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Hyperosmotic and thermal stresses activate p38-MAPK in the perfused amphibian heart

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Summary

We assessed the activation of p38-MAPK (mitogen-activated protein kinase) by osmotic and thermal stresses in the isolated perfused amphibian (*Rana ridibunda*) heart. Hyperosmotic stress induced the rapid activation of the kinase. In particular, in the presence of 0.5 mol l^{-1} sorbitol, p38-MAPK was maximally phosphorylated (by approximately twelvefold) at 15 min, while excess of NaCl (206 mmol l^{-1} final concentration) or KCl (16 mmol l^{-1} final concentration) stimulated a less potent activation, maximised (by approximately eightfold and fourfold) within 2 min and 30 s, respectively, relative to control values. The effect of all three compounds examined was reversible, since the kinase phosphorylation levels decreased upon reperfusion of the heart with normal bicarbonate-buffered saline. Conversely, hypotonicity did not induce any p38-MAPK activation. Furthermore, both hypothermia and hyperthermia induced considerable phosphorylation of the kinase, by four- and 7.5-fold, respectively, relative to control values. Immunohistochemical studies elucidated the localisation pattern of phospho-p38-MAPK and also revealed enhanced atrial natriuretic peptide (ANP) immunoreactivity in osmotically stressed hearts. Interestingly, SB 203580 (1 μmol l^{-1}) not only completely blocked the activation of p38-MAPK by all these interventions, but also abolished the enhanced ANP immunoreactivity induced by 0.5 mol l^{-1} sorbitol. These findings indicate the possible involvement of ANP in the mechanisms regulating responses under such stressful conditions.

Keywords: p38-MAPK, osmotic stress, thermal stress, atrial natriuretic peptide, immunolocalisation, amphibian heart, *Rana ridibunda*.

Introduction

In physiologically stressful situations, survival of vertebrates is dependent upon uninterrupted heart function. Cardiac muscle is therefore particularly interesting because of its ability to maintain pump performance even under extreme conditions. Hearts of ectotherms face a far greater range of environmental variants, such as osmolarity and temperature, than those of endotherms (for reviews, see Driedzic and Gesser, 1994; Pinder et al., 1992). Thus, the amphibian heart represents an excellent experimental model for the elucidation of the physiological and molecular mechanisms involved in the response to osmotic and thermal stress.

Osmolarity and temperature are two variables that characterise the physiological environment and have been shown to exert profound influences on the electrophysiological behaviour of the heart (Nagai and Iriki, 1984; Layne et al., 1989; Gennser et al., 1990). Amphibians, as ectotherms, face a wide range of water and temperature imbalances in their physiological environment (Hutchison and Dupr, 1992; Hoffman and Katz, 1997). For these organisms, skin is a site for gas exchange and high permeability to water. Evaporation, which links water and temperature regulation, limits amphibian activity in time and space (Pough, 1983; Katz et al., 1986). As it is well established that osmolarity and temperature constitute severe environmental constraints on cardiac function in ectothermic animals, we examined the effect of hyperosmotic and thermal stresses on a classical ‘stress-responsive’ protein kinase, p38-MAPK, in the isolated perfused amphibian heart.

Mitogen-activated protein kinases (MAPKs) are members of a major intracellular signal transduction pathway that has been demonstrated to play an important role in various physiological processes (Seger and Krebs, 1995; Robinson and Cobb, 1997; Widmann et al., 1999). Three subfamilies of these serine/threonine kinases have been clearly identified in mammals: the extracellularly responsive kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38-MAPKs. The third subfamily, p38-MAPK, is activated by various forms of environmental stress including hyperosmolarity and heat shock (for reviews, see Bogoyevitch, 2000; Kyriakis and Avruch, 2001). The respective MAPKs in the amphibian

heart have been recently characterised in our laboratory (Aggeli et al., 2001a,b). In the isolated perfused *Rana ridibunda* heart the one isoform of ERK (p43) detected was activated by phorbol esters (1 μmol l^{-1} 4β-phorbol 12 myristate 13-acetate, PMA) and mechanical overload (increased perfusion pressure). The two isoforms of JNKs identified (p46-JNK1 and p52-JNK2) were found to be phosphorylated in response to 0.5 mol l^{-1} sorbitol, mechanical overload and reoxygenation following anoxia. p38-MAPK was also stimulated by mechanical overload, but most potently activated by 0.5 mol l^{-1} sorbitol.

Activated MAPKs are characterised by their localisation in both the cytoplasm and nucleus, where they interact with their substrates (Bogoyevitch, 2000; Aggeli et al., 2001b). In particular, they were found to phosphorylate other protein kinases (MAPKAPK2 and 3) (Rouse et al., 1994) or cytoskeletal proteins, or to modulate gene expression *via* activation of transcription factors (ATF2, Elk1) (Raingeaud et al., 1995, 1996). Thus, certain forms of stress that have been found to induce p38-MAPK stimulation lead to the transcriptional activation of genes that contribute to appropriate compensatory responses, including the atrial natriuretic peptide (ANP) gene (Thuerauf et al., 1998).

ANP is a peptide hormone involved in the regulation of extracellular fluid volume and electrolyte balance (Yashujima et al., 1985; Glass et al., 1996) (for reviews, see Ruskoaho, 1992; Silberbach and Roberts, 2001). The presence of this hormone precursor has already been demonstrated in both atrial and ventricular tissue from *Rana ridibunda* (Gilles et al., 1990; Netchitailo et al., 1988). Therefore, it was quite intriguing to investigate the localisation pattern of ANP in osmotically stressed amphibian hearts, since this hormone may exert a potentially protective modulatory role under such stressful conditions.

Overall, our results demonstrate that p38-MAPK is activated by various forms of hyperosmotic stress as well as by thermal stress, and that these responses differ quantitively and qualitatively. Furthermore, our immunohistochemical studies provide evidence that, under the hyperosmotic conditions examined, the presence of ANP is enhanced. All these findings taken together could indicate a possible involvement of p38-MAPK and ANP in the preservation of amphibian heart homeostasis under similar situations *in vivo*.

Materials and methods

Materials

Most biochemicals used were obtained from Sigma Chemical Co. (St Louis, USA). The enhanced chemiluminescence (ECL) kit was from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA) and the alkaline phosphatase Kwik kit from Lipshaw (Pittsburgh, USA). [γ-^{32}ATP] was from NEN Life Sciences (1130 Brussels, Belgium). Bradford protein assay reagent was from Bio-Rad (Hercules, California 94547, USA). Nitrocellulose (0.45 μm) was obtained from Schleicher & Schuell (Keene NH, USA). SB 203580 was obtained from Calbiochem-Novabiochem (La Jolla, CA, USA).

Rabbit polyclonal antibody to the total p38-MAPK, as well as the antibody specific for the dually phosphorylated p38-MAPK, were obtained from New England Biolabs (Beverly, MA, USA). Rabbit polyclonal antibody specific for human ANP (1–28) was purchased from Biogenesis Ltd (Poole, UK). Prestained molecular mass markers were from New England Biolabs. Biotinylated anti-rabbit antibody was from Dako A/S (DK-2600 Glostrup, Denmark). X-OMAT AR 13 cm×18 cm and Elite chrome 100 films were purchased from Eastman Kodak Company (New York, USA). MAPKAPK2 (46–400) was kindly provided by Prof. P. H. Sugden (Imperial College, London, UK).

Animals

Frogs (*Rana ridibunda* Pallas) weighing 100–120 g from the vicinity of Thessaloniki, Greece, were supplied by a local dealer. The frogs were kept in containers in fresh water and used one week after arrival. Care of the animals conformed to Good Laboratory Practice.

Heart perfusions

Hearts from *Rana ridibunda* (weighing 100–120 g) were perfused with the non-recirculating Langendorff mode at a pressure of 4.5 kPa (31.5 mmHg) with bicarbonate-buffered saline (23.8 mmol l^{-1} $NaHCO_3$, 103 mmol l^{-1} NaCl, 1.8 mmol l^{-1} $CaCl_2$, 2.5 mmol l^{-1} KCl, 1.8 mmol l^{-1} $MgCl_2$, 0.6 NaH_2PO_4, pH 7.4 at 25 °C) supplemented with 10 mmol l^{-1} glucose and equilibrated with 95 % O_2/5 % CO_2. The temperature of the hearts and perfusates was maintained at 25 °C by the use of a water-jacketed apparatus. All hearts were equilibrated for 15 min under these conditions. After the equilibration period, hearts were perfused either with excess of NaCl (206 mmol l^{-1} final concentration) or KCl (16 mmol l^{-1} final concentration) in bicarbonate-buffered saline for time periods varying from 30 s up to 45 min. 'Control' hearts were perfused with the bicarbonate-buffered saline described above at 25 °C for respective time periods. As positive controls, hearts perfused with 0.5 mol l^{-1} sorbitol for 15 min were used.

In another series of experiments, after p38-MAPK maximal activation by each compound was reached, hearts were further perfused (reperfused) with normal bicarbonate-buffered saline for time periods varying from 30 s up to 45 min. When the inhibitor SB 203580 was used, it was added throughout the experiment at a concentration of 1 μmol l^{-1}. In parallel, hearts were perfused either with dimethylsulphoxide (DMSO) solvent or with 1 μmol l^{-1} SB 203580 alone, in order to examine whether these chemicals affect any of the variables measured.

To examine the effect of thermal stress, the temperature of perfusate was quickly changed to either 15 °C or 42 °C at the end of the equilibration period, by using a second water-jacketed apparatus. Hearts were perfused at the desired temperature for time periods varying from 1 min (for 15 °C) or 30 s (for 42 °C) up to 60 min.

At the end of the perfusions, hearts were 'freeze-clamped' between aluminium tongs cooled in liquid N_2, and after the removal of the atria, ventricles were pulverised under liquid N_2 and powders were stored at –80 °C.

Tissue extractions

Heart powders were homogenised with 3 ml g^{-1} of buffer [20 mmol l^{-1} Tris-HCl, pH 7.5, 20 mmol l^{-1} β-glycerophosphate, 20 mmol l^{-1} NaF, 2 mmol l^{-1} EDTA, 0.2 mmol l^{-1} Na_3VO_4, 5 mmol l^{-1} dithiothreitol (DTT), 10 mmol l^{-1} benzamidine, 200 μmol l^{-1} leupeptin, 120 μmol l^{-1} pepstatin A, 10 μmol l^{-1} trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane, 300 μmol l^{-1} phenyl methyl sulphonyl fluoride (PMSF), 0.5 % (v/v) Triton X-100] and extracted on ice for 30 min. The samples were centrifuged (10,000 ***g***, 5 min, 4 °C) and the supernatants boiled with 0.33 volumes of 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 BioRad Bradford assay (Bradford, 1976).

SDS–PAGE and immunoblot analysis

Proteins were separated by SDS–PAGE on 10 % (w/v) acrylamide, 0.275 % (w/v) bisacrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 μm). Membranes were then incubated in TBST (20 mmol l^{-1} Tris-HCl, pH 7.5, 137 mmol l^{-1} NaCl, 0.1 % (v/v) Tween 20) containing 5 % (w/v) non-fat milk powder for 30 min at room temperature. Subsequently, the membranes were incubated with the appropriate antibody according to the manufacturer's instructions. After washing in TBST (3× 5 min) the blots were incubated with horseradish peroxidase-linked anti-rabbit IgG antibodies (1:5000 dilution in TBST containing 1 % (w/v) non-fat milk powder, 1 h, room temperature). The blots were washed again in TBST (3× 5 min) and the bands detected using ECL with exposure to X-OMAT AR film. Blots were quantified by laser scanning densitometry.

In-gel kinase assays

Proteins (200 μg per lane) were separated on 10 % (w/v) SDS–polyacrylamide gels with 6 % (w/v) stacking gel. The 10 % (w/v) gels were formed in the presence of 0.5 mg ml^{-1} glutathione S-transferase-conjugated MAPK-activated protein kinase 2 (GST–MAPKAPK2) (46–400) for the assay of p38-MAPK (Rouse et al., 1994). After electrophoresis, SDS was removed from the gels by washing in 20 % (v/v) propan-2-ol in 50 mmol l^{-1} Tris-HCl, pH 8.0 (3× 30 min). The propan-2-ol was removed by washing in 50 mmol l^{-1} Tris-HCl, pH 8.0, 5 mmol l^{-1} 2-mercaptoethanol (3× 30 min). Proteins were denatured in 6 mol l^{-1} guanidine-HCl, 50 mmol l^{-1} Tris-HCl, pH 8.0, 5 mmol l^{-1} 2-mercaptoethanol (two incubations for 30 min each) and then renatured in 50 mmol l^{-1} Tris-HCl (pH 8.0), 5 mmol l^{-1} 2-mercaptoethanol, 0.04 % (v/v) Tween 40 (incubation sequence: 1× 30 min, 2× 1 h, 1× 18 h, 1× 30 min, 4 °C). The gels were equilibrated to room temperature with 40 mmol l^{-1} Hepes, pH 8.0, 2 mmol l^{-1} DTT, 10 mmol l^{-1} $MgCl_2$ (two incubations for 30 min each) and then further incubated for 3 h with [γ-^{32}P]ATP (46.25×10^4 Bq per gel) in 5 ml of 40 mmol l^{-1} Hepes, pH 8.0, 0.5 mmol l^{-1} EGTA, 10 mmol l^{-1} $MgCl_2$, 50 μmol l^{-1} ATP, 0.1 μmol l^{-1} c-AMP dependent protein kinase inhibitor (PKI). The reaction was stopped and gels were washed with 1 % (w/v) disodium pyrophosphate, 5 % (w/v) trichloroacetic acid. The gels were dried onto 3MM Whatman chromatography paper and autoradiographed. In-gel kinase activities were quantified by laser scanning densitometry.

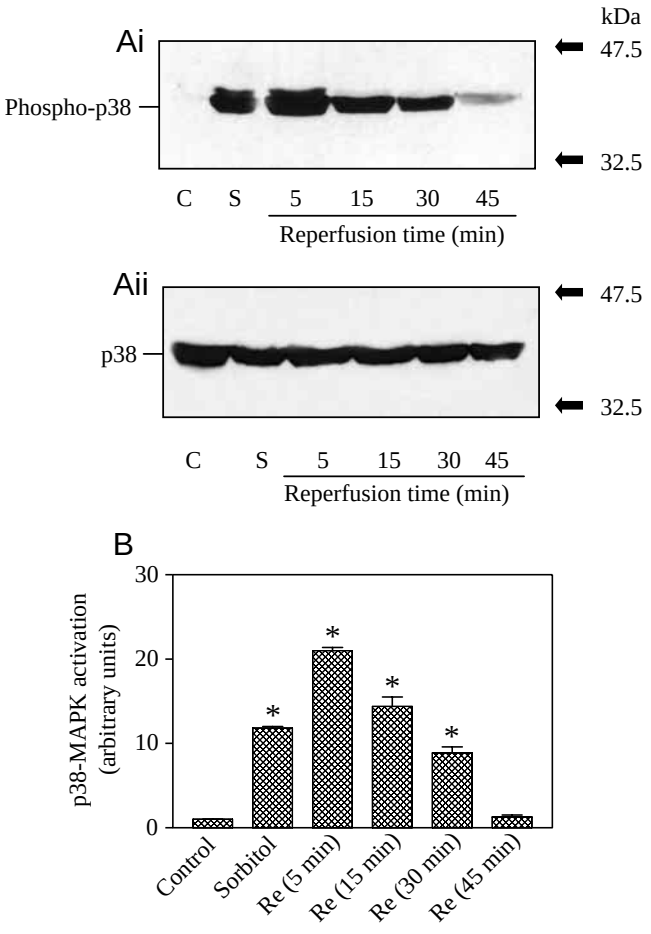


Fig. 1. Time course of p38-MAPK phosphorylation in the amphibian heart, in response to reperfusion after sorbitol treatment. (Ai) Phospho-p38-MAPK was detected in extracts (100 μg of protein) from control hearts (C), hearts perfused with 0.5 mol l^{-1} sorbitol for 15 min (S), or hearts reperfused for the indicated times with normal bicarbonate-buffered saline following a 15 min perfusion with 0.5 mol l^{-1} sorbitol. Molecular mass markers (kDa) are shown to the right. (Aii) Identical samples immunoblotted for total p38-MAPK levels, as a control for loading. (B) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Results are means ± S.E.M. for three independent experiments performed with similar findings. Re, reperfusion. The western blots are representative of three independent experiments. *Significantly different from control value (P<0.001).

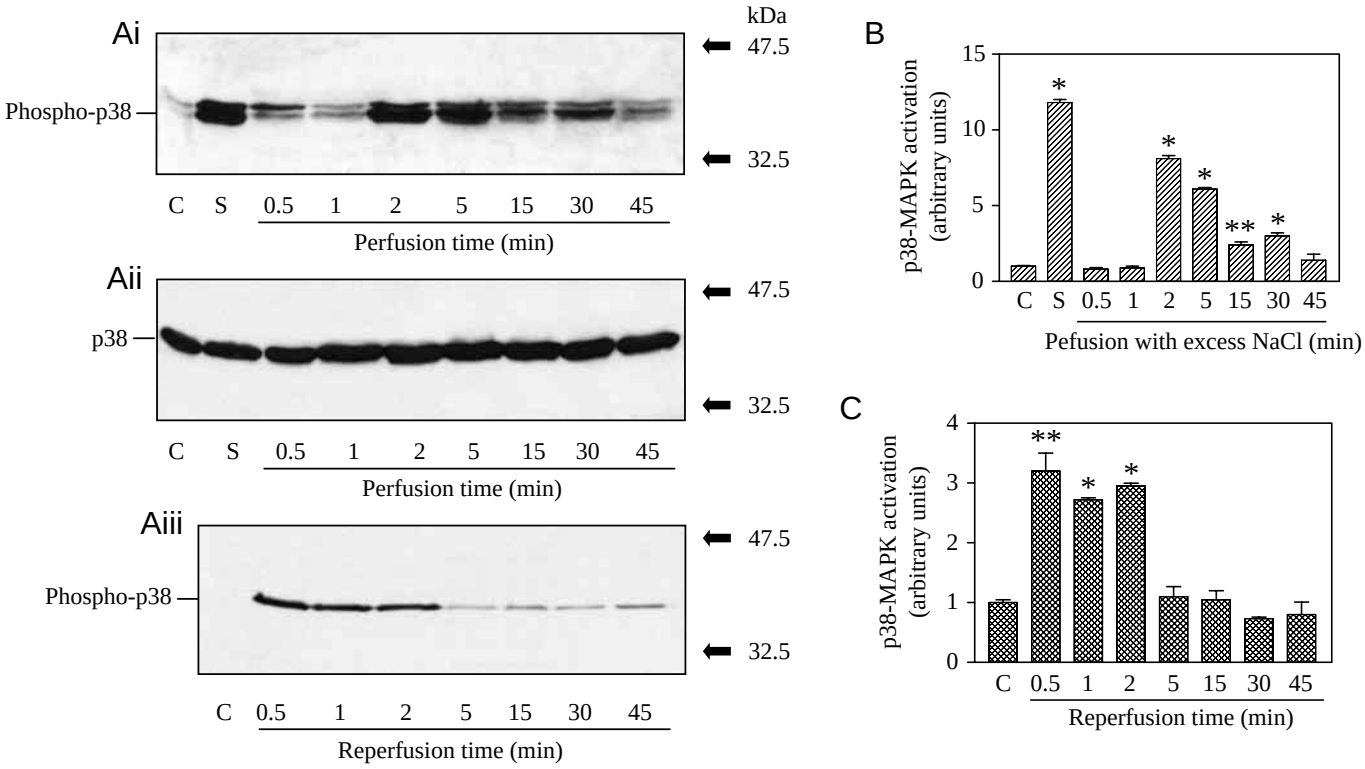


Fig. 2. Time course of the effect of excessive NaCl concentration upon p38-MAPK phosphorylation in the amphibian heart. (Ai) Phospho-p38-MAPK was detected in extracts (100 μg of protein) from control hearts (C), hearts perfused with 0.5 mol l^{-1} sorbitol for 15 min (S), or hearts perfused with excess of NaCl (206 mmol l^{-1} final concentration) in bicarbonate-buffered saline for various time periods. (Aii) Total p38-MAPK levels in identical samples, as a control for loading. (Aiii) Phospho-p38-MAPK was also detected in extracts (100 μg of protein) from hearts reperfused with normal buffer after a 2 min perfusion with excess of NaCl. The western blots shown are representative of three independent experiments. Molecular mass markers (kDa) are shown to the right. (B,C) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. The results are means ± S.E.M. for three independent experiments. Asterisks indicate values significantly different from control values; *P<0.001, **P<0.01.

Immunolocalisation of phospho-p38-MAPK and atrial natriuretic peptide

At the end of the perfusions, atria were removed and ventricles immersed in Uvasol/isopentane pre-cooled in liquid N_2, then stored at –80 °C. Tissues were sectioned with a cryostat at a thickness of 5–6 μm, fixed with ice-cold acetone (10 min, room temperature), and specimens stored at –30 °C until use. Alternatively, ventricles were fixed in 10 % (v/v) formaldehyde, dehydrated, embedded in paraffin and sectioned at a thickness of 5–6 μm. Prior to immunohistochemical staining, they were deparaffinised in xylene and rehydrated in graded alcohol. Tissue sections were washed in TBST and non-specific binding sites blocked with 3 % (w/v) bovine serum albumin (BSA) in TBST (1 h, room temperature). Cryo-sections were incubated with primary antibody specific for phospho-p38-MAPK according to the method previously described (Aggeli et al., 2001b), and paraffin-embedded sections for the ANP immunolocalisation pattern, according to the manufacturer's instructions. Cryo-sections were processed in parallel with the same antibody in order to obtain comparable patterns that would reveal the localisation of both p38-MAPK and ANP. Sections were incubated with primary antibody specific for human ANP (1–28) diluted in 3 % BSA (w/v) in TBST (overnight, 4 °C). All sections were immunostained by the alkaline phosphatase method using a Kwik kit, according to the manufacturer's instructions. The alkaline phosphatase label was visualised by exposing the sections to Fast Red chromogen and nuclei were counterstained with Haematoxylin. Slides were mounted, examined with a Zeiss Axioplan microscope equipped with Nomarski filter and photographed with a Kodak Elite chrome 100 film.

Statistical evaluations

Western blots shown are representative of at least three independent experiments. Each data point represents the mean ± S.E.M. of samples from at least three separate hearts perfused under the respective conditions. Comparisons between control and treatments were performed using Student's paired t-test. A value of P<0.05 was considered to be statistically significant. p38-MAPK activation in 'control' hearts was set at 1, and the stimulated p38-MAPK activation in treated hearts was expressed as fold activation over 'control' hearts.

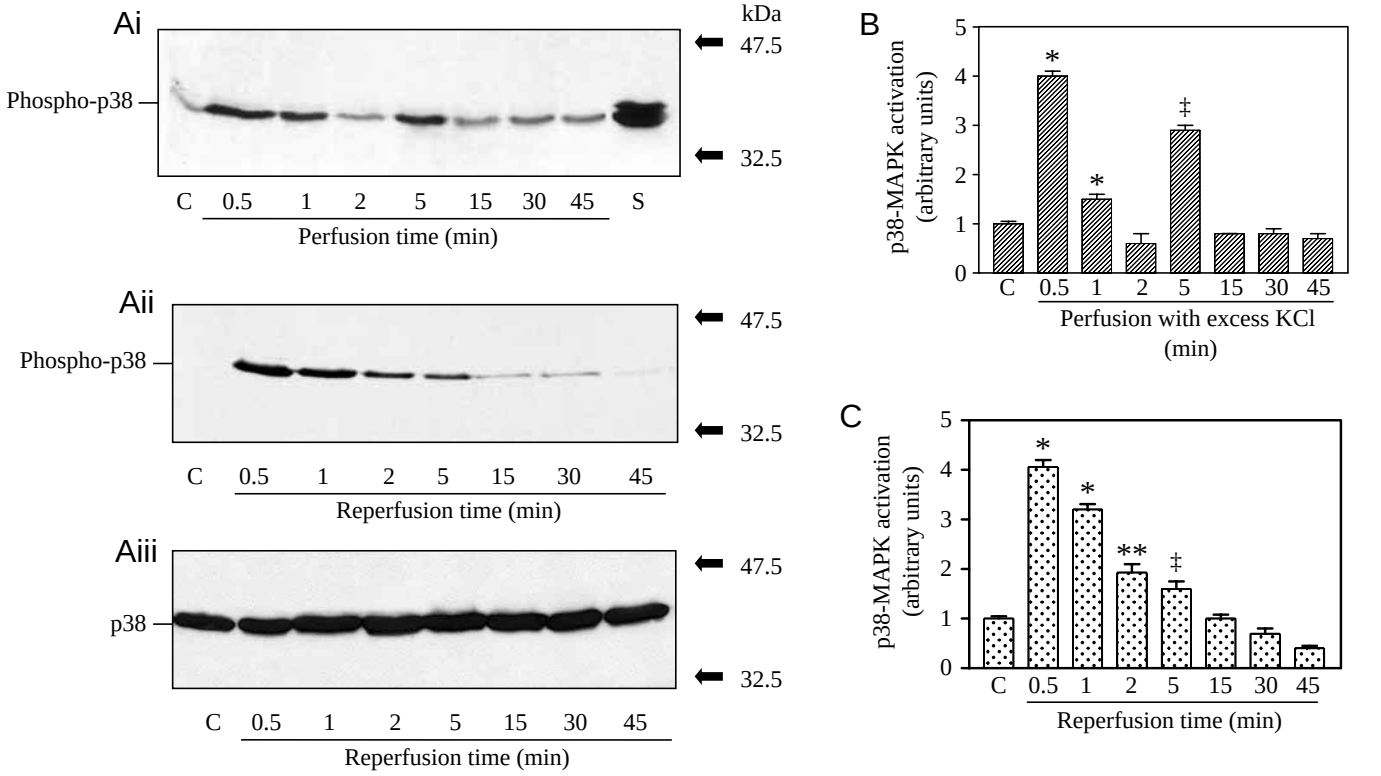


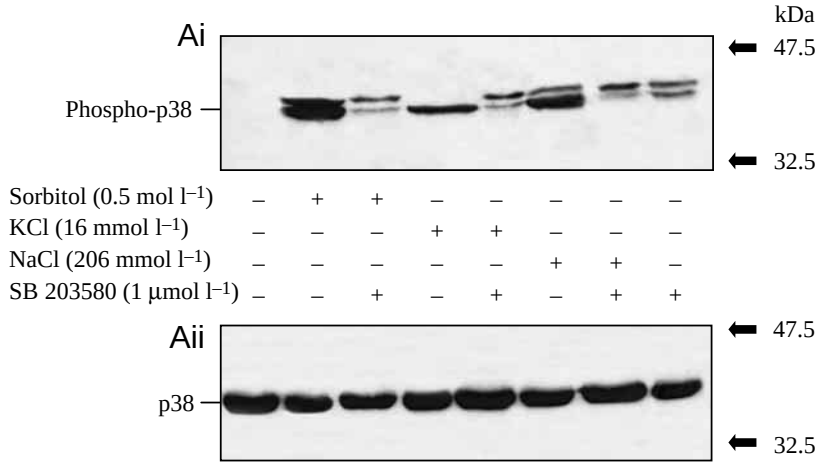
Fig. 3. Time course of the effect of excessive KCl concentration upon p38-MAPK phosphorylation in the amphibian heart. (Ai) Phospho-p38-MAPK was detected in extracts (100 μg of protein) from control hearts (C), hearts perfused with 0.5 mol l^{-1} sorbitol for 15 min (S), or hearts perfused with 16 mmol l^{-1} KCl in bicarbonate-buffered saline. (Aii) Phospho-p38-MAPK was also detected in extracts (100 μg) from hearts perfused for 30 s with excess of KCl and reperfused with normal bicarbonate-buffered saline. (Aiii) Total p38-MAPK levels were detected in identical samples as a control for loading. The western blots shown are representative of three independent experiments. Molecular mass markers (kDa) are shown to the right. (B,C) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Values are means ± S.E.M. for three independent experiments performed with similar results. Asterisks indicate values significantly different from control values; **P*<0.001, ***P*<0.01, ‡*P*<0.05.

Results

We have previously established that 0.5 mol l^{-1} sorbitol potently activates two isoforms of amphibian heart p38-MAPK (Aggeli et al., 2001a). Following a perfusion for 15 min with this hypertonic buffer, we examined the effect of reperfusion with normal bicarbonate-buffered saline, for periods of 5–45 min. The results clearly showed that p38-MAPK phosphorylation by 0.5 mol l^{-1} sorbitol was reversible (Fig. 1Ai,B). Interestingly, a reperfusion period of 5 min with normal buffer almost doubled the activation levels of the kinase (by approximately 21.7±0.4-fold relative to control), with phosphorylation then showing a progressive decline, reaching control values after a period of 45 min (Fig. 1). Equivalent protein loading was confirmed by probing identical samples with an antibody recognising total p38-MAPK levels (Fig. 1Aii). This also confirmed that the kinase expression did not change during this relatively prolonged treatment.

To determine the time course of the effect of extracellular osmolarity on the phosphorylation of p38-MAPK, frog hearts were perfused with various hypertonic solutions for various time periods from 30 s to 45 min. Excess of 0.6 % (w/v) NaCl (206 mmol l^{-1} final concentration) maximally activated p38-MAPK at 2 min (by approximately 8±0.2-fold, relative to controls). The kinase phosphorylation levels remained considerably elevated over a 30 min perfusion (approximately 3.1±0.2-fold, relative to controls), and returned to control values by 45 min (Fig. 2Ai,B). Reperfusion with normal bicarbonate-buffered saline, following a 2 min perfusion period with this hypertonic excess NaCl, resulted in the gradual decrease of p38-MAPK phosphorylation, which reached basal levels within 5 min (Fig. 2Aiii,C). Fig. 2Aii shows that there were no changes in the total cellular pool of p38-MAPK during the time course of the experiment, and therefore provides a control for protein loading under these conditions.

Another condition tested, excess of 0.1 % (w/v) KCl (16 mmol l^{-1} final concentration), rapidly increased p38-MAPK phosphorylation to maximal values after 30 s (approximately 4±0.1-fold increase, relative to controls). The osmolarity of the perfusate is slightly increased under these conditions, which principally affect the highly conserved concentration of [K^+], an important intracellular electrolyte. A second peak of phosphorylation was observed at 5 min (approximately 3±0.1-fold increase, relative to controls), with a progressive decline thereafter (Fig. 3Ai,B). Reperfusion with



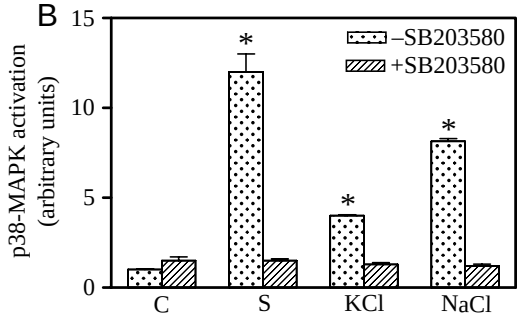
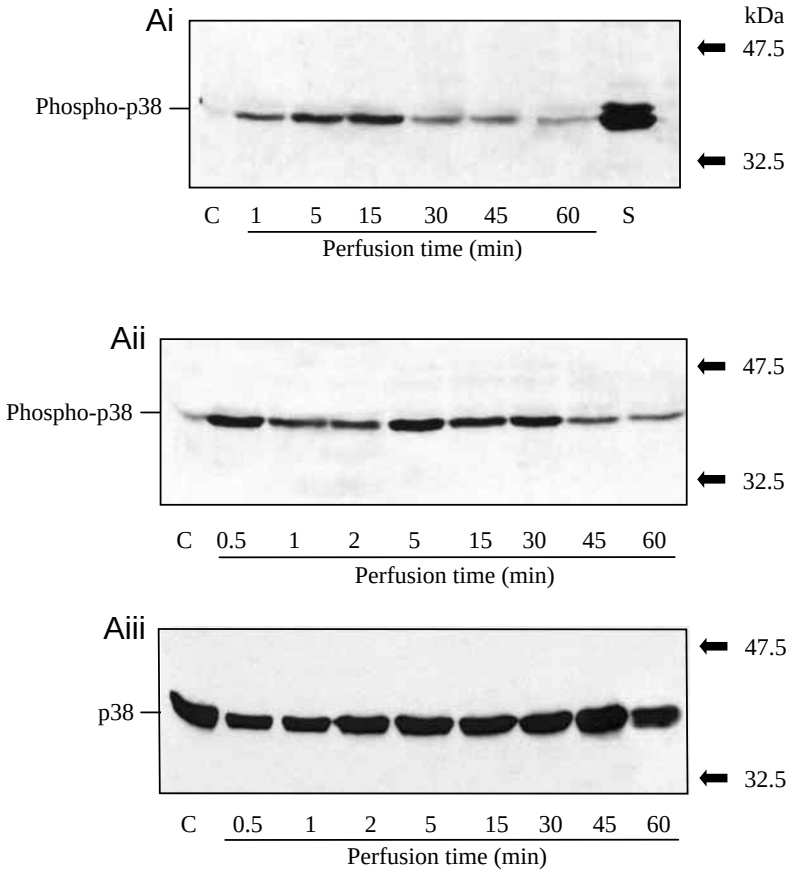


Fig. 4. Effect of SB 203580 on p38-MAPK activation by hyperosmotic stress. SB 203580 (1 μmol l^{-1}) was added after a 15 min equilibration period and was present throughout the experiment. Phosphorylated (Ai) and total (Aii) p38-MAPK levels were assayed in extracts (100 μg of protein) from control hearts, as well as from hearts perfused under the various hyperosmotic conditions indicated, in the absence (–) or presence (+) of the inhibitor. The western blots shown are representative of three independent experiments performed with similar findings. Molecular mass markers (kDa) are shown to the right. (B) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Values are means ± S.E.M. for three independent experiments. *Value significantly different from control value (P<0.001).



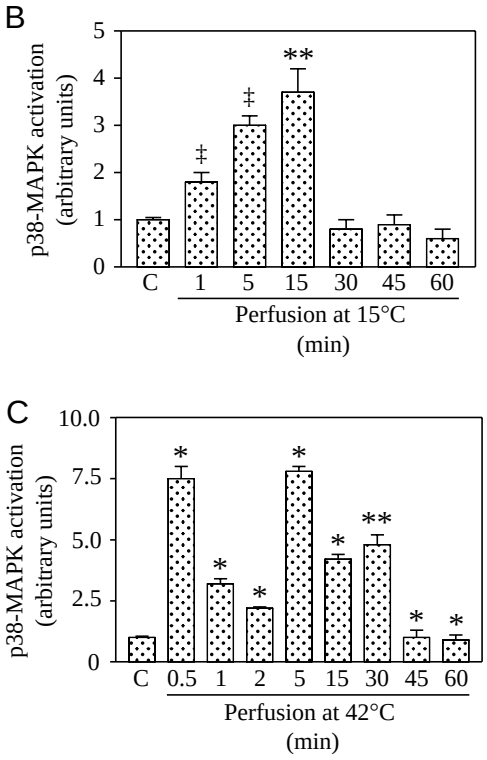


Fig. 5. Time course of the effect of thermal stress upon p38-MAPK phosphorylation in the amphibian heart. Phosphorylated p38-MAPK was detected in extracts (100 μg of protein) from control hearts perfused at 25 °C (C), hearts perfused with 0.5 mol l^{-1} sorbitol for 15 min at 25 °C (S), or hearts perfused at 15 °C (Ai) or 42 °C (Aii) for the indicated times. (Aiii) Total p38-MAPK levels detected in samples from control hearts perfused at 25 °C (C) or hearts perfused at 42 °C for the indicated times, as a μcontrol for loading. The western blots shown are representative of three independent experiments. Molecular mass markers (kDa) are shown to the right. (B,C) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Values are means ± S.E.M. for three independent experiments performed with similar results. Values significantly different from controls are indicated; *P<0.05, **P<0.01, $^{‡}P$<0.001.

normal bicarbonate-buffered saline, following a 30 s perfusion period with excessive KCl, resulted in the gradual decrease of p38-MAPK phosphorylation levels, which reached basal values at 15 min (Fig. 3Aii,C). Equal protein loading was verified by immunoblot analysis using an antibody for total levels of p38-MAPK (independent of phosphorylation state) (Fig. 3Aiii). Hypotonicity [net 0.6 % NaCl (w/v)] had no effect on the phosphorylation of p38-MAPK (data not shown).

The specific p38-MAPK inhibitor, SB 203580, has been extensively used to demonstrate the direct involvement of this kinase in the transduction of particular physiologically stressful signals. In the present study, SB 203580 (1 μmol l^{-1}) completely blocked the kinase activation by all the treatments mentioned above (Fig. 4Ai,B). SB 203580 (1 μmol l^{-1}) alone had a minor effect on the kinase phosphorylation levels, which was accounted for when evaluating the inhibitor effect on p38-MAPK activation. Equivalent protein loading was confirmed by probing identical samples with an antibody recognising total p38-MAPK levels (Fig. 4Aii).

Cold stress as well as heat stress activated p38-MAPK in a time-dependent manner. Hypothermia (15 °C) induced a relatively moderate phosphorylation of the kinase, which was maximal at 15 min (approximately 4±0.5-fold increase, relative to control hearts perfused at 25 °C) and decreased thereafter (Fig. 5Ai,B). In contrast, hyperthermia (42 °C) induced a biphasic response of this kinase. In particular, there was a rapid maximal phosphorylation of p38-MAPK (approximately 7.5±0.5-fold increase, relative to control hearts perfused at 25 °C), after 30 s, while a second maximum (approximately 7.6±0.4-fold increase, relative to control hearts perfused at 25 °C) was detected after 5 min (Fig. 5Aii,C). p38-MAPK phosphorylation reached control values at 60 min (Fig. 5Aii,C). Fig. 5Aiii shows that there were no changes in the total cellular pool of p38-MAPK and is therefore a control for protein loading under these conditions.

p38-MAPK activity was also examined using in-gel kinase assays, with MAPKAPK2 being the substrate phosphorylated. Our results clearly showed that hyperosmotic stress (NaCl, 206 mmol l^{-1} for 2 min), as well as hyperthermic stress (42 °C for 30 s), not only induced the phosphorylation (hence activation) but also a considerable increase of p38-MAPK activity (4.6±0.5- or 5±0.3-fold, respectively), relative to controls (Fig. 6A,B).

Upon activation by mechanical overload, amphibian heart p38-MAPK immunoreactive complexes are localised widely in the cytoplasm as well as around the elongated myocyte nuclei (Aggeli et al., 2001b). In order to investigate the localisation pattern of the activated kinase immunohistochemically, under conditions of osmotic stress, frog hearts were perfused with hypertonic medium, in the absence or presence of the specific inhibitor SB 203580 (1 μmol l^{-1}), then sectioned and processed using an antibody specific for the phosphorylated p38-MAPK. No immunoreactivity was detected in control hearts (Fig. 7C) or in specimens incubated either with the secondary antibody or

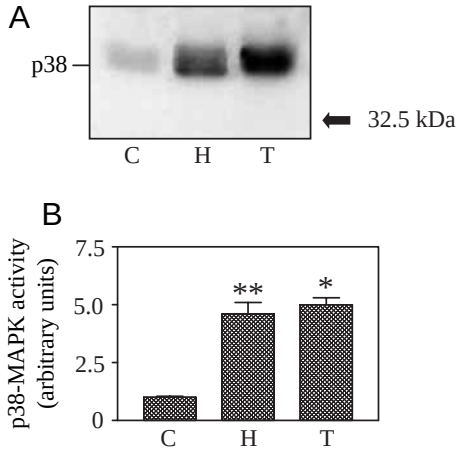


Fig. 6. p38-MAPK activity in response to hyperosmolarity (NaCl 206 mmol l^{-1} for 2 min) and hyperthermia (42 °C for 30 s) in the perfused amphibian heart. (A) The kinase activity was assayed by the in-gel kinase method in extracts (200 μg of protein) from control hearts (C), hearts perfused with hypertonic medium (H) or perfused at increased temperature (T), as described in the Materials and Methods. A typical autoradiogram is shown, representative of at least three independent experiments. The molecular mass marker (kDa) is shown to the right. (B) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Values are means ± S.E.M. for three independent experiments performed with similar results. Asterisks indicate values significantly different from control values; *P<0.001, **P<0.01.

with the chromogen alone (data not shown). In specimens from hearts perfused with 0.5 mol l^{-1} sorbitol for 15 min (Fig. 7, S), excessive NaCl (206 mmol l^{-1}) for 2 min (Fig. 7, NaCl), or excessive KCl (16 mmol l^{-1}) for 30 s (Fig. 7, KCl), immunoreactivity staining was observed within the cytoplasm as well as in the perinuclear region. A similar immunoreactivity pattern was detected in specimens from hearts perfused under these conditions and then reperfused with normal buffer for 5 min (Fig. 7, S/Rep) or 30 s (Fig. 7, NaCl/Rep and KCl/Rep, respectively). In particular, perinuclear clustering of deposits indicating phospho-p38-MAPK immunoproducts was more intense in specimens from hearts subjected to sorbitol treatment (Fig. 7, S), while in the case of excessive NaCl or KCl, most immunoproducts detected were scattered throughout the cytoplasm (Fig. 7, NaCl, KCl). No immunoreactivity was detected in hearts perfused with 0.5 mol l^{-1} sorbitol for 15 min, in the presence of 1 μmol l^{-1} SB 203580 (Fig. 7, S+SB).

ANP is a peptide hormone involved in osmoregulation, and its localisation pattern was examined in respective sections from osmotically stressed amphibian hearts. We used an antibody detecting the human (1–28) biologically active form of the peptide. Immunohistochemical studies were done on paraffin-embedded sections (Fig. 8A) as well as in cryo-sections (Fig. 8B), as described in Materials and methods. In specimens from hearts perfused with 0.5 mol l^{-1} sorbitol for 15 min (Fig. 8, S), excessive NaCl (206 mmol l^{-1}) for 2 min (Fig. 8, NaCl), or excessive KCl (16 mmol l^{-1}) for 30 s

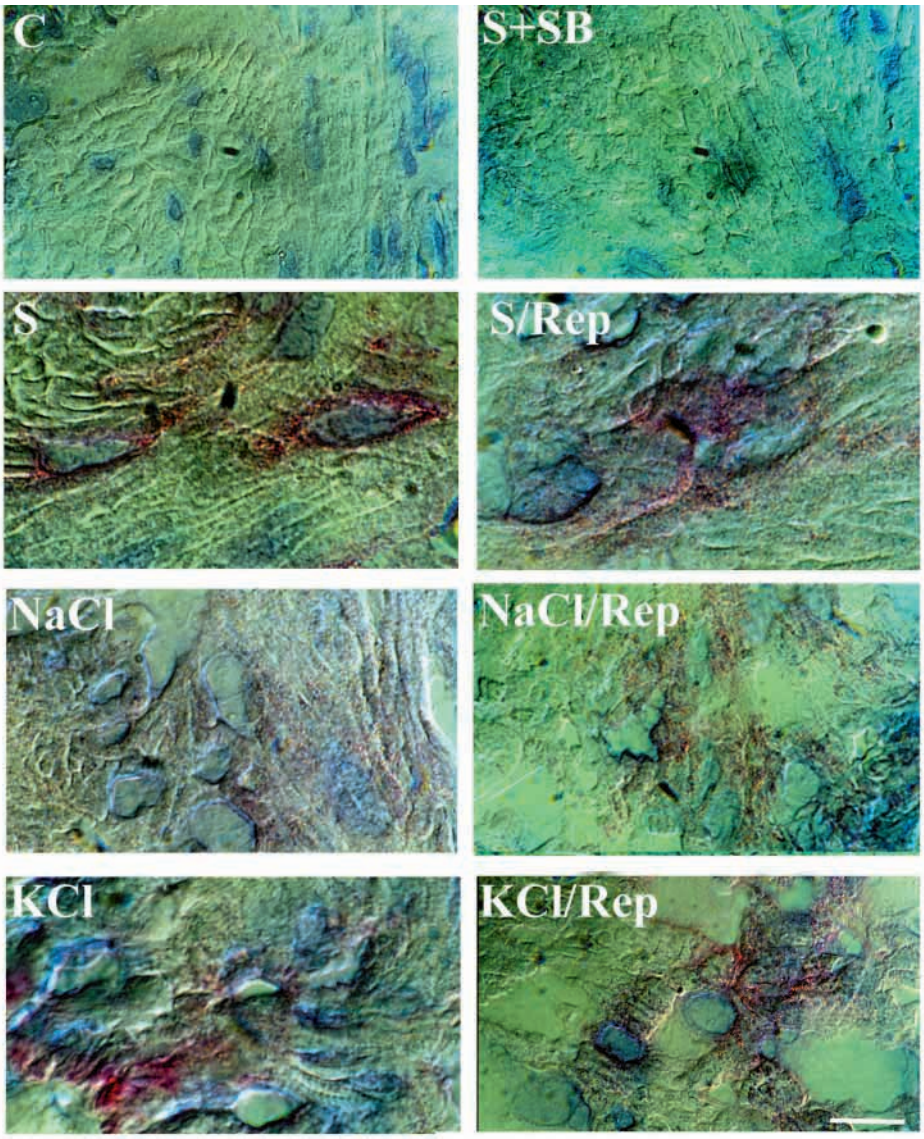


Fig. 7. Immunohistochemical localisation of phosphorylated p38-MAPK in the ventricle of isolated amphibian heart perfused under various hyperosmotic conditions. Hearts were perfused under normal conditions (C), with 0.5 mol l^{-1} sorbitol in the presence of 1 μmol l^{-1} SB203580 (S+SB), or subjected to diverse forms of osmotic stress such as 0.5 mol l^{-1} sorbitol for 15 min (S), 0.5 mol l^{-1} sorbitol (15 min) followed by reperfusion (5 min) (S/Rep), 206 mmol l^{-1} NaCl for 2 min (NaCl), 206 mmol l^{-1} NaCl (2 min) followed by reperfusion (30 s) (NaCl/Rep), 16 mmol l^{-1} KCl for 30 s (KCl), 16 mmol l^{-1} KCl (30 s) followed by reperfusion (30 s) (KCl/Rep). After the removal of atria, ventricles were cryosectioned longitudinally and fixed with ice-cold acetone. Specimens were incubated with phospho-p38-MAPK antibody (1:200 dilution) and counterstained with Haematoxylin. The figure shows representative photographs from three independent experiments performed with similar results. Immunoreaction deposits are visualised with Fast Red chromogen. Bar, 20 μm.

(Fig. 8, KCl), the antibody produced a discreet and specific granular pattern of ANP immunoreactivity staining. The ANP-immunoproducts observed were localised in the perinuclear region but also widely dispersed in the cytoplasm. Osmotically stressed hearts contained a significant amount of immunoreactivity compared to control ones. Interestingly, SB203580 (1 μmol l^{-1}) abolished the ANP immunoreactivity stimulated by 0.5 mol l^{-1} sorbitol (Fig. 8, S+SB). In specimens from control hearts (Fig. 8, C), as well as in sections incubated only with the secondary antibody (Fig. 8, NC), no immunoreactivity was detected.

Discussion

Ectotherms modulate their metabolic demands according to the status of their environment. These changes in metabolic demands require compensatory changes in cardiac output (Nagai and Iriki, 1984). Heart muscle has been shown to be osmotically sensitive to the tonicity of its surrounding bathing solutions (Roos, 1986; Houser and Freeman, 1979), with an electrophysiological behaviour that is also profoundly affected by temperature (Gennser, 1990). Therefore, the amphibian heart constitutes an intriguing candidate experimental system for studying the effects of environmental stress on multiple aspects of physiology at the cellular and molecular levels. Among the various signal transduction pathways involved in the responses to environmental stress, MAPKs have been shown to play a significant role (Kyriakis and Avruch, 1996; Karin, 1998; Schaeffer and Weber, 1999). In particular, p38-MAPK has been characterised as the principal stress kinase responsive to fluctuations in ambient osmolality and temperature (Zhang and Cohen, 1996; Kultz, 1997; Gon et al., 1998). In the present study, we have investigated the possible involvement of p38-MAPK in the response of the amphibian heart to osmotic and thermal stresses.

All three hyperosmotic stress signals tested (0.5 mol l^{-1} sorbitol, 206 mmol l^{-1} NaCl and 16 mmol l^{-1} KCl) caused rapid and reversible p38-MAPK phosphorylation (hence activation). Perfusion with 0.5 mol l^{-1} sorbitol, a known p38-MAPK activator, had a considerable sustained effect on the kinase phosphorylation levels (Aggeli et al., 2001a). Excess NaCl (206 mmol l^{-1}) caused a less robust response and excess KCl (16 mmol l^{-1}) a moderate one. The concentrations of the compounds used were chosen to reflect

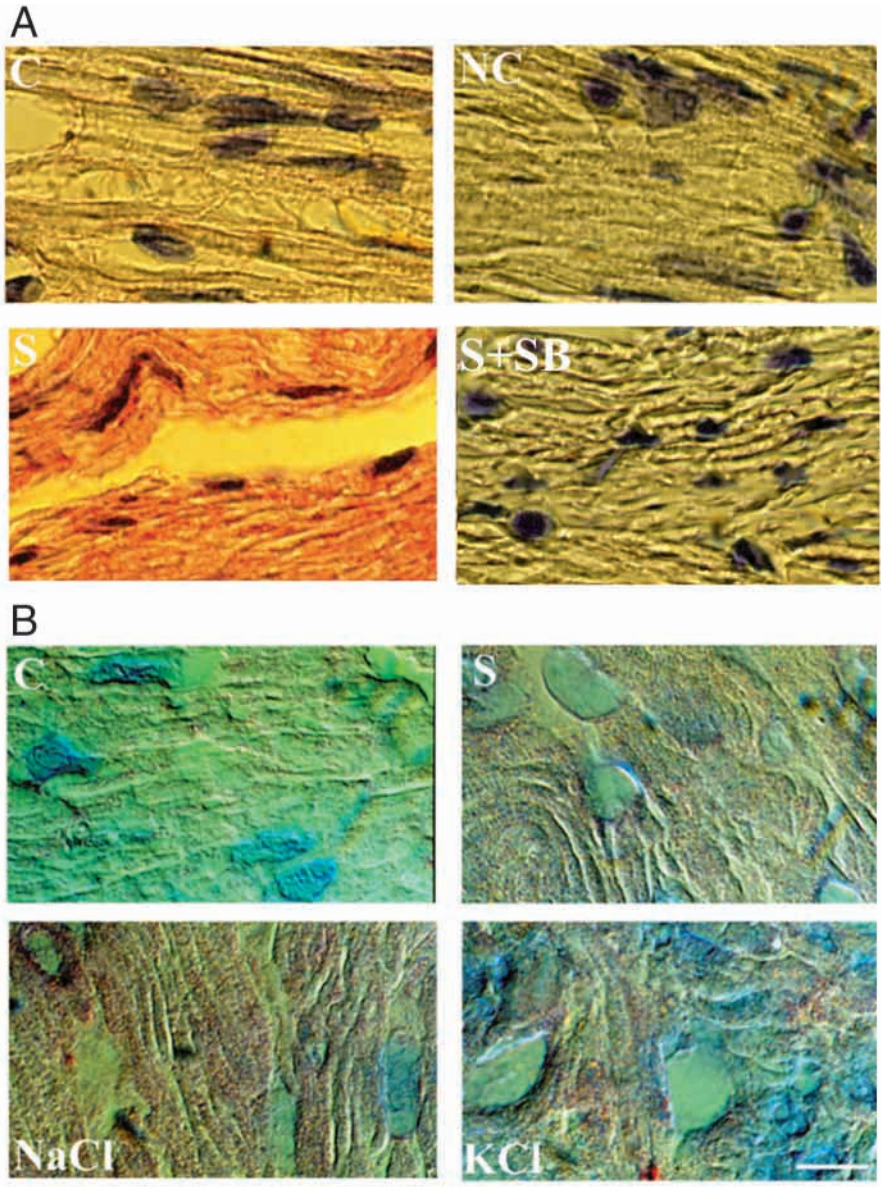


Fig. 8. ANP immunolocalisation in the ventricle of isolated amphibian heart perfused under various hyperosmotic conditions. Hearts were perfused under normal conditions (C) or subjected to various forms of hyperosmotic stress such as 0.5 mol l^{-1} sorbitol for 15 min in the absence (S) or presence of 1 μmol l^{-1} SB203580 (S+SB), 206 mmol l^{-1} NaCl for 2 min (NaCl) and 16 mmol l^{-1} KCl for 30 s (KCl), respectively. (A) Paraffin- or (B) cryo-sections were incubated with an antibody specific for human ANP (1–28) (1:500 dilution) and counterstained with Haematoxylin. In sections incubated only with secondary antibody (negative control, NC) no immunoreactivity was detected. The figure shows representative photographs from three independent experiments performed with similar results. Immunoreaction deposits are visualised with Fast Red chromogen. Bar, 20 μm.

their normal values in this particular experimental model. Sorbitol, as well as excess NaCl, caused a considerable increase in perfusate osmolality (osmotic stress). On the other hand, excess KCl actually represents a concentration imbalance of the specific electrolyte. Quite surprisingly, the effect of sorbitol on the kinase phosphorylation levels was doubled after a 5 min reperfusion period with normal bicarbonate-buffered saline and was sustained for 30 min (Fig. 1). In contrast, the increased kinase activation levels induced by perfusion with excess NaCl or KCl rapidly decreased and reached control values after a 5 or 15 min reperfusion period with normal bicarbonate-buffered saline, respectively (Figs 2, 3).

Since any disturbance of intracellular and extracellular osmolarity is paralleled by alterations of cell volume (Lang et al., 1998), the role of the latter should be considered in order to elucidate the possible physiological significance of the diverse responses observed. In stressful situations, cells restore their conserved ionic milieu, chiefly by adjusting the levels of compatible osmolytes (Somero and Yancey, 1997), to provide environments ‘compatible’ for macromolecular structure and function (Brown, 1976). Amphibians in particular, facing water stress, possess a solute-adaptation strategy capable of allowing the organism to cope with wide ranges of cyclic water stress, using polyhydric alcohols-polyols (principally glycerol), various amino acids and urea, as osmolytes (Brown, 1976; Katz et al., 1984). The considerable effect of sorbitol on p38-MAPK activation compared to that of NaCl and KCl could be because sorbitol is not the principal osmolyte for amphibian species (Yancey et al., 1982), whereas the other two osmotically active compounds tested constitute electrolytes that are familiar to the cellular physiology of the experimental model studied, thereby leading to more moderate effects. Nevertheless, a common characteristic of all interventions investigated was the immediate phosphorylation of p38-MAPK, which temporally coincided with the activation of volume-regulatory proteins (O’Neil and Klein, 1992; O’Donnell et al., 1995). This observation is consistent with the idea that p38 activation is directly related to the initial cell response to osmotic shock. Our results fit with several studies which have shown that osmotic shock results in marked phosphorylation of MAPK family members in several types of mammalian cells, including vascular endothelial cells (Duzgan et al., 2000), fibroblasts (Krump et al., 1997), intestinal cells (Matsuda et al., 1995), renal medullary cells (Zhang and Cohen, 1996), astrocytes, neutrophils and glial cells (Sinning et al., 1997).

Furthermore, SB 203580 (1 μmol l^{-1}) was found to abolish p38-MAPK phosphorylation induced by the various hyperosmotic stresses tested (Fig. 4). Among the several isoforms of the kinase that have been identified (Zervos et al., 1995; Li et al., 1996; Goedert et al., 1997; Kumar et al., 1997), only two (α and β_1) are strongly inhibited by SB 203580 (Goedert et al., 1997; Kumar et al., 1997). Although it was not possible to determine whether any specific p38-MAPK isoform is activated under the conditions examined in this study, our results demonstrate the possibility that these two isoforms are the ones detected in the isolated perfused *Rana ridibunda* heart, responsive to hyperosmotic stress stimulation and equally sensitive to this specific inhibitor.

Hypotonic stress had no effect on p38-MAPK activation. Our results corroborate those of Sadoshima et al. (1996), who observed that in rat cardiac myocytes p38-MAPK was not activated by hypo-osmolar conditions, although Tilly et al. (1996) have reported that hypo-osmotic stress activated p38-MAPK in a human intestine cell line. It is unknown how cells initially sense low osmolarity and convert it into intracellular signals, as this signalling mechanism is distinct from that of hyperosmolar stress in mammalian cells (Sadoshima et al., 1996).

Osmolarity regulation is profoundly affected by temperature variation, particularly in ectotherms, so we examined the effect of thermal stress (hypothermia and hyperthermia) on p38-MAPK activation. Hyperthermia (42 °C) induced p38-MAPK activation in an immediate (maximum at 30 s), sustained (over 30 min) and considerable (approximately 7.5-fold, relative to controls) way, whereas the effect of hypothermia was not so intense (Fig. 5). Cardiac activity of ectotherms is known to be relatively resistant to low temperatures (Gennser et al., 1990; Rocha and Branco, 1998), which act directly on pacemaker cells, but also influence cardiovascular nerves and reflexes (Courtice, 1990). Thus, the moderate response of p38-MAPK to hypothermia could be attributed to the fact that amphibians are routinely subjected to hypothermic stress and have consequently developed multiple adaptive responses in order to preserve their function under analogous conditions (Layne et al., 1989) (for a review, see Driedzic and Gesser, 1994). On the other hand, the pleiotropic effects of heat are likely to lead to the activation of multiple protein kinases, including p38-MAPK, which may then regulate stress response (thermotolerance) or apoptosis, or facilitate the repair of damaged proteins and other cellular components (Woessmann et al., 1999). However, the detailed regulation of heat shock response through activation of these signalling pathways remains to be determined. The choice of the specific temperature values was based on their environmental relevance for this frog species. That is, the biogeographical range inhabited by *Rana ridibunda* justifies both the hypothermic and hyperthermic temperatures investigated.

DeBold et al. (1981) and DeBold and Salerno (1983) were the first to report the production of a hormone involved in the regulation of extracellular fluid volume and electrolyte balance by atria of various animal species. In frogs, immunoreactive atrial natriuretic peptide (ANP) is detected in both atrial and ventricular cardiac myocytes (Mifune et al., 1996). Since important sequence homologies between the C-terminal regions of mammalian and amphibian ANP have been suggested by several investigators (Netchitailo et al., 1987; Gilles et al., 1990; Bruno and Coviello, 1992), an antibody specific for human ANP (1–28) was used to detect the presence of this hormone in osmotically stressed *Rana ridibunda* hearts. The enhanced ANP immunolocalisation pattern observed (Fig. 8) is in accordance with previous studies reporting an increase in ANP secretion by increased extracellular osmolality in rat atria, with ANP release being stimulated regardless of the added solute (Gibbs, 1987). The mechanism by which increased osmolality enhances ANP release, however, remains obscure.

Since hyperosmotic stress was also found to induce p38-MAPK phosphorylation in the isolated perfused amphibian heart (Fig. 7), our findings indicate a possible involvement of both p38-MAPK and ANP in the regulation of the biochemical events triggered under such stressful stimuli *in vivo*. Furthermore, the complete inhibition by 1 μmol l^{-1} SB203580 of both p38-MAPK phosphorylation and ANP accumulation in hearts perfused with 0.5 mol l^{-1} sorbitol supports the suggestion that these processes may be linked.

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