α₁- and β-adrenoceptor stimulation differentially activate p38-MAPK and atrial natriuretic peptide production in the perfused amphibian heart

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Summary

We investigated the activation of p38-MAPK by various adrenergic agents in the perfused *Rana ridibunda* heart. Phenylephrine ($50\,\mu mol\,l^{-1}$) rapidly induced the differential activation of all three mitogen-activated protein kinase (MAPK) subfamilies (ERK, JNKs and p38-MAPK) in this experimental system. Focusing on p38-MAPK response to phenylephrine, we found that the kinase phosphorylation reached maximal values at 30 s, declining thereafter to basal values at 15 min. p38-MAPK activation by phenylephrine was verified as exclusively α₁-AR-mediated. Furthermore, SB203580 ($1\,\mu mol\,l^{-1}$) abolished the kinase phosphorylation by phenylephrine. Isoproterenol ($50\,\mu mol\,l^{-1}$) was also shown to activate p38-MAPK in a time- and temperature-dependent manner. A marked, sustained p38-MAPK activation profile was observed at 25 °C, while at 18 °C the kinase response to isoproterenol was modest. Isoproterenol effect on p38-MAPK stimulation was β-AR-mediated. Immunohistochemical studies revealed the enhanced presence of phosphorylated p38-MAPK and atrial natriuretic peptide (ANP) in both phenylephrine- and isoproterenol-stimulated hearts, a reaction completely blocked by the respective specific antagonists, or the specific p38-MAPK inhibitor SB203580. These findings indicate a functional correlation between p38-MAPK activation and ANP accumulation in the perfused amphibian heart.

Key words: p38-MAPK, adrenoceptor, amphibian heart, atrial natriuretic peptide, adrenergic agonist, *Rana ridibunda*.

Introduction

A significant role for adrenergic agonists has been established in the mammalian myocardium, since these compounds are involved in the regulation of its metabolic, contractile and conduction properties through stimulation of α- and β-adrenoceptors (ARs) (Endoh, 1991; Li et al., 1997; Varma and Deng, 2000). An emerging number of reports indicate that mammalian heart α- and β-AR signal transduction pathways are diverse, leading to a variety of actions (Terzic et al., 1993; Xiao and Lakatta, 1993; Altschuld et al., 1995; Zhong and Minneman, 1999; Varma and Deng, 2000).

The neurotransmitter released in amphibians is epinephrine, which exerts its actions mainly *via* β-ARs (Stene-Larson and Helle, 1978; Herman and Sandoval, 1983; Herman and Mata, 1985). However, existing data concerning the morphological and functional relationships of amphibian heart α- and β-adrenoceptors is limited and, to a certain extent, controversial. Thus, although it is generally accepted that the anuran heart contains predominantly β-ARs (Stene-Larson and Helle, 1978), which are considered to be 'beta-2-like' (Stene-Larson and Helle, 1978; Hieble and Ruffolo, 1991), the presence of α-ARs has been questioned by several investigators (Benfey,

1982; Ask, 1983). However, in a recent study we confirmed the presence of α₁-ARs in *Rana ridibunda* hearts by competitive binding assay. In addition, we showed that these receptors are functional and coupled to phosphoinositide hydrolysis (Lazou et al., 2002).

A large number of reports have already demonstrated that MAPKs (mitogen-activated protein kinases) are also included among the multiple signal transduction pathways activated by AR-stimulation in the mammalian heart (Bogoyevitch et al., 1993, 1995; Yamazaki et al., 1997; Clerk et al., 1998; Lazou et al., 1998). In this large family of widely expressed protein kinases, three subfamilies have been clearly identified: extracellular signal-related kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 reactivating kinases (p38-MAPKs). ERKs are largely involved in cell growth, division and differentiation, whereas JNKs and p38-MAPKs respond preferentially to cellular stresses (Schaeffer and Weber, 1999; Widmann et al., 1999). Members of all three subfamilies are present in the heart (Widmann et al., 1999; Bogoyevitch, 2000; Kyriakis and Avruch, 2001); however, the exact role of each subfamily has not yet been clarified and their coupling to ARs

appears to be cell-type-specific. Therefore, it was of great interest to assess their possible involvement in AR signalling in the amphibian heart, given that its physiology is fundamentally different from that of mammals. In preliminary experiments we assessed the activation of all three MAPK subfamilies by adrenergic agonists in the amphibian heart. However, since ERKs responded in a constant and sustained manner and JNKs activation was moderate, we focussed on p38-MAPK activation under these conditions.

In rat hearts, an additional effect mediated by α- as well as β-AR is the release of atrial natriuretic peptide (ANP) after infusion or heart perfusion with respective agonists (Currie and Newman, 1986; Garcia et al., 1986). However, the role of this hormone as a modulator of the various AR-stimulated effects on heart muscle has not yet been clarified (Ruskoaho, 1992). We have already shown the presence of ANP in the isolated perfused amphibian heart (Aggeli et al., 2002). Since the MAPKs signalling pathway was recently suggested to be involved in its regulation (Thuerauf et al., 1998; Ng et al., 2001), we also studied the ANP presence and localisation pattern in sections from AR-stimulated frog hearts.

Our results demonstrate, for the first time in the amphibian heart, that both α- and β-adrenergic agonists activate p38-MAPK in a diverse, time-dependent manner. Activation by phenylephrine is rapid and transient, whereas activation by isoproterenol is more sustained and temperature-dependent. In addition, the enchanced presence of ANP observed in both phenylephrine (PHE)- and isoproterenol (ISO)-stimulated amphibian hearts was blocked by $1\,\mu mol\,l^{-1}$ SB203580. These results provide evidence of a direct link between p38-MAPK signalling pathway and ANP production under these conditions.

Materials and methods
Materials

Most biochemicals used were obtained from Sigma Chemical Co. (St Louis, USA). The enhanced chemiluminescence (ECL) kit was from Amersham International (Uppsala 751 84, Sweden) and alkaline phosphatase Kwik kit from Lipshaw (Pittsburgh, USA). Bradford protein assay reagent was from Bio-Rad (Hercules, California 94547, USA). Nitrocellulose ($0.45\,\mu m$) was obtained from Schleicher & Schuell (Keene N.H. 03431, USA). SB203580 was obtained from Calbiochem-Novabiochem (La Jolla, CA, USA).

Rabbit polyclonal antibodies specific for total ERKs and p38-MAPK, as well as for the dual-phosphorylated ERKs, p38-MAPK and JNKs, were obtained from New England Biolabs (Beverly, MA, USA). The antibody raised against a peptide corresponding to an amino acid sequence mapping at the carboxy terminus of JNK1 of human origin, was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibody to 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 13×18 cm and Elite chrome 100 films were purchased from Eastman Kodak Company (New York, USA).

Animals

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

Heart perfusions

R. ridibunda hearts were perfused in 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, $1.8\,mmol\,l^{-1}$ $MgCl_2$, $0.6\,mmol\,l^{-1}$ 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. At the end of the equilibration period, hearts were perfused with $50\,\mu mol\,l^{-1}$ of either PHE or ISO for a further 0.5–60 min period, in the presence or absence of various antagonists. When used, adrenergic antagonists were added 15 min prior to the α_1- or β-AR agonists and were present throughout the perfusion with the latter. In particular, depending on the time point of maximal p38-MAPK activation, perfusion with antagonists in the presence of PHE or ISO lasted 0.5 or 5 min, respectively. Similarly, hearts perfused with $0.5\,mol\,l^{-1}$ sorbitol for 15 min after the equilibration period were used as positive controls. When the inhibitor SB203580 was used, it was added 15 min before the addition of PHE and was present at a concentration of $1\,\mu mol\,l^{-1}$ throughout the perfusion with this α_1-AR agonist. PHE (a selective α_1-AR agonist), ISO (a nonselective β-AR agonist) and propranolol (a nonselective β-AR antagonist) were dissolved in $100\,\mu mol\,l^{-1}$ L-ascorbic acid and used fresh daily. Prazosin (a selective α_1-AR antagonist), yohimbine (a selective α_2-AR antagonist) and phentolamine (a nonselective α-AR antagonist) were dissolved in dimethylsulfoxide (DMSO). In control experiments, we established the effect of ascorbic acid, DMSO or various AR-antagonists on the variables measured.

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 pulverized under liquid N_2 and powders were stored at –80 °C.

Tissue extractions

Heart powders were homogenized 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, $200\,\mu mol\,l^{-1}$ leupeptin, $120\,\mu mol\,l^{-1}$ pepstatin A, $10\,\mu mol\,l^{-1}$ trans-epoxy succinyl-L-leucylamido-

Ai

Aii

Aiii

Bi

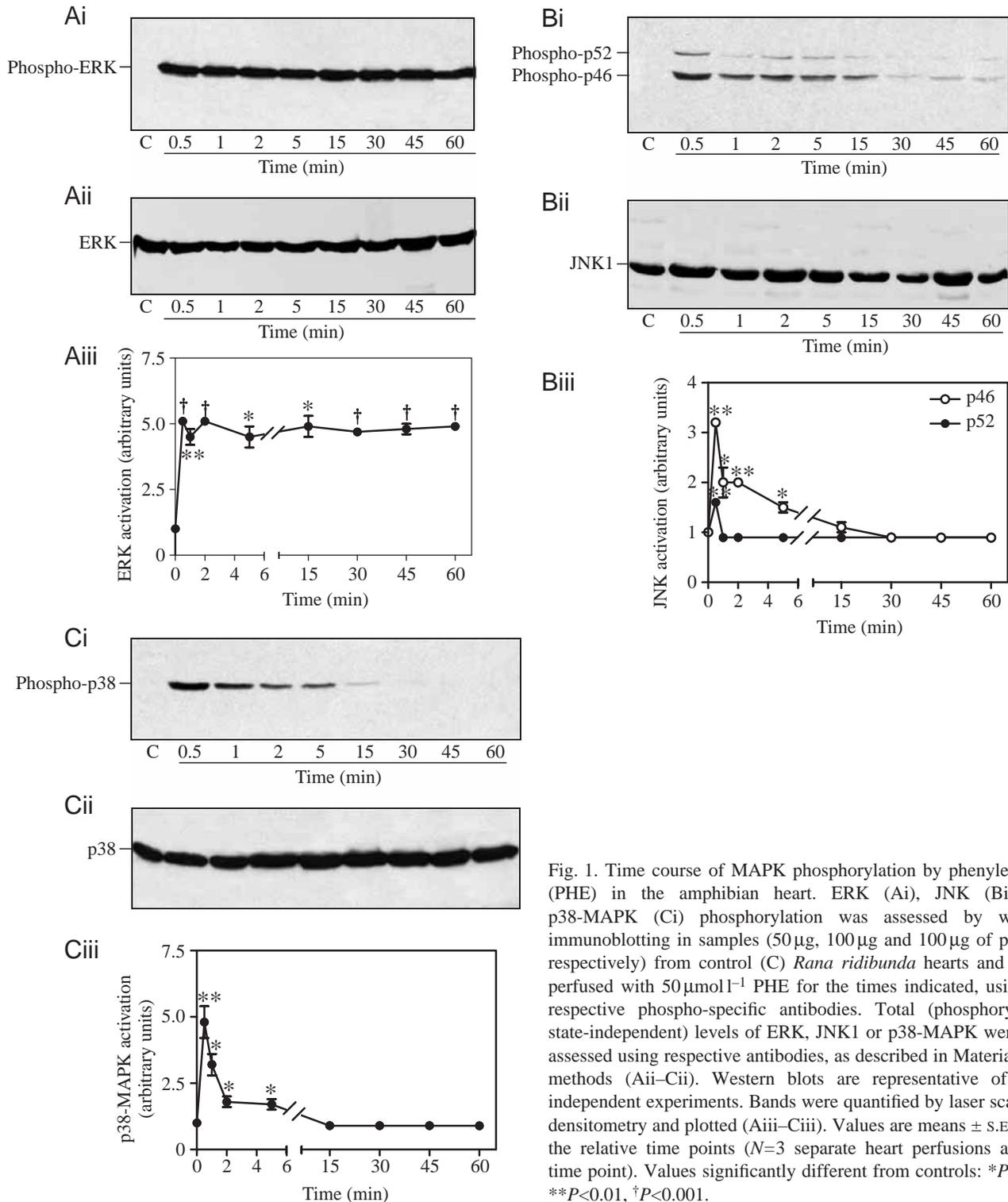Bii

Biii

Ci

Cii

Ciii

Fig. 1. Time course of MAPK phosphorylation by phenylephrine (PHE) in the amphibian heart. ERK (Ai), JNK (Bi) and p38-MAPK (Ci) phosphorylation was assessed by western immunoblotting in samples (50 μg, 100 μg and 100 μg of protein, respectively) from control (C) *Rana ridibunda* hearts and hearts perfused with 50 μmol l^{-1} PHE for the times indicated, using the respective phospho-specific antibodies. Total (phosphorylation state-independent) levels of ERK, JNK1 or p38-MAPK were also assessed using respective antibodies, as described in Materials and methods (Aii–Cii). Western blots are representative of three independent experiments. Bands were quantified by laser scanning densitometry and plotted (Aiii–Ciii). Values are means ± S.E.M. for the relative time points (*N*=3 separate heart perfusions at each time point). Values significantly different from controls: *P<0.05, **P<0.01, $^{†}P$<0.001.

(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.

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 TBS-T [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 TBS-T (3× 5 min) the blots were incubated with horseradish peroxidase-linked anti-rabbit IgG antibodies (1:5000 dilution in TBS-T containing 1 % (w/v) non-fat milk powder, 1 h, room temperature). The blots were washed again in TBS-T (3× 5 min) and the bands were detected using ECL with exposure to X-OMAT AR film. Blots were quantified by laser scanning densitometry.

Immunolocalisation of phospho-p38 MAPK and atrial natriuretic peptide

At the end of the perfusions, atria were removed and ventricles were immersed in Uvasol/isopentane pre-cooled in liquid N₂ and stored at –80 °C. Tissues were sectioned with a cryostat at a thickness of 5–6 μm, fixed with ice-cold acetone (10 min, at room temperature) and specimens were stored at –30 °C until use. Tissue sections were washed in TBS-T and non-specific binding sites were blocked with 3 % (w/v) BSA in TBS-T (1 h, at room temperature). Specimens were incubated with primary antibody specific either for phospho-p38-MAPK or for human ANP (1–28), diluted in 3 % BSA (w/v) in TBS-T (overnight, 4 °C), according to the method previously described (Aggeli et al., 2002). 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 and photographed with a Kodak Elite chrome 100 film.

Statistical evaluations

All data are presented as means ± S.E.M. 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. MAPK activation in 'control' hearts was set at 1, and the stimulated MAPK activation in treated hearts was expressed as -fold activation over 'control' hearts.

Results

Activation of ERK, JNKs and p38-MAPK was studied in extracts from frog hearts perfused under control conditions or in the presence of 50 μmol l⁻¹ PHE (a selective α₁-AR agonist). Initially, we determined the time course of PHE effects on the phosphorylation of these kinases, using western blotting with antibodies detecting the respective dual-phosphorylated species. PHE induced a rapid (30 s) maximal activation of the three subfamilies examined (5.1±0.4-fold for p43-ERK, 3.2±0.3-fold for p46-JNK, 1.6±0.1-fold for p52-JNK and
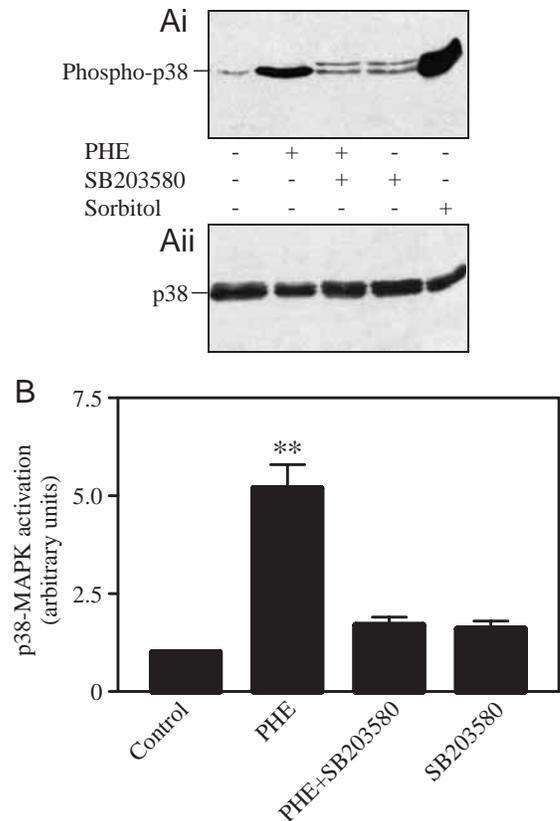

Fig. 2. Effect of SB203580 on p38-MAPK activation by phenylephrine (PHE). SB203580 (1 μmol l⁻¹) was added after a 15 min equilibration period and was present throughout the experiment. Samples (100 μg of protein) from control *Rana ridibunda* hearts, hearts perfused with 0.5 mol l⁻¹ sorbitol and hearts perfused with 50 μmol l⁻¹ PHE in the presence or absence of SB203580 were assayed for p38-MAPK phosphorylation (Ai) as well as for total p38-MAPK immunoreactivity (Aii). The experiment was repeated on two further occasions with similar results. Bands were quantified by laser scanning densitometry and plotted (B). **Values significantly different from controls ($P<0.01$).

4.8±0.6-fold for p38-MAPK, relative to controls). The ERK response remained elevated over 60 min (4.9±0.2-fold, relative to controls), whereas JNK and p38-MAPK phosphorylation levels declined considerably after 5 min, reaching basal values at 30 and 15 min respectively (Fig. 1Ai–Ci,Aiii–Ciii). Equivalent protein loading was confirmed using antibodies recognising total (phosphorylation state-independent) levels of ERK, JNK1 or p38-MAPK (Fig. 1Aii–Cii, respectively). In control experiments, no activation of the kinases examined was observed even in samples from hearts perfused for as long as 60 min (data not shown).

Although ERK phosphorylation by PHE was quite robust, the activation profile observed was constant and sustained over the time points examined, which is a common, widespread response of ERKs to various stimuli examined (Steinberg, 2000). On the other hand, JNK phosphorylation by this agonist was low compared to previous reports of the response of this kinase to various stressful stimuli such as hyperosmotic stress

or mechanical overload (Aggeli et al., 2001a,b). Therefore, we focused on the response of p38-MAPK to adrenergic stimulation.

SB203580 ($1\,\mu mol\,l^{-1}$ in DMSO), a widely used specific inhibitor of p38-MAPK shown to selectively inhibit the kinase activation in this physiological setting (Aggeli et al., 2001a), abolished p38-MAPK phosphorylation by $50\,\mu mol\,l^{-1}$ of PHE (Fig. 2Ai,B). To ensure that equal amounts of protein were loaded, western blots were also performed using an antibody detecting total (phosphorylation state-independent) kinase levels (Fig. 2Aii). Inclusion of SB203580 alone in the perfusion medium had a slight effect on p38-MAPK phosphorylation, which was taken into consideration during the evaluation of the inhibitor's effect on the kinase activation.

To confirm that the effect of PHE on p38-MAPK phosphorylation was α_1-receptor-mediated, hearts were perfused with $50\,\mu mol\,l^{-1}$ of this agonist in the presence of various AR antagonists. Prazosin ($1\,\mu mol\,l^{-1}$), a selective α_1-AR antagonist, and phentolamine ($1\,\mu mol\,l^{-1}$), a nonselective α-AR reversible antagonist, completely inhibited p38-MAPK phosphorylation (hence activation) by PHE (Fig. 3Ai,Aii,C). In addition, phosphorylation levels of the kinase were not altered in the presence of $1\,\mu mol\,l^{-1}$ yohimbine, a specific α_2-AR antagonist (Fig. 3Aiii,C), nor in the presence of $1\,\mu mol\,l^{-1}$ propranolol, a nonselective β-AR antagonist (Fig. 3Aiv,C). These results indicate that in this experimental model, p38-MAPK activation by PHE is mediated *via* α_1-ARs. In Fig. 3B, total p38-MAPK immunoreactivity in identical heart samples is shown.

The time course of the ISO effect on p38-MAPK phosphorylation was also examined using antibodies detecting the respective dual-phosphorylated species. Western blotting was performed with extracts from hearts perfused under control conditions or in the presence of $50\,\mu mol\,l^{-1}$ ISO. At 25°C, the normal perfusion temperature used in our experiments, a strong activation of p38-MAPK was detected from as early as 30 s (4.5 ± 0.3-fold, relative to controls), which maximised at 5 min (6.5 ± 0.4-fold, relative to controls). The kinase phosphorylation was sustained at considerable levels for at least 30 min (6.0 ± 0.4-fold, relative to controls), reaching basal values after 60 min (Fig. 4A,C).

The cardiac effects of ISO in amphibians are markedly dependent on temperature (Volkmann, 1985). Therefore, p38-MAPK activation by this agonist was also examined at a lower temperature (18°C). Under these conditions, the kinase phosphorylation profile was different, with a slight but significant increase detected from as early as

1 min (2.3 ± 0.2-fold, relative to controls, $P<0.01$) and maximal activation attained at 5 min (4.7 ± 0.3-fold, relative to controls). Subsequently, p38-MAPK phosphorylation levels declined, reaching basal values at 30 min (1.6 ± 0.1-fold, relative to controls) (Fig. 4Bi,C). Equivalent protein loading was confirmed using antibodies recognising total (phosphorylation state-independent) levels of p38-MAPK (Fig. 4Bii). No
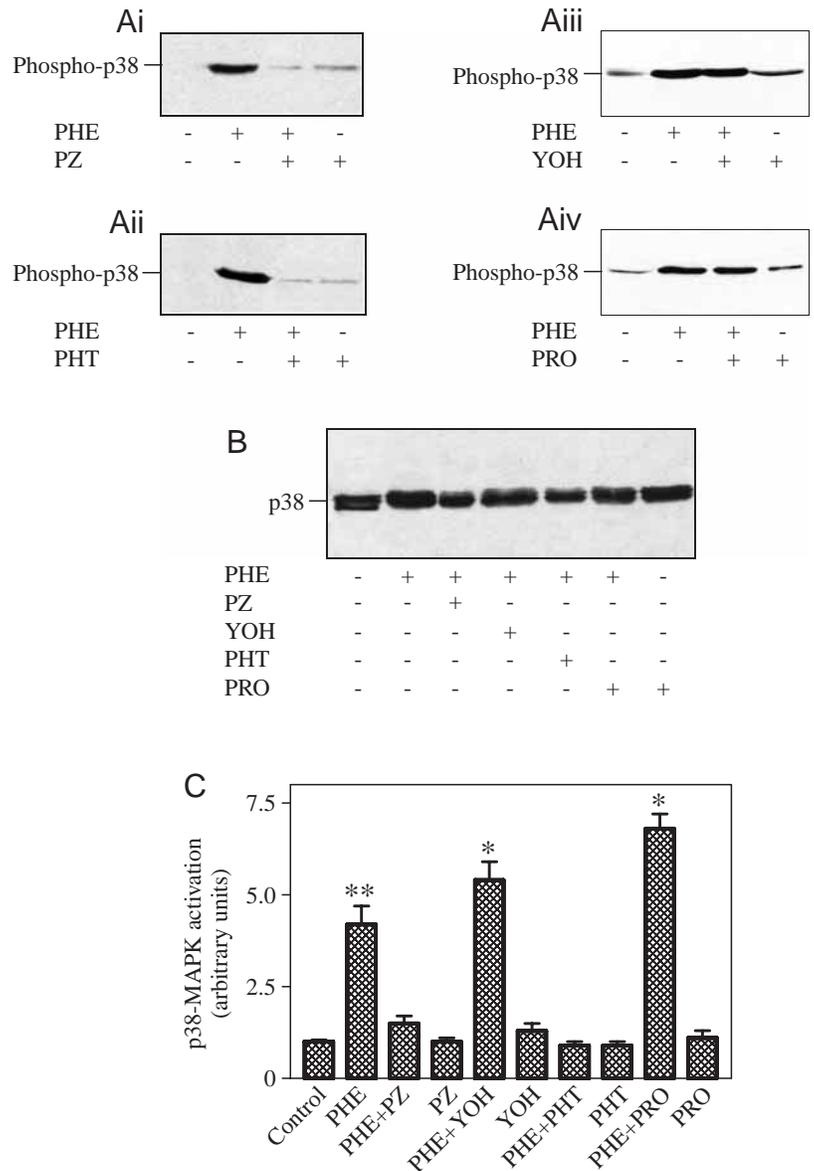
Fig. 3. Effect of α- and β-adrenergic antagonists on p38-MAPK stimulation by phenylephrine (PHE). (A) p38-MAPK phosphorylation was assessed by western immunoblotting in samples (100 μg of protein) from control *Rana ridibunda* hearts and hearts perfused with $50\,\mu mol\,l^{-1}$ PHE in the presence or absence of $1\,\mu mol\,l^{-1}$ prazosin (PZ) (Ai), $1\,\mu mol\,l^{-1}$ phentolamine (PHT) (Aii), $1\,\mu mol\,l^{-1}$ yohimbine (YOH) (Aiii) and $1\,\mu mol\,l^{-1}$ propranolol (PRO) (Aiv). Western blot analysis was performed as described in Materials and methods. Identical samples were also assayed for total p38-MAPK immunoreactivity (B). Each experiment was repeated on two further occasions with similar results. Bands were quantified by laser scanning densitometry and plotted (C). Asterisks indicate values significantly different from controls: *$P<0.05$, **$P<0.01$.
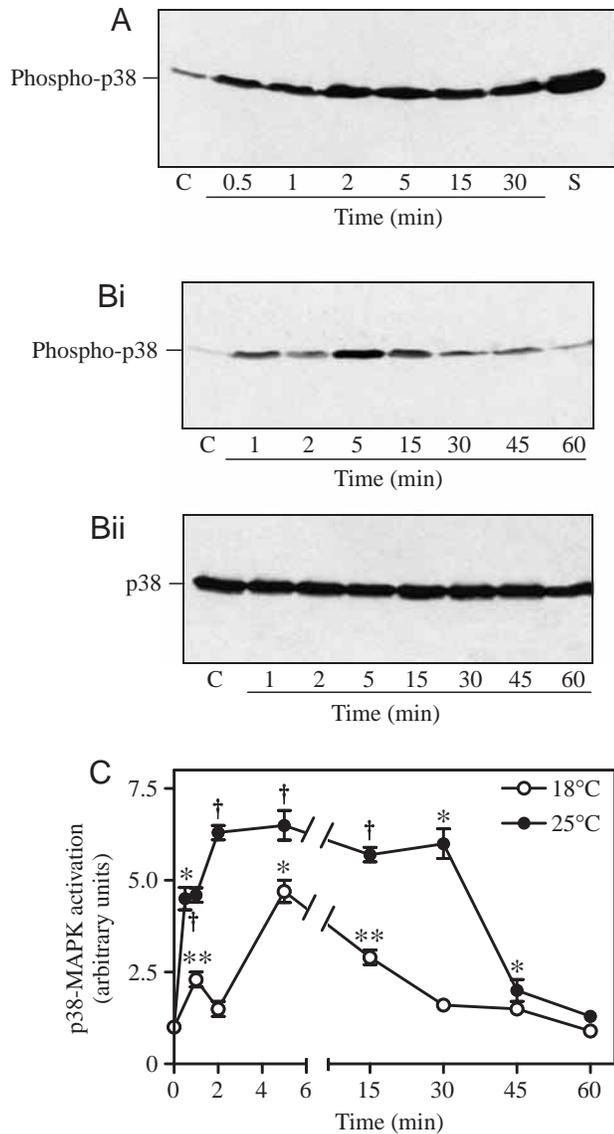
A

Phospho-p38 —

C 0.5 1 2 5 15 30 S

Time (min)

Bi

Phospho-p38 —

C 1 2 5 15 30 45 60

Time (min)

Bii

p38 —

C 1 2 5 15 30 45 60

Time (min)

C

Fig. 4. Time course of p38-MAPK activation by isoproterenol (ISO). p38-MAPK phosphorylation was assessed by western immunoblotting in samples ($100\,\mu g$ of protein) from control (C) *Rana ridibunda* hearts, hearts perfused with $0.5\,mol\,l^{-1}$ sorbitol (S) and hearts perfused with $50\,\mu mol\,l^{-1}$ ISO for the times indicated at 25° (A) and $18^\circ C$ (Bi), using a phospho-specific p38-MAPK antibody. Total kinase levels were also assayed (Bii). Bands were quantified by laser scanning densitometry and plotted (C). Values are means ± S.E.M. for the relative time points (*N*=3 separate heart perfusions at each time point). Values significantly different from controls: *P<0.05, **P<0.01, †P<0.001.

temperature-dependent effect was observed in respective samples from PHE-stimulated hearts (data not shown).

We confirmed that p38-MAPK activation by ISO was mediated through β-ARs, by perfusion of hearts (at $25\,^\circ C$) with $50\,\mu mol\,l^{-1}$ of this agonist in the presence of $1\,\mu mol\,l^{-1}$ propranolol (a β-AR antagonist). This antagonist completely inhibited p38-MAPK phosphorylation by ISO (Fig. 5Ai,B), while prazosin ($10\,\mu mol\,l^{-1}$), a selective α_1-AR antagonist, had

no inhibitory effect on the kinase activation by this agonist (Fig. 5Aii,B). Equivalent protein loading was verified in identical heart samples, with an antibody detecting total kinase levels (Fig. 5Aiii).

In order to investigate the localisation pattern of the activated p38-MAPK immunohistochemically, under α_1- or β-AR stimulation, frog hearts were perfused with $50\,\mu mol\,l^{-1}$ of either PHE (for $30\,s$) or ISO (for $5\,min$), in the presence or absence of the respective specific antagonists or $1\,\mu mol\,l^{-1}$ SB203580. After the removal of atria, the ventricle was sectioned and the respective specimens were processed using an antibody specific for the dual-phosphorylated p38-MAPK. No immunoreactivity was detected in control hearts (Figs 6A, 7A), nor in specimens incubated either with the secondary antibody or with the chromogen alone (data not shown). In specimens from hearts perfused with $50\,\mu mol\,l^{-1}$ of either PHE or ISO, immunoreactivity staining was observed within the cytoplasm as well as in the perinuclear region (Figs 6B, 7B). In accordance with the results of the biochemical studies, PHE-induced phospho-p38-MAPK immunostaining was abolished by prazosin and SB203580, while propranolol and SB3203580 completely blocked the ISO-stimulated activation of the kinase (Figs 6C,E, 7D,E). Therefore, PHE effect on p38-MAPK activation was confirmed to be α_1-AR mediated and the ISO effect, β-AR mediated.

Atrial natriuretic peptide (ANP), particularly the circulating 28-amino-acid, biologically active form of this hormone, has been previously reported to be released by α- as well as by β-adrenergic stimulation in rat atria, although the physiological significance of this effect remains questionable. Thus, it was of interest to examine the production and localisation pattern of ANP in sections from *Rana ridibunda* hearts perfused with PHE or ISO. For this purpose, we used an antibody detecting the human (1–28) active form of the peptide, as important homologies exist between the C-terminal regions of mammalian and amphibian ANP (Netchitailo et al., 1988). Our immunohistochemical study in cryosections from hearts revealed considerably enhanced ANP staining in both PHE- and ISO-perfused hearts compared to the controls (Figs 8B, 9B, respectively). A discreet pattern of ANP immunoreactivity was observed in the perinuclear region as well as widely in the cytoplasm. We confirmed that this effect stimulated by PHE was attributed to α_1-ARs, by the significantly decreased staining observed in sections from hearts perfused with this agonist ($50\,\mu mol\,l^{-1}$) in the presence of $1\,\mu mol\,l^{-1}$ prazosin (Fig. 8C), while propranolol ($1\,\mu mol\,l^{-1}$) had no effect on the ANP immunoreactivity detected (Fig. 8D). Furthermore, the increase in ANP immunoreactivity complexes observed in sections from hearts perfused with ISO ($50\,\mu mol\,l^{-1}$), was verified to be β-AR mediated, as $1\,\mu mol\,l^{-1}$ propranolol inhibited this effect (Fig. 9D) in contrast to $10\,\mu mol\,l^{-1}$ prazosin, which resulted in no such inhibition (Fig. 9C). Interestingly, in the presence of SB203580 ($1\,\mu mol\,l^{-1}$), both PHE- and ISO-stimulated ANP accumulation were completely inhibited (Figs 8E, 9E). When sections

were incubated with secondary antibody only, no immunoreactivity was detected (data not shown).

Discussion

We have examined and compared the activation of MAPKs by α_1 and β adrenoceptors in the frog heart. Although α_1 adrenoceptors in amphibian heart have been the subject of debate, we recently showed that their presence suggests a functional role in the frog heart (Lazou et al., 2002). In addition, MAPK activation by β adrenoceptors is not well characterised, even in mammalian heart. Therefore, we first studied the phosphorylation patterns of ERK, JNKs and p38-MAPK by PHE. While ERK activation was markedly constant and sustained, activation of JNKs was quite modest (Fig. 1A,B). On the other hand, maximal phosphorylation of p38-MAPK was rapid, and decreased progressively within 2 min, comparable to the JNKs activation profile (Fig. 1C). This diversity in the extent of activation of MAPKs suggests that they have different α_1-AR mediated regulation mechanisms, while the similar time course of the JNKs and p38-MAPK responses indicates that these two cascades are activated in parallel by PHE. This feature also characterises the respective kinases in rat heart (Lazou et al., 1998), indicative of a possible synergistic effect of these pathways.

Focusing on p38-MAPK, SB203580 was found to abolish the activation of this kinase by PHE in both biochemical and immunohistochemical studies (Figs 2, 6E). Among the several isoforms of the kinase that have been identified (Kumar et al., 1997; Zhong and Minneman, 1999), only two are strongly inhibited by SB203580; α and β_1 (Li et al., 1996; Zhong and Minneman, 1999). Although it was not possible to determine whether any specific p38-MAPK isoform is activated under the conditions examined in this experimental model, our results demonstrate that at least one of the above two is present and responsive to α_1 adrenergic stimulation.

We also investigated the ability of a range of pharmacological inhibitors to interfere with PHE-induced activation of p38-MAPK. Neither propranolol (nonselective β antagonist, $1\,\mu\mathrm{mol}\,l^{-1}$) nor yohimbine ($\alpha_2$ antagonist, $1\,\mu\mathrm{mol}\,l^{-1}$) had any inhibitory effect on the kinase phosphorylation by PHE. In addition, phentolamine (nonselective α antagonist, $1\,\mu\mathrm{mol}\,l^{-1}$) as well as prazosin ($\alpha_1$ selective antagonist, $1\,\mu\mathrm{mol}\,l^{-1}$) both abolished the kinase activation, demonstrating that PHE-induced p38-MAPK activation is α_1-AR mediated (Fig. 3). These data further support a functional role for α_1 adrenoceptors in frog heart.

The temporal profile of p38 MAPK response to ISO was entirely different from the one induced by PHE, with the maximal kinase phosphorylation levels reaching higher values (6.5 ± 0.4 and 4.8 ± 0.6-fold relative to controls, respectively) and remaining elevated over a considerably longer period of time (Figs 1C *versus* 4A). Stimulation with ISO was found to considerably increase the contractility of our experimental setting, whereas PHE did not. This effect could account to a certain extent for the different p38-MAPK activation profiles induced by the stimulation of the two types of adrenoceptors. However, recent data show a rapid phosphorylation profile of p38 MAPK by mechanical overload (Aggeli et al., 2001b), in contrast to the sustained one triggered by β-AR stimulation. Therefore, factors other than contractility may account for the distinct response of the kinase under α_1- and β-AR stimulation. That ISO-induced p38-MAPK activation is mediated *via* β-ARs was also confirmed by its complete inhibition by propranolol (β-AR antagonist) and the
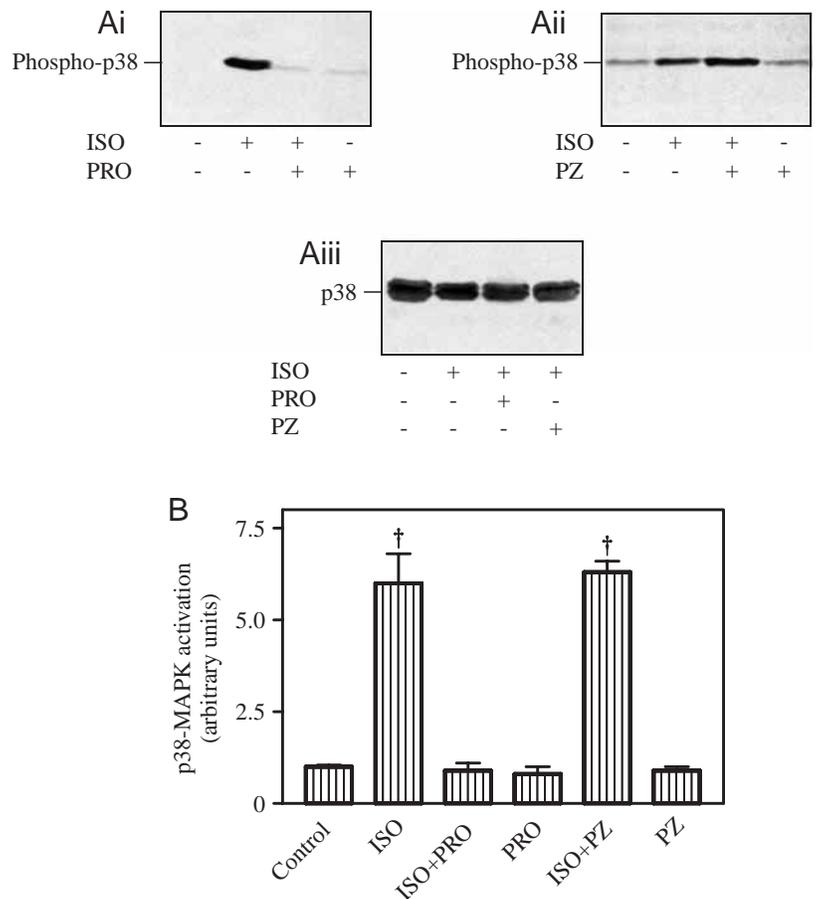

Fig. 5. Effect of α- and β-AR antagonists on p38-MAPK stimulation by isoproterenol (ISO). p38-MAPK phosphorylation was assessed by western immunoblotting in samples ($100\,\mu g$ of protein) from control *Rana ridibunda* hearts and hearts perfused with $50\,\mu\mathrm{mol}\,l^{-1}$ ISO in the presence or absence of either $1\,\mu\mathrm{mol}\,l^{-1}$ propranolol (PRO) (Ai) or $10\,\mu\mathrm{mol}\,l^{-1}$ prazosin (PZ) (Aii). Western blot analysis was performed as described in Materials and methods. Identical samples were also assayed for total p38-MAPK immunoreactivity (Aiii). Values are means $\pm$ S.E.M. Each experiment was repeated on at least two further occasions with similar results. Bands were quantified by laser scanning densitometry and plotted (B). Values significantly different from controls: $^{\dagger}P<0.001$.
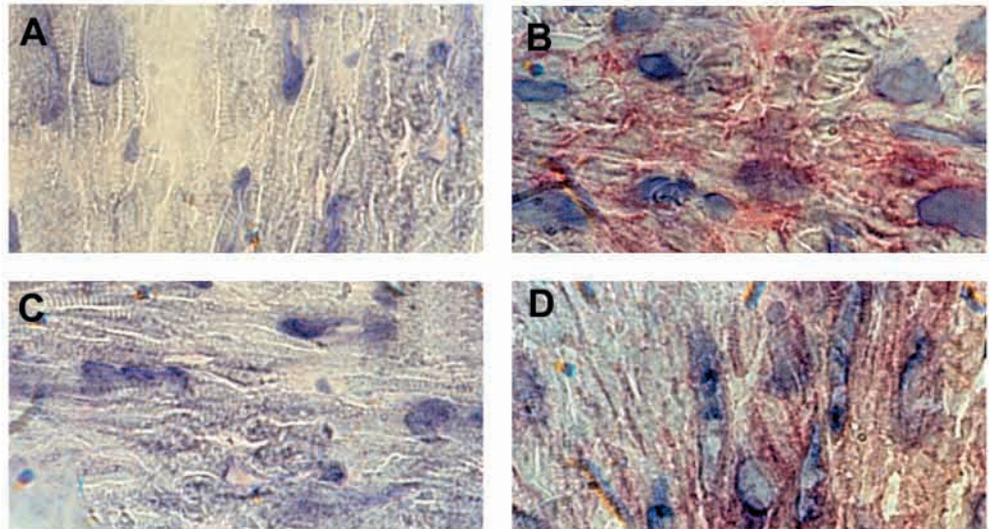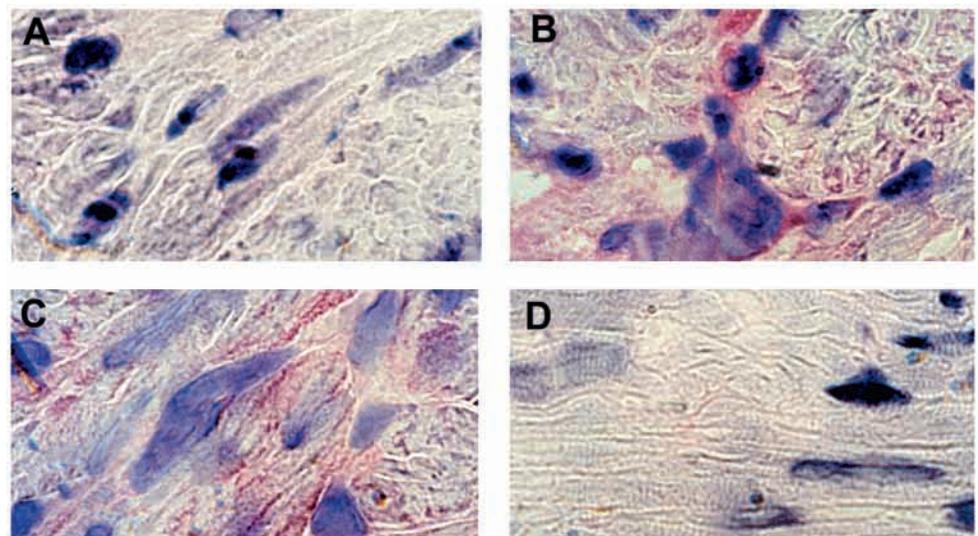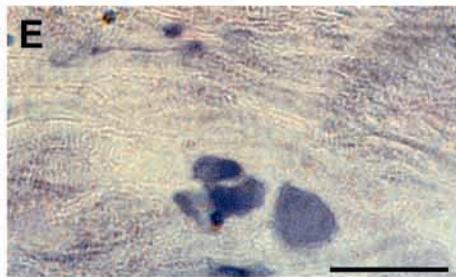
Fig. 6. Immunohistochemical localisation of phosphorylated p38-MAPK in the ventricle of isolated amphibian heart perfused under normal conditions (A) and with $50\,\mu\mathrm{mol\,l^{-1}}$ of phenylephrine (PHE) for 0.5 min in the absence (B) or presence of either $1\,\mu\mathrm{mol\,l^{-1}}$ prazosin (C), $1\,\mu\mathrm{mol\,l^{-1}}$ propranolol (D) or $1\,\mu\mathrm{mol\,l^{-1}}$ SB203580 (E). Cryosections were incubated with phospho-p38 MAPK specific antibody (1:200 dilution) and counterstained with Haematoxylin. Representative photographs from three independent experiments performed with similar results are shown. Immunolocalisation deposits are visualised with Fast Red chromogen. Bar, 20 μm.
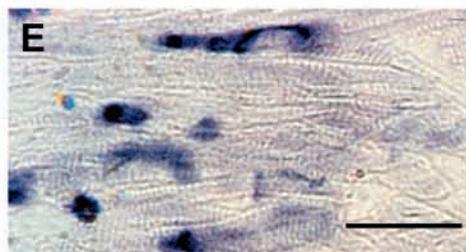

Fig. 7. Immunohistochemical localisation of phosphorylated p38-MAPK in the ventricle of isolated amphibian heart perfused under normal conditions (A) and with $50\,\mu\mathrm{mol\,l^{-1}}$ of isoproterenol (ISO) for 5 min in the absence (B) or presence of either $10\,\mu\mathrm{mol\,l^{-1}}$ prazosin (C), $1\,\mu\mathrm{mol\,l^{-1}}$ propranolol (D) or $1\,\mu\mathrm{mol\,l^{-1}}$ SB203580 (E). Cryosections were incubated with phospho-p38 MAPK specific antibody (1:200 dilution) and counterstained with Haematoxylin. Representative photographs from three independent experiments performed with similar results are shown. Immunolocalisation deposits are visualised with Fast Red chromogen. Bar, 20 μm.
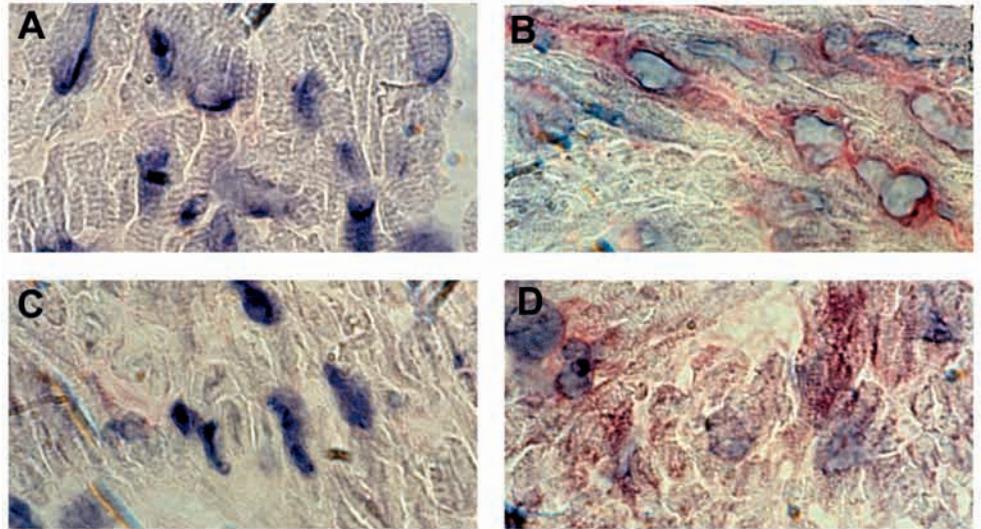
Fig. 8. ANP immunolocalisation in the ventricle of isolated amphibian heart perfused with phenylephrine (PHE) and various adrenoceptor antagonists or SB203580. Hearts were perfused under normal conditions (A) and with $50 \, \mu mol \, l^{-1}$ of PHE for 0.5 min in the absence (B) or presence of either $1 \, \mu mol \, l^{-1}$ prazosin (C), $1 \, \mu mol \, l^{-1}$ propranolol (D) or $1 \, \mu mol \, l^{-1}$ SB203580 (E). Cryosections were incubated with an antibody specific for human atrial natriuretic peptide (ANP 1–28) (1:500 dilution) and counterstained with Haematoxylin. Immunoreaction deposits for ANP were visualised with Fast Red Chromogen. Representative photographs from three independent experiments are shown. Bar, $20 \, \mu m$.
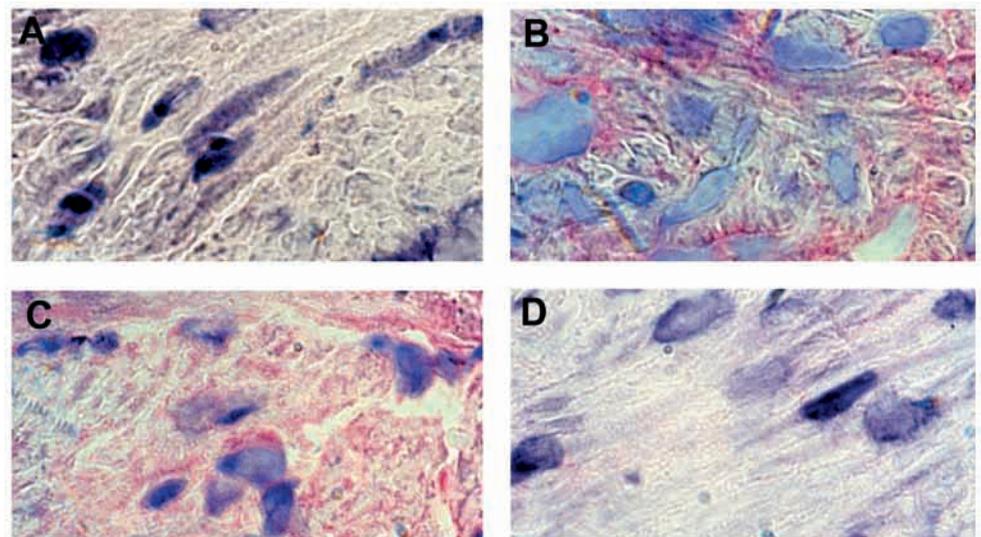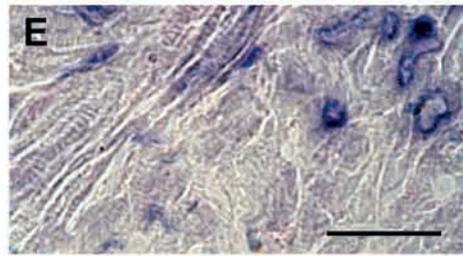

Fig. 9. ANP immunolocalisation in the ventricle of isolated amphibian heart perfused with isoproterenol (ISO) and various adrenoceptor antagonists or SB203580. Hearts were perfused under normal conditions (A) and with $50 \, \mu mol \, l^{-1}$ of ISO for 5 min in the absence (B) or presence of either $10 \, \mu mol \, l^{-1}$ prazosin (C), $1 \, \mu mol \, l^{-1}$ propranolol (D) or $1 \, \mu mol \, l^{-1}$ SB203580 (E). Cryosections were incubated with an antibody specific for human ANP (1–28) (1:500 dilution) and counterstained with Haematoxylin. Immunoreaction deposits for ANP were visualised with Fast Red chromogen. Representative photographs from three independent experiments are shown. Bar, $20 \, \mu m$.
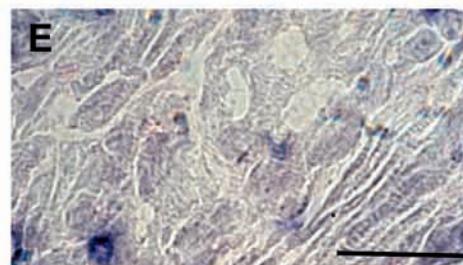
lack of an analogous effect by prazosin (α_1-AR antagonist) (Fig. 5). The immunohistochemical data correlated with the above results. Thus, ISO was found to induce a more intense p38-MAPK response than PHE (Figs 7B *versus* 6B), and these effects were mediated by β- and α_1-ARs, respectively (Figs 7C,D *versus* 6C,D). p38-MAPK response to ISO was found to be temperature-dependent, a feature not observed with PHE stimulation. Previous studies have shown that ISO induces subcellular damage in ectotherms at 25 °C (Volkmann, 1985; Herman et al., 1986). Therefore, the sustained, marked p38-MAPK phosphorylation by ISO observed at 25 °C (Fig. 4), which was completely different from the response at 18 °C (Fig. 4), may indicate that the kinase exerts a protective role under such conditions. A protective role for p38-MAPK has been also described in adult rat cardiomyocytes stimulated with ISO (Communal et al., 2000).

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. Adrenergic compounds exert a positive inotropic effect on vertebrate cardiac muscle and ANP has been shown to function as a potential modulator of systemic blood pressure in AR-stimulated rat heart (Lang et al., 1985; Currie and Newman, 1986; Garcia et al., 1986). In frogs, immunoreactive ANP is detected in both atrial and ventricular cardiac myocytes (Mifune et al., 1996). Therefore, it was interesting to investigate the immunolocalisation pattern of this hormone in specimens from control as well as AR-stimulated amphibian heart ventricles. The enhanced presence of ANP observed in specimens from hearts perfused with PHE as well as ISO could reflect an involvement of this peptide hormone in the cardiac muscle response to the haemodynamic changes induced under such conditions (Figs 8B, 9B). Furthermore, since ANP immunostaining was considerably decreased in specimens from hearts perfused with PHE or ISO in the presence of their respective antagonists (Figs 8C, 9D), ANP accumulation seems to constitute a direct stimulation effect by AR, in this specific experimental model.

Certain forms of stress inducing p38-MAPK activation have been found to lead to the transcriptional activation of several genes with a probable compensatory effect, including the ANP gene (Thuerauf et al., 1998). In light of the observed inhibition of ANP immunoreactivity by SB203580, a p38-MAPK pathway regulatory role in AR-stimulated ANP production could be proposed. However, since the increase in ANP immunoreactivity induced by PHE or ISO is quite rapid, it is more likely to be a consequence of the release of this peptide hormone from its storage granules (Mifune et al., 1996; Ruskoaho, 1992), rather than an enhancement of ANP synthesis. It is therefore evident that further investigation is required in order to detect any involvement of p38-MAPK in the transcriptional activation of ANP. In addition, since the localisation patterns of p38 MAPK and ANP seem to be similar to a certain extent, a more thorough immunohistochemical study *via* electron transmission microscopy, could reveal any physical correlation between them.

In conclusion, the present study demonstrates that stimulation by both α_1- and β-AR differentially activates p38-MAPK in the amphibian heart. Furthermore, a functional link between α_1- as well as β-AR-induced p38-MAPK stimulation and the enhanced presence of ANP is documented, suggestive of a regulatory role of this hormonal modulator, under these conditions.

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