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European Journal of Pharmacology 360 (1998) 91–98

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Protein kinase C-mediated up-regulation of Na^+/Ca^{2+}-exchanger in rat hepatocytes determined by a new Na^+/Ca^{2+}-exchanger inhibitor, KB-R7943

Akira Ikari *, Hideki Sakai, Noriaki Takeguchi

Department of Pharmaceutical Physiology, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Toyama, 930-0194 Japan

Received 18 May 1998; revised 31 August 1998; accepted 4 September 1998

Abstract

The regulatory mechanism of the plasma membrane Na^+/Ca^{2+}-exchanger in isolated rat hepatocytes was studied using microspectrofluorometry and $^{45}Ca^{2+}$ uptake methods. Exposure of single hepatocytes to low-Na^+ solutions induced an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) which depended on the presence of extracellular Ca^{2+}. 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate (KB-R7943), a novel selective inhibitor of Na^+/Ca^{2+}-exchangers, inhibited the initial rate of $[Ca^{2+}]_i$ increase induced by exposure to the low-Na^+ solution ($IC_{50} = 2$ μM). KB-R7943 also reduced the initial rate of $^{45}Ca^{2+}$ uptake ($IC_{50} = 4$ μM). The increase in $[Ca^{2+}]_i$ induced by exposure to the low-Na^+ solution was inhibited by pre-incubation with 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7, 50 μM), but not with *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide (H-8, 60 μM) or a tyrosine kinase inhibitor, genistein (100 μM). Furthermore, taurocholate and phorbol-12,13-dibutyrate, both of which activate protein kinase C, promoted the increase in $[Ca^{2+}]_i$. These $[Ca^{2+}]_i$ increases were sensitive to KB-R7943. Our results indicate that the Na^+/Ca^{2+}-exchanger is up-regulated via protein kinase C. The activity of Na^+/Ca^{2+}-exchangers is not evident under normal physiological conditions, suggesting that the exchanger may be activated under pathophysiological conditions.

Keywords: Na^+/Ca^{2+}-exchange; Hepatocyte; Protein kinase C

1. Introduction

The Na^+/Ca^{2+}-exchanger is one of the major Ca^{2+} extrusion systems for maintenance of Ca^{2+} homeostasis in excitable cells such as cardiac and smooth muscle cells. This exchanger also plays an important role in regulating Ca^{2+} re-absorption in the nephron (Lederer et al., 1996; Philipson et al., 1996; Matsuda et al., 1997). That is, operation of the exchanger is bidirectional; the direction of Na^+ and Ca^{2+} fluxes is controlled by the magnitude and polarity of transmembrane electrical potentials and ionic gradients (Eisner and Lederer, 1985; Kiang et al., 1992; Studer and Borle, 1992; Kiang and Smallridge, 1994; Fontana et al., 1995). In liver cells (hepatocytes), differently from excitable or renal cells, the Na^+/Ca^{2+}-exchanger is apparently not active under normal physiological conditions (Lidofsky et al., 1990). The operation of this hepatic exchanger is promoted by hypoxia and oxidative stress (Carini et al., 1997), which is also observed in human epidermoid A-431 cells (Kiang et al., 1992; Kiang and Smallridge, 1994). The physiological regulator of the exchanger in hepatocytes has so far not been reported.

A number of heavy metals and amiloride derivatives are known to inhibit the Na^+/Ca^{2+}-exchanger (Siegl et al., 1984; Kaczorowski et al., 1989; Plasman et al., 1991; Kiang et al., 1992, 1998; Kiang and Smallridge, 1994; Dai et al., 1996). They, however, also inhibit other ion transport systems such as voltage-gated Na^+ channels (Kleyman and Cragoe, 1988) and T-type and L-type Ca^{2+} channels (Suarez-Kurtz and Kaczorowski, 1988; Garcia et al., 1990). Use of these inhibitors, therefore, made it very difficult to separate the true activity of Na^+/Ca^{2+}-exchanger from those of other Ca^{2+}-transporting transporters and channels, especially in hepatocytes.

* Corresponding author. Tel.: +81-764-34-2281 ext. 2668; Fax: +81-764-34-5051; E-mail: g4720203@ms.toyama-mpu.ac.jp

0014-2999/98/$ - see front matter
PII: S0014-2999(98)00659-1

In this study, using KB-R7943, a novel selective Na^+/Ca^{2+}-exchanger inhibitor, we have found evidence that the plasma membrane Na^+/Ca^{2+}-exchanger in rat hepatocytes is regulated by protein kinase C.

2. Materials and methods

2.1. Solutions

HEPES-buffered Krebs–Henseleit solution used for dye-loading, fluorescence measurement and $^{45}Ca^{2+}$ uptake contained (mM) 120 NaCl, 4.8 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 1.3 $CaCl_2$, 2 mg ml^{-1} D-glucose and 20 HEPES (pH 7.4). For low-Na^+ solutions, NaCl was replaced by equimolar *N*-methyl-D-glucamine plus HCl; the pH was adjusted to 7.4 with Tris.

2.2. Preparation of isolated hepatocytes

The procedures described below were performed in accordance with the guidelines presented by the Animal Care and Use Committee of Toyama Medical and Pharmaceutical University. Hepatocytes were prepared from male rats of the Sprague–Dawley strain, weighing 160–250 g. The rats were anaesthetized with pentobarbital sodium (40 mg/kg, i.p.). At the end of the dissection procedure the rats were killed by decapitation. After the blood was flushed from the liver, the liver was perfused with a digestion solution containing 0.043% collagenase for 5–7 min as described elsewhere (Koike et al., 1992). Cell viability, judged by the Trypan Blue extrusion, was more than 90%. Isolated hepatocytes were suspended in Krebs–Henseleit solution (Krebs and Henseleit, 1932) supplemented with 2 mg ml^{-1} bovine serum albumin.

2.3. Measurement of $[Ca^{2+}]_i$ by ratio microspectrofluorometry

$[Ca^{2+}]_i$ was monitored using fura-2 as a fluorescent Ca^{2+} indicator. Briefly, hepatocytes were pre-incubated in the dye loading solution under O_2/CO_2 (19:1) for 10 min at 37°C (Takeguchi et al., 1993). Then, 5 μM fura-2 acetoxymethyl ester and Pluronic F127 (0.025%, w/v) were added to the cell suspension (2.0×10^6 cells/ml), followed by incubation for 30 min at 37°C. The dye-loaded cells were washed twice in ice-cold HEPES-buffered Krebs–Henseleit solution and stored in this solution containing 2 mg ml^{-1} bovine serum albumin on ice until use. The cells were placed on a thin glass coverslip, which was set as the bottom glass of a chamber (Japan Spectroscopic, SC-20) with 400 μl of the HEPES-buffered Krebs–Henseleit solution. The total fluorescence intensity from single cells was monitored at excitation wavelengths of 340 and 380 nm with an emission wavelength of 505 nm (interference filter) using a photon-counting technique (Spex Fluorolog-2 spectrofluorometer, Edison, NJ, USA). After corrections for background fluorescence, the intensity ratio (340 nm/380 nm) and $[Ca^{2+}]_i$ were calculated as described previously (Koike et al., 1992). When 3′,4′-dichlorobenzamil was used, the fluorescence intensity ratio of fura-2 was calculated at excitation wavelengths of 340 and 360 nm, because 3′,4′-dichlorobenzamil has a peak absorbance at 380 nm.

2.4. Measurement of $^{45}Ca^{2+}$ uptake

An isolated hepatocyte suspension was placed on 24-well dishes coated with poly-L-lysine for 5 min. The cells were incubated at 23°C for 5 min in 0.5 ml of HEPES-buffered Krebs–Henseleit solution supplemented with 100 μM $CaCl_2$. $^{45}Ca^{2+}$ uptake was initiated by switching the medium to 1 mM Na^+ HEPES-buffered solution containing 1.3 mM $CaCl_2$ and $^{45}Ca^{2+}$ (1.2 μM, 19 kBq/well). Thirty seconds after the addition of $^{45}Ca^{2+}$, the uptake was stopped by several washes with the cold HEPES-buffered solution. Then the cells were solubilized with 0.1 N NaOH, and aliquots were taken for determination of radioactivity.

2.5. Statistical analysis

Results of multiple observations were presented as the means ± S.E.M. (number of observations). The significance of differences between mean values was evaluated with Student's *t*-test and $P < 0.05$ was accepted to indicate statistical significance. The IC_{50} values were calculated using the KaleidaGraph programme, version 3.0.4 (Synergy Software, Reading, PA, USA).

2.6. Chemicals

2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl] isothiourea methanesulfonate (KB-R7943) was a generous gift from Kanebo (Osaka, Japan). 3′,4′-dichlorobenzamil was synthesized as described elsewhere (Cragoe et al., 1967). Fura-2 acetoxymethyl ester was obtained from Dojindo Laboratories (Kumamoto, Japan), Pluronic F127 from Molecular Probes (Eugene, OR, USA) and *N*-methyl-D-glucamine from Tokyo Kasei (Tokyo, Japan). Phenylephrine, vasopressin and taurocholate were obtained from Sigma Chemical (St. Louis, MO, USA), dilthiazem, phorbol-12,13-dibutyrate (PDB) and thapsigargin from Wako Pure Chemical (Osaka, Japan). Dibutyryl cAMP was obtained from Daiichi Pharmaceutical (Tokyo, Japan), genistein and *N*-(2-[methylamino]ethyl)-5-isoquinolinesulfonamide (H-8) from Funakoshi (Tokyo, Japan), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) from Seikagaku Kogyo (Tokyo, Japan), ATP from Oriental Yeast (Tokyo, Japan) and verapamil from Eisai (Tokyo, Japan). KB-R7943, 3′,4′-dichlorobenzamil and thapsigargin were dissolved in dimethyl sulfoxide. Genistein, PDB and verapamil were dissolved in ethanol. Concentrations of dimethyl sulfoxide

and ethanol in final aqueous solutions never exceeded 0.5% (v/v). To test for possible effects of various signal-modulating agents on $[Ca^{2+}]_i$, hepatocytes were pre-incubated for 60 min with a protein kinase A and a protein kinase C inhibitor, H-7 (50 μM), a protein kinase A inhibitor, H-8 (60 μM), or a tyrosine kinase inhibitor, genistein (100 μM). $^{45}CaCl_2$ was purchased from DuPont NEN (Boston, MA, USA). All other chemicals were of the highest grade of purity available.

3. Results

3.1. Effect of extracellular Na^+ on $[Ca^{2+}]_i$

Fig. 1 shows the effect of lowering the extracellular Na^+ concentration ($[Na^+]_o$) on $[Ca^{2+}]_i$ of single hepatocytes. When $[Na^+]_o$ was decreased (1–60 mM), $[Ca^{2+}]_i$ showed a transient peak followed by a steady plateau phase. After reestablishment of the 120 mM Na^+ solution, $[Ca^{2+}]_i$ returned to its basal level (Fig. 1b). The increases of $[Ca^{2+}]_i$ both at peak and plateau phases were, approximately, inversely correlated with a decrease in log $[Na^+]_o$ (Fig. 1a). The increases in $[Ca^{2+}]_i$ depended on the presence of extracellular Ca^{2+}, because only a very small change of $[Ca^{2+}]_i$ was observed in the Ca^{2+}-free solution (1 mM Na^+) supplemented with 0.5 mM EGTA ($\Delta[Ca^{2+}]_i = 3.8 \pm 1.6$ nM, $n = 5$).

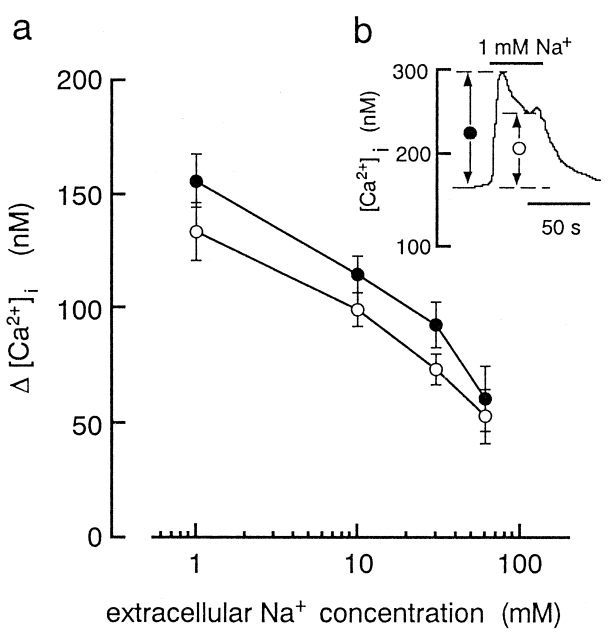


Fig. 1. Increases in $[Ca^{2+}]_i$ induced by lowering $[Na^+]_o$. $[Ca^{2+}]_i$ was measured as a function of the extracellular Na^+ concentration in the presence of 1.3 mM Ca^{2+}. (a) Closed and open circles show the differences in $[Ca^{2+}]_i$ levels shown at peak and plateau phases, respectively. (b) A typical example of change in $[Ca^{2+}]_i$ when a single hepatocyte was exposed to 1 mM Na^+ solution. The number of experiments was 5–12.

3.2. Effect of KB-R7943 on $[Ca^{2+}]_i$ increase induced by exposure to 1 mM Na^+ solution

Pretreatment of hepatocytes with 100 μM KB-R7943 did not significantly affect the resting value (control; 173.3 ± 12.1 nM, $n = 6$: KB-R7943; 165.3 ± 17.7 nM, $n = 6$; $P > 0.05$).

Fig. 2a shows the inhibitory effect of KB-R7943, a novel Na^+/Ca^{2+}-exchanger inhibitor, on the initial increase rate of $[Ca^{2+}]_i$ induced by exposure to 1 mM Na^+ solution. To minimize or exclude a possible contribution of Ca^{2+} mobilization from intracellular stores, we examined the initial rise in $[Ca^{2+}]_i$ for 20 s. The IC_{50} of KB-R7943 was 2 μM (Fig. 2a). Under the identical conditions, 3′,4′-dichlorobenzamil reduced the initial increase rate of $[Ca^{2+}]_i$ ($IC_{50} = 33$ μM), indicating that KB-R7943 was more potent than 3′,4′-dichlorobenzamil.

Thapsigargin, an endoplasmic/sarcoplasmic reticulum Ca^{2+}-ATPase specific inhibitor, slowly increased $[Ca^{2+}]_i$ from its resting value of 156 ± 7 nM to a broad plateau value of 349 ± 14 nM ($n = 8$) under the standard conditions. After this treatment with thapsigargin, exposure to 1 mM Na^+ solution induced a further increase in $[Ca^{2+}]_i$, despite empty cytoplasmic Ca^{2+} stores. This increase was also inhibited by KB-R7943 (Fig. 2b, $IC_{50} = 5$ μM). These results indicate that the exposure to extracellular low Na^+ solution induces Ca^{2+} influx via a plasma membrane Na^+/Ca^{2+}-exchanger.

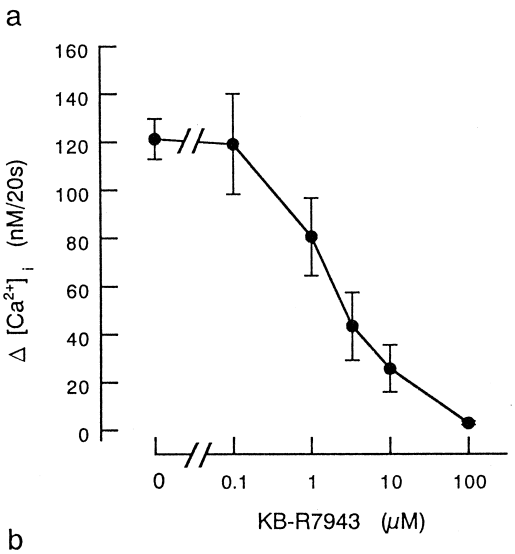
$\Delta[Ca^{2+}]_i$, at both the initial peak and plateau phases described above (Fig. 1a) decreased similarly, inversely with the increase in $[Na^+]_o$. Fig. 2c shows that KB-R7943 in a concentration-dependent manner also lowered this plateau $\Delta[Ca^{2+}]_i$ ($IC_{50} = 4.5$ μM), indicating that most of the plateau $\Delta[Ca^{2+}]_i$ is also due to operation of the plasma membrane Na^+/Ca^{2+}-exchanger.

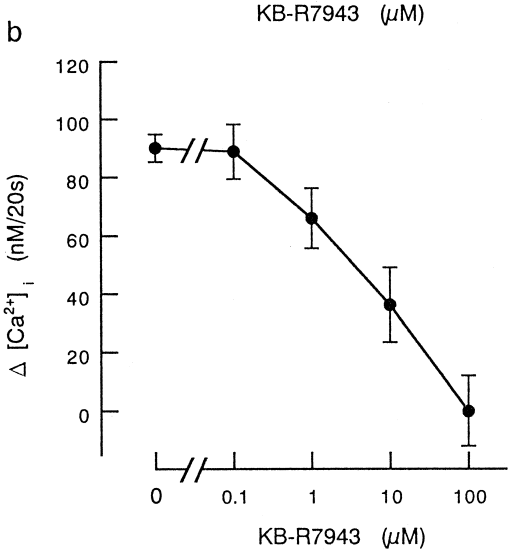
3.3. Effect of KB-R7943 on Na^+-dependent $^{45}Ca^{2+}$ uptake

KB-R7943 inhibited $^{45}Ca^{2+}$ uptake in a concentration-dependent manner after exposure to 1 mM Na^+ solution (Fig. 3). $^{45}Ca^{2+}$ uptake was measured for the initial 30 s after exposure to 1 mM Na^+ solution. The IC_{50} was 4 μM. This value is near the IC_{50} values of 2, 5, and 4.5 μM obtained from the experiments shown in Fig. 2.

3.4. Selectivity of KB-R7943

Here, we tested the effect of KB-R7943 on plasma membrane Ca^{2+} channels, such as messenger-operated, receptor-operated and store-operated Ca^{2+} channels. 1 mM dibutyryl cAMP activates messenger-operated Ca^{2+} channel (Ikari et al., 1997). This increase in $[Ca^{2+}]_i$ was little reduced by 100 μM KB-R7943 (6.3 ± 1.1%, $n = 4$). The addition of extracellular vasopressin increased $[Ca^{2+}]_i$ in hepatocytes, with a peak followed by a plateau phase. The initial peak phase is due to Ca^{2+} release from intracellular





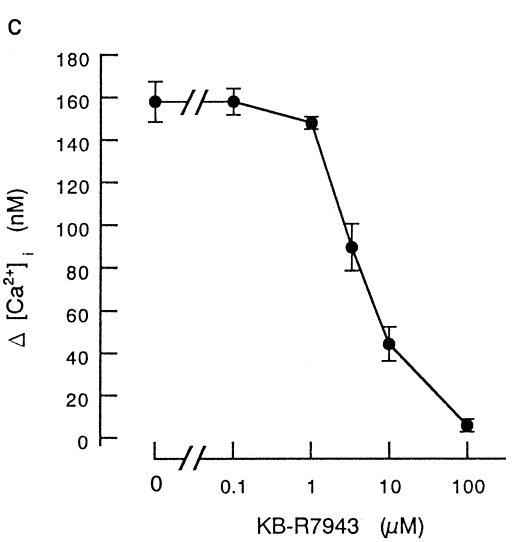


Fig. 2. Effect of KB-R7943 on the initial increase of $[Ca^{2+}]_i$ induced by the exposure to 1 mM Na^+ solution. Hepatocytes were incubated for 7 min with different concentrations of KB-R7943 in the absence of thapsigargin (a, c) or in the presence of 2 μM thapsigargin (b). Then, the standard solution was changed to the 1 mM Na^+ solution. The initial increase of $[Ca^{2+}]_i$ for 20 s (a and b) and the plateau $\Delta[Ca^{2+}]_i$ (c, shown in Fig. 1b) are plotted against the KB-R7943 concentration. The number of experiments was 4–8.

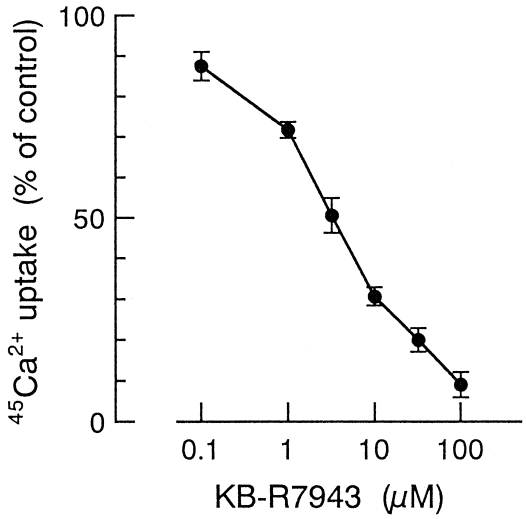


Fig. 3. Effect of KB-R7943 on $^{45}Ca^{2+}$ uptake induced by exposure to 1 mM Na^+ solution. Hepatocytes were incubated for 5 min with different concentrations of KB-R7943. $^{45}Ca^{2+}$ uptake for 30 s under exposure to 1 mM Na^+ solution in the presence of KB-R7943 is expressed as percentage of the control in the absence of KB-R7943. The number of experiments was 7–16.

stores and the plateau phase is due to Ca^{2+} influx from the extracellular solution (Koike et al., 1992; Ikari et al., 1997). We added 100 μM KB-R7943 during the plateau phase, which induced only a slight decrease in $[Ca^{2+}]_i$ ($12.3 \pm 4.3\%$, $n = 4$). Thapsigargin activates the store-operated Ca^{2+} influx channel. Incubation of hepatocytes with 2 μM thapsigargin induced the peak and plateau phases. The addition of 100 μM KB-R7943 at the plateau phase caused a slight decrease in $[Ca^{2+}]_i$ ($14.3 \pm 1.8\%$, $n = 4$). These results indicate that KB-R7943 only slightly closed the messenger-, receptor- and store-operated Ca^{2+} influx channels.

3.5. Effect of Ca^{2+} channel blockers on $[Ca^{2+}]_i$ response to 1 mM Na^+ solution

To rule out any participation of plasma membrane Ca^{2+} channels in response to the removal of extracellular Na^+, we examined the effect of verapamil and diltiazem on $[Ca^{2+}]_i$. These L-type Ca^{2+} channel blockers do not in-

hibit plasmalemmal electrogenic Na^+/Ca^{2+}-exchange activity, but inhibit the electroneutral Na^+/Ca^{2+}-exchange system found in mitochondria isolated from heart and brain (Kaczorowski et al., 1989). The addition of 50 μM verapamil or diltiazem during the plateau phase reached after exposure to 1 mM Na^+ solution, slightly decreased $[Ca^{2+}]_i$. This decrease in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) for 30 s was not significantly different ($P > 0.05$) from that in the untreated control ($\Delta[Ca^{2+}]_i = 7.8 \pm 5.0$ nM, $n = 6$ for control; $\Delta[Ca^{2+}]_i = 12.0 \pm 3.4$ nM, $n = 5$ for verapamil; $\Delta[Ca^{2+}]_i = 18.0 \pm 9.4$ nM, $n = 5$ for diltiazem). These results indicate that exposure to 1 mM Na^+ solution did not significantly open plasma membrane Ca^{2+} channels in rat hepatocytes.

It has been reported that La^{3+} is a potent inhibitor of the Na^+/Ca^{2+}-exchanger in a number of cells: for example, in barnacle cells, La^{3+} inhibited the Ca^{2+} influx mode of the Na^+/Ca^{2+}-exchanger with an IC_{50} of 0.38 mM (Hector et al., 1989). We found that La^{3+} also lowered the plateau phase reached after exposure to 1 mM Na^+ solution in rat hepatocytes. The IC_{50} was 0.56 mM ($n = 4$–6). This value nears the IC_{50} value obtained in barnacle muscle cells.

3.6. Regulation of Na^+/Ca^{2+}-exchanger by protein kinase C-mediated phosphorylation

Fig. 4 shows the effects of protein kinase inhibitors on $[Ca^{2+}]_i$. H-7 (50 μM), an inhibitor of protein kinase A and protein kinase C (Hidaka et al., 1984), did not affect the resting level of $[Ca^{2+}]_i$, but effectively lowered the plateau level induced by exposure to 1 mM Na^+ solution. No further significant decrease was observed on addition of 50 μM KB-R7943 during the plateau phase. Pre-incubation for 60 min with H-8 (60 μM), a protein kinase A inhibitor (Hidaka et al., 1984), and genistein (100 μM), a tyrosine

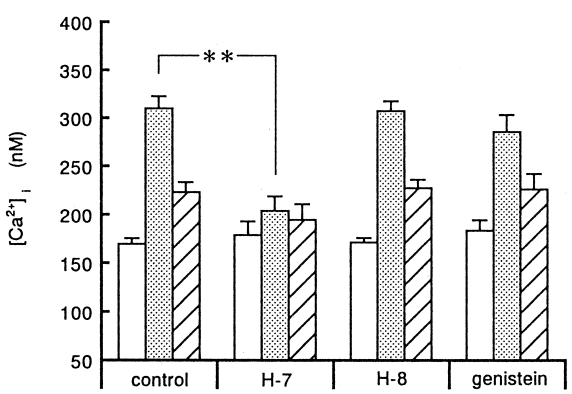


Fig. 4. Effects of various protein kinase inhibitors on $[Ca^{2+}]_i$ increase induced by exposure to 1 mM Na^+ solution. Hepatocytes were pre-incubated with 50 μM H-7, 60 μM H-8 or 100 μM genistein for 60 min before measurements. Open bar show resting $[Ca^{2+}]_i$ values in the standard solution, shaded bar show the plateau values induced by 1 mM Na^+ solution and hatched bar show secondary plateau values in the presence of 50 μM KB-R7943 which was added to the plateau phase. The number of experiments was 4–14. $**P < 0.01$, significantly different from control.

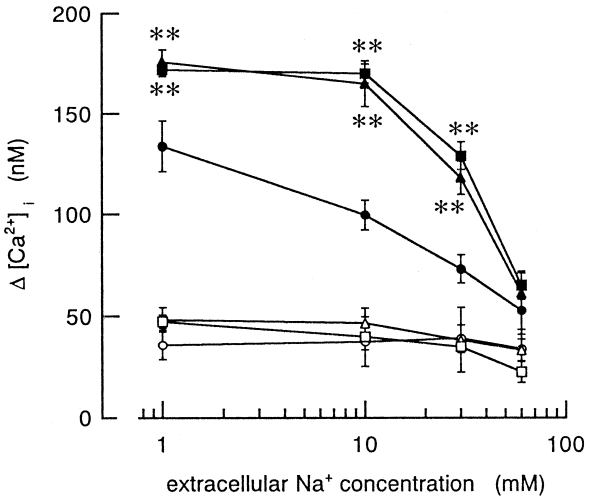


Fig. 5. Effects of PDB and taurocholate on the increase of $[Ca^{2+}]_i$ induced by exposure to low Na^+ solution. Hepatocytes were incubated in the presence of 1 μM PDB (△,▲) or 10 μM taurocholate (□,■) or in the absence of these protein kinase C activators (○,●) for 5 min, followed by exposure to various low Na^+ solutions, which increased $[Ca^{2+}]_i$ with a peak and a plateau phase. Hepatocytes were further incubated in the presence (open symbols) and absence (closed symbols) of 50 μM KB-R7943 for 5 min in the same low-Na^+ solutions. The change in $[Ca^{2+}]_i$ from the initial resting value to the final plateau value was plotted against the Na^+ concentration. The number of experiments was 3–6. $**P < 0.01$, significantly different from the value in the absence of protein kinase C activators.

kinase inhibitor (Linassier et al., 1990), did not affect the resting and the plateau $[Ca^{2+}]_i$ levels. The subsequent addition of KB-R7943 to the plateau phase significantly decreased the plateau levels.

Taurocholate, a bile acid, was reported to induce activation of protein kinase C in rat hepatocytes (Stravitz et al., 1996). We examined whether taurocholate and PDB, a potent protein kinase C activator, increased $[Ca^{2+}]_i$ (Fig. 5). Incubation with PDB (1 μM) or taurocholate (10 μM) for 5 min did not significantly affect the resting $[Ca^{2+}]_i$ (control; 165.5 ± 5.2 nM, $n = 36$: PDB; 185.1 ± 8.2, $n = 19$: taurocholate; 194.0 ± 4.4, $n = 21$; $P > 0.05$). After incubation of the hepatocytes with PDB or taurocholate, the extracellular solution was changed to low-Na^+ solutions. Fig. 5 shows that the plateau levels were increased by treatments with PDB and taurocholate, and the these increases were almost completely prevented by 50 μM KB-R7943.

3.7. Effects of KB-R7943 on Ca^{2+} efflux induced by exposure to the Ca^{2+}-free, normal Na^+ solution

When the extracellular solution was changed from the normal solution to the Ca^{2+}-free, normal Na^+ solution containing 0.5 mM EGTA, $[Ca^{2+}]_i$ in single hepatocytes decreased. Fig. 6 shows that KB-R7943 prevented this decrease of $[Ca^{2+}]_i$ in a concentration-dependent manner. The rate of decrease in $[Ca^{2+}]_i$ was plotted against the KB-R7943 concentration (Fig. 6b). The IC_{50} was 18 μM. This value was higher than that found for the Ca^{2+} influx

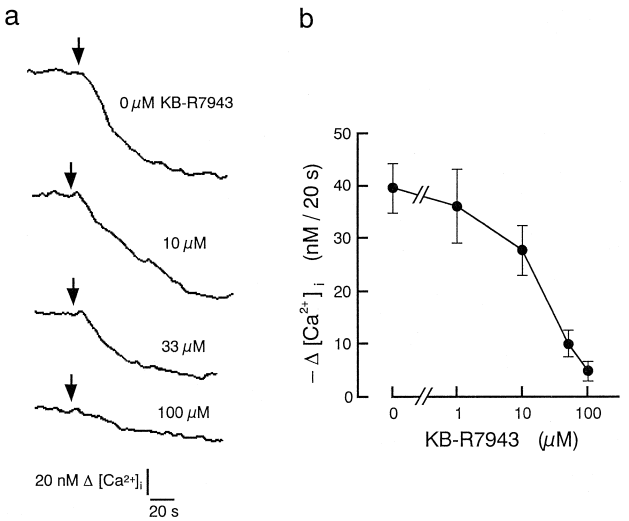


Fig. 6. Effect of KB-R7943 on Ca^{2+} efflux. (a) Hepatocytes were incubated with different concentrations of KB-R7943 for 5 min. At the arrow, the standard solution was changed to the Ca^{2+}-free, normal Na^+ solution supplemented with 0.5 mM EGTA. Representative traces of $[Ca^{2+}]_i$ of single hepatocytes are shown as a function of the KB-R7943 concentration. (b) The rate of decrease in $[Ca^{2+}]_i$ is plotted against the KB-R7943 concentration. The number of experiments was 4–18.

(IC_{50} = 2–5 μM), indicating that KB-R7943 inhibits the reverse mode operation (Ca^{2+} influx) of Na^+/Ca^{2+}-exchanger more potently than that of the normal mode (Ca^{2+} efflux).

4. Discussion

A cardiac form of Na^+/Ca^{2+}-exchanger (abbreviated as NCX1), which was identified as the first gene, was reported to be present at only detectable levels in liver (Kofuji et al., 1992; Lee et al., 1994): for example, the relative levels in various tissues, as determined from Northern blot analysis, were 0.03, 0.5, 1.0 and 1.0 for liver, large intestine, kidney cortex and heart. Another isoform (NCX2), expressed in brain and skeletal muscle, was not detected in liver (Li et al., 1994). However, molecular cloning has shown that two alternative splicing forms from the NCX1 gene are predominantly expressed in liver, aorta, stomach, and kidney (Nakasaki et al., 1993).

In this study, we have shown that the exposure of hepatocytes to low Na^+ solution increases both $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ uptake, which is due to operation of the plasma membrane Na^+/Ca^{2+}-exchanger. Although a decrease in $[Na^+]_o$ has been shown to evoke Ca^{2+} mobilization from intracellular stores in some mammalian cells (Smith et al., 1989; Dwyer et al., 1991), this type of Ca^{2+} mobilization was negligibly small in our present systems because (1) in the absence of extracellular Ca^{2+}, the same manipulation did not induce changes in $[Ca^{2+}]_i$, and (2) pretreatment with thapsigargin, which depleted the intracellular stores, induced a similar increase in $[Ca^{2+}]_i$ by exposure to 1 mM Na^+ solution. Furthermore, the $^{45}Ca^{2+}$ uptake induced by exposure to 1 mM Na^+ solution was almost completely inhibited by KB-R7943: that is, only the Na^+/Ca^{2+}-exchanger operated under these conditions, excluding possibilities of significant involvement of other Ca^{2+} influx pathways. Iwamoto et al. (1996) have shown that 10 μM KB-R7943 did not significantly influence Na^+/H^+ exchange and the passive $^{22}Na^+$ uptake in cardiomyocytes, the dihydropyridine-sensitive $^{45}Ca^{2+}$ uptake in smooth muscle cells, sarcolemmal and sarcoplasmic reticulum Ca^{2+}-ATPase, or sarcolemmal Na^+,K^+-ATPase. We also showed that 50 μM KB-R7943 did not alter the increase in $[Ca^{2+}]_i$ induced by vasopressin or thapsigargin.

These results indicate that KB-R7943 selectively inhibits the Na^+/Ca^{2+}-exchanger operating in the Ca^{2+} influx mode when exposed to low-Na^+ solutions in hepatocytes. The IC_{50} was 2–5 μM.

We also examined the effects of KB-R7943 on the Na^+/Ca^{2+}-exchanger operating in the Ca^{2+} efflux mode. When the extracellular solution was changed to Ca^{2+}-free solution, $[Ca^{2+}]_i$ decreased. KB-R7943 almost completely inhibited this Ca^{2+} efflux, but the effect (IC_{50} = 18 μM) was weaker than that on Ca^{2+} influx (IC_{50} = 2–5 μM). This Ca^{2+} influx and efflux mode-dependent effects of KB-R7943 are in agreement with previous results obtained for the cardiac Na^+/Ca^{2+}-exchanger: that is, IC_{50} values of KB-R7943 for Ca^{2+} influx and efflux were 1.2–2.4 μM and > 30 μM, respectively (Iwamoto et al., 1996). The reason for this mode-dependent effect of KB-R7943 must await further studies.

Under normal physiological conditions, the Na^+/Ca^{2+}-exchanger in excitable cells and chromaffin cells operates in the Ca^{2+} efflux mode, while it operates in the Ca^{2+}-influx mode in renal cells (Lederer et al., 1996; Philipson et al., 1996; Matsuda et al., 1997). The direction of Ca^{2+} movement is determined by several factors, including cell membrane potential and intracellular and extracellular concentrations of Na^+ and Ca^{2+} (Eisner and Lederer, 1985). In hepatocytes, the Na^+/Ca^{2+}-exchanger was not active under normal conditions (Lidofsky et al., 1990), suggesting that the combination of related factors generated balanced conditions for the movement of Na^+ and Ca^{2+} through the exchanger. Consistent with this result, KB-R7943 did not affect the resting $[Ca^{2+}]_i$ in our system, as described in Section 3.2. It has been reported that the Na^+/Ca^{2+}-exchanger operates under hypoxic conditions or during oxidative stress in hepatocytes (Carini et al., 1995, 1997) and also in human epidermoid A-431 cells (Kiang and Smallridge, 1994). Therefore, the hepatic exchanger may be active under pathophysiological conditions such that Na^+ and/or Ca^{2+} homeostasis is disturbed. For example, if the activity of the Na^+,K^+-ATPase decreases, $[Na^+]_i$ would increase and the Na^+/Ca^{2+} exchanger should then extrude intracellular Na^+. The role of the hepatic Na^+/Ca^{2+} exchanger in preventing Na^+ overload can be very significant (Carini et al., 1997).

It has been reported that protein kinases modulate the activity of the Na^+/Ca^{2+}-exchanger in several systems. Protein kinase C up-regulates the Na^+/Ca^{2+}-exchanger in rat neonatal cardiomyocytes (Iwamoto et al., 1996) and rat aortic smooth muscle cells (Iwamoto et al., 1995), but down-regulates it in bovine chromaffin cells (Lin et al., 1994; Tokumura et al., 1998). Furthermore, protein kinase A up-regulates the exchanger in bovine chromaffin cells (Houchi et al., 1995), but down-regulates it in arterial myocytes (Smith and Smith, 1995) and human mesangial cells (Mene et al., 1991). Tyrosine kinase up-regulates it in cultured bovine adrenal chromaffin cells (Tokumura et al., 1998).

So far, there has been no report about protein kinase-mediated regulation of the Na^+/Ca^{2+}-exchanger in hepatocytes. In the present study, we have shown that (1) PDB and taurocholate, which activate protein kinase C, enhanced the activity of the Na^+/Ca^{2+}-exchanger in hepatocytes exposed to a low-Na^+ extracellular solution, (2) an inhibitor of protein kinase A and protein kinase C, H-7, abolished the activity and (3) a protein kinase A inhibitor, H-8, and a tyrosine kinase inhibitor, genistein, did not affect the activity. Therefore, the Na^+/Ca^{2+}-exchanger in hepatocytes is up-regulated by protein kinase C, but not by protein kinase A or tyrosine kinase.

Protein kinases A and C have been reported to up- or down-regulate Na^+/Ca^{2+}-exchangers depending on the different cell types, as described above. These findings suggest that distinct isoforms of the Na^+/Ca^{2+}-exchanger are involved in their activation or inactivation by various protein kinases. In fact, Kofuji et al. (1994) proposed that an unusual arrangement of exons in the Na^+/Ca^{2+}-exchanger (NCX1) gene could allow for generating 32 different Na^+/Ca^{2+}-exchanger mRNAs. Further studies are necessary to clarify the role of phosphorylation in the modulation of Na^+/Ca^{2+}-exchange activity in hepatocytes.

Recently, it was reported that taurocholate down-regulated cholesterol 7a-hydroxylase transcription in rat hepatocytes (Stravitz et al., 1996). It will be interesting to find if the Na^+/Ca^{2+}-exchanger activated by protein kinase C-mediated phosphorylation is involved in bile acid-induced hepatotoxicity and cholestasis.

In conclusion, we could detect the activity of the Na^+/Ca^{2+}-exchanger in the plasma membrane of isolated rat hepatocytes, using KB-R7943. KB-R7943 will provide a new tool to study the role of the Na^+/Ca^{2+}-exchanger in many other types of cells.

Acknowledgements

This work was supported by a Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists (to A.I.).

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