PRESENCE OF TWO 3'-5'-CYCLIC AMP PHOSPHODIESTERASES IN RAT KIDNEY AND FROG BLADDER EPITHELIAL CELLS EXTRACTS

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SUMMARY. The phosphodiesterase activity extracted from frog bladder epithelial cells and rat kidney may be described as the sum of the activities of two phosphodiesterases (I and II). Phosphodiesterases I and II may be partially separated by gel filtration or by centrifugation in sucrose gradients. Phosphodiesterase I (molecular weight about 80,000) is stable on storage and heat denaturation and has a low affinity for cyclic AMP (Km about 3×10^{-5} M). Phosphodiesterase II has a lower molecular weight (about 40,000) and an affinity for cyclic AMP ten times higher. It is thermolabile and more susceptible to inhibition by ATP than phosphodiesterase I. The possibility that phosphodiesterases I and II correspond to two forms of the same enzyme is discussed.

The role of 3'5' cyclic AMP as second messenger for an increasing number of hormonal actions is now established by ample documentary evidence (1). According to the general scheme proposed by SUTHERLAND and coll, the intracellular concentration of 3'5' cyclic AMP is regulated by two enzymes : an adenylcyclase which catalyses its formation from ATP and a phosphodiesterase catalysing its conversion to 5' AMP. The properties of the 3'5' cyclic AMP phosphodiesterase extracted and purified from the heart and brain have been described (2,3,4,5,6,7 and 8). In this article we describe the properties of the phosphodiesterase activity present in cytoplasmic and particulate fractions prepared from frog bladder epithelial cells and rat kidney, two organs in which hormone-regulated production of 3'5' cyclic AMP has been clearly demonstrated (9,10 and 11).

MATERIAL AND METHODS - Products used : ^{3}H 3'5' cyclic AMP (1.4 Ci./mM) and 3'5' cyclic AMP were purchased from SCHWARTZ BIORESEARCH Inc., Crotalus atrox venon from SIGMA, synthetic Lysinevasopressin from SANDOZ, Norepinephrine from RHONE-POULENC and Theophylline from MANN. All other reagents were A grade.

Preparation of extracts : Frog bladders (Rana esculenta) were dissected from the pithed animals, rinsed in a Ringer solution, blotted on gauze and homogenized in 2 ml of cold TRIS-HCl pH 7.4 10 mM by means of a glass homogenizer equipped with a Teflon pestle. The connective tissue was removed by filtration on gauze. This crude extract was centrifugated at 20,000 g for 15 min. The supernatant (referred to as the cytoplasmic fraction) was separated and the pellet (particulate fraction) was washed once and suspended in 2 ml of the same buffer. The two fractions were dialyzed overnight at 4° C against 2 L of buffer. Holtzman rats were anaes-

thetized with ether and bled by heart section. The kidneys were excised, freed from adipose and connective tissue and homogenized in 4 vol. distillated water. The homogenate was centrifugated for 30 min. at 35,000 g. The supernatant was mixed (V/V) with a saturated $SO_4 (NH_4)_2$ solution. Precipitation was allowed to proceed for 2 h at 0° C. The pellet was separated by centrifugation for 20 min. at 35,000 g, dissolved in the initial volume of TRIS-HCl buffer pH 8.0, 5mM and submitted to overnight dialysis against 2 L TRIS-HCl pH 8.0, 5 mM, $MgCl_2$ 0.5 mM, KCl 0.25 mM. This enzyme referred to as the $SO_4 (NH_4)_2$ fraction in the results, was stored at -20° C. Filtration of the $SO_4 (NH_4)_2$ fraction was performed on a G 100 Sephadex column (1.5 x 75 cm) using 2 ml of enzyme for each run. The eluate was collected in 80 1.5 ml fractions. The column was calibrated using calf Intestine Alcaline Phosphatase (MW 100,000), the activity of which was measured by the method of MALAMY and HORECKER (12) ; Bovin serum albumin (MW 68,000) was determined by absorbance at 280 nm. Density gradient experiments were performed using linear 5 to 30 % sucrose gradient (total volume 5 ml, amount of enzyme applied : 0.2 ml). After centrifugation for 17 h at 125,000 g, the tube content was collected in 20 fractions.

Measurement of phosphodiesterase activity : Phosphodiesterase activity was measured either by the conversion of ^3H 3'5' AMP, or by the release of inorganic phosphate from the 5' AMP by the treatment with a snake venom (Crotalus atrox) phosphatase. When tritiated 3'5' cyclic AMP was used, the incubation medium (final volume 0.1 ml) contained TRIS-HCl pH 8.0, 25 mM, $MgCl_2$ 2.5 mM, KCl 1.25 mM, ^3H cyclic AMP (0.3 µci) and 3'5' cyclic AMP in various amounts (final concentrations ranged from 2.10^{-7} to 8.10^{-5} M). The reaction was initiated by the addition of enzyme, lasted from 5 to 210 min. and was stopped by boiling for 3 min. Each tube was then incubated for 10 min. at 30° C in the presence of snake venom, 50 µg and 5' AMP, 0.05 µM. (Under these conditions, conversion of 5' AMP is complete and the phosphodiesterase activity of the snake venom, negligible). 3'5' cyclic AMP was separated from its metabolites by thin layer chromatography on cellulose plates using the system C_2H_5OH, CH_3-COO (NH_4) M (70/30 V/V). The tritium content of the different spots was measured by liquid scintillation. The % conversion of 3'5' cyclic AMP was determined on duplicates or triplicates and corrected for the values measured on blanks incubated without enzyme. When phosphodiesterase was measured by the release of inorganic phosphate from 5' AMP, the reaction after snake venom treatment was stopped by adding 50 µl of 50 % CCl_3COOH and 0.5 mg bovine serum albumin. Inorganic phosphate in the supernatant was measured by the LOWRY and LOPEZ method (13). Each experimental series contained blanks without enzyme and blanks without 3'5' cyclic AMP. The protein contents of the different fractions were measured by the method developped by LOWRY & al. (14).

RESULTS AND DISCUSSION. Fig. 1 shows the results of a typical experiment using

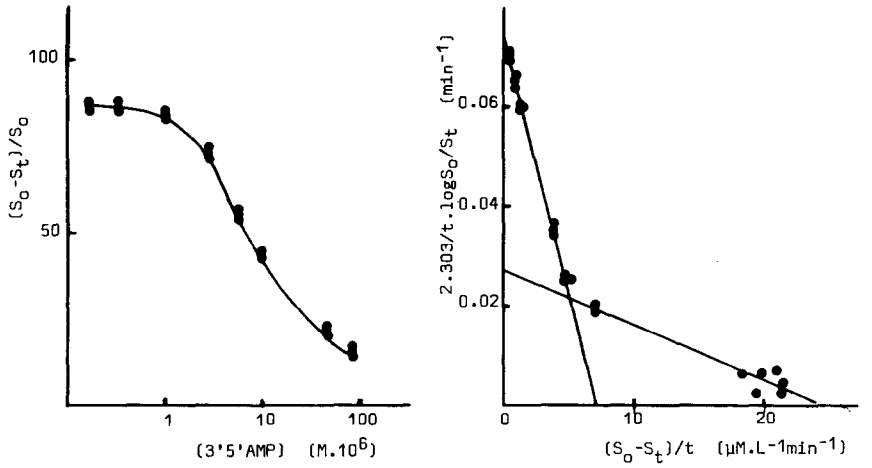

Fig. 1 : 3'-5'-cyclic AMP phosphodiesterase activity of the cytoplasmic fraction of an extract from frog bladder epithelial cells.
Left part : the % conversion of 3'-5'-cyclic AMP is plotted against the initial concentration of 3'-5'-cyclic AMP in the medium (So).
St is the concentration at the end of the incubation period (30 min.).
Incubation conditions are described in the text, the amount of enzyme was 21 µg.
Right part : the results of the same experiment are plotted using the integrated form of the Michaelis equation (explanations in the text).

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the cytoplasmic fraction of a frog bladder extract as the source of enzyme. It clearly indicates that the % conversion of 3'5' cyclic AMP decreases when the initial substrate concentration increases. This effect which is observed for a low substrate concentration (0,5 µM) indicates the presence in the enzyme preparation of a phosphodiesterase with a high affinity for 3'5' cyclic AMP. Since under our experimental conditions a significant fraction of the substrate is transformed, the mean velocity of the reaction (So - St)/t (in which So and St are the substrate concentrations at the beginning and end of the incubation period, and t the incubation time) does not correspond to a correct estimation of the initial rate of the reaction and cannot therefore be used directly to determine the velocity - substrate concentration relationships. An estimate of the latter was made using the integrated form of the Michaelis equation (15). 2. 303/t log (So/St) = - 1/Km x (So - St)/t + Vm. As shown on Fig. 1, when 2.303/t. log (So/St) is plotted against (So - St)/t, the experimental curve may be described by two linear relationships, one of them corresponding to the lowest substrate concentrations (from 0.2 µM to 5 µM) and the other to the highest (5 µM to 80 µM). It is thus possible, in a first approximation, to describe the total phosphodiesterase activity of the preparation as the sum of the activities of two phosphodiesterases (PDE I and PDE II) with different Vm and different Km for 3'5' cyclic AMP.

The values of Vm and Km can be deduced from the slopes and the x - axis intercepts of the two experimental straight lines. The same pattern was obtained for all experiments using crude extracts or SO_4 $(NH_4)_2$ fractions. In both rat kidney and frog bladder epithelial cell extracts, the specific activity of the two phosphodiesterases (Vm_1 and Vm_2 expressed as µµM/min/mg protein) is about ten times higher in the cytoplasmic than in the particulate fraction. Taking into account the relative protein content of the two fractions, it may be estimated that 90 % of the total phosphodiesterase activity of crude extracts is present in soluble form. The ratio Vm_1/Vm_2, which is an estimation of the relative amounts of PDE I and II, is similar in the cytoplasmic and particulate fractions. Both for PDE I and II, the apparent Km for 3'5' cyclic AMP is the same for soluble and insoluble forms of the enzyme. The mean Km values obtained for PDE I are 35 $\pm$ 17 µM (n = 7) and 95 $\pm$ 63 µM (n = 4) for frog bladder and rat kidney extracts respectively. The

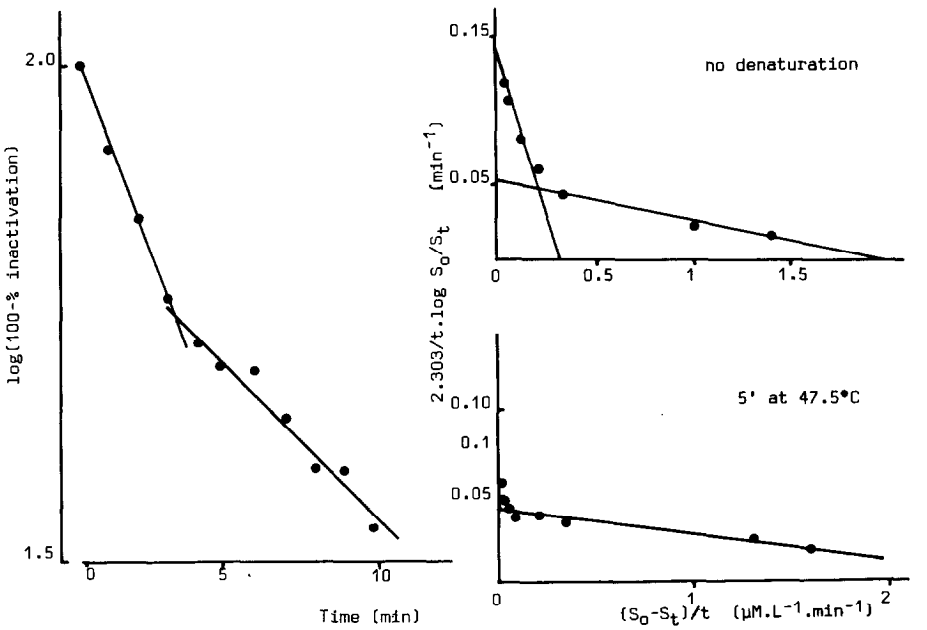

Fig.2: Thermal inactivation of the $SO4(NH_4)_2$ fraction.
Left part :inactivation curve of total phosphodiesterase activity.300 µl aliquotes of the $SO_4(NH4)2$ fraction without 3'5'cyclic AMP were incubated at 47.5°C for different times and rapidly cooled at 4°C. Phosphodiesterase activity was then measured at 30°C. Incubation conditions are described in the text.Substrate concentration was 2.10^{-5} M.Conversion percentages were below 35%.The decrease in substrate concentration was not taken into account when calculating reaction velocity.The inactivation curve clearly indicates the presence of two components of different thermostability.
Right part : Comparison of the substrate concentration-velocity relationships (see Fig. 1) for the native and partially inactivated enzyme.As shown on the curves ,heat treatment at 47.5°C for 5min. results in almost complete inactivation of Phosphodiesterase II.

corresponding values for PDE II are 1.5 ± 0.4 mM (n = 7) and 2.7 ± 0.9 µM (n = 4). All further experiments were performed using the $SO_4 (NH_4)_2$ fraction from rat kidney. This partial purification (15 - fold increase in specific activity) does not lead to the disappearance of the overall phosphodiesterase activity's peculiar kinetic properties. Exposure of the enzyme preparation to 47.5° C results in progressive inactivation ; the inactivation curve (Fig. 2) clearly indicates the presence of two components of different thermostability. The kinetic properties of the total PDE activity are sharply modified during thermal inactivation. As indicated in Fig. 2, a 5 min. inactivation at 47.5° C leads to the almost complete disappearance of PDE II. Furthermore the elution profile from a Sephadex G 100 column indicates the presence of two peaks (Fig. 3). The heaviest fraction corresponds to PDE I and the lightest to PDE II, as indicated by the kinetics curves of Fig. 3. A rough estimation of molecular weights gives about 80,000 for PDE I

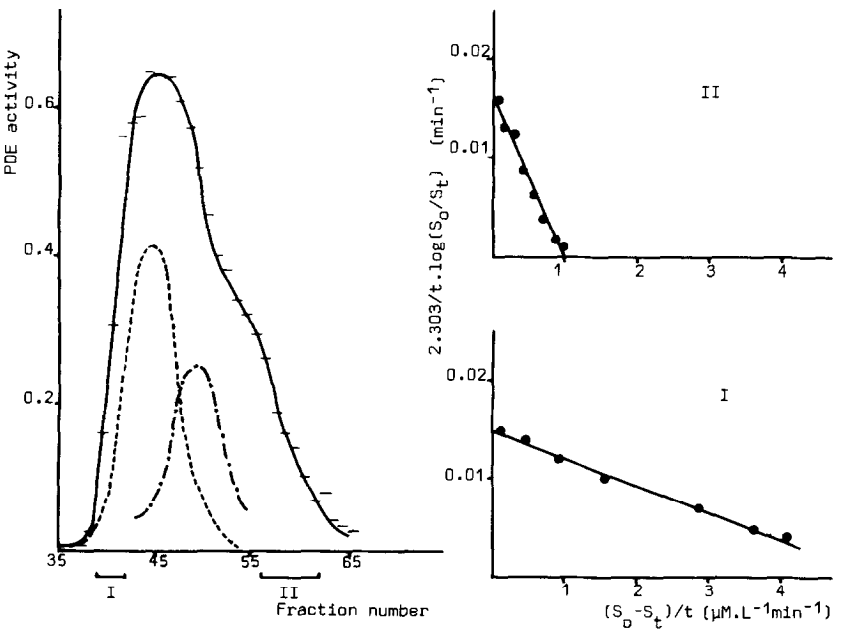

Fig. 3 : Gel filtration of the $SO_4(NH_4)_2$ fraction :
Left part : Elution profiles of phosphodiesterase activity (solid line) and of the two molecules used for calibration, alcaline phosphatase (-----), and bovine serum albumine (-.-.-.-). Phosphodiesterase activity was determined using a single 3'5' cyclic AMP concentration (5.10^{-7}M). Incubation conditions are described in the text. Incubation time was 20 min. At this low substrate concentration the velocity of the reaction can be considered proportional to the substrate concentration, both for phosphodiesterase I and II. Under these conditions the velocity constant (Vm/Km).t can be estimated by log So/St with So as the initial substrate concentration and St as the substrate concentration at the end of the incubation period.
Right part : Substrate velocity relationships (see Fig. 1) for the heaviest and lightest fractions indicated on the elution profile by I and II respectively. The two curves obtained indicate the complete separation of phosphodiesterases I and II.

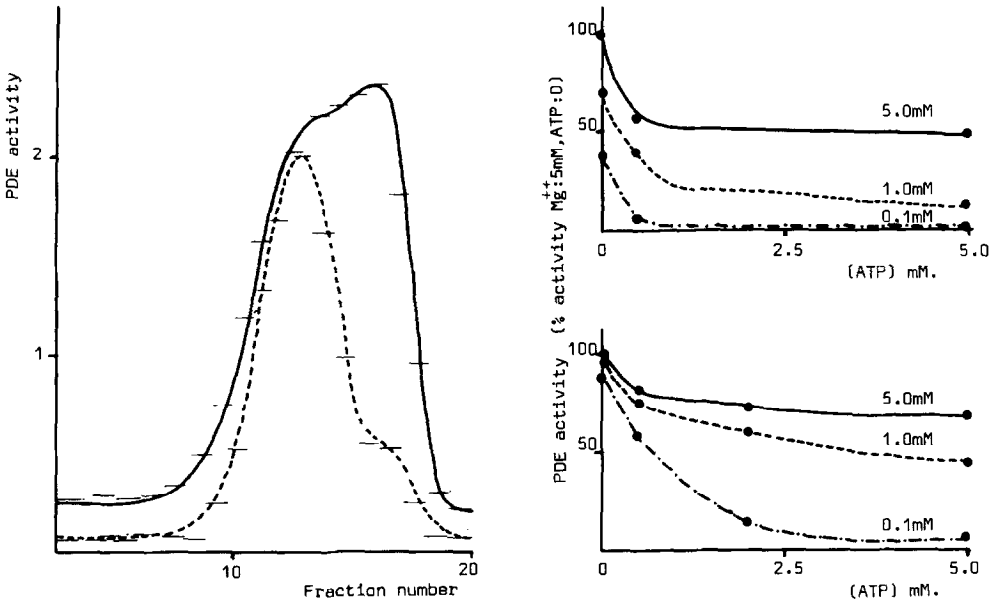

Fig. 4 : (Left part). Sedimentation profile of phosphodiesterase activity in a sucrose gradient. Collection of fractions and incubation conditions are described under "methods". Phosphodiesterase activity was measured at 5.10^{-7}M 3'5' cyclic AMP, and determined as indicated in the legend of Fig. 3. Solid line : profile obtained with the native enzyme. Dotted line : profile obtained with enzyme inactivated for 10 min. at 47.5° C. Note the almost complete disappearance of phosphodiesterase activity in the lightest fractions.

Fig; 5 : (Right part).ATP inhibition and magnesium dependence for phosphodiesterase II (upper curves) and the $SO_4(NH_4)_2$ fraction (lower curves). Phosphodiesterase activity was measured with 3'5' cyclic AMP 5.10^{-4} M. It is expressed as a % of the activity measured without ATP and with Mg^{++} 5 mM.At this high substrate concentration the activity of the whole $SO_4(NH_4)_2$ fraction essentially reflects the activity of PDE I.

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and 40 to 50,000 for PDE II. This difference in molecular weight was confirmed by the sucrose gradient centrifugation experiments (Fig. 4). The profile obtained shows the presence of two incompletely separated peaks. Thermal inactivation at 47.5° C for 10 min. leads to the disappearance of the lightest fraction (PDE II). Fractions containing almost exclusively PDE I or PDE II were obtained by separating the first 1/3 of the heaviest peak and the last 1/4 of the lightest peak of the elution profile of a G 100 Sephadex column allowing a study of some of the properties of PDE I and PDE II. Stored at - 20° C in diluted form, PDE II is unstable (50 % inactivation in a week) ; all experiments with PDE II were performed within 48 h after separation. Both PDE I and PDE II are inhibited by ATP (Fig. 5). As shown by the curves, 1°) the magnitude of the inhibition by ATP increases

when Mg^{++} concentration is decreased ; 2°) for a given Mg^{++} concentration the inhibition is more pronounced for PDE II than for PDE I, and 3°) in the absence of ATP the reduction of Mg^{++} concentration from 5 mM to 0.1 mM leads to an important decrease in the activity of PDE II (40 % of the activity at 5 mM Mg^{++}) while at 0.1 mM Mg^{++}, the activity of PDE I is still 80 % of the maximum activity at 5 mM. PDE I and II are inhibited by Theophylline (Fig. 6). At the substrate concentration used for this experiment (0.5 mM 3'5' cyclic AMP) the inhibition of PDE I is more pronounced. Assuming that the inhibition is of a purely competitive type, the estimated Ki for Theophylline determined from inhibition by the four highest inhibitor concentrations, is 8.1 µM for PDE I and 4.2 µM for PDE II. Thus the difference in affinity for Theophylline is much lower than the difference in affinity for 3'5' cyclic AMP (see above). Treatment of PDE I and II with either Norepinephrine (10 µM) or Lysine - vasopressin (0.37 µM) does not modify their activities.

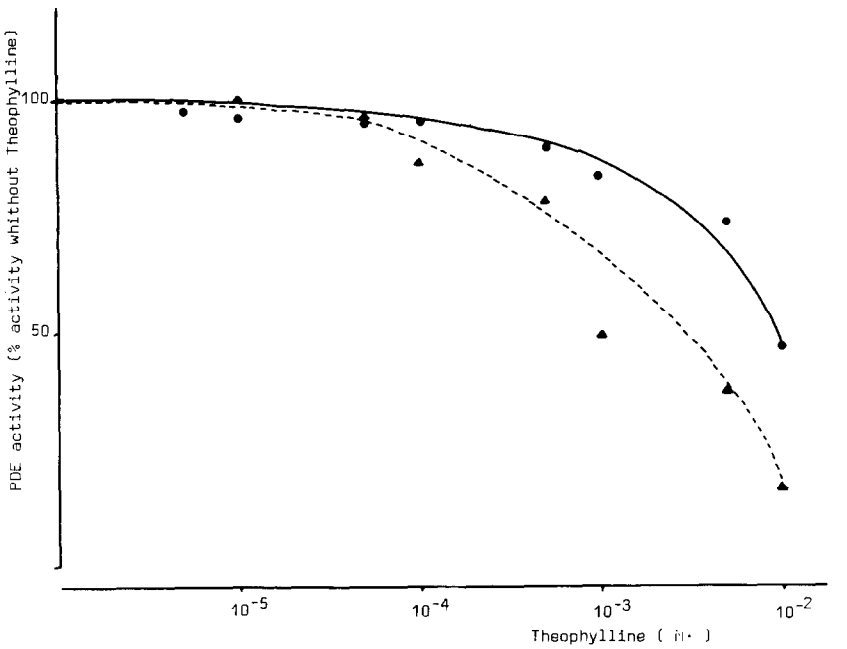

Fig. 6 : Inhibition by Theophylline of phosphodiesterases I and II. Phosphodiesterase activity was measured at 5.10^{-4} M of 3'5' cyclic AMP. Solid line : phosphodiesterase II ; dotted line : phosphodiesterase I.

The results reported in this paper indicate the presence in frog badder epithelial cells and Rat kidney homogenates of two phosphodiesterases which can be partially separated and which differ from each other as regards molecular weight, affinity for 3'5' cyclic AMP, thermostability and inhibition by ATP.

Whether or not these two phosphodiesterases correspond to iso-enzymes or two forms of the same enzyme requires further experiments to determine. In this respect it may be noted that ROSEN (16) recently described the separation of two phosphodiesterase activities from frog erythrocytes. In addition CHEUNG recently reported the presence of a PDE activator in snake venom (17) and brain tissue. Furthermore the activated and non activated forms of PDE differ in molecular weight and can be separated by filtration on Sephadex columns. It is likely that phosphodiesterases I and II from rat kidney correspond to the same two activated and non activated forms described by CHEUNG (18,19).

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