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Activation of multiple MAPK pathways (ERKs, JNKs, p38-MAPK) by diverse stimuli in the amphibian heart

Ioanna-Katerina S. Aggeli,[1] Catherine Gaitanaki,[1] Antigone Lazou[2] and Isidoros Beis[1]

[1]*Department of Animal and Human Physiology, School of Biology, Faculty of Sciences, University of Athens, Athens;*
[2]*Laboratory of Animal Physiology, School of Biology, Aristotle University of Thessaloniki, Thessaloniki, Greece*

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Abstract

We investigated the expression and activation of three MAPK subfamilies in the isolated perfused amphibian heart. ERK was detected as a 43 kDa band; p38-MAPK was detected as a band corresponding to 38 kDa and JNKs were detected as two bands corresponding to 46 and 52 kDa, respectively. PMA induced the activation of the ERK pathway as assessed by determining the phosphorylation state of ERK and the upstream component MEK1/2. PD98059 abolished this activation. p38-MAPK was phosphorylated by sorbitol (almost 12-fold, maximal within 10–15 min) and JNKs were phosphorylated and activated by sorbitol or anoxia/reoxygenation (approximately 4- and 2.5-fold, respectively). SB203580 completely blocked the activation of p38-MAPK by sorbitol. These results indicate that the MAPK pathways activated by phorbol esters, hyperosmotic stress or anoxia/reoxygenation in the amphibian heart may have an important role in this experimental system. (Mol Cell Biochem **221**: 63–69, 2001)

Key words: mitogen-activated protein kinase, ERKs, JNKs, p38-MAPK, amphibian heart

Abbreviations: DTT – dithiothreitol; DMSO – dimethylsulfoxide; ECL – enhanced chemiluminescence; ERK – extracellular signal-regulated kinase; JNK – c-Jun N-terminal kinase; MAPK – mitogen-activated protein kinase; MEK – MAP kinase kinase; MEKK – MAPK kinase kinase; p38-MAPK, p38 mitogen activated protein kinase; PKI – c-AMP dependent protein kinase inhibitor; PMA– 4β-phorbol 12-myristate 13-acetate; PMSF – phenyl methyl sulphonyl fluoride; SAPK – stress activated protein kinase

Introduction

Mitogen-activated protein kinases (MAPKs) are proline directed serine/threonine protein kinases that transduce signals from the cell membrane to the nucleus in response to a variety of different stimuli and participate in various intracellular signalling pathways that control a wide spectrum of cellular processes including cell growth, differentiation and stress responses (reviewed in [1–3]). MAPK signalling pathways have been implicated in the regulation of the physiological responses of many organisms as diverse as yeast, insects or mammals. The basic assembly of MAPK pathways is a three component module conserved from yeast to humans. This protein kinase cascade comprises of MAPK kinase kinase (also referred to as MEKK), MAPK kinase (also referred to as MEK) and MAPK [4].

Three major subfamilies have been characterized, including the extracellular signal- regulated kinases (ERKs), the c-jun N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPKs) and the p38 mitogen activated pro-

Address for offprints: I. Beis, Department of Animal and Human Physiology, School of Biology, Faculty of Sciences, University of Athens, Athens, Greece 157 84

tein kinases (p38-MAPKs) [1, 5–7]. Although ERKs are mainly involved in mediating anabolic processes such as cell division, growth and differentiation, JNKs and p38-MAPKs are generally associated with cellular response to diverse stresses. Interaction between these pathways takes place at various levels. At stimulus level we find that one stimulus can activate more than one of these parallel pathways. Within one pathway multiple signals may converge at one point. Interaction can also take place at substrate level [8].

Studies on the characterization and function of MAPK modules have been largely conducted using principally mammalian models and to a lesser extent a number of non-mammalian experimental systems including *X. laevis*, *D. melanogaster* and *C. elegans* (reviewed in [3]). Therefore, although these kinases are highly conserved, there is limited information available to indicate their existence in lower vertebrates.

Here, we provide evidence for the expression and activation of the different MAPK subfamilies (ERKs, JNKs, and p38-MAPKs) in the isolated perfused amphibian heart. Lower vertebrates represent an approach to terrestrial life quite different from that adopted by mammals. These organisms that often face various environmental stresses, may thus prove to be a valuable experimental tool that will provide interesting insights on the function and role of MAPK pathways in important physiological processes. Furthermore, isolated perfused heart has been routinely used as a probe for various physiological studies and in contrast to the cell cultures may provide information about the roles of these kinases at the whole organ level.

Materials and methods

Materials

Enhanced chemiluminescence (ECL) kit was from Amersham International (Uppsala, Sweden), [γ-^{32}ATP] was from NEN Life Sciences (Brussels, Belgium). Bradford protein assay reagent was from Bio-Rad (Hercules, CA, USA). Nitrocellulose (0.45 μm) was obtained from Schleicher & Schuell (Keene, NH, USA). SB203580 and PD98059 were obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). General laboratory chemicals were obtained from Sigma Chemical Co (St. Louis, MO, USA).

Rabbit polyclonal antibodies to p44/42 ERKs, MEK1/2 and p38-MAPK were from New England Biolabs (Beverly, MA, USA). The antibodies specific to the dually phosphorylated ERKs, MEK1/2, p38-MAPK, and JNKs, were also obtained from New England Biolabs. 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). Prestained molecular mass markers were from New England Biolabs. Biotinylated anti-rabbit antibody was from DAKO A/S (Glostrup, Denmark). Film (X-OMAT AR 13 × 18 cm) was purchased from Eastman Kodak Company (Rochester, NY, USA). GST (glutathione-S-transerase)-c-Jun (1–135) was kindly provided by Professor P.H. Sugden (Imperial College, London, UK).

Heart perfusions

Amphibian (*Rana ridibunda*, 100–120 g) hearts were perfused with the non-recirculating Langendorff mode at a pressure of 4.5 kPa (31.5 mmHg) with bicarbonate-buffered saline (23.8 mM $NaHCO_3$, 103 mM NaCl, 1.8 mM $CaCl_2$, 2.5 mM KCl, 1.8 mM $MgCl_2$, 0.6 mM NaH_2PO_4 (pH 7.4 at 25°C)) supplemented with 10 mM 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 perfused for 30 min equilibration period under these conditions. 4β-phorbol 12-myristate 13-acetate (PMA) (1 μM) or sorbitol (0.5 M) were added after the equilibration period and hearts were further perfused for increasing time periods varying from 30 sec up to 60 min or 30 sec up to 45 min, respectively. When the inhibitors PD98059 or SB203580 were used, they were added throughout the experiment at a concentration of 25 or 10 μM, respectively. In parallel, hearts were perfused either with the dimethylsulfoxide (DMSO) solvent or the respective inhibitor alone, in order to examine if they affect any of the variables measured. Hearts were subjected to anoxia, which is a more physiological stimulus for the frog, for 15 min by perfusion with bicarbonate-buffered saline equilibrated with 95%N_2/5%CO_2 followed by reoxygenation for increasing time periods varying from 5 up to 45 min.

Adult male (250–300 g) Wistar rat hearts were perfused with the non-recirculating Langendorff mode as previously described [9]. As positive controls, rat hearts perfused with 1 μM PMA for 5 min, or 0.5 M sorbitol for 30 min after the equilibration period were used. Rat hearts were also subjected to global ischemia followed by reperfusion as previously described [9].

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

Preparation of heart samples

Heart powders were homogenized with 3 ml/g of buffer (20 mM Tris-HCl, pH 7.5, 20 mM glycerophosphate, 20 mM

NaF, 2 mM EDTA, 0.2 mM Na_3VO_4, 5 mM dithiothreitol (DTT), 10 mM benzamidine, 200 μM leupeptin, 120 μM pepstatin A, 10 μM trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane, 300 μM 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 were boiled with 0.33 vol of SDS/PAGE sample buffer (0.33 M 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 [10].

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). Western blots for p44/42 ERK, MEK1/2, p38-MAPK and JNK1 were performed with antibodies that detect total kinase (phosphorylation-state independent) levels. Western blots for the activated forms of the kinases examined were performed with antibodies against the dually phosphorylated species, according to the manufacturer's instructions. The bands were detected using the ECL method with exposure to X-OMAT AR film. Blots were quantified by laser scanning densitometry.

JNK activity assay

Proteins (200 μg loaded/lane) were separated on 10% (w/v) SDS-polyacrylamide gels in the presence of 0.5 mg/ml GST-c-Jun (1–135) with 6% (w/v) stacking gel. After electrophoresis, SDS was removed from the gels by washing in 20% (v/v) propan-2-ol in 50 mM Tris-HCl, pH 8.0 (3 × 30 min). The propan-2-ol was removed by washing in 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol (3 × 30 min). Proteins were denatured in 6 M guanidine-HCl, 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol (2 × 30 min) and then renatured in 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 0.04% (v/v) Tween 40 (1 × 30 min, 2 × 1 h, 1 × 18 h, 1 × 30 min, 4°C). The gels were equilibrated to room temperature with 40 mM Hepes (pH 8.0), 2 mM DTT, 10 mM $MgCl_2$ (2 × 30 min) and incubated for 3 h with 12.5 μCi/gel [γ-^{32}P]ATP in 5 ml of 40 mM Hepes (pH 8.0), 0.5 mM EGTA, 10 mM $MgCl_2$, 50 μM ATP, 0.1 μM PKI (c-AMP dependent protein kinase inhibitor). 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.

Results

Antibodies to p44/42 MAPK detected a 43 kDa band in the frog heart while two bands of 44 kDa (ERK1) and 42 kDa (ERK2) are detected in the rat heart (Fig. 1A, top panel). This p43 protein was phosphorylated in response to stimulation with 1 μM PMA which is a powerful activator of cardiac ERKs in mammalian systems (Fig. 1A, bottom panel). The

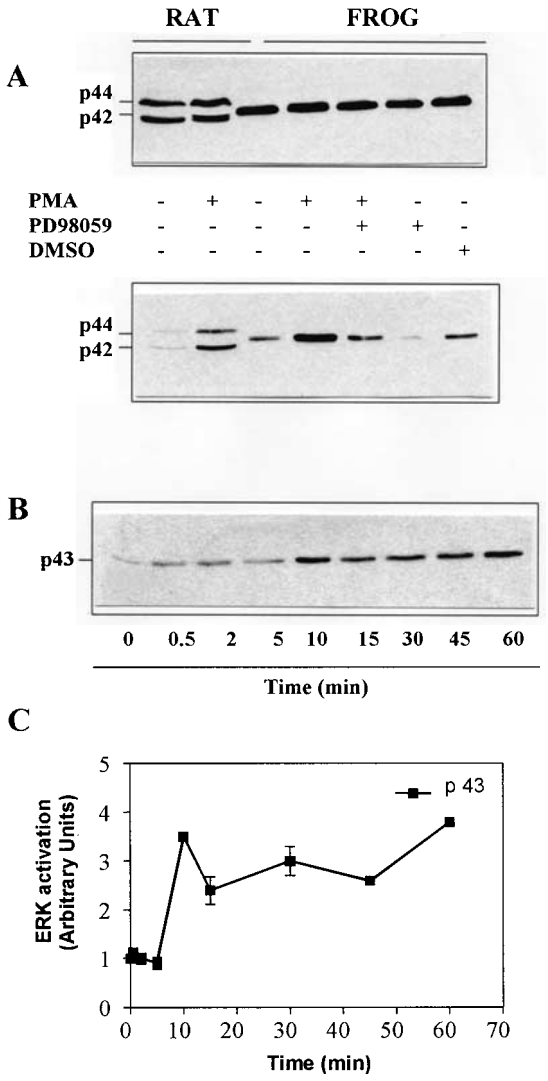

Fig. 1. Expression and phosphorylation of ERK in the perfused frog heart. (A) ERKs were detected in extracts (50 μg) from control frog or rat hearts, hearts perfused with 1 μM PMA for 10 or 5 min (for frog or rat, respectively) and frog hearts perfused with 25 μM PD98059 or 0.1% (v/v) DMSO (the solvent for PD98059) by Western blotting using antibodies specific for p44/42 ERK (top panel) or for the dually phosphorylated ERK1/2 (bottom panel). (B) Time course of ERK phosphorylation by 1 μM PMA in the perfused frog heart. (C) Densitometric analysis of phospho-ERK bands by laser scanning. Results are means ± S.E. for 3 independent observations. Western blots shown are representative of 3 independent experiments.

degree of phosphorylation was comparable to that induced by 1 μM PMA in the rat heart and was completely blocked by 25 μM PD98059, an inhibitor of the ERK pathway. Maximal phosphorylation of the 43 kDa ERK (approximately 4-fold relative to controls) was observed at 10 min and was sustained over the 60 min period studied (Figs 1B and 1C). Activation of MEK1/2, the upstream component of the pathway, was also examined. Antibodies to MEK1/2 detected a double band of approximately 45 kDa (Fig. 2, top panel). However, only the upper band was responsive to PMA treatment as evidenced by immunoblotting using anti-phospho MEK1/2 antibodies. PD98059 abolished this phosphorylation (Fig. 2, bottom panel). Phosphorylation of MEK in the frog heart followed a parallel time course with the 43 kDa ERK (results not shown) and the extent of phosphorylation was also comparable to that observed in the rat heart under the same conditions (Fig. 2, bottom panel).

Antibodies to p38-MAPK detected a 38 kDa band which co-migrated with the rat p38-MAPK (Fig. 3A, top panel). p38-MAPKs are activated by dual phosphorylation in response to various forms of stress (osmotic stress, UV irradiation, ischemia/reperfusion) in many cell types. To determine whether stress also activates p38-MAPK in the amphibian heart, the phosphorylation state of p38-MAPKs was assessed by immunoblotting with an antibody specific for the dually phosphorylated (activated) form of the kinase. Sorbitol (0.5 M) induced a rapid increase in p38-MAPK phosphorylation that was comparable to that induced in the rat heart (Fig. 3A, bottom panel). Interestingly, an additional protein band corresponding to ~39 kDa was also detected, exclusively in the frog heart. The phosphorylation of p38 and p39 was completely blocked by 10 μM SB203580, a specific p38-MAPK inhibitor (Fig. 3A, bottom panel). This finding indicates that p39 possibly represents a different isoform of p38-MAPK, equally sensitive to SB203580. The time course for the activation of p38-MAPK by sorbitol showed that phosphorylation was maximal within 10–15 min (approximately 12-fold relative to controls) and remained elevated at almost the same level for at least 45 min (Figs 3B and 3C). On the other hand, the degree of p38-MAPK phosphorylation showed no significant change by either anoxia alone or anoxia/reoxygenation (data not shown).

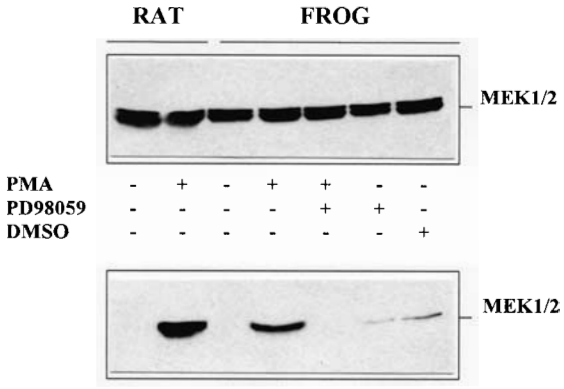

Fig. 2. Expression and phosphorylation of MEK1/2 in the perfused frog heart. (A) MEK 1/2 were detected in extracts (200 μg) from control frog or rat hearts, hearts perfused with 1 μM PMA for 10 or 5 min (for frog or rat, respectively) and frog hearts perfused with 25 μM PD98059 or 0.1% (v/v) DMSO (the solvent for PD98059) by Western blotting using antibodies specific for total MEK1/2 (top panel) or for the dually phosphorylated MEK1/2 (bottom panel). Western blots shown are representative of 3 independent experiments.

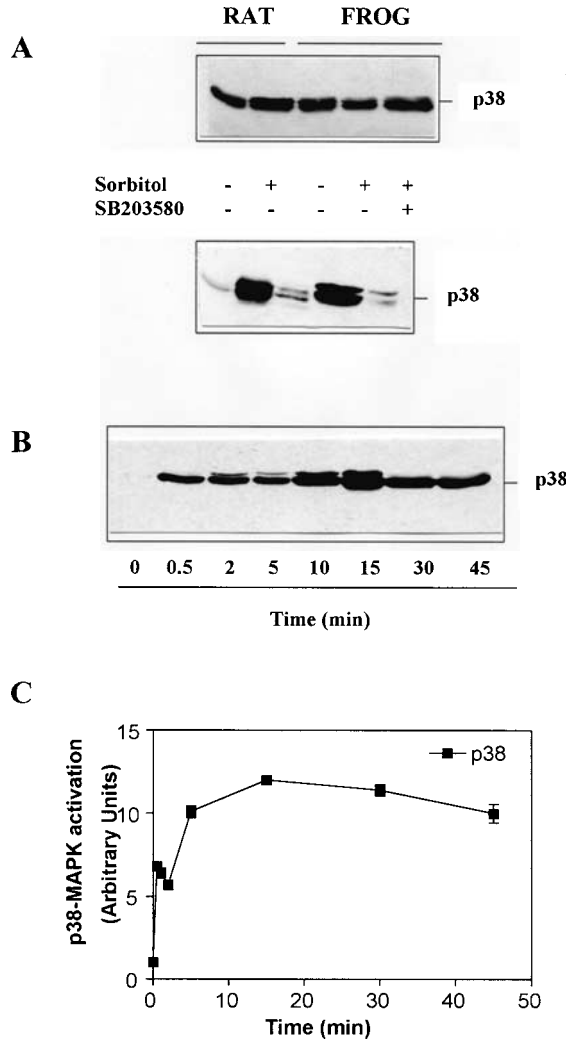

Fig. 3. Expression and phosphorylation of p38-MAPK in the perfused frog heart. (A) p38-MAPK was detected in extracts (100 μg) from control frog or rat hearts, hearts perfused with 0.5 M sorbitol for 15 or 30 min (for frog or rat, respectively) and frog hearts perfused with 10 μM SB203580 by Western blotting using antibodies specific for p38-MAPK (top panel) or for the dually phosphorylated p38-MAPK (bottom panel). (B) Time course of p38-MAPK phosphorylation by 0.5 M sorbitol in the perfused frog heart. (C) Densitometric analysis of phospho p38-MAPK band by laser scanning. Results are means ± S.E. for 3 independent observations. Western blots shown are representative of 3 independent experiments.

Using in-gel kinase assays, two bands of JNK activity were detected in the frog heart corresponding to molecular masses of 46 and 52 kDa, respectively (Fig. 4A). The mean activities of both isoforms were significantly increased by sorbitol treatment as well as upon reoxygenation following anoxia (approximately 4- and 2.5-fold respectively). On Western blots, JNK1 antibodies detected one band corresponding to molecular mass of approximately 46 kDa in the frog heart samples which co-migrated with the rat heart isoform (Fig. 4B). Furthermore, using antibodies to the dually phosphorylated forms of JNKs, two main bands corresponding to molecular masses of 46 and 52 kDa respectively, were detected (Fig. 4C), the phosphorylation state of which increased in response to exposure to sorbitol. This increase was rapid, evident as early as 30 sec and remained elevated over 45 min. Maximal response was observed at 30 min (Fig. 4C). The phosphorylation state of both bands also increased upon reoxygenation following 15 min of anoxia. In particular, anoxia/reoxygenation-induced activation of both JNKs was evident at 5 min, maximized at 15 min, with a progressive decline after 30 min (Fig. 4D). The additional band detected, corresponding to approximately 47 kDa, which was also responsive to sorbitol treatment and anoxia/reoxygenation, may represent another isoform of these kinases.

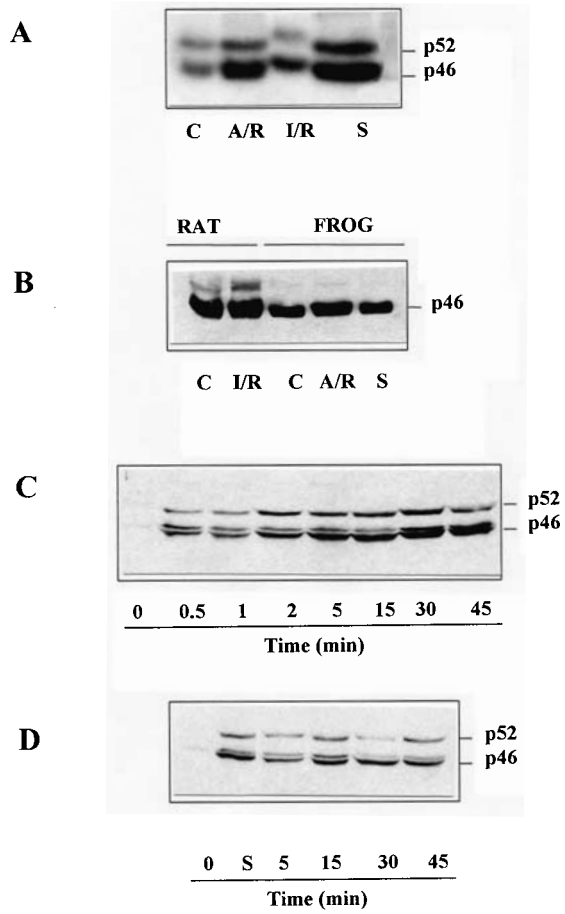

Fig. 4. Expression of JNKs in the perfused frog heart. (A) JNK activity was assayed by the in gel kinase assay in extracts (200 μg) from control frog hearts (C), frog hearts perfused with 0.5 M sorbitol (S) for 15 min and hearts subjected to ischemia/reperfusion (I/R, for rat) or anoxia/reoxygenation (A/R, for frog) as described in the Materials and methods section. A typical autoradiogram is shown, representative of 3 independent experiments. (B) Western blot analysis of samples (150 μg) from control frog and rat hearts (C), frog hearts perfused with 0.5 M sorbitol (S) for 15 min and hearts subjected to ischemia/reperfusion (I/R, for rat) or anoxia/reoxygenation (A/R, for frog) with an antibody specific for JNK1. (C) Time course of JNK phosphorylation by 0.5 M sorbitol in the perfused frog heart using an antibody specific for the dually phosphorylated JNKs. (D) Time course of JNK phosphorylation upon reoxygenation after 15 min of anoxia in the perfused frog heart. As positive control frog hearts perfused with 0.5 M sorbitol (S) were used. Western blots shown are representative of 3 independent experiments.

Discussion

Activation of the MAPK pathways has been intensively investigated in numerous cell types (reviewed in [3]). In this study we have demonstrated that the three MAPK subfamilies, thoroughly examined in rat hearts (reviewed in [11]), are also expressed in the amphibian heart and activated by diverse stimuli. MAPKs are activated by dual phosphorylation on a Thr-Xaa-Tyr motif by dual specificity MAPK kinases. Here, we have examined the phosphorylation of ERKs, JNKs and p38-MAPKs, a process equivalent to the activation of these kinases.

Only one kinase (p43) of the ERK subfamily is expressed in the frog heart. Consistent with findings in mammalian cardiac cells [12–14], p43 was strongly activated by 1 μM PMA (Fig. 1A). The time course of this response in the frog heart is similar to that reported for the rat heart [12]. This activation was parallel to the MEK1/2 activation (Fig. 2) and inhibited by the selective MEK1/2 inhibitor, PD98059, consistent with signal transduction through the well characterized ERK cascade.

p38-MAPK was detected as a 38 kDa band (Fig. 3A). It is widely known that osmotic shock induces a strong phosphorylation of p38-MAPK subfamily in many cell types (reviewed in [1, 2, 5]). Accordingly, sorbitol strongly phosphorylated p38-MAPK in the frog heart (Figs 3A and 3B). The lack of p38-MAPK activation either by anoxia or upon reoxygenation following anoxia in the frog heart, contrasts with the activation of mammalian heart p38-MAPK either by ischemia or ischemia/reperfusion [9, 15, 16]. However, our result is in accordance with a recent study by Greenway and Storey [17] reporting that in wood frogs exposed to anoxia, no significant activation of p38-MAPK was observed either in liver or in kidney.

The nature of the p39 kDa band detected exclusively in the frog heart, also responsive to sorbitol, remains unclear. As multiple isoforms of p38-MAPK have been identified in

mammals [18–20], further investigation is required in order to propose the expression of different p38-MAPK isoforms in the amphibian heart. Furthermore, our results demonstrate that SB203580 inhibits sorbitol-induced phosphorylation of p38-MAPK in this experimental setting (Fig. 3A). Although several investigators propose that pyridinylimidazoles inhibit the enzymatic activity rather than the activation (phosphorylation) of p38-MAPK [21, 22], we have clearly demonstrated that SB203580 inhibits stimulus-induced phosphorylation of p38-MAPK. Recent studies support our finding, suggesting that this inhibition may be due to their binding to the inactive form of the kinase, resulting to a significant reduction of the kinase activation rate [23, 24].

On Western blots, using antibodies specific for the dually phosphorylated JNKs, two bands corresponding to molecular masses of 46 and 52 kDa respectively, were detected (Fig. 4C). The identities of these kinases were confirmed as p46 and p52 JNKs by the in gel kinase assay with c-Jun as substrate (Fig. 4A). Consistent with findings in the perfused rat heart, reporting JNK activation by osmotic shock or ischemia/reperfusion [15, 16], both JNKs were also activated by sorbitol and anoxia/reoxygenation in the frog heart (Figs 4C and 4D).

From the results presented here, it appears that despite the fundamental differences in the physiology of the two species used, the response of each MAPK subfamily to diverse stimuli is in general analogous in both mammals and amphibians. The roles of the different MAPK subfamilies in cell function are still under investigation. In many cells, ERK activation is associated with cell survival [25, 26], whereas JNKs and p38-MAPK may promote apoptosis [25, 27–30]. The roles of the different MAPKs in the amphibian heart remain to be elucidated but their activation is indicative of their importance in the physiology of these organisms.

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