Protein phosphatase type 2C active at physiological Mg^{2+}: stimulation by unsaturated fatty acids

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Abstract Type 2C serine/threonine protein phosphatases (PP2C) so far require unphysiologically large amounts of Mg^{2+} ions for activity. Activators and inhibitors are not available, targeting subunits unknown. Studying the regulation of PP2C isozymes in bovine retinae, we found that the activity of PP2C increased specifically by the addition of mono- and polyunsaturated fatty acids. Activation was most pronounced at low Mg^{2+} levels (10-fold stimulation of PP2Cα by 0.5 mM arachidonic acid at 0.7 mM Mg^{2+}). Sensitivity of PP2Cβ was 30–50% less, revealing for the first time enzymatic differences among the PP2C isozymes. Combining unsaturated fatty acids with physiological Mg^{2+} concentrations resulted in PP2C activity that by far exceeded the dephosphorylation rates obtained otherwise. This suggests that PP2C activity has been severely underestimated in the past. In the presence of fatty acids, Ca^{2+} ions became inhibitory in the micromolar range. We conclude that unsaturated fatty acids may play a role in the regulation of PP2C activity.

Key words: Arachidonic acid; Calcium; Enzyme activation; Magnesium; Protein phosphatase; Protein phosphorylation

1. Introduction

Protein phosphorylation mediates the action of multiple intracellular signaling pathways. Four major types of serine/threonine protein phosphatases (PP) have been described [1–3]: PP1, PP2A, PP2B (calcineurin) and PP2C (PPM). Type 2C phosphatases are characterized by the requirement of Mg^{2+} ions for activity [4]. Two isoforms of PP2C, α and β, are known from a variety of mammalian tissues [5,6]. They share 75% sequence identity, have the same enzymatic characteristics, and are considered monomeric (43–48 kDa). Both isozymes are ubiquitously expressed. In addition, PP2C also exists as a module in diverse proteins, e.g. adenylyl cyclases [7]. In contrast to the other Ser/Thr protein phosphatases, there is no knowledge on how the activity, localization and substrate specificity of PP2C are governed. And unlike the other Ser/Thr phosphatases, there is no activator or inhibitor available to specifically affect PP2C activity.

Arachidonic acid (20:4) is one of the best characterized cellular lipid second messengers [8]. Its precursors and metabolites are embedded in Ca^{2+}- and G-protein coupled pathways. Arachidonic acid itself directly regulates the activity of various enzymes such as protein kinase C, and modulates the activity of ion channels. Elevated levels of arachidonic acid are found in various diseases including diabetes and ischemia. Arachidonic acid is known to inhibit myosin light chain phosphatase activity [9,10] and to activate PP5 [11].

PP2C is present in bovine retina [12]. α- and β-isozymes are predominantly localized in the photoreceptor outer segments; they both decrease upon retinal degeneration [12]. Photoreceptor cells are specialized sensory neurons with an extensive area of folded membranes. Their lipid content is at least half of the dry weight. We now report that PP2C can be activated by polyunsaturated fatty acids such as arachidonic acid. Upon addition of fatty acids, PP2C no longer requires unphysiologically high Mg^{2+} concentrations for activity, and Ca^{2+} ions become inhibitory in the micromolar range. The stimulation of PP2C in vitro by fatty acids suggests that this enzyme may be regulated by an endogenous lipid messenger in vivo.

2. Materials and methods

2.1. Preparation of native and recombinant PP2C

Enzyme preparations were carried out at 4°C with buffers containing 0.02% NaN_3 and 0.1% (v/v) 2-mercaptoethanol. Chromatography on DEAE Sephacel and heparin-Sepharose was performed as 'batch' procedures. Beads were pelleted at $700\times g$ for 1 min. Eighty bovine retinae were shaken for 90 s in 80 ml sucrose medium (20 mM Tris-HCl, pH 7.5, 600 mM sucrose, 10 mM glucose, 0.2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine). The soluble extract ($48\,000\times g$, 1 h) was applied to DEAE Sephacel (50 ml) equilibrated in buffer A (20 mM Tris-HCl, pH 7.0, 1 mM EDTA, 5% glycerol). After gentle rocking (1 h) and centrifugation, DEAE was washed with buffer B (buffer A containing 0.1 M NaCl) prior to elution of PP2C with 45 ml buffer C (buffer A containing 0.5 M NaCl; 45 min). Supernatant containing PP2C activity was diluted 1:5 with buffer D (20 mM Tris-HCl, pH 7.0, 1 mM EDTA), applied to 10 ml heparin-Sepharose CL-6B equilibrated in buffer A, and shaken for 90 min. PP1 could be removed upon binding to heparin-Sepharose [12]. PP2C activity was recovered in the heparin-Sepharose flow through fraction, concentrated via Centriplus (Amicon) and further purified on Mono Q HR5/5 (buffer A, 0.5 ml/min) using 0.7 M NaCl for elution. Chromatography on Superdex 75 prep grade 26/60 (1 ml/min) equilibrated in buffer E (20 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 5% glycerol, 50 mM NaCl) finally separated PP2C from PP2A (>100 kDa). Recombinant PP2C isozymes were obtained from cDNA clones encoding PP2Cα and PP2Cβ from bovine retina, and expressed in *Escherichia coli* BL21(DE3)pLysS [12]. His-tag containing proteins were purified from the soluble extract by affinity chromatography on Ni^{2+}-NTA agarose [12].

2.2. Measurement of PP2C activity

PP2C activity was measured using ^{32}P-labeled casein as a substrate [4]. Phosphorylation of bovine casein was performed with cAMP-dependent protein kinase in the presence of 50 μM cAMP and 37 MBq [γ-^{32}P]ATP (110 TBq/mmol). The labeled protein was separated from unincorporated nucleotides by gel filtration (Sephadex G-50) and stored at 4°C. PP2C assays were performed in 20 mM Tris-HCl, pH 7.0, 0.1% 2-mercaptoethanol, magnesium acetate as indicated, 0.3 mg/ml BSA and 1 μM [^{32}P]casein ($\sim 5\times 10^4$ cpm) in a final vol-

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Abbreviations: PP1, PP2A, and PP2C, types 1, 2A, and 2C eukaryotic serine/threonine-specific protein phosphatases; BSA, bovine serum albumin

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ume of 30 μl at 30°C for 10 min. Reactions were terminated by the addition of 200 μl 20% trichloroacetic acid and placed on ice for 5 min. After centrifugation at 10 000×*g* (5 min), 200 μl of the supernatant was analyzed for [^{32}P]phosphate content. Activity measurements were kept within the linear range of time and protein.

2.3. Materials and miscellaneous techniques

Radioisotopes and chromatography supply were from Amersham. Casein (C-4765), cAMP-dependent protein kinase (P-4890), and fatty acids were purchased from Sigma, palmitic acid and stearic acid from Merck. Fatty acids were dissolved and diluted in DMSO. Protein concentration was determined by Bio-Rad using BSA as a standard. Enzyme preparations were analyzed on 10% SDS-PAGE minigels.

3. Results and discussion

3.1. Stimulation of PP2C activity by unsaturated fatty acids

We set out to search for the regulatory components of vertebrate PP2C. For that purpose, native PP2C was partially purified from a soluble extract of bovine retinae. PP2C preparations were free of other protein phosphatases and proteases. Recombinant PP2Cα and PP2Cβ were used as an enzyme source to check for isozyme-specific effects of the lipids.

Activity of recombinant PP2Cα (PP2Cβ) increased up to 11-fold (5-fold) upon addition of arachidonic acid (Fig. 1A). Stimulation was concentration-dependent. In the presence of 0.7 mM Mg^{2+}, dephosphorylation of [^{32}P]casein by PP2Cα and PP2Cβ was maximal at a lipid concentration of ∼0.5 mM. This optimum was found to be independent of the Mg^{2+} ion concentration. However, the concentration of Mg^{2+} ions strongly influenced the rate of stimulation of PP2C activity by arachidonic acid (Fig. 1B). Activation was most pronounced at physiological Mg^{2+} levels. Activation was barely visible at the artificially high Mg^{2+} concentrations (>10 mM) so far used for determination of PP2C activity.

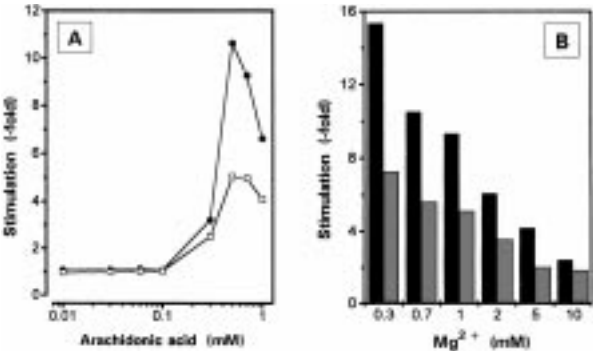

Fig. 1. Effect of arachidonic acid on PP2C activity. A: Dose-response curve for recombinant PP2Cα (●) and PP2Cβ (□). Phosphatase activity was determined as described in Section 2 in the presence of 0.7 mM Mg^{2+}, and arachidonic acid as indicated. Specific activity in the absence of fatty acid was 9.2 (10.6) nmol P_i/min/mg for PP2Cα (PP2Cβ). B: Activation of recombinant PP2Cα (black bars) and PP2Cβ (gray bars) by 0.5 mM arachidonic acid as a function of the Mg^{2+} concentration.

This might explain why the stimulation of PP2C activity by fatty acids has previously been overlooked.

To analyze the structural requirements for lipids that enable activation of PP2C systematically, various fatty acids and analogs were tested at the concentrations yielding optimal response (0.5 mM fatty acid, 0.7 mM Mg^{2+}). The activity of recombinant PP2C isozymes was not influenced by saturated fatty acids from C12 to C22 (Table 1). Monounsaturated and polyunsaturated fatty acids, however, significantly stimulated enzyme activity (Table 1). Introduction of double bonds causes bending, shortening and an increase in volume. For a chain length of C18, introduction of one double bond was sufficient to yield maximal stimulation of PP2C activity. The

Table 1
Effects of fatty acids on PP2C activity

Lipid additive	Carbon chain	Relative activity (-fold)		
		rec. α	rec. β	native
Lauric acid	12:0	1.4	1.2	2.7
Myristic acid	14:0	1.3	1.1	2.1
Pentadecanoic acid	15:0	1.3	1.1	1.9
Palmitic acid	16:0	1.1	1.0	1.9
Palmitoleic acid	16:1	10.0	7.2	3.5
Heptadecanoic acid	17:0	1.2	1.1	1.5
Stearic acid	18:0	1.1	1.0	1.6
Oleic acid	18:1	12.1	7.7	3.1
Linoleic acid	18:2	11.6	6.9	4.1
γ-Linolenic acid	18:3	10.1	6.4	4.0
Arachidic acid	20:0	1.9	1.4	0.9
Eicosatrienoic acid	20:3	7.6	3.4	2.6
Arachidonic acid	20:4	10.1	5.2	4.0
Behenic acid	22:0	1.4	1.0	0.9
Erucic acid	22:1	2.0	1.7	1.7
Docosahexanoic acid	22:6	8.9	4.6	3.1

Phosphatase activity was assayed as described in Section 2 in the presence of 0.7 mM Mg^{2+} and 0.5 mM fatty acid as indicated. Control activity in the absence of lipids released 9.3 (10.7, and 0.25) nmol P_i/min/mg for recombinant PP2Cα (recombinant PP2Cβ, and native PP2C). The enzyme purified from bovine retinae ('native' PP2C) contained equimolar amounts of PP2Cα and PP2Cβ as verified by Western blots using antibodies specific for either one of the isozymes.

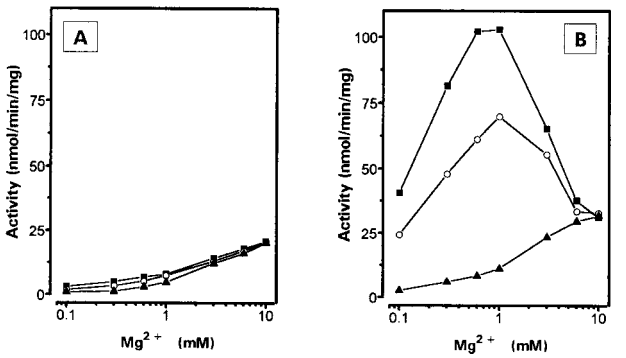

Fig. 2. Addition of fatty acids alters the cation dependence of PP2C. Mg^{2+} dependence of recombinant PP2Cα in the absence of fatty acids (A; 12.5 ng protein/assay) or in the presence of 0.5 mM oleic acid (B; 6.3 ng protein/assay). The concentration of Ca^{2+} ions added was 0 (■), 0.1 mM (○) or 0.3 mM (▲).

data suggest that stimulation of PP2C activity by unsaturated fatty acids is not simply a hydrophobic effect. Maximal activation was observed at 0.5 mM of the unsaturated fatty acids, independent of chain length and degree of unsaturation (data not shown). That concentration is above the critical micellar concentration of any of the unsaturated fatty acids (30–100 μM). Micelle formation alone, however, was not sufficient for stimulation of PP2C activity.

Unsaturated fatty acids that stimulate the activity of PP2Cα also increased the activity of PP2Cβ (Table 1). Quantitatively, however, the extent of activation revealed unexpected differences: the maximal rate of stimulation for PP2Cβ by unsaturated fatty acids was 30–50% less than that of PP2Cα (Fig. 1A, Table 1). Until now, PP2C isozymes from various sources were enzymatically indistinguishable, although the proteins share 75% sequence identity. Amino acids 1–300 are considered to be the catalytic core of 2Cα and 2Cβ, whereas the carboxy-terminal 90 amino acids are expected to be responsible for regulation and localization [13]. Dissimilarities between PP2Cα and PP2Cβ cluster within the C-termini (20% identity within 15 amino acids). It will be interesting to see whether the difference in sensitivity to fatty acids by PP2Cα compared to PP2Cβ is due to the difference in amino acids located at the C-termini, resides in the potential myristoylation sites, or is of a more complex nature.

Native PP2C contained α- and β-isozymes in equal amounts as verified by Western blotting using antibodies specific for PP2Cα and PP2Cβ, respectively (data not shown). Unlike the recombinant isozymes, saturated C12 and C14 fatty acids were able to activate native PP2C activity 2–3-fold (Table 1). All other described structure-activity relationships for lipids and recombinant PP2C were found also to be applicable to native PP2C (Table 1). The maximal rate of stimulation, however, was considerably less for the native enzyme. This might be explained by the possible presence of endogenous fatty acids in the preparation originating from bovine retinae. Furthermore, the fatty acid environment to which recombinant PP2C had been exposed upon bacterial expression is different compared to that from vertebrates.

3.2. Influence of fatty acids on the divalent cation dependence

PP2C is a metalloenzyme, binding Mg^{2+} and Mn^{2+} [13]. Enzyme activity, however, is barely measurable at physiological Mg^{2+} concentrations (Fig. 2A). Protocols for in vitro assays, therefore, recommend 10–20 mM Mg^{2+} [4]. Ca^{2+} ions do not support dephosphorylation reactions carried out by PP2C; on the contrary, they have been found to inhibit Mg-sustained PP2C activity [12,14].

We discovered that unsaturated fatty acids strongly affect the response of PP2C to divalent cations. The addition of 0.5 mM oleic acid resulted in a high PP2C activity at physiological Mg^{2+} concentrations (Fig. 2B). This amounts to a 10-fold decrease in the required Mg^{2+} concentration compared to control assays in the absence of fatty acids (Fig. 2A vs. 2B). Furthermore, the rate of PP2C activity achieved upon combining the addition of unsaturated fatty acids with the presence of low (0.5–1 mM) Mg^{2+} by far exceeded the dephosphorylation rates obtained otherwise (Fig. 2A vs. 2B). In the absence of Mg^{2+}, fatty acids had no effect on PP2C activity (data not shown).

Addition of fatty acids did not render Ca^{2+} ions capable of supporting PP2C activity (data not shown). However, the inhibition of Mg^{2+}-sustained activity by Ca^{2+} ions became much more potent (Fig. 2A vs. 2B). In the presence of unsaturated fatty acids and low Mg^{2+} concentrations, Ca^{2+} ions strongly inhibited PP2C activity ($IC_{50} = 120$ μM Ca^{2+}, Fig. 2B). Enzyme activity was completely blocked at 0.3 mM Ca^{2+}. Casein, the universal in vitro substrate for PP2C, is known to chelate Ca^{2+} ions. It is, therefore, conceivable that once the physiological substrate of PP2C is identified, inhibition of PP2C by Ca^{2+} ions might be even more pronounced.

4. Conclusions

In summary, the data presented demonstrate that unsaturated fatty acids are essential for PP2C activity in vitro. It is unknown at present whether one specific fatty acid is responsible for enzyme activity in vivo. As suggested and exemplified by the retinal tissue described here, however, differential localization of the fatty acids might be the determining factor: in vertebrate retinae, a striking concentration gradient exists between docosahexaenoic acid (major component in photoreceptor outer segments) and palmitic acid (found in the adjacent pigment epithelium cells). This uneven distribution of long chain fatty acids is the basis for continuous supply of photoreceptors with 11-*cis* retinal [15]. The molecular interaction of PP2C with those fatty acids (Table 1; activation by docosahexaenoic acid, no effect of palmitic acid) raises the possibility of regulation of PP2C activity via differential subcellular distribution of specific unsaturated fatty acids. The findings presented are suggestive of a role of fatty acids for PP2C activity of similar functional importance as the proteinaceous regulatory and targeting subunits known for PP1, PP2A and PP2B.

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