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An improved procedure for identifying and quantitating protein phosphatases in mammalian tissues

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The type 2A protein phosphatases in mammalian tissue extracts are inhibited completely and specifically by 1–2 nM okadaic acid. In contrast, type 1 protein phosphatases are hardly affected at these concentrations, complete inhibition requiring 1 μM okadaic acid. These observations have been exploited to develop an improved procedure for the identification and quantitation of type 1, type 2A and type 2C protein phosphatases in tissue extracts.

Protein phosphatase; Protein phosphorylation; Inhibitor protein; Okadaic acid; Tumor promoter

1. INTRODUCTION

It is generally accepted that 4 major classes of serine/threonine-specific protein phosphatase (PP) catalytic subunits are present in the cytoplasmic compartment of mammalian cells. These enzymes have been subdivided into two groups depending on whether they dephosphorylate the β-subunit of phosphorylase kinase specifically and are inhibited by the thermostable proteins inhibitor-1 and inhibitor-2 (type 1) or whether they dephosphorylate the α-subunit of phosphorylase kinase preferentially and are insensitive to inhibitors 1 and 2 (type 2). The type 2 phosphatases comprise 3 distinct groups of enzymes, termed 2A, 2B and 2C, which are most simply distinguished by their requirement for divalent cations. Type 2A phosphatases are active in the absence of divalent cations, whereas types 2B and 2C have absolute requirements for Ca^{2+} and Mg^{2+}, respectively. Type 1, 2A and 2C have relatively broad and overlapping substrate specificities in vitro, while type 2B enzymes display high activity towards relatively few substrates (reviewed in [1]).

Type 1 and type 2A protein phosphatases are the only enzymes with significant activity towards glycogen phosphorylase [2]. Type 1 activity in tissue extracts has therefore been taken as the proportion of phosphorylase phosphatase activity that can be inactivated by inhibitor 1 or inhibitor 2, and type 2A as the activity which is resistant to the inhibitors. However, this procedure might underestimate type 1 and overestimate type 2A activity, since complete inhibition of the native forms of some type 1 enzymes requires prolonged incubation with inhibitors 1 and 2 [3,4], while others may be resistant to them [5]. Furthermore, measurement of type 2C activity in tissue extracts has been impossible, because this enzyme accounts for only a minor proportion of the phosphatase activity towards the substrates that have so far been examined [2].

In this report a simple procedure is introduced for identifying and quantitating type 1, 2A and 2C protein phosphatases in mammalian tissue extracts, which is based on the use of the tumour promotor okadaic acid, a potent and specific inhibitor of type 1 and type 2A protein phosphatases [6,7].

2. EXPERIMENTAL PROCEDURES

2.1. *Materials*

Okadaic acid, a generous gift from Dr Y. Tsukitani, Fujisawa Chemical Company, Japan, was dissolved in dimethyl sulphoxide to give a 5 mM solution, and diluted in aqueous buffers

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before use. The catalytic subunits of type 1 and type 2A protein phosphatase [8], type 2C protein phosphatase [9] and inhibitors 1 and 2 [10] were purified from rabbit skeletal muscle.

2.2. *Tissue extracts*

Rabbit skeletal muscle and brain, rat liver and bovine adrenal medulla were homogenized in 2.5 or 3 ml buffer/g wet weight as described [2], except for the further inclusion of a proteinase inhibitor cocktail [11]. Homogenates of skeletal muscle were centrifuged for 45 min at 5000 × *g*, and those of other tissues for 15 min at 16 000 × *g*. Extracts were prepared from rat adipocytes as in [12]. Supernatants were decanted and used for assay of protein phosphatases. Protein concentrations in the extracts determined according to Bradford [13] were: rabbit skeletal muscle, 15–20 mg/ml; rabbit brain, 4–5 mg/ml; rat liver, 18–22 mg/ml; rat adipocyte, 2.6–3.4 mg/ml; bovine adrenal medulla, 12 mg/ml. Glycogen particles were prepared from rat liver as in [11].

2.3. *Preparation of ^{32}P-labelled substrates and phosphatase assays.*

^{32}P-labelled rabbit skeletal muscle phosphorylase kinase (1.66 mol phosphate/$\alpha\beta\gamma\delta$ unit) [14] and bovine casein (4 nmol phosphate/mg [9] were prepared by phosphorylation with cyclic AMP-dependent protein kinase, and ^{32}P-labelled muscle phosphorylase (1.0 mol phosphate/mol subunit) [8] and muscle glycogen synthase (1.8 mol phosphate/mol subunit [14]) by phosphorylation with phosphorylase kinase and glycogen synthase kinase 3, respectively. The specific radioactivity of each substrate was $\approx 10^6$ dpm/nmol. Type 1 and 2A protein phosphatases were assayed in the absence of divalent cations and presence of 0.1 mM EGTA [8] and type 2C in the presence of Mg^{2+} [9] using 10 μM phosphorylase, 6 μM ^{32}P-casein, 1 μM phosphorylase kinase and 1 μM glycogen synthase. One unit of activity, U, was that amount which catalysed the dephosphorylation of 1.0 μmol of substrate in one min. When inhibitor 1 or inhibitor 2 were included, diluted extracts were preincubated with these proteins for 15 min prior to initiating the reactions with substrate [10].

3. RESULTS

Dephosphorylation of glycogen phosphorylase by the purified type 1 and type 2A catalytic subunits from rabbit skeletal muscle was potently inhibited by okadaic acid. However, the concentration required for 50% inhibition (IC_{50}) of the type 2A enzyme depended on the phosphatase concentration in the assay, ranging from 0.04 nM at 0.03 mU/ml to 2.5 nM at 3.0 mU/ml (fig.1). These levels of okadaic acid are similar to the concentration of type 2A phosphatase in the assays, indicating an extremely strong interaction with the tumour promoter (K_d < 0.01 nM). By contrast, the IC_{50} for the type 1 catalytic subunit (12 nM) was not significantly affected by increases in phosphatase concentration from 0.03 to 3 mU/ml (fig.1). In order to maximise the difference in sensitivity to okadaic acid between type 1 and type 2A phosphatases, tissue extracts were therefore assayed at the highest possible dilution (<0.1 mU/ml phosphorylase phosphatase).

To examine whether the native forms of type 1 and type 2A phosphatases were inhibited by okadaic acid with similar potency to the catalytic subunits, the effects of this tumour promoter were studied in extracts of several mammalian tissues and with several substrates. Provided that the extracts were assayed at < 0.1 mU/ml phosphorylase phosphatase, the inhibitor 2 insensitive activity measured in the absence of divalent cations (i.e. type 2A) was inhibited by okadaic acid with an IC_{50} of $\approx$ 0.1 nM, essentially complete inhibition occurring at 1–2 nM okadaic acid (fig.2). By contrast, activity not blocked by 1 nM okadaic acid (i.e. type 1), was only inhibited at much higher concentrations ($IC_{50} \approx$ 15–20 nM). Similar results were ob-

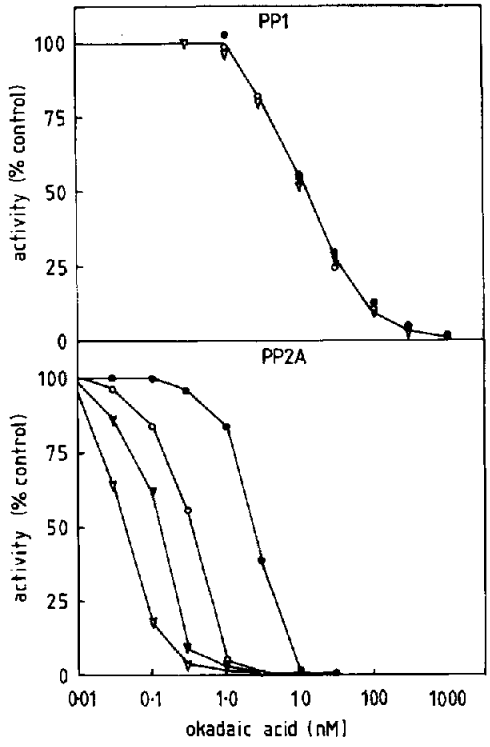

Fig.1. Influence of enzyme concentration on inhibition of the purified catalytic subunits of rabbit skeletal muscle type 1 (PP1) and type 2A (PP2A) phosphatases by okadaic acid. Assays were carried out with glycogen phosphorylase as substrate. Phosphatase concentrations in the assays were 0.03 (▽), 0.1 (▼), 0.3 (○) and 3.0 (●) mU/ml.

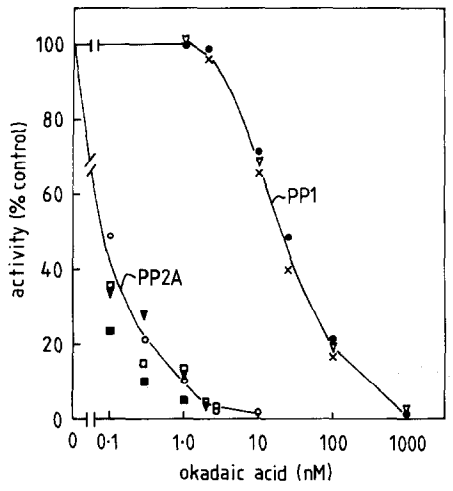

Fig.2. Effect of okadaic acid on type 1 (PP1) and type 2A (PP2A) protein phosphatases in tissue extracts. Assays were performed at 600-fold dilutions of the extracts in the presence of 1 nM okadaic acid (PP1) or after preincubation for 15 min with 0.2 μM inhibitor-2 (PP-2A). Phosphorylase phosphatase activity was measured in extracts of bovine adrenal medulla (○), rat liver (▽, PP1; ▼, PP2A) and rabbit brain (□); phosphorylase kinase phosphatase in an extract of rabbit skeletal muscle (×), glycogen synthase phosphatase with the rat liver glycogen fraction (●) and casein phosphatase in an extract of rabbit brain cortex (■). The experiments were repeated using extracts from three different animals with very similar results.

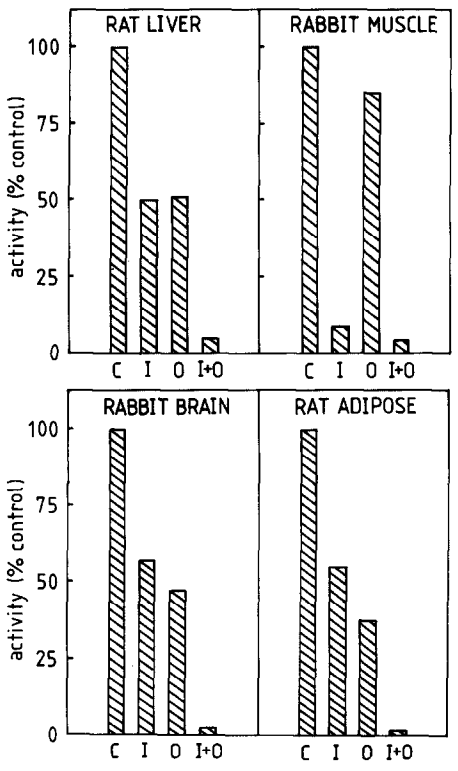

Fig.3. Effect of okadaic acid and inhibitor-2 on phosphorylase phosphatase (PhP) activity in tissue extracts. Assays were performed in the absence of divalent cations at 600-fold dilution (muscle, liver, brain) or 60-fold dilution (adipocyte) in the presence of 2 nM okadaic acid (O), after preincubation for 15 min with 0.2 μM inhibitor-2 (I), 15 min preincubation with inhibitor-2 plus okadaic acid (I+O), or in the absence of okadaic acid and inhibitor-2 (C, control). Control activities (undiluted extracts) were: 50 mU/ml (rabbit muscle), 25 mU/ml (rat liver), 24 mU/ml (rabbit brain), 2.2 mU/ml (rat adipose). The experiments were repeated using extracts from 3 different animals with very similar results.

tained in each tissue examined, and with phosphorylase, phosphorylase kinase, glycogen synthase or casein as substrates (fig.2).

The experiments in fig.2 indicated that the native forms of type 1 and type 2A phosphatases in several mammalian tissues were inhibited by okadaic acid with similar potency to the catalytic subunits, and that okadaic acid could therefore be used as a specific inhibitor of type 2A enzymes. Consistent with this conclusion, the effects of okadaic acid (2 nM) and inhibitor 2 (0.2 μM on phosphorylase phosphatase activity in several mammalian tissue extracts was additive. Activity not inhibited by 2 nM okadaic acid could be suppressed almost completely by inhibitor 2, while activity not inhibited by inhibitor 2 was blocked by 2 nM okadaic acid (fig.3). Identical results were obtained when inhibitor-1 (0.1 μM) replaced inhibitor 2 (not shown). Additive effects of okadaic acid (2 nM) and inhibitor 1 (0.1 μM), and complete inhibition in the presence of both, was also observed when phosphorylase kinase or glycogen synthase were employed as substrates (not shown).

Type 1 and type 2A protein phosphatases are both inhibited completely above 1 μM okadaic acid (figs 1,2) suggesting the possibility of quantitating type 2C activity in tissue extracts by including high levels of this tumour promoter in the assays. In the presence of 5 μM okadaic acid, casein phosphatase activity in skeletal muscle extracts was completely dependent on Mg^{2+} with an $A_{0.5}$ value of ≈ 1.3 mM (fig.4A). The same result was obtained using phosphorylase kinase as a substrate, or other tissues such as liver, brain and adipose (not shown). Furthermore, the Mg^{2+} dependent and okadaic acid-insensitive activity in muscle extracts was specific for the α-subunit of phosphorylase

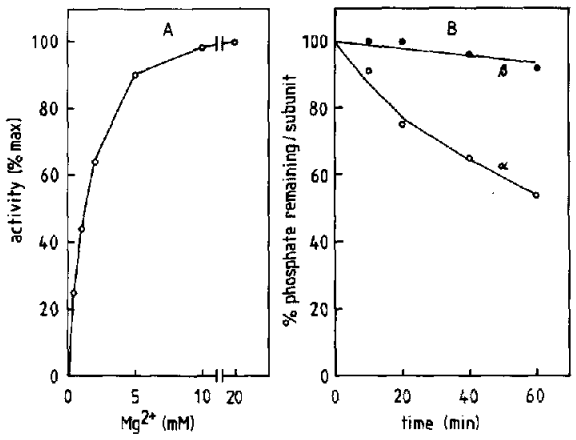

Fig.4. Desphosphorylation of casein (A) and phosphorylase kinase (B) by rabbit skeletal muscle extracts in the presence of 20 mM Mg^{2+} and 5 μM okadaic acid. Assays were performed at 30-fold (A) or 20-fold (B) dilution of the extracts. In A, 100% activity corresponds to the casein phosphatase activity in undiluted extracts (1.5 mU/ml). Similar results were obtained with extracts prepared from three different animals. In B., the phosphorylase kinase substrate contained 0.85 mol phosphatase/β-subunit and 0.81 mol phosphate/α-subunit, and release of phosphate from the α (○) and β (●) subunits was quantitated as in [14].

kinase (fig.4B). In contrast, the β-subunit was dephosphorylated selectively in the absence of okadaic acid and Mg^{2+} (not shown), as expected from the results in fig.3. Other properties of the Mg^{2+}-dependent activity, such as its low phosphorylase phosphatase activity (not shown) were also identical to the type 2C enzymes.

4. DISCUSSION

Okadaic acid, a potent tumour promoter in two stage mouse skin carcinogenesis experiments [15] is a specific inhibitor of type 1 and type 2A protein phosphatases [6]. PP2B is only inhibited at much higher concentrations [6], while PP2C [6], pyruvate dehydrogenase phosphatase [7], acid and alkaline phosphatases [6], a protein tyrosine phosphatase [6] and eight different protein kinases tested [7] (including protein kinase C[15]) are unaffected. Nor does okadaic acid inhibit non-specifically a large number of enzymes involved in carbohydrate and lipid metabolism [7].

In this paper we have demonstrated a further use for okadaic acid in identifying and quantitating protein phosphatases in tissue extracts. It is a specific inhibitor of type 2A phosphatases at 1–2 nM, while above 1 μM it inactivates type 1 as well as the type 2A enzymes, allowing measurement of type 2C phosphatases in tissue extracts, which has previously been impossible. The combined use of okadaic acid and inhibitor proteins allows type 1 and type 2A phosphatases to be quantitated in two independent ways. Type 1 phosphatase can be taken as the activity which is sensitive to the inhibitor proteins, or as the activity at 5 μM okadaic acid subtracted from that measured at 1–2 nM okadaic acid. Type 2A phosphatase can be taken as the activity which is unaffected by the inhibitor proteins, or as the amount which is blocked by 1–2 nM okadaic acid. Both methods agree to ± 10% in skeletal muscle, liver, brain and adipose extracts (fig.3), and are most accurate when extracts contain similar amounts of each phosphatase. If one enzyme accounts for 90% or more of the activity, accurate quantitation of the other is still difficult because large errors are introduced if the inhibitor proteins or okadaic acid are only 95% effective. Accurate quantitation of type 1, type 2A and type 2C phosphatases in tissue extracts will facilitate studies of the effects of hormones on their activities.

Two further conclusions may be drawn from this study. Firstly, >90% of type 1 phosphatase activity in each tissue extract is inhibited by either inhibitor 1 (0.1 μM) or inhibitor 2 (0.2 μM) after 15 min preincubation, validating the use of these proteins to estimate type 1 activity. However, an advantage of okadaic acid is that it is effective instantaneously and no preincubation is required. Secondly, complete inhibition at 1–5 μM okadaic acid confirms that type 1 and type 2A phosphatases are the only enzymes in tissue extracts with activity towards phosphorylase, phosphorylase kinase, glycogen synthase and casein, in the absence of divalent cations.

The similar effects of okadaic acid in each mammalian tissue is consistent with recent structural information which indicates that the major type 1, type 2A and type 2C catalytic subunits are the same gene products in skeletal muscle and liver [16–18]. Northern blotting indicates that the major mRNAs encoding the type 1 and type 2A catalytic subunits are 1.6 kb and 2.0 kb, respectively, in a wide variety of tissues, including brain [19].

The native type 2A enzymes in which the catalytic subunits are complexed to other proteins (i.e. those forms present in tissue extracts) are as sensitive to okadaic acid as the free catalytic subunit (figs 1 and 2). This finding is in disagreement with observations made by other investigators, who reported that the native forms of some type 2A phosphatases were up to 100-fold less sensitive [6,20]. The reason for this discrepancy is unclear, although the results presented in fig.1 show it is critical to compare the effects of okadaic acid at identical phosphatase concentrations. The purified glycogen associated [3] and myofibrillar [21] forms of protein phosphatase 1 are also inhibited by okadaic acid in a similar manner to the type 1 catalytic subunit (not shown).

The $A_{0.5}$ for Mg^{2+} of the type 2C protein phosphatase in skeletal muscle extracts was ≈ 1.3 mM with casein as substrate (fig.4). compared to 2.5–3 mM for the homogeneous $2C_2$ isoform (not shown). This observation suggests that a cofactor which increases the affinity of the enzyme for Mg^{2+} may be lost during purification.

Okadaic acid can enter intact cells, and when added to adipocytes or hepatocytes, produces marked increases in the phosphorylation states of many proteins and alters carbohydrate and lipid metabolism in a manner consistent with its action as a specific protein phosphatase inhibitor [7]. However, although okadaic acid should bind preferentially to type 2A phosphatases, it cannot be used to determine whether type 1 or type 2A phosphatases are the relevant enzymes acting on a particular phosphoprotein in vivo. The intracellular concentrations of these enzymes are both 0.1–1.0 μM and similar levels of okadaic acid will be required to inhibit both enzymes in intact cells.

We have recently used the new methodology described in this article to identify the protein phosphatases acting on acetyl-CoA carboxylase in adipose tissue and liver (Cohen, P. and Hardie, D.G., unpublished) and tyrosine hydroxylase in adrenal medulla and brain [22]. In the following paper we demonstrate that these procedures can even be applied to yeast [23], suggesting they are likely to be useful for the analysis of protein phosphatases in all eukaryotic cells.

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