ELSEVIER

European Journal of Pharmacology 420 (2001) 1–8

ejp

www.elsevier.nl/locate/ejphar

Potent and selective inhibition of the human Na^+/H^+ exchanger isoform NHE1 by a novel aminoguanidine derivative T-162559

Tomohiro Kawamoto [a,*], Hiroyuki Kimura [a], Keiji Kusumoto [b], Shoji Fukumoto [c], Mitsuru Shiraishi [c], Toshifumi Watanabe [b], Hidekazu Sawada [a]

[a] *Discovery Research Laboratories IV, Pharmaceutical Discovery Research Division, Takeda Chemical Industries, Ltd., 17-85, Jusohonmachi 2-chome, Yodogawa-ku, Osaka 532-8686, Japan*

[b] *Pharmacology Research Laboratories I, Pharmaceutical Research Division, Takeda Chemical Industries, Ltd., Osaka, Japan*

[c] *Medicinal Chemistry Research Laboratories, Pharmaceutical Research Division, Takeda Chemical Industries, Ltd., Osaka, Japan*

Received 18 January 2001; received in revised form 6 April 2001; accepted 11 April 2001

Abstract

We isolated Na^+/H^+ exchanger (NHE)-deficient Chinese hamster ovary (CHO-K1) cells stably expressing human NHE isoforms (hNHE1, hNHE2 and hNHE3) and established an assay system for measuring their Na^+/H^+ exchange activity by monitoring intracellular pH alterations. Using this assay system, we demonstrated that the acylguanidine derivatives, cariporide and eniporide, cause selective inhibition of hNHE1 (IC_{50} value of 30 nM for cariporide, IC_{50} value of 4.5 nM for eniporide). Furthermore, we found that a novel synthetic aminoguanidine derivative, T-162559 ((5*E*,7*S*)-[7-(5-fluoro-2-methylphenyl)-4-methyl-7,8-dihydro-5(6*H*)-quinolinylidene-amino] guanidine dimethanesulfonate), causes a selective inhibition of hNHE1 with more potent activity than cariporide and eniporide (IC_{50} value of 0.96 nM). This compound did not affect Na^+/HCO_3^- cotransport and Na^+/Ca^{2+} exchange. © 2001 Published by Elsevier Science B.V.

Keywords: T-162559; Na^+/H^+ exchanger isoform; Na^+/H^+ exchanger inhibitor

1. Introduction

Na^+/H^+ exchangers (NHE) are highly conserved in mammalian cells and participate in various cellular functions such as the maintenance of intracellular pH and cell volume. During ischemia-reperfusion, excessive activation of NHE by a decrease in intracellular pH (pHi) causes a significant elevation of intracellular Na^+ (MacLeod, 1991). The increase in intracellular Na^+ leads to Ca^{2+} overload through the reverse-mode activation of the Na^+/Ca^{2+} exchanger (NCX) (Lazdunski et al., 1985; Guarnieri, 1987; Wier, 1990). Ca^{2+} overload is thought to be a major mechanism involved in the development of irreversible cellular damage during ischemia-reperfusion. Inhibition of Na^+/H^+ exchange may protect ischemic cells against Ca^{2+} overload. Therefore, a number of NHE inhibitors have been under the development for therapeutic use in the treatment of ischemia-reperfusion injury (Scholz et al., 1995; Gumina et al., 1998; Counillon et al., 1993a; Yamamoto et al., 2000).

Recent molecular cloning studies have demonstrated the existence of five distinct isoforms of the Na^+/H^+ exchanger—NHE1, NHE2, NHE3, NHE5, and NHE6—in humans (Takaichi et al., 1992; Malakooti et al., 1999; Brant et al., 1995, Baird et al., 1999; Numata et al., 1998). NHE1 is ubiquitously expressed in various tissues, whereas NHE2, NHE3, and NHE5 exhibit tissue-specific expression (Takaichi et al.,1992; Malakooti et al., 1999; Brant et al., 1995, Baird et al., 1999). By contrast to the other NHE isoforms, NHE6 is not expressed at the plasma membrane but in mitochondria (Numata et al., 1998). Acylguanidine derivatives such as cariporide and eniporide have been reported to be potent and selective inhibitors of NHE1, which is the dominant isoform in cardiac tissue, and these compounds have cardioprotective effects in ischemia-reperfusion models (Counillon et al., 1993a; Scholz et al., 1995; Gumina et al., 1998; Linz et al., 1998).

* Corresponding author. Tel.: +81-6-6300-6596; fax: +81-6-6300-6254.

E-mail address: Kawamoto_Tomohiro @takeda.co.jp (T. Kawamoto).

0014-2999/01/$ - see front matter © 2001 Published by Elsevier Science B.V.
PII: S0014-2999(01)00991-8

In this study, we isolated cells stably expressing human NHE isoforms (hNHE1, hNHE2, and hNHE3) and established an assay system for measuring their Na^+/H^+ exchange activity by monitoring intracellular pH alterations. Using this assay system, we clarified the selectivity of cariporide and eniporide for human NHE isoforms. Furthermore, we investigated the effects of a novel synthetic NHE inhibitor, the aminoguanidine derivative T-162559 ((5*E*,7*S*)-[7-(5-fluoro-2-methylphenyl)-4-methyl-7,8-dihydro-5(6*H*)-quinolinylideneamino] guanidine dimethanesulfonate)), on human NHE isoforms. In this study, we showed that T-162559 is a selective inhibitor of the hNHE1 isoform with more potent activity than acylguanidine derivatives such as cariporide and eniporide.

2. Materials and methods

2.1. Compounds

T-162559 ((5*E*,7*S*)-[7-(5-fluoro-2-methylphenyl)-4-methyl-7,8-dihydro-5(6*H*)-quinolinylideneamino]guanidine dimethanesulfonate), T-162561 ((5*E*,7*R*)-[7-(5-fluoro-2-methylphenyl)-4-methyl-7,8-dihydro-5(6*H*)-quinolinylideneamino] guanidine dimethanesulfonate), cariporide, and eniporide were synthesized at Takeda Chemical Industries, Ltd., (Osaka, Japan). These compounds were dissolved in dimethyl sulfoxide (DMSO). (Fig. 1).

2.2. Plasmids

cDNAs encoding hNHE1 (Fliegel et al., 1993) hNHE3 (Brant et al., 1995) and human Na^+/HCO_3^- cotransporter 1 (hNBC1) (Burnham et al., 1997) were isolated by polymerase chain reaction (PCR) and subcloned into the mammalian expression vector pMSRα-neo, which contained the SRα promoter. cDNAs encoding hNHE2 (Malakooti et al., 1999) and human NCX1 (Komuro et al., 1992) were isolated by PCR and subcloned into the mammalian expression vector pcDNA3.1/Zeo (Invitrogen), which contained the cytomegalovirus (CMV) promoter.

2.3. Cell culture

The CHO-K1 cells and their NHE-deficient derivatives were maintained in Ham's F-12 medium (Life Technologies, USA) containing 10% fetal bovine serum and 50 μg/ml gentamicin (Life Technologies) in a humidified 5% CO_2 incubator at 37°C.

2.4. Isolation of NHE-deficient mutant

The NHE-deficient mutant was isolated by the proton suicide technique (Pouyssegur et al., 1984). Briefly, CHO-K1 cells were first mutagenized with 150 μg/ml ethylmethane sulfonate (Aldrich) in the culture medium for 16 h. Then, cells were trypsinized and incubated in Li^+ saline solution (130 mM LiCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM glucose, 20 mM HEPES, pH 7.4) for 2 h at 37°C. After Li^+ loading, Li^+ saline solution was removed by centrifugation and the cell pellet was washed and then incubated in choline-Cl saline solution (130 mM choline-Cl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM glucose, 20 mM 2-(*N*-morpholino) ethanesulfonic acid, pH 5.5) for 60 min at 37°C. Cells were centrifuged and the cell pellet was suspended and transferred to the culture medium. After 4 days of culture, resistant cells were trypsinized and subjected to two cycles of selection. NHE-deficient clones were selected and a single clone was isolated by limited dilution.

2.5. Transfection

Transfection was performed with NHE-deficient CHO-K1 cells (8×10^6 cells/800 μl) for hNHE1, hNHE2, hNHE3, and hNBC1, and with wild-type CHO-K1 cells (8×10^6 cells/800 μl) for hNCX1 by electroporation using a Gene Pulser (Bio-Rad Laboratories) at 250 mV and 960 μF. Stable transformants of hNHE1, hNHE3, and hNBC1 were selected in the presence of 500 μg/ml geneticin (Life Technologies). Stable transformants of

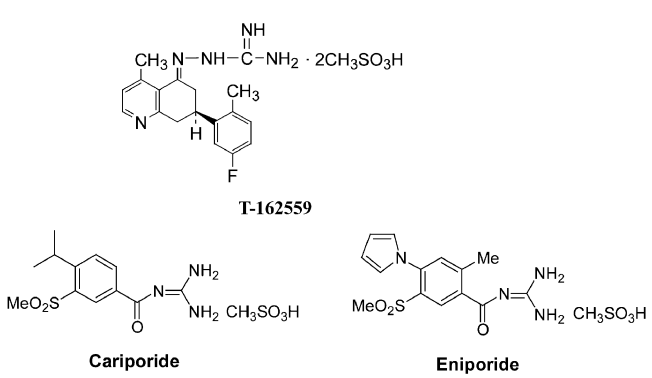

Fig. 1. Chemical structures of T-162559, cariporide, and eniporide.

hNHE2 and hNCX1 were selected in the presence of 250 μg/ml zeocin (Invitrogen).

2.6. Measurement of Na^+/H^+ exchange activity

Na^+/H^+ exchange activity was determined using the membrane-permeable acetoxymethyl (AM) ester form of the pH-sensitive fluorescent indicator 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) to measure the Na^+-dependent recovery of pHi from NH_4Cl prepulse acidification (Nakanishi et al., 1991). Aliquots of 5×10^4 cells were seeded in WhiteClini Plates (white 96-well plates, Labsystems, Finland) and cultured under 5% CO_2 at 37°C overnight. After removal of the medium, the cells were loaded with 5 μM BCECF-AM (Wako, Japan) for 30 min at 37°C. Cells were washed with Na^+ solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM glucose, 10 mM HEPES, pH 7.4), followed by incubation in Na^+ solution containing 25 mM NH_4Cl for 10 min at 37°C. Acid loading was performed by washing cells with Na^+-free solution (140 mM *N*-methyl-D(−)-glucamine, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM glucose, 10 mM HEPES, pH 7.4). Then, 47.5 μl of Na^+-free solution and 2.5 μl of test compounds were added to the cells, followed by incubation for 5 min. Fluorescence was measured by using the fluorescence drug screening system, FDSS-2000 (Hamamatsu Photonics, Japan). Aliquots of 200 μl of Na^+ solution were added to the cells and the fluorescence ratio (450/490 nm) was monitored. The initial velocity of pHi recovery via NHE was calculated using linear regression of the initial data points (10 s for NHE1, 40 s for NHE2 and NHE3). An inhibitory effect of NHE inhibitor was evaluated by a reduction of the velocity in the presence of the test compound. For calculation of the inhibition, pHi recovery with DMSO in cells expressing NHE isoforms was equated with 100% activity, and pHi recovery with DMSO in NHE-deficient cells was equated with 0% activity.

2.7. Measurement of Na^+:$HCO3^-$ cotransport activity

Na^+/HCO_3^- cotransport activity was determined using BCECF-AM to measure the Na^+-dependent recovery of pHi from NH_4Cl prepulse acidification (Burnham et al., 1997). Cells were seeded at 5×10^4 cells in WhiteClini Plates (white 96-well plates, Labsystems) and cultured under 5% CO_2 at 37°C overnight. After removal of the medium, cells were loaded with 5 μM BCECF-AM (Wako) for 30 min at 37°C. Cells were washed with solution A (140 mM tetramethyl ammonium chloride, 25 mM $KHCO_3$, 0.8 mM K_2HPO_4, 0.2 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4), and then incubated in solution B (100 mM tetramethyl ammonium chloride, 40 mM NH_4Cl, 25 mM $KHCO_3$, 0.8 mM K_2HPO_4, 0.2 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4) for 10 min at 37°C. Acid loading was performed by washing cells with solution A. Then 47.5 μl of solution A and 2.5 μl of test compounds were added to the cells, followed by incubation for 5 min. Fluorescence was mea-

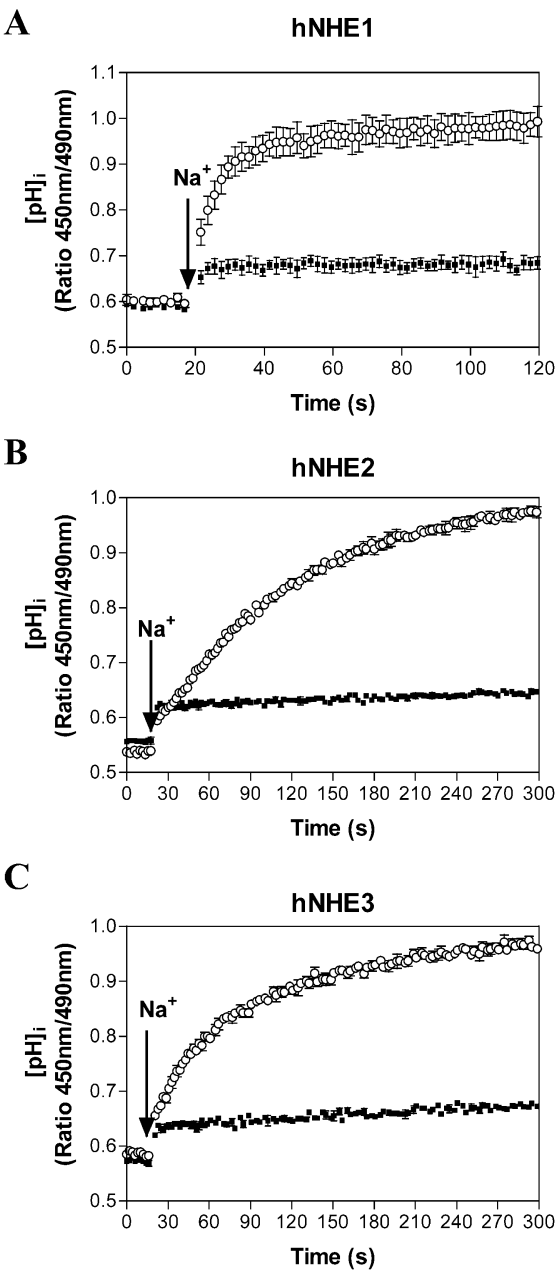

Fig. 2. Na^+-dependent recovery of the intracellular pH in cells stably expressing human NHE isoforms. NHE-deficient CHO-K1 cells were transfected with human NHE isoform expression plasmids and stable transformants were isolated. Cells were loaded with BCECF-AM and then acidified by NH_4Cl prepulse. Recovery of pHi was initiated by the addition of external Na^+ at the time indicated by the arrow. The results shown are for cells expressing hNHE isoforms (○) and nontransfected cells (■). Each data point represents the mean ± S.E. ($n = 3$).

sured with a FDSS-2000 (Hamamatsu Photonics). Aliquots of 200 μl of solution C (115 mM NaCl, 25 mM KCl, 25 mM $NaHCO_3$, 0.8 mM K_2HPO_4, 0.2 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4) were added to the cells and fluorescence ratio (450/490 nm) was monitored for 5 min.

2.8. Measurement of Na^+/Ca^{2+} exchange activity

Na^+/Ca^{2+} exchange activity was determined using the membrane-permeable AM ester form of the Ca^{2+}-sensitive fluorescent indicator FuraPE3 to measure cytoplasmic Ca^{2+} alterations (Fang et al., 1998). Cells were seeded at 5×10^4 cells in Opaque Plates (white 96-well plates, Corning Coster) and cultured under 5% CO_2 at 37°C overnight. After removal of the medium, cells were loaded with 20 μM FuraPE3-AM (Wako) for 60 min at 37°C. Cells were washed three times with physiological salt solution (PSS) (120 mM NaCl, 20 mM $NaHCO_3$, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, pH 7.4). Then, 188 μl of PSS and 2.0 μl of test compounds were added to the cells, followed by incubation for 5 min. Fluorescence was measured with a FDSS-2000 system (Hamamatsu Photonics). Aliquots of 10 μl of phosphate-buffered saline (PBS) containing 200 μM gramicidin (Sigma) were added to the cells and the fluorescence ratio (340/380 nm) was monitored for 15 min.

3. Results

3.1. Functional expression of human NHE isoforms in NHE-deficient CHO-K1 cells

Na^+/H^+ exchangers are endogenously expressed in most mammalian cells. Therefore, we attempted to isolate a NHE-deficient CHO-K1 mutant by the proton suicide technique as previously described (Pouyssegur et al., 1984). The cell growth of the NHE-deficient mutant obtained in this study was not significantly different from that of the wild-type in the regular medium buffered at pH 7.4 with CO_2/HCO_3^- (data not shown).

NHE-deficient CHO-K1 cells were transfected with human NHE isoform expression plasmids and stable transformants were isolated. Na^+/H^+ exchange activity was determined using BCECF-AM to measure the Na^+-dependent recovery of intracellular pH from NH_4Cl prepulse acidification. Intracellular pH was measured with a fluorescence drug screening system, FDSS-2000 (Hamamatsu Photonics), which was able to simultaneously measure the fluorescence alterations in real time in a 96-well microplate using a charge coupled device (CCD) camera.

Human NHE isoforms, hNHE1, hNHE2, and hNHE3, expressed in NHE-deficient CHO-K1 cells showed functional Na^+/H^+ exchange activity, whereas NHE-deficient cells showed no change in pHi after the addition of external Na^+ (Fig. 2). In particular, NHE1-expressing cells

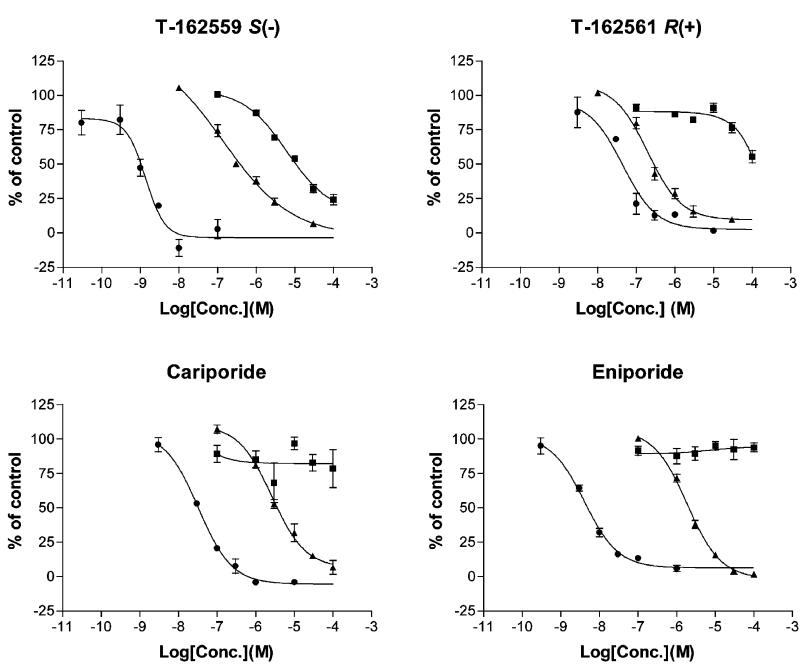

Fig. 3. Dose–response curves for inhibition of hNHE1, hNHE2, and hNHE3 by NHE inhibitors. NHE-deficient CHO-K1 cells expressing hNHE isoforms were loaded with BCECF-AM and then acidified by NH_4Cl prepulse, followed by treatment with increasing concentrations of test compounds for 5 min. Initial velocity of pHi recovery for NHE expressing cells from NH_4Cl prepulse acidification was calculated using linear regression in the initial data points (10 s for NHE1, 40 s for NHE2 and NHE3). An inhibitory effect of NHE inhibitor was evaluated by a reduction of the velocity of pHi recovery in the presence of the test compound. The results are expressed as percentages of the initial velocity of pHi recovery in cells expressing the human NHE isoforms, hNHE1 (●), hNHE2 (▲), and hNHE3 (■). All data are means ± S.E. ($n = 3$).

Table 1
Inhibitory activities of NHE inhibitors against human NHE isoforms

Compound	IC_{50} values (nM)		
	hNHE1	hNHE2	hNHE3
T-162559 *S* (−)	0.96	430	11000
T-162561 *R* (+)	35	310	> 30000
Cariporide	30	4300	> 100000
Eniporide	4.5	2000	> 100000

showed a rapid recovery of pHi from NH_4Cl prepulse acidification as compared with hNHE2 and hNHE3-expressing cells (Fig. 2). This result supports the conclusion that NHE1 is the major isoform involved in the recovery of pHi and the entry of Na^+ during cellular acidosis.

3.2. Selective inhibition of hNHE1 isoform by cariporide, eniporide, and T-162559

We investigated the selectivity of NHE inhibitors, cariporide and eniporide, for human NHE isoforms. In the Na^+-dependent pHi recovery assay, cariporide and eniporide inhibited hNHE1 in a concentration-dependent manner (Fig. 3). The IC_{50} values of cariporide and eniporide for hNHE1 were 30 and 4.5 nM, respectively (Table 1). Cariporide and eniporide inhibited hNHE2 with low affinity (IC_{50} values of 4.3 and 2.0 μM, respectively). In addition, these compounds did not inhibit hNHE3 at 100 μM.

Furthermore, we evaluated the potency of a novel synthetic NHE inhibitor, the aminoguanidine derivative T-162559 ((5*E*,7*S*)-[7-(5-fluoro-2-methylphenyl)-4-methyl-7,8-dihydro-5(6*H*)-quinolinylideneamino]guanidine dimethanesulfonate), on human NHE isoforms. In the Na^+-dependent pHi recovery assay, T-162559 inhibited hNHE1 in a concentration-dependent manner (Fig. 3). The IC_{50} value of T-162559 for hNHE1 was 0.96 nM, which was about 30 and 4.7-fold lower than those of cariporide and eniporide (Table 1). T-162559 showed only weak inhibitory effects on hNHE2 and hNHE3 (IC_{50} values of 430 and 11 μM, respectively). The IC_{50} values of this compound for hNHE2 and hNHE3 were about 450- and 11,000-fold higher than that of hNHE1, respectively (Table 1). In addition, we also evaluated the potency of T-162561, the *R* enantiomer of T-162559 (Fig. 3). The inhibitory effect of T-162561 on hNHE1 was about 36-fold less potent than that of the *S* enantiomer, T-162559 (IC_{50} value of T-162561 for hNHE1 was 35 nM), whereas the IC_{50} value of T-162561 for hNHE2 was similar to that of T-162559 (Table 1).

3.3. Effects of T-162559 on the other ion transporters, hNBC1 and hNCX1

To investigate the effects of T-162559 on the other ion transporters, we isolated cells stably expressing the human Na^+/HCO_3^- cotransporter (hNBC1) and the human cardiac Na^+/Ca^{2+} exchanger (hNCX1), respectively.

The Na^+/HCO_3^- cotransport activity was measured by monitoring Na^+-dependent pH recovery from NH_4Cl prepulse acidification in the presence of HCO_3^-. After the addition of external Na^+, a significant increase in pHi was observed in cells expressing hNBC1, whereas nontransfected NHE-deficient CHO-K1 cells showed only a slight increase in pHi (Fig. 4A). In this assay system, T-162559 did not significantly affect the initial velocity of pHi recovery via hNBC1 at 100 μM (Fig. 4A).

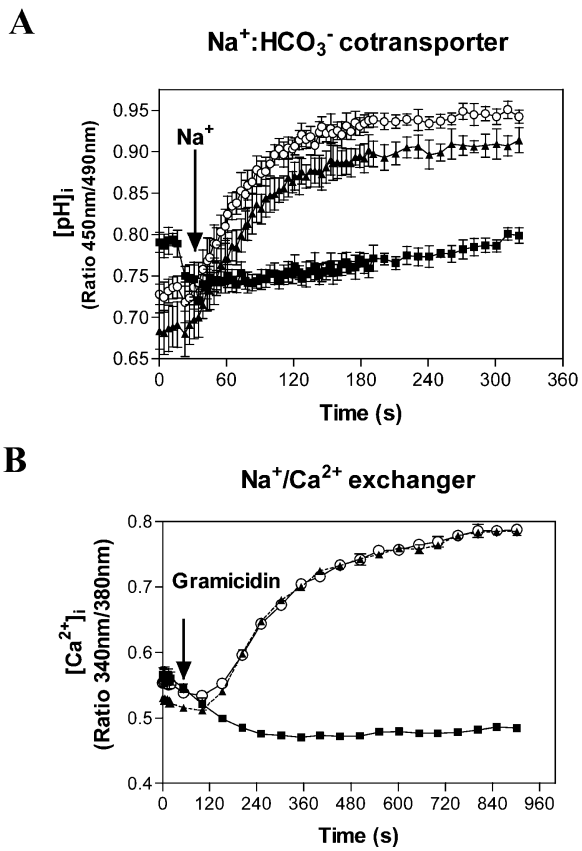

Fig. 4. Effects of T-162559 on human Na^+/HCO_3^- cotransporter (A) and human cardiac Na^+/Ca^{2+} exchanger (B). (A) NHE-deficient CHO-K1 cells were transfected with hNBC1 expression plasmid and a stable transformant was isolated. Cells were loaded with BCECF-AM and acidified by NH_4Cl prepulse, followed by incubation with 100 μM T-162559 or DMSO for 5 min. Recovery of pHi was initiated by the addition of external Na^+ at the time indicated by the arrow in the presence of HCO_3^-. The results shown are for cells expressing hNBC1 in the presence of 100 μM T-162559 (○) or DMSO control (▲), and for nontransfected cells (■). (B) CHO-K1 cells were transfected with hNCX1 expression plasmid and a stable transformant was isolated. Cells were loaded with FuraPE3-AM, followed by incubation with 100 μM T-162559 or DMSO for 5 min. The reverse-mode of Na^+/Ca^{2+} exchange was activated by the addition of gramicidin at the time indicated by the arrow and the increase in intracellular Ca^{2+} was monitored for 15 min. The results shown are for cells expressing hNCX1 in the presence of 100 μM T-162559 (○) or DMSO control (▲), and for nontransfected cells (■). Each data point represents the mean ± S.E. ($n = 3$).

Na^+/Ca^{2+} exchange activity of hNCX1 expressed in CHO-K1 cells was quantified as Ca^{2+} influx driven by the increase in intracellular Na^+ in the presence of the Na^+ ionophore gramicidin. After the reverse-mode activation of hNCX1 by the increase in intracellular Na^+, a slow and sustained increase in intracellular Ca^{2+} was observed in cells transfected with hNCX1, whereas nontransfected CHO-K1 cells showed no change in intracellular Ca^{2+} (Fig. 4B). In this assay system, T-162559 did not affect hNCX1 at 100 μM, as shown in Fig. 4B.

4. Discussion

Na^+/H^+ exchange plays an important role in the development of irreversible cellular damage during ischemia-reperfusion. The primary mechanism of myocardial protection by NHE1 inhibitors in ischemia-reperfusion is likely to be a delay in the progression of ischemic injury, resulting in decreased infarct size even after relatively long periods (> 4 h) of ischemia (Avkiran, 1999). Therefore, a number of NHE inhibitors have been under development for therapeutic use in the treatment of ischemia-reperfusion injury (Scholz et al., 1995; Gumina et al., 1998; Counillon et al., 1993a; Yamamoto et al., 2000). Recent NHE inhibitors, cariporide and eniporide, which show cardioprotective effects in several ischemia-reperfusion models, have been reported to be potent and selective inhibitors of the NHE1 isoform (Scholz et al., 1995; Gumina et al., 1998; Linz et al., 1998). Cariporide is the first NHE inhibitor that has been subjected to clinical trial. Clinical study with cariporide showed cardioprotective effects in patients with acute anterior myocardial infarction treated with direct percutaneous transluminal coronary angioplasty (Rupprecht et al., 2000). The guard during ischemia against necrosis (GUARDIAN) clinical trial with cariporide documented the safety of the drug and suggested that a high degree of inhibition of NHE could prevent cell necrosis in the setting of ischemia-reperfusion (Theroux et al., 2000). Eniporide is also currently under clinical development for use in patients with acute myocardial infarction caused by angioplasty or thrombolysis (Karmazyn, 2000).

In this study, we isolated cells stably expressing hNHE isoforms (hNHE1, hNHE2, and hNHE3), hNBC1 and hNCX1, and established an assay system for measuring their activities by monitoring fluorescence alterations in cells cultured in 96-well plates. We also confirmed the inhibition of Na^+/HCO_3^- cotransport by 1 mM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) (Burnham et al., 1997) and the inhibiton of Na^+/Ca^{2+} exchange by 10 μM KB-R7943 (Iwamoto et al., 1996) in this assay system (data not shown). This system is very useful for the rapid screening of inhibitors of ion transporters such as NHE, NBC, and NCX, which are an important therapeutic target for the treatment of ischemia-reperfusion injury and should contribute to the development of novel drugs for limiting myocardial injury induced by ischemia and reperfusion.

We clarified the selectivity of cariporide and eniporide for human NHE isoforms in the Na^+-dependent pHi recovery assay. These compounds showed selective inhibition of the human NHE1 isoform. The IC_{50} values obtained for human NHE isoforms were similar to those previously reported for the rat NHE isoforms (Scholz et al., 1995; Gumina et al., 1998). These results are consistent with the observation that amiloride binding domain in the fourth transmembrane segment of NHE isoforms is conserved between human and rat sequences (Wakabayashi et al., 1997; Counillon et al., 1993b, 1997).

Furthermore, we found that a novel synthetic NHE inhibitor, the aminoguanidine derivative T-162559, is a selective inhibitor of hNHE1 with a greater potency than cariporide and eniporide (IC_{50} value of 0.96 nM). We have not measured inhibitory activities against hNHE1 of other recent NHE inhibitors, such as S3226 (Schwark et al., 1998), SL 59.1227 (Lorrain et al., 2000),Y-12533 (Aihara et al., 2000) and SM-20550 (Yamamoto et al., 2000), in our assay system. The reported IC_{50} values of these compounds for NHE1 are 3.6 μM (S3226), 3.3 nM (SL 59.1227), 17 nM (TY-12533), and approximately 10 nM (SM-20550). Therefore, T-162559 may be a most potent NHE1 inhibitor. The inhibitory effect of the *R* enantiomer T-162561 on hNHE1 was about 36-fold less potent than that of the *S* enantiomer, T-162559, whereas the IC_{50} value of the *R* enantiomer for hNHE2 was similar to that of the *S* enantiomer (Table 1). This result suggests that the configuration of the asymmetric carbon of T-162559 is important for potent binding to hNHE1. Previously reported NHE inhibitors, including amiloride, cariporide, and eniporide, are mostly acylguanidine derivatives. In this study, we found that the aminoguanidine derivative T-162559 is a potent non-acylguanidine type NHE1 inhibitor.

As shown in Fig. 2, NHE1-expressing cells showed rapid recovery from cellular acidosis as compared with hNHE2- and hNHE3-expressing cells. In addition, NHE1 is the dominant isoform in cardiac tissue and its mRNA expression is elevated in response to cardiac injury (Humphreys et al., 1999; Dyck et al., 1995; Gan et al., 1999; Piper et al., 1996). The sarcolemmal NHE activity of human ventricular myocytes is due to the NHE1 isoform and is inhibited by cariporide (Yokoyama et al., 2000). Sarcolemmal NHE activity is significantly greater in recipient hearts with chronic end-stage heart failure than it is in unused donor hearts (Yokoyama et al., 2000). The other human NHE isoforms at the plasma membrane exhibit tissue-specific expression. hNHE2 is expressed in skeletal muscle, colon, and kidney (Malakooti et al., 1999). hNHE3 is expressed in kidney, testis, and small intestine (Brant et al., 1995). The recently cloned hNHE5 is predominantly expressed in brain (Baird et al., 1999). The expression of hNHE2, hNHE3, and hNHE5 has not been detected in

heart by Northern blot. Therefore, selective inhibition of the NHE1 isoform may offer an effective approach for the treatment of cardiac injury during ischemia-reperfusion.

Amiloride and its derivatives are nonspecific NHE inhibitors because these compounds have been shown to inhibit the Ca^{2+} current, Na^+ current, K^+ current and Na^+/Ca^{2+} exchange (Pierce et al., 1993; Lai et al., 1994). Na^+/HCO_3^- cotransport and Na^+/Ca^{2+} exchange are also thought to be important mechanisms involved in the pathophysiological process of ischemia-reperfusion injury (MacLeod, 1991; Lazdunski et al., 1985; Guarnieri, 1987; Wier, 1990; Schafer et al., 2000). T-162559 did not affect Na^+/HCO_3^- cotransport and Na^+/Ca^{2+} exchange at 100 μM (Fig. 4). These results indicate that T-162559 is a highly potent and specific inhibitor of hNHE1. In a rat model of myocardial infarction induced by coronary occlusion and reperfusion, intravenous administration of T-162559 resulted in a significant reduction in infarct size which was more potent than cariporide and eniporide (Igata et al., 2001). Therefore, this compound might be a potential drug for the treatment of ischemia-reperfusion injury and other cardiac diseases (Karmazyn et al., 1999).

Acknowledgements

We thank H. Igata and A. Abe for their helpful discussions, and C. Kawahara for her technical assistance.

References

Aihara, K., Hisa, H., Sato, T., Yoneyama, F., Sasamori, J., Yamaguchi, F., Yoneyama, S., Mizuno, Y., Takahashi, A., Nagai, A., Kimura, T., Kogi, K., Satoh, S., 2000. Cardioprotective effect of TY-12533, a novel Na^+/H^+ exchange inhibitor, on ischemia/reperfusion injury. Eur. J. Pharmacol. 404, 221–229.

Avkiran, M., 1999. Rational basis for use of sodium-hydrogen exchange inhibitors in myocardial ischemia. Am. J. Cardiol. 83, G10–G18.

Baird, N.R., Orlowski, J., Szabo, E.Z., Zaun, H.C., Schultheis, P.J., Menon, A.G., Shull, G.E., 1999. Molecular cloning, genomic organization, and functional expression of Na^+/H^+ exchanger isoform 5 (NHE5) from human brain. J. Biol. Chem. 274, 4377–4382.

Brant, S.R., Yun, C.H., Donowitz, M., Donowitz, Ts.M., 1995. Cloning, tissue distribution, and functional analysis of the human Na^+/H^+ exchanger isoform, NHE3. Am. J. Physiol. 269, C198–C206.

Burnham, C.E., Amlal, H., Wang, Z., Shull, G.E., Soleimani, M., 1997. Cloning and functional expression of a human kidney Na^+:HCO_3^- cotransporter. J. Biol. Chem. 272, 19111–19114.

Counillon, L., Scholz, W., Lang, H.J., Pouyssegur, J., 1993a. Pharmacological characterization of stably transfected Na^+/H^+ antiporter isoforms using amiloride analogs and a new inhibitor exhibiting anti-ischemic properties. Mol. Pharmacol. 44, 1041–1045.

Counillon, A., Franchi, A., Pouyssegur, J., 1993b. A point mutation of the Na^+/H^+ exchanger gene (NHE1) and amplification of the mutated allele confer amiloride resistance upon chronic acidosis. Proc. Natl. Acad. Sci. U. S. A. 90, 4508–4512.

Counillon, L., Noel, J., Reithmeier, R.A., Pouyssegur, J., 1997. Random mutagenesis reveals a novel site involved in inhibitor interaction within the fourth transmembrane segment of the Na^+/H^+ exchanger-1. Biochemistry 36, 2951–2959.

Dyck, J.R., Maddaford, T.G., Pierce, G.N., Fliegel, L., 1995. Induction of expression of the sodium-hydrogen exchanger in rat myocardium. Cardiovasc. Res. 29, 203–208.

Fang, Y., Condrescu, M., Reeves, J.P., 1998. Regulation of Na^+/Ca^{2+} exchange activity by cytosolic Ca^{2+} in transfected Chinese hamster ovary cells. Am. J. Physiol. 275, C50–C55.

Fliegel, L., Dyck, J.R., Wang, H., Fong, C., Haworth, R.S., 1993. Cloning and analysis of the human myocardial Na^+/H^+ exchanger. Mol. Cell. Biochem. 125, 137–143.

Gan, X.T., Chakrabati, S., Karmazyn, M., 1999. Modulation of Na^+–H^+ exchange isoform 1 mRNA expression in isolated rat hearts. Am. J. Physiol. 277, H993–H998.

Guarnieri, T., 1987. Intracellular sodium–calcium dissociation in early contractile failure in hypoxic ferret papillary muscle. J. Physiol. 388, 449–465.

Gumina, R.J., Mizumura, T., Beier, N., Schelling, P., Schultz, J.J., Gross, G.J., 1998. A new sodium/hydrogen exchange inhibitor, EMD 85131, limits infarct size in dogs when administered before or after coronary artery occlusion. J. Pharmacol. Exp. Ther. 286, 175–183.

Humphreys, R.A., Haist, J.V., Chakrabarti, S., Feng, Q., Arnold, J.M., Karmazyn, M., 1999. Orally administered NHE1 inhibitor cariporide reduces acute responses to coronary occlusion and reperfusion. Am. J. Physiol. 276, H749–H757.

Igata, H., Fujiwara, S., Kaneko, M., Abe, A., Kusumoto, K., Fukumoto, S., Shiraishi, M., Watanabe, T., 2001. Potent and long-lasting cardioprotective effects of T-162559, a new Na–H exchange inhibitor, in rats. Jpn. J. Pharmacol. 85 (Suppl. I), 78.

Iwamoto, T., Watano, T., Shigekawa, M., 1996. A novel isothiourea derivative selectively inhibits the reverse mode of Na^+/Ca^{2+} exchange in cells expressing NCX1. J. Biol. Chem. 271, 22391–22397.

Karmazyn, M., 2000. Pharmacology and clinical assessment of cariporide for the treatment coronary artery diseases. Expert Opin. Invest. Drugs 9, 1099–1108.

Karmazyn, M., Gan, X.T., Humphreys, R.A., Yoshida, H., Kusumoto, K., 1999. The myocardial Na^+–H^+ exchange: structure, regulation, and its role in heart disease. Circ. Res. 85, 777–786.

Komuro, I., Wenninger, K.E., Philipson, K.D., Izumo, S., 1992. Molecular cloning and characterization of the human cardiac Na^+/Ca^{2+} exchanger cDNA. Proc. Natl. Acad. Sci. U. S. A. 89, 4769–4773.

Lai, Z.F., Hotokebuchi, N., Cragoe Jr., E.J., Nishi, K., 1994. Effects of 5-(*N*,*N*-hexamethylene)amiloride on action potentials, intracellular Na, and pH of guinea pig ventricular muscle in vitro. J. Cardiovasc. Pharmacol. 23, 259–267.

Lazdunski, M., Frelin, C., Vigne, P., 1985. The sodium/hydrogen exchange system in cardiac cells: its biochemical and pharmacological properties and its role in regulating internal concentrations of sodium and internal pH. J. Mol. Cell. Cardiol. 17, 1029–1042.

Linz, W., Albus, U., Crause, P., Jung, W., Weichert, A., Scholkens, B.A., Scholz, W., 1998. Dose-dependent reduction of myocardial infact mass in rabbits by the NHE-1 inhibitor, cariporide (HOE642). Clin. Exp. Hypertens. 20, 733–749.

Lorrain, J., Briand, V., Favennec, E., Duval, N., Grosset, A., Janiak, P., Hoornaert, C., Cremer, G., Latham, C., O'Connor, S.E., 2000. Pharmacological profile of SL 59.1227, a novel inhibitor of the sodium/hydrogen exchanger. Br. J. Pharmacol. 131, 1188–1194.

MacLeod, K.T., 1991. Regulation and interaction of intracellular calcium, sodium and hydrogen ions in cardiac muscle. Cardioscience 2, 71–85.

Malakooti, J., Dahdal, R.Y., Schmidt, L., Layden, T.J., Dudeja, P.K., Ramaswamy, K., 1999. Molecular cloning, tissue distribution, and functional expression of the human Na^+/H^+ exchanger NHE2. Am. J. Physiol. 277, G383–G390.

Nakanishi, T., Seguchi, M., Tsuchiya, T., Cragoe Jr., E.J., Takao, A., Momma, K., 1991. Effect of partial Na pump and Na^+–H^+ exchange inhibition on $[Ca^{2+}]i$ during acidosis in cardiac cells. Am. J. Physiol. 261, C758–C766.

Numata, M., Petrecca, K., Lake, N., Orlowski, J., 1998. Identification of a mitochondrial Na^+/H^+ exchanger. J. Biol. Chem. 273, 6951–6959.

Pierce, G.N., Cole, W.C., Liu, K., Massaeli, H., Maddaford, T.G., Chen, Y.J., McPherson, C.D., Jain, S., Sontag, D., 1993. Modulation of cardiac performance by amiloride and several selected derivatives of amiloride. J. Pharmacol. Exp. Ther. 265, 1280–1291.

Piper, H.M., Balser, C., Ladilov, Y.V., Schafer, M., Siegemund, B., Ruiz-Meana, M., Garcia, D., 1996. The role of Na^+/H^+ exchange in ischemia-reperfusion. Basic Res. Cardiol. 91, 191–202.

Pouyssegur, J., Sardet, C., Franchi, A., L'Allemain, G., Paris, S., 1984. A specific mutation abolishing Na^+/H^+ antiport activity in hamster fibroblasts precludes growth at neutral and acidic pH. Proc. Natl. Acad. Sci. U. S. A. 81, 4833–4837.

Rupprecht, H.J., vom Dahl, J., Terres, W., Seyfarth, K.M., Richardt, G., Schultheibeta, H.P., Buerke, M., Sheehan, F.H., Drexler, H., 2000. Cardioprotective effects of the Na^+/H^+ exchange inhibitor cariporide in patients with acute anterior myocardial infarction undergoing direct PTCA. Circulation 101, 2902–2908.

Schafer, C., Ladilov, Y.V., Siegmund, B., Piper, H.M., 2000. Importance of bicarbonate transport for protection of cardiomyocytes against reoxygenation injury. Am. J. Physiol. 278, H1457–H1463.

Scholz, W., Albus, U., Counillon, L., Gogelein, H., Lang, H., Linz, W., Weichrt, A., Scholkens, B., 1995. Protective effects of HOE642, a selective sodium–hydrogen exchange subtype 1 inhibitor, on cardiac ischemia and reperfusion. Cardiovasc. Res. 29, 260–268.

Schwark, J.R., Jansen, H.W., Lang, H.J., Krick, W., Burckhardt, G., Hropot, M., 1998. S3226, a novel inhibitor of Na^+/H^+ exchanger subtype 3 in various cell types. Pfluegers Arch. 436, 797–800.

Takaichi, K., Wang, D., Balkovetz, D.F., Warnock, D.G., 1992. Cloning, sequencing, and expression of Na^+/H^+ antiporter cDNAs from human tissues. Am. J. Physiol. 262, C1069–C1076.

Theroux, P., Chaitman, B.R., Danchin, N., Erhardt, L., Meinertz, T., Schroeder, J.S., Tognoni, G., White, H.D., Willerson, J.T., Jessel, A., 2000. Inhibition of the sodium–hydrogen exchanger with cariporide to prevent myocardial infarction in high-risk ischemic situations. Main results of the GUARDIAN trial. Guard during ischemia against necrosis (GUARDIAN) Investigators. Circulation 102, 3032–3038.

Wakabayashi, S., Shigekawa, M., Pouyssegur, J., 1997. Molecular physiology of vertebrate Na^+/H^+ exchangers. Physiol. Rev. 77, 51–74.

Wier, W.G., 1990. Cytoplasmic $[Ca^{2+}]$ in mammalian venticle: dynamic control by cellular processes. Annu. Rev. Physiol. 52, 467–485.

Yamamoto, S., Matsui, K., Kitano, M., Ohashi, N., 2000. SM-20550, a new Na^+/H^+ exchange inhibitor and its cardioprotective effect in ischemic/reperfused isolated rat hearts by preventing Ca^{2+}-overload. J. Cardiovasc. Pharmacol. 35, 855–862.

Yokoyama, H., Gunasegaram, S., Harding, S.E., Avkiran, M., 2000. Sarcolemmal Na^+/H^+ exchanger activity and expression in human ventricular myocardium. J. Am. Coll. Cardiol. 36, 534–540.