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THEOPHYLLINE EFFECT ON THE CYCLIC AMP DEGRADING MULTIENZYME SEQUENCE

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Abstract—Membrane-bound 3′:5′-cyclic nucleotide phosphodiesterase (EC 3.1.4.17) is closely associated physically with nucleotidase and deaminase, thus forming an enzyme cluster of unique catalytic behaviour [H. Wombacher, *Archs. Biochem. Biophys.* **201**, 8 (1980)]. This multienzyme cluster, which was found in the microsomal fraction of beef adrenal cortex, catalyses the degradation of cyclic AMP, via AMP and adenosine, to inosine. The present study shows how theophylline, a well-known inhibitor of the phosphodiesterase, acts on the membrane-bound multienzyme sequence. The findings were as follows. Firstly, as expected, theophylline inhibited the phosphodiesterase competitively. In particular, the high-affinity enzyme was inhibited by mM concentrations of theophylline. Phosphodiesterase activity was tentatively ascribed to two enzymes, one with a low K_m [0.3 μM], one with a high K_m [60 μM]. Secondly, theophylline inhibited the nucleotidase activity to a great extent. A detailed kinetic analysis showed the inhibition to be hyperbolic noncompetitive ($\alpha = 1$, $\beta = 0.35$ and $K_i = 0.25$ mM). Thirdly, theophylline did not inhibit the deaminase activity of the multienzyme sequence. A model of theophylline inhibition is suggested explaining how an effector could modulate the kinetic behaviour of an enzyme cluster by acting at a single allosteric site. Finally, in view of the existence of the cyclic AMP degrading multienzyme sequence and the effect of theophylline on it, the possibility is discussed that physiologically active adenosine is derived from cyclic AMP.

Methylxanthines, especially theophylline, have distinct pharmacological effects on heart, brain and other organs in several species including man [1]. Following the finding of Butcher and Sutherland [2], that theophylline is an inhibitor of the cyclic nucleotide phosphodiesterase (EC 3.1.4.17), it has been assumed that many, if not all, actions of the methylxanthines are secondary to the elevated level of cyclic AMP, and that the physiological effect of cyclic AMP is exclusively mediated through regulation of the activity of cyclic AMP-dependent protein kinases, first discovered by Walsh *et al.* [3]. However, any correlation between the cyclic AMP effect and theophylline as an inhibitor of phosphodiesterase must be made with caution. Sattin and Rall [4] reported that theophylline blocks adenosine-elicited accumulation of cyclic AMP in brain tissue. The existence of the adenosine receptor and the antagonistic effect of theophylline on it are well documented (see reviews: Burnstock [5] and Snyder *et al.* [6]). Another effect of theophylline, the more or less direct mobilization of calcium in cell compartments, has been discussed by Rasmussen [7]. A particularly interesting facet of the theophylline action was the finding that nucelotidase activity is significantly inhibited by theophylline, first reported by Bastomsky *et al.* [8] and more recently studied by Tsuzuki and Newburgh [9] and Fredholm *et al.* [10]. The present study reports the effect of theophylline on the multienzyme sequence which degrades cyclic AMP to inosine via AMP and adenosine, as described previously [11].

MATERIALS AND METHODS

Chemicals

[U-^{14}C]Adenosine 5′-monophosphate, ammonium salt [sp. act. 538 mCi/mmole, batch 16], [U-^{14}C]adenosine (sp. act. 549 mCi/mmole, batch 27), [8-^{3}H]adenosine 3′,5′-monophosphate (sp. act. 30 Ci/mmole, batch 27) were obtained from Amersham/England. The products were within the specifications given by the manufacturer when analysed as described earlier [12, 13]. Creatine kinase, creatine phosphate and all unlabelled nucleotides and nucleosides were obtained from Boehringer (Mannheim, West Germany). Bovine serum albumin was purchased from Behring AG (Marburg, West Germany). Thin-layer plates, silica gel 60 F_{254} (0.25 mm), cellulose (0.1 mm), F_{254} and all other analytical-grade chemicals were obtained from Merck (Darmstadt, West Germany). A gas flow proportional counter with a plotter (Berthold, Wildbad, West Germany) was used for radioactive analysis of the chromatograms. Liquid scintillation counting was performed with Unisolve (Zinsser, Frankfurt, West Germany) as a premixed scintillator solution in a Packard Model 3380 (Downers Grove, IL).

Preparation of the membranes

The microsomal material was prepared from adrenal cortex tissue as previously described [11].

Assay procedure

The assay was performed as previously described

[11–14]. The incubation mixture contained 40 mM Tris–HCl, pH 7.2, 2.5 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin, the radioactive labelled substrate (cyclic AMP, AMP or adenosine) and theophylline. The reaction was started by the addition of the microsomal material (12 μg protein) to give a final volume of 50 μl. Incubation was performed at 30° and stopped by the addition of 10 μl of "cold" substrate and the corresponding intermediates (1 mM) with immediate heating for 0.5 min at 100°. Incubation time was adjusted to ensure initial rate measurements, i.e. no more than about 5% of substrate was hydrolyzed. Then, the metabolites were chromatographed by thin-layer chromatography. After scraping off the separated metabolites radioactivity was determined by liquid scintillation counting.

Chromatographic systems. System 1: ethanol, saturated aqueous solution of $Na_2B_4O_7$, 5 M ammonium acetate, 0.5 M $EDTANa_2$ (220:80:20:0.5 by vol.) on silica gel 60. System 2: saturated aqueous solution of ammonium sulfate, 1 M sodium citrate, isopropanol (80:18:2 by vol.) on cellulose.

Protein determination. Protein was determined by a micro-version of the method of Lowry *et al.* [15] with bovine serum albumin as the standard.

RESULTS

Cyclic AMP metabolism, via 5′-AMP and adenosine, to the end product inosine by a membrane-bound multienzyme cluster has been previously demonstrated [11]. The kinetic behaviour of the phosphodiesterase reaction, the rate-limiting step of the reaction sequence, has been studied by measuring the nucleoside production. The rate of product formation during the steady-state phase is shown in Fig. 1. According to the method of Spears *et al.* [16] a theoretical curve of phosphodiesterase activity has been calculated (Fig. 1, dotted line), assuming the existence of two phosphodiesterases. The experimental values show a good approximation to the theoretical values, giving a K_m value of 60 μM for the low affinity enzyme and 0.3 μM for the high affinity enzyme, with V_{max} values of 1 (μM min^{-1}) and 5 (nM min^{-1}), respectively. These values of K_m and V_{max} of the low and high affinity enzyme differ significantly from the graphically derived values obtained from the douple-reciprocal plot commonly used. As already noted by Spears *et al.* [16] the simple double-reciprocal graphical derivation is inaccurate, because the assumption is not correct that the low affinity enzyme does not contribute appreciably to the rate of the high affinity enzyme at μM and lower substrate concentrations.

Theophylline decreased the rate of nucleoside formation over the whole concentration range of the substrate, cyclic AMP (Fig. 1). To determine whether the decreased nucleoside formation was due solely to the known inhibitory effect of theophylline on the phosphodiesterase, the nucleotidase function of the multienzyme sequence was blocked by ATP,

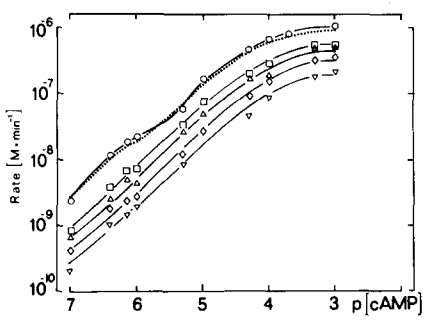

Fig. 1. Effect of theophylline on the rate of degradation of cyclic AMP to the nucleosides, adenosine and inosine. The initial rate of nucleoside formation was determined at varying cyclic AMP concentrations [p(cAMP) is defined as negative logarithm of cyclic AMP concentration expressed in M]. In addition, theophylline was present at different fixed concentrations. · · · ·, Calculated curve of phosphodiesterase activity assuming a low- and high K_m-enzyme according to the method of Spears *et al.* [16]; —○—, experimentally observed curve of PDE activity without theophylline; —□—, in the presence of 0.5 mM theophylline; —△—, in the presence of 1 mM theophylline; —◇—, in the presence of 2 mM theophylline; —▽—, in the presence of 5 mM theophylline. The assay was performed as described under Materials and Methods. [^{3}H]Cyclic AMP was used as a tracer. The radioactivity ranged from 80 nCi at low cyclic AMP concentration up to 400 nCi/assay at higher cyclic AMP concentrations. Values were means of four experiments. Chromatographic system 1, where the nucleosides run closely together, was used to separate the products. The sum of the nucleosides was determined by scraping off the corresponding areas and measuring the radioactivity by liquid scintillation counting.

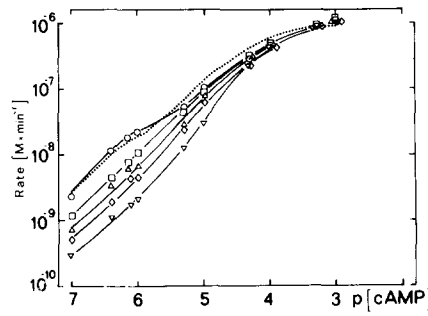

Fig. 2. Effect of theophylline on the rate of hydrolysis of cyclic AMP to 5′-AMP. The initial rate of AMP formation by the membrane-bound multienzyme sequence was determined at varying substrate concentrations in the presence and absence of theophylline. · · · ·, Theoretical curve of phosphodiesterase activity calculated as described in Fig. 1; —○—, without theophylline, in the presence of ATP; —□—, 0.5 mM theophylline, in the presence of ATP; —△—, 1 mM theophylline, in the presence of ATP; —◇—, 2 mM theophylline, in the presence of ATP, —▽—, 5 mM theophylline, in the presence of ATP. Since the phosphodiesterase activity was to be determined by measuring AMP formation, nucleotidase function of the multienzyme sequence had to be blocked by ATP (2 mM). In order to keep the ATP concentration constant during the initial phase of the reaction in the presence of ATPase activity in the membrane material, experiments were performed using an ATP regenerating system (40 μg creatine kinase and 1 μmole creatine phosphate/assay). All other assay conditions and the subsequent chromatography and evaluation were as described under Materials and Methods. Values are means of four experiments.

as described earlier [11]. Thus the phosphodiesterase activity and the effect of theophylline on it were represented by the rate of AMP formation (Fig. 2).

However, a slight inhibitory effect of ATP on the phosphodiesterase activity has to be taken into account. As already described [11], this slight inhibition by ATP appears only in the region of the low affinity enzyme. For the estimation of this ATP-effect on the phosphodiesterase activity, the calculated theoretical curve from Fig. 1 is included for reference in Fig. 2. In spite of this limitation Fig. 2 clearly shows that theophylline inhibited especially the high affinity phosphodiesterase enzyme. Fig. 2 shows that increasing substrate concentrations of cyclic AMP reduce the inhibition, and at mM concentrations of cyclic AMP the inhibition of phosphodiesterase is abolished. Hence the question arose of how to explain the inhibition of nucleoside formation at mM concentrations of cyclic AMP, as shown in Fig. 1. Inhibition of the deaminase in multienzyme sequence was excluded, because no inhibition of this enzyme by theophylline could be demonstrated. (Deaminase activity was tested with adenosine as substrate and [^{14}C]adenosine as a tracer. In this case chromatographic system 2 was used to separate adenosine from inosine [12]. All other conditions were as described under Materials and Methods.) Hence, it was concluded that theophylline inhibits the nucleotidase activity of the multienzyme sequence as well as the phosphodiesterase activity.

Summarizing, theophylline reduces the rate of cyclic AMP degradation to inosine by inhibiting the phosphodiesterase activity and the nucleotidase activity. Phosphodiesterase activity was significantly inhibited at low substrate concentrations. Increasing substrate concentrations diminished the theophylline effect in a competitive manner (Fig. 2). In contrast, the nucleotidase activity could not be restored by increasing substrate concentrations as documented by the reduced V_{max} values (Fig. 1).

A further detailed analysis of the inhibition of the nucleotidase activity by theophylline was performed. 5′-AMP instead of cyclic AMP was used as the substrate to start the reaction sequence. The results are shown as a double-reciprocal plot [Fig. 3] and a Dixon plot [17] [Fig. 4].

Assuming rapid-equilibrium conditions and a two-site model, inhibition may be expressed in a general way

$$\begin{array}{ccccc} E & + S & \overset{K_m}{\rightleftharpoons} & ES & \overset{k_2}{\longrightarrow} E + P \\ + & & & + & \\ I & & & I & \\ {\scriptstyle K_i}\downarrow\uparrow & & {\scriptstyle \alpha K_i}\uparrow\downarrow & & {\scriptstyle \beta k_2} \\ EI & + S & \underset{\alpha K_m}{\rightleftharpoons} & ESI & \longrightarrow EI + P \end{array}$$

According to Segel [18] the general rate equation derived from it under rapid equilibrium condition reads

$$\frac{v}{V_{max}} = \frac{[S]}{\alpha K_m \cdot \left(\frac{[I] + K_i}{\beta[I] + \alpha K_i}\right) + [S] \cdot \left(\frac{[I] + \alpha K_i}{\beta[I] + \alpha K_i}\right)}. \quad (1)$$

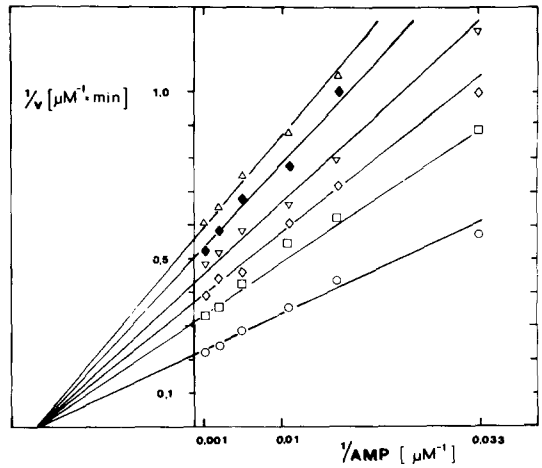

Fig. 3. Effect of theophylline on nucleotidase activity [double-reciprocal plot, resp. Lineweaver–Burk (L–B) plot]. The initial rate of AMP hydrolysis was determined at AMP concentrations from 5 μM to 720 μM (○) and in the presence of different theophylline concentrations (□ 0.2 mM, ◇ 0.4 mM, ▽ 0.8 mM, ◆ 1.6 mM, △ 3.2 mM). Reaction was started with 5′-AMP as substrate, using [^{14}C]5′-AMP [60 nCi/assay] as a tracer. The assay conditions were the same as for cyclic AMP, described under Materials and Methods. The products, adenosine and inosine, were separated by chromatographic system 1 and determined as described under Materials and Methods. Values are means of four experiments.

At any [I] the overall rate at which product is produced is $k_2[ES] + \beta k_2[ESI]$, where $0 < \beta < 1$. From the equilibria it is to be seen that an infinitely high substrate concentration will not drive all the enzyme to the ES form. At any inhibitor concentration a portion of the enzyme will exist as the less productive ESI form. Consequently, V_{max} will decrease in the presence of partial noncompetitive

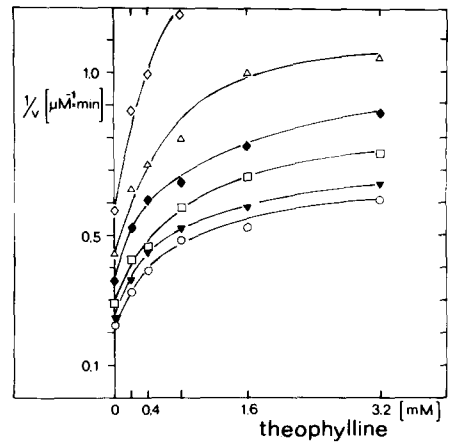

Fig. 4. Effect of theophylline on nucleotidase activity (Dixon plot [17]). 1/v-Values at different fixed concentrations of substrate (○ 0.72 mM AMP, ▼ 0.35 mM AMP, □ 0.18 mM AMP, ◆ 0.09 mM AMP, △ 0.06 mM AMP, ◇ 0.03 mM AMP) were plotted vs theophylline concentrations. For determination of nucleotidase activity, see Fig. 3.

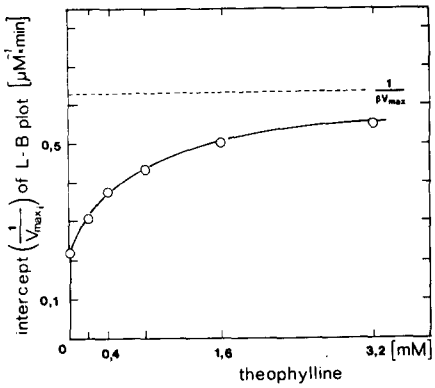

Fig. 5. $1/v$-Axis intercept (taken from Fig. 3) replotted vs theophylline concentrations.

inhibitor. However, the partial noncompetitive inhibition of theophylline cannot be distinguished from simple noncompetitive inhibition by the double-reciprocal plot (Fig. 3) where $1/v$ vs $1/[S]$ in the absence and the presence of theophylline is plotted. Therefore, the Dixon plot [17] (Fig. 4) where $1/v$ is plotted vs [I] at fixed [S] is needed to indicate the hyperbolic characteristic of the inhibition. This is expected when I converts the enzyme to a modified, but functional enzyme, EI, with a decreased rate of product formation from the ESI form. For further information the double-reciprocal plot was analysed. The reciprocal form of the general equation (1) reads

$$\frac{1}{v} = \frac{\alpha K_m}{V_{max}} \cdot \left(\frac{[I] + K_i}{\beta[I] + \alpha K_i}\right) \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \cdot \left(\frac{[I] + \alpha K_i}{\beta[I] + \alpha K_i}\right). \quad (2)$$

On the basis of Eqn. 2 the system ($1 < \alpha < \infty$, $0 < \beta < 1$) can be distinguished from the system ($1 < \alpha < \infty$, $\beta = 0$) by replots of the slope vs [I] and $1/v$-axis intercept vs [I]. Both plots are hyperbolic. In the present case the "intercept-hyperbolic" noncompetitive inhibition of theophylline is shown in Fig. 5. Due to the hyperbolic form the limits which allow α, β and K_i to be determined are difficult to identify. Therefore, the relationship is converted to a straight line by plotting $1/\Delta$-intercept vs $1/[I]$. The Δ-intercept is used to raise the horizontal axis of Fig. 5 so that the hyperbola starts at the origin.

$\Delta(1/v$-axis intercept)

$$= \frac{1}{V_{max}} \cdot \left(\frac{[I] + \alpha K_i}{\beta[I] + \alpha K_i}\right) - \frac{1}{V_{max}}. \quad (3)$$

Hence

$$1/\Delta\text{-intercept} = \frac{\alpha K_i \cdot V_{max}}{(1-\beta)} \cdot \frac{1}{[I]} + \frac{\beta V_{max}}{(1-\beta)}. \quad (4)$$

The data of Fig. 5 are plotted in Fig. 6 according to Eqn. (4); this permits the determination of K_i (= 0.25 mM) and β (= 0.35). The value of α is taken from the double-reciprocal plot, Fig. 3. It is seen that the K_m-value remains unchanged in the presence of varying inhibitor concentrations. The series of intersecting lines in a point on the $1/[S]$ axis documents the simple non-competitive character of inhibition, i.e. $\alpha = 1$. Summarizing, the theophylline inhibition of nucleotidase function, within the multienzyme sequence that degrades cyclic AMP to inosine, can be described as "hyperbolic mixed-type inhibition".

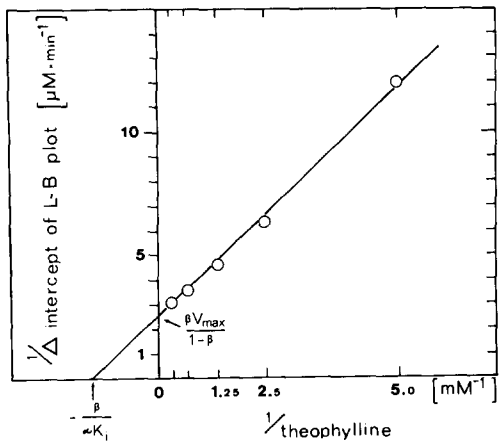

Fig. 6. Secondary replot of $1/\Delta$-intercept for the hyperbolic mixed-type inhibition of theophylline. [Δ means the $1/v$-intercept, which is calculated from $1/V_{max}$ of the L–B plot (Fig. 3) at different fixed concentrations of theophylline minus $1/V_{max}$ in the absence of theophylline.]

DISCUSSION

The description of PDE activity in the multienzyme sequence

The good approximation of the theoretical data to the experimental curve (Fig. 1) was obtained by assuming the existence of two phosphodiesterases of low and high affinity to the common substrate, cyclic AMP, as earlier suggested [16]. However, the existence of only one enzyme with such an unusual kinetic cannot be excluded. Russel *et al.* [19] have proposed a negative cooperativity model for one enzyme to explain the phenomenon. On the other hand, using the phosphodiesterase data obtained from a work of Thompson and Appleman [20] for a systematic kinetic analysis, Kohn and Garfinkel [21] have mentioned that the information content of the data is insufficient to discriminate between rival mechanism. As many as 10 rate laws were found that fit the experimental data within 3%. Moreover, a model of positive cooperativity is reported, where phosphodiesterase is substrate-activated, thus producing rate vs substrate concentration curves similar to those of negative cooperativity. At present the question of a so-called multiphasic kinetic of the phosphodiesterase cannot be solved by kinetic means only. Therefore, on the basis of the fair compatibility of the experimental data with the existence of a high- and low-K_m enzyme, the multiphasic kinetic is tentatively explained in these terms. However, the commonly used procedure to yield the apparent K_m-values by plotting the experimental data in a double-reciprocal plot and extrapolating these curves (at low substrate concentrations) should not be used, as already noted by Spears *et al.* [16].

Also, the unusual kinetic of the phosphodiesterase could be due to the fact that phosphodiesterase is part of a multienzyme cluster. It has to be taken into account that some aspects of catalytic behaviour can result from protein–protein interaction of the constituents of an enzyme cluster.

The theophylline effect on the phosphodiesterase function

If the effect of theophylline on the phosphodiesterase function in the multienzyme sequence is to be analysed, two difficulties arise which are not easy to solve. Firstly, all data include a slight inhibitory effect of ATP on the phosphodiesterase activity as described under Results. Secondly, the concept of two phosphodiesterases requires that the theophylline effect on each enzyme should be studied. As these difficulties could not be overcome, the results have to be interpreted with limitation. Thus, the interpretation of the theophylline effect is reduced to a phenomenological description. As shown in Fig. 2, the data resemble those for competitive inhibition, where increasing substrate concentrations are able to remove the inhibitory effect. If classical competitive inhibition is assumed, the ligands, cyclic AMP and theophylline, compete for the same site, the active site of the enzyme. The formation of an ESI complex is not allowed. A further analysis of the data is not presented under Results due to the restrictions mentioned above. However, if the restrictions were ignored and the data were analysed in a double-reciprocal plot, an interesting facet of the competitive inhibition would be revealed. In particular, if the slopes of the straight lines of the double-reciprocal plot (which suggested simple competitive inhibition) were plotted vs inhibitor concentrations a hyperbolic curve resulted, which indicated the existence of an ESI complex (figures not shown). This type of inhibition had to be interpreted as partial competitive or hyperbolic competitive. Since the ESI complex can form product, as assumed in this type of inhibition, the rate can never be driven to zero. This inhibition is more compatible with the experimentally found activity which remained in the presence of high theophylline concentrations, than the classical competitive inhibition which demands that infinitely high inhibitor concentrations drive the enzyme to the EI form and the rate of catalysis is reduced to zero. The molecular interpretation of the hyperbolic competitive inhibition means that theophylline binds to a site on the enzyme other than the active site and in so doing causes a reduction in the affinity of the enzyme for the substrate, but does not affect the rate of catalytic breakdown of the enzyme-substrate complexes to give a product. This possible analysis is included in the discussion in order to shed light on the complex situation, and especially to show that the phenomenologically found competitive inhibition, as to be seen in Fig. 2, does not necessarily mean that an ESI complex does not exist.

The effect of theophylline on the nucleotidase function

Bastomsky *et al.* [8] first reported that theophylline is a nucleotidase inhibitor. Studying cyclic AMP hydrolysis in thyroid homogenate, the major products were found to be dephosphorylated, but in the presence of theophylline appreciable amounts of 5'-AMP accumulated. When AMP was used as the substrate, theophylline inhibited its hydrolysis. Newman and McIlwain [22] more recently reported that the drug-induced increase in the adenosine content of brain slices of guinea-pig was significantly reduced by theophylline. Tsuzuki and Newburgh [9] studied the inhibition of nucleotidase of rat brain by theophylline more explicitly. Fredholm *et al.* [10] demonstrated the inhibitory effect of theophylline on nucleotidase and phosphodiesterase of rabbit renal cortex and medulla. The inhibition of nucleotidase was described as simple noncompetitive. All these findings are very similar and completely compatible with our result that theophylline inhibits cyclic AMP degradation by a negative effect on the phosphodiesterase function, as well as on the nucleotidase function in the membrane-bound multienzyme sequence that degrades cyclic AMP. Likewise the result that deaminase activity was not inhibited is also in line with the previous findings [9, 10]. As demonstrated in the present work, theophylline acted as a noncompetitive inhibitor of nucleotidase. This requires the existence of an ESI complex. In other words theophylline acts at a binding site other than the active site. If it is proposed that each enzyme of the multienzyme sequence has the potential to alter its conformation and hence its catalytic properties in response to a single effector ligand acting at a single site, the catalytic capacity of each enzyme activity in the sequence may be increased or decreased, i.e. K_m and V_{max} values or both of each enzyme in the cluster could be influenced positively or negatively by a single effector. A review by Gaertner [23] of the unique catalytic properties of enzyme clusters provides examples for such a coordinate effect. In the present case it is suggested that theophylline exerts its effect in such a manner. Correspondingly, theophylline, as a common effector of the phosphodiesterase function and nucleotidase function, binds to that constituent of the multienzyme sequence that carries the phosphodiesterase function. By binding to the active site of phosphodiesterase (corresponding to a pure competitive inhibition) or by binding to an allosteric site on the

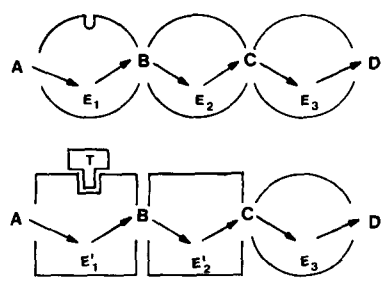

Fig. 7. A model of theophylline inhibition of the multienzyme sequence. By the action of theophylline [T] on a single allosteric site a coordinated conformational change is induced which results in an inactivation of the first two enzymes in the multienzyme sequence. E_1, E_2 and E_3 represent phosphodiesterase, nucleotidase and deaminase in the multienzyme sequence. The active form of the enzyme is presented by a circle and the inactive form by a square. The symbols A, B, C and D respectively, mean substrate, intermediates and end-product of the consecutive reactions.

phosphodiesterase (corresponding to the hyperbolic competitive inhibition) theophylline reduces the phosphodiesterase activity and additionally the nucleotidase activity of the multienzyme cluster. This inhibition of the nucleotidase activity, however, is in accordance with the hyperbolic noncompetitive inhibition mechanism. This molecular interpretation of the theophylline effect takes into account the direct neighbourhood of the enzymes in question and hence heterologous protein–protein interaction [24] which leads to reduced catalytic activity of phosphodiesterase and nucleotidase. This concept is illustrated by a model (Fig. 7).

It should be mentioned that an attempt was made to separate physically the phosphodiesterase and the nucleotidase from each other. This could lead to more direct evidence for the theophylline binding. However, the isolation of these enzymatically active constituents of the enzyme cluster was accompanied by inactivation. There is reason to believe that the active conformation is stabilized by protein–protein and membrane interaction.

An additional physiological aspect of cyclic AMP metabolism

A further aspect arises from the finding that the multienzyme sequence is able to "channel" the cyclic AMP metabolism, and from the finding that not only the phosphodiesterase but also the nucleotidase function is inhibited by theophylline. Biochemical and electrophysiological experiments reveal a remarkable biological activity of adenosine in a variety of tissues (reviews [5, 6, 25]), and nucleotidase may be a crucial enzyme in this process. Indeed, if the role of adenosine as an intercellular communication molecule is accepted, the question has to be answered where and how is it formed and how is the effective extracellular level of adenosine in the tissue controlled? Under the assumption of a metabolic channeling of cyclic AMP to the physiologically active adenosine, the increase and decrease of adenosine could be regulated indirectly through the cyclic AMP concentration. However, we do not yet know the circumstances *in vivo* that favour adenosine, rather than inosine, as the end product of the reaction sequence, as found with the microsomal material. Inosine formation could serve for the inactivation of the physiologically active adenosine and as a salvage pathway for ATP regeneration in the cell.

The idea that the physiologically active adenosine could be derived from cyclic AMP is further supported by some reports, which in part also studied the theophylline effect [22, 27]. Furthermore, such a physiologically relevant linkage between cyclic AMP and adenosine has been documented [29, 30] and is even required for certain theoretical reasons [21, 28].

If all the facts are brought together there is some evidence for the hypothesis that physiologically active adenosine is derived from cyclic AMP and that it may regulate further functionally meaningful reactions in the tissue. The concept that reduces the physiological function of the cyclic AMP solely to the stimulation of cyclic AMP protein kinases in the cell may be too restrictive.

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