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EFFECT OF PHOSPHATASE INHIBITION OF *IN VITRO* DOPAMINE SULFATION AND 3′-PHOSPHOADENOSINE-5′-PHOSPHOSULFATE CATABOLISM IN HUMAN BRAIN

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Abstract—The effects of inhibition of phosphatase activity in 100,000 *g* supernatant solution from human frontal cortex on dopamine (DA) conjugation were examined using the phosphatase substrate *p*-nitrophenyl phosphate ($pNPO_4$). The increases in DA sulfation seen in the presence of $pNPO_4$ suggested that inhibition of phosphatase activity in high speed supernatant solutions of brain may substantially alter the pattern of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) metabolism and subsequently the rate of DA sulfation. Accordingly, the effects of the pyrophosphate analog phosphonoacetic acid (PAA) on the extent of DA sulfation and PAPS metabolism were examined in 100,000 *g* supernatant solution from human frontal cortex. At concentrations up to 10 mM, PAA markedly reduced PAPS hydrolysis to inorganic sulfate and 3′-phosphoadenosine-5′-phosphate (PAP) and significantly extended the linear time period for the sulfation of DA. These findings suggest that the phosphatase enzymes that degrade PAPS to produce the end product inhibitor, PAP, and possibly other break-down products of PAP, play an important role in determining the observed levels of phenol sulfotransferase activity in tissue from human brain *in vitro*.

Previous investigations by Anderson and Weinshilboum [1, 2] have suggested that apparent differences in tissue levels of phenol sulfotransferase (PST)† and non-linear relationships between the rate of sulfation and protein concentration may be due to the presence of endogenous inhibitors. This is based on the observation that human kidney homogenates, red blood cell lysates, and various rat tissues are capable of inhibiting human erythrocyte PST. Two putative inhibitors were described, one which was heat-labile and possessed an apparent molecular weight greater than 65,000, and a second which was heat, acid and base-stable and had a molecular weight of less than 2000 daltons.

Recent studies [3] in this laboratory have demonstrated that the primary pathway of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) metabolism in 100,000 *g* supernatant solution from human frontal cortex involves a rapid hydrolysis of [^{35}S]PAPS to produce ^{35}S-inorganic sulfate, while a negligible amount of PAPS is utilized to conjugate dopamine (DA) and other acceptor substrates. These findings suggest that a major catabolic pathway of PAPS in crude high-speed supernatant fractions of human brain probably involves the action of phosphatase-like enzymes, which act to hydrolyze PAPS to yield inorganic sulfate (SO_4) and the inhibitory product 3′-phosphoadenosine-5′-phosphate (PAP) [4].

To evaluate the contribution of the phosphatase hydrolytic enzymes to the metabolism of PAPS, the structural analog of inorganic pyrophosphate, phosphonoacetic acid (PAA), was used to selectively inhibit phosphatase activity [5] because pyrophosphate has been reported to be an effective inhibitor of alkaline phosphatase [6]. Results of these experiments confirm previous findings that PAPS is rapidly hydrolyzed to PAP and inorganic sulfate in human brain and that the hydrolytic enzymes, the phosphatases, may represent the high molecular weight, heat-labile endogenous inhibitor previously reported by Anderson and Weinshilboum [1, 2].

MATERIALS AND METHODS

Preparation of 100,000 g *supernatant solution from human frontal cortex*. Sections of frontal cortex from human brain were obtained at autopsy within 12 hr of death. Tissue was stored at −80° until use. Postmortem changes in the activity of PST were expected to be minimal, as it has been demonstrated previously that no correlation exists between enzyme activity and storage time [7]. Approximately 10–20 g of cortical tissue was thawed and disrupted with a Teflon–glass homogenizer in 5 vol. of 10 mM triethanolamine, pH 7.4, containing 0.25 M sucrose, 5 mM 2-mercaptoethanol and 500 μM $MgCl_2$. The homogenate was centrifuged at 12,000 *g* for 30 min, and the resulting supernatant fraction was recentrifuged at 100,000 *g* for 60 min. The microsomal pellet was discarded, and aliquots of the high-speed supernatant solution containing PST activity were

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† Abbreviations: PST, phenol sulfotransferase; DA, dopamine; $pNPO_4$, *p*-nitrophenol phosphate; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PAA, phosphonoacetic acid; PAP, 3′-phosphoadenosine-5′-phosphate; SO_4, inorganic sulfate; and APS, adenosine-5′-phosphosulfate.

stored at −80° until needed. All procedures used during the tissue preparation were carried out at 4°.

Assay of PST activity and determination of PAPS metabolism. Dopamine sulfation and PAPS catabolism were measured using a modification of the Ecteola-cellulose assay [3] procedure described by Borchardt *et al.* [8]. Incubations were carried out in the presence of 10 μM DA, 1 μM [^{35}S]PAPS (0.8 μCi/nmole), 1 mM pargyline (a monoamine oxidase inhibitor) and 100,000 *g* supernatant solution in a final volume of 0.5 ml of 50 mM triethanolamine (TEA) buffer, pH 7.4. It should be noted that the presence of PAA in the reaction mixture interferes with the widely-used barium precipitation assay of Foldes and Meek [7]. Following incubation at 37°, aliquots of the incubation mixtures were placed on EC columns (2 cm bed height, formate counter-ion, equilibrated with 5 mM NH_4HCO_3) prepared in small pasteur pipets. Dopamine sulfate was eluted with 3 ml of water, while SO_4 and PAPS were eluted in sequence with 6 ml of 20 and 200 mM NH_4HCO_3 respectively.

Materials. 3′-Phosphoadenosine-5′-phospho[^{35}S] sulfate (0.1 to 5.0 Ci/mmole) and Formula 963 liquid scintillation fluid were purchased from the New England Nuclear Corp., Boston, MA. Triethanolamine free base and 2-mercaptoethanol were obtained from the J. T. Baker Chemical Co., Phillipsburg, NJ. Triethanolamine-HCl, dopamine-HCl, *p*-nitrophenyl phosphate ($pNPO_4$) and pargyline-HCl were purchased from the Sigma Chemical Co., St. Louis, MO. Phosphonoacetic acid was obtained from the Ventron Co., Alfa Division, Danvers, MA.

RESULTS

To etablish whether a phosphatase-like enzyme is actually involved in PAPS hydrolysis, the effects of the phosphatase substrate, $pNPO_4$, on dopamine sulfation were examined (Fig. 1). Inclusion of a phosphatase substrate in the incubation medium would be expected to decrease the hydrolysis of PAPS by the phosphatases and, thereby, increase the production of dopamine sulfate by making more PAPS available for sulfoconjugation. As illustrated in Fig. 1, a 3-fold increase in the formation of dopamine sulfate was observed during an incubation performed in the presence of 100 mM $pNPO_4$.

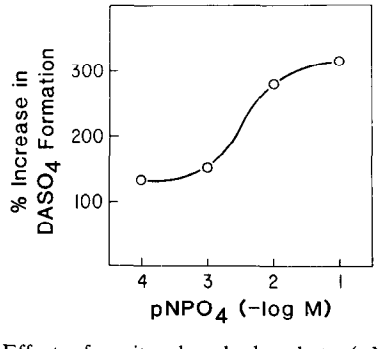

Fig. 1. Effect of *p*-nitrophenyl phosphate ($pNPO_4$) on dopamine sulfation by 100,000 *g* supernatant solution from human frontal cortex. All assays were carried out in the presence of 20 μM dopamine, 1 mM pargyline, and 1 μM [^{35}S]PAPS in a total volume of 50 mM TEA buffer, pH 7.4, with an incubation period of 60 min. Dopamine sulfation was measured as described in Methods. In samples incubated in the absence of $pNPO_4$, 9.6 pmoles of dopamine sulfate was formed. $DASO_4$ = dopamine sulfate.

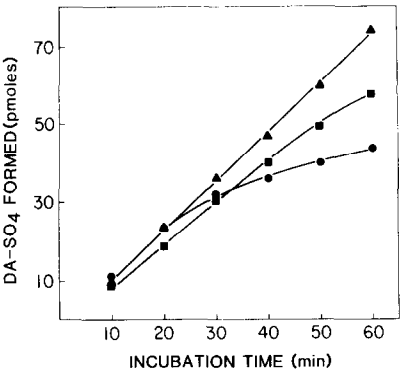

Fig. 2. Effect of phosphonoacetic acid on dopamine sulfation as a function of time. All assays were carried out in the presence of 20 μM DA, 1 μM [^{35}S]PAPS and 1 mM pargyline, with no PAA (●—●), 4 mM PAA (■—■) or 10 mM PAA (▲—▲). Dopamine sulfate formation was quantitated as described in Methods.

The potential use of $pNPO_4$ for assaying PST activity by preventing PAPS breakdown is somewhat limited since the product of hydrolysis of $pNPO_4$, *p*-nitrophenol, could potentially interfere with the sulfation reaction by acting as a competing PST substrate. To find an inhibitor of phosphatase which did not interfere with PST activity, the effects of PAA on PAPS metabolism and dopamine sulfate production were investigated. The results of these experiments, illustrated in Fig. 2, demonstrate that, in the absence of PAA and at saturating concentrations of both DA and PAPS, the rate of DA sulfation was apparently linear over a 30-min period under the assay condition employed. After this time, only slight increases in the amount of sulfated product were observed. In the presence of 4 mM PAA, the formation of dopamine sulfate was linear for approximately 50 min, with 40% more dopamine sulfate formed than when PAA was absent at this time point. When the concentration of PAA was increased to 10 mM, the reaction remained linear for 60 min and an 80% increase in the amount of product formed was observed.

The time course of PAPS degradation over a 60-min incubation period performed in the presence and absence of PAA is shown in Fig. 3, A and B respectively. In the absence of PAA, sulfate production was linear for approximately 40 min and accounted for approximately 45% of the total PAPS initially present at this time point. By 60 min, approximately 70% of the total PAPS had been degraded to SO_4. The effects of addition of 10 mM PAA to the incubation mixture are illustrated in Fig. 3A, and reveal that SO_4 production accounted for between 6 and 22% of the PAPS initially present. Over this incubation period, PAPS hydrolysis

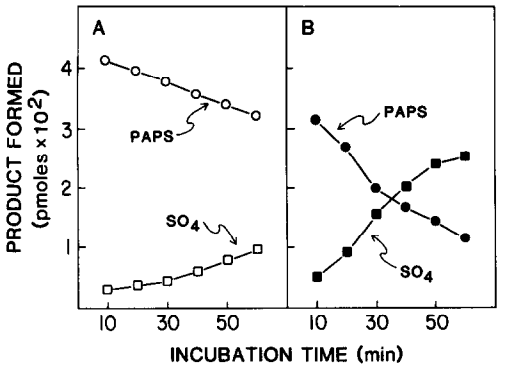

Fig. 3. Effect of incubation time on SO_4 production and PAPS utilization in the presence (A) and absence (B) of 10 mM phosphonoacetic acid. Reaction conditions were as described for Fig. 1. SO_4 and PAPS were quantified as described in Methods.

exhibited a linear decrease, with approximately 9% utilization at 10 min and less than 30% utilization at 60 min.

The relationship between changes in the concentration of PAA and the formation of dopamine sulfate for a 60-min incubation period is shown in Fig. 4A. Dopamine sulfate formation increased from approximately 9 pmoles/hr to 27 pmoles/hr when 2 mM PAA was included in the incubation mixture, this rate remaining constant up to a concentration of 8 mM PAA. Panel B of Fig. 4 depicts the effects of various PAA concentrations on SO_4 production and PAPS utilization for the same 60-min period. As expected, SO_4 production declined from 70% to approximately 20% of the total radiolabel recovered as the concentration of PAA increased, while the amount of unreacted [^{35}S]PAPS increased steadily from 28 to 70% over the same concentration range.

DISCUSSION

The data presented in this paper demonstrate that inclusion of $pNPO_4$ or PAA in reaction mixtures utilizing 100,000 *g* supernatant solution as a source of PST increased the amount of DA sulfation while decreasing PAPS catabolism. The linear period of DA sulfation was extended from 30 to 60 min, resulting in a substantial increase in the amount of product formed. Concomitant with the increase in dopamine sulfate production was a decrease in hydrolysis of PAPS to SO_4. In the tissues employed in this study, clearly, enzymatic hydrolysis of PAPS accounted for the majority of sulfate donor utilization. The effect of PAA on PAPS degradation appeared to be saturable, with maximal effect occurring at 2 mM. It is unclear at this point whether the residual production of SO_4 and PAPS utilization at apparent saturating concentrations of PAA was due to incomplete inhibition of phosphatase activity or to the presence of alternative pathways of PAPS metabolism. Inorganic SO_4 could also be produced by two alternative two-step pathways, the first involving the action of 5′-nucleotidase [9, 10] to form a mixed sulfate-phosphate anhydride and adenosine-3′-phosphate, with subsequent hydrolysis of the anhydride by pyrophosphatase to form SO_4 and orthophosphate. The second possibility involves the action of a 3′-nucleotidase [11] to form adenosine-5′-phosphosulfate (APS), with subsequent action of phosphatase or APS-sulfohydrolase [12, 13] to generate SO_4. The former pathway seems unlikely, as 5′-nucleotidase is a plasma membrane-associated enzyme [14, 15] and our investigation was carried out using the cytosolic fraction of human brain. It is unclear how much 3′-nucleotidase activity might be present in the enzyme fraction used in these experiments, as all assays were performed at pH 7.4 and this nucleotidase has been reported to have a substantially more acidic pH optimum [16].

Anderson and Weinshilboum [1] have proposed that high and low molecular weight endogenous inhibitors of PST may be present in various tissues, basing their argument upon non-linear relationships between the rate of sulfate conjugation and protein concentration, as well as the ability of various other tissue preparations to decrease the velocity of sulfation by human erythrocyte PST. Although endogenous inhibitors of PST may exist *in vivo*, the data presented in this study support the hypothesis that the high rate of PAPS hydrolysis caused by phosphatases results in the accumulation of the end product inhibitor, PAP, as well as subsequent breakdown products of PAA which all may inhibit PST activity and promote an apparent decrease in the amount of sulfation of DA. PAP has been reported to be a potent inhibitor of human brain M-PST,

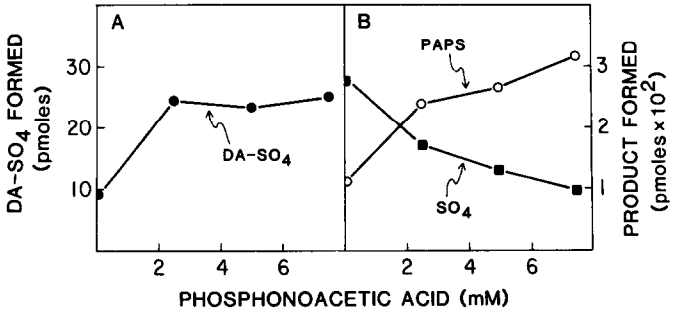

Fig. 4. Effect of phosphonoacetic acid on dopamine sulfation SO_4 production and PAPS utilization. Assay conditions were as described in the legend of Fig. 1.

possessing a K_i value of approximately 0.1 μM [17]. Since this value is similar to the K_{ia} for PAPS binding to PST, it can be calculated that, if 50% of PAPS is converted to PAP in the reaction mixture, then the reaction rate will be reduced to approximately the same extent. It is also likely that PAP is degraded to AMP or adenosine-3'-monophosphate which can similarly inhibit DA sulfation. The exact nature of the inhibiting species is not known, but PAPS breakdown to PAP apparently is the initial event in formation of these dead-end inhibitors.

The marked decrease in SO_4 production and PAPS utilization observed in the presence of PAA suggests that the observed levels of PST activity in a given tissue represent a dynamic balance between the actual sulfation process, availability of PAPS, and the steady-state level of the inhibitors PAP and PAP catabolites. It is likely that previous reports of endogenous inhibitors of PST [1, 2] can be attributed to tissue differences in the balance between PST and phosphatase activities, rather than the presence of a true inhibitor of PST. Accordingly, the high molecular weight inhibitor previously reported may actually represent the phosphatases which are responsible for the break-down of PAPS to the end product inhibitor, PAP, and subsequently PAP metabolites.

The findings reported in this paper also serve to emphasize the importance of competing pathways of PAPS utilization, especially when dealing with comparisons of enzyme activities among different species of tissues. Whether the rapid hydrolysis of PAPS to the inhibitor, PAP, occurs extensively *in vivo* of course is not known, but in light of the apparent importance of PAP inhibition of PST, it may be necessary to reeevaluate the literature with respect to observed levels of PST activity and PAPS utilization.

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