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Extracellular pH changes activate the p38-MAPK signalling pathway in the amphibian heart

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Summary

We investigated the activation of the p38-MAPK signalling pathway during extracellular pH changes in the isolated perfused amphibian heart. Extracellular alkalosis (pH 8.5 or 9.5) maximally activated p38-MAPK within 2 min (4.17- and 3.20-fold, respectively) and this effect was reversible since the kinase phosphorylation levels decreased upon reperfusing the heart with normal Tris–Tyrode's buffer. Extracellular acidosis also activated p38-MAPK moderately, but persistently (1.65-fold, at 1 min and 1.91-fold, at 60 min). The alkalosis-induced p38-MAPK activation depended upon the Na^+/H^+ exchanger (NHE) and Na^+/K^+-ATPase, because it was abolished when the NHE inhibitors amiloride and HOE642 and the Na^+/K^+-ATPase inhibitor, ouabain, were used. Our studies also showed that extracellular alkalosis (pH 8.5) induced MAPKAPK2 phosphorylation (2.59-fold, 2 min) and HSP27 phosphorylation (5.33-fold, 2 min) in a p38-MAPK-dependent manner, as it was inhibited with 1 μmol l^{-1} SB203580. Furthermore, immunohistochemical studies of the phosphorylated forms of p38-MAPK and HSP27 revealed that these proteins were localised in the perinuclear region and dispersedly in the cytoplasm of ventricular cells during alkalosis. Finally, alkalosis induced the increase of HSP70 protein levels (1.52-fold, 5 min), but independently of p38-MAPK activation. These data indicate that the p38-MAPK signalling pathway is activated by extracellular pH changes and in the case of alkalosis this activation may have a protective role.

Key words: alkalosis, acidosis, p38-MAPK, HSP27, amphibian heart, *Rana ridibunda*, signal transduction, cellular signalling.

Introduction

Intracellular pH is a very important physiological parameter since it affects proper protein folding and, therefore, enzyme activity and cellular processes, such as protein synthesis (Roos and Boron, 1981; Fuller et al., 1989; Gaitanaki et al., 1990). In mammals, intracellular acidosis affects cardiac contractile function (Jeffrey et al., 1987) and is closely related to ischemia–anoxia or ischemia/re-oxygenation and the cell death that these phenomena cause (Webster et al., 1999; Kubasiak et al., 2002; Lagadic-Gossmann et al., 2004). By contrast, alkalosis has been reported to be induced during the action of peptide hormones and growth factors (Klip et al., 1986; Ives and Daniel, 1987) and is correlated with increases in protein synthesis rates (Fuller et al., 1989; Gaitanaki et al., 1990), transport of proteins to certain organelles (Khaled et al., 1999) and programmed cell death (Lagadic-Gossmann et al., 2004).

The above-mentioned physiological changes can be affected through the process of signal transduction. Among the most important signalling pathways are those involving the mitogen activated protein kinases (MAPKs). This kinase family is divided into four subfamilies: the ERKs, the JNKs, the p38-MAPK and the BMK1/ERK5 (for a review, see Kyriakis and Avruch, 2001). p38-MAPK is activated by dual phosphorylation at the Thr and Tyr residues of the Thr-Gly-Tyr motif which lies at the activation loop of the molecule (Davis, 1994). The p38-MAPK activation can be induced by cytokines (Freshney et al., 1994; Goedert et al., 1997), or oxidative (Clerk et al., 1998; Gaitanaki et al., 2003) and mechanical (Aggeli et al., 2001b; Aikawa et al., 2001) stresses.

The substrates of p38-MAPK are cytoplasmic proteins and other kinases such as the MAPK-activated protein kinases 2 and 3 (MAPKAPK2 and 3) or transcription factors (Stokoe et al., 1992; Rouse et al., 1994; Han et al., 1997; Thuerauf et al., 1998). In addition, known substrates for p38-MAPK are members of the heat shock family, such as HSP27, which are part of the protein folding machinery of the cell, helping proteins to acquire their proper conformation and maintain this under stressful conditions (Snoeckx et al., 2001; Sreedhar and Csermely, 2004). For the small heat shock protein HSP27 a growing body of evidence supports its participation in the p38-MAPK signalling pathway (Stokoe et al., 1992; Rouse et al., 1994; Gaitanaki et al., 2003) and its contribution to stabilising the cytoskeleton and

protecting the cells against damage (Huot et al., 1996; Concannon et al., 2003; Sreedhar and Csermely, 2004).

p38-MAPK signalling pathway has been extensively studied in the mammalian heart since it is implicated in hypertrophy (Wang et al., 1998) and the negative effects of ischemia/reperfusion (Yin et al., 1997; Kaiser et al., 2004). However, it is interesting to study this pathway in other vertebrate systems, which differ structurally and physiologically from mammals. Such a system is the amphibian heart, and recent studies in our laboratory have shown that amphibian heart p38-MAPK is activated by α_1- and β-adrenergic agonists, hyperosmotic, mechanical and oxidative stress (Aggeli et al., 2001a; Aggeli et al., 2001b; Aggeli et al., 2002a; Aggeli et al., 2002b; Gaitanaki et al., 2003). Contrary to the mammalian heart, frog heart p38-MAPK is not activated by anoxia or anoxia/re-oxygenation (Aggeli et al., 2001a) as amphibians frequently encounter hypoxic environmental conditions and therefore they have adapted.

In the present study we investigated the time-dependent activation of the amphibian heart p38-MAPK pathway during changes in the extracellular pH value. We used two pH values for alkalosis, a moderate one (8.5) and an extreme one (9.5), and a pH value for acidosis (6.5) that is not very severe for the frog cells. We also investigated the effect of the p38-MAPK specific inhibitor SB203580 and the impact of inhibiting Na^+/H^+ exchanger (NHE) (by using amiloride and HOE642) and Na^+/K^+-ATPase (by using ouabain) on the alkalosis-induced phosphorylation of p38-MAPK. What is more, we examined the effects of alkalosis on the MAPKAPK2 and HSP27 phosphorylation and the protein levels of the chaperone HSP70 and investigated whether any of these changes are p38-MAPK dependent.

Our results provide the first evidence that the amphibian heart p38-MAPK signalling pathway is activated by changes in the extracellular pH and that this activation seems to be protective for the cardiac cells.

Materials and methods

Materials

Most biochemicals used were from Applichem GmbH (Ottoweg 10b, D-64291 Darmstandt, Germany). The enhanced chemiluminescence kit was from Amersham International (Uppsala, Sweden) and the alkaline phosphatase Kwik kit was from Lipshaw (Pittsburgh, PA, USA). Bradford protein assay reagent was from Bio-Rad (Hercules, CA, USA). Nitrocellulose (0.45 μm) was purchased from Schleider & Schuell (Keene, NH, USA). Isopentane (#1.06056) was from Merck (64271 Darmstandt, Germany). The p38-MAPK inhibitor SB203580 was obtained from Alexis Corporation (CH-4415 Lausen, Switzerland) and stock solutions (10 mmol l^{-1}) were prepared in dimethyl sulfoxide (DMSO). Ouabain (O 3125; a Na^+/K^+-ATPase inhibitor) and amiloride hydrochloride hydrate (A 7410; a NHE1 inhibitor) were purchased from Sigma Chemical Co (St Louis, MO, USA). HOE642, a NHE1 inhibitor, was kindly provided by Aventis Pharma Deutschland GmbH. Stock solutions of amiloride (75 mmol l^{-1}) and HOE642 (50 mmol l^{-1}) were prepared in DMSO, whereas ouabain was directly diluted in the perfusion buffer.

Rabbit polyclonal antibodies specific for the total and the dually phosphorylated p38-MAPK (#9212 and #9211, respectively), total and phosphorylated (Thr 334) MAPKAPK2 (#3042 and #3041, respectively), phosphorylated (Ser 82) HSP27 (#2401) and total HSP70 (#4872) were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-actin antibody (A2103) was from Sigma Chemical Co. Pre-stained molecular mass markers were from New England Biolabs (P7708S; Ipswich, MA, USA). Biotinylated anti-rabbit antibody was from DAKO A/S (Glostrup, Denmark). X-OMAT AR 13 cm×18 cm and Elite chrome 100 film were purchased from Eastman Kodak Company (New York, NY, USA).

Animals

Frogs (*Rana ridibunda* Pallas*)* weighing 120–150 g were caught in the vicinity of Thessaloniki, Greece and supplied by a local dealer. They were kept in containers in fresh water and received humane care in accordance to the Guidelines for the Care and Use of Laboratory Animals published by the Greek Government (160/1991) based on EC regulation (86/609).

Heart perfusions

Hearts were perfused with the non-recirculating Langendorff mode at a pressure of 4.5 kPa (31.5 mmHg) and at 25°C using a water-jacketed apparatus. Perfusions were conducted with two different Tyrode's solutions with different pH values. For perfusing hearts under alkaline conditions (pH 8.5 or 9.5) a Tris–Tyrode's buffer consisting of 10 mmol l^{-1} Tris, 140 mmol l^{-1} NaCl, 6 mmol l^{-1} KCl, 1 mmol l^{-1} $MgCl_2$, 1.8 mmol l^{-1} $CaCl_2$ and 25 mmol l^{-1} glucose was used. Perfusions under acidic conditions (pH 6.5) were conducted with a MES–Tyrode's buffer which contained 10 mmol l^{-1} MES, 120 mmol l^{-1} NaCl, 6 mmol l^{-1} KCl, 1 mmol l^{-1} $MgCl_2$, 1.8 mmol l^{-1} $CaCl_2$ and 25 mmol l^{-1} glucose. pH was adjusted to 6.5 with NaOH and the appropriate quantity of NaCl was added for a final concentration of 140 mmol l^{-1}. Both buffers were gassed throughout the experiments with 100% O_2. Hearts were first equilibrated with the corresponding solutions at normal pH (7.35) for 30 min and then perfused at the specified conditions for periods of time ranging from 1 min to 60 min.

In addition, hearts perfused for 15 min with 0.5 mol l^{-1} sorbitol (in either of the above-mentioned solutions with a physiological pH of 7.35) after the equilibration period were used as positive controls (Aggeli et al., 2001a). Perfusions were also conducted in the presence of different ion channel inhibitors (100 μmol l^{-1} amiloride, 5 μmol l^{-1} HOE642 or 100 μmol l^{-1} ouabain) or in the presence of the specific p38-MAPK inhibitor SB203580 (1 μmol l^{-1}), during both the equilibration period and the perfusion with an alkaline (pH 8.5) Tyrode's buffer for 2 min.

At the end of the perfusions, atria were removed and ventricles, after being immersed in liquid N_2, were pulverized under liquid N_2. Powders were stored at –80°C.

Tissue extractions

Heart powders were homogenized with 3 ml g^{-1} of buffer [10 mmol l^{-1} Hepes, pH 7.9, 10 mmol l^{-1} KCl, 0.1 mmol l^{-1} EGTA, 0.1 mmol l^{-1} EDTA, 1.5 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} NaF, 1 mmol l^{-1} Na_3VO_4, 20 mmol l^{-1} β-glycerophosphate, 0.5 mmol l^{-1} phenyl methyl sulfonyl fluoride (PMSF), 1 mmol l^{-1} dithithreitol (DTT), 4 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin] and extracted on ice for 30 min. The samples were centrifuged (10 000 ***g***, 10 min, 4°C) and the supernatants boiled with 0.33 volumes of sodium dodecyl sulphate–polyacrylamide electrophoresis (SDS–PAGE) sample buffer [0.33 mol l^{-1} Tris–HCl, pH 6.8, 10% (w/v) SDS, 13% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, 0.2% (w/v) bromophenol blue]. Protein concentrations were determined using the Bio-Rad Bradford assay.

SDS–PAGE and immunoblot analysis

Proteins were separated by SDS–PAGE on 10% (w/v) or 15% (w/v) acrylamide, 0.275% (w/v) bis-acrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 μm). Membranes were then incubated in TBS-T [20 mmol l^{-1} Tris–HCl, pH 7.6, 137 mmol l^{-1} NaCl, 0.05% (v/v) Tween 20] containing 1% (w/v) bovine serum albumin (BSA) for 30 min at room temperature. The membranes were subsequently incubated with the appropriate primary antibody according to the manufacturer's instructions. After washing in TBS-T (4×5 min), membranes were incubated with horseradish peroxidase-conjugated secondary antibody [1:5000 dilution in TBS-T containing 1% (w/v) BSA; 1 h at room temperature]. The blots were then washed again in TBS-T (4×5 min), and the bands were detected using the enhanced chemiluminescence (ECL) reaction with exposure to X-OMAT AR films. Blots were quantified by laser scanning densitometry.

Immunolocalisation of phospho-p38-MAPK and phospho-HSP27

At the end of the perfusion atria were removed and ventricles were immersed in isopentane pre-cooled in liquid N_2, and stored at –80°C. Tissues were sectioned (5–6 μm thickness) using a cryostat, fixed with ice-cold acetone for 10 min and stored at –30°C until use. Tissue sections were first washed in TBS-T [containing 0.1% (v/v) Tween 20] and then incubated with 3% (w/v) BSA in TBS-T for 1 h at room temperature. Subsequently, sections were incubated overnight with the primary antibodies specific for phospho-p38-MAPK and phospho-HSP27 (1:200 dilution in 3% (w/v) BSA in TBS-T) at 4°C, according to the method previously described (Aggeli et al., 2002a). All sections were immunostained by the alkaline phosphatase method using a Kwik kit according to the manufacturer's instructions. The alkaline phosphatase label was visualized by exposing the sections to Fast Red chromogen, and nuclei were counterstained with Haematoxylin. Slides were mounted, examined with a Zeiss Axioplan microscope and photographed with a Kodak Elite chrome 100 film.

Statistical analysis

All data are presented as means ± s.e.m. Comparisons between control and treatments were performed using the unpaired Student's *t*-test. A value of $P<0.05$ was considered to be statistically significant. Kinase and HSP27 phosphorylation levels or HSP70 protein levels were normalised against total protein levels and in 'control' hearts were set at 1, whereas the stimulated kinase and HSP27 phosphorylation or HSP70 protein levels in treated hearts were expressed as 'fold' activation or increase over control hearts.

Results

As the first step in the present study we examined the phosphorylation (hence activation) of p38-MAPK induced by alkalosis in the isolated amphibian heart. It is widely known that p38-MAPK is activated by dual phosphorylation of Thr and Tyr residues within the Thr-Gly-Tyr motif (Kyriakis and Avruch, 2001) and this can be monitored by immunoblot analysis using a specific antibody that recognizes this dually phosphorylated form of the kinase.

Perfusion of the amphibian heart with Tyrode's buffers of different alkaline pH values, for increasing periods varying from 1 up to 60 min, resulted in a strong but transient increase of p38-MAPK phosphorylation levels. In particular, pH 8.5, induced a rapid and intense p38-MAPK phosphorylation (within 1 min), with a maximum value attained within 2 min (~4.17±0.89-fold relative to control hearts perfused with Tris–Tyrode's buffer pH 7.35, $P<0.05$) and progressively declined thereafter, reaching control values at 60 min of treatment (Fig. 1A top, B). As a positive control, extract from hearts perfused for 15 min with 0.5 mol l^{-1} sorbitol diluted in the corresponding normal Tris–Tyrode's solution (pH 7.35) was used (Fig. 1A top, B). In addition, reperfusing the heart following the maximal activation time-point, for increasing periods (varying from 2 up to 60 min), showed that this p38-MAPK activation was sustained for at least 5 min after the onset of the reperfusion, with a progressive decline thereafter, reaching control values within 30 min of reperfusion (Fig. 1C top, D). This result indicates that the phosphorylation of the kinase induced by alkalosis was reversible. Furthermore, SB203580 (1 μmol l^{-1}), a selective p38-MAPK inhibitor, abolished the alkalosis-induced phosphorylation of the kinase (Fig. 2A top, B). Equivalent protein loading was confirmed by probing identical samples with antibodies recognizing the total p38-MAPK (Fig. 1A bottom) or actin (Fig 1C bottom, Fig 2A bottom) protein levels.

Using a more alkaline Tris–Tyrode's perfusion buffer (pH 9.5), p38-MAPK was activated in a similar but less intense manner (maximum at 2 min, ~3.20±0.46-fold relative to control values, $P<0.05$) (Fig. 3A top, B).

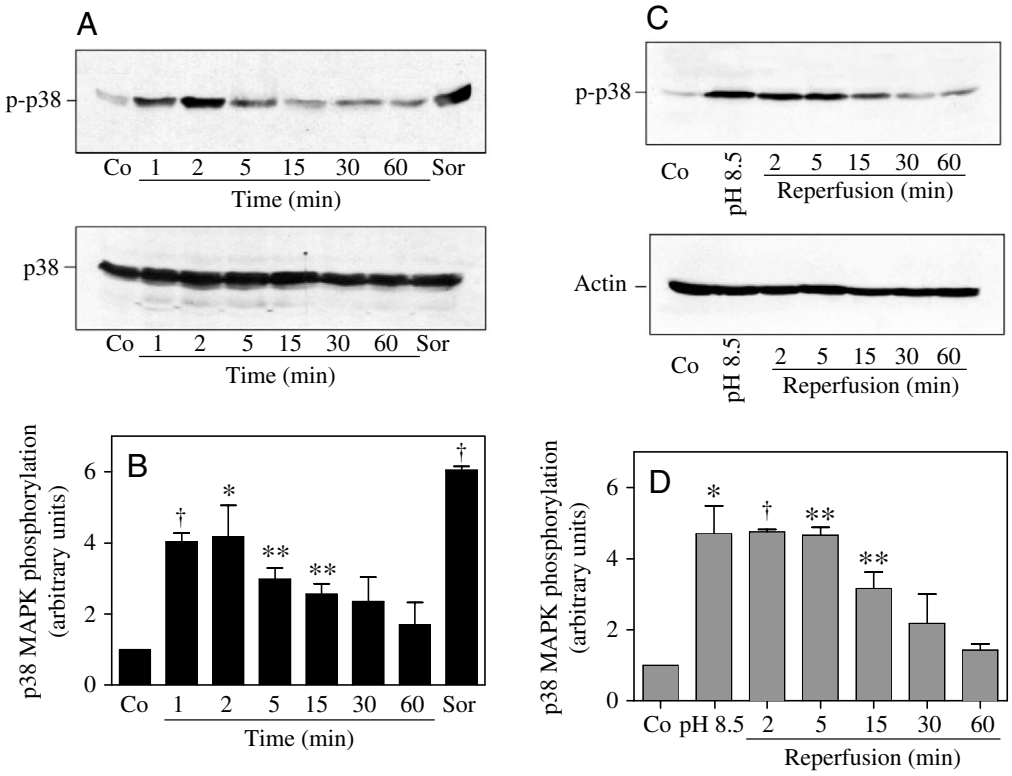

Fig. 1. Phosphorylation of p38-MAPK by extracellular alkalosis (pH 8.5). (A) Protein (50 μg) from *Rana ridibunda* hearts perfused without (Co) or with Tris–Tyrode's buffer (pH 8.5) for the times indicated was assessed by immunoblot analysis using a phosphospecific anti-p38-MAPK antibody (top) or total p38-MAPK antibody as a control for equal loading (bottom). Extract from hearts perfused with 0.5 mol l^{-1} sorbitol (Sor) for 15 min was used as a positive control. (C) Time course of p38-MAPK phosphorylation induced by reperfusing hearts subjected to extracellular alkalosis (pH 8.5, 2 min; top). Equal loading was assessed by blotting identical samples with an anti-actin-specific antibody (bottom). (B,D) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Values are means ± s.e.m. for three independent experiments performed with similar findings. **P*<0.05, ***P*<0.01, †*P*<0.001 *vs* control value.

By contrast, extracellular acidosis (pH 6.5) induced the p38-MAPK phosphorylation in a different time-dependent manner. The kinase was moderately activated, but this activation was prolonged (at 1 min: ~1.65±0.02-fold relative to control values, *P*<0.001; at 60 min: ~1.91±0.08-fold relative to control values, *P*<0.01) (Fig. 3C top, D). Extract from hearts perfused

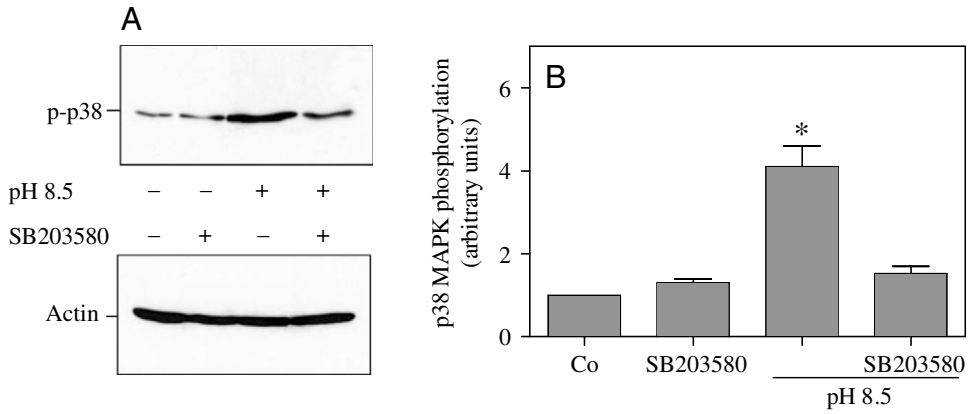

Fig. 2. Effect of the specific inhibitor SB203580 on the p38-MAPK phosphorylation induced by extracellular alkalosis (pH 8.5). (A) Protein (50 μg) from hearts perfused without (Co) or with an alkaline (pH 8.5) Tris–Tyrode's solution for 2 min in the absence (–) or presence (+) of 1 μmol l^{-1} SB203580 was assessed by immunoblot using phosphospecific anti-p38-MAPK (top) or anti-actin antibody (bottom). (B) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Results are means ± s.e.m. for three independent experiments. **P*<0.05 *vs* control value.

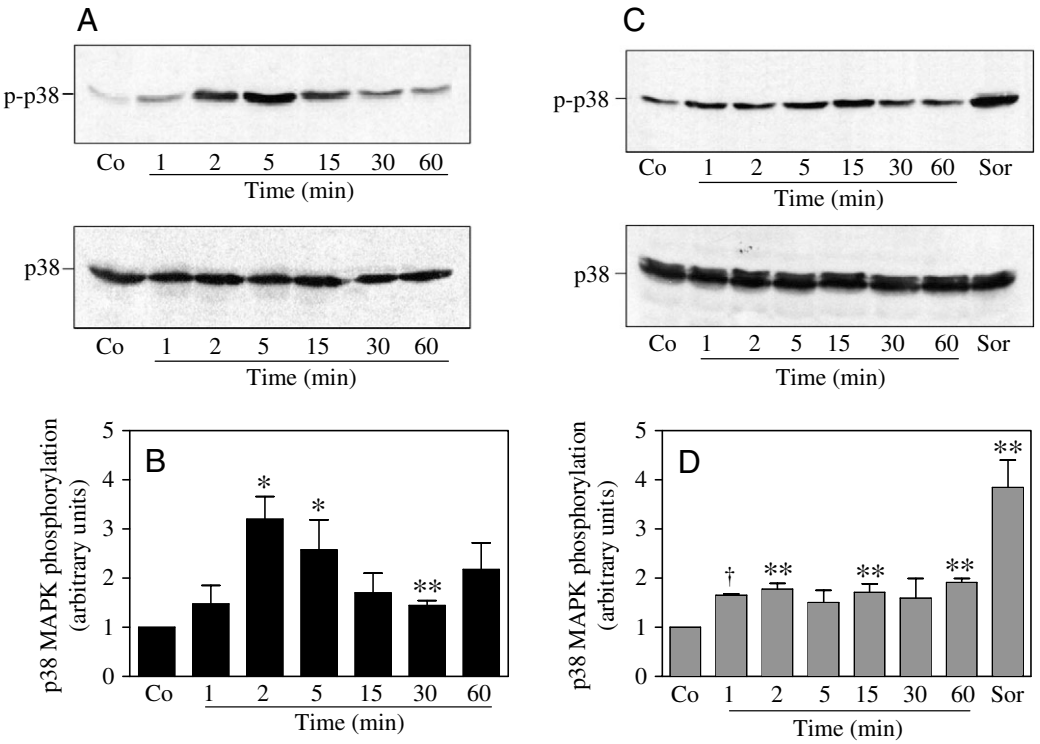

Fig. 3. Phosphorylation of p38-MAPK by more intense extracellular alkalosis (pH 9.5) (A top, B) or acidosis (pH 6.5) (C top, D). (A) Phospho-p38-MAPK was detected in extracts (50 μg of protein) from control hearts (Co) and hearts perfused with a Tris–Tyrode's perfusion buffer of pH 9.5 for the indicated times (top). Total p38-MAPK levels were detected in identical samples as a control for loading (bottom). (C) The p38-MAPK phosphorylation was also measured by immunoblot analysis in samples from hearts subjected to extracellular acidosis (pH 6.5) for increasing periods of time using the MES–Tyrode's perfusion buffer (top), as described in Materials and methods. Equal loading was assessed, as previously, using a p38-MAPK antibody (bottom). As a positive control, extract from hearts perfused with 0.5 mol l^{-1} sorbitol (Sor) for 15 min was used. (B,D) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Results are means ± s.e.m. for three independent experiments. *P<0.05, **P<0.01, $^{\dagger}P$<0.001 *vs* control value.

for 15 min with 0.5 mol l^{-1} sorbitol diluted in the corresponding normal MES–Tyrode's (pH 7.35) buffer was also used as a positive control. Total p38-MAPK levels of identical samples were detected so as to confirm the equivalent protein loading (Fig. 3C bottom).

We next investigated whether the alkalosis-induced activation of p38-MAPK depends on proteins, which are involved in the cellular pH regulation. Such proteins are NHE and Na^+/K^+-ATPase, which regulate pH directly or indirectly, respectively (Hoffmann and Simonsen, 1989; Bers et al., 2003). In order to assess their role in the p38-MAPK activation by extracellular alkalosis, we perfused amphibian hearts with normal and alkaline (pH 8.5) Tris–Tyrode's solutions in the presence of the following inhibitors: HOE642 (5 μmol l^{-1}) and amiloride (100 μmol l^{-1}), so as to inhibit the NHE, and ouabain (100 μmol l^{-1}) to inhibit the Na^+/K^+-ATPase. These results showed that all three inhibitors used alone induce a significant phosphorylation of the kinase (Fig. 4A top, B). However, when the apparent phosphorylation levels were subtracted from the ones obtained during extracellular alkalosis (pH 8.5, 2 min) in the presence of the inhibitors mentioned above, the net effect was that amiloride and HOE642 abolished the alkalosis-induced phosphorylation of the kinase, while the inhibition exerted by ouabain was partial (Fig. 4C). From the three inhibitors used, the most effective in down-regulating the alkalosis-induced p38-MAPK activation seemed to be HOE642 (Fig. 4C).

We also examined the phosphorylation state of two potent members of the p38-MAPK signalling pathway, MAPKAPK2 and HSP27, under alkaline conditions. MAPKAPK2 has been established as a direct substrate of p38-MAPK, whereas HSP27 is phosphorylated at up to three sites (Ser15, Ser78 and Ser82) by MAPKAPK2 and the related kinase MAPKAPK3. Therefore, we conducted an immunoblot analysis using antibodies specifically raised against the phosphorylated forms of MAPKAPK2 (Thr334) and HSP27 (Ser82). The results of these experiments revealed that extracellular alkalosis (pH 8.5) induced a rapid phosphorylation of MAPKAPK2, which was maximized within 2 min (~2.59±0.28-fold relative to control values, P<0.01) and declined thereafter, reaching control values within 30 min of treatment (Fig. 5A top). As a positive control, extract from hearts perfused with 0.5 mol l^{-1} sorbitol for 15 min was used (Fig. 5A top) and equal loading was assessed with an anti-MAPKAPK2 antibody (Fig. 5A bottom).

Extracellular alkalosis induced a significant increase in

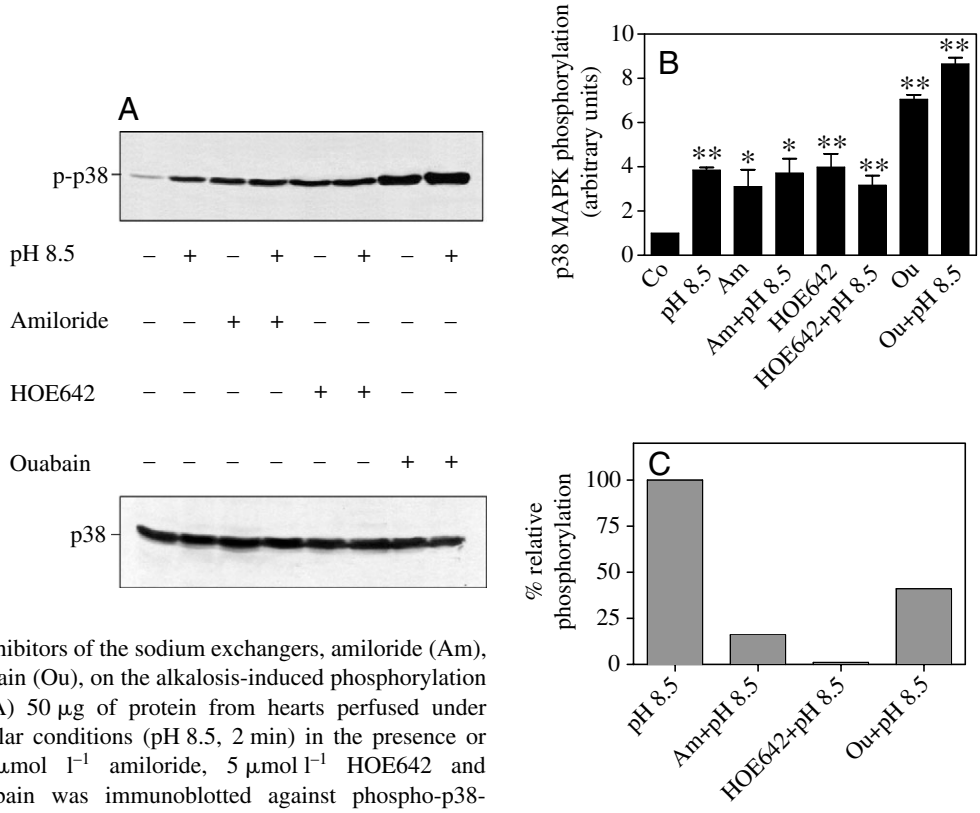

Fig. 4. Effect of inhibitors of the sodium exchangers, amiloride (Am), HOE642 and ouabain (Ou), on the alkalosis-induced phosphorylation of p38-MAPK. (A) 50 μg of protein from hearts perfused under alkaline extracellular conditions (pH 8.5, 2 min) in the presence or absence of 100 μmol l^{-1} amiloride, 5 μmol l^{-1} HOE642 and 100 μmol l^{-1} ouabain was immunoblotted against phospho-p38-MAPK (top) and total p38-MAPK (bottom) using the corresponding antibodies. (B) Densitometric analysis of phospho-p38-MAPK by laser scanning. Co, control. (C) Relation of the net p38-MAPK phosphorylation induced by each inhibitor in the presence of extracellular alkalosis to the alkalosis-induced p38-MAPK phosphorylation. Values are means ± s.e.m. for three independent experiments. **P*<0.05, ***P*<0.01 *vs* control value.

HSP27 phosphorylation levels within 2 min of perfusion (~5.33±0.81-fold relative to control values, $P<0.05$) (Fig. 6A top, B). Moreover, the selective p38-MAPK inhibitor SB203580 even at a low concentration (1 μmol l^{-1}) abolished this phosphorylation, revealing that HSP27 is indeed a member of the p38-MAPK pathway and lies downstream from the kinase (Fig. 6A top, B).

In order to investigate the localisation pattern of the activated p38-MAPK and HSP27 immunohistochemically under alkaline conditions, frog hearts were perfused with

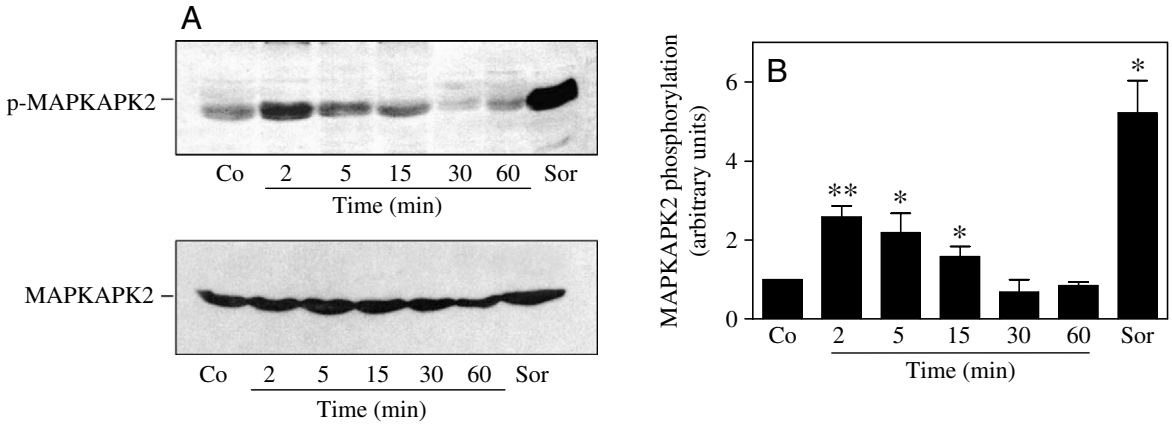

Fig. 5. (A) Extracts (100 μg of protein) from hearts perfused with an alkaline (pH 8.5) Tris–Tyrode's perfusion buffer for the indicated times were assayed for MAPKAPK2 phosphorylation through immunoblot analysis using an antibody specific for the phosphorylated form of MAPKAPK2 (top). Samples from hearts perfused with 0.5 mol l^{-1} sorbitol (Sor) for 15 min were used as positive control. Equal loading was assessed in identical samples using an antibody against total MAPKAPK2 (bottom). (B) Densitometric analysis of phospho-MAPKAPK2 bands by laser scanning. Results are means ± s.e.m. for three independent experiments. **P*<0.05, ***P*<0.01 *vs* control value.

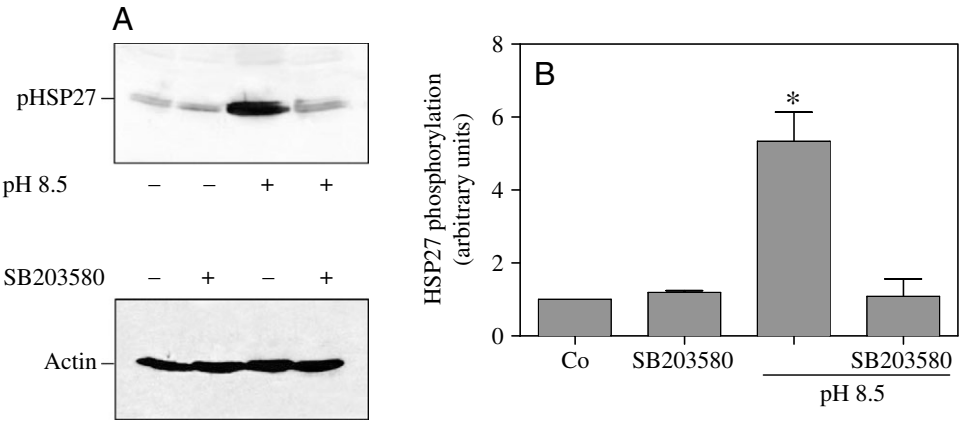

Fig. 6. Phosphorylation of HSP27 induced by extracellular alkalosis (pH 8.5) and effect of the p38-MAPK specific inhibitor SB203580. (A) Protein (100 μg) from hearts perfused without (Co) or with a Tris–Tyrode's buffer of pH 8.5 for 2 min in the presence or absence of 1 μmol l^{-1} SB203580 was used to perform western blot analysis with antibodies specific for the phosphorylated HSP27 (top) or actin (bottom). (B) Densitometric analysis of phospho-HSP27 by laser scanning. Values are means ± s.e.m. for three independent experiments. *P<0.05 *vs* control value.

Tris–Tyrode's buffers of pH 8.5 or 9.5. After the removal of atria, the ventricle was sectioned and the respective specimens were processed using antibodies specific for the phosphorylated forms of p38-MAPK and HSP27. In control hearts, slight immunoreactivity was observed for both of the proteins (Fig. 7A,D), corresponding to their basal levels,

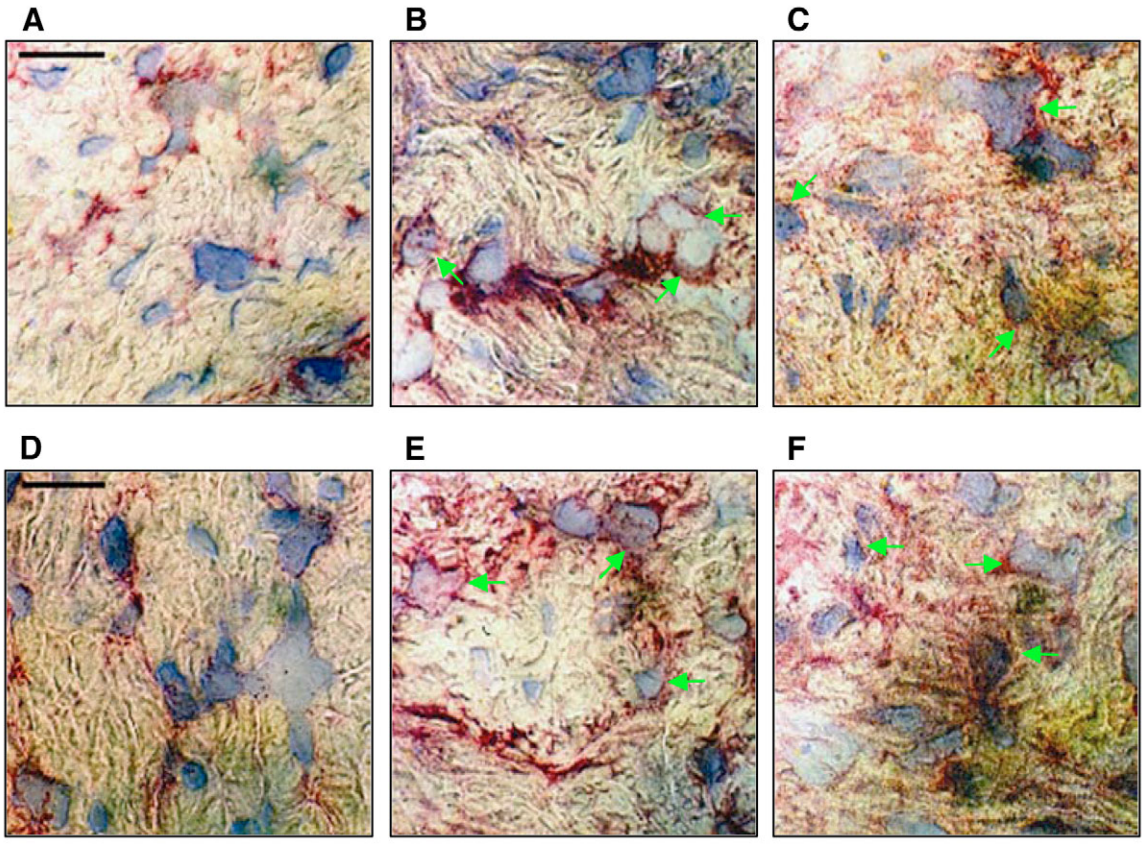

Fig. 7. Immunohistochemical localisation of phospho-p38-MAPK (A–C) and phospho-HSP27 (D–F) in the ventricle of isolated amphibian heart perfused with Tris–Tyrode's perfusion buffers of various pHs: a normal (control heart) (A,D) or alkaline [(B,E), pH 8.5 or (C,F), pH 9.5] for 2 min. Cryosections were incubated with a phosphospecific anti-p38-MAPK (1:200 dilution) or a phosphospecific anti-HSP27 (1:200 dilution) antibody and processed as described in Materials and methods. Immunolocalisation deposits were visualized with Fast Red chromogen. Representative photographs of three independent experiments are shown. Green arrows indicate the perinuclear localisation. Scale bars, 20 μm.

whereas no immunostaining was detected in specimens incubated either with the secondary antibody or with the chromogen alone (data not shown). However, in specimens perfused with either of the alkaline Tris–Tyrode's buffers strong immunoreactive staining for the phosphorylated p38-MAPK was observed within the cytoplasm as well as in the perinuclear region (green arrows; Fig. 7B,C). The anti-phospho-HSP27 antibody produced a similar pattern of phospho-HSP27 immunostaining, with immunocomplexes in the perinuclear region and also widely dispersed in the cytoplasm (Fig. 7E,F).

Furthermore, raising the extracellular pH caused a moderate and transient increase in the levels of HSP70, another heat shock protein, which participates in the protection of the cell against stress (Snoeckx et al., 2001). This increase was significant within the first 5 min of treatment (~1.52±0.18-fold relative to control values, $P<0.05$) and declined immediately thereafter (Fig. 8A,B). However, this induction in the HSP70 protein levels did not seem to be p38-MAPK dependent, as treatment with SB203580 did not influence this response (Fig. 8C).

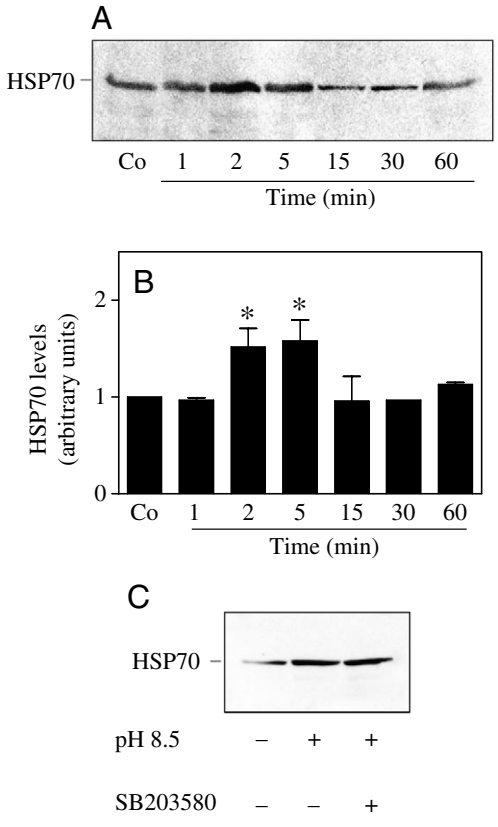

Fig. 8. Measurement of HSP70 protein levels by immunoblot analysis under extracellular alkaline (pH 8.5) conditions. (A) Protein (50 μg) from hearts perfused with a Tris–Tyrode's solution of pH 8.5 for the indicated times was immunoassayed with an anti-HSP70 antibody. Co, control. (B) Densitometric analysis of HSP70 bands by laser scanning. Results are means ± s.e.m. for three independent experiments. *$P<0.05$ *vs* control value. (C) Effect of the p38-MAPK specific inhibitor SB203580 (1 μmol l^{-1}) on the protein levels of HSP70 in the presence of extracellular alkalosis.

Discussion

Ectotherms, like amphibians, are subjected to greater environmental pressures than endotherms as a result of their distinct physiology. Among the physiological parameters that are affected by fluctuations in environmental conditions, such as temperature, osmolarity and oxygen availability is the intracellular pH. Since pH is important for normal cellular function, it is of great importance for cell survival. In particular, in cardiac myocytes, the mobility of H^+ is low, and it could be suggested that significant intracellular pH gradients can arise when a local pH disturbance occurs in these cells (Swietach and Vaughan-Jones, 2005a; Swietach and Vaughan-Jones, 2005b). The development of such gradients may have important implications for pH-sensitive processes within ventricular myocytes, such as contraction (Jeffrey et al., 1987).

In the mammalian and amphibian heart, stressful conditions can activate intracellular signalling pathways including that of p38-MAPK (Clerk et al., 1998; Aikawa et al., 2001; Gaitanaki et al., 2003). However, its role in cell survival is contradictory; depending on the system and the stimulus it is either protective or apoptotic (Wada and Penninger, 2004).

In the present study we investigated the effect of extracellular pH changes on the activation of the p38-MAPK signalling pathway in the heart of the amphibian *Rana ridibunda*. Previous studies in the mammalian heart have shown that extracellular pH changes by 1.0 unit cause respective alterations in the intracellular pH values by approximately 0.25 units (Fuller et al., 1989; Zheng et al., 2005). Even though extracellular pH changes of such magnitude are unlikely to occur *in vivo*, with the exception of some pathological states, we used them in the aforementioned context i.e. to induce changes in the intracellular pH of the amphibian cardiac cells.

Our results indicate that p38-MAPK activation in the amphibian heart follows a different pattern according to the type of extracellular pH change. Acidosis at pH 6.5 caused a moderate but prolonged phosphorylation of the kinase (Fig. 3), whereas alkalosis-induced p38-MAPK activation was intense and transient (Figs 1, 3). What is more, p38-MAPK phosphorylation was greater when we used mild (pH 8.5) rather than extreme (pH 9.5) alkalosis (Figs 1, 3) indicating that the stimulus intensity is also important for the kinase activation. The results for alkalosis were supported by immunolocalisation studies where the immunocomplexes of phospho-p38-MAPK were found both diffusely in the cytoplasm and in the perinuclear region (Fig. 7). The alkalosis-induced phosphorylation of p38-MAPK was reversible and this was shown with reperfusion experiments using a normal Tris–Tyrode's solution after perfusing hearts with an alkaline (pH 8.5) buffer for 2 min (Fig. 1). The fact that the kinase activation did not reach control values immediately, but only

after 30 min of reperfusion, can be attributed to the delayed restoration of the normal acid–base balance in the cell.

The apparent differences in the activation of p38-MAPK during acidosis and alkalosis reveal the specificity of the pathway in the amphibian heart; it is mainly activated by alkalosis and not acidosis. This result is significant physiologically since acidosis is closely related to hypoxia (Webster et al., 1999; Kubasiak et al., 2002), an environmental condition that amphibians frequently confront in nature and to which they have adapted. In support of this, previous studies in our laboratory showed that amphibian p38-MAPK was not activated by anoxia or anoxia/reoxygenation (Aggeli et al., 2001a). However, the moderate p38-MAPK activation induced by acidosis may indicate that the amphibian ventricular myocytes are in a 'stand-by' condition possibly prepared for a more severe stress stimulus. Alkalinization, on the other hand, is associated with the effects of α_1-adrenergic stimulation (Fuller et al., 1991) and hypertonic stress (Befroy et al., 1999), stimuli known to activate p38-MAPK in the amphibian heart (Aggeli et al., 2002a; Aggeli et al., 2002b).

In agreement with our results, extracellular acidosis at pH 6.5 also caused a moderate activation of the p38-MAPK in mammalian cardiomyocytes (Zheng et al., 2005). However, when these authors used a more severe extracellular acidosis (pH 5.5) p38-MAPK activation was more prominent. In addition, exposure of rat skeletal muscles to respiratory acidosis did not increase p38-MAPK phosphorylation (Wretman et al., 2001), whereas acidic extracellular medium activated the kinase in LLC-PK_1-$FBPase^+$ cells (Feifel et al., 2002), mouse melanoma cells (Kato et al., 2005) and Barrett's oesophageal adenocarcinoma cells (Sarosi, Jr et al., 2005).

As far as alkalosis is concerned, studies on p38-MAPK activation have shown that weak base-induced intracellular alkalinization in U937 cells (Shrode et al., 1997) and perfusion of rat hearts with alkaline Tris–Tyrode's buffer (C. Gaitanaki and I. Beis, unpublished data) activated the kinase. By contrast, experiments by Susa and Wakabayashi (Susa and Wakabayashi, 2003) using vascular smooth muscle cells showed that p38-MAPK was not activated by extracellular alkalosis. However, the maximum pH value used in the previous study was 7.9 and this might not be as intense as it is required for p38-MAPK activation. These results indicate that p38-MAPK activation by extracellular pH changes depends on the kind and severity of the stimulus and the cell type.

Moreover, the specificity of the alkalosis-induced p38-MAPK phosphorylation in our model is evident from the fact that it was abolished by SB203580, a specific kinase inhibitor (Fig. 2). SB203580 is known to react with the mammalian p38-MAPK alpha and beta isoforms (Kumar et al., 2003), indicating that the corresponding isoforms are the ones detected in the *R. ridibunda* heart. These isoforms have been shown to be either anti- or proapoptotic in the mammalian heart (Wang et al., 1998; Clerk et al., 2003; Kumar et al., 2003). Therefore, it was intriguing to investigate whether alkalosis-induced p38-MAPK activation is implicated or not in cell survival in our experimental model. For this reason, we tried to assess the effects of alkalosis on the heat shock proteins HSP27 and HSP70 that are generally known to be protective for the cardiac cell, and we also investigated whether any of these effects were p38-MAPK dependent.

The small heat shock protein HSP27 is a MAPKAPK2 substrate (Stokoe et al., 1992; Rouse et al., 1994) and is implicated in cytoprotection as it interacts with F-actin fibres and helps in cytoskeleton stabilization under stressful conditions (Huot et al., 1996). Furthermore, HSP27 prevents cytochrome *c* release from mitochondria and its integration in the apoptosome, thereby interfering with the mitochondrial apoptotic pathway (Bruey et al., 2000; Paul et al., 2002). In a previous study we showed that oxidative stress-induced MAPKAPK2 and HSP27 phosphorylation in the amphibian heart is p38-MAPK dependent (Gaitanaki et al., 2003). Similarly, alkalosis-induced activation of MAPKAPK2 followed the activation pattern of p38-MAPK (Fig. 5) and HSP27 phosphorylation increased immediately after perfusion of hearts with the alkaline Tyrode's buffer, a response that was abolished by the p38-MAPK-specific inhibitor SB203580 (Fig. 6). The previous result was in accordance with immunohistochemical studies (Fig. 7), which showed that the phosphorylated form of HSP27 was localised in the cytoplasm and the perinuclear region immediately after the extracellular alkalosis stimulus, a pattern similar to that observed for phospho-p38-MAPK. Therefore, alkalosis induces a direct pathway from p38-MAPK to MAPKAPK2 to HSP27, which might be protective for cardiac cells.

The other heat shock protein examined was HSP70. HSP70 comprises a family of molecular chaperones that are divided into constituent and inducible isoforms, all of which are important for cardioprotection under conditions of stress (Snoeckx et al., 2001). HSP70 family members function in the preservation of proper protein conformation and promote the degradation of abnormally folded proteins (Sreedhar and Csermely, 2004). In addition, they participate in the folding of the newly synthesized proteins and this might be significant in the case of alkalosis since previous studies have shown that this stimulus increases protein synthesis in the mammalian heart (Fuller et al., 1989; Fuller et al., 1991). In our system, alkalosis induced a slight, but significant, increase in HSP70 protein levels. This response was rapid and transient and it seemed to follow the p38-MAPK activation. However, SB203580, the specific p38-MAPK inhibitor, did not affect the increase in HSP70 protein levels induced by alkalosis (Fig. 8) and therefore this response was p38-MAPK independent.

We finally tried to examine whether the alkalosis-induced activation of p38-MAPK is affected by pH regulating mechanisms. Intracellular pH regulation is mainly exerted through channels such as the sarcollemal NHE, which exchanges intracellular H^+ for Na^+, and the Na^+/K^+-ATPase pump, which has an indirect impact on intracellular pH regulation as it affects NHE function by extruding the extra

Na^+ accumulated in the cell (Hoffmann and Simonsen, 1989; Bers et al., 2003). Inhibition of the basal activity of NHE with amiloride and HOE642 attenuated the alkalosis-induced activation of p38-MAPK, and inhibition of Na^+/K^+-ATPase with ouabain caused a partial inhibition (Fig. 4). The almost complete decrease in the alkalosis-induced p38-MAPK activation by NHE inhibition might be due to the local increase in intracellular H^+ concentration which counteracts the effects of alkalinization, whereas the partial inhibition effected by ouabain might be due to the indirect nature of pH regulation by Na^+/K^+-ATPase. The fact that all the inhibitors alone activated p38-MAPK (Fig. 4) could be attributed to the communication of the corresponding channels with signal transduction pathways as is the case for Na^+/K^+-ATPase (Kometiani et al., 1998; Xie, 2003).

In conclusion, extracellular pH changes differentially activate p38-MAPK in the amphibian heart. The diverse patterns of p38-MAPK activation by acidosis and alkalosis reflect the physiological adaptations of amphibians to their natural environment. In the case of alkalosis, protective cellular mechanisms, in the form of the molecular chaperones HSP27 and HSP70, are triggered. This protection can be assumed to have two aspects: protection of the existing proteins from misfolding caused by alterations in cellular pH and promotion of the nascent polypeptide chains' proper conformation, since alkalosis increases the protein synthesis rate (Fuller et al., 1989; Fuller et al., 1991). p38-MAPK is implicated in these cell survival mechanisms through the small heat shock protein HSP27 which lies downstream in the kinase signalling pathway.

List of abbreviations

DMSO	dimethylsulfoxide
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ERK	extracellular signal-regulated kinase
Hsp	heat shock protein
JNK	c-Jun *N*-terminal kinase
MAPK	mitogen-activated protein kinase
MAPKAPK	MAPK-activated protein kinase
NHE	Na^+/H^+ exchanger
p38-MAPK/RK	p38 reactivating kinase
PAGE	polyacrylamide gel electrophoresis
PMSF	phenyl methyl sulfonyl fluoride
TBS	Tris-buffered saline

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References

Aggeli, I.-K. S., Gaitanaki, C., Lazou, A. and Beis, I. (2001a). Activation of multiple MAPK pathways (ERKs, JNKs, p38-MAPK) by diverse stimuli in the amphibian heart. *Mol. Cell. Biochem.* **221**, 63-69.

Aggeli, I.-K. S., Gaitanaki, C., Lazou, A. and Beis, I. (2001b). Stimulation of multiple MAPK pathways by mechanical overload in the perfused amphibian heart. *Am. J. Physiol.* **281**, R1689-R1698.

Aggeli, I.-K. S., Gaitanaki, C., Lazou, A. and Beis, I. (2002a). Hyperosmotic and thermal stresses activate p38-MAPK in the perfused amphibian heart. *J. Exp. Biol.* **205**, 443-454.

Aggeli, I.-K. S., Gaitanaki, C., Lazou, A. and Beis, I. (2002b). α_1- and β-adrenoreceptor stimulation differentially activate p38-MAPK and atrial natriuretic peptide production in the perfused amphibian heart. *J. Exp. Biol.* **205**, 2387-2397.

Aikawa, R., Nagai, T., Tanaka, M., Zou, Y., Ishihara, T., Takano, H., Hasegawa, H., Akazawa, H., Mizukami, M., Nagai, R. et al. (2001). Reactive oxygen species in mechanical stress-induced cardiac hypertrophy. *Biochem. Biophys. Res. Commun.* **289**, 901-907.

Befroy, D. E., Powell, T., Radda, G. K. and Clarke, K. (1999). Osmotic shock: modulation of contractile function, pHi, and ischemic damage in perfused guinea pig heart. *Am. J. Physiol.* **276**, H1236-H1244.

Bers, D. M., Barry, W. H. and Despa, S. (2003). Intracellular Na^+ regulation in cardiac myocytes. *Cardiovasc. Res.* **57**, 897-912.

Bruey, J. M., Ducasse, C., Bonniaud, P., Ravagnan, L., Susin, S. A., Diaz-Latoud, C., Gurbuxani, S., Arrigo, A. P., Kroemer, G., Solary, E. et al. (2000). Hsp27 negatively regulates cell death by interacting with cytochrome *c*. *Nat. Cell Biol.* **2**, 645-652.

Clerk, A., Fuller, S. J., Michael, A. and Sugden, P. H. (1998). Stimulation of "stress-regulated" mitogen-activated protein kinases (Stress-activated protein kinases/c-Jun N-terminal Kinases and p38-Mitogen-activated protein kinases) in perfused rat hearts by oxidative and other stresses. *J. Biol. Chem.* **273**, 7228-7234.

Clerk, A., Cole, S. M., Cullingford, T. E., Harrison, J. G., Jormakka, M. and Valks, D. M. (2003). Regulation of cardiac myocyte cell death. *Pharmacol. Therapeut.* **97**, 223-261.

Concannon, C. G., Gorman, A. M. and Samall, A. (2003). On the role of Hsp27 in regulating apoptosis. *Apoptosis* **8**, 61-70.

Davis, R. J. (1994). MAPKs: new JNK expands the group. *Trends Biochem. Sci.* **19**, 470-473.

Feifel, E., Obexer, P., Andratsch, M., Euler, S., Taylor, L., Tang, A., Wei, Y., Schramek, H., Curthoys, N. M. and Gstraunthaler, G. (2002). p38 MAPK mediates acid-induced transcription of PEPCK in LLC-PK_1-$FBPase^+$ cells. *Am. J. Physiol.* **283**, F678-F688.

Freshney, N. W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J. and Saklatvala, J. (1994). Interleukin-1 activates a novel protein cascade that results in the phosphorylation of Hsp27. *Cell* **78**, 1039-1049.

Fuller, S. J., Gaitanaki, C. and Sugden, P. H. (1989). Effects of increasing extracellular pH on protein synthesis and protein degradation in the perfused working rat heart. *Biochem. J.* **259**, 173-179.

Fuller. S. J., Gaitanaki, C., Hatchett, R. J. and Sugden, P. H. (1991). Acute α_1-adrenergic stimulation of cardiac protein synthesis may involve increased pH and protein kinase activity. *Biochem. J.* **273**, 347-353.

Gaitanaki, C., Sugden, P. H. and Fuller, S. J. (1990). Stimulation of protein synthesis by raised extracellular pH in cardiac myocytes and perfused hearts. *FEBS Lett.* **260**, 42-44.

Gaitanaki, C., Stathopoulou, K., Stavridou, C. and Beis, I. (2003). Oxidative stress stimulates multiple MAPK signalling pathways and phosphorylation of the small HSP27 in the perfused amphibian heart. *J. Exp. Biol.* **206**, 2759-2769.

Goedert, M., Cuenda, A., Craxton, M., Jakes, R. and Cohen, P. (1997). Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases. *EMBO J.* **16**, 3563-3571.

Han, J., Jiang, Y., Li, Z., Kravchenco, V. V. and Ulevitch, R. J. (1997). Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. *Nature* **386**, 296-299.

Hoffmann, E. K. and Simonsen, L. O. (1989). Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiol. Rev.* **69**, 315-382.

Huot, J., Houle, F., Spitz, D. R. and Landry, J. (1996). HSP27 phosphorylation-mediated resistance against actin fragmentation and cell death induced by oxidative stress. *Cancer Res.* **56**, 273-279.

Ives, H. E. and Daniel, T. O. (1987). Interrelationship between growth factor-

induced pH changes and intracellular Ca^{2+}. *Proc. Natl. Acad. Sci. USA* **84**, 1950-1954.

Jeffrey, F. M. H., Malloy, C. R. and Radda, G. K. (1987). Influence of intracellular acidosis on contractile function in the working rat heart. *Am. J. Physiol.* **253**, H1499-H1505.

Kaiser, R. A., Bueno, O. F., Lips, D. J., Doevendans, P. A., Jones, F., Kimball, T. F. and Molkentin, J. D. (2004). Targeted inhibition of p38-mitogen activated protein kinase antagonizes cardiac injury and cell death following ischemia-reperfusion in vivo. *J. Biol. Chem.* **279**, 15524-15530.

Kato, Y., Lambert, C. A., Collige, A. C., Mineur, P., Noël, A., Frankenne, F., Foidart, J.-M., Baba, M., Hata, R.-I., Miyazaki, K. et al. (2005). Acidic extracellular pH induces matrix metalloproteinase-9 expression in mouse metastatic melanoma cells through the phospholipase D–mitogen-activated protein kinase signaling. *J. Biol. Chem.* **280**, 10938-10944.

Khaled, A. R., Kim, K., Hofmeister, R., Muegee, K. and Durum, S. K. (1999). Withdrawal of IL-7 induces Bax translocation from cytosol to mitochondria through a rise in intracellular pH. *Proc. Natl. Acad. Sci. USA* **96**, 14476-14481.

Klip, A., Ramlal, T. and Cragoe, E. J., Jr (1986). Insulin-induced cytoplasmic alkalinization and glucose transport in muscle cells. *Am. J. Physiol.* **250**, C720-C728.

Kometiani, P., Li, J., Gnudi, L., Kahn, B. B., Askari, A. and Xie, Z. (1998). Multiple signal transduction pathways link Na^+/K^+-ATPase to growth-related genes in cardiac-myocytes. *J. Biol. Chem.* **273**, 15249-15256.

Kubasiak, L. A., Hernandez, O. M., Bishopric, N. H. and Webster, K. A. (2002). Hypoxia and acidosis activate cardiac myocyte death through the Bcl-2 family protein BNIP3. *Proc. Natl. Acad. Sci. USA* **99**, 12825-12830.

Kumar, S., Boehm, J. and Lee, J. C. (2003). p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nat. Rev. Drug Discov.* **2**, 717-726.

Kyriakis, J. M. and Avruch, J. (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev.* **81**, 807-869.

Lagadic-Gossmann, D., Huc, L. and Lecureur, V. (2004). Alterations of intracellular pH homeostasis in apoptosis: origins and roles. *Cell Death Differ.* **11**, 953-961.

Paul, C., Manero, F., Gonin, S., Kretz-Remy, C., Virot, S. and Arrigo, A. P. (2002). Hsp27 as a negative regulator of cytochrome *c* release. *Mol. Cell. Biol.* **22**, 816-834.

Roos, A. and Boron, W. F. (1981). Intracellular pH. *Physiol. Rev.* **61**, 297-434.

Rouse, J., Cohen, P., Trigon, S., Moragne, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T. and Nebreda, A. R. (1994). A novel kinase cascade triggered stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* **78**, 1027-1037.

Sarosi, G. A., Jr, Jaiswal, K., Herndon, E., Lopez-Guzman, C., Spechler, S. J. and Souza R. F. Acid increases MAPK-mediated proliferation in Barrett's esophageal adenocarcinoma cells via intracellular acidification through a Cl^-/HCO_3^- exchanger. *Am. J. Physiol. Gastrointest. Liver Physiol.* **289**, G991-G996.

Shrode, L. M., Rubie, E. A., Woodgett, J. R. and Grinstein, S. (1997). Cytosolic alkalinization increases stress-activated protein kinase/c-jun NH_2-terminal kinase (SAPK/JNK) activity and p38 mitogen-activated protein kinase activity by a calcium-independent mechanism. *J. Biol. Chem.* **272**, 13653-13659.

Snoeckx, L. H. E. H., Cornelussen, R. N., Van Nieuwenhoven, F. A., Reneman, R. S. and Van der Vusse, G. J. (2001). Heat shock proteins and cardiovascular pathophysiology. *Physiol. Rev.* **81**, 1461-1497.

Sreedhar, A. S. and Csermely, P. (2004). Heat shock proteins in the regulation of apoptosis: new strategies in tumor therapy. A comprehensive review. *Pharmacol. Therapeut.* **101**, 227-257.

Stokoe, D., Engel, K., Campbell, D. G., Cohen, P. and Gaestel, M. (1992). Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylationof the small mammalian heat shock proteins. *FEBS Lett.* **313**, 307-313.

Susa, S. and Wakabayashi, I. (2003). Extracellular alkalosis activates ERK mitogen-activated protein kinase of vascular smooth muscle cells through NADPH-mediated formation of reactive oxygen species. *FEBS Lett.* **554**, 399-402.

Swietach, P. and Vaughan-Jones, R. D. (2005a). Spatial regulation of intracellular pH in the ventricular myocyte. *Ann. NY Acad. Sci.* **1047**, 271-282.

Swietach, P. and Vaughan-Jones, R. D. (2005b). Relationship between intracellular pH and proton mobility in rat and guinea-pig ventricular myocytes. *J. Physiol.* **566**, 793-806.

Thuerauf, D. J., Arnold, N. D. and Zechner, D. (1998). p38 mitogen-activated protein kinase mediates the transcriptional induction of atrial natriuretic factor gene through a serum response element. A potential role for the transcription factor ATF6. *J. Biol. Chem.* **273**, 20636-20643.

Wada, T. and Penninger, J. M. (2004). Mitogen-activated protein kinases in apoptosis regulation. *Oncogene* **23**, 2838-2849.

Wang, Y., Huang, S., Sah, V. P., Ros, J., Jr and Brown, J. H. (1998). Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38-mitogen activated protein kinase family. *J. Biol. Chem.* **273**, 2161-2168.

Webster, K. A., Discher, D. J., Kaiser, S., Hernandez, O., Sato, B. and Bishopric, N. H. (1999). Hypoxia activated apoptosis of cardiac myocytes requires reoxygenation or a pH shift and is independent of p53. *J. Clin. Invest.* **104**, 239-252.

Wretman, C., Lionikas, A., Widegren, U., Lännergren, J., Westerblad, H. and Henriksson, J. (2001). Effects of concentric and eccentric contractions on phosphorylation of $MAPK^{erk1/2}$ and $MAPK^{p38}$ in isolated skeletal muscle. *J. Physiol.* **535**, 155-164.

Xie, Z. (2003). Molecular mechanisms of Na^+/K^+-ATPase-mediated signal transduction. *Ann. NY Acad. Sci.* **986**, 497-503.

Yin, T., Sandhu, G., Wolfgang, C. D., Burrier, A., Webb, R. L., Rigel, D. F., Hai, T. and Whelan, J. (1997). Tissue-specific pattern of stress kinase activation in ischemic/reperfused heart and kidney. *J. Biol. Chem.* **172**, 19943-19950.

Zheng, M., Reynolds, C., Jo, S.-H., Wersto, R., Han, Q. and Xiao, R.-P. (2005). Intracellular acidosis-activated p38 MAPK signaling and its essential role in cardiomyocyte hypoxic injury. *FASEB J.* **19**, 109-111.