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Characterization of sabiporide, a new specific NHE-1 inhibitor exhibiting slow dissociation kinetics and cardioprotective effects

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Abstract

Sabiporide, a new benzoguanidine, was characterized on fibroblasts stably expressing the Na^+/H^+ exchanger isoforms NHE-1, NHE-2 and NHE-3. $^{22}Na^+$ uptake experiments show that this compound possesses a K_i of $5 \pm 1.2 \times 10^{-8}$ M for NHE-1, and discriminates efficiently between the NHE-1, -2 and -3 isoforms (K_i for NHE-2: $3 \pm 0.9 \times 10^{-6}$ M, and K_i>1 mM for NHE-3). Similar K_i values are obtained on rat cardiomyocytes and human platelets expressing NHE-1 (K_i's of $7 \pm 1 \times 10^{-9}$ and $2.7 \pm 0.4 \times 10^{-8}$ M respectively). Interestingly, when compared with amiloride and cariporide, sabiporide inhibition persists even after this molecule had been rinsed out (half time of 7 h for sabiporide, and of 1 and 2.5 min for amiloride and cariporide, respectively), the decay of all these molecules exhibiting a complex multiexponential behavior. Thus, sabiporide, which possesses remarkable cardioprotective properties, is a specific NHE-1 inhibitor possessing unique binding kinetics.

Keywords: Sabiporide; Slow dissociation kinetics; Cardioprotective effect

1. Introduction

The Na^+/H^+ exchanger NHE-1 isoform (NHE-1 stands for Na^+/H^+ Exchanger 1) is ubiquitously expressed and plays the crucial role of regulating intracellular pH by catalyzing the exchange of one extracellular sodium ion against one intracellular proton (for review, see Counillon and Pouyssegur, 2000). This exchange, which is electroneutral does not depend on the membrane potential, and is energized by the Na^+ gradient created by the Na^+/K^+ ATPase. Ischemic conditions induce sustained intracellular acidifications (for review, see Dennis et al., 1991), which strongly activate the Na^+/H^+ exchanger. Under these conditions, the Na^+/H^+ exchanger activity, which has been selected by evolution to protect the cells in the case of intracellular acidification, contributes to a massive Na^+ overload. This results in increased ATP consumption by any residual Na^+/K^+ ATPase activity, and in Ca^{2+} influx through reverse activity of the Na^+/Ca^{2+} exchanger, which in normal conditions functions as a Ca^{2+} extruder. In the case of coronary occlusion, these effects are potentially lethal, since Na^+ and Ca^{2+} overload mediated by the exchangers trigger myocardial stunning and/or arrythmia (Avkiran, 1999). Although it has been demonstrated that NHE-1 is not the only sodium-loading acid extruder functioning in the heart cells (Dart and Vaughan-Jones, 1992), the inhibition of this exchanger has clearly been shown to be cardioprotective both in vitro and in animal studies (see for example Avkiran, 1999; Engelhardt et al., 2001). Continuously providing patients suffering from coronary artery disease with NHE-1 inhibitors may therefore be beneficial in the case of acute coronary occlusion. This led to the strategy of designing potent and specific NHE-1 inhibitors, which can be used as cardioprotective compounds. However, to be clinically efficient, these molecules have to possess particular features, such has a high ability to discriminate between the NHE1, NHE-2 and NHE-3 isoforms, the latter two of which mediate transepithelial vectorial transport of Na^+ and acid–base equivalents. For

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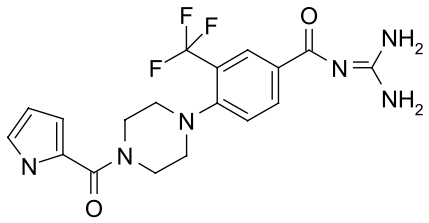

Fig. 1. The structure of the sabiporide molecule.

chronic treatment, these molecules must also have a long half life in vivo. In addition, side effects may arise due to the fact that NHE-1 is highly expressed in neuronal cells, which are extremely sensitive to intracellular pH variations. Thus potential anti-ischemic candidates, which are able to cross the blood–brain barrier, have been shown to exhibit central nervous system side-effects such as somnolence. Amongst the NHE-1 inhibitors, acylguanidinium derivatives, which act in competition with sodium and possess K_i values ranging between the micromolar and the submicromolar level (for review, see Cragoe et al., 1992) have been widely investigated. Unrelated molecules such as imidazolpiperidines might be also potentially interesting (Lorrain et al., 2000). Several inhibitors possessing a high affinity for NHE-1, and a good ability to discriminate between the NHE-isoforms have been developed and tested for their pharmacological properties (see for example Counillon et al., 1993; Scholz et al., 1995; Knight et al., 2001). The results obtained from clinical trials have not been totally conclusive, although protective effects could be detected (Theroux, 2000; Karmazyn, 2000; Avkiran and Marber, 2002). It is thought that the lack of significant therapeutic effects is due to too low dosages of the molecule used in order to avoid central nervous system side effects, and to the fact that the in vivo concentrations decline too fast for efficiency upon chronic administration. In this report, we describe the characterization of a new Na^+/H^+ exchange inhibitor, Sabiporide (Fig. 1) which in addition to its high specificity for NHE-1 exhibits long-lasting inhibitory effects on the antiporter, even after the molecule has been removed from the medium. Thus, this new molecule may solve some of the problems pertaining to the previously tested NHE-1 inhibitors.

2. Materials and methods

2.1. Cell culture

Mutant Chinese Hamster fibroblast (PS120 cell line Pouysségur et al., 1984) stably transfected with either the NHE-1, NHE-2 and NHE-3 isoforms were grown in Dulbecco's modified Eagle's medium supplemented with 50 μg/ml streptomycin, 50 units/ml penicillin, and 7.5% fetal calf serum at 37 °C, in a humidified atmosphere of 5% CO_2 and 95% air.

2.2. Dose–response curves on fibroblasts transfected with NHE-1, -2 and -3

Fibroblasts were seeded on 24 well plates 2 days before the experiment. For the measurement, the cells were acid loaded at 37 °C for 60 min with a solution containing 50 mM NH_4Cl, 70 mM choline chloride, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and buffered at pH 7.4 with 15 mM MOPS (3-*N* morpholinopropanesulfonic acid). The cells were then washed twice in for seconds with 120 mM choline chloride medium buffered at pH 7.4 with 15 mM HEPES–Tris, and loaded with the uptake medium containing 0.2–1 μCi/ml of carrier-free $^{22}Na^+$, 1 mM ouabain, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 120 mM choline chloride, and buffered at pH 7.4 with 15 mM HEPES–Tris. To test the potency of the inhibitor, different concentrations of the inhibitor were added to the uptake medium. Following 3 min of uptake at 37 °C, the cells were then rinsed four times with ice-cold phosphate buffer saline (PBS) in less than 10 s. After cell lysis in 0.1 N NaOH, the radioactivity was measured using either scintillation or γ counting.

2.3. Rinseout kinetics

The cells contained in 24 well plates were exposed to 10^{-7} M of sabiporide or cariporide, and 10^{-5} M of amiloride for 5 min during the NH_4Cl loading procedure (see above), and then rinsed twice with 500 μl per well for each rinse. Following different times after the rinseout, the cells were acidified as described above, and $^{22}Na^+$ uptake was conducted for 3 min at 37 °C to ensure initial rates conditions. The cells were then lysed in 0.1 N NaOH and the radioactivity was counted using either scintillation or γ counting. For each time point, the residual inhibition was calculated as the percentage of the maximal activity of NHE-1 without inhibitor. The data were fitted using SigmaPlot 4.0 (Jandel) software.

2.4. Isolation of rat ventricular myocytes

Single ventricular myocytes were prepared according to the protocol described by Jourdon and Feuvray (1993) from hearts of Wistar rats (200–250 g each) anaesthetized with 50 mg/kg of body weight of i.p. penthotal, using a combination of enzymatic (0.35 mg/ml collagenase (Yakult), 0.05 mg/ml protease type XIV (Sigma)) and mechanical dispersion. Following dissociation, calcium-tolerant rod-shaped ventricular myocytes were plated on to collagen-coated 35 mm culture dishes (Falcon) for analysis.

2.5. Intracellular acidification and pH recovery

Experiments were carried out in HEPES-buffered Tyrode solution (140 mM NaCl, 5.4 mM KCl, $CaCl_2$ 1 mM, $MgCl_2$ 1.2 mM, Glucose 11 mM, buffered at pH 7.4 with 10 mM HEPES) at room temperature. For NH_4Cl-induced

acidification, ammonium chloride was added to this solution to a final concentration of 50 mM. The pH recovery following the ammonium pulse was carried out in the same Tyrode solution containing various concentrations of Sabiporide.

2.6. Intracellular pH measurements

The pH_i of isolated myocytes was monitored using the pH-sensitive dye BCECF/AM (2′,7′-bis(carboxyethyl)-5-6-carboxyfluoresceine/acetoxy methylester) which was incubated for 5 min with the cells at concentration of 5 μM. The cells were then rinsed in tyrode solution and loaded with the tyrode/NH_4Cl solution for 10 min. Following the acid load, the myocytes were perfused with the tyrode solution containing various concentrations of the inhibitors.

During all these steps, which were performed at room temperature, pH variations were monitored by the measurement of BCECF/AM fluorescence (Bidet et al., 1987). The imaging system consisted of a Zeiss ICM 405 inverted microscope with a Zeiss 40 × objective, coupled to a video camera. Fluorescence excitation was provided by a 75 W Xenon lamp (Osram) and was computer-controlled by a shutter. The excitation beam was filtered through 450 and 490 nm narrow band interference filters paired with appropriate quartz neutral-density filters mounted in a computer-controlled motorized wheel. Therefore, the cells were excited successively at 490 and 450 nm, and each image was digitized and stored on the computer hard disk. Image treatment was performed using the Axon® Imaging Workbench (AIW 4.0) software. In our conditions, 3–5 rod-shaped calcium tolerant myocytes were usually present per microscopic field and used in each experiment. For slope calculations and statistical analysis, the data were processed using a Microsoft Excel macro designed for this purpose in the laboratory.

At the end of the experimental series, the fluorescence signals relative to pH_i were calibrated using the K^+/H^+ exchanging ionophore nigericin. For this purpose the cells were perfused with KCl solutions (140 mM KCl, 20 mM Hepes and 10μM nigericin) adjusted to pH 7.4, 6.8 and 6.4. From these measurements, a three-point calibration curve was constructed by plotting the gray level ratios (490/450 nm) as function of the pH values. The pH_i values were then obtained by linear interpolation of the gray level values and the calculated values were adjusted according to the experimental calibration.

For each inhibitor concentration, the cells underwent a control acidification without inhibitor and the slope of the pH recovery was determined. Then following a second acidification, the slope in the presence of a given concentration of inhibitor was measured. The ratio (slope of the pH recovery in the presence of inhibitor/slope of the pH recovery in the control condition) was then expressed as a percent of the exchanger's activity in the presence of a given concentration of the inhibitor, and dose–response curves were constructed from these percentage calculations for several concentrations of the inhibitors. We also verified that the cells responded with the same pH recovery slope for up to three successive intracellular acidificatory treatments, and that their buffering capacity did not vary significantly during the course of the measurements.

2.7. Statistical analysis

All the results presented in this study are the compilation of at least three completely independent experiments. The experimental points for $^{22}Na^+$ uptake experiments have been determined at least in triplicate, and each slope measurement was carried out on two to five myocytes at the same time. Standard errors are given in the numbers, and are also reported on the graphs.

2.8. Chemicals

The NHE-1 inhibitors sabiporide Cl (Lot 8960020), and cariporide (HOE 642; Lot A), were synthesized by Boehringer Ingelheim Pharma and were dissolved in purified water immediately before use. Amiloride was purchased from Sigma, and dissolved to a 0.1-mM concentration in dimethylsulfoxide (DMSO). For the experiments, the concentration of DMSO was kept below 0.1%.

2.9. Preparation of platelets

Whole blood from five healthy volunteers of either sex were used. Blood was collected using an anticoagulant solution containing trisodium citrate (final concentration, 0.315%). The whole blood was centrifuged at 170 × *g* for 15 min at room temperature and the platelet-rich plasma (PRP) was collected (approximately 30–50% of the original volume of whole blood). The platelets in the PRP were loaded with BCECF/AM in a concentration of 4 μg/ml at 37 °C for 30 min. Following this, the platelets were centrifuged (700 × *g* for 10 min at room temperature). The pellet was gently washed with *N*-methyl glucamine (NMG) buffer, and the platelets were resuspended in NMG buffer and adjusted to a final concentration of $75–125 \times 10^6$ cells/ml. The concentration of the platelets was measured with a Coulter counter (Technicon H.1E; Bayer Diagnostics, Germany).

2.10. Fluorescence and pH measurements

Measurements were performed using a fluorescence spectrophotometer (model LS 50B; Perkin Elmer, UK) under continuous magnetic stirring at 37 °C. A randomized order for the fluorescence measurements for each concentration of a given drug was employed. Measurements were automatically taken every 2.3 s for 420 s, using appropriate emission and recording wavenlength for BCECF fluorescence (see above).

After a control period 0.3–1000 nM of sabiporide was administered to the cell suspension, followed by administration of nigericin to a final concentration of 5 μM. After 60 s, bovine serum albumin (BSA) was added (final concentration: 5 mg/ml) to scavenge nigericin and after another 60 s NaCl was added to a final concentration of 50 mM to activate the sodium-proton exchanger. The drugs or vehicle (15 μl), nigericin (7.5 μl of a 1-mM solution), BSA (37.5 μl of a 20% solution), and NaCl or vehicle (37.5 μl of a 2-M solution) were added directly into the platelet suspension (1.5 ml) in the cuvette 60, 120, 180 and 240 s after the start of the measurement, respectively.

Fluorescence data were recorded and analyzed by a commercially available software (FL winlabs; Perkin Elmer). The average of 10 points immediately prior to the administration of the NaCl was used as a base line for the calculation of the area under the curves (AUCs). The AUCs for 60 s from the point of NaCl addition (240–300 s) were calculated for each experiment. The ratio of AUCs of pH_i recovery with drug pretreatment against AUCs of pH_i recovery with vehicle treatment was represented as percentage of control as mentioned below. All results are given as means ± S.E.M. The relative inhibition of the NHE-1-dependent pH recovery was calculated as the percentage of the difference between the AUC of the control minus the AUC of the vehicle.

The dose–response curves were fitted with a four-parametric logistic equation to yield IC_{50} values.

2.11. Determination of infact size afer ischemia/reperfusion in isolated rabbit hearts

Twenty-three New Zealand White (NZW) rabbits of either sex were anaesthetised using 30 mg/kg i.v. pentobarbital. A tracheotomy was performed and the animals were ventilated using a Harvard Apparatus rodent ventilator. A mid-sternal thoracotomy was performed and the heart was exposed. The aorta was dissected and the heart was rapidly excised, placed in physiologic saline, washed and mounted to the Langendorff apparatus within less than 30 s. The hearts were perfused using a modified Krebs–Henseleit buffer solution containing (mM): NaCl (126.0), KCl (4.7), $MgSO_4$ (1.2), KH_2PO_4 (1.2), $CaCl_2$ (2.5), D-glucose (10.0), $NaHCO_3$ (17.0) and adjusted to a pH of 7.4. The perfusion buffer was bubbled with O_2/CO_2 at a ratio of 95%:5%.

A latex balloon connected to a pressure transducer was inserted into the left ventricle through the left atrium and was adjusted to a preload of approximately 10 mm Hg. The aortic flow was measured using an ultrasonic flowprobe (Transsonics, Ithaca, NY) and the hearts were immersed in a buffer-filled double-walled glass waterjacket at 37 °C. A suture was placed around the left main coronary artery approximately 3 mm distal to its origin.

After the heart had stabilized the right atrium was stimulated at a frequency of 4 Hz (240 bpm), which was continued until the end of the experiment. Fifteen minutes later sabiporide ($N=12$) in a final concentration of 3.0 μM or vehicle (purified water; $N=11$) was added to the perfusion buffer. The infusion was continued for the entire duration of the experiments. Ten minutes into the sabiporide perfusion the suture was tightened and following 30 min of ischemia the heart was reperfused for 2 h. At the end of the reperfusion period the heart was arrested by perfusion with 1 M KCl solution, the suture was re-tightened and the non-ischemic region was stained using black ink. The heart was then removed from the Langendorff apparatus and cut into approximately 3 mm thick horizontal slices. The heart slices were then stained for 15 min with 1% triphenoltetrazolim-chloride in PBS at pH 7.0 and at 37 °C. The proximal face of the slice was then photographed using a digital camera with a macro lens and the digital image was planimetrically evaluated using a commercial computer program (Photoshop 5.0, Adobe Systems, San Jose, CA). The area unstained by the ink was defined as the area at risk and the white areas within the area at risk were defined as the infarct size.

2.12. Ethics

The experimental protocols used in this work comply with the Declarations of Helsinki and Kyoto for materials obtained from human subjects. For experiments involving animals, the European Community guidelines have been adhered to, and the experimental protocols have been approved by our respective ethics committees.

3. Results

3.1. Sabiporide dose–response curves for the NHE-1, NHE-2 and NHE-3 isoforms

Different concentrations of sabiporide were tested for their ability to inhibit the $^{22}Na^+$ uptake mediated by the NHE-1, -2 or -3 isoforms stably transfected in PS120 cells. In the experimental conditions used in this study, the exchangers were fully activated by intracellular protons, and the uptake time was short enough so that the measured inhibition of the initial rates of Na^+/H^+ activity corresponded to initial rates of the exchangers at their maximal activation by intracellular protons. Using this technique, the estimated K_i's for sabiporide were $5 \pm 1.2 \times 10^{-8}$ M for the NHE-1 isoform and $3 \pm 0.9 \times 10^{-6}$ M for NHE-2 (Fig. 2). It was impossible to determine the value for the half inhibition constant for the NHE-3 isoform, due to the fact that at millimolar concentrations, NHE-3 was still not inhibited by the molecule. Thus, sabiporide is highly discriminative between each isoform, since it completely blocks NHE-1 at concentrations at which NHE-2 retains about 30% of its activity and NHE-3 is still completely active.

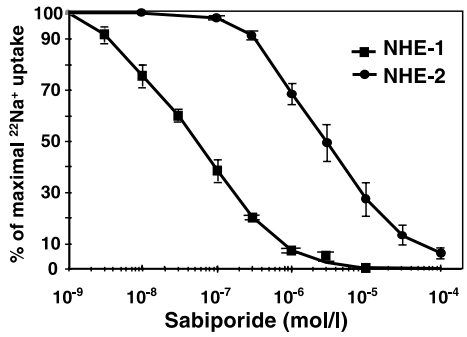

Fig. 2. Dose–response curves for the inhibition of the NHE-1, NHE-2 and NHE-3 isoforms by sabiporide. Cells were grown to confluence in 24-well plates, and then loaded with H^+ using the NH_4Cl prepulse technique. As described in the Section 2, initial rates of $^{22}Na^+$ uptake was determined at 37 °C in the presence of various concentrations of sabiporide for the three isoforms expressed in PS120 fibroblasts. Note the difference between NHE-1 and NHE-2 and the absence of detectable inhibition for NHE-3.

3.2. Sabiporide inhibition of the Na^+/H^+ exchanger present in rat cardiomyocytes

We used a modified version of the Langendorf technique to prepare rat cardiomyocytes (Jourdon and Feuvray, 1993). Following BCECF loading, the cardiomyocytes were acidified using the ammonium prepulse technique (Boron and De Weer, 1976), and the percentages of inhibition corresponding to various doses of sabiporide were measured by comparing the slopes of pH recovery in the presence of the inhibitor with those determined from control acidifications. The half inhibition constant for sabiporide on the Na^+/H^+ exchanger expressed in rat cardiomyocytes is $7 \pm 1 \times 10^{-9}$ M (Fig. 3). Although this value might seem different from the K_i value determined by $^{22}Na^+$ uptake, it is important to notice that the measurement techniques are completely different. Initial rates of Amiloride-sensitive $^{22}Na^+$ uptake directly reflect the activity of the exchanger, while kinetics of pH variation integrate the activity of multiple effectors. In this context, the values obtained by these different experimental approaches are self-consistent.

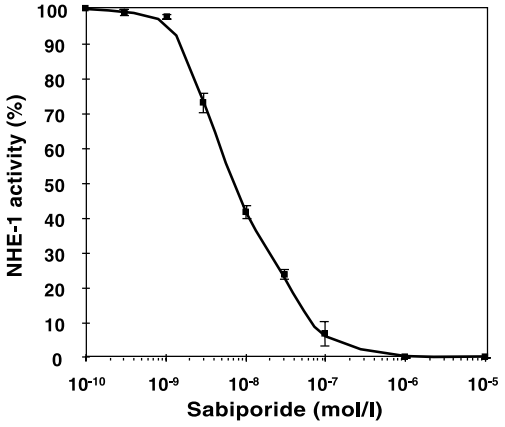

Fig. 3. Dose–response curves for the inhibition of the NHE-1 expressed in rat cardiomyocytes. Calcium-tolerant rat cardiomyocytes obtained using a modified version of the Langendorf technique were acidified using the ammonium prepulse technique. The initial rates of pH recovery mediated by the Na^+/H^+ exchanger at room temperature were determined in the presence of different concentrations of sabiporide using BCECF fluorescence imaging to monitor pH variations as described in Section 2.

3.3. Dose–response curves of sabiporide inhibition on platelets

To obtain an independent estimate of the K_i value of Sabiporide on human cells, the dose–response curve of inhibition of this molecule was determined using fluorescence measurements on platelets obtained from healthy human volunteers. The results (percentage of control) of the areas under curves (AUCs) over 60 s for all concentrations of the drug are shown in Fig. 4. The IC_{50} values for sabiporide in the human platelets was $2.75 \pm 4.7 \times 10^{-8}$ M. Although this value is in the same order of magnitude than the result obtained on rat cardiomyocytes, the slight difference observed can be easily explained by the difference in the measurement technique, and by interspecies variations between the NHE-1 isoform expressed in humans and rats. A similar K_i variation has been observed between human and dog platelets (data not shown).

3.4. Washout kinetics of sabiporide

Using our experimental setup to investigate the sabiporide inhibition on cardiomyocytes, we observed that following exposure to inhibitory concentrations of Sabiporide, it was impossible to re-activate the exchanger, even if the cells have been rinsed for substantial amounts of time (Fig. 5). This phenomenon was not observed with amiloride and cariporide. These results therefore suggest that the sabiporide compound possesses a long lasting inhibitory effect on the Na^+/H^+ exchanger, presumably by having a very low k_{off} for this molecule. To further investigate these effects,

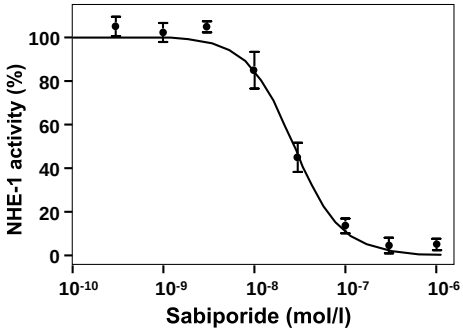

Fig. 4. Dose–response curves for the inhibition of the NHE-1 expressed in human platelets. Platelets obtained from healthy human volunteers were acidified using the K^+/H^+ ionophore nigericin. For various concentrations of sabiporide, pH recovery mediated by the Na^+/H^+ exchanger was measured using BCECF fluorescence, as described in Section 2. The percentages of inhibition were determined as the ratio of the area under the pH recovery curve (AUC) in the presence of the inhibitor, with the same area measured in control conditions.

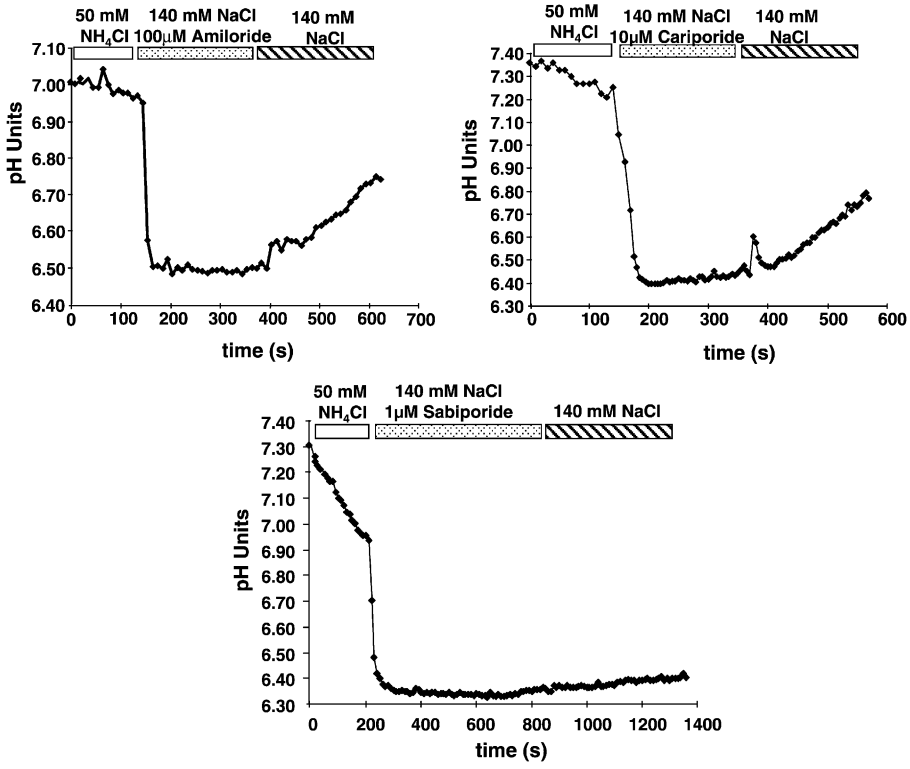

Fig. 5. Long-lasting inhibitory effect of Sabiporide. pH variations were monitored on cardiomyocytes using BCECF fluorescence measurements. Following the ammonium-loading, the myocytes were perfused with tyrode medium containing the various inhibitors (10^{-5} M for cariporide and 10^{-6} M for sabiporide, and 10^{-4} M for amiloride), and then the medium was replaced by the same perfusate without inhibitors. Note the rapid pH recovery after amiloride and cariporide removal, as compared to the absence of significant pH variation following the removal of sabiporide. Each trace corresponds to the average of recordings from at least three different myocytes.

we decided to conduct washout kinetics of sabiporide (10^{-7} M) using $^{22}Na^{+}$ uptake experiments at 37 °C. For this purpose, PS120 fibroblasts transfected with NHE-1 were submitted to 5 min exposure to sabiporide, and after different rinsing times, the cells were acidified using the ammonium prepulse technique, and the residual activity of the exchanger measured by determining the initial rates of $^{22}Na^{+}$ uptake. For comparison, the same rinsing kinetics have been performed using equipotent concentrations of cariporide (10^{-7} M) and amiloride (10^{-5} M). Fig. 6 shows that the decays of the sabiporide, cariporide and amiloride inhibition are exponential as it can be expected for a rinseout kinetic in a nearly infinite volume. However, it can be observed that the rates of the decay of inhibition are extremely different between the classical inhibitors of NHE-1 and for sabiporide. Non-linear fitting of the observed decay curves show a complex multiexponential behavior. The observed half times are roughly of 2.5 min for cariporide and 1.5 min for amiloride, compared to 7 ± 3 h for sabiporide.

3.5. Cardioprotective effect of sabiporide

To evaluate the protective effects of sabiporide on ischemia/reperfusion-induced necrosis, isolated rabbit hearts (pretreated or not with 3 μM sabiporide) were submitted to 30 min of ischemia by reversibly suturing the left main coronary artery, followed by 2 h reperfusion. The anti-necrotic properties of sabiporide were then evaluated by comparing the size of the area at risk with the infarct size. The results are expressed in Fig. 7 as the percentage of the

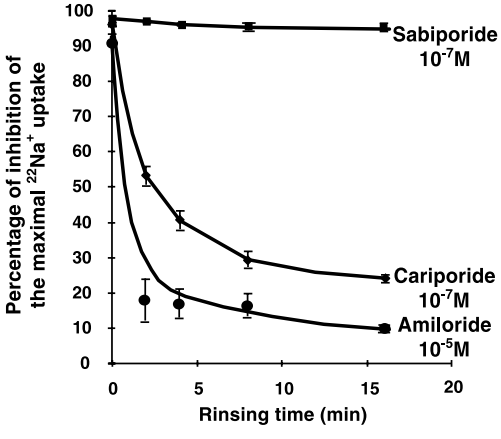

Fig. 6. Decay of the inhibition of NHE-1 by amiloride, cariporide and sabiporide. Fibroblasts expressing NHE-1 preincubated for 5 min with each of the inhibitors (10^{-7} M for cariporide and sabiporide, 10^{-5} M for amiloride), and then rinsed for various periods of time. Initial rates of NHE-1 activity were then measured using $^{22}Na^{+}$ uptake. Note the very slow decay of inhibition for sabiporide as compared to the other inhibitors.

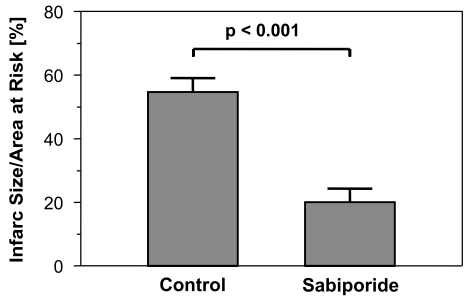

Fig. 7. Cardioprotective properties of sabiporide: isolated rabbit hearts were submitted to 30 min of ischemia followed by two hours reperfusion, in the presence or in the absence of 3 μM sabiporide, which was perfused 15 min before the begining of ischemia. The anti-necrotic properties of sabiporide are showed by the percent of the infarct size versus the area at risk, both for the control and treated hearts. Note the important reduction of the infarct size for the treated hearts.

ratio of these two values. These results clearly demonstrate the anti-necrotic effect of sabiporide, the percentage of infarct size/area at risk being reduced by 63% in the presence of the drug when compared to the control conditions.

4. Discussion

In this article, we describe the effects of a new molecule, sabiporide on the inhibition of the Na^+/H^+ exchangers. When assayed in fibroblasts expressing either the NHE-1, NHE-2 or NHE-3 isoforms, this inhibitor blocks NHE-1 with a K_i of $5 \pm 1.2 \times 10^{-8}$ M, and NHE-2 with a K_i of $3 \pm 0.9 \times 10^{-6}$ M while NHE-3 is totally unaffected even at millimolar concentrations. Thus, sabiporide is a selective NHE-1 inhibitor possessing an affinity comparable to that of cariporide on the ubiquitously expressed NHE-1 isoform. Preliminary experiments in the presence of 140 mM extracellular sodium showed that the apparent affinity of sabiporide for NHE-1 was significantly decreased when compared to the above mentioned conditions, where sodium was used as a tracer. This suggests that sodium and sabiporide compete at least partially on the Na^+/H^+ exchanger. To further investigate the effects of this inhibitor, we determined the ability of different concentrations of sabiporide to block the initial rates of NHE-1-mediated pH recovery in rat cardiomyocytes, and human platelets. The obtained IC_{50} values are respectively $7 \pm 1 \times 10^{-9}$ M for the rat myocytes and $2.7 \pm 4.7 \times 10^{-8}$ M for the human platelets, consistent with the K_i value obtained from $^{22}Na^+$ uptake measurements for the human NHE-1.

We decided to investigate the binding kinetics of this molecule on the exchanger. As depicted in Figs. 5 and 6, the inhibition of NHE-1 by sabiporide persists for long periods of time after the inhibitor had been rinsed out, especially when compared to amiloride and cariporide, which inhibition has been shown consistently to be reversible in human ventricular myocytes (Yokoyama et al., 2000). Nonlinear analysis of the data showed that the best fit was multi-exponential, suggesting a complex dissociation mechanism. Two hypotheses, which might be interrelated, can account for this long-lasting inhibition. The simplest hypothesis is that the molecule binds to a highly hydrophobic part of the exchanger and has a very low off-kinetic constant, resulting in a very long half-time on the molecule. A complementary hypothesis is that this compound, as it is the case for the majority of the acylguanidinium derivatives (see for example Benos et al., 1983) can partition in the plasma membrane, which may constitute a storage compartment for the molecule. This might result in a slow release of the compound, producing inhibitory effects over a long period of time. However, the first hypothesis might account for the major part of this long lasting effect, for the following reasons: first, sabiporide is not noticeably more hydrophobic than other NHE- inhibitors, and therefore, if partition in a membrane compartment was involved, all the NHE- inhibitors should exhibit such long-lasting inhibition kinetics, which is not the case. Second, when compared to the bulky hydrophobic core of the Na^+/H^+ exchanger, sabiporide is comparatively small. It is therefore difficult to imagine how the guanidinium group of this molecule could reach the functional sites of the exchanger, which are embedded in the transmembrane part of the protein. To confirm that the long-lasting inhibitory effect is not due to the partitioning of the molecule in the plasma membrane further studies using radiolabelled sabiporide, and competition experiments have to be performed.

The fact that this inhibitor possesses long lasting effects when tested in vitro, is likely to correspond to improved clinical properties, since we did not notice any particular toxic effects for fibroblasts in culture, cardiomyocytes, or when administered to animals at concentrations up to 130 mg/kg of body weight in drinking water (Touret, Tanneur and Counillon, unpublished results). Firstly, when administered during relatively short periods of time, this molecule can accumulate on the Na^+/H^+ exchangers, and can resist long periods of rinseout by the blood flow or by cardioplegic solution in the myocardium. Additional beneficial effects may be due to the fact that this molecule can remain bound to the Na^+/H^+ exchanger over long periods of time, despite the fact that its soluble form has been eliminated from the plasma.

Taken together, the data concerning the specificity and the particular behavior of this new molecule on the Na^+/H^+ exchanger make it a particularly interesting NHE-1 specific inhibitor, which may be a promising candidate as a new therapeutic agent. The results shown in Fig. 7 already demonstrate that when used on isolated hearts submitted to ischemia/reperfusion injury, sabiporide possesses excellent anti-necrotic properties, the administration of 3 μM of this drug 15 min before ischemia resulting in a relative decrease of 63% for the infarct size when compared to the control conditions. The effects of this molecule will be tested soon in preclinical and clinical studies.

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