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Differences in the effects of Na^+–H^+ exchange inhibitors on cardiac function and apoptosis in guinea-pig ischemia-reperfused hearts

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Abstract

The protective effects of the Na^+–H^+ exchange (NHE) inhibitors SM-198110 (2-[[(aminoiminomethyl) amino] carbonyl]-4-chloro-1*H*-indole-1-propanesulfonic acid monohydrate) and SM-197378 (*N*-(aminoiminomethyl)-1-methyl-7-(sulfooxy)-4-(trifluoromethyl)-1*H*-indole-2-carboxamide monohydrate) were investigated in perfused Langendorff guinea-pig hearts subjected to ischemia (40 min) and reperfusion (40 min). The recovery of left ventricular developed pressure (LVDP) from ischemia by reperfusion was 39.0% in the control, while in the hearts pretreated with SM-198110 or SM-197378 (10^{-7} M), it was about 100%. The ATP level, monitored simultaneously by ^{31}P-nuclear magnetic resonance spectrometry, was already higher than the control value at the end of the ischemic period, and the elevation in Na^+ or Ca^{2+} fluorometric signals induced during ischemia was suppressed. In post-treated hearts, the LVDP recovery rate was significantly higher with SM-198110 than with SM-197378. By in vitro electron paramagnetic resonance spectrometry, SM-197378 was found to directly quench the active oxygen radical, whereas SM-198110 had no effect. Numbers of apoptotic cardiomyocytes after ischemia (1 h) followed by reperfusion (5 h) were significantly lower in SM-197378-treated than in SM-198110-treated hearts, consistent with the level of activity of caspase-3. These results suggest that the antioxidant effects of NHE inhibitors have an important role in apoptosis during ischemia–reperfusion, but apoptosis is not a major manifestation of cardiac function during postischemic recovery, and that NHE-sensitive mechanisms of reperfusion injury promote both necrotic and apoptotic processes death.

Keywords: Na^+–H^+ exchange inhibitor; LVDP (Left ventricular developed pressure); ^{31}P-NMR (nuclear magnetic resonance); EPR (Electron paramagnetic resonance) spectrometry; Ischemia–reperfusion injury; Apoptosis; (Guinea pig)

1. Introduction

Ischemia–reperfusion injury is a major clinical problem causing myocardial damage, arrhythmias and stunning via an increase in the intracellular Na^+ concentration, intracellular Ca^{2+} concentration and intracellular pH (pH_i), mediated by activation of Na^+–H^+exchange (NHE) (Karmazyn, 2001). Reperfusion induces microvascular injury or ventricular arrhythmia and accelerates or promotes cell death (necrosis, Opie, 1989; or apoptosis, Wang et al., 2002). The intracellular accumulation of Ca^{2+} is abnormally high during ischemia (Hotta et al., 1998) and especially soon after reperfusion is suggested to be a mechanism for reperfusion injury (Brooks et al., 1995). The increase in intracellular Ca^{2+} is mediated in part by a rise in intracellular Na^+ resulting from activation of NHE (Tani and Neely,

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1989). The use of inhibitors of NHE has been proposed to reduce arrhythmia and myocardial infarction by preventing Na^+ overload and consequently Ca^{2+} overload. In fact, ethylisopropyl-amiloride (EIPA)(Bugge et al., 1996), 3-methylsulfonyl-4-piperidinobenzoil-guanidine methanesulfonate (HOE694) (Toint and Opie, 1993), cariporide (HOE642) (Aye et al., 1999), 2-methyl-5-methlsulphonyl-1-(1-pyrrollyl)-benzoyl-guanidine (EDM85131) (Gumina et al., 1998), *N*-(aminoimino-methyl)-1,4-dimethyl-1*H*-indole-2-carboxamide methanesulfonic acid (SM20550) (Yamamoto et al., 2000a; Yamada et al., 2001; Ito et al., 1999; Hotta et al., 2001a, 2001b), and *N*-(aminoiminomethyl)-11-5,6,7,8-tetrahydro-8-oxo-4*H*-pyrrolo [3,2,1-kl] [1] benzazocine-2-carboxamide monomethansulfonate monohydrate (SMP300) (Yamamoto et al., 2000b) have been reported to inhibit stunning, reperfusion arrhythmia, and myocardial infarction in several animal models of ischemia and reperfusion.

In this study, two newly synthesized compounds, SM-198110 (2-[[(aminoiminomethyl) amino] carbonyl]-4-chloro-1*H*-indole-1-propanesulfonic acid monohydrate) and SM-197378 [*N*-(aminoiminomethyl)-1-methyl-7-(sulfooxy)-4-(trifluoromethyl)-1*H*-indole-2-carboxamide monohydrate] (Fig. 1A), were evaluated for their cardioprotective

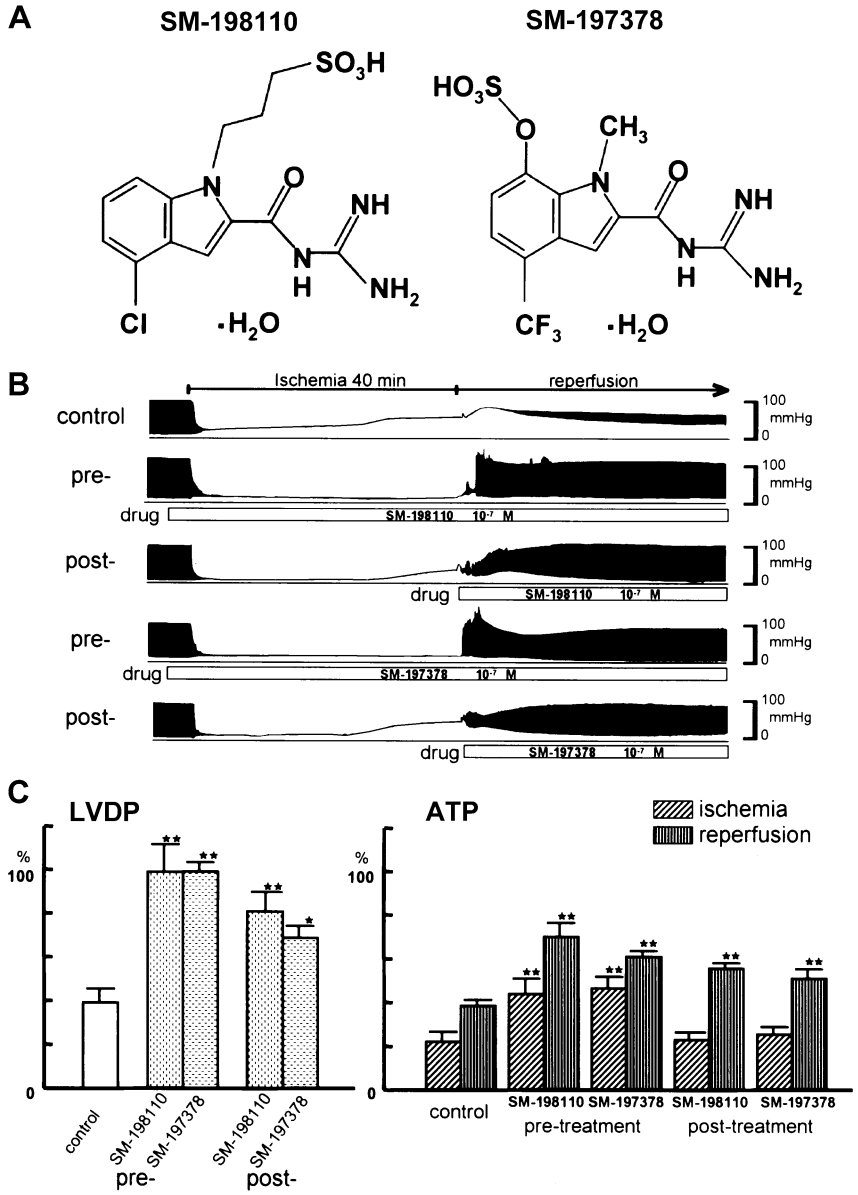

Fig. 1. (A) Structural formula of the Na^+–H^+ exchange inhibitors, SM-198110 and SM-197378. (B) Beneficial effects of SM-198110 or SM-197378 (10^{-7} M) during ischemia–reperfusion in Langendorff heart preparations. Treatment with SM-198110 or SM-197378 enhanced the recovery of LVDP after reperfusion compared to control. (C) Post-ischemia recovery of left ventricular developed pressure (LVDP) by reperfusion of untreated heart (control), and hearts treated with SM-198110 or SM-197378 (left) and the changes in high-energy phosphates (ATP) during 40 min of ischemia and a subsequent 40 min of reperfusion (right). *P<0.05, **P<0.01—significantly different from the control value. Data are expressed as the mean values from six preparations. Vertical lines represent S.E.M.

effect on ischemia–reperfusion injury in isolated perfused guinea-pig hearts. Furthermore, the effect on myocardium infarction of SM-198110 or SM-197378 was investigated in hearts pretreated (Pregroup) or post-treated (Post-group) with these agents. In addition to having a direct protective effect, SM-198110 or SM-197378 caused a change in the intracellular levels of ATP, Na^+ and Ca^{2+} during ischemia and reperfusion measured simultaneously with heart function. In connection with our investigation of Ca^{2+} systems, mitochondrial Ca^{2+} uptake by acidification and Ca^{2+} concentration changes similar to those of reperfusion after global ischemia in Langendorff heart preparations were measured following pretreatment with SM-198110 or SM-197378. Additionally, the ability of both drugs to scavenge active oxygen radicals was measured. There is now evidence that apoptosis, or programmed cell death, is an important response of the myocardium to ischemia, which precedes cell necrosis and appears to contribute to the overall sequelae of cardiac injury. The role of NHE in this response can be delineated with NHE inhibitors (Karmazyn, 2001). Therefore, we examined which protective action is associated with the beneficial effects of the NHE inhibitors, SM-198110 and SM-197378, with respect to the reduction in terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling assay (TUNEL)-positive myocytes and activation of caspase-3 related with NHE1 expression (Wu et al., 2003).

2. Materials and methods

2.1. Cardiac myocytes

2.1.1. Measurement of intracellular pH (pH_i) and Na^+–H^+ exchange (NHE) inhibitory activity

Cardiac myocytes were isolated from Sprague–Dawley rats (6–7 weeks), according to the enzymatic digestion method described previously (Yamamoto et al., 2000a). For the measurement of pH_i, myocytes were loaded with BCECF-AM (3′-*o*-acetyl-2′, 7′-bis(carboxymethyl)-4 or 5-carboxy fluorescein, diacetoxymethyl ester) (3 μM) for 30 min at room temperature. Myocytes loaded with BCECF were then allowed to settle on a laminin-coated glass coverslip at the bottom of a small chamber, which was mounted on the stage of an inverted microscope (Nikon, Tokyo, Japan). After adhering to the coverslip, myocytes were superfused (3 ml/min) with normal HEPES (2-[4-(2-hydroxyethyl)-1-piperatinyl] ethanesulfonic acid) solution (composition in mM: NaCl 137, KCl 5.9, $CaCl_2$ 2.2, $MgCl_2$ 1.2, glucose 14, HEPES 10, pH adjusted to 7.4 with Tris). Intracellular BCECF was illuminated at 450 and 490 nm and the BCECF ratio (490 nm/450 nm) of emitted light signal at 530 nm was measured with a fluorescence image analyzer (Argus-50, Hamamatsu Photonics, Hamamatsu, Japan). The emission intensity ratio (BCECF ratio) was used as an index of pH_i. The activity of drugs in inhibiting NHE was determined by the inhibition of pH_i recovery from NH_4Cl prepulse-induced intracellular acidosis. This measurement was performed under HCO_3^--free conditions, in which pH_i recovery from acidosis is restricted to the activity of the NHE. The test drugs or vehicle was present for 3 min prior to the NH_4Cl prepulse and throughout the entire experimental period (n=14–21).

2.1.2. Measurement of Na^+–Ca^{2+} exchange (NCE) inhibitory activity

The NCE activity in rat cardiomyocytes was determined by measuring Na^+-dependent Ca^{2+} influx. The cardiac myocytes were loaded with fura 2-AM (5 μM) for 20 min at room temperature. The activity of drugs in inhibiting NCE was determined by the inhibition of the increase in the fura-2 ratio (340 nm /380 nm, emitted at 550 nm) after perfusion of Na^+-free normal HEPES buffer (NaCl was eliminated and replaced with a molar equivalent of choline chloride). The test drugs or vehicle was present for 2 min prior to exposure to Na^+-free buffer and throughout the entire experimental period.

2.2. Langendorff guinea-pig hearts

2.2.1. Heart preparation and examination procedures

Hartley strain guinea pigs of either sex, weighing 300–350 g, were anesthetized with diethyl ether and heparinized (250 IU, i.p.). The heart was rapidly excised and the aorta was cannulated. The Langendorff heart preparations were then perfused with Krebs–Henseleit solution (KH solution, pH 7.4, at 37 °C) containing in mM: NaCl 115, $NaHCO_3$ 25, KCl 4.7, $CaCl_2$ 2.0, $MgCl_2$ 1.2, KH_2PO_4 1.2 and glucose 10. The KH solution was presaturated with a gas mixture containing 95% O_2 and 5% CO_2, and the heart was perfused at a constant pressure of 75 cm H_2O for NMR spectrometry or at a flow rate of 7 ml/min, using a peristaltic pump, for fluorometry. Subsequent to an equilibration period of 30 min, to stabilize mechanical function, the perfused hearts were exposed to 40 min of global ischemia by clamping the perfusion flow line, and were then reperfused for 40 min with the drug-containing medium. In the pretreatment groups, SM-198110 or SM-197378 (10^{-7} M) was introduced into the perfusate on line 4 min before the start of ischemia, and in post-treatment groups, SM-198110 or SM-197378 (10^{-7} M) was added only to the perfusate during reperfusion. To examine TUNEL and caspase-3 activity, the hearts were subjected to 1 h of ischemia and 5 h of reperfusion. Drugs (10^{-7} M) were administered for 4 min before ischemia and for 5 h throughout reperfusion.

2.2.2. Measurement of myocardial function

Coronary flow (FR) in the NMR analysis was measured continuously with an in-line flow probe connected to an ultrasonic flow meter (transonic T101, Advance, Ithaca, NY). A Latex balloon (Hirokawa, Niigata, Japan) was introduced into the left ventricle via the left atrium and

connected to a strain-gauge transducer (MIP-5100, Baxer, Tokyo, Japan) for measurement of isovolumic left ventricular pressure. The left ventricular end-diastolic pressure (LVEDP) was adjusted to 10 mm Hg during the equilibration period in each heart, and the volume of the balloon was not changed during the experiments. The left ventricular developed pressure (LVDP) was calculated by subtracting LVEDP from left ventricular systolic pressure.

2.2.3. ^{31}P-NMR measurement and data analysis

Myocardial temperature was maintained at 37±0.5 °C by means of a water-jacketed perfusion line and a continuous stream of air around the NMR sample tube. The heart connected to the Langendorff perfusion line was placed in a standard 20-mm NMR tube with the apex approximately 2.5 cm from the bottom of the tube, and the tube was inserted into the NMR coil. The effluent was removed from above the heart by a peristaltic pump, leaving the heart submerged in a fixed volume of perfusate. In the ischemia–reperfusion experiments, ^{31}P-NMR spectra were monitored along with simultaneous recording of ventricular pressure, as described previously (Hotta et al., 1998, 2001; Koike et al., 1996), and paced at a rate of 3–4 Hz stimulation via a 3 M KCl agar electrode. ^{31}P-NMR spectra were obtained at 161.8 MHz on a GSX 400 spectrometer (JEOL Datum, Tokyo, Japan) equipped with a 9.4-T vertical-bore magnet. For each spectrum, 90 free-induction decays (4 min) were accumulated after 45° flip-angle pulses (18 μs) using 4096 data points and a 15.015 kHz spectral width with a repetition time of 2 s. Accumulated free-induction decays were filtered exponentially, resulting in a broadening by 30 Hz.

PCr, Pi and -ATP were quantified by comparison with a capillary tube of standard methylenediphosphonic acid (0.25 M) fixed inside the NMR tube. Phosphate peaks, expressed as percentages of control values, were determined by measuring the area under each resonance peak. The subsequent relative intensities of each peak were used for quantitative analysis. Datum Station ALICE software (JEOL Datum) was used to determine the area under each peak using a personal computer. The intracellular pH (pH_i) was calculated from the chemical shift between the phosphocreatine (PCr) and inorganic phosphate (Pi) resonances using the following equation: $pH=6.90-\log[(\delta_o-5.85)/(3.29-\delta_o)]$, where δ_o is the chemical shift of Pi from PCr expressed as parts per million (ppm).

2.2.4. Cytosolic ion fluorometry in Langendorff hearts

Cytosolic Na^+ and Ca^{2+} fluorometry was performed with a fluorometer (CAF-100 or CAM230 with the biological fiberscope, FB30; Japan Spectroscopic, Tokyo, Japan) as previously described (Hotta et al., 1998). The changes in the content of each ion during ischemia and reperfusion in Langendorff guinea-pig hearts, preloaded with a specific fluorescent indicator for each ion, were measured during simultaneous recording of mechanical performance (LVDP). The hearts were field-stimulated at a driving rate of 3–4 Hz using a pair of platinum plate electrodes. After loading with each indicator (added at 0.025% cremophore EL), the heart was perfused in a non-recirculating mode for 10 min to remove excess indicator fura-2 AM from the extracellular space. Subsequent to an equilibration period of 20 min, the changes in ion content and LVDP during ischemia (40 min) and reperfusion (40 min) were measured as described for the protocol with ^{31}P NMR. Drug was introduced into the perfusate online 4 min before the start of ischemia. The hearts incubated with fura-2 AM (2 μM, for Ca^{2+} measurements, 30 min as loading time) and SBFI AM (6 μM, for Na^+ measurements, 90 min as loading time) were measured at 500 nm and the ion content is expressed as the ratio of fluorescence strength (R340/380) excited at 340 nm (F340) and 380 nm (F380). Because of the difficulty of determining the real dissociation constant of fura-2 or SBFI for Ca^{2+} or Na^+ in myocardial cells, in the present study we used the ratio of R340/380 as an indicator of cytosolic Ca^{2+} or Na^+.

2.3. Mitochondria

2.3.1. Intramitochondrial fluorometric measurements according to the change in the $Ca2^+$ concentration or pH of the perfusate

Mitochondria were isolated from guinea-pig hearts as previously described (Hotta et al., 1999, 2001b). The suspensions were incubated at 24 °C in a normal medium with a composition similar to the intracellular ionic composition (100 nM Ca^{2+}, 10 mM Na^+ and 110 mM K^+) containing respiratory substrates (composition in mM: sucrose 250, $MgCl_2$ 1, KH_2PO_4 1, succinate 10, malate 5, and MOPS (3-morpholinopropanesulfonic acid) 20 adjusted to pH 7.4 with KOH) and the Ca^{2+} fluoroprobe fura-2AM (10 μM) and 0.025% cremophor EL. A 0.5-ml aliquot on mitochondria was allowed to settle on a glass coverslip of the stage of an inverted microscope (CAM230, Japan Spectroscopic) for 30 min. The coverslip had been treated with poly-L-lysine to promote mitochondrial adhesion. After dye loading, the mitochondria on the coverslip were mounted on the stage of an inverted microscope and washed continuously with a dye-free solution for 10 min, before being washed continuously with 10 ml of medium. Fura-2 Ca^{2+} signals were measured at 500 nm as the ratio of fluorescence (R340/380) excited at 340 nm (F340) and 380 nm (F380). After fura-2 was loaded for 30 min, the F340 fluorescence of the mitochondria increased by five- to sevenfold compared with that of unloaded mitochondria. The Ca^{2+} signal of mitochondria preloaded with high Ca^{2+} concentrations (10 μM) for over 5 min was markedly increased by lowering the Ca^{2+} concentration from a high to a physiologically low level (100 nM) or by acidifying the perfusate (pH 7.5→6.5). We then determined whether SM-198110 or SM-197378 suppressed the Ca^{2+} increase induced by these manipulations. The final mitochondrial protein concentration was adjusted to 30–35 mg/ml by dilution.

2.4. Examination procedures for detection of apoptosis

2.4.1. Histological examination and assessment of internucleosomal DNA cleavage by the TUNEL method and measurement of caspase-3 activity

The Langendorff heart preparations were perfused with normal Krebs–Henseleit solution (KH solution, pH 7.4, at 37 °C) presaturated with a gas mixture containing 95% O_2 and 5% CO_2. Subsequent to an equilibration period of 30 min, required to stabilize mechanical function, the preparations were perfused with a modified KH solution in which glucose 10 mM was replaced by 2-deoxy-D-glucose 5 mM and glucose 5 mM 4 min before ischemia for 1 h. After reperfusion for 5 h the left ventricle was cut into several pieces. SM-198110 or SM-197378 (10^{-7} M) was introduced into the modified perfusate for 4 min before the start of ischemia and was added to the drug-containing medium used for reperfusion. The changes in cellular levels of high-energy phosphates in the heart together with simultaneous recordings of LVDP, LVEDP and FR were monitored by ^{31}P-NMR. The proximal portions of the isolated Langendorff hearts were fixed for 2 h at room temperature with phosphate-buffered solution containing 2% paraformaldehyde and embedded in paraffin for subsequent routine histological examination and the TUNEL method (Itoh et al., 1995; Tong et al., 2002).

Other sections of each heart were stored in liquid nitrogen for the measurement of caspase-3 activity using a CPP32/Caspase-3 fluorometric protease assay kit (Casciola-Rosen et al., 1996). The assay is based on detection of the cleavage of substrate DEVD-AFC (AFC: 7-amino-4-trifluoromethyl coumarin). DEVD-AFC emits blue light (λ_{max}=400 nm), but upon cleavage with CPP32 or related caspases, free AFC emits a yellow-green fluorescence (λ_{max}=505 nm), which can be quantified using a fluorometer. Comparison of the fluorescence of AFC from an apoptotic sample with an uninduced control allows determination of the fold-increase in CPP32 activity. The uninduced control was a heart preparation which has been perfused with normal solution (control-1).

2.5. Electron paramagnetic resonance (EPR) spectrometry

2.5.1. Measurement of the quenching effect of superoxide anion radicals (O_2^-) and hydroxyl radicals (OH)

SM-198110 and SM-197378 were suspended in 15 mM NaOH and their activities in systems producing superoxide anion radical (O_2^-) and hydroxyl radical (OH) were determined using EPR spectrometry, as reported previously (Hotta et al., 2002). The final concentration of SM-198110 or SM-197378 used to test their activity in quenching the production of both radicals was 2.5×10^{-6} to 2.5×10^{-3} M.

The conditions for EPR spectrometry (JES-RE, JEOL) to estimate the concentration of superoxide anion radicals were as follows: magnetic field: 335.7±5 mT; power: 4 mW, 9.414 GHz; modulation: 100 kHz, 1×0.079 mT; response: 0.1 s; temperature: 25 °C; amplitude: 160; sweep time: 2 min. For the hydroxyl radicals, the magnetic field, power, modulation, response, temperature, amplitude, and sweep time were 335.7±5 mT; 4 mW, 9.414 GHz; 100 kHz, 1×0.079 mT, 0.1 s, 25 °C, 160 and 2 min, respectively.

2.5.2. Superoxide anion radicals (O_2^-)

For the analysis of O_2^-, hypoxanthine, diethylene triamine penta acetic acid (DETAPAC), SM-198110, SM-197378 or vehicle (15 mM NaOH), 5-dimethyl-pyrroline-1-oxide (DMPO) and xanthine oxidase (XOD) were added to a test tube and mixed for 10 s. The mixture was transferred to a special flat cell for the analysis of the DMPO spin adducts of O_2^-. Measurements were recorded 45 s after the addition of XOD. Signal intensity was evaluated from the peak height of the first signal of the DMPO-O_2^- spin adduct relative to the intensity of the Mn^{2+} signal, with an internal standard being used to correct the measurement error.

2.5.3. Hydroxyl radicals (OH)

For the analysis of OH, $FeSO_4$ solution, hydrogen peroxide (H_2O_2), SM-198110, SM-197378 or vehicle (15 mM NaOH), and DMPO were mixed in a test tube. The amount of DMPO-OH spin adduct that was formed was estimated exactly 45 s after DMPO was added. The signal intensity was evaluated from the peak height of the second signal of the quartet of the DMPO-OH spin adduct relative to the intensity of the Mn^{2+} signal. Standard hydroxyl radical scavengers, such as ascorbic acid, glutathione and tocopherol, were also measured in this system to estimate the concentrations of agents required to inhibit the relative peak height of the DMPO-OH spin adduct. The control contained 15 mM NaOH.

2.6. Chemicals

Fura-2 AM and BCECF-AM were obtained from Dojindo Laboratories (Kumamoto, Japan). SBFI AM, Methylenediphosphonic acid and cremophore EL were purchased from Molecular Probes (Eugene, OR, USA). 5,5-Dimethyl-1-pyrroline-1-oxide (DMPO) was obtained from Labotec (Tokyo, Japan). Hypoxanthine, cyclosporin A (CsA), diethylene triamine penta acetic acid (DETAPAC) and xanthine oxidase (XOD) were from Sigma (St. Louis, MO, USA). The CPP32/Caspase-3 fluorometric protease assay kit was purchased from MBL (Nagoya, Japan).

2.7. Animals

Throughout the experiments, all animals were handled in accordance with the guidelines for animal experimentation set by the Japanese Association for Laboratory Animal Science.

2.8. Statistical analysis

All values are presented as means±S.E.M unless otherwise specified. The unpaired *t* test or analysis of variance (ANOVA) test followed by Dunnett's method was used for the comparison of means between groups. Statistical significance was defined as $p<0.05$.

3. Results

3.1. Na^+–H^+ exchange inhibitory activity of SM-198110 and SM-197378

SM-198110 and SM-197378 suppressed the pH_i recovery from acidosis in rat myocytes, in a concentration-dependent manner, and their IC_{50} values were 83 and 37 nM, respectively. SM-198110 and SM-197378 at 10 μM did not show inhibitory activity against Na^+–Ca^{2+} exchange.

3.2. Beneficial effects of SM-198110 and SM-197378 during ischemia–reperfusion in Langendorff heart preparations

3.2.1. Postischemic recovery of contraction

Fig. 1B shows the sequential changes in contractility (LVDP) in the drug-free group (control), the pretreated group during ischemia–reperfusion and the post-treated group during reperfusion. In the control hearts, LVDP recovered to less than 39.0% of the preischemic level (as 100%) after 40 min of reperfusion. A significant recovery of LVDP to 99.0% of the preischemic value ($P<0.01$) was obtained in SM-198110-pretreated hearts. In the SM-197378-pretreated hearts, there was a greater recovery of LVDP, to 98.8% of the preischemic value ($P<0.01$), than in control hearts. In the post-treated group of Langendorff heart preparations exposed to SM-198110 or SM-197378 (10^{-7} M) during reperfusion, the rate of recovery of LVDP was 81.0±9.0% and 69.0±6.0% ($P<0.05$ compared to control), respectively (Fig. 1C). As shown in Fig. 1B, the administration of SM-198110 during reperfusion increased LVDP gradually, while the LDVP in the presence of SM-197378 was 10% lower at 40 min than at 20 min after reperfusion. At 80 min after reperfusion, the recovery of LVDP was enhanced significantly ($P<0.05$, $n=6$) compared to that in the SM-197378 post-treated groups (SM-198110: 79.8±5.9%, 45.9±4.5 mm Hg; SM-197378: 65.0±8.1%, 30.4±4.4 mm Hg). The beneficial effect of SM-198110 during reperfusion was greater than that of SM-197378. The LVEDP and flow rate (FR) after 80 min of reperfusion did not differ significantly between the two treatment groups.

3.2.2. Postischemic end-diastolic pressure

The LVEDP at the end of 40 min of ischemia (ischemic contracture) was lower in hearts pretreated with SM-198110 and SM-197378 during ischemia and reperfusion than in the untreated or post-treated hearts (Fig. 1B). The main feature in the recovery of postischemic function was the difference in end-diastolic pressure between the control and drug-treated hearts. In the control group, LVEDP tended to increase during the first 5 min of reperfusion, but thereafter it decreased again and remained low throughout reperfusion. During reperfusion in the hearts pretreated with 10^{-7} M SM-198110 or SM-197378, there was no further increase in diastolic pressure, which gradually returned to the preischemic value (end reperfusion, 12.0±3.2 mm Hg, $P< 0.01$; in SM-198110-pretreated hearts or 12.5±2.3 mm Hg, $P<0.01$; in SM-197378-pretreated hearts versus 41.5±1.9 mm Hg in control hearts). In post-treated hearts during reperfusion, there were no differences at the end of 40 min of ischemia from the control, but after 40 min of reperfusion, both post-treated hearts showed a similar functional outcome, significantly better (SM-198110: 23.5±3.4 mm Hg, SM-197378: 27.0±4.0 mm Hg) than that observed in the control group. No difference between the two treated hearts could be found at 80 min of reperfusion (SM-198110: 21.5±4.1 mm Hg, SM-197378: 28.2±3.8 mm Hg, not significant). The LVEDP values obtained at the end of reperfusion were significantly lower than in the control group and showed an improved recovery of LVDP

Table 1
Left ventricular developed pressure (LVDP), ventricular end diastolic pressure (LVEDP), and coronary flow rate (FR) changes during ischemia–reperfusion of Langendorff hearts

	n	LVDP (mm Hg)			LVEDP (mm Hg)			FR (ml/min)		
		Pre	Isch	Rep	Pre	Isch	Rep	Pre	Isch	Rep
Control	6	63.3±7.8	0	24.7±5.3	10	39.3±4.1	41.5±1.9	10.4±0.5	0	4.8±0.7
SM-198110 (pre-)	6	59.2±4.2	0	58.8±7.4[a]	10	30.0±2.6[b]	12.0±3.2[a]	10.1±0.8	0	8.2±0.5[b]
SM-198110 (post-)	6	57.4±4.6	0	44.2±5.7[a]	10	40.2±1.6	23.5±3.4[a]	10.5±1.4	0	7.5±0.9[b]
SM-197378 (pre-)	6	54.0±6.3	0	52.2±4.5[a]	10	29.3±2.8[b]	12.5±2.3[a]	10.1±1.2	0	8.0±0.4[b]
SM-197378 (post-)	6	53.3±5.6	0	34.3±6.0[b]	10	38.5±1.2	27.0±4.0[a]	10.2±1.0	0	7.0±1.0[b]

Changes during 40 min of ischemia and a subsequent 40 min of reperfusion. Values are the means ±S.E.M. Preischemia (Pre), ischemia (Isch), reperfusion (Rep). Drug was introduced into the perfusion on-line 4 min before the start of ischemia (pre-), Drug was introduced into the perfusate for reperfusion (post-).

[a] $P<0.01$, significantly different from control values.

[b] $P<0.05$, significantly different from control values.

during the first 30 min of reperfusion. Actual values (mm Hg) of LVDP and LVEDP are shown in Table 1.

3.2.3. Postischemic coronary flow rate

At the end of reperfusion in the control hearts, the coronary flow (FR) was 4.8±0.7 ml/min, significantly lower than the preischemic level of 10.4±0.5 ml/min. However, compared to the control, hearts pre-and post-treated with SM-198110 and SM-197378 showed significant differences ($p<0.05$) at the end of reperfusion (see Table 1). In post-treated hearts, after 80 min of reperfusion, the flow rate of SM-197378-treated hearts decreased gradually, but there was no significant difference between the groups (SM-198110: 8.2±0.6 ml/min, SM-197378: 6.6±0.7 ml/min).

3.3. Effect of SM-198110 and SM-197378 on high-energy phosphates during ischemia and reperfusion determined by ^{31}P-NMR spectroscopy

As mentioned before, these experiments were carried out in Krebs–Henseleit (KH)-perfused hearts; ischemia and reperfusion were measured for 40 min. The effects of SM-198110 and SM-197378 were investigated, and the drug was introduced into the perfusate 4 min before global ischemia was induced by stopping the perfusate (pretreated groups) or the heart was reperfused with the drug-containing medium (post-treated groups). High-energy phosphate, β-ATP, values are presented graphically as percentages relative to the preischemic baseline values with the postischemia recovery rate of LVDP in Fig. 1C. During 20 min of ischemia in the

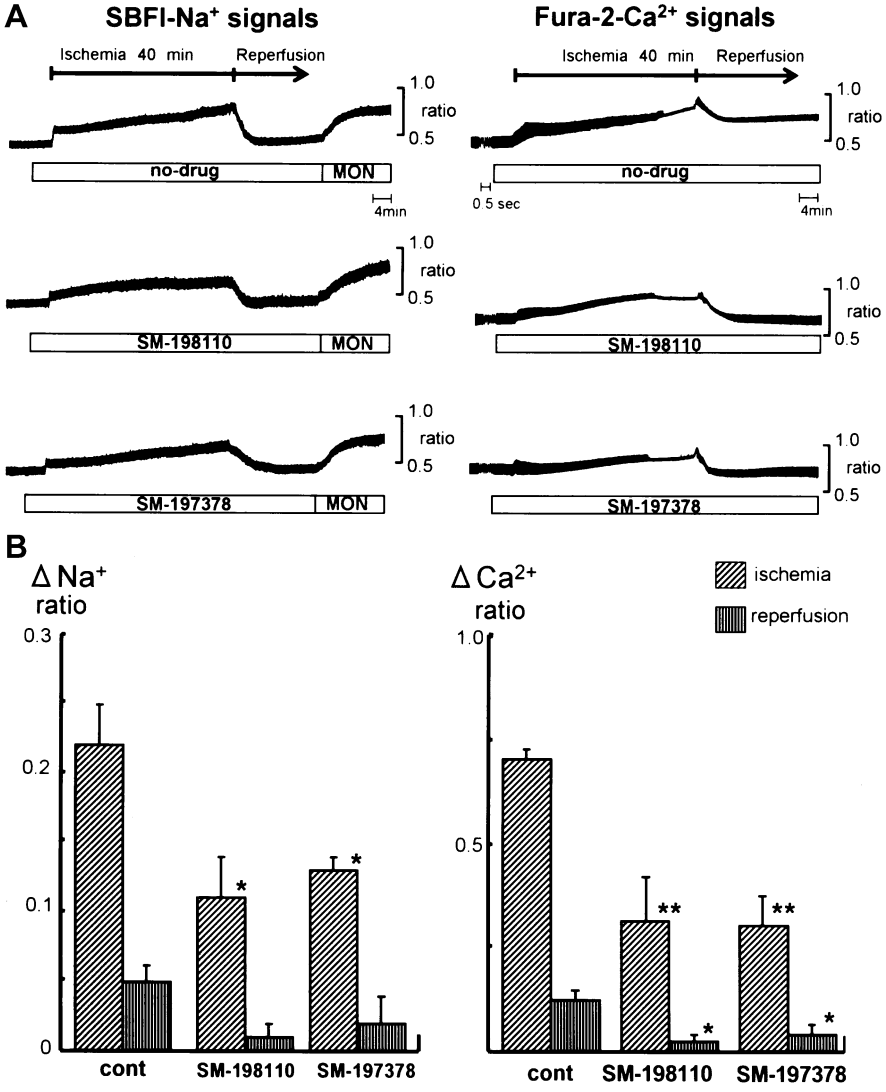

Fig. 2. (A) Effects of SM-198110 or SM-197378 (10^{-7} M) on Ca^{2+} signals (right) or Na^{+} signals (left) during ischemia–reperfusion. The Ca^{2+} or Na^{+} signals in the presence and absence of SM-198110 or SM-197378 (10^{-7} M) during ischemia–reperfusion were superimposable. The ischemia-induced Na^{+} signals returned to the preischemia level on reperfusion, while they increased on addition of the Na-ionophore monensin (MON, 10^{-5} M). (B) Changes in cytosolic Ca^{2+} signals (right) and Na^{+} signals (left) in a preischemic (control) heart, after 40 min of ischemia, and at 30 min after reperfusion (details in the Results section). *$P<0.05$, **$P<0.01$—significantly different from the control (drug-free) values. Data are expressed as the mean values from five preparations. Vertical lines represent S.E.M.

pretreated group, β-ATP and PCr did not change significantly. At the end of 40 min of ischemia, significant differences in the preservation of ATP were seen only in the hearts pretreated with SM-198110 and SM-197378 (SM-198110: 44.0±7.0% with preischemia as 100%, $P<0.01$) (SM-197378: 46.0±6.0%, $P<0.01$) compared with the control (22.0±4.0%). During reperfusion, there was a resynthesis of ATP in pretreated hearts. Differences between drug-treated and control hearts were significant at 20 min of reperfusion and thereafter. The level of ATP at the end of 40 min reperfusion was significantly higher in the SM-198110 ($P<0.01$) and SM-197378 groups ($P<0.01$) than in the control group (SM-198110: 70.0±6.0%, SM-197378: 61.0±3.0%, control: 38.0±3.0%, with preischemia as 100%). A rapid decrease in PCr was observed during ischemia in all groups. After 40 min of reperfusion, the level of ATP, which was rapidly resynthesized in post-treated hearts, increased to 55.0±3.0% (SM-198110: $P<0.01$) or 51.0±4.0% (SM-197378: $P<0.01$) compared with the controls. In post-treated groups, the difference between the two was not significant after 80 min (SM-198110: 54.0±3.0%, SM-197378: 50.0±4.7%) of reperfusion.

Changes in pH_i during ischemia and reperfusion were calculated as described in the Materials and methods. The initial (preischemia) level of pH_i was almost identical in each group (7.35±0.04). At the end of 36–40 min of ischemia, the pH_i did not differ significantly between the control, pretreated or post-treated hearts (control: 5.86±0.04, pre-SM-198110: 5.8±0.03, pre-SM-197378: 5.84±0.04, post-SM-198110: 5.92±0.04, post-SM-198110: 5.87±0.04) During reperfusion, the recovery from intracellular acidification observed in SM-198110-pretreated or SM-197378-pretreated hearts was faster than that of the control. However, pH_i values in these hearts at 40 min of reperfusion were not significantly different from the control value.

3.4. Effect of SM-198110 or SM-197378 on fura-2 Ca^{2+} and SBFI-Na^+ signals during ischemia and reperfusion assessed by fluorometry

Fig. 2A show a typical change in Ca^{2+}- or Na^+-selective fluorescence signals in drug-free and pre- or post-treated hearts exposed to ischemia–reperfusion. The obtained

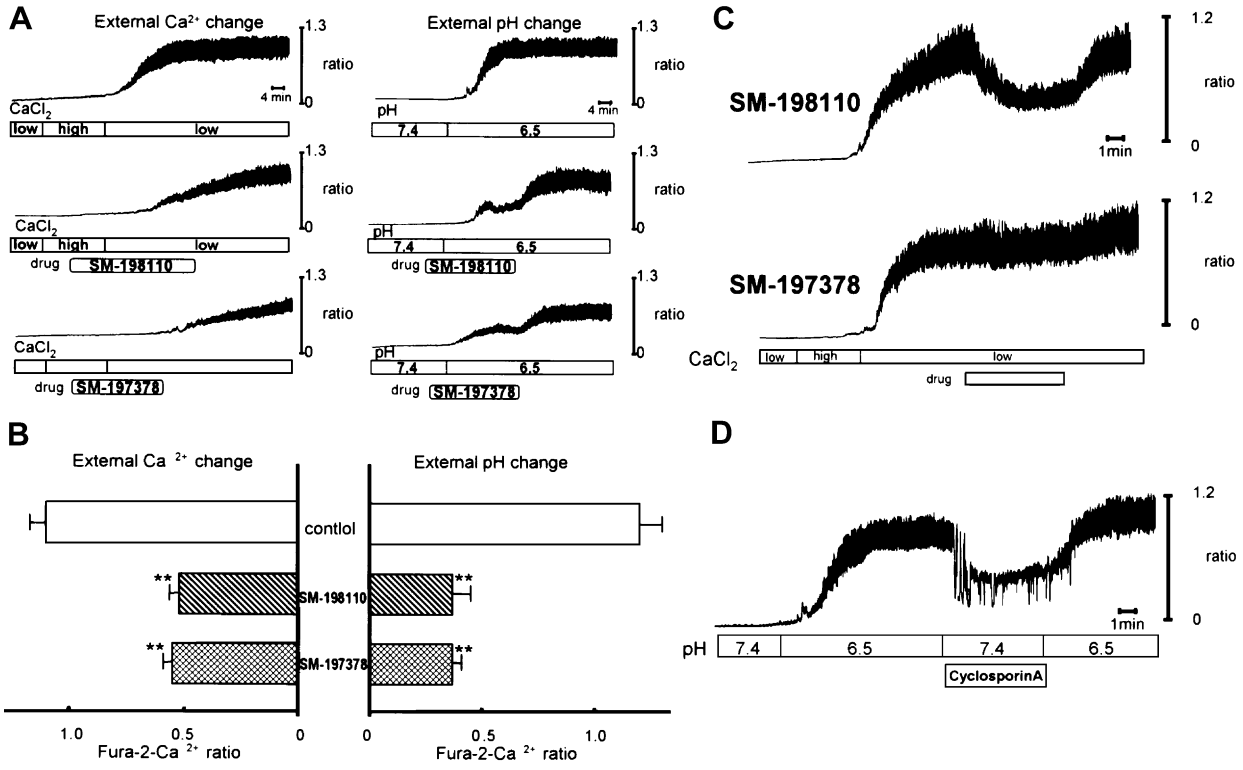

Fig. 3. (A) Recordings of intramitochondrial Ca^{2+} from the fura-2-loaded mitochondria preparations affected by changing the Ca^{2+} concentrations or pH of the perfusate. The mitochondrial Ca^{2+}signal ($[Ca^{2+}]_m$) gradually increased with extremely high Ca^{2+}(10 μM), and perfusion with a low Ca^{2+} content solution (100 nM) rapidly increased the intramitochondrial Ca^{2+} level (left, top). Perfusate acidification (pH 6.5) in the low Ca^{2+} content solution (100 nM) produced a more rapid Ca^{2+} signal elevation than that seen with changes in Ca^{2+} content (right, top). SM-198110: 10^{-6} M (middle) or SM197378: 10^{-6} M (bottom) reduced the increase in intramitochondrial Ca^{2+} signals induced by a change of perfusate. (B) Comparison of inhibition of Ca^{2+} uptake into mitochondria in the presence of SM-198110 or SM-197378. The mitochondrial Ca^{2+} level was elevated by an external Ca^{2+} concentration change (left) or external acidification (right). The $[Ca^{2+}]_m$ elevation was reduced by SM-198110 or SM197378. $^{**}P<0.01$—significantly different from the control (drug-free) values. Values are expressed as the mean values from 3–5 preparations; Vertical lines represent S.E.M. (C) The $[Ca^{2+}]_m$ elevation that brought an external Ca^{2+} concentration change was reduced by infusion of SM-198110 or SM-197378. The decrease in $[Ca^{2+}]_m$ caused by the administration of SM-198110 was greater than that produced by SM-197378. (D) The $[Ca^{2+}]_m$ elevation brought about by perfusate acidification decreased on the addition of mitochondrial PTP inhibitor cyclosporin A (10^{-4} M).

values are summarized in Fig. 2B. SM-198110 or SM-197378 had a significant restorative effect. During the final period of ischemia, the diastolic Ca^{2+} level, elevated due to treatment with SM-198110 or SM-197378, decreased from 0.56±0.02% (control) to 0.25±0.08% ($P<0.01$) and 0.24±0.05% ($P<0.01$), respectively. However, Na^+ levels decreased from 0.22±0.03% (control) to 0.11±0.03% (SM-198110, $P<0.05$) and 0.13±0.02% (SM-197378, $P<0.05$). These values are expressed as percentages of diastolic Ca^{2+} and Na^+ levels during the preischemic period. In the period of reperfusion with new KH solution, the increased systolic level of transient Ca^{2+} (T_{Ca}) and the Na^+ signal (the amplitude of transient Na^+ could not be detected from the SBFI-Na^+ signal) were significantly reduced in the presence of the proton antiport inhibitor SM-198110 or SM-197378 (10^{-7} M). The reperfusion-induced SBFI-Na^+ signals returned to the preischemic level but increased on addition of 10^{-5} M Na^+-ionophore monensin (MON), as previously described (Hotta et al., 1998, 2001a).

3.5. Intramitochondrial Ca^{2+} ($[Ca^{2+}]$ m) measurements with fura-2 AM

The intramitochondrial fura-2 Ca^{2+} signal ($[Ca^{2+}]_m$) increased steadily and linearly but was only 10% higher at extremely high perfusate Ca^{2+} concentrations (1 μM–1 mM) (Fig. 3A). The displacement of the normal matrix Ca^{2+} concentration (100 nM) with perfusate produced a rapid and intense increase of up to about 8.5-fold (0.13→1.1±0.07), with the change in $[Ca^{2+}]_m$ caused by displacement of the perfusate at a high Ca^{2+} concentration being regarded as 100%. Pretreatment of mitochondria with SM-198110 or SM-197378 (10^{-6} M) markedly suppressed the increase in $[Ca^{2+}]_m$, which occurred in the drug-free perfusate, by 47.3% or 50.0% (SM-19811010^{-6} M; 0.13→0.52±0.04, SM-198110 10^{-5} M; 0.13→0.32±0.03, SM-19737810^{-6} M; 0.13→0.55±0.04, SM-197378 10^{-5} M; 0.13→0.35±0.04) (Fig. 3B).

Changing the pH from 7.4 to 6.5 in a low-Ca^{2+} perfusate (100 nM) elevated $[Ca^{2+}]_m$ to the maximal extent, up to about 10 times that in the pH 7.4 perfusate, which was regarded as 100% (Fig. 3A). The appreciable $[Ca^{2+}]_m$ elevation induced by acidification (0.13→1.3±0.1) was reduced by pretreatment with SM-198110 at 10^{-6} M (0.13→0.37±0.08) or SM-197378 at 10^{-6} M (0.13→0.37±0.04). Furthermore, it was greater and more rapid than that by perfusion with induced the low-Ca^{2+} perfusate.

As shown in Fig. 3C, the addition of SM-198110 or SM-197378 (10^{-5} M) to the perfusate increased steadily and constantly the change in the concentration of perfusate Ca^{2+} released from mitochondria. The effect of SM-198110 was greater than that of SM-197378. These effects were similar to those seen with cyclosporin A (CsA, 10^{-4} M), which inhibits the mitochondrial permeability transition pore (PTP) (Fig. 3D).

3.6. Detection of apoptosis

Examples of apoptotic tissues obtained after the various treatments are shown in Fig. 4. Apoptotic cells were first observed in the ventricle after 5 h of reperfusion after 1 h of ischemia with KH solution containing 2-deoxy-D-glucose.

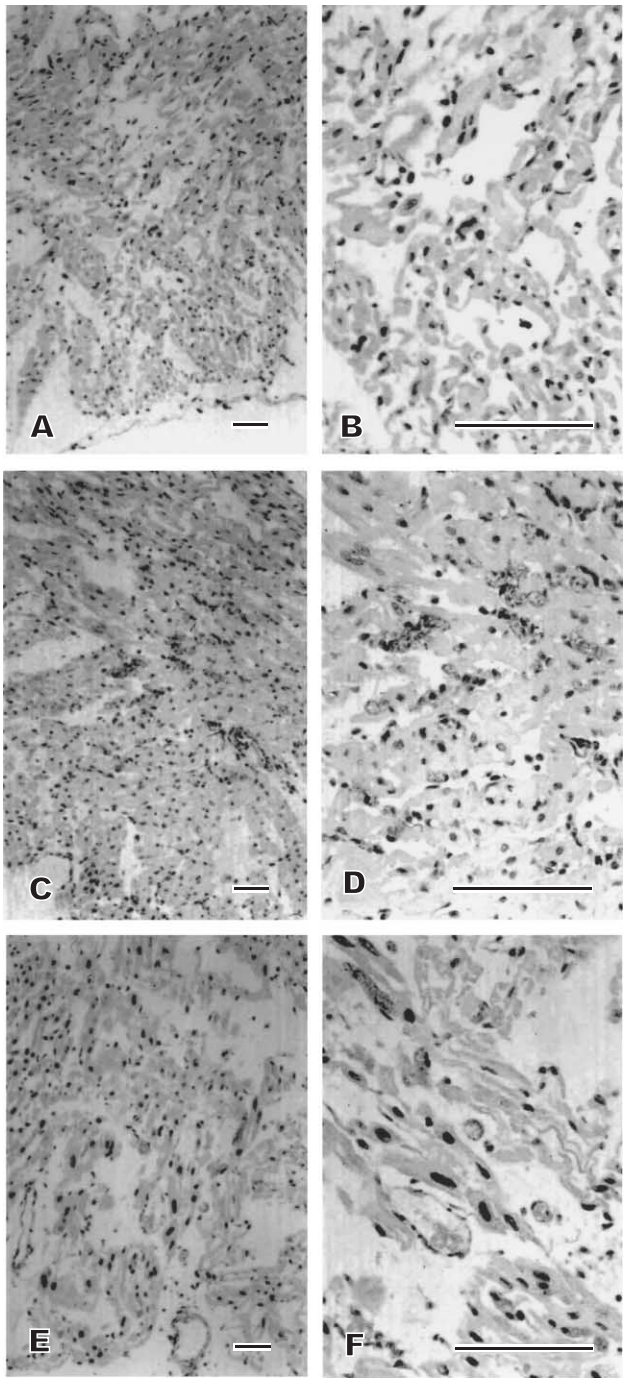

Fig. 4. Representative light micrographs (A, C, E: ×60; B, D, F: ×240) illustrating apoptotic cells. TUNEL-positive cardiomyocytes were detected in the outer two-thirds of the left ventricular wall. No stained cells were observed in control hearts not subjected to ischemia. (A and B) Control, (C and D) SM-198110 (10^{-7} M), (E and F) SM-197378 (10^{-7} M). Scale=100 μm.

As shown in Fig. 5A, 5 h of reperfusion resulted in an average of 27.7±2.4 cells per 100 microscopic fields, but 4 h of reperfusion did not produce apoptotic cells. SM-198110 and SM-197378 (10^{-7} M) reduced the incidence of apoptosis in the ischemic and reperfused myocardium to an average of 14.5±1.7 (P<0.05) and 5.0 ± 0.9 (P<0.05) cells per 100 microscopic fields respectively, compared with the control. The effect on apoptotic cells of SM-197378 was greater than that of SM-198110 (P<0.05).

The caspase-3 activity in control and apoptotic samples was measured using the same frozen tissues with the TUNEL method. Control-1 was a heart preparation perfused for 30 min with normal KH solution (considered as 1.0), while control-2 was a heart subjected to 1 h of ischemia and 5 h of reperfusion with a drug-free solution (TUNEL: control). The decrease in caspase-3 activity in the heart pretreated with SM-198110 or SM-197378 [control-1: 1.0, control-2: 6.5±1.0, SM-198110: 6.2±0.9 (P<0.05 for control-2), SM-197378: 2.3±0.5 (not significant for control-2, P<0.05 for SM-198110-treated heart)] was similar to the result of the TUNEL assay (Fig. 5B).

As shown in Table 2, which lists changes in mechanical function and high-energy phosphates, the LVDP, FR and β-ATP were significantly different in the hearts treated with SM-198110 at 10^{-7} M after 5 h of reperfusion compared with the control (P< 0.05), but SM-197378 at 10^{-7} M

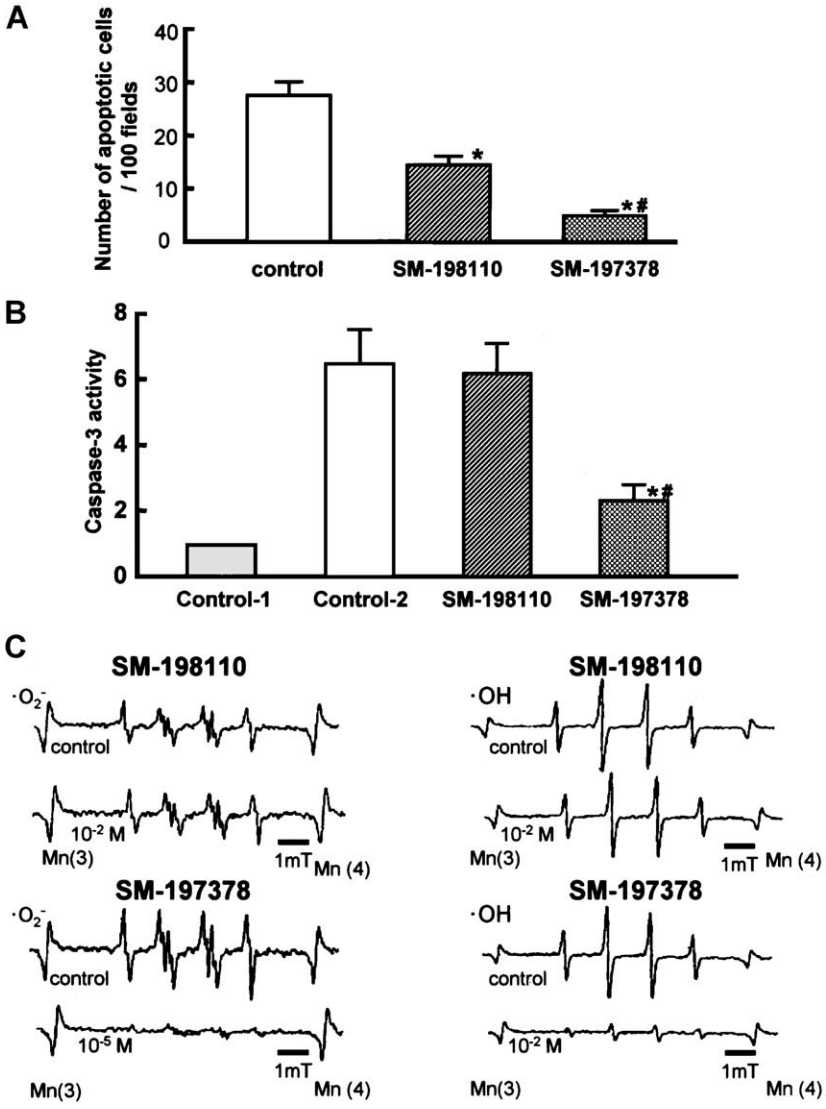

Fig. 5. (A) The incidence of apoptosis observed with the TUNEL technique described in Materials and methods. Values are expressed as the means from five preparations±S.E.M and indicate the number of positively stained cells identified per 100 microscopic fields. *P<0.05—significantly different compared with control (drug-free) values. #P<0.05—significantly different between SM-198110 and SM-197378-treated groups. (B) Control-1: The uninduced control was a heart preparation perfused at 30 min with normal KH solution. Control-2: SM-198110 or SM-197378 and the same heart tissue were used with the TUNEL method. Values are expressed as the means for five preparations±S.E.M and indicate caspase-3 activity. *P<0.05—significantly different compared with control (drug-free) values. #P<0.05—significantly different between SM-198110 and SM-197378-treated groups. (C) Representative EPR spectra of a spin adduct showing in vitro quenching of superoxide anion radical (O_2^-) (left) or hydroxyl radical (OH) production (right) by SM-198110 or SM-197378 (upper) compared with the control (drug-free).

Table 2
Left ventricular developed pressure (LVDP), ventricular end-diastolic pressure (LVEDP), coronary flow rate (FR) and high phosphorous energy (PCr, β-ATP) changes during ischemia–reperfusion of Langendorff hearts for the TUNEL method and caspase-3-activity measurements

	n	LVDP (mm Hg)			LVEDP (mm Hg)			FR (ml/min)			ATP (%)			PCr (%)		
		Pre	Isch	Rep	Pre	Isch	Rep	Pre	Isch	Rep	Pre	Isch	Rep	Pre	Isch	Rep
Control	5	62.3±8.6	0	2.0±1.2	10	34.2±4.1	41.6±2.0	10.4±1.0	0	0.5±0.1	100	12.6±2.0	12.4±2.2	100	0	4.8±2.3
SM-198110	5	58.4±7.4	0	7.4±1.5[a]	10	25.8±2.4	36.0±2.9	10.5±1.3	0	3.1±0.8[a]	100	19.6±2.7	22.8±2.3[a]	100	0	39.2±11.4[a]
SM-197378	5	57.6±6.9	0	3.2±1.0	10	27.8±2.9	39.6±3.0	10.7±1.5	0	1.5±0.7	100	18.8±2.9	20.2±2.8	100	0	27.8±7.7[a]

Changes during 1 h of ischemia and a subsequent 5 h of reperfusion. Values are the means ± S.E.M. Preischemia (Pre), ischemia (Isch), reperfusion (Rep).
[a] $P<0.05$, significantly different from control values.

caused no appreciable difference. The PCr signal was stronger in the hearts pretreated with either drug (SM-198110 or SM-197378) than in the control hearts ($P<0.05$). From these results, the preventive effects of SM-198110 on the recovery of LVDP and tissue levels of high-energy phosphate were greater than those of SM-197378.

3.7. In vitro free radical-quenching activity of SM-198110 or SM-197378

SM-198110 and SM-197378 showed quenching activity against OH or O_2^- radicals. Representative spectra of active oxygen radicals quenched with SM-198110 at 10^{-2} M (OH), SM-198110 at 10^{-2} M (O_2^-), and SM-197378 at 10^{-5} M (OH), and SM-197378 at 10^{-2} M (O_2^-) are shown in Fig. 5C. In the experiment on quenching activity, SM-197378 at final concentrations of 10^{-7} to 10^{-2} M dose dependently quenched the generation of OH or O_2^-. The IC_{50} (concentration causing a 50% scavenging effect) of SM-197378 was 2.4×10^{-3} M (OH) and 1.1×10^{-6} M (O_2^-). However, the IC_{50} value of SM-198110 could not be measured, because SM-198110 did not cause 50% inhibition. The IC_{50} of vitamin C was 2.6×10^{-3} M (OH) and 6.3×10^{-5} M (O_2^-); the radical quenching activity of SM-197378 was greater than that of vitamin C. SM-198110 and SM-197378 were added to the system as suspensions in 15 mM NaOH.

4. Discussion

In the present study, we investigated the beneficial effects of two newly synthesized NHE inhibitors, SM-198110 and SM-197378, on ischemia–reperfusion injury in isolated guinea-pig Langendorff hearts. In pretreated hearts, SM-198110 and SM-197378 had a similar pronounced effect on the recovery of LVDP (SM-198110: 99.0%, SM-197378: 98.8% vs. drug-free heart, control 39.0%) and a significant preserving effect (SM-198110: 44.0%, SM-197378: 46.0% with the preischemia control as 100%) on the tissue levels of high-energy phosphate (ATP and phosphocreatine) measured in the final stage of ischemia and after reperfusion compared with control, drug-free hearts (22.0%) (Fig. 1C). In hearts post-treated with both drugs during reperfusion only, the rate of recovery of LVDP was high and accompanied the restoration of high-energy phosphate levels. The beneficial effect of SM-198110 during reperfusion after ischemia was greater than that of SM-197378 (Table 2). These findings strongly indicated that mitochondrial function, in close association with the synthesis of high-energy phosphates, is an important part of the regulatory mechanisms involved in myocardial ischemic injury.

The changes in intracellular Ca^{2+} transient signals (T_{Ca}) or Na^+ signals that accompanied LVDP during ischemia–reperfusion of Langendorff guinea-pig hearts were measured simultaneously using a fura-2-Ca^{2+} or SBFI-Na^+ probe

(Fig. 2). From the SBFI-Na signals ($[Na^+]_i$), it is evident that $[Na^+]_i$ increased rapidly during the early stage of ischemia and remained at a constant level until the final stage. The fura-2 T_{Ca} signals increased markedly during the first stage of ischemia, followed by a marked increase in the basal level during the last 40 min of ischemia. Therefore, at this stage of ischemia, the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was evidently increased by Na^+–Ca^{2+} exchange (NCE) mediated by an activated NHE system. The treatment of guinea-pig hearts with NHE inhibitors and NCE inhibitors (Hotta et al., 2002) significantly attenuated this increase in Na or Ca signals.

On reperfusion in Langendorff guinea-pig hearts, the $[Ca^{2+}]_i$ level, which had increased during global ischemia, rapidly returned to the control level, as previously reported (Hotta et al., 1998, 2001a). The fura-2 Ca^{2+} signals ($[Ca^{2+}]_m$) in isolated myocardial mitochondria preloaded with abnormally high Ca^{2+} levels, using a superfusion technique, increased rapidly, reaching a maximum level during perfusion of a solution containing a cytosolic level of Ca^{2+} (~100 nM) (Fig. 3). In addition, the elevation of $[Ca^{2+}]_m$ was quickly detected on acidification of the perfusate, similar to the final stage of global ischemia in the Langendorff hearts. These increases in the perfused mitochondrial preparation were attenuated by pretreatment with SM-198110 or SM-197378 and drugs having a beneficial effect in Langendorff hearts (Hotta et al., 1998, 1999, 2001a). From these findings, it was suggested that for Ca^{2+} pumping at the time of reperfusion, the mitochondria play an essential role in cellular Ca^{2+} homeostasis for the maintenance of cellular function of the heart, acting as a Ca^{2+} scavenger in the cytosol (Tani and Neely, 1989; Brooks et al., 1995). Factors that induce Ca^{2+} overload in mitochondria via sarcolemmal Ca^{2+} influx and exchange mechanisms with Na^+, K^+, Ca^{2+}, and H^+ will lead to a loss of contractility, associated with an extremely low level of free energy change, as predicted from the reduced ATP · PCr/Pi ratio measured by ^{31}P-NMR (Koike et al., 1996; Hotta et al., 2001a).

Mitochondria play a major role in the regulation of both physiological and pathological cell death (Kroemer et al., 1998). Irrespective of its exact composition, the mitochondrial PTP complex contains multiple targets for pharmacological investigations, involved in different pathways of apoptosis induction as a sensor for stress and damage, as well as for certain signals connected to receptors. There is now evidence that apoptosis, or programmed cell death, is an important response of the myocardium to ischemia and reperfusion which is rapid, precedes cell necrosis and appears to contribute to the overall sequelae of cardiac injury. The role of NHE in this response has been shown; that is, the NHE inhibitor cariporide significantly attenuated the development of early apoptosis in cardiomyocytes after global ischemia and reperfusion in rat hearts (Chakrabarti et al., 1997; Humphreys et al., 1999).

We have demonstrated that approximately 27.7% of cardiomyocytes were TUNEL-positive in drug-free guinea-pig hearts subjected to 1 h of ischemia followed by 5 h of reperfusion with KH solution containing 5 mM 2-deoxy-D-glucose (Fig. 4 and 5A). The proportion of TUNEL-positive cardiomyocytes after 4 h of reperfusion was approximately 10%, and thus a longer period of reperfusion may lead to accelerated apoptosis. The administration of SM-198110 or SM-197378 following 5 h of reperfusion significantly affected the number of TUNEL-positive cardiomyocytes (SM-198110: 14.5%, SM-197378: 5%) compared with that in drug-free hearts. Although apoptosis was detected using the TUNEL assay, no visible DNA ladders, characteristic of apoptosis, could be found in myocardial tissues, as reported by Otani et al. (2000). Our inability to demonstrate DNA ladders could be attributed to a smaller number of apoptotic cardiomyocytes found in the TUNEL assay. However, we could measure caspase-3 activity in the tissue used for the TUNEL assay (Fig. 5B). The effect of SM-197378 on the number of TUNEL-positive cardiomyocytes and caspase-3 activity after ischemia and reperfusion was less than that of SM-198110 (with no quenching activity for OH and O_2^- radicals by in vitro EPR). However, SM-198110 significantly improved the recovery of left ventricular function (LVDP) and coronary flow (FR), with increases in the levels of high-energy phosphates (ATP, PCr), compared with those in drug-free hearts during a 5-h longer reperfusion. The beneficial effect of SM-198110 provided a greater protection of ischemic myocardium and of hearts subject to reperfusion procedures than did SM-197378. However, the IC_{50} of the selective NHE inhibitor SM-198110 was 83 nM, which was lower than that of SM-197378, 37 nM. By in vitro EPR spectrometry (Fig. 5C), the free radical-quenching activity (IC_{50}) of SM-197378 was found to be 2.4×10^{-3} M (OH) and 1.1×10^{-6} M (O_2^-), more than that of vitamin C, while the IC_{50} of SM-198110 could not be measured. More studies are required to more adequately resolve the relation between apoptotic caspase activity and the NHE-inhibitory effect of both agents.

Treatment with the caspase inhibitor zVAD.fmk reduced in the improvement of hemodynamic parameters associated with the inhibition of cardiomyocyte apoptosis (Yaoita et al., 1998). Furthermore, Wu et al. (2003) found that apoptosis is associated with caspase-dependent NHE1 degradation in cell cultures and whole animals. Otani et al. (2000) also reported necrotic changes without typical apoptotic features in cardiomyocytes after reperfusion, detected by electron microscopy. Such necrotic changes were prevented by the NHE inhibitor, cariporide. They suggested that apoptosis is not a major manifestation of cardiomyocyte cell death in ischemic-reperfused myocardium and that a cariporide-sensitive mechanism of reperfusion injury promotes both the necrotic and apoptotic process. It is also possible that apoptosis precedes the necrotic changes that are provoked by the

apoptosis-induced disintegration of mitochondrial function (Griffiths and Halestrap, 1995; McConkey and Orrenius, 1997). If this is the case, inhibition of the apoptotic cascade could contribute to overall cardiomyocyte cell death. Further investigation of the different cardiac effects of the NHE inhibitors, SM-198110 and SM-197378, should provide valuable information on the normal physiology of cell death, and help in the design of cardioprotective drugs.

Acknowledgments

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