Requirement of Pyk2 for the activation of the MAP kinase cascade induced by Ca^{2+} (but not by PKC or G protein) in PC12 cells

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Abstract The role of the Ca^{2+}-activated tyrosine kinase, Pyk2, in the pleiotropic coupling of nerve cell stimulation to the MAP kinase cascade still remains undefined. Using a panel of PC12 clones, one of which was defective in Pyk2, we demonstrate (1) that the MAP kinase response induced by a $[Ca^{2+}]_i$ rise (following application of the Ca^{2+} ionophore, ionomycin) is inappreciable in the defective clone and is re-established after Pyk2 transfection; and (2) that the responses to both protein kinase C and P_{2y2} receptor activation occur normally even in the defective cells. We conclude that Pyk2 is the key mediator in the pathway activated by Ca^{2+} but has minor roles with the other types of stimulation.

Key words: PC12; Mitogen-activated protein kinase; Calcium; Signal transduction; Pyk2; P_{2y2} receptor

1. Introduction

In nerve cells, growth, differentiation and plasticity occur under the control of a complex network of transduction pathways ultimately converging to induce activation of the mitogen-activated protein kinase (MAPK) signalling cascade [1,2]. Previous studies have shown that Pyk2, a tyrosine kinase (TK) also called CAKβ, RAFTK and CADTK, has a role in the MAPK response initiated by the increase of the cytosolic Ca^{2+}, $[Ca^{2+}]_i$ [3–5]. So far, however, it is not clear whether the Pyk2 pathway accounts entirely or not for the latter response and whether the TK is also involved in pathways initiated by other types of cell stimuli and/or intracellular signals.

A direct answer to these question is given here based on results obtained in a panel of clones isolated from the neurosecretory cell line PC12 [6]. One of these clones (PC12-27), spontaneously defective in Pyk2, was employed as such and after transfection-induced expression of the TK.

2. Materials and methods

The PC12 line and the clones isolated therefrom (#27, 15 and 37) were grown in DMEM, 10% horse serum and 5% fetal calf serum (see [6,7]). PC12-27 cells were transfected with a plasmid containing the complete open reading frame of Pyk2 [8] using the polyethyleneimine (25 kDa, Aldrich, Milwaukee, WI, USA) method [9]). Stable subclones were isolated based on their hygromycin resistance. Immunocytochemistry was carried out with the purified anti-Pyk2 antibody [10]. Details on fixation, embedding and confocal microscope analysis are given in [7].

For Northern blots, extraction of total RNA was performed according to the single step procedure [11]. The hybridization probe for Pyk2 mRNA (a 1500 base fragment) was excised (at *Hin*DIII sites) from the TK cDNA [8] labeled with [α^{32}P]dCTP (3000 Ci/mmol, Redivue, Amersham, UK). Hybridization, washes and autoradiography were carried out as in [7].

For Western blots, cells were lysed, and their protein content assayed by the BCA procedure (Pierce, Rockford, IL, USA). Samples were prepared, run and blotted as in [7]. Anti-Pyk2 immunolabeling was performed with either the purified antibody of [10] or the monoclonal from Transduction Labs (Lexington, KY, USA). Blots were developed using the ECL procedure (Redivue, Amersham, UK). Images were acquired and quantitated using the IMAGEQUANT software (Molecular Dynamics, UK).

3. Results and discussion

Several clones of PC12 cells, isolated by the conventional technique of limiting dilution [6], were screened for Pyk2 expression by Northern and Western blotting. In all preparations the levels of Pyk2 mRNA appeared similar (Fig. 1A), whereas in one clone (#27) Pyk2 protein was inappreciable, indicating a post-transcriptional defect (Fig. 1B). In two subclones of PC12-27 (#27a and #27b), stably transfected with the Pyk2 mRNA [7], the expression of Pyk2 protein was restored to levels comparable to the non-defective PC12 cells (Fig. 1C). These results were confirmed by confocal immunocytochemistry. Parental and non-defective PC12 clone cells exhibited Pyk2 immunoreactivity distributed throughout the cytoplasm (Fig. 1D), whereas the cells of clone 27 appeared completely negative (Fig. 1E) and those of the transfected subclones were indistinguishable from non-defective PC12 cells (Fig. 1F). On the other hand, expression of focal adhesion kinase (FAK), the only other known cytosolic TK closely related to Pyk2 [5], was found to be similar in all clones, including PC12-27 (not shown). Apparently, therefore (and at variance with a clone of fibroblasts defective in FAK described recently [12]), no compensatory increased expression of the related TK takes place in the Pyk2-defective PC12-27 cells.

Addition to the various PC12 preparations of the Ca^{2+} ionophore, ionomycin (1–3 μM, 5 min, applied in the Ca^{2+}-containing medium), induced $[Ca^{2+}]_i$ to rise to μM concentrations (see [13] and not shown). Concomitantly, Pyk2 became tyrosine phosphorylated (and thus activated) in parental and non-defective clone cells, as revealed by staining with a phosphorylation state-specific antibody (not shown). Signalling consequences of this activation were investigated by studying MAPKs. Since the levels of one of the family members, ERK1, were variable from one PC12 clone to another (Fig.

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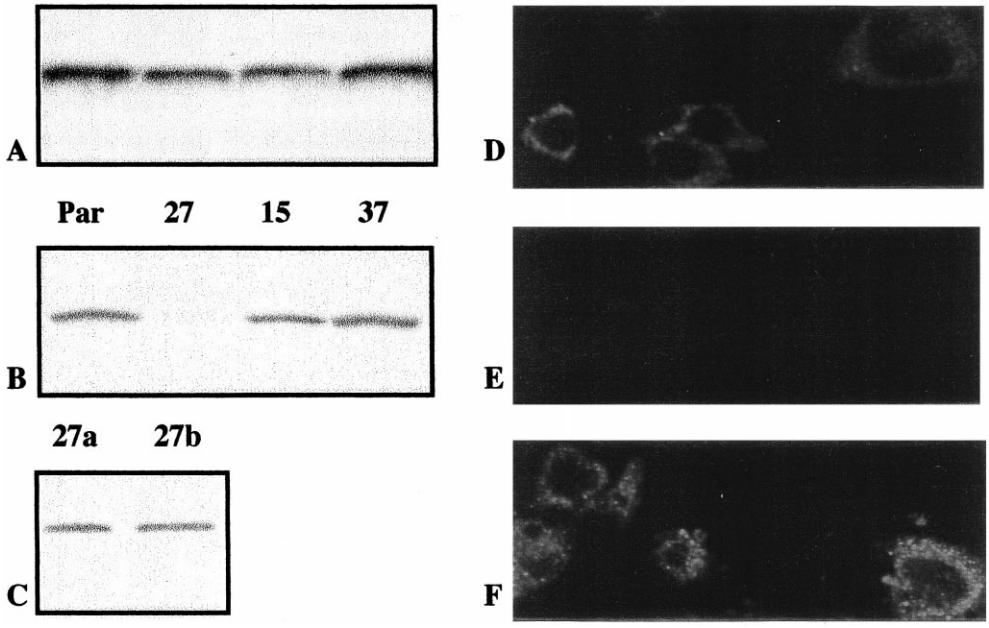

Fig. 1. Pyk2 Northern (A) and Western (B, C) blots and immunocytochemistry (D–F) in PC12 parental (Par) cells and clones isolated therefrom. Parental (Par) PC12 cells and clones 27, 15 and 37 [6] were used. A: Pyk2 Northern blots. Each lane contains 10 μg of total RNA. Representative of three separate experiments. B: Pyk2 Western blots. Each lane was loaded with 50 μg protein. Representative of over 10 experiments. C: Western blots as in B, performed on two subclones (27a and 27b) isolated from PC12-27 after stable transfection with Pyk2 cDNA. D–F: Immunocytochemistry carried out with the purified anti-Pyk2 antibody [10]. D = parental PC12; E = PC12-27; F = PC12-27a. Magnification 550×.

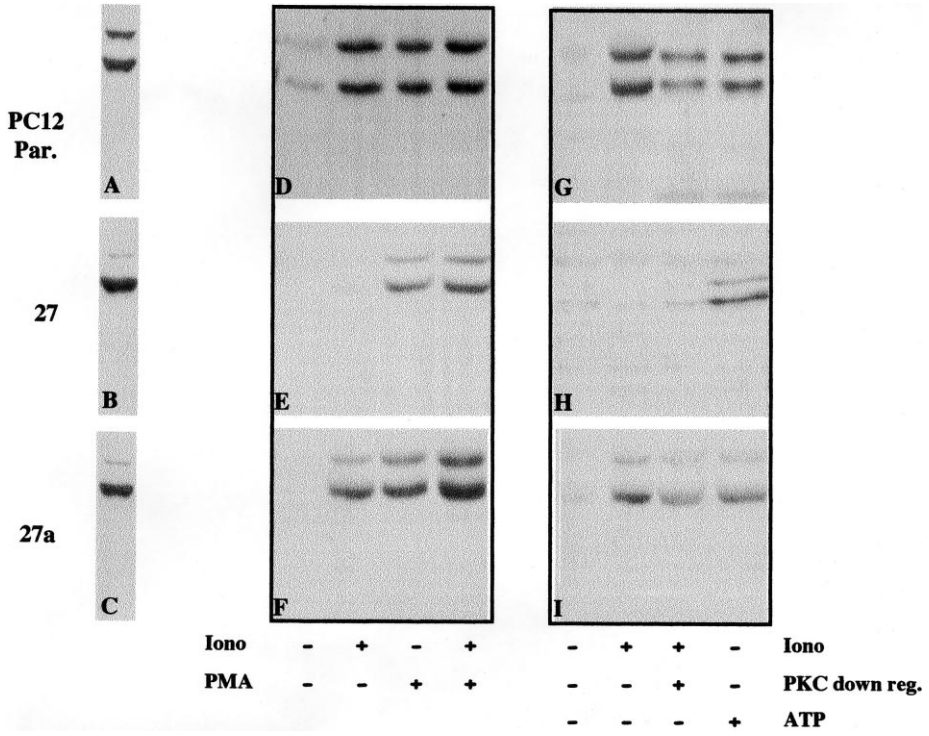

Fig. 2. ERK expression in parental PC12, PC12-27 and PC12-27a cells, and ERK phosphorylation of the same cells in response to the indicated treatments. A–C: Western blots performed as in Fig. 1B, using anti-ERK1 and 2 antibody (Zymed, San Francisco, CA, USA). A = parental PC12; B = PC12-27; C = PC12-27a. D–I: Western blots with anti-active MAPK antibody (Promega, Madison WI, USA) performed as in Fig. 1B. However, saturation of the nitrocellulose and washes were in PBS with 3% BSA, 0.05% Tween 20. Cells were serum-starved for 18 h in DMEM, 0.5% horse serum, 0.25% fetal calf serum, then washed in PBS and finally treated with the indicated stimuli for 5 min. Ionomycin was applied at 1 μM, PMA at 50 nM, ATP at 100 μM. Down-regulation of PKC was induced by treatment with PMA, 50 nM, for 12 h. D,G = parental PC12; E,H = PC12-27; F,I = PC12-27a. Representative of 3–5 experiments.

Fig. 3. ERK-2 phosphorylation in PC12-27 cells incubated in Ca^{2+}-free medium and stimulated with ATP. Where indicated, PKC down-regulation was achieved with PMA 50 nM, 12 h. BAPTA loading by incubation with the acetoxymethylester, 50 μM, 15 min. Before exposure to ATP (100 μM, 5 min) the cells were transferred to Ca^{2+}-free KRH medium. Representative of three experiments.

2A and data not shown), we concentrated our attention on the phosphorylation of the other, ERK2.

The effects of three treatments, with ionomycin, phorbol myristate acetate (PMA) and a combination of the two, are illustrated in Fig. 2D–F. In the parental PC12 cells, the ionophore induced a marked phosphorylation of ERK2, similar to that induced by PMA (Fig. 2D). The effects of the combination (ionomycin+PMA) were only slightly stronger than those of ionomycin alone (Fig. 2D). In the Pyk2-defective PC12-27 cells, no appreciable ERK2 phosphorylation response was observed. The response to PMA was relatively weak, albeit clearly present, with some reinforcement by the combination with ionomycin (Fig. 2E). In the Pyk2-transfected subclones, the effects of ionomycin were largely restored, and a clear reinforcement of the response was observed in the combined presence of PMA and ionomycin (Fig. 2F). None of the responses was influenced by the pretreatment of the cells with the calmodulin antagonist, W7 (50 nM, 10 min) which, in addition, had no appreciable effect on the level of ERK2 phosphorylation when administered alone to resting cells (not shown).

In a further series of experiments, the role of protein kinase C (PKC) in the $[Ca^{2+}]_i$-induced responses was examined. In both the parental cells (Fig. 2G) and the Pyk2-transfected PC12-27 subclone (Fig. 2I), the ERK2 phosphorylations induced by ionomycin were considerably decreased by prolonged pre-exposure to PMA, a treatment which induces the down-regulation of classical and novel PKCs. Finally, the response to ATP (known to act through P_{2y2}, a receptor that in the employed PC12 cells is coupled to phospholipase C through both pertussis toxin-sensitive and -insensitive G protein(s), see [13]) was prominent in all three cell types, including the defective PC12-27 clone (Fig. 2G–I), with no appreciable change induced by long-term PMA treatment (not shown).

The role of $[Ca^{2+}]_i$ in the ATP response of PC12-27 cells was further studied by using a Ca^{2+}-free, EGTA-containing incubation medium (Fig. 3). In these conditions, prolonged PMA treatment remained without appreciable effect on the ATP-induced ERK2 phosphorylation. Only when the PKC down-regulation was associated with an efficient clamping of $[Ca^{2+}]_i$ near the resting level, obtained by pre-loading of the cells with the chelator BAPTA [14], was the response to ATP inhibited, however only slightly (Fig. 3).

The results reported here demonstrate that in PC12 cells, the most widely employed nerve cell model, Pyk2 plays the key role in the linking of $[Ca^{2+}]_i$ increase to the MAPK cascade, and thus to the processes regulated thereby. This does not exclude contributions of other pathways, which however could only work by modulation of the Pyk2-initiated pathway. On the other hand, PMA was still able to induce a MAPK response in the cells defective in Pyk2. This result excludes Pyk2 as a necessary step in the pathway initiated by PKC, which therefore appears to operate, at least in part, in parallel to the Pyk2 pathway.

Of interest were also the results with ATP. The persistence of the ATP-induced activation of ERK2 in Pyk2-defective cells indicates that the TK is not necessary for the response to stimulation of P_{2Y2} receptors, a conclusion incompatible with those proposed by others, based however on indirect evidence [15,16]. Moreover, the results obtained in Pyk2-defective cells with down-regulated PKC and buffered $[Ca^{2+}]_i$ exclude the involvement of both PKC and the EGF receptor, whose transphosphorylation requires high $[Ca^{2+}]_i$ to occur [17]. At the moment the pathway(s) sustaining the ATP-induced response remain(s) undefined. Possible participants may be G protein subunits, α and βγ heterodimers, dissociated by receptor activation [18–23], working also via the activation of other TKs, such as Src [20–23], and also FAK, which is normally expressed in our PC12-27 clone and which is known to be activated by mechanism(s) different from those of Pyk2 [10].

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