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CHARACTERIZATION OF D-MYO-INOSITOL 1,4,5-TRISPHOSPHATE PHOSPHATASE IN RAT BRAIN

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Rat brain homogenates contain significant amounts of inositol 1,4,5-trisphosphate phosphatase in both 180,000xg (60 min) particulate and supernatant fractions. As other membrane-bound enzymes (e.g. guanylate cyclase), particulate inositol 1,4,5-trisphosphate phosphatase activity is highly sensitive to low concentrations of Triton X-100 (0.03 %). Higher concentrations of detergent (1 %) partially solubilized the enzyme. Thiol blocking agents (e.g. p-hydroxymercuribenzoate) inactivate inositol 1,4,5-trisphosphate phosphatase activity (an effect reversed with 2-mercaptoethanol). It is thus suggested that enzymatic activity requires the presence of -SH groups.

Inositol 1,4,5-trisphosphate (IP_3), which is formed by receptor-coupled hydrolysis of phosphatidylinositol bisphosphate, appears to be the intracellular signal responsible for mobilizing intracellular calcium (1). The intracellular concentration of IP_3 is determined by the rate of its synthesis by phospholipase C and the rate of its catabolism by IP_3 phosphatase(s). Previous work on cyclic nucleotide phosphodiesterases has demonstrated the importance of the catabolic pathway of cAMP as a target for positive or negative regulation of the cAMP system (2). Dephosphorylation reactions of IP_3 by phosphatase(s) could similarly determine the intensity and duration of IP_3 response to hormones or neurotransmitters and be involved in the regulation of IP_3 signal. In human erythrocytes and in rat liver, IP_3 phosphatase is mainly associated to plasma membranes. The enzyme specifically hydrolyzes IP_3 to inositol

ABBREVIATIONS:
IP_3 : D-myo-inositol 1,4,5-trisphosphate; DTT : dithiothreitol; PMSF : phenylmethylsulfonyl fluoride; PHMB : p-hydroxymercuribenzoate; DTNB : 5,5'-dithiobis-2-nitrobenzoic acid; NEM : N-ethylmaleimide.

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bisphosphate at the 5-phosphate of the IP_3 molecule (3-7). In contrast, a IP_3 5-phosphatase apparently soluble of molecular weight of 38,000 had recently been purified in human platelets (8). As phosphatidylinositol turnover is very active in the brain and as lithium salts inhibit inositol 1-phosphate phosphatase (9) and are widely used in psychiatric treatment, we investigated brain enzymes involved in this metabolism. We report here the presence in rat brain of a IP_3 phosphatase activity.

MATERIALS AND METHODS

Materials. PMSF, leupeptin, and IP_3 were purchased from Sigma Chemical Co; PHMB, DTNB and DTT from Serva; Triton X-100,GSH and GSSG from Boehringer; AG_1-X8 Dowex (formate form) from Bio-Rad; polyethyleneimine-cellulose thin layer chromatography plates from Merck; Sephadex G-25 from Pharmacia.

Preparation of human erythrocyte membranes and [^{32}P] labelled IP_3.
Erythrocyte membranes and [4,5-^{32}P] Ins(1,4,5)P_3 or [^{32}P]IP_3 were prepared according to published procedures (4,10). [^{32}P]IP_3, isolated by Dowex formate chromatography, was identified by autoradiography after thin layer polyethyleimine-cellulose chromatography (11). [^{32}P]IP_3 was a substrate of the erythrocyte Ins(1,4,5)P_3 5-phosphatase with Michaelis-Menten kinetics (Km=30 µM) in agreement with Downes et al. (4). The concentration of IP_3 was determined by phosphate content measurement (12).

Soluble and particulate fractions from rat brain.
IP_3 phosphatase in soluble and particulate fractions from rat brain was prepared as follows. In general, 3 g of fresh brains from Sprague-Dawley male rats were homogenized at 0-4°C in 12 ml of the following buffer medium : 20 mM Tris/HCl (pH 7.5), 0.4 mM PMSF, 5 µM leupeptin, 0.25 mM sucrose. This preparation was centrifuged at 180,000xg for 1 hour. The supernatant fluid was removed and the pellet fraction resuspended in the homogenizing medium (final volume equal to the whole homogenate volume) and centrifuged for 30 min at 180,000xg. The washed pellet was resuspended in hypotonic medium 20 mM Tris/HCl (pH 7.5), 0.4mM PMSF and again centrifuged at 180,000xg (1 hour). This pellet is referred to as the particulate fraction. Alternatively, the washed pellet (after the second centrifugation) was resuspended (final volume equal to the whole homogenate volume) in hypotonic medium containing 10% glycerol and 1 % Triton X-100. This preparation was kept on ice for 1 hour before centrifugation at 180,000xg (1 hour). The supernatant of the last centrifugation is referred to as the Triton-dispersed preparation. Active fractions were stored at -80°C in small aliquots which were thawed just before the enzymatic assay. Protein concentration was measured by the method of Lowry et al. (13).

Incubation and assay of inositol 1,4,5-trisphosphate phosphatase.
The incubation mixture contained the phosphatase in 50 mM Na-Hepes (pH 7.4), 2 mM $MgCl_2$, 5 mM C_2H_6OS, 1 mg bovine serum albumin/ml and 500-1000 cpm [^{32}P]IP_3 (0.028 nmole or more) in 0.1 ml. The reaction was initiated by adding the enzyme for 5-7 min incubation at 37°C. The incubation was terminated by adding 0.1 ml ice cold 20 % trichloroacetic acid. After 15 min, the precipitates were pelleted by centrifugation (30 min, 3000 rpm) at 4°C. 0.170 ml of each supernatant was extracted three times with 0.5 ml of diethyl ether to remove the trichloroacetic

acid and then neutralized with 2 µl of concentrated NH_4OH. The inositol phosphates were separated on 0.2 ml Dowex 1-X8 (formate) as described by Downes and Michell (3). Enzyme activities were calculated from the [^{32}P]Pi eluted in the phosphate peak on Dowex formate chromatography and expressed as pmoles/min.

RESULTS

Apparent distribution of IP_3 phosphatase activity in soluble and particulate fractions from rat brain.

IP_3 phosphatase activity was observed in both soluble and particulate fractions from rat brain. Both Mg^{2+} or Mn^{2+} could serve as metal ions for the dephosphorylation reaction of IP_3 and 1 mM Li^+ did not affect phosphatase (not shown). Enzyme activities were linear with respect to protein concentration (Fig. 1A). Table 1 shows the distribution of IP_3 phosphatase between 180,000xg (60 min) particulate and supernatant fluid fractions. The enzyme appeared to be confined primarily to the particulate fraction (70 % of total activity when assayed in the absence of Triton X-100). When the brain washed pellet (after the two first centrifugations) was resuspended in hypotonic medium in the presence of 10 % glycerol and 1% Triton X-100, centrifuged at 180,000xg (60 min), 40-50 % of the activity measured in total extract before centrifugation remained in the supernatant fluid (not shown). Activity of several membrane-bound enzymes (e.g. guanylate cyclase or tyrosine protein kinase) are often sensitive to nonionic detergents (14,15). This was also observed with rat brain particulate IP_3 phosphatase : 0.02-0.04 % Triton X-100 stimulated the particulate activity by 2-5 fold. Enzyme activities measured in crude homogenates but not in soluble fractions were also sensitive to the presence of detergent in the incubation mixture (Table 1).

Downes et al. reported that the 5-phosphate of IP_3 is selectively removed from IP_3 by erythrocyte membranes (4). They also showed that [^{32}P] is unequally distributed between the 4- and 5- phosphate of IP_3 (the 1-phosphate is not labelled in human erythrocytes, ref. 4) with 60-80 % of [^{32}P] being in the 5-phosphate. In a time course study (refs

TABLE 1

IP_3 PHOSPHATASE ACTIVITY IN RAT BRAIN TISSUE

Fraction assayed	0.03 % Triton X-100 in the incubation mixture	
	—	+
Homogenate	3924 ± 131	13832 ± 1471
Supernatant fluid	512 ± 25	512 ± 4
Particles	2796 ± 71	12753 ± 59

IP_3 phosphatase activity was measured on freshly prepared homogenate, supernatant and particulate fractions from rat brain (3 g of tissue) as described in the Methods section. Total activity, nmoles/min ± SEM. Results are means of triplicate determinations and are representative of experiments with three different preparations.

4 and 16 and Fig. 1B), the proportion of [^{32}P] actually in the 5-phosphate of IP_3 ([^{32}P] labelled from human red cells) can be measured by the [^{32}P]Pi released by erythrocyte membranes. Fig. 1B shows a kinetic study of the Triton-dispersed rat brain particulate fraction. Activity

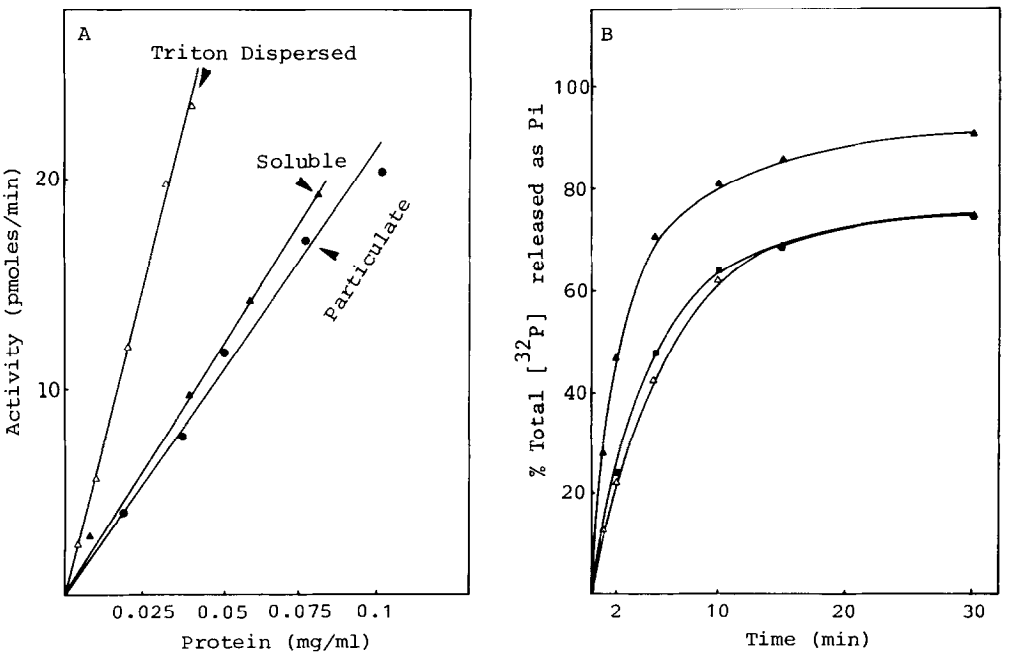

FIGURE 1. IP_3 phosphatase activity as a function of protein concentration and incubation time. A, effect of protein concentration of rat brain soluble, particulate and Triton-dispersed preparation. The reaction mixture was incubated for 5 min at 37°C, B, time course study of rat brain soluble (▲-▲), Triton-dispersed preparation (△-△) and in human erythrocyte membranes (■-■).

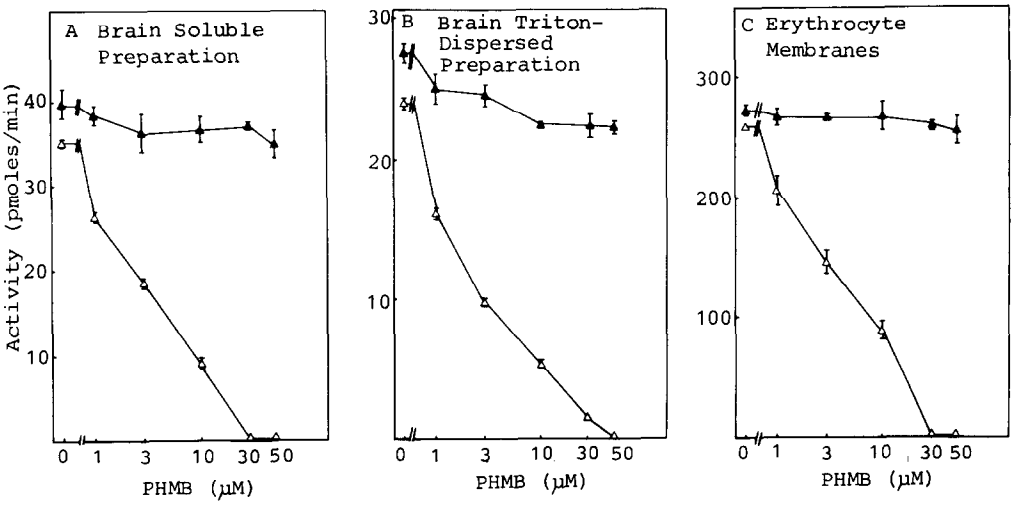

FIGURE 2. Inactivation of IP_3 phosphatase activity by PHMB. Samples of enzyme of rat brain soluble (A), Triton-dispersed preparation (B) and human erythrocyte membranes (C) were incubated with the indicated concentration of PHMB in the presence (▲-▲) and absence (△-△) of 2 mM 2-mercaptoethanol. Activity, pmoles/min ± SEM.

is expressed as the proportion of [^{32}P] released as organic phosphate (16). The data were compared with IP_3 phosphatase of erythrocytes and rat brain soluble fraction. Activity-curves of erythrocytes and brain particulate fraction run parallel and reached a plateau at 73% [^{32}P] released. This shows that 1) 73 % of [^{32}P] in our preparation of IP_3 is in the 5-phosphate position; 2) that brain particulate solubilized activity is specific for the 5-position contrarily to the soluble activity. Substrate-velocity relationships of erythrocytes and Triton-dispersed particulate preparation of brain showed Michaelis-Menten kinetics with apparent Km for IP_3 of 30 μM and 50 μM, respectively (not shown).

Reversible inactivation of IP_3 phosphatase by sulfhydryl reagents.

IP_3 phosphatase activity of rat brain soluble, particulate or Triton-dispersed particulate preparation was inactivated by PHMB (50 % inactivation at 3-10 μM PHMB, Fig. 2). Addition of millimolar 2-mercaptoethanol or DTT to the medium restored activity. Inactivation of enzyme preincubated with PHMB at 0°C (10 min) persisted after removal of free PHMB by chromatography on Sephadex G-25. 2 mM DTT produced maximal

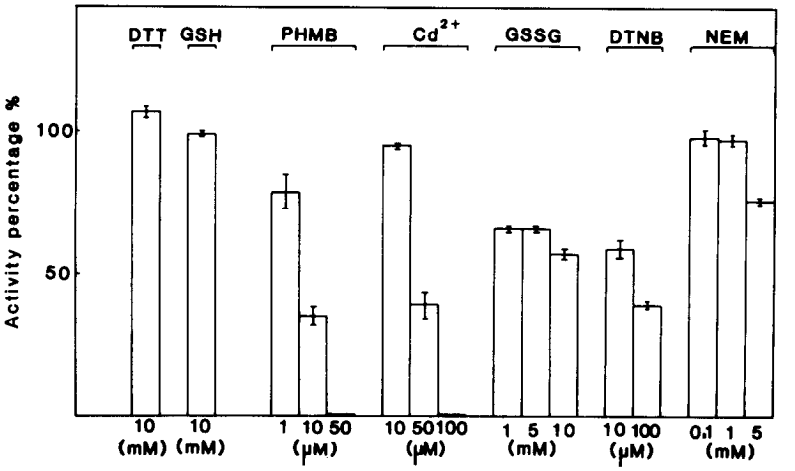

FIGURE 3. Inactivation of IP_3 phosphatase activity of erythrocyte membranes by reducing and sulfhydryl reagents. Activities are expressed relative to that of enzyme incubated in the absence of reagent = 100.

reactivation (data not shown). The effect of PHMB was similarly observed with the erythrocyte IP_3 phosphatase (Fig. 2). It could be generalized to other compounds capable of forming mixed disulfides or covalent mercaptide bonds : DTNB, NEM or GSSG (Fig. 3). Activity was also reduced in the presence of the dithiol modifier, Cd^{2+} (17). Thiol reducing agents 2-mercaptoethanol, DTT or GSH did not affect IP_3 phosphatase activity measured by brain or erythrocyte membranes phosphatase.

DISCUSSION

The three important features of this report can be summarized as follows : 1) rat brain homogenates contain significant amounts of IP_3 phosphatase activity in both 180,000xg (60 min) particulate and supernatant fractions. When assayed in the absence of detergent, 70-80 % of total activity is recovered in the particulate fraction, 2) particulate IP_3 phosphatase activity is highly sensitive to low concentrations of Triton X-100 (0.03 %). Higher concentrations of detergent (1 %) partially solubilized the enzyme with no loss of its properties (at least for the kinetics and inhibition by PHMB), and 3) the observation that thiol blocking agents inactivate IP_3 phosphatase activity (an effect reversed with 2-mercaptoethanol), suggests that enzymatic activity requires the presence of -SH group(s).

In liver and in erythrocytes, IP_3 phosphatase is largely associated with plasma membranes (3-7). This is in contrast with platelets where most activity remains "soluble" after high speed centrifugation (8). Whether or not in brain the soluble phosphatase that dephosphorylates IP_3 is different from the particulate activity has not been clear. We have not ruled out the possibility that the soluble form is generated from the particulate enzyme fraction by endogenous proteolysis. Moreover, the soluble fraction of rat brain homogenates probably contains other phosphatases that are less specific towards the 5-phosphate of IP_3. For example, rat liver soluble fraction shows phosphatase activity that dephosphorylates inositol 1,4-bisphosphate and inositol 1-phosphate (5-7). Purification of IP_3 phosphatase activity of both soluble and particulate fractions of rat brain should clarify the situation. The Triton-solubilized particulate phosphatase should be helpful for that purpose.

Storey et al. reported that Cd^{2+} is a potent inhibitor of rat liver IP_3 phosphatase activity (6). In light of a decrease of phosphatase activity by thiol inhibitors (this study), it is suggested that as for guanylate cyclase (17), the Cd^{2+} inhibition is due to its interaction with vicinal dithiols. Whether or not thiols and dilsulfides are involved in receptor-mediated and regulation of IP_3 signal in intact cells is unknown. It is noteworthy that processes involving oxidation-reduction and modification of critical sulfhydryl groups of guanylate cyclase may be a mechanism for altering its activity in intact cells (18,19).

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