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Kinetic parameters of 3′,5′-cAMP phosphodiesterase in cat sciatic nerve*

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Electrophysiological evidence indicates that cAMP may play a role in the modulation of neurotransmitter release at the neuromuscular junction. For example, lipid-soluble derivatives of cAMP and drugs which stimulate adenylate cyclase or inhibit cAMP phosphodiesterase have been shown to increase the frequency of spontaneous miniature endplate potentials, the quantal content and mobilization of stimulus-evoked release, and to produce stimulus-bound repetitive firing of the nerve ending [1, 2]. However, there have been few biochemical studies of cyclic nucleotide synthesizing and degrading enzymes at the motor nerve ending. Bray *et al.* [3] found that adenylate cyclase and phosphodiesterase accumulate proximal to a ligature placed around the sciatic nerve in chickens. This suggested that the enzymes are carried by rapid axonal transport toward the nerve ending. In addition, Pardos and Lentz [4] have shown by electron microscopic examination of newt motor-nerve endings stained for phosphodiesterase that the enzyme is bound to the membranes of the nerve endings and to vesicles within them. This paper describes the biochemical characteristics of 3′,5′-cAMP phosphodiesterase (cAMP PDE) in cat sciatic nerve and demonstrates that this enzyme is transported toward the nerve ending.

Methods

Tissue preparation. A portion of the sciatic nerve was removed from cats anesthetized with 70 mg α-chloralose/kg, i.v. The sciatic nerve was taken from the sciatic notch to its bifurcation into the tibial and common peroneal nerves. The extracted nerve was kept frozen in Tris–Mg buffer (TMB) which contained 160 mM Tris–HCl and 20 mM $MgCl_2$ (pH 8.0). Just before enzyme assay, the tissue was thawed and disheathed. The remaining nerve fibers were minced and then homogenized to 10% in TMB using a Potter–Elvehjem homogenizer with a Teflon pestle. The resulting suspension was called the tissue homogenate.

For 3′,5′-cAMP PDE measurements, the tissue homogenate was separated by centrifugation into soluble and particulate fractions. This was accomplished by spinning the tissue homogenate at 2000 rpm × 5 min in a Sorvall refrigerated centrifuge. The resulting supernatant fraction was then spun at 105,000 *g* × 50 min in a refrigerated Beckman ultracentrifuge. The resulting high speed supernatant fraction which contained all of the soluble cAMP PDE was decanted and kept on ice for later assay. The particulate fraction was washed by resuspending the high and low speed pellets together to 10% (w/v) in TMB. This suspension was then spun at 105,000 *g* × 50 min. The resulting supernatant fraction was discarded. The resulting pellet, containing membrane bound or particulate cAMP PDE, was resuspended to 10% in TMB and assayed.

cAMP phosphodiesterase assay. cAMP PDE was assayed according to the following modifications of the two-step method described by Thompson and Appleman [5]. The reaction mixture (total volume 200 μl) contained 40 mM Tris, 40 mM $MgCl_2$, cyclic-[^{3}H]3′,5′-AMP to yield 8 pmoles and approximately 150,000 cpm, unlabeled cAMP, and 50 μl of enzyme sample. These enzyme samples contained approximately 1.15 mg/ml of protein for the high affinity supernatant measurements, 2.4 mg/ml protein for the high affinity pellet, 1.6 mg/ml protein for low affinity supernatant, and 3.4 mg/ml protein for the low affinity pellet. Incubations at 30° were carried out for 10–30 min. After boiling the reaction tubes for 2.0 min to stop the reaction, 50 μl of snake venom (*Ophiophagus hannah*) solution (1 mg/ml) was added. After a 10-min incubation with snake venom, 50 μl of aqueous [^{14}C]adenosine containing approximately 1000 cpm was added to each sample to monitor isotope recovery for the anion-exchange column. Bio-Rad columns AG 1-X2 (2.0 cm high) were prepared in glass Pasteur pipettes with glass wool as a support. Each sample was applied to a separate column and washed with 0.6 ml of distilled H_2O; the eluate was discarded. Then 2.5 ml H_2O was applied to each column and the eluates were collected in separate scintillation vials. Scintillation mixture was added to these vials and the [^{3}H]- and [^{14}C]- adenosine were measured in a liquid scintillation spectrometer. The reaction rates of enzyme activities were linear with respect to time and protein concentration range. Proteins were assayed by the method of Lowry *et al.* [6].

Based on the kinetic parameters obtained for the high and low affinity enzyme forms of cAMP PDE from cat sciatic nerve, further enzyme assays were performed at substrate concentrations appropriate for measuring either high affinity (0.05 μM) or low affinity (150 μM) forms of the enzyme. By using these substrate concentrations, the contribution of either the high or low affinity enzyme form to measurements of the other was negligible.

Transport studies. Cats were anesthetized with 25 mg/kg of ketamine hydrochloride, i.m. Under aseptic conditions, the sciatic nerve was exposed at the sciatic notch and ligated with a silk thread at approximately 5 cm from the spinal cord. The wound was closed. The cats were killed 48 hr after ligation, and 1-mm segments of the sciatic nerve were removed at distances of 1 and 6 mm proximal and distal to the ligation. The segments were homogenized and assayed for cAMP PDE. Only the high affinity forms of the enzymes were assayed due to limitations imposed by the size of the tissue samples. The final concentration of cAMP used to assay the ligated segments was 0.05 μM.

Unlabeled cAMP was purchased from the Sigma Chemical Co., St. Louis, MO. The cyclic-[^{3}H]3′,5′-AMP (34.6 Ci/mmole) and the [^{14}C]adenosine (54.5 mCi/mmole) were purchased from the New England Nuclear Corp., Boston, MA. The anion exchange resin AG 1-X2, 200–400 mesh, was obtained from Bio-Rad Laboratories, Richmond, CA. All other chemicals were reagent grade.

Results and discussion

Initial enzyme assays using cat sciatic nerve indicated the presence of both high and low affinity forms of cAMP PDE. These enzyme activities were investigated further in both the soluble and particulate fractions which were separated as described under Methods. Based on specific activity measurements for the tissue homogenate before fractionation, specific activities for both the high and low affinity forms of cAMP PDE were found to be approximately 60% soluble and 40% particulate after separation. As the Lineweaver–Burk plots in Fig. 1 show, both high and low affinity enzyme forms were identified in each fraction.

Table 1 shows the results of the enzyme kinetic measurements. The high affinity form of cAMP PDE was measured in a substrate concentration range from 0.04 to 2.0 μM in

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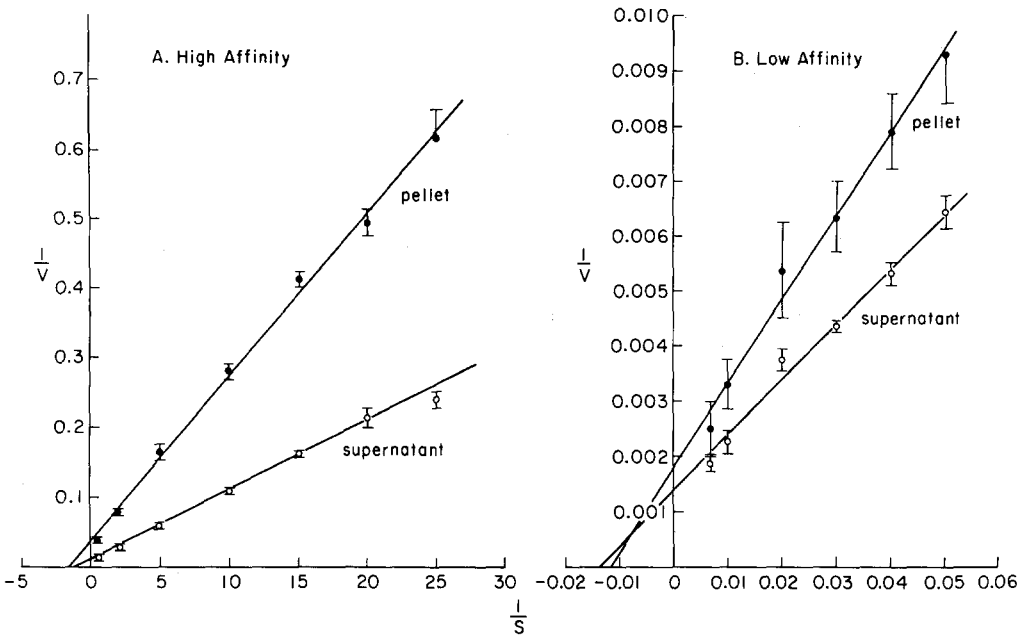


Fig. 1. Kinetic properties of cAMP PDE. These data show Lineweaver–Burk plots for the kinetic properties of cAMP PDE from cat sciatic nerve. Values are means ± S.E. for six to nine determinations. The cat sciatic nerve tissue homogenate was prepared and separated into pellet and supernatant fractions as described under Methods. Substrate concentration is represented by S, which was calculated by varying the amount of unlabeled cAMP which was added to 8 pmoles/assay of [^{3}H]cAMP. Velocity is represented by V which is the rate of formation of the product of the enzyme reaction and was measured as pmoles of [^{3}H]adenosine/(min · mg).

the soluble and particulate fractions with resulting K_m values of 0.81 and 0.60 μM respectively. The correlating V_{max} values were 84.0 pmoles/(min · mg) (soluble) and 25.6 pmoles/(min · mg) (particulate). Low affinity cAMP PDE was measured in a substrate concentration range from 20 to 150 μM in both fractions. The soluble enzyme form had a K_m of 74.0 μM and V_{max} of 733 pmoles/(min · mg). Similarly, the particulate enzyme form had a K_m of 84.7 μM and a V_{max} of 555 pmoles/(min · mg).

The transport studies, done in three animals, demonstrated that both soluble and particulate forms of high affinity cAMP PDE in the proximal segment were transported toward the nerve ending. Table 2 shows the results from one animal and is representative of all three. As can be seen, enzyme activity was greatest 1 mm proximal to a ligature as compared to other segments of the same ligated nerve. The soluble high affinity cAMP PDE showed more than a 2-fold increase, while the particulate fraction showed a 37% increase in enzyme activity.

Table 1. Kinetic parameters of cat sciatic nerve cAMP phosphodiesterase*

	K_m (μM)	V_{max} [pmoles/(min · mg)]
Soluble		
High affinity	0.81	84.0
Low affinity	74.0	733.0
Particulate		
High affinity	0.60	25.6
Low affinity	84.7	555.0

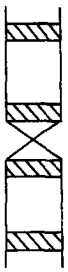
* Values were determined from the graph of Fig. 1. The maximum velocity (V_{max}) is defined as the reciprocal of the y axis intercept in the Lineweaver–Burk plot. The value for K_m is defined as the negative reciprocal of the x axis intercept in the Lineweaver–Burk plot and is expressed in terms of μM concentration of substrate.

The enzyme cAMP PDE has been found in virtually every tissue that has been tested. However, enzyme properties such as subcellular distribution, ionic requirements, and kinetic parameters appear to vary with the tissue being studied [7–10]. Most of the literature available describes cAMP PDE of the 100,000 g supernatant (a cytosolic) fraction of mammalian cells. In our studies of cat sciatic nerve, we found 60% of the enzyme activity in this soluble fraction, while 40% was measured in the pellet. Since the particulate fraction contained a substantial portion of the cAMP PDE activity, we continued to study both the soluble and particulate forms of the enzyme throughout our experiments. Detailed subcellular fractionation studies would be necessary to determine the exact location of the particulate enzyme activity. It is possible that a large portion of the particulate activity could be associated with mitochondria, as was reported for other neural tissue [11–14]. On the other hand, an electron microscopic examination of newt motor nerve endings stained for phosphodiesterase showed this enzyme bound to membranes of the axon and to vesicles within the nerve endings [4].

The kinetic data shown in Fig. 1 indicate the presence of both a high and low affinity form of the enzyme in both the soluble and particulate fractions. This characterization of a dual K_m has been reported for several other tissues including rat and rabbit skeletal muscle. The actual kinetic values given here approximate those reported for the rat brain cortex [9]. Further, Table 1 shows that the high affinity and low affinity enzymes for cat sciatic nerve appear to have similar kinetic properties in both their soluble and particulate forms.

Multiple forms of cAMP PDE have been previously

Table 2. Axonal transport of cAMP phosphodiesterase within cat sciatic nerve

Schematic diagram	Distance from ligation (mm)	High affinity enzyme activity* [pmoles/(min · mg protein)] Soluble	Particulate
Spinal cord			
	6	2.47	3.22
	1 0	5.50	4.41
	1	3.83	3.44
	6	3.42	3.73
Nerve terminal			

* High affinity cAMP PDE activity for both soluble and particulate fractions was measured at a substrate concentration of 0.05 μM cAMP. The results are from one animal; they are representative of measurements from three animals.

separated by gel filtration techniques [9]. These forms were shown to vary with respect to substrate specificity, tissue, and ionic requirements. Other work has shown that the calcium sensitivity of some forms of PDE is linked to an activator protein described as calmodulin [15]. We observed that various concentrations of calcium added to the assay mixture did not induce additional cAMP PDE activity for high or low affinity forms in either the soluble or particulate fraction. If these forms are calcium-sensitive enzymes, then the calcium present in the tissue itself must be sufficient for their functions. Thus, a calcium-sensitive form of the enzyme may not predominate.

Demonstration of cAMP PDE in cat sciatic nerve and evidence of its transport toward the nerve ending are compatible with the electrophysiological evidence indicating that cAMP may play a role in the modulation of neurotransmitter release at the neuromuscular junction. This study provides the enzyme kinetic parameters which will be the basis for further pharmacological studies correlating drug effects on the enzyme to the electrophysiology.

In summary, cAMP PDE from cat sciatic nerve was 60% soluble and 40% particulate and was transported towards the nerve ending. It occurred in both subcellular fractions in both its high and low affinity forms. The high affinity enzyme showed a K_m of 0.81 μM and a V_{max} of 84.0 pmoles/(min · mg) for the soluble form and a K_m of 0.60 μM with a V_{max} of 25.6 pmoles/(min · mg) for the particulate form. The low affinity enzyme had a K_m of 74.0 μM with a V_{max} of 733 pmoles/(min · mg) (soluble), and a K_m of 84.7 with a V_{max} of 555 pmoles/(min · mg) (particulate).

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