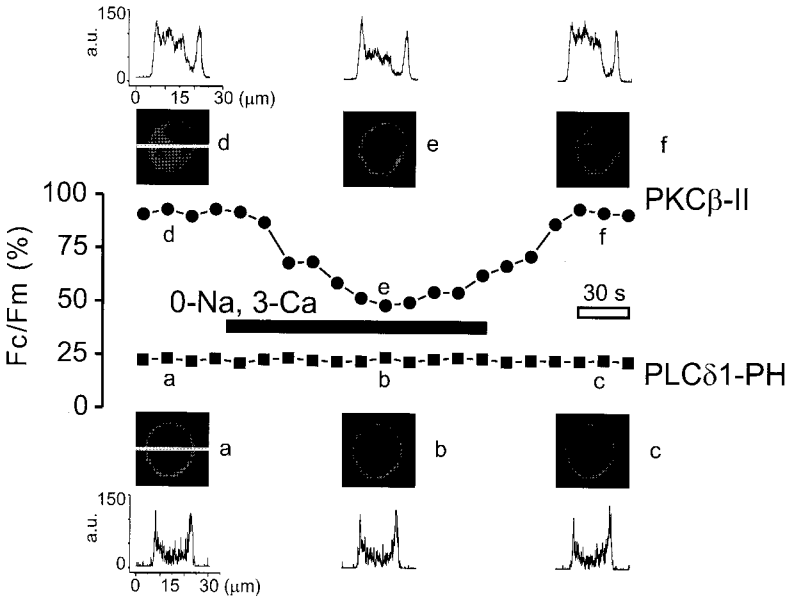

Fig. 2. Using a confocal imaging, changes of the protein kinase C activity and the PIP_2 concentration during the Ca^{2+} transients were monitored via PKC β -II-GFP and PLC δ 1-PH-GFP. Fluorescence intensity obtained along the horizontal axis was shown as histogram with corresponding confocal image. The normalized fluorescence intensity (F_c/F_m, %) was calculated at every 10 sec and plotted against time. F_c, fluorescence intensity of the cytosol; F_m, fluorescence intensity of the plasma membrane. (Upper panel) PKC β -II-GFP, initially located in the cytosol, rapidly translocated to the plasma membrane upon Ca^{2+} influx via reverse NCX1. PKC β -II-GFP returned to the cytosol when NCX1 was turned off. (Lower panel) No significant translocation of PH domain was monitored during the Ca^{2+} transient.

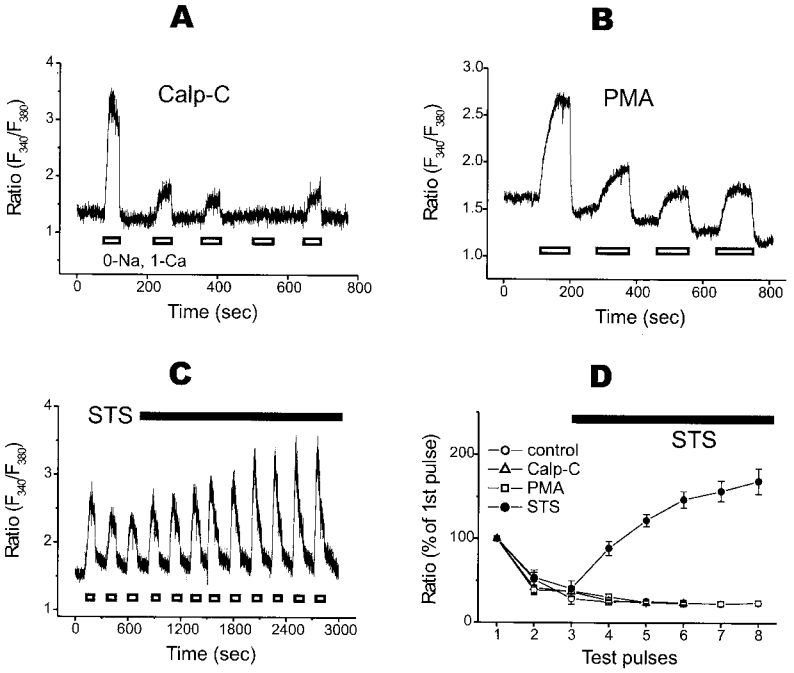

Fig. 3. The effects of protein kinase C modulators on rundown. (A) Pretreatment of the cells with a selective PKC inhibitor, calphostin C (1 μM), did not inhibit rundown of the Ca^{2+} transients. (B) Pretreatment of a strong PKC activator, PMA (0.1 μ M), did not change rundown. (C) Normal rundown was recorded at the beginning of the recording (the first 3 repeats), and the 0.1 μM STS was applied to the cells. The post-treatment of staurosporine (STS) recovered the Ca^{2+} transients and the final amplitudes were higher than the initial one. (D) Summarized data are illustrated. Control, rundown from control cells (n=8); Calp-C, pretreatment with calphostin C (n=6); PMA, pretreatment of phorbol ester (n=6); STS, post-treatment of STS after the 3rd transient (n=4). The black bars in C and D denote the period of time with STS.

Calmodulin-independency

In addition to PKC, other Ca^{2+}-dependent signaling molecules can be activated by the Ca^{2+} influx through reverse-NCX1. Although data are not shown, application of a calmodulin inhibitor (W7, 30 μM) and a CAMK-II inhibitor (KN-93, 10 μM) was without effect on rundown, excluding the possible contributions of calmodulin-dependent signaling pathways.

DISCUSSION

The present study demonstrated that a long-lived $[Ca^{2+}]_i$-dependent rundown of reverse NCX1 is independent of PIP_2 depletion and PKC activation, in accordance with a recent work by Hilgemann and colleagues (2008). In our study, relatively low concentration of a non-selective PKC inhibitor, STS, effectively prevented rundown. However, PKC inhibition by STS seems unlikely to play role, since a highly specific PKC inhibitor (calphostin C) was not effective as

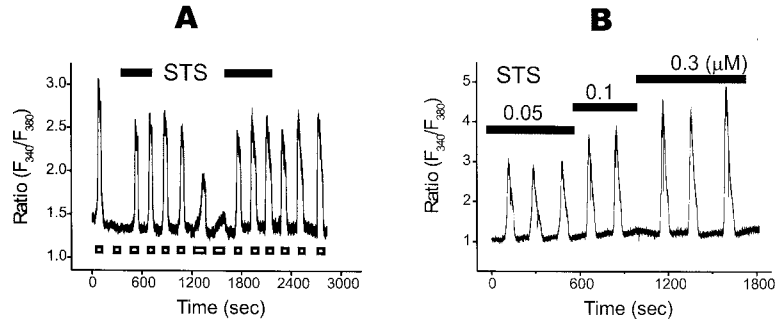

Fig. 4. Staurosporine (STS) stimulated Na^+/Ca^{2+} exchanger in a reversible and a dose-dependent manner. (A) In this recording, the initial Ca^{2+} transient was followed by a complete rundown of NCX1 (i.e. the 2nd transient was completely disappeared). When STS (0.1 μM) was briefly applied prior to the 3rd one, it rapidly restored the Ca^{2+} transients. After the washing of STS, the next rundown was processed. By the second application of STS, restoration of the transients started again. (B) A lower concentration of STS (50 nM), that was applied before the initial stimulation, significantly inhibited rundown (compare the amplitudes of the initial 3 Ca^{2+} transients). When the concentrations of STS were increased up to 0.3 μM, the amplitudes of the transients were increased dose-dependently.

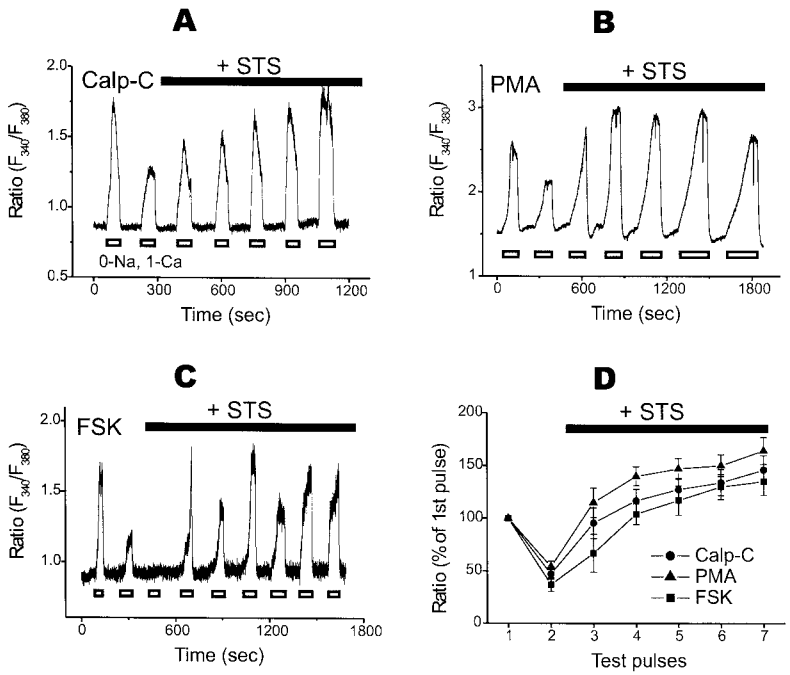

Fig. 5. STS-increased Na^+/Ca^{2+} exchanger is independent of protein kinase C and/or protein kinase A. (A~C) Even in the presence of calphostin C (1 μM), PMA (0.1 μM), and a PKA activator forskolin (FSK, 1 μM), rundown of NCX1 was normally developed (i.e. the 2nd or 3rd transient was markedly decreased). However, the addition of STS (0.1 μM) with each chemical almost completely restored the Ca^{2+} transients. STS was applied after the recording of the 2nd Ca^{2+} transient. (D) Summarized data were illustrated with time courses of rundown. Calp-C, calphostin C with STS (n=4); PMA, PMA with STS (n=7); FSK, forskolin with STS (n=5).

STS. Although detailed mechanisms of the Ca^{2+}-dependent rundown and the stimulation of NCX1 by STS were not fully elucidated in this study, it is striking to note that STS effectively prevented rundown in intact cells. Therefore, STS can be used as a practical tool to prevent rundown of NCX1, thus enabling us to monitor NCX1's activities for a long period of time.

Staurosporine, an indolo[2, 3-alpha] carbazole, was first discovered and extracted from the bacterium *Streptomyces* sp. STS has been known to be a potent, but non-selective inhibitor of PKC (IC_{50} of ~1 nM), and considered as the lead compound for the medicinal synthesis of selective PKC inhibitors with anticancer activities. STS analogues inhibit conventional PKC isoenzymes more potently than novel and atypical PKCs (Gescher, 2000). Besides of PKCs, STS can inhibit not only other protein kinases (PKA, Ca^{2+}/calmodulin-dependent kinase II, cyclin dependent kinases), but also ion channels (Kv1.3, L-type Ca^{2+} channel, voltage-gated K^+ channel) (Ruëgg and Burgess, 1989; Yanagihara et al, 1991; Choi et al, 1999; Ko et al, 2005; Park et al, 2005). Cellular mechanisms of STS-induced inhibition of the channels are largely unknown, and direct blockade or PKC-independent pathways have been suggested as a plausible mechanism (Choi et al, 1999; Ko et al, 2005). As suggested for ion channels, it is of an interest to note that NCX1, an ion transporter, can also be regulated by STS with a similar process.

To fully understand the mechanisms of STS-sensitive, Ca^{2+}-dependent rundown of NCX1, the following possibilities and questions should be addressed in further studies. First of all, STS-induced mitochondrial depolarization can be a plausible explanation for the PKC-independent action of STS, because the activity of reverse NCX1 has been suggested to be regulated by mitochondrial Ca^{2+} uptake (Opuni and Reeves, 2000). STS has widely been used to induce apoptosis of many cells, in which the release of cytochrome c from mitochondria and loss of mitochondrial potential are important mechanisms (Yang et al, 1997). Similar to our present study, a decrease of reverse NCX1 activity with repeated stimulation has been reported in intact CHO-NCX1

cells (Opuni and Reeves, 2000): The inhibition of mitochondrial Ca^{2+} uptake by mitochondrial uncouplers prevented the decrease, and an inhibitory signal generated by mitochondrial Ca^{2+} sequestration has been suggested to play a role, albeit the identity of the signal has not been revealed. Therefore, it is necessary to simultaneously monitor the changes of mitochondrial potential with NCX1's activity to clarify the action mechanisms of STS. Second, a question of whether rundown of NCX1 is due to internalization of NCX1 proteins should be proved (Egger et al, 2005; Shen et al, 2007). This possibility, however, might be excluded because a recent work on the capacitance measurement along with NCX1 current recording clearly demonstrated that a strong membrane fusion, but not the membrane fission, occurs when cytosolic Ca^{2+} is increased by reverse NCX1 (Yaradanakul et al, 2008). Since a long-lasting, irreversible rundown of NCX1 current was accompanied with massive fusion, it is unclear whether the cellular mechanisms of NCX rundown are independent of endocytosis. Nonetheless, it is not clear whether a membrane domain enriched with NCX1 proteins can specifically be internalized during the period of massive fusion. Third, the removal of Na^+-inactivation by STS should be addressed. It has been known that, intracellular Na^+-induced inactivation of outward NCX1 current is ablated by allosteric Ca^{2+} activation (Hilgemann et al, 1992b; Reeves and Condrescu, 2003). If STS interferes with the process of Na^+-inactivation, it can ablate the inactivation and enhance the reverse exchanger, as intracellular Ca^{2+} does. Fourth, whether the suppression of calcineurin can prevent rundown should be tested, because the inhibition of NCX1 by calcineurin has been reported (Katanosaka et al, 2005). The Ca^{2+} entry by reverse NCX1 can activate calcineurin, a Ca^{2+} and calmodulin-dependent phosphatases. Although the relationship between the exchanger's activity and the phosphorylation state of NCX1 proteins has been a matter of debate (Iwamoto et al, 1998; Katanosaka et al, 2005; Zhang et al, 2006), the increased calcineurin activity can affect other target proteins that may regulate NCX1's function or trafficking of the exchanger.

In summary, the present results suggest that the STS-induced recovery of NCX1's rundown is mediated by PKC-independent pathways. The signaling pathways of rundown and action mechanisms of STS remain largely unclear, however PIP_2 depletion seems unlikely to play roles. To clearly understand the mechanisms involved, the roles of mitochondria, trafficking of NCX1 proteins, and other Ca^{2+}-activated signaling molecules should also be determined in future studies.

ACKNOWLEDGEMENT

We thank Kenneth D. Philipson (UCLA, USA) for BHK-NCX1 cells, Dong Min Shin (Yonsei University, Korea) for cDNAs of PKC β II-GFP and PLC δ 1-PH-GFP, and Jee-Eun Lee for cell culture. This work was supported by the Korea Research Foundation Grant (KRF-2005-041-E00016).

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