INHIBITION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE BY ADENOSINE 5'-TRIPHOSPHATE AND INORGANIC PYROPHOSPHATE

Wai Yiu Cheung

Department of Biophysics and Physical Biochemistry
Johnson Research Foundation, University of Pennsylvania
Philadelphia, Pennsylvania

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Sutherland and Rall (1958) discovered a cyclic nucleotide phosphodiesterase which hydrolyzes adenosine 3',5'-phosphate (cyclic AMP) to adenosine 5'-phosphate. Later Rall and Sutherland (1962) reported an adenyl cyclase catalyzing the formation of cyclic AMP from adenosine 5'-triphosphate (ATP) with concomittant release of inorganic pyrophosphate (PP). Brain cortex is rich in both enzymes, phosphodiesterase being present in much greater excess than adenyl cyclase (Butcher and Sutherland, 1962; Sutherland et al., 1962). Rall and Kakiuchi (1965) have shown that cyclic AMP increased markedly in the brain cortex shortly after decapitation and in brain slices incubated in the presence of neurohormones. The fact that cyclic AMP accumulated with phosphodiesterase and adenyl cyclase present in marked disparity suggests that either its level is subject to control mechanisms or the enzymes involved in its metabolism are localized in subcellular structures distinct from each other. Cyclic AMP thus formed would not be freely accessible to phosphodiesterase. Results reported here show that both ATP and PP are effective inhibitors of phosphodiesterase. The fact that ATP and PP are substrate and product of adenyl cyclase, respectively, suggests that their inhibition on phosphodiesterase might represent a mechanism of physiological relevance.

MATERIALS AND METHODS

Brains from young male rats, Wistar strain, were homogenized in 5 volumes of glass-distilled water. The homogenate, with pH 6.8, was dialyzed overnight

against 200-400 volumes of 20 mM Tris-HCl, pH 7.5. The dialyzed extract was centrifuged for 30 min at 30,000 x g, and the supernatant fluid was used immediately or stored at -20°. Unless further diluted, the extract could be kept at -20° for several weeks with no appreciable loss of enzymic activity. The reaction mixture contained in μmoles, in a final volume of 1 ml: 40 Tris-HCl, pH 7.5; 1.8 $MgSO_4$; 2 cyclic AMP; protein and ATP or PP as indicated. The temperature was 25°. The reaction was started by the addition of substrate after a 10 min pre-incubation. At the end of 30 min, 0.1 ml of 3 N $HClO_4$ was added to terminate the reaction. The pH was brought back to neutrality with $KHCO_3$ crystals and kept at 0° for 30 min. Insoluble $KClO_4$ was removed by low speed centrifugation and an aliquot of the supernatant fluid assayed for 5'-AMP using the coupled reaction of myokinase, pyruvate kinase, and lactate dehydrogenase (Adams, 1963). Separate experiments established that ATP and PP at the concentrations used did not appreciably interfere with the enzymes employed in the 5'-AMP assay. Protein was determined by the phenol reagent as described by Lowry et al. (1951). One unit of enzyme is defined as the amount of protein that hydrolyzes 1 μmole cyclic AMP in 30 min at 25°.

RESULTS AND DISCUSSION

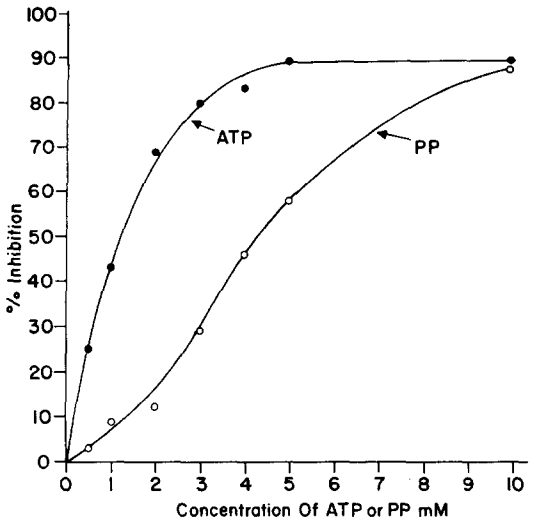

Fig. 1. Inhibition of phosphodiesterase by ATP and PP. Standard assay conditions as described under Materials and Methods: protein concentration, 0.2 mg with specific activity 1.4 units/mg.

Figure 1 depicts that the inhibition curve of phosphodiesterase by ATP is hyperbolic and reaches a saturation level of 90°/o inhibition at 5 mM with half maximum around 1 mM. The inhibition by PP is less severe, and the curve tends to be sigmoid. An asymptote of 90°/o inhibition is virtually reached at 10 mM with half maximum at about 4 mM.

Table I shows the effects of ATP and PP are additive. The extent of inhibition in the presence of both inhibitors agrees very well with the sum of that obtained through individual additions. It should be pointed out that while 90°/o inhibition usually represents maximum, greater inhibition by either ATP or PP could be obtained as shown in Table I.

Additions (in mM)	°/o Inhibition	Additions (in mM)	°/o Inhibition
None	0	10 PP	91
1 ATP	43	1 ATP + 1 PP	58 (54)
2 ATP	73	1 ATP + 3 PP	80 (78)
10 ATP	96	2 ATP + 1 PP	81 (84)
1 PP	11	2 ATP + 3 PP	90 (100)
3 PP	35	10 ATP + 10 PP	100 (100)

TABLE I. Additive inhibition of phosphodiesterase by ATP and PP. Figures in parenthesis are calculated from individual addition of ATP or PP. Standard assay conditions with 0.3 mg protein, sp. act. 1.2 units/mg.

Figures 2 and 3 indicate the nature of the inhibition by ATP or PP respectively. In Figure 2 a Km of 0.20 mM with no inhibitor increases to 0.33 mM at 3 mM ATP, indicating competitive inhibition. The fact that the intercept does not fall on the ordinate suggests that this is not a simple competitive inhibition but a mixed type (Dixon and Webb, 1964).

Figure 3 illustrates that the inhibition by PP exhibits characteristics similar to those of ATP in Fig. 2. Again the Km increases from 0.28 mM with no PP to 0.50 mM in the presence of 5 mM PP. In both Figs 2 and 3, Vmax varies as a function of inhibitor concentrations.

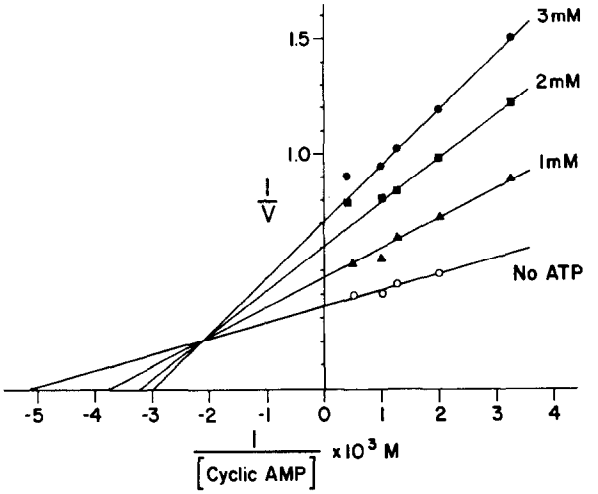

Fig. 2. Nature of ATP inhibition. Standard assay conditions with 0.37 mg protein, specific activity 1.2 units/mg protein; ATP concentrations as indicated; v = Δ O.D./tube/30 min at 25°.

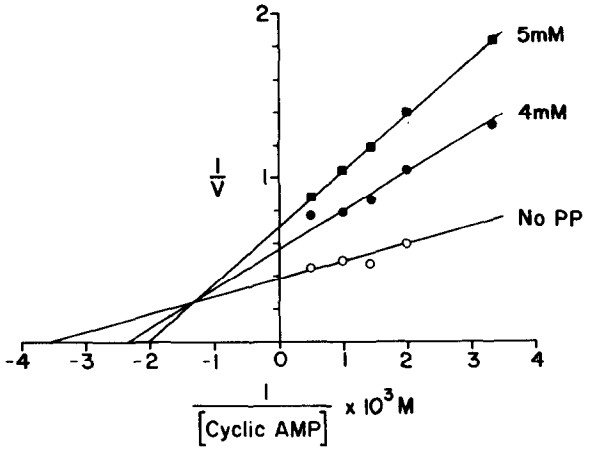

Fig. 3. Nature of PP inhibition. Conditions identical to those of Figure 2; PP concentrations as indicated, v = Δ O.D./tube/30 min at 25°.

Gorden (1950) has noted an extremely active inorganic pyrophosphatase in the rat brain, but PP itself if detectable is present in very small amounts in tissues examined for it (Kornberg, 1962). In view of the possible prefer-

ential distribution of ATP in the synaptosome fraction in the brain as claimed by Whittaker (1966), a localized and transient accumulation of PP might not be unlikely. Pertinent to our discussion is the finding (Cheung and Salganicoff, to be published) that considerable phosphodiesterase activity of the rat brain is associated with a fraction comparable to the synaptosome fraction described by Whittaker.

Studies along different lines by Chance et al. (1966) on rats similar to those used in our experiment showed that the level of ATP in the brain is 1.8 $\pm$ 0.2 μmoles/g tissue. Intracellular concentration of ATP could conceivably reach 4 mM, estimated on the basis that the water content of the brain is 80°/o and the intracellular water 55°/o. Taking these observations into consideration, it would appear that phosphodiesterase might exist in vivo in a greatly inhibited state.

The fact that both ATP and PP inhibit phosphodiesterase suggests that durring synthesis of cyclic ATP, conditions do not favor activities leading to its destruction. A transient accumulation of the nucleotide in the presence of an overwhelming phosphodiesterase thus becomes possible. Several effects have been attributed to cyclic AMP, among them is the activation of phosphorylase b kinase (Krebs et al., 1959), a key enzyme in the regulation of glycogenolysis. Once cyclic AMP is formed and its influence felt, it is important to have its action terminated so that stored energy will not be called upon longer than necessary. An excess of the phosphodiesterase would be a warranty against any unnecessary delay which would result in a waste of energy. By suppressing phosphodiesterase activity, ATP and PP keep the potentiality under proper control. The stimulation of cyclic AMP on phosphorylase b kinase has been cited merely to illustrate the advantage for the cell to have a large reserved capability and not to imply it as the main function in the brain.

Butcher and Sutherland (1962) observed that caffeine competitively inhibits a cyclic nucleotide phosphodiesterase from beef heart. In our hands, caffeine at 20 mM gave 50°/o inhibition and is therefore much less potent

than either PP or ATP. Of the compounds reported to inhibit phosphodiesterase, the most potent is ATP, followed by PP. The inhibition of phosphodiesterase by both ATP and PP might well be a regulatory mechanism operative in the intact cell.

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