Activation of p21-activated protein kinase α (αPAK) by hyperosmotic shock in neonatal ventricular myocytes

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Abstract The p21-activated protein kinases (PAKs) may participate in signalling from Cdc42/Rac1 to the stress-regulated MAPKs (SAPKs/JNKs and p38-/HOG-1-related-MAPKs). We characterized the expression and regulation of αPAK in cultured ventricular myocytes. αPAK was specifically immunoprecipitated from myocyte extracts. High basal αPAK activity was detected in unstimulated myocytes. Its activity was increased rapidly (< 30 s) by hyperosmotic shock in the presence of okadaic acid, and was maximal by 3 min (187 ± 7% relative to unstimulated cells). Endothelin-1 and interleukin-1β, which also activate SAPKs/JNKs, did not increase αPAK activity and presumably act through different PAK isoforms or other mechanisms.

Key words: p21-activated protein kinase α; Hyperosmotic shock; Cardiac myocyte

1. Introduction

Mitogen-activated protein kinase (MAPK) cascades are activated in eukaryotic cells in response to external stimuli and are important in signalling to the nucleus (reviewed [1–4]). Three MAPK cascades have been identified in rat heart and cultured ventricular myocytes from neonatal rat hearts. The extracellularly regulated kinases (ERKs) are activated by growth factors and hypertrophic agonists such as endothelin-1 (ET-1) [5,6]. The stress-activated protein kinases (SAPKs or JNKs, for c-Jun N-terminal kinases) are activated by cellular stress (hyperosmotic stress, protein synthesis inhibitors or ischaemia/reperfusion) and by ET-1 [7,8]. The p38- or HOG-1-related MAPK(s) is activated by ischaemia [8].

The p21-activated protein kinases (PAKs) are a family of protein Ser-/Thr-kinases that may be involved in the upstream activation of the SAPK/JNK and p38-MAPK cascades (reviewed [4]). At least three members have been identified. αPAK (also known as PAK1 and human PAK65 [9]) was first identified in rat brain as a kinase which interacted with the active (GTP-bound) forms of the Rho family G proteins, Cdc42 and Rac1 [10]. This interaction results in the autophosphorylation and activation of αPAK. Other PAKs include βPAK [11] (also known as mouse PAK3 [12]) and γPAK [13] (also known as PAK I [14]).

Very little is known about the activation of PAKs, though γPAK is activated in platelets by thrombin [13]. The downstream targets of the PAKs and the coupling mechanism to the SAPK/JNK and p38-MAPK cascades are currently unknown. Here, we have characterized the expression of one PAK isoforms (αPAK) in neonatal rat ventricular myocytes in primary culture, and studied the activation of this protein kinase in response to stimuli which activate the SAPK/JNK cascade in these cells.

2. Materials and methods

2.1. Materials

The polyclonal αPAK antibody αPAK(N-20) raised to residues 2–21 was from Santa Cruz Biotechnology. [γ-^{32}P]ATP, prestained molecular mass markers, biotinylated anti-rabbit IgG, ECL blotting reagents and Hyperfilm MP were from Amersham International. SDS-PAGE reagents were from Bio-Rad. Nitrocellulose (Schleicher & Schuell, 0.45 μm) was from Anderman & Co. Protein A-Sepharose and other biochemicals were from Sigma. Myocytes were isolated from the ventricles of neonatal Sprague-Dawley rat hearts essentially as previously described [15] and cultured at a density of 1400 cells/mm^2 for 90 h on 60 mm gelatin-coated dishes with one medium change at 66 h. The cells were incubated for 24 h in serum-free medium and then assayed for αPAK activity after exposure to agonists.

2.2. Assay of αPAK

Myocytes were unstimulated (i.e. no medium change), exposed to a medium change (control cells), subjected to hyperosmotic shock (0.5 M sorbitol) in the presence or absence of the PP1/PP2A protein phosphatase inhibitor okadaic acid (0.2 μM), or exposed to ET-1 (0.1 μM) or interleukin-1β (IL-1β, 100 ng/ml) at 37°C. The medium was removed and the cells scraped into 0.4 ml of ice-cold extraction buffer consisting of 20 mM *n*-octyl β-D-glucopyranoside and 1% (v/v) Triton X-100 in buffer A (10 mM Tris-HCl pH 7.4, 5 mM EDTA, 50 mM NaF, 50 mM NaCl, 2 mM Na_3VO_4, 0.1% (w/v) fatty acid-free bovine serum albumin, 20 μg/ml aprotinin). The samples were extracted on ice (10 min) and centrifuged (5 min, 10 000 × *g*, 4°C). The supernatants were incubated with 4 μl (0.4 μg) αPAK antibody in the presence or absence of competing peptide (0.8 μg) on a rotating wheel (2 h, 4°C). Protein A-Sepharose was added (20 μl of a 50% slurry in buffer A) and the samples rotated for another 1 h. The samples were centrifuged (1 min, 10 000 × *g*, 4°C), the supernatants removed and the pellet washed in buffer A (3 × 150 μl, 4°C). The pellets were then washed in 0.4 ml αPAK assay buffer (20 mM HEPES pH 7.6, 25 mM β-glycerophosphate, 20 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1 mM Na_3VO_4, 2 mM dithiothreitol) and resuspended in 50 μl αPAK assay buffer containing 10 μg myelin basic protein (MBP). Samples were assayed for activity with 10 μl [γ-^{32}P]ATP (20 μM, 25 μCi/ml) for 20 min at 30°C. The entire assay mixtures were spotted onto Whatman 3MM papers which were washed in 5% trichloroacetic acid (4 × 15 min). ^{32}P incorporation into MBP was determined by Čerenkov counting.

2.3. Western blotting

Myocytes were extracted in extraction buffer (150 μl) as above and supernatants retained. For total extracts, the supernatants were boiled with 0.33 vol. SDS sample buffer (10% (w/v) SDS, 13% (v/v) glycerol, 300 mM Tris-HCl pH 6.8, 130 mM dithiothreitol, 0.2% bromophenol blue). αPAK was immunoprecipitated from supernatants with 4 μl of antibody and 20 μl 50% (v/v) Protein A-Sepharose in buffer A as described above. The washed immunoprecipitates (resuspended in 150 μl Buffer A) or the αPAK immunodepleted supernatants were boiled with 0.33 vol. SDS sample buffer. Proteins (25 μl) were separated by SDS-PAGE on 8% (w/v) polyacrylamide gels and transferred

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electrophoretically to nitrocellulose [16]. Non-specific binding sites were blocked with 5% (w/v) non-fat milk powder in phosphate-buffered saline containing 0.05% Tween-20 (PBST) and the blots were incubated with αPAK antibody (1:100 dilution in blocking solution, overnight, 4°C). After washing in PBST (3×5 min) the blots were incubated with horseradish peroxidase-linked anti-rabbit IgG (1:5000 dilution in PBST containing 1% (w/v) non-fat milk powder, 1 h, room temperature). The blots were washed again in PBST (3×5 min) and the bands were detected using the Amersham International ECL method with exposure to Hyperfilm MP.

3. Results

3.1. Immunoprecipitation of αPAK

Four bands of approx. 62, 65, 68 and 76 kDa were detected on Western blots of total myocyte extracts probed with αPAK antibodies (Fig. 1A). This antibody immunoprecipitated only the 65 and 68 kDa proteins from cell extracts and the immunoprecipitation was quantitative (Fig. 1B). The 65 and 68 kDa bands were not detected when the immunoprecipitation procedure was carried out in the absence of antibody or with antibody in the presence of competing peptide (Fig. 1C). These data indicate that the immunoprecipitation of the 65 and 68 kDa doublet was specific and represents αPAK. The 62 and 76 kDa bands detected on Western blots may represent either non-specific proteins detected only in the denatured state, or proteins related to αPAK for which the antibody has a lower affinity.

3.2. Activation of αPAK by hyperosmotic shock

αPAK activities were assayed after immunoprecipitation from myocyte extracts using MBP as substrate. In the absence of precipitating antibody, there was minimal phosphorylation of MBP (Table 1). Immunoprecipitation of αPAK and subsequent assay showed a high basal level of αPAK activity in unstimulated (no medium change) myocytes (Table 1). Replacing the medium with fresh serum-free medium did not induce any significant further increase in αPAK activity at 1 min, but there was an increase in activity in cells subjected to 0.5 M sorbitol in the presence or absence of 0.2 μM okadaic acid (Table 1, Fig. 2). In both control cells and cells subjected to hyperosmotic shock, phosphorylation of MBP was essentially abolished when the immunoprecipitation procedure was carried out in the presence of competing peptide (Table 1). Assay of αPAK in control and stimulated cells was therefore specific.

Although in control cells exposed to fresh serum-free medium there was no increase in αPAK activity above basal levels at 1 min, by 3 min there was a slight increase in activity ($122 \pm 3\%$, $p < 0.05$) which was sustained up to 15 min (Fig. 2). Hyperosmotic shock (0.5 M sorbitol) in conjunction with okadaic acid (0.2 μM) potently activates the SAPKs/JNKs in ventricular myocytes (A. Clerk and P.H. Sugden, unpublished). Exposure of cells to sorbitol+okadaic acid induced a rapid increase in αPAK activity within 30 s ($144 \pm 6\%$), which was maximal by 3 min ($187 \pm 7\%$) and sustained over 15 min (Fig. 2). ET-1 and IL-1β also activate SAPKs/JNKs in these myocytes ([7], and A. Clerk and P.H. Sugden, unpublished). However, neither agonist increased αPAK activity significantly above control levels at either 3 or 15 min (data not shown).

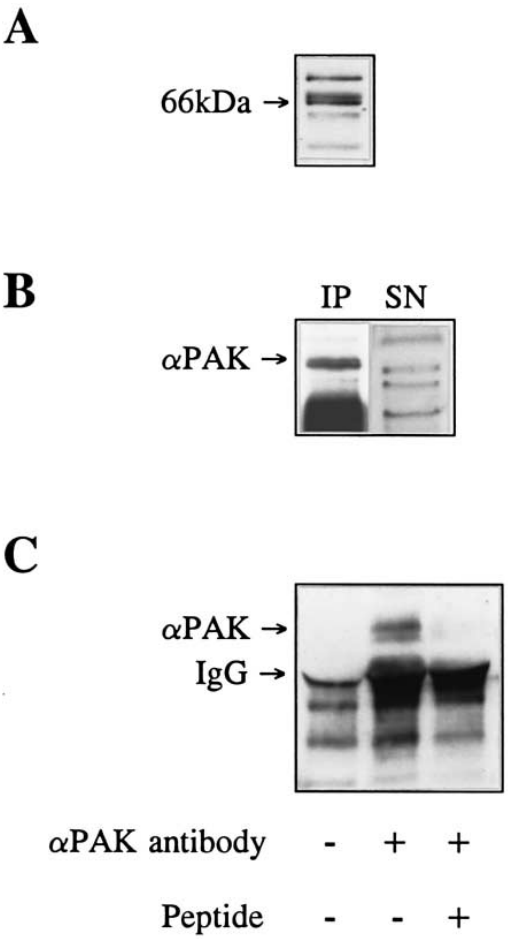

Fig. 1. Western blots of αPAK in myocyte extracts. (A) Total extract (50 μg protein) was immunoblotted for αPAK. The position of the 66 kDa molecular mass marker is shown on the right. (B) Immunoblots of αPAK in the immunoprecipitate (IP) and immunodepleted supernatant (SN). (C) Specificity of αPAK immunoprecipitation using the competing peptide epitope.

Table 1
αPAK immunokinase assays

	αPAK activity (cpm ^{32}P incorporated into MBP)	
	No peptide	+peptide
No antibody	2546	n.d.
Unstimulated (no medium change)	44177	n.d.
Control (1 min after medium change)	48011	4484
0.5 M sorbitol (1 min)	67519	4734

αPAK activity was measured as described in Section 2. To ensure assay specificity, immunoprecipitations were carried out in the presence of the competing peptide. A single representative experiment (out of two independent experiments) is shown. n.d., not determined.

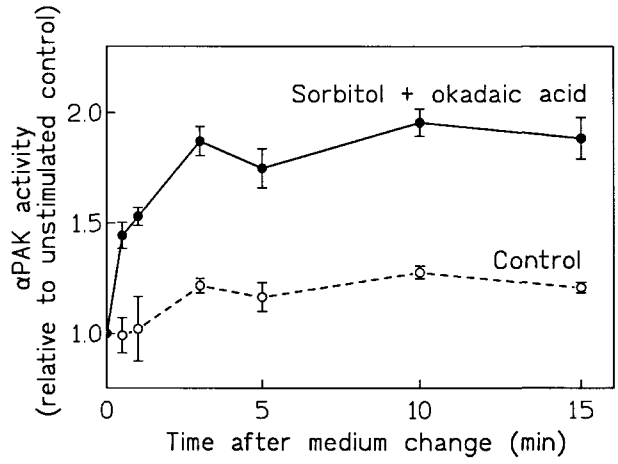

Fig. 2. Time course of αPAK activation by sorbitol+okadaic acid. Myocytes were incubated under control conditions (○) or were exposed to 0.5 M sorbitol+0.2 μM okadaic acid (●). αPAK was assayed by an immunokinase method as described in Section 2. The media were changed in both incubations.

4. Discussion

The apparent relative molecular mass of αPAK on SDS gels is 65–68 kDa ([10,11] and Fig. 1A). The antibody used here was specific for αPAK with no cross-reactivity with βPAK or γPAK according to the suppliers (Santa Cruz Biotechnology). Of the four bands detected on Western blots of myocyte extracts, only the 65 and 68 kDa proteins blots were detected after immunoprecipitation (Fig. 1B). These bands were specific (they were not detected when competing peptide was included in the immunoprecipitation, Fig. 1C) and probably represent the two forms of αPAK previously identified [10]. The 68 kDa protein may be a phosphorylated form of αPAK, which has a reduced mobility compared with the non-phosphorylated form [11]. However, the relative levels of the 65 and 68 kDa proteins did not change on exposure of the cells to hyperosmotic shock (data not shown). Thus, if the 68 kDa protein is a phosphorylated form of αPAK, hyperosmotic shock did not increase its level of phosphorylation. αPAK is highly expressed in brain [10], but has also been identified in COS-7 and Swiss 3T3 cells in association with the Nck adaptor protein [17], and in neutrophils [18]. Here, we have detected αPAK in cultured ventricular myocytes. We have not been able to ascertain the abundance relative to other PAK isoforms because of lack of suitable antibodies.

There have been few studies on activation of PAKs. Cdc42/Rac1 inducible autophosphorylation and activation of recombinant PAKs expressed in a variety of cells has been demonstrated in vitro and in vivo using MBP as a substrate [11,12,19–21]. However, endogenous PAK activation has so far only been demonstrated in platelets, where thrombin (which binds to a G-protein coupled receptor) activates γPAK within 30 s [13]. Our study of αPAK in neonatal ventricular myocytes has shown a high basal level of activity of this isoform in unstimulated myocytes. The Rho-family of small G proteins are implicated in re-organisation of the cytoarchitecture (reviewed [22]). It may be that they (and consequently αPAK) are activated in these neonatal myocytes which, in culture, are continually undergoing cytoskeletal/myofibrillar reorganisation.

Despite the high basal activity of αPAK, hyperosmotic stress combined with okadaic acid induced further activation of the kinase (almost 2-fold, Fig. 2). The rapid and sustained activation of αPAK (maximal within 3 min) is consistent its putative role in SAPK/JNK activation (detectable from 5 min, sustained over 4 h [7]). In contrast, neither ET-1 (which, like thrombin, acts through a G protein-coupled receptor [23]) nor IL-1β increased the activity of αPAK, although both agonists stimulate the SAPKs/JNKs in myocytes in culture ([7]; and A. Clerk and P.H. Sugden, unpublished). The receptors for these agonists presumably couple to the SAPK/JNK cascade either through different PAK isoforms, or using different proteins entirely. Other proteins implicated in the activation of the SAPK/JNK cascade include the mixed lineage kinases and germinal center kinase (reviewed [4]), which remain to be investigated.

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