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Bradykinin B_1 and B_2 receptors differentially regulate cardiac Na^+–H^+ exchanger, Na^+–Ca^{2+} exchanger and Na^+–HCO_3^- symporter

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Abstract

Bradykinin B_1 and B_2 receptors are up-regulated in the infarcted myocardium, and both receptors are involved in the regulation of intracellular pH and Ca^{2+}. The present study investigated the role of bradykinin B_1 and B_2 receptors in the regulation of Na^+–H^+ exchanger (NHE-1), Na^+–Ca^{2+} exchanger (NCE-1) and Na^+–HCO_3^- symporter (NBC-1) in the infarcted myocardium. NHE-1, NCE-1 and NBC-1 mRNA expression was determined by Northern blot analysis and the protein levels by Western blot analysis. Measurements were performed 1, 7 and 14 days after induction of myocardial infarction. Localization of NHE-1, NCE-1 and NBC-1 within the myocardium was studied using confocal microscopy. Cardiac morphology was measured in picrosiris-red-stained hearts. Rats were treated with placebo, the bradykinin B_2 receptor antagonist icatibant (0.5 mg/kg/day) or the bradykinin B_1 receptor antagonist des-Arg^9-[Leu^8]bradykinin (1 mg/kg/day). Treatment was started 1 week prior to surgery and continued until 1, 7 and 14 days post infarction. NHE-1, NCE-1 and NBC-1 mRNA expression and protein levels were increased 1 day and reached maximum values on day 7 post infarction. NHE-1 was localized in the plasma membrane, NCE-1 in the membrane of the sarcoplasmatic reticulum and NBC-1 near the Z-line. Icatibant reduced NHE-1 and inhibited NCE-1 mRNA- and protein up-regulation, while des-Arg^9-[Leu^8]bradykinin had no effect on NHE-1 and NCE-1 expression and translation. Transcriptional and translational up-regulation of NBC-1 was unaffected by the bradykinin B_1 and B_2 receptor antagonists. Icatibant, but not des-Arg^9-[Leu^8]bradykinin, limited infarct size and reduced left ventricular dilation, septal thickening and interstitial fibrosis post infarction. Bradykinin B_2 receptors are involved in transcriptional and translational regulation of NHE-1 and NCE-1 in the ischemic myocardium. Chronic B_2 receptor blockade might exert an anti-ischemic effect via limitation of NHE-1-mediated acidosis and NCE-1-mediated Ca^{2+}-overload.

Keywords: Myocardial infarction; Bradykinin receptor; Membrane transporter; (Rat)

1. Introduction

The ischemic myocardium is characterized by an intracellular acidosis and Ca^{2+}-overload, which both have been shown to be involved in ventricular dysfunction and to induce tissue injury and cell necrosis (Orchard and Kentish, 1990). A number of pH- and Ca^{2+}-regulatory proteins, which are part of the plasma membrane of myocytes, control intracellular ion homeostasis. In the mammalian myocardium, the Na^+–H^+ exchanger isoform-1 (NHE-1) and the Na^+–HCO_3^- symporter isoform-1 (NBC-1) are the major transporter systems that regulate intracellular pH (Lazdunski et al., 1985; Thomas, 1989). During ischemia, the primary function of the cardiac NHE-1 is to extrude H^+ in exchange for Na^+ whereas the cardiac NBC-1 imports HCO_3^- together with Na^+. Thus, both transporters, when activated, induce an intracellular alkalinization but also participate in elevating the intracellular Na^+-load (Frelin et al., 1984; Dart and Vaughan-Jones, 1992). In myocardial cells, accumulated Na^+ can be exchanged for Ca^{2+} via the Na^+–Ca^{2+} exchanger isoform-1 (NCE-1) (Kim et al., 1987; Siffert and Akkerman, 1989). The resulting rise in intracellular Ca^{2+} has been shown to activate intracellular Ca^{2+}-dependent enzymes causing myocardial damage (Sandmann et al., 2001a). These findings are in agreement with observations that inhibition of the Na^+/H^+- and Na^+/Ca^{2+}-antiport reduced acidosis and Ca^{2+}-overload induced cardiac damage (Karmazyn, 1988; Spitznagel et al., 2000). Additionally, in previous studies, we found that inhibition of the angio-

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tensin-converting enzyme prevented the ischemia-induced up-regulation of the cardiac NHE-1 and NBC-1 (Sandmann et al., 2001b). Since the angiotensin-converting enzyme is identical with the bradykinin-degrading enzyme, kininase II, in cardiac membranes (Blais et al., 1997), there is a possible contribution of kinins in the regulation of these cardiac transporters.

Increasing evidence suggests that the kallikrein–kinin system is activated after acute myocardial infarction. It has been demonstrated that tissue damage and inflammatory response during cardiac ischemia activate plasma and tissue kallikreins, which generate bradykinin and the related peptide kallidin (or lys-bradykinin) from high- and low-molecular-weight kininogen precursors (Farmer and Burch, 1992; Hall, 1992). The biological action of the kinins is mediated by activation of at least two subtypes of G-protein-coupled bradykinin receptors, B_1 and B_2 (Carretero, 1999; Nsa Allogho et al., 1995). The bradykinin B_1 receptor is only weakly expressed under physiological conditions, but is induced by pathophysiological stimuli (Su et al., 2000). Activation of the bradykinin B_1 receptor has been reported to induce vasodilation, to inhibit proliferation and inflammation, to stimulate fibrosis, to be involved in cardiac preconditioning and to promote angiogenesis (for review, see Linz et al., 1995). In contrast, the bradykinin B_2 receptor, which is constitutively expressed in most tissues, is considered to be the major mediator of the bradykinin-induced effects in the cardiovascular system (Carretero, 1999).

A study by Tschope et al. showed that mRNA and protein levels of both bradykinin receptors are up-regulated in the rat myocardium during the acute phase following infarction, suggesting that bradykinin is involved in functional and structural alterations of the ischemic myocardium (Tschope et al., 1999, 2000). In addition, studies demonstrated that the cardioprotective action of angiotensin-converting enzyme inhibitors during ischemia can in part be attributed to a potentiation of endogenous bradykinin, implying a role of the kinin system against acute ischemic injury (Linz et al., 1995; Hirsch et al., 1991). Whether this effect of bradykinin in the acute phase following infarction is mediated via affecting pH- and Ca^{2+}-regulating transport systems has to be clarified. Findings of recent studies demonstrating that bradykinin induced an acidification in endothelial cells (Bentley and Jarrott, 2000) and a Ca^{2+}-mobilization in efferent arterioles of rat kidneys (Su et al., 2000) suggest a contribution of bradykinin receptors to the control of intracellular pH and Ca^{2+}. However, little is known, which bradykinin receptor subtype is involved in the regulation of intramyocardial pH and Ca^{2+}.

The purpose of the present study was to investigate the role of bradykinin B_1 and B_2 receptors on transcriptional and translational regulation of the cardiac transporters NHE-1, NCE-1 and NBC-1 using specific receptor antagonists. To determine temporal and regional differences in the expression and translation of the transporters, measurements were performed at different time points post myocardial infarction in the infarcted and non-infarcted myocardium. The intracellular distribution of H^+ and Ca^{2+} depends on local formation and the presence of intramyocardial compartments suggests that the regulatory ion transporter mechanisms of the myocardium should be present near the region of ion origin. To verify this hypothesis, we studied the localization of NHE-1, NCE-1 and NBC-1 in the cardiac tissue using confocal microscopy.

2. Methods

2.1. Animals and study design

Male, normotensive Wistar rats (Charles River Viga, Sulzfeld, Germany), initially weighing 230–270 g, were used. All experiments were performed in accordance with the German law on animal protection as revised in 1993. The animals were housed in individual cages at controlled temperature and humidity under a 12-h light/dark cycle. Rats had free access to a standard diet (Altromin®, Altromin, Lage-Lippe, Germany) and to drinking water.

The animals were randomly divided into four groups: group 1, sham-operated without treatment; group 2, myocardial infarction subjected to placebo treatment (0.9% saline); group 3, myocardial infarction subjected to treatment with the bradykinin B_2 receptor antagonist icatibant (0.5 mg/kg/day); group 4, myocardial infarction subjected to treatment with the bradykinin B_1 receptor antagonist des-Arg^9-[Leu^8]bradykinin (1 mg/kg/day). Icatibant was kindly provided by Aventis (Frankfurt, Germany) and des-Arg^9-[Leu^8]bradykinin was purchased from Sigma (Deisenhofen, Germany). Both drugs were given as a solution in water via subcutaneously implanted minipumps. The doses of the bradykinin B_1 and B_2 receptor antagonist were chosen according to the literature, ensuring effective receptor blockade (Regoli and Barabe, 1980; Campbell et al., 1999). Treatment was initiated 1 week prior to induction of myocardial infarction in all groups and continued until sacrifice. The number of animals per group was five to six.

At the day of surgery, rats were anaesthetized by injection of ketamine–xylazine (35 mg/2 mg/kg i.p.) and artificially ventilated (70 ventilations/min, 200 mm H_2O, 2.5 ml/ventilation) with room air, using a positive-pressure respirator. A left thoracotomy was performed via the fourth intercostal space as described previously (Sandmann et al., 2001c). The left coronary artery was intrathoracally ligated with a sterile 6-0 silk suture (Ethibond, Ethicon, Norderstedt, Germany) near its origin between the pulmonary outflow tract and the edge of the left atrium. Myocardial infarction was considered successful when the anterior wall of the left ventricle became cyanotic and the electrocardiogram showed obvious ST-segment elevation. The lungs were inflated by increasing positive end-expiratory pressure and the thorax was closed. Animals in group 1 were subjected to the same procedure except the suture around

the coronary artery was not tied. At the end of the surgery, analgesia was induced by a subcutaneous injection of buprenorphine-HCl (0.2 mg/kg). Animals were kept under a warming lamp until they were awake.

On days 1, 7 and 14 post infarction, the hearts were rapidly excised from sham-operated and infarcted animals, the ventricles separated from the atria and dissected into the cardiac tissue regions: right ventricle, interventricular septum and left ventricular free wall. This procedure was performed within 2 min to minimize possible postmortem artifacts. The left ventricular free wall consisted of scar tissue and area at risk. Tissue samples were rapidly frozen in liquid nitrogen and stored at −80 °C until use. At the three time points post infarction, gene expression and protein translation of the cardiac transporters NHE-1, NCE-1 and NBC-1 were determined. On day 7 post infarction, the localization of the transporters in the cardiac tissue was investigate using confocal microscopy. Fourteen days post infarction, cardiac morphometric examinations were performed in picrosirius-red-stained hearts.

2.2. RNA extraction and Northern blot analysis

For measurement of gene expression, total RNA was isolated from frozen tissues using the Trizol reaction kit as described in the manufacturer's instructions (Life Technologies, Karlsruhe, Germany; Chomczynski and Sacchi, 1987). The RNA concentration was quantified by densitometric measurement of UV absorption at 260 nm. For Northern blot analysis, 5 μg of RNA from each tissue sample was fractionated by denaturing formaldehyde agarose (1%) gel electrophoresis. Subsequently, the RNA was transferred onto a Hybond-N^+ membrane (Amersham Pharmacia Biotech, Braunschweig, Germany) using a capillary blotting technique according to the manufacturer's instructions. The RNA was fixed to the membrane with UV radiation (0.15 J/cm^2). RNA size markers (0.2–10 kb, Sigma) were visualized with UV light after staining with ethidium bromide. Hybridization reactions were carried out in Rapid-Hyb buffer (Amersham Pharmacia Biotech) using ^{32}P-labelled probes. The probes were labeled using T4 polynucleotide kinase (New England Biolabs, Frankfurt, Germany) and γ^{32}P-ATP (37 MBq, 10 mCi/ml, NEN Life Science, Zaventem, Belgium).

Following a pre-hybridisation period of 20 min, the hybridisation with oligomer probes for the cardiac transporters NHE-1, NCE-1 and NBC-1 were performed for 2 h at 42 °C. Membranes were washed using high stringency conditions: one time for 2 min in 2× SSC/0.1% sodium dodocyl sulfate, followed by three 10-min washes in the same buffer. The blots were then exposed on autoradiography film overnight. The sequences of the 20-mer oligonucleotides used for detection of the mRNA were 5′-AAGGTGGTCCAGGAACTGTG-3′ for the cardiac NHE-1, 5′-TCTGGGACCACGTAAACACA-3′ for cardiac NCE-1 5′-AAGAAGATGATCAAGCTGCC-3′ for the cardiac NBC-1 and 5′-ATGCCAGAGTCTCGTTCGTT-3′ for the 18S mRNA.

For quantification, the blots were scanned and evaluated using the public domain NIH image program. The NHE-1, NCE-1 and NBC-1 mRNA signals were normalized to the 18S mRNA signals to compensate differences in RNA loading.

2.3. Protein extraction and western blot analysis

For measurement of protein levels, the tissue samples were homogenized in 5 ml of ice-cold lysis buffer containing 50 mM Tris–HCl (pH 7.4), 500 mM EDTA, 150 mM NaCl, 0.1% Triton X-100 and freshly added proteinase inhibitor (100 μg/ml PMSF, 1 mg/ml Trasylol), followed by centrifugation at 14,000 rpm at 4 °C for 1 min. The supernatant was collected and aliquots were mixed with loading buffer (1 M Tris–HCl (pH 6.8), 1% sodium dodocyl sulfate, 30% glycerol, 800 mM DTT, 2% bromphenol blue), solubilized for 10 min at 95 °C and then centrifuged for 30 min at 14,000 rpm, 4 °C. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard. All steps were carried out at 4 °C.

For Western blotting, 100 μg of total protein per lane was separated by a 10% sodium dodocyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred onto Hybond-C Super transfer membrane (Amersham, Life Science, Germany). The membrane was washed three times for 20 min in TTBS (0.1% Tween-20, 100 mM Tris–HCl, 150 mM NaCl, pH 7.5), blocked for 1 h in 5% nonfat milk/TTBS and incubated with the primary rabbit polyclonal antibodies for the cardiac NHE-1 (1:1000, Alpha Diagnostic, Bio Trend Chemicals, Germany), for the cardiac NCE-1 (1:1500, Swant, Switzerland) or for the cardiac NBC-1 (1:300, Chemicon, Hofheim, Germany). After three washes in TTBS, the membrane was incubated with a 1:1000 dilution of the anti-rabbit horseradish peroxidase-coupled secondary antibody (Amersham Pharmacia Biotech) for 30 min at room temperature. Following extensive washes in TTBS, cardiac NHE-1, NCE-1 and NBC-1 were detected using ECL-reagents (Amersham Pharmacia Biotech) and exposed to ECL film according to the manufacturer's instructions. Each membrane was counter blotted with 1:5000 dilution of monoclonal anti-β-actin antibodies (Sigma) to ensure same amounts of protein loading on the membranes. Because β-actin has been shown to be unchanged in ischemic rat hearts, the signals were used as an internal control (Gallinat et al., 1998). To measure protein levels, the Western blots were scanned and digitized on an optical scanner (EPSON GT-8000, Seiko, Tokyo, Japan). Quantification of Western blots was performed on a computer using the NIH image analysis system (Scion, Frederick, MD, USA). For all Western blots, the density of the target protein band was divided by that of the β-actin band to correct differences in protein transfer. The protein levels were expressed as percentage of sham-operated animals.

2.4. Immunohistological procedures

Seven days after induction of myocardial infarction, hearts of sham-operated and infarcted animals were fixed by perfusion with 4% phosphate-buffered formaldehyde, rapidly excised and stored in the same formaldehyde solution. Following removal of the atria and large vessels, the ventricles were cut at the midsagittal level of the heart in a standardized fashion (Sandmann et al., 2000) and embedded in paraffin. For immunohistological stainings, serial 4-μm sections were cut from the apical side of the heart, deparaffinized and incubated for 10 min at 96 °C in citrate buffer (DAKO, Hamburg, Germany) to unmask antigens. After two washes in PBS (100 mM NaOH, 100 mM NaH_2PO_4, 70 mM NaCl, pH 7.4), the sections were incubated for 1 h at room temperature in 1% Na-borhydride (MERCK, Darmstadt, Germany) in PBS to reduce background fluorescence. Then, the sections were washed two times in TBS (200 mM NaCl, 4 mM KCl, 20 mM Tris–HCl, pH 7.4), blocked for 1 h at room temperature in 0.1% bovine serum albumin/0.2% glycine/TBS and incubated for 30 min at room temperature in a 1:10 dilution of immunoblock solution in TBS (Roth, Karlsruhe, Germany). The sections were then incubated overnight at 4 °C with a 1:200 dilution of a primary mouse monoclonal antibody (Alpha Diagnostic, Bio Trend Chemicals) for the cardiac NHE-1 or with primary rabbit polyclonal antibodies (Chemicon) for the cardiac NCE-1 and NBC-1. After two washes in 0.1% Tween-20/TBS and three washes in TBS, the sections were incubated for 1 h at 37 °C with a 1:1000 dilution of the secondary antibodies goat anti-mouse Alexa-Fluor-546 or goat anti-rabbit Alexa-Fluor-488 (Molecular Probes, Göttingen, Germany). For double fluorescence staining, glycoproteins of the plasma membrane of interstitial cells were labeled using a 1:10000 dilution of wheat germ agglu-

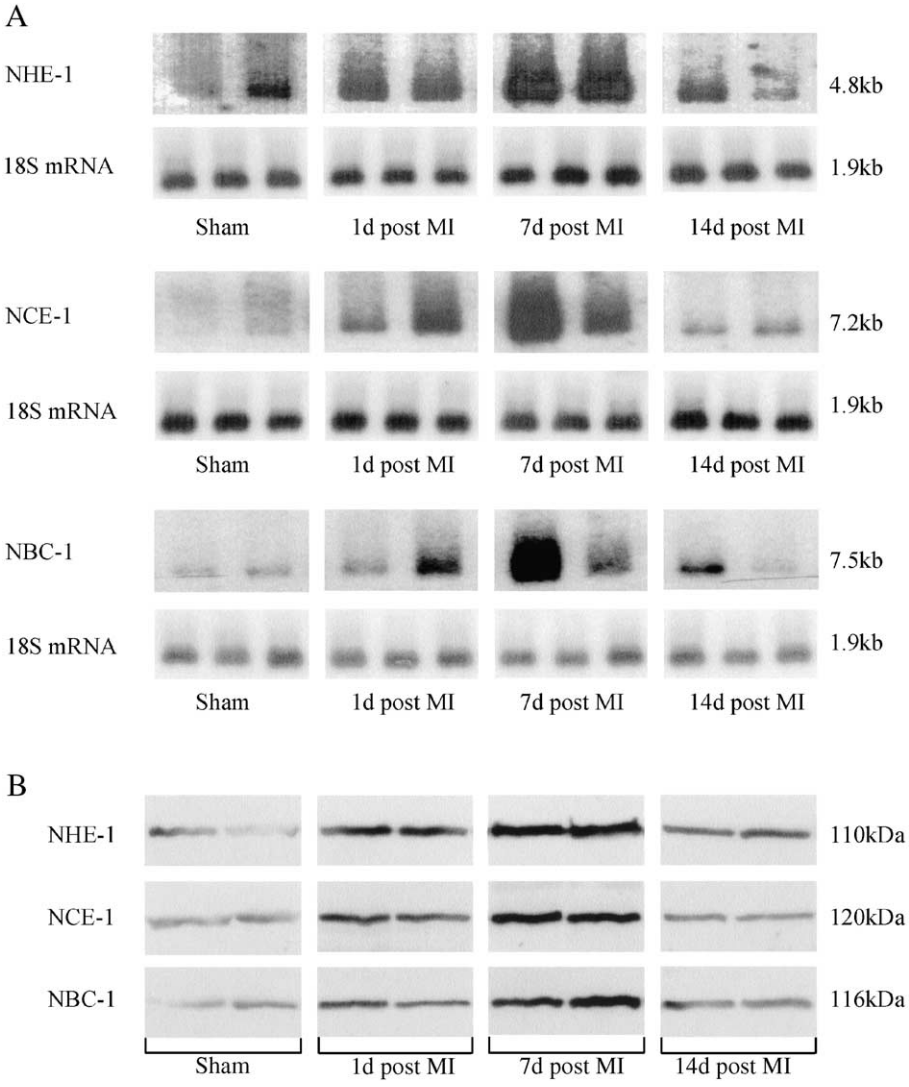


Fig. 1. Representative experiments using Northern blot analysis (A) and Western blot analysis (B) showing up-regulation of NHE-1, NCE-1 and NBC-1 mRNA expression and protein levels in the myocardium of sham-operated and placebo-treated infarcted animals on days 1, 7 and 14 post myocardial infarction. As the house keeping gene, the 18S mRNA expression was used as the internal control.

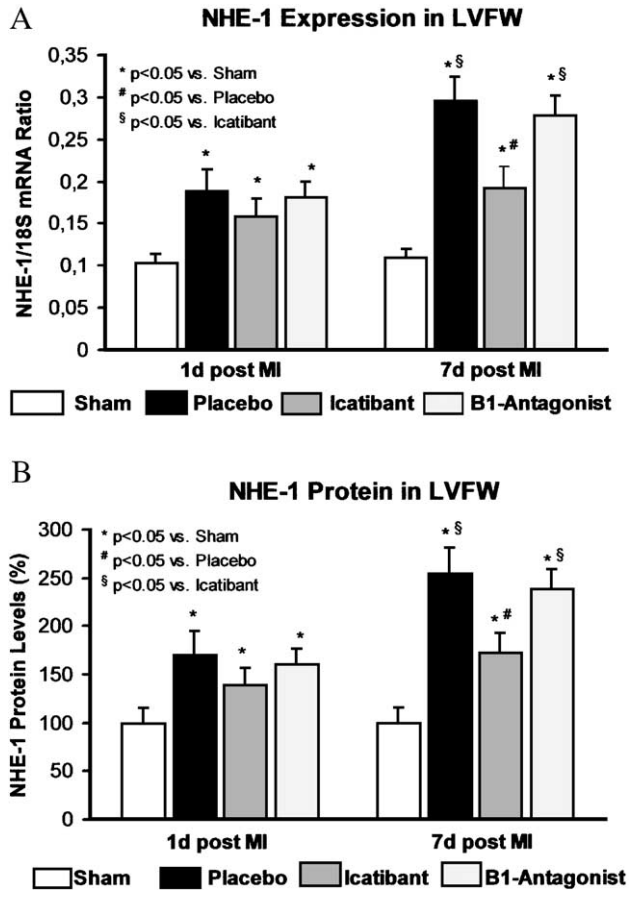


Fig. 2. Effects of chronic treatment with the bradykinin B_2 receptor antagonist icatibant (0.5 mg/kg/day) and the bradykinin B_1 receptor antagonist des-Arg^9-[Leu^8]bradykinin (1 mg/kg/day) on mRNA expression (A) and protein levels (B) of NHE-1 in left ventricular free wall (LVFW) on days 1 and 7 post myocardial infarction compared to sham-operated and placebo-treated infarcted animals. *$P<0.05$ compared to sham operation; $^{\#}P<0.05$ compared to placebo with myocardial infarction; $^{\S}P<0.05$ compared to icatibant with myocardial infarction. Data represent mean ± S.E.M., $n=5-6$.

tinin tetramethyl-rhodamine-isothiocyanate (WGA-TRITC, Molecular Probes) to ensure visual orientation in cardiac tissue. In double immunofluorescence stainings, the contractile filaments where visualized to antibodies against β-actin or with antibodies against the sarcoplasmatic reticulum Ca^{2+}-ATPase type 2 (SERCA 2). All sections were then washed four times in PBS and two times in TBS, mounted with SlowFade® light in glycerol (Molecular Probes) and sealed with acetone glue. For determination of the localization of the three transporters within the cardiac tissue, the sections were evaluated using a ISS 510 confocal laser scanning microscope (Carl-Zeiss-Jena, Jena, Germany) with individual channel recordings at onscreen magnification of 400 × .

2.5. Morphometric examinations

Fourteen days post myocardial infarction, hearts of sham-operated and infarcted animals were rapidly excised, stored in phosphate-buffered formaldehyde and prepared as described above. The ventricles were then cut in a standardized fashion into five transversal slices from the apex to the basis using a special Plexiglas box adapted to rat hearts. The five slices obtained were weighed separately after removal of clotted blood from the ventricles. The weight of all five slices was added to obtain the total heart weight which was related to body weight. The slices were then embedded in paraffin via routine histological procedures. Serial 4-μm sections were cut from slice 3 which has been shown to have the largest left ventricular circumference (Passier et al., 1996) and routinely stained with the collagen-specific stain picrosirius red (Sirius Red, C.I. 3570, Polysciences, Warrington PA, USA, in aqueous picric acid), respectively. Using this method, interstitial collagen types I and III were clearly detectable within the collagen accumulating tissue showing a red color and the remaining tissue a yellow color. The histology of picrosirius-red-stained sections showed a transmural myocardial infarction. Infarct size,

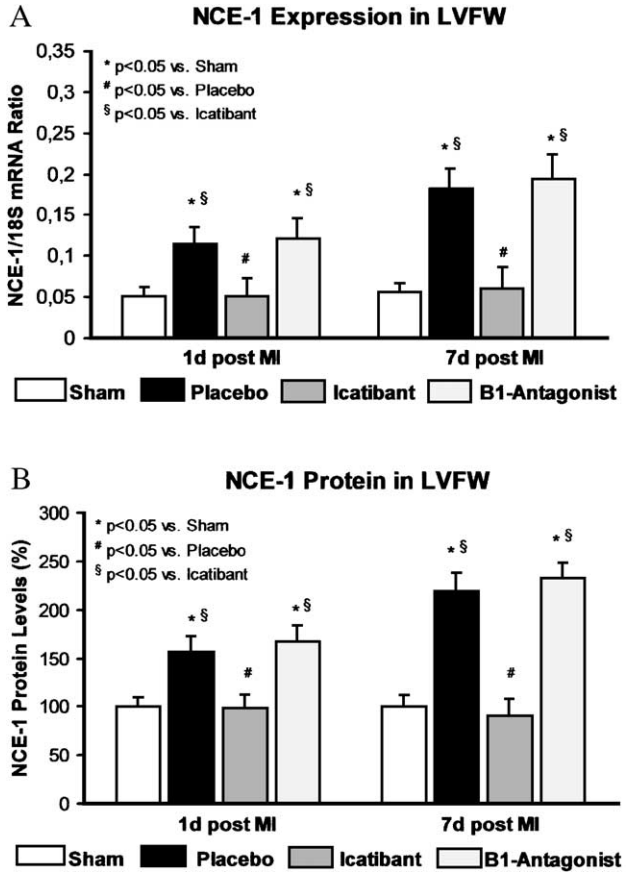
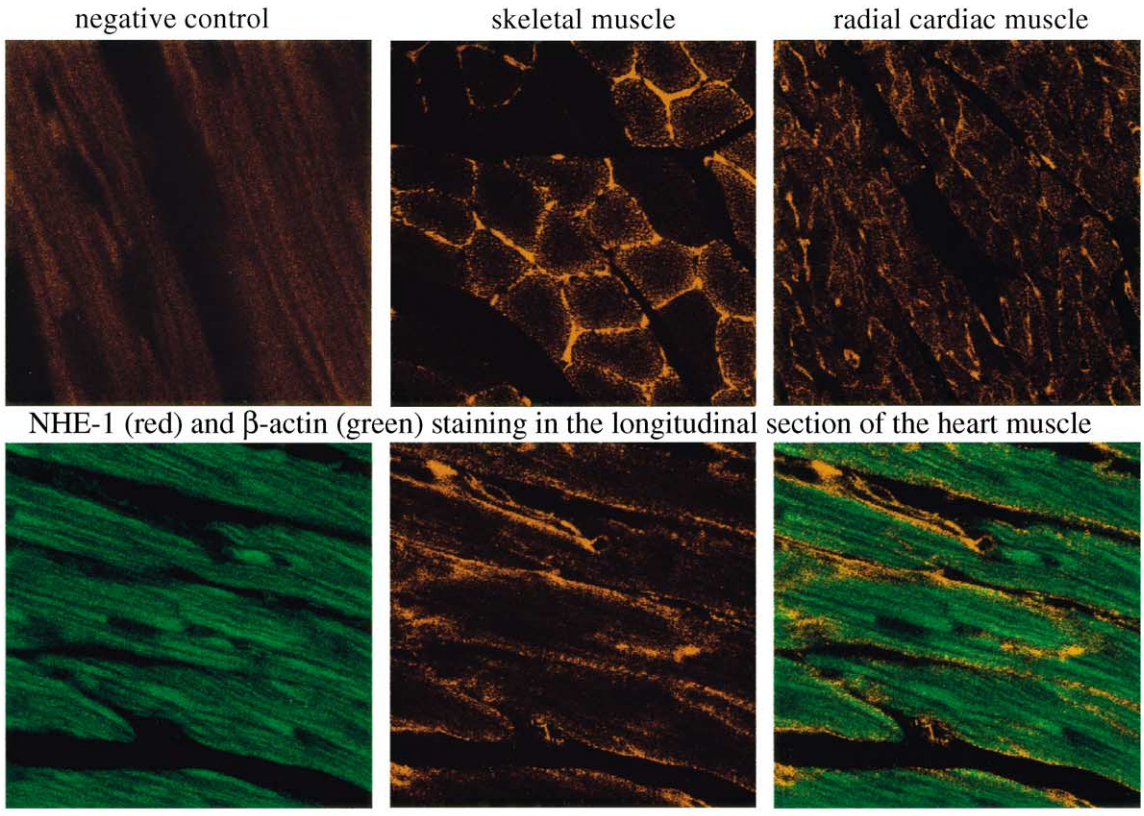
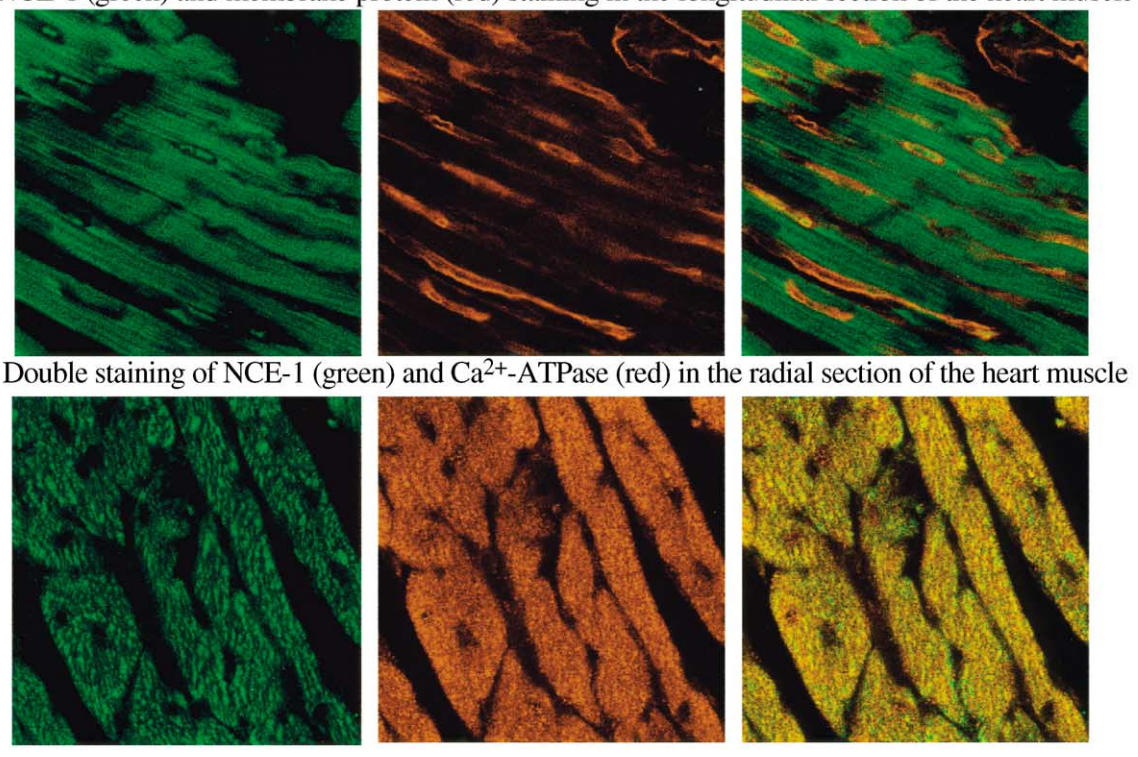


Fig. 3. Effects of chronic treatment with the bradykinin B_2 receptor antagonist icatibant (0.5 mg/kg/day) and the bradykinin B_1 receptor antagonist des-Arg^9-[Leu^8]bradykinin (1 mg/kg/day) on mRNA expression (Fig. 2A) and protein levels (Fig. 2B) of NCE-1 in left ventricular free wall (LVFW) on days 1 and 7 post myocardial infarction compared to sham-operated and placebo-treated infarcted animals. *$P<0.05$ compared to sham operation; $^{\#}P<0.05$ compared to placebo with myocardial infarction; $^{\S}P<0.05$ compared to icatibant with myocardial infarction. Data represent mean ± S.E.M., $n=5-6$.

A


negative control
skeletal muscle
radial cardiac muscle
NHE-1 (red) and β-actin (green) staining in the longitudinal section of the heart muscle

B


NCE-1 (green) and membrane protein (red) staining in the longitudinal section of the heart muscle
Double staining of NCE-1 (green) and Ca2+-ATPase (red) in the radial section of the heart muscle

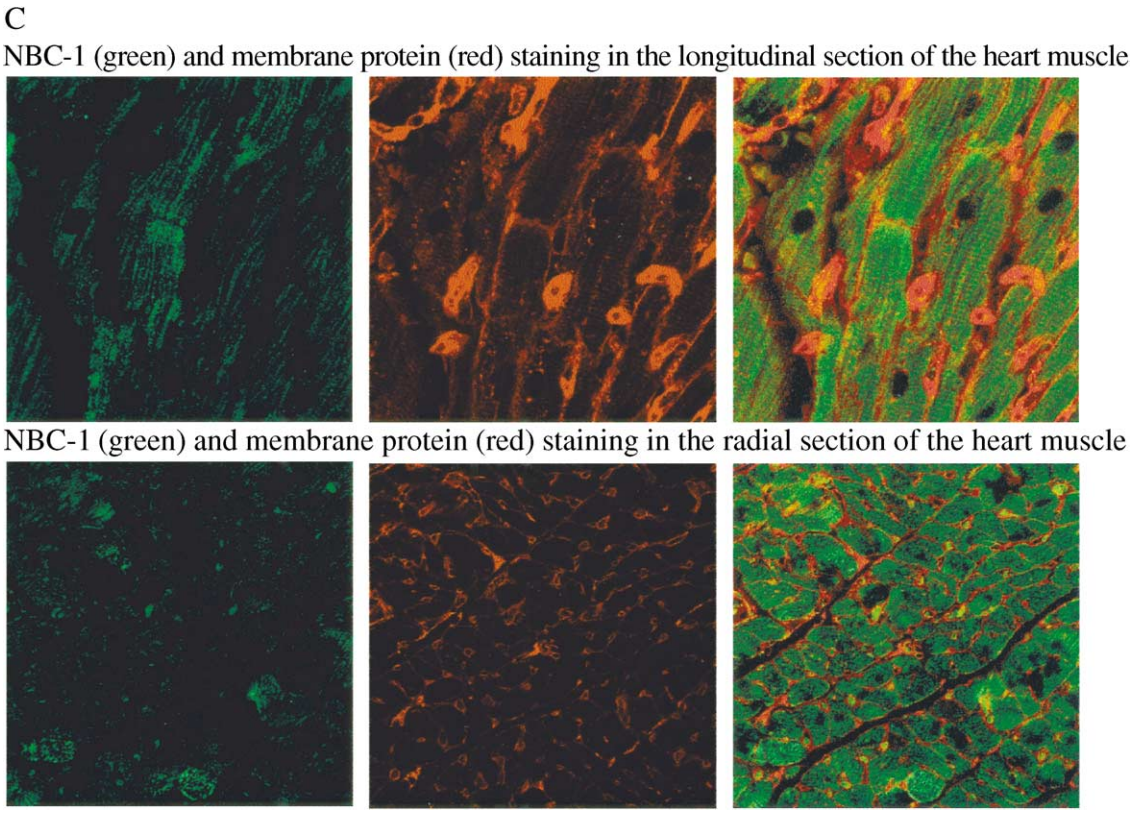


Fig. 4. Photomicrographs using confocal laser scanning microscopy show double immunofluorescence-labeled stainings of NHE-1 (A), NCE-1 (B) and NBC-1 (C) in longitudinal and radial cardiac muscle section. (A) Red-labeled immunostainings of the upper lane show the negative control (left panel), the NHE-1 in skeletal muscle (middle lane) and the NHE-1 in radial cardiac muscle section (right lane). Photomicrographs demonstrate that the NHE-1 is predominantly localized in the plasma membrane of skeletal and cardiac muscle cells. The lower lane shows double immunofluorescence stainings of the cardiac NHE-1 (red) and of cardiac β-actin (green) in the longitudinal section of the heart muscle. (B) Immunostainings of the longitudinal section (upper lane) of the heart muscle show that the cardiac NCE-1 (green) is predominantly localized in the membrane of the sarcoplasmatic reticulum close to the contractile filaments whereas membrane proteins were depicted by WGA-affinity (red). Control stainings (lower lane) demonstrate that the cardiac NCE-1 (green) was colocalized with the sarcoplasmatic reticulum Ca^{2+}–ATPase type 2 (SERCA 2, red) as double immunofluorescence stainings resulted in a yellow color. (C) Immunostainings show that the cardiac NBC-1 (green) is predominantly localized at the apical side of myocytes near the Z-line. WGA-binding depicts the extracellular space.

left ventricular dilation, septal thickness and interstitial collagen volume fraction were estimated using a computerized surface determination method (Quantimet 570 morphometer including morphometry software, Leica, Cambridge Instruments, Cambridge, UK). Briefly, the sections were scanned at 25-fold lens magnification and the borderline between the infarcted area and the remaining myocardial muscle was marked exactly with a pointer. Infarct size was calculated by the computer program as the percentage of the left ventricular circumference. Left ventricular dilation and septal thickness were determined according to the method as published previously (Sandmann et al., 1998). Interstitial collagen volume fraction of the non-infarcted myocardium was evaluated by the software basing on density of the picrosirius-red-stained collagen fibers of tissue areas in direct light and was calculated as the sum of all connective tissue areas divided by the sum of all connective tissue and muscle areas of the non-infarcted myocardium. All histological and morphometric measurements were performed in a blinded fashion by a single observer.

2.6. Statistics

Statistical evaluation was performed using one-way analysis of variance (ANOVA). Means shown to be different between individual groups were compared using the post hoc unpaired Student's *t*-test or the Bonferroni test. A probability of $P < 0.05$ or less was considered as significant. Results are expressed as mean ± standard error of the mean (S.E.M.). Further details of statistical analysis are given in the legends to the figures.

3. Results

3.1. Transcriptional and translational regulation of the transporters NHE-1, NCE-1 and NBC-1 in the infarcted myocardium

Evaluation of the mRNA expression of the cardiac NHE-1, NCE-1 and NBC-1 using Northern blot analysis resulted in

single bands of the predicted size for NHE-1 of 4.8 kb, for NCE-1 of 7.2 kb and for NBC-1 of 7.5 kb. The 18S mRNA expression yielded a single band of 1.9 kb and did not change in the rat myocardium during the early phase after myocardial infarction. Therefore, the 18S mRNA signals were used as an internal loading control, and the mRNA levels of the cardiac NHE-1, NCE-1 and NBC-1 were normalized to the 18S mRNA signals and expressed by folds of increase compared to basal levels of sham-operated animals.

In the left ventricular free wall, NHE-1, NCE-1 and NBC-1 mRNA abundance was significantly increased following infarction as compared to sham-operated animals. The message of NHE-1 was increased twofold on day 1, was elevated approximately threefold on day 7 post myocardial infarction (Fig. 1A) and decreased to baseline values on day 14 post infarction. Similarly, the message of the NCE-1 was elevated on day 1, was increased twofold on day 7 and decreased on day 14 post infarction (Fig. 1A). In addition, the mRNA expression of the NBC-1 was increased on day 1, further increased on day 7 and decreased to baseline values on day 14 post infarction (Fig. 1A). In contrast, the NHE-1, NCE-1 and NBC-1 mRNA expression in the interventricular septum and right ventricle were unchanged throughout the post infarction period when compared to sham-operated animals (data not shown). In sham-operated animals, NHE-1, NCE-1 and NBC-1 mRNA expressions were unchanged at all times post surgery compared to healthy rats (data not shown). These results are in agreement with earlier findings (Sandmann et al., 2001b) demonstrating that myocardial infarction induced an up-regulation of these three transporters in the infarcted but not in the non-infarcted region of the rat myocardium.

Protein levels of the cardiac NHE-1, NCE-1 and NBC-1 were measured in the right ventricle, interventricular septum and left ventricular free wall of sham-operated and infarcted animals on days 1, 7 and 14 post coronary occlusion using Western blot analysis. The density of the digitized signals was quantified as percentage of the signal density of sham-operated animals which was set to 100%, respectively. In the rat myocardium, we detected a single band of ~ 110 kDa for cardiac NHE-1, of ~ 120 kDa for cardiac NCE-1 and of ~ 116 kDa for cardiac NBC-1.

As demonstrated for mRNA expression, the protein levels of the cardiac NHE-1, NCE-1 and NBC-1 were increased in the left ventricular free wall of placebo-treated infarcted animals on day 1 and reached maximum values on day 7 post infarction compared to sham-operated animals. Fourteen days post infarction, protein levels of NHE-1, NCE-1 and NBC-1 showed values of sham-operated animals (Fig. 1B). The protein levels of the three cardiac transporters were not significantly different in the right ventricle and interventricular septum of infarcted and sham-operated rat hearts at all time points measured (data not shown). In healthy animals, protein levels of the three cardiac transporters were not significantly different from those of sham-operated animals (data not shown). Thus, myocardial infarction induced a translational up-regulation of the cardiac NHE-1, NCE-1 and NBC-1 in the infarcted region of the rat myocardium.

3.2. Effects of bradykinin B_1 and B_2 receptor antagonists on NHE-1, NCE-1 and NBC-1 mRNA expression and protein levels

To determine whether bradykinin is involved in the mRNA and protein up-regulation of the cardiac NHE-1, NCE-1 and NBC-1 at the three time points post sham surgery or induction of myocardial infarction, animals were pretreated 1 week before and up to sacrifice post operation with the selective bradykinin B_2 or B_1 receptor antagonists, icatibant or des-Arg^9-[Leu^8]bradykinin, respectively. In sham-operated animals, the gene expression and protein translation of the NHE-1, NCE-1 and NBC-1 in the right ventricle, interventricular septum and left ventricular free wall were unaffected by chronic treatment with icatibant and des-Arg^9-[Leu^8]bradykinin.

In infarcted animals, chronic treatment with the bradykinin B_2 receptor antagonist, icatibant, significantly reduced the infarction-induced increase in cardiac NHE-1 mRNA expression (Fig. 2A) and protein levels (Fig. 2B) in the left ventricular free wall on day 7 post infarction when compared to placebo-treated animals. However, mRNA expression and protein levels of this transporter remained significantly elevated in the left ventricular free wall of icatibant-treated animals on days 1 and 7 post infarction when compared to sham-operated animals. Fourteen days following myocardial infarction, mRNA expression and protein levels of the cardiac NHE-1 showed values of sham-operated animals and were unaffected by chronic icatibant treatment. In contrast, pretreatment of infarcted animals with the bradykinin B_1 receptor antagonist, des-Arg^9-[Leu^8]bradykinin, had no effect on gene expression (Fig. 2A) and protein translation (Fig. 2B) of the cardiac NHE-1 in the left ventricular free wall measured at the tree time points post infarction (significantly increased compared to sham-operated as well as to icatibant-treated animals on day 7 post infarction). NHE-1 mRNA and protein levels in the right ventricle and interventricular septum were unaffected by chronic placebo-, icatibant- and des-Arg^9-[Leu^8]bradykinin-treatment.

Pretreatment with icatibant completely abolished the infarction-induced increase in NCE-1 mRNA expression (Fig. 3A) and protein levels (Fig. 3B) in the left ventricular free wall measured at the three time points post infarction compared to placebo treatment. Thus, 1 and 7 days following infarction, mRNA expression and protein levels of this cardiac transporter were not significantly different from those of sham-operated animals. On day 14 post infarction, NCE-1 mRNA and protein levels reversed to values of sham-operated animals and was not affected by icatibant. Chronic treatment with des-Arg^9-[Leu^8]bradykinin did not affect NCE-1 mRNA (Fig. 3A) and protein (Fig. 3B) up-

regulation in the left ventricular free wall on days 1, 7 and 14 post infarction and was significantly increased compared to icatibant-treated animals. No significant differences in NCE-1 mRNA and protein values were observed between placebo-, icatibant- and des-Arg^9-[Leu^8]bradykinin-treated infarcted animals in the right ventricle and interventricular septum at any time point measured.

The infarction-induced increase in NBC-1 mRNA expression and protein levels in the left ventricular free wall was unaffected by chronic treatment with icatibant or des-Arg^9-[Leu^8]bradykinin measured on days 1, 7 and 14 post infarction (data not shown). NBC-1 mRNA and protein concentrations in the right ventricle and interventricular septum of placebo-treated infarcted animals did not significantly differ from those of chronically icatibant- or des-Arg^9-[Leu^8]bradykinin-treated infarcted animals.

3.3. Localization of the NHE-1, NCE-1 and NBC-1 in cardiac tissue

To investigate the localization of the cardiac NHE-1, NCE-1 and NBC-1 within the myocardium, indirect double immunofluorescence stainings were analyzed using confocal laser scanning microscopy. Fig. 4 shows immunostainings of heart muscle sections where the cardiac NHE-1 was stained in red color and the cardiac NCE-1 and NBC-1 were stained in green color. As shown in the upper lane of Fig. 4A, the cardiac NHE-1 was predominantly localized in the plasma membrane of skeletal muscle cells and of cardiac muscle cells. The lower lane of Fig. 4A shows double immunofluorescence stainings of a longitudinal section of the heart muscle where β-actin (green) was detected within myocytes and the cardiac NHE-1 (red) appeared in the plasma membrane of myocytes.

In contrast, Fig. 4B demonstrates that the cardiac NCE-1 (green) was localized within cardiac myocytes as shown by double immunofluorescence stainings of a longitudinal section (upper lane) of the heart muscle with membrane glycoproteins (red). In control stainings (lower lane), the NCE-1 was not colocalized with β-actin (data not shown), but with the sarcoplasmatic reticulum Ca^{2+}-ATPase type 2 (SERCA 2) as evidenced by a yellow color in the red–green double immunofluorescence stainings.

Fig. 4C shows double immunofluorescence stainings of the cardiac NBC-1 (green) and membrane glycoproteins (red) in the longitudinal (upper lane) and radial (lower lane) section of the heart muscle. Photomicrographs demonstrate that this transporter was predominantly localized at the apical side of myocytes near the Z-line.

3.4. Effects of B_1 and B_2 receptor antagonists on cardiac morphometry

Fourteen days following myocardial infarction, the ratio of total heart weight to body weight was increase in placebo-treated animals compared to sham-operated animals. The heart weight to body weight ratio of infarcted animals treated with the bradykinin B_2 receptor antagonist, icatibant, was decreased compared to placebo-treated animals (Table 1). In contrast, treatment with the bradykinin B_1 receptor antagonist, des-Arg^9-[Leu^8]bradykinin, had no effect on the ratio of total heart weight to body weight of infarcted animals (Table 1). The infarct size was 46.2 ± 2.1% in placebo-treated animals and was significantly reduced in icatibant-treated animals, whereas des-Arg^9-[Leu^8]bradykinin had no effect on infarct size (Table 1). The left ventricle was dilated in all animals with myocardial infarction compared to sham-operated animals (Table 1). The left ventricular dilation of icatibant-treated animals but not of des-Arg^9-[Leu^8]bradykinin-treated animals was significantly reduced when compared to placebo-treated animals (Table 1). Fourteen days post infarction, septal thickness and interstitial collagen volume fraction of the non-infarcted myocardium were markedly increased in placebo-treated infarcted animals when compared to sham-operated animals (Table 1). Treatment with icatibant inhibited septal thickening (not significantly elevated compared to sham-operated animals) and reduced interstitial fibrosis in infarcted animals, whereas treatment of infarcted animals with des-Arg^9-[Leu^8]bradykinin did not affect septal thickness and interstitial collagen volume fraction (Table 1). In these animals, both parameters were significantly increased compared to icatibant-treated animals. Thus, chronic bradykinin B_2 receptor blockade with icatibant limited infarct size and reduced the infarction-induced myocardial remodeling process.

Table 1
Morphometric data of rat hearts 14 days after induction of myocardial infarction or sham surgery

	Sham	Placebo	Icatibant	B_1 antagonist
THW/BW (g)	0.23 ± 0.01	0.29 ± 0.02[a]	0.26 ± 0.01[b]	0.28 ± 0.01
Infarct size (%)		46.2 ± 2.1	39.8 ± 1.4[b]	45.8 ± 1.8[c]
LVD (mm)	5.02 ± 0.02	7.11 ± 0.04[a]	5.75 ± 0.03[a,b]	7.09 ± 0.03[a]
ST (mm)	1.51 ± 0.05	1.99 ± 0.07[a]	1.62 ± 0.06[b]	1.97 ± 0.05[a,c]
ICVF (%)	2.60 ± 0.68	9.12 ± 1.45[a]	5.61 ± 0.89[a,b]	8.89 ± 1.07[a,c]

Indicated are animals that underwent sham surgery (sham) or induction of myocardial infarction treated either with placebo (0.9% saline), with the bradykinin B_2 receptor antagonist icatibant (0.5 mg/kg/day) or with the bradykinin B_1 receptor antagonist des-Arg^9-[Leu^8]bradykinin (1 mg/kg/day) beginning 1 week prior to induction of myocardial infarction. Data are presented as mean ± S.E.M. of total heart weight to body weight (THW/BW), infarct size, left ventricular dilation (LVD), septal thickness (ST) and interstitial collagen volume fraction of the non-infarcted myocardium (ICVF).

[a] Significant versus sham ($P < 0.05$).

[b] Significant versus placebo ($P < 0.05$).

[c] Significant versus icatibant, $n = 6$.

4. Discussion

In the present study, we demonstrate for the first time that the bradykinin B_2 receptor, but not the bradykinin B_1 receptor, is involved in the myocardial infarction-induced

transcriptional and translational up-regulation of the cardiac NHE-1 and NCE-1 in the ischemic (left ventricular free wall) myocardium during the early phase following infarction. Pretreatment with the bradykinin B_2 receptor antagonist, icatibant, prevented the up-regulation of these cardiac transporters, while the bradykinin B_1 receptor antagonist, des-Arg^9-[Leu^8]bradykinin, had no effect on the up-regulation of the cardiac transporters. In contrast, mRNA- and protein up-regulation of the cardiac NBC-1 post infarction was neither influenced by icatibant nor by des-Arg^9-[Leu^8]-bradykinin. The exact mechanisms underlying the transcriptional and translational up-regulation of the cardiac NHE-1, NCE-1 and NBC-1 are not clear. However, in the present experiments, permanent occlusion of the left coronary artery resulted in an ischemia of the left ventricular free wall known to produce an intracellular acidosis within this region (Cobbe and Poole-Wilson, 1982). A well-established consequence of tissue acidosis is changes in gene expression levels and protein translation or degradation (Akiba et al., 1987; Hwang and Curthoys, 1991). Thus, it is possible that in our experiments acidosis induced mRNA and protein up-regulation of the cardiac transporters in the ischemic (left ventricular free wall) but not in the remaining (right ventricle and interventricular septum) myocardium.

4.1. Role of cardiac NHE-1, NCE-1 and NBC-1 during myocardial ischemia

During ischemia, the cardiac NHE-1 regulates intracellular pH by extruding one proton in exchange for one sodium ion. Thus, the NHE-1 also participates in establishing an intramyocardial Na^+-load accounting for 50% of the basal permeability of the membrane to Na^+ (Frelin et al., 1984). As a result of the activation of the cardiac NHE-1 following intracellular acidosis, accumulated Na^+ can be exchanged for Ca^{2+} via the cardiac NCE-1 at a 3:1 ratio. As a consequence, changes in intracellular pH result in an increase in intracellular Ca^{2+} because of the close interaction of these two exchangers with respect to cytosolic Na^+ (“pH paradox”) (Frelin et al., 1984; Seifter and Aronson, 1986). It has been demonstrated previously that the NHE-1-mediated acidosis induced an intracellular Ca^{2+}-overload via the NCE-1 which has been shown to be associated with cell death and tissue injury (Siffert and Akkerman, 1989; Meng et al., 1991). Therefore, the up-regulation of cardiac NHE-1 and NCE-1 (Kim et al., 1987) in the ischemic myocardium following infarction results in an increased incidence of cardiac damage and myocardial dysfunction (Karmazyn, 1988; Meng et al., 1991).

The primary function of the cardiac NBC-1 is to import $3HCO_3^-$ together with $1Na^+$, leading to an intracellular alkalinization (Sandmann et al., 2001b; Camilion de Hurtado et al., 1996). The fact that this transporter has been shown to be colocalized directly with the Na^+–K^+ pump within the plasma membrane (Marino et al., 1999) suggests that the NBC-induced Na^+ accumulation could be removed immediately from the cytosol via the coupled Na^+–K^+ pump in exchange to K^+ (3:2 ratio). The Na^+–K^+ pump-mediated Na^+ efflux causes a hyperpolarisation of the cell membrane which is known to protect the myocardium against arrhythmias. Thus, an enhanced expression and translation of the cardiac NBC-1 in the ischemic myocardium could play a cardioprotective role.

4.2. Contribution of the bradykinin B_2 receptor to the regulation of cardiac NHE-1, NCE-1 and NBC-1

Evidence of several studies demonstrating the existence of a local kallikrein–kinin system in the heart (Nolly et al., 1994), an increased bradykinin production during ischemia (Kimura et al., 1973; Matsuki et al., 1987) as well as an up-regulation of the bradykinin receptor subtypes, B_2 and B_1, following myocardial infarction (Tschope et al., 1999, 2000) implicates that bradykinin might act as an autocrine/paracrine hormone in the up-regulation of cardiac transporters during ischemia. Indeed, the findings of the present study demonstrate that chronic blockade of the bradykinin B_2 receptor with icatibant abolished mRNA and protein up-regulation of the cardiac NHE-1 and NCE-1 post infarction whereas chronic bradykinin B_1 receptor blockade did not. These results suggest that the bradykinin B_2 receptor and not the bradykinin B_1 receptor is involved in the transcriptional and translational up-regulation of the cardiac NHE-1 and NCE-1 during ischemia. On the other hand, the fact that the up-regulation of the NHE-1 was totally abolished by icatibant on day 1 post infarction, whereas on day 7 post infarction this inhibition was not complete, suggests that bradykinin-independent mechanisms also contribute to the regulation of the cardiac NHE-1 at later time points following infarction. For instance, in rat ventricular myocytes, it has been demonstrated that α_1-adrenergic stimulation acutely causes an alkalinization of pH_i and an enhanced rate of NHE-mediated recovery from an acid load (Terzic et al., 1992). Endothelin (Karmazyn, 1996) and thrombin (Yasutake et al., 1996) have also been shown to activate the cardiac NHE-1 of rat myocytes. In addition, we previously reported that angiotensin II activates the cardiac NHE-1 via stimulation of its angiotensin AT_1 receptor (Sandmann et al., 2001b).

A stimulatory effect of bradykinin on NHE activity has previously been demonstrated in endothelial cells by Fleming et al. (1994). In these experiments, the resulting decrease in intracellular pH was associated with an increase in intracellular Ca^{2+}. This study has evoked speculations that bradykinin indirectly mediates an increase in intracellular Ca^{2+} via an association with the NCE. In the present study, we show for the first time a direct stimulatory action of bradykinin on the cardiac NCE-1 expression. Moreover, we demonstrate that the bradykinin-induced effects on this transporter in the ischemic myocardium were mediated through the bradykinin B_2 receptor since they were abolished by chronic blockade of this bradykinin receptor by icatibant.

As the NHE-1 and the NCE-1 participate in the regulation of intramyocardial pH and Ca^{2+}, our findings dem-

onstrate that bradykinin contributes to infarction-induced myocardial acidosis and Ca^{2+}-overload via stimulation of its bradykinin B_2 receptor and, thereby, is involved in cardiac tissue injury and myocardial dysfunction. Chronic blockade of this bradykinin receptor subtype by icatibant during infarction reduced the infarction-induced up-regulation of the cardiac NHE-1 and NCE-1 and, thus, prevented the myocardial Ca^{2+}-overload via inhibition of the NHE–NCE pathway. On the other hand, the finding that the infarction-induced up-regulation of the cardiac NBC-1 was not affected by bradykinin or by the bradykinin B_2 receptor antagonist, icatibant, suggest that the ischemic tissue recovers from an intracellular acidosis via activation of this cardiac transporter. As the NBC-1 exerts a three times more capability to buffer intracellular H^+ and the accumulating Na^+ can be exporter via the linked Na^+–K^+ pump without Ca^{2+}-overload implicates a protective effect of icatibant against ischemia-induced acidosis and Ca^{2+}-overload. From these results could be speculated that the myocardial cell damage induced by acidosis or Ca^{2+}-overload following myocardial infarction might be prevented by chronic blockade of the bradykinin B_2 receptor resulting in a reduction of infarct size by icatibant.

Kinins have been shown to be involved in myocardial energy metabolism. As showed by Zhang et al. (1997, 1999), incubation of coronary vessels and myocardial tissue sections with an angiotensin-converting enzyme inhibitor or kininogen decreased myocardial oxygen consumption; this effect was blocked by a bradykinin B_2 receptor antagonist. Bradykinin has also been shown to induce the release of NO from the myocardium via a bradykinin B_2 receptor-mediated mechanism (Loke et al., 1999). Since NO is known to participate in the regulation of myocardial glucose, lactate and fatty acid metabolism (Toda et al., 2000), bradykinin preserves cardiac metabolism during ischemia. In addition, bradykinin has been shown to increase the production of myocardial high-energy phosphates and glycogen content, along with a reduction in lactate dehydrogenase and creatinine kinase activity in the ischemic heart, effects that were both blocked by a bradykinin B_2 receptor antagonist (Schölkens et al., 1988; Schölkens and Linz, 1992). Thus, kinins reduce oxygen consumption and facilitate energy utilization of the ischemic myocardium via a bradykinin B_2 receptor-mediated mechanism and, thereby, exert a cardioprotective action. In this study, we demonstrate that chronic bradykinin B_2 receptor blockade during ischemia also exerts a cardioprotective action and an infarct size limiting effect via reduction of the ischemia-induced acidosis and Ca^{2+}-overload in the myocardium.

4.3. Effects of bradykinin B_1 and B_2 receptor antagonists on cardiac morphology

Fourteen days after permanent ligation of the left coronary artery, placebo-treated animals had developed a dilative cardiac hypertrophy evidenced by a left ventricular dilation and an increase in the ratio of total heart weight to body weight associated with an elevation in interstitial collagen content and an increase in septal thickness. The myocardial infarction-induced alterations in cardiac morphology and the engendered myocardial fibrosis in placebo-treated animals indicate an increased myocardial stiffness accounting in part for the deterioration in the left ventricular performance leading to the development of congestive heart failure (Gay, 1990).

Chronic treatment of infarcted animals with the bradykinin B_2 receptor antagonist, icatibant, but not with the bradykinin B_1 receptor antagonist, des-Arg^9-[Leu^8]bradykinin, reduced infarct size and left ventricular dilation and decreased the ratio of total heart weight to body weight and interstital fibrosis. In addition, septal thickening following infarction was totally inhibited by icatibant as septal thickness was not significantly elevated over the one of sham-operated animals. Thus, chronic bradykinin B_2 receptor blockade limited the cardiac remodeling process post infarction indicating an improvement of ventricular function. The infarct size limiting property of icatibant seems to be related to the reduction of intramyocardial Ca^{2+}-overload via inhibition of the NHE–NCE pathway in the ischemic myocardium during the acute phase post infarction as shown by the present study. Additionally, the reduction of infarct size by icatibant might be related to inhibition of bradykinin B_2 receptor-mediated pro-inflammatory action of bradykinin during the early phase post infarction (Tiffany and Burch, 1989). It could be speculated that icatibant reduced the inflammatory reaction within the infarcted myocardium via decreasing of the tumor necrosis factor-α and interleucine-1 release by macrophages. Additionally, the infarct size reduction by icatibant might be related to the activation of ATP-dependent K^+ channels (Pan et al., 2000) resulting in a shortening of action potential duration as seen in preconditioning.

The ability of icatibant to decrease cardiac hypertrophy and myocardial fibrosis seems to be partially related to the modulation of the mitogenic and pro-arrhythmic catecholamines (Hatta et al., 1999). Bradykinin has been shown to induce the release of norepinephrine from sympathetic nerve endings via stimulation of its bradykinin B_2 receptor (Seyedi et al., 1997). As norepinephrine is known to facilitate collagen synthesis in cardiac fibroblast and to induce a positive inotropic response, it could be possible that, in our experiments, chronic bradykinin B_2 receptor blockade limited cardiac fibrosis and preserved ventricular contractility via reduction of norepinephrine release in the heart. In addition, an anti-arrhythmic effect of icatibant seems to be mediated via inhibition of the bradykinin B_2 receptor-mediated stimulation of the Na^+–K^+-ATPase (Dodson and Rhoden, 2001). Bradykinin B_2 receptor stimulation by bradykinin has also been shown to increase intracellular Ca^{2+} in cardiac fibroblasts and to activate protein kinase C, implicating that icatibant decreased Ca^{2+}-dependent collagen synthesis and reduced production of cellular proteins (Farmer and Burch, 1992).

4.4. Localization of the cardiac NHE-1, NCE-1 and NBC-1 within the myocardium

Using mouse monoclonal antibodies in indirect immunofluorescence stainings, we identified the fully glycosylated ~ 110 kDa isoform of the cardiac NHE-1 to be predominantly localized in the myocyte plasma membrane (Fig. 4A) of the infarcted myocardium. In contrast, Petrecca et al. (1999) demonstrated three bands of different isoforms of the cardiac NHE-1 in the healthy myocardium using polyclonal antibodies: the fully glycosylated isoform of ~ 110 kDa, the dimerized isoform of ~ 200 kDa and the non-glycosylated isoform of ~ 90 kDa. In these experiments, the ~ 110 kDa isoform of the cardiac NHE-1 was predominantly localized at the intercalated disk region in close proximity to the gap junction protein. This isoform of the cardiac NHE-1 has been shown to mainly contribute to the regulation of intracellular H^+ during acidic load. As the ischemic myocardium is characterized by an intracellular acidosis, our findings suggest an important role of the ~ 110 kDa isoform of this transporter in H^+-transport mechanisms between the cytosol and the extracellular space during cardiac ischemia.

In contrast, we found that the cardiac NCE-1 was predominantly localized in the membrane of the sarcoplasmatic reticulum close to the contractile filaments (Fig. 4B). In the failing myocardium, the Ca^{2+} transient is prolonged (Gwathmey et al., 1987) and the Ca^{2+} uptake activity of the sarcoplasmatic reticulum is reduced due to a down-regulation of SERCA 2 (Flesch et al., 1996), which both result in an increase in resting intramyocardial Ca^{2+} concentration and diastolic dysfunction (Beuckelmann et al., 1992). On the other hand, the present study and earlier findings (Studer et al., 1994) showed an up-regulation of the cardiac NCE-1 in the ischemic myocardium. Because the cardiac NCE-1 is known to work in the reverse mode during Ca^{2+}-overload to prevent intracellular Ca^{2+} accumulation, our findings that the cardiac NCE-1 is located in the sarcoplasmatic reticulum indicate a possible contribution of this transporter to the Ca^{2+} uptake mechanism of the sarcoplasmatic reticulum. One could speculate that the cardiac NCE-1 is involved in Ca^{2+} transport mechanisms between myofibrils and the sarcoplasmatic reticulum and, thereby, regulates intramyocardial Ca^{2+} to protect contractile function of the ischemic myocardium.

Fig. 4C clearly shows that the cardiac NBC-1 is enriched at the apical side of myocytes near the Z-line, implying an involvement of this cardiac transporter in pH-regulating mechanisms in the connecting region between adjacent myocytes. An accumulation of H^+ in the myocardium has been shown to reduce the sensitivity of the contractile filaments to Ca^{2+} and, thereby, lower myofilament contractility (Garlick et al., 1979; Momomura et al., 1985). Acidosis is known to disturb the attachment of β-actin to the Z-line as well as to interrupt the major connecting protein between myosin and the Z-line, known as titin (Opie, 1998). In addition, as the gap junctions allow the physical communication of the cytosol of one myocyte with that of the neighboring myocyte the regulation of intramyocardial pH with precision in this cardiac region is of special importance for cardiac structure and the functioning heart muscle. Furthermore, as the longitudinal connection of adjacent myocytes is important to ensure mechanical continuity between cardiac myocytes, our findings suggest that the cardiac NBC-1 preserves myocardial structural integrity via protection the ischemic myocardium against acidosis-induced cardiac disruption and contractile dysfunction.

References

Akiba, T., Pocco, V.K., Warnock, D.G., 1987. Parallel adaptation of the rabbit renal cortical sodium/proton antiporter and sodium/bicarbonate cotransporter in metabolic acidosis and alkalosis. J. Clin. Invest. 80, 308–315.

Bentley, K.R., Jarrott, B., 2000. Lipopolysaccharide decreases bradykinin receptor-induced acidification responses in cultured bovine aortic endothelial cells. Eur. J. Pharmacol. 402 (1–2), 11–18.

Beuckelmann, D.J., Näbauer, M., Erdmann, E., 1992. Intracellular calcium handling in isolated ventricular myocytes from patients with terminal heart failure. Circulation 85, 1046–1055.

Blais Jr., C., Drapeau, G., Raymond, P., Lamontagne, D., Gervais, N., Venneman, I., Adam, A., 1997. Contribution of angiotensin-converting enzyme to the cardiac metabolism of bradykinin: an interspecies study. Am. J. Physiol. 273, H2263–H2271.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Camilion de Hurtado, M.C., Alvarez, B.V., Perez, N.G., Cingolani, H.E., 1996. Role of an electrogenic Na^+–HCO_3^- cotransport in determining myocardial pH_i after an increase in heart rate. Circ. Res. 79 (4), 698–704.

Campbell, D.J., Kladis, A., Briscoe, T.A., Zhuo, J., 1999. Type 2 bradykinin-receptor antagonism does not modify kinin or angiotensin peptide levels. Hypertension 33, 1233–1236.

Carretero, O.A., 1999. Kinins in the heart. In: Share, L. (Ed.), Contemporary Endocrinology: Hormones and the Heart in Heath and Disease. Humana Press, Totowa, NJ, pp. 137–158.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Cobbe, S.M., Poole-Wilson, P.A., 1982. The time of onset and severity of acidosis in myocardial ischaemia. J. Mol. Cell. Cardiol. 12, 745–760.

Dart, C., Vaughan-Jones, R.D., 1992. Na^+–HCO_3^- symport in the sheep cardiac Purkinje fibre. J. Physiol. 451, 365–385.

Dodson, A.M., Rhoden, K.J., 2001. Bradykinin increases Na^+–K^+ pump activity in cultured guinea-pig tracheal smooth muscle cells. Br. J. Pharmacol. 133, 1339–1345.

Farmer, S.G., Burch, R.M., 1992. Biochemical and molecular pharmacology of kinin receptors. Annu. Rev. Pharmacol. Toxicol. 32, 511–536.

Fleming, I., Hecker, M., Busse, R., 1994. Intracellular alkalinization induced by bradykinin sustains activation of the constitutive nitric oxide synthase in endothelial cells. Circ. Res. 74, 1220–1226.

Flesch, M., Schwinger, R.H., Schnabel, P., Schiffer, F., van Gelder, I., Bavendiek, U., Sudkamp, M., Kuhn-Regnier, F., Bohm, M., 1996. Sarcoplasmatic reticulum Ca^{2+}-ATPase and phospholamban mRNA and protein in end-stage human heart failure due to ischemic or dilated cardiomyopathy. J. Mol. Med. 74, 321–332.

Frelin, C., Vigne, P., Lazdunski, M., 1984. The role of the Na^+/H^+ ex-

change system in cardiac cells in relation to the control of the internal Na^+ concentration. A molecular basis for the antagonistic effect of ouabain and amiloride on the heart. J. Biol. Chem. 259, 8880–8885.

Gallinat, S., Yu, M., Dorst, A., Unger, Th., Herdegen, Th., 1998. Sciatic nerve transection evokes lasting up-regulation of angiotensin AT_2 and AT_1 receptor mRNA in adult rat dorsal root ganglia and sciatic nerves. Mol. Brain Res. 57, 111–122.

Garlick, P.B., Radda, G.K., Seeley, P.J., 1979. Studies of acidosis in ischemic heart by phosphorus nuclear magnetic resonance. Biochem. J. 184, 547–554.

Gay, R.G., 1990. Early and late effects of captopril treatment after large myocardial infarction in rats. JACC 16, 967–977.

Gwathmey, J.K., Copelas, L., MacKinnon, R., Schoen, F.J., Feldman, M.D., Grossman, W., Morgan, J.P., 1987. Abnormal intracellular calcium handling in myocardium from patients with end-stage heart failure. Circ. Res. 61, 70–76.

Hall, J.M., 1992. Bradykinin receptors: pharmacological properties and biological roles. Pharmacol. Ther. 56, 131–190.

Hatta, E., Maruyama, R., Marshall, S.J., Imamura, M., Levi, R., 1999. Bradykinin promotes ischemic norepinephrine release in guinea pig and human hearts. J. Pharmacol. Exp. Ther. 288, 919–927.

Hirsch, A.T., Talsness, C.E., Schunkert, H., Paul, M., Dzau, V.J., 1991. Tissue-specific activation of cardiac angiotensin converting enzyme in experimental heart failure. Circ. Res. 69 (2), 475–482.

Hwang, J.J., Curthoys, N.P., 1991. Effect of acute alterations in acid–base balance on rat renal glutaminase and phosphoenolpyruvate carboxykinase gene expression. J. Biol. Chem. 266, 9236–9292.

Karmazyn, M., 1988. Amiloride enhances postischemic ventricular recovery: possible role of Na^+–H^+ exchange. Am. J. Physiol. 255, H608–H615.

Karmazyn, M., 1996. Role of sodium–hydrogen exchange in mediating myocardial ischemic and reperfusion injury. Mechanisms and therapeutic implications. In: Fliegel, L. (Ed.), The Na^+/H^+ Exchanger. Springer R.G. Landes, Austin, TX, pp. 189–215.

Kim, D., Cragoe, E.J., Smith, T.W., 1987. Relations among sodium pump inhibition, Na–Ca and Na–H exchange activities and Ca–H interactions in cultured chick heart cells. Circ. Res. 60, 185–193.

Kimura, E., Hashimoto, K., Furakawa, S., Hayakawa, H., 1973. Changes in bradykinin level in coronary sinus blood after experimental occlusion of a coronary artery. Am. Heart J. 85, 635–647.

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

Linz, W., Wiemer, G., Gohlke, P., Unger, Th., Schölkens, B.A., 1995. Contribution of kinins to the cardiovascular actions of angiotensin-converting enzyme inhibitors. Pharmacol. Rev. 47, 25–49.

Loke, K.E., Curran, C.M.L., Messina, E.J., Laycock, S.K., Shesely, E.G., Carretero, O.A., Hintze, T.H., 1999. Role of nitric oxide in the control of cardiac oxygen consumption in B_2-kinin receptor knockout mice. Hypertension 34, 563–567.

Marino, C.R., Jeanes, V., Boron, W.R., Schmitt, B.M., 1999. Expression and distribution of Na^+–HCO_3^- cotransporter in human pancreas. Am. J. Physiol. 277 (2 Pt. 1), G487–G494.

Matsuki, T., Shoji, T., Yoshida, S., Kudoh, Y., Motoe, M., Inoue, M., Nakata, T., Hosoda, S., Shimamoto, K., Yellon, D., Iimura, O., 1987. Sympathetically induced myocardial ischemia causes the heart to release plasma kinin. Cardiovasc. Res. 21, 428–432.

Meng, H.P., Lonsberry, B.B., Pierce, G.N., 1991. Influence of perfusate pH on the postischemic recovery of cardiac contractile function: involvement of sodium–hydrogen exchange. J. Pharmacol. Exp. Ther. 258, 772–777.

Momomura, S.I., Ingwall, J.S., Parker, J.A., Sahagian, P., Ferguson, J.J., Grossman, W., 1985. The relationship of high energy phosphates, tissue pH and regional blood flow to diastolic distensibility in the ischemic dog myocardium. Circ. Res. 57, 822–835.

Nolly, H., Carbini, L.A., Scicli, G., Carretero, O.A., Scicli, A.G., 1994. A local kallikrein–kinin system is present in rat hearts. Hypertension 23, 919–923.

Nsa Allogho, S., Gobeil, F., Pheng, L.H., Nguyen-Le, X.K., Neugebauer, W., Regoli, D., 1995. Kinin B_1 and B_2 receptors in the mouse. Can. J. Physiol. Pharm. 73, 1764–1859.

Orchard, C.H., Kentish, J.C., 1990. Effects of changes of pH on the contractile function of cardiac muscle. Am. J. Physiol. 258, C967–C981.

Opie, L.H., 1998. The Heart. Physiology, from Cell to Circulation, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Pan, H.-L., Chen, S.-R., Scicli, G.M., Carretero, O.A., 2000. Cardiac interstitial bradykinin release during ischemia is enhanced by ischemic preconditioning. Am. J. Physiol. 279, H116–H121.

Passier, R.C.J.J., Smits, J.F.M., Verluyten, M.J.A., Daemen, M.J.A.P., 1996. Expression and localisation of renin and angiotensinogen in rat heart after myocardial infarction. Am. J. Physiol. 271, H1040–H1048.

Petrecca, K., Atanasiu, R., Grinstein, S., Orlowski, J., Shrier, A., 1999. Subcellular localization of the Na^+/H^+ exchanger NHE1 in rat myocardium. Am. J. Physiol. 276, H709–H717.

Regoli, D., Barabe, J., 1980. Pharmacology of bradykinin and related kinins. Pharmacol. Rev. 32, 1–46.

Sandmann, S., Spitznagel, H., Chung, O., Xia, Q.G., Illner, S., Jänichen, G., Rossius, B., Daemen, M.J.A.P., Unger, Th., 1998. Effects of the calcium channel antagonist mibefradil on haemodynamic and morphological parameters in myocardial infarction-induced cardiac failure in rats. Cardiovasc. Res. 39, 339–350.

Sandmann, S., Bohle, R.M., Dreyer, Th., Unger, Th., 2000. The T-type calcium channel blocker mibefradil reduced interstitial and perivascular fibrosis and improved haemodynamic parameters in myocardial infarction-induced cardiac failure in rats. Virchows Arch. 436 (2), 147–157.

Sandmann, S., Yu, M., Unger, Th., 2001a. Transcriptional and translational regulation of calpain in the rat heart after myocardial infarction—effects of AT(1) and AT(2) receptor antagonists and ACE inhibitor. Br. J. Pharmacol. 132 (3), 767–777.

Sandmann, S., Yu, M., Kaschina, E., Blume, A., Bouzinova, E., Aalkjaer, C., Unger, Th., 2001b. Differential effects of angiotensin AT_1 and AT_2 receptors on the expression, translation and function of the Na^+–H^+ exchanger and Na^+–HCO_3^- symporter in the rat heart after myocardial infarction. J. Am. Coll. Cardiol. 37 (8), 2154–2165.

Sandmann, S., Claas, R., Cleutjens, J.P., Daemen, M.J., Unger, Th., 2001c. Calcium channel blockade limits cardiac remodeling and improves cardiac function in myocardial infarction-induced heart failure in rats. J. Cardiovasc. Pharmacol. 37 (1), 64–77.

Schölkens, B.A., Linz, W., 1992. Bradykinin-mediated metabolic effects in isolated perfused rat hearts. Agents Actions Suppl. 38, 36–42.

Schölkens, B.A., Linz, W., König, W., 1988. Effects of the angiotensin converting enzyme inhibitor, ramipril, in isolated ischemic rat heart are abolished by a bradykinin antagonist. J. Hypertens. 6 (4), S25–S28.

Seifter, J.L., Aronson, P.S., 1986. Properties and physiological roles of plasma membrane sodium–hydrogen exchanger. J. Clin. Invest. 78, 859–864.

Seyedi, N., Win, T., Lander, H.M., Levi, R., 1997. Bradykinin B2 receptor activation augments norepinephrine exocytosis from cardiac sympathetic nerve endings: mediation by autocrine/paracrine mechanisms. Circ. Res. 81, 774–784.

Siffert, W., Akkerman, J.W.N., 1989. Na^+/H^+ exchange and Ca^{2+} influx. FEBS 259, 1–4.

Spitznagel, H., Chung, O., Xia, Q.G., Rossius, B., Illner, S., Jähnichen, G., Sandmann, S., Reinecke, A., Daemen, M.J.A.P., Unger, Th., 2000. Cardioprotective effects of the Na^+/H^+-exchanger inhibitor cardiporide in infarct-induced heart failure. Cardiovasc. Res. 46, 102–110.

Studer, R., Reinecke, H., Bilger, J., Eschenhagen, T., Böhm, M., Hasenfuß, G., Just, H., Holtz, J., Drexler, H., 1994. Gene expression of the cardiac Na^+–Ca^{2+} exchanger in end-stage human heart failure. Circ. Res. 75, 43–453.

Su, J.B., Hoüel, R., Heloire, F., Barbe, F., Beverelli, F., Sambin, L., Castaigne, A., Berdeaux, A., Crozatier, B., Hittinger, L., 2000. Stimulation of bradykinin B_1 receptors induces vasodilation in conductance and

resistance coronary vessels in conscious dogs. Comparison with B_2 receptor stimulation. Circulation 101, 1848–1853.

Terzic, A., Puceat, M., Clement, O., Scamps, F., Vassort, G., 1992. Alpha1-adrenergic effects on intracellular pH and calcium and on myofilaments in single rat cardiac cells. J. Physiol. 447, 275–292.

Thomas, R.C., 1989. Cell growth factors, bicarbonate and pH_i response. Nature 337, 601.

Tiffany, C.W., Burch, R.M., 1989. Bradykinin stimulates tumor necrosis factor and interleukin-1 release from macrophages. FEBS Lett. 247, 189–192.

Toda, H., Thompson, C.I., Recchia, F.A., Loke, K.E., Ochoa, M., Smith, C.J., Shesely, E.G., Kaley, G., Hintze, T.H., 2000. Myocardial glucose uptake is regulated by nitric oxide via endothelial nitric oxide synthase in Langendorff mouse heart. Circ. Res. 86, 270–274.

Tschope, C., Koch, M., Spillmann, F., Heringer-Walther, S., Mochmann, H.C., Stauss, H., Bader, M., Unger, T., Schultheiss, H.P., Walther, T., 1999. Upregulation of the cardiac bradykinin B2 receptors after myocardial infarction. Immunopharmacology 44 (1–2), 111–117.

Tschope, C., Heringer-Walther, S., Koch, M., Spillmann, F., Wendorf, M., Leitner, E., Schultheiss, H.P., Walther, T., 2000. Upregulation of bradykinin B1-receptor expression after myocardial infarction. Br. J. Pharmacol. 129 (8), 1537–1538.

Yasutake, M., Haworth, R.S., King, A., Avkiran, M., 1996. Thrombin activates the sarcolemmal Na^+–H^+ exchanger. Evidence for a receptor-mediated mechanism involving protein kinase C. Circ. Res. 79 (4), 705–715.

Zhang, X., Xie, Y.-W., Nasjletti, A., Xu, X., Wolin, M.S., Hintze, T.H., 1997. ACE inhibitors promote nitric oxide accumulation to modulate oxygen consumption. Circulation 95, 176–182.

Zhang, X., Recchia, F.A., Bernstein, R., Xu, X., Nasjletti, A., Hintze, T.H., 1999. Kinin-mediated coronary nitric oxide production contributes to the therapeutic action of angiotensin-converting enzyme and neutral endopeptidase inhibitors and amlodipine in the treatment of heart failure. J. Pharmacol. Exp. Ther. 288, 742–751.