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PRELIMINARY COMMUNICATIONS

EFFECT OF THEOPHYLLINE ON THE BINDING OF cAMP TO SOLUBLE PROTEIN FROM TRACHEAL SMOOTH MUSCLE

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Bronchial smooth muscle relaxes upon lowering the intracellular level of free calcium which is modulated by stimulation of beta-2 adrenergic receptors, activation of adenylate cyclase, inhibition of phosphodiesterase(PDE), or activation of protein kinase by cAMP (1). Theophylline(THEO), a clinically useful bronchodilator, has generally been thought to produce its pharmacological action through the elevation of the intracellular concentration of cAMP secondary to PDE inhibition. THEO in vitro is a competitive inhibitor of both low and high K_m phosphodiesterases (2). However, its therapeutic effect as a bronchodilator may not be due to inhibition of PDE, since therapeutic THEO blood levels (50-100 μM) are well below the concentration necessary to significantly inhibit phosphodiesterases, including PDE extracted from tracheal smooth muscle (3). Furthermore, THEO (10 mM) causes relaxation of carbachol contracted bovine trachealis muscle without elevating cAMP levels (2). We studied the effect of THEO on the binding of cAMP by the soluble protein fraction of bovine trachealis muscle.

METHODS

Fresh bovine trachea were transported from the abattoir to the laboratory in oxygenated, ice cold Krebs-Ringer solution. The trachealis muscle was dissected free of mucosa and fascia and homogenized at 4° in normal saline (1 g/4 ml). The homogenate was centrifuged at 20,000 g for 10 min. The supernatant fluid was decanted and used as the source of trachealis cAMP-binding protein (TBP).

The cAMP-binding assay was carried out at 4° in 4.5 mM sodium EDTA, 2.4 mM potassium phosphate buffer, pH 6.8, and 39 nM [^{3}H]cAMP (42.5 Ci/mmole) in a 100 μl reaction volume containing 50 μl TBP. After incubation for 1 hr, 50 μl of 10% (w/v) Norit A charcoal in 10 mM sodium EDTA, 4.8 mM potassium phosphate buffer, pH 6.8, and 2% albumin were added to the reaction mixture. The resulting suspension was centrifuged, and 50 μl of the supernatant fluid were added to 4.5 ml of scintillant fluid. The radioactivity of the sample as measured in a liquid scintillation spectrometer represents the [^{3}H]cAMP bound to TBP.

RESULTS AND DISCUSSION

The TBP in 50 μl of various crude extracts of bovine trachealis muscle (10 μg protein/μl) had the ability to bind 20-30 percent of [^{3}H]cAMP. Binding of [^{3}H]cAMP to TBP was also demonstrable when separation of bound from free [^{3}H]cAMP is carried out by molecular sieving on Bio-Gel P2 (exclusion limit > 1000 mol. wt.) instead of the charcoal procedure. Unlabeled cAMP (0.02 to 1.2 μM), but not 5'-AMP, 5'-GMP or cGMP, competes with the binding of [^{3}H]cAMP to TBP (Fig. 1). The competitive binding curve obtained with unlabeled cAMP was shifted to the left in the presence of THEO (Fig. 1) indicating that THEO increases the affinity of TBP for cAMP. The THEO-induced increase in the binding of [^{3}H]cAMP to TBP is dose dependent and has a half maximal effect at 100 μM (Fig. 2). At a concentration of 100 μM THEO, caffeine, theobromine, 3-methylxanthine, and xanthine increased the binding of [^{3}H]cAMP to TBP by 110, 52, 40, 31, and 3 percent respectively.

When the amount of [^{3}H]cAMP bound to TBP was plotted as a function of the amount of TBP, a concave downward curve was obtained and THEO decreased the curvature of this line (Fig. 3). This suggested the presence of an inhibitor of cAMP-binding (cAMP-BI) in the crude extract. If the protein in the crude extract was separated from endogenous low molecular compounds by molecular sieving through a Bio-Gel P2 column, the protein fraction still bound [^{3}H]cAMP, but THEO no longer increased the amount of [^{3}H]cAMP bound.

When the binding of [^{3}H]cAMP to TBP was analyzed by means of a Scatchard plot, a biphasic plot was obtained (Fig. 4). This suggests that the crude trachealis extract contains two different cAMP-binding sites on either the same or different proteins. THEO increased the slope of the initial segment of the biphasic Scatchard plot, indicating an increase in a cAMP-binding site's affinity for cAMP. Although low concentrations of cGMP do not interfere with the binding of cAMP in a classical competitive manner (Fig. 1), cGMP does antagonize the effect of THEO on the binding of [^{3}H]cAMP (Fig. 2). These data suggest that the cAMP-BI is a low molecular compound and that THEO antagonizes the inhibitory effect of the cAMP-BI, thereby increasing the binding of [^{3}H]cAMP to TBP. If cAMP-BI is in reality cGMP, this would be a biochemical mechanism consistent with the concept of biological dualism, i.e. the Yin-Yang hypothesis (4,5).

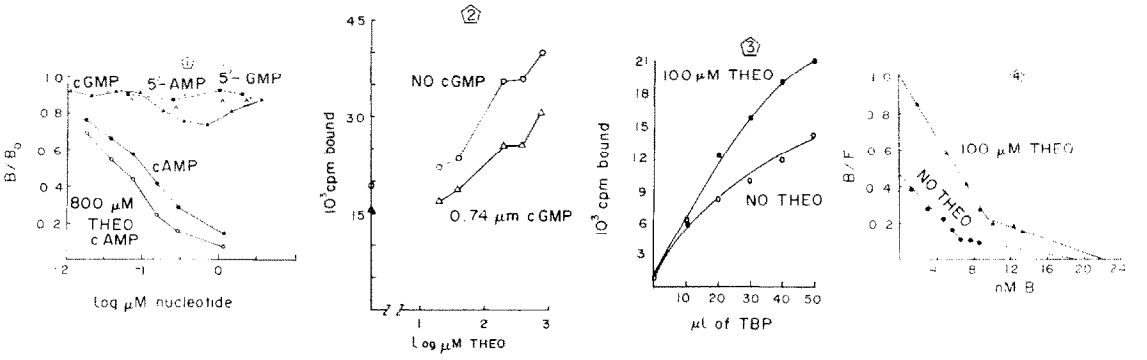

Fig. 1. Ratio of radioactivity bound to TBP in the presence of unlabeled nucleotide to radioactivity bound to TBP in the absence of unlabeled nucleotide, i.e. (B/Bo) versus the log of the concentration of the unlabeled nucleotide. THEO displaces the competitive binding curve of cAMP to the left, indicating an increase in the affinity for [^{3}H]cAMP by TBP. THEO does not affect the binding of free [^{3}H]cAMP to charcoal.
Fig. 2. Binding of [^{3}H]cAMP by TBP as a function of the log of the THEO concentration. Cyclic-GMP (0.72 μM) shifts the dose-response curve of THEO to the right, indicating that cGMP antagonizes the effect of THEO. THEO concentrations above 1mM do not cause any further increase in the binding of [^{3}H]cAMP.
Fig. 3. Binding of [^{3}H]cAMP as a function of the amount of TBP. In the presence of 100 μM THEO, the binding of [^{3}H]cAMP and the linearity of response are increased.
Fig. 4. Scatchard plot in the presence and absence of 100 μM THEO. THEO increases the slope of the initial segment of the Scatchard plot, suggesting that THEO may increase the affinity of TBP for [^{3}H]cAMP.

Since the half maximal effect of THEO on TBP is well below the K_i values of THEO for phosphodiesterases and since the binding of [^{3}H]cAMP to TBP is carried out in the absence of divalent cations, it is unlikely that TBP is a PDE. The identity of TBP and cAMP-BI, with the possibility of TBP being a cAMP-binding protein(s) associated with a protein kinase, is under investigation.

The biochemical mechanism by which THEO produces bronchodilation may be due to its ability to increase the binding of cAMP to cAMP-binding protein(s), possibly by antagonizing a cGMP mediated inhibition of the binding of cAMP. This concept and the methodology described above may provide a new approach for the development of drugs which can activate a cAMP-mediated cellular event without requiring a rise in the intracellular level of cAMP. Also, it is possible that other drugs which are thought to selectively inhibit a particular tissue PDE may in fact have an additional component to their mechanism of action, i.e. de-inhibition of the binding of cAMP to cAMP-binding protein(s) by interfering with the binding of an endogenous (-)allosteric inhibitor.

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