TANNIN UNCOUPLES THE ß-ADRENERGIC RECEPTOR (ßAR) FROM G PROTEINS

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Abstract

Tannin, isolated from cotton bracts, inhibits chloride (Cl^-) secretion and secondary water transport in airway epithelium. The mechanism for this inhibition was examined using bovine tracheal epithelial cells and ^{3}H-dihydroalprenolol. Tannin (25 μg/ml) exposure rapidly desensitized airway cells to isoproterenol-stimulated cAMP release. This desensitization was due in part to tannin-induced decreases in ß-adrenergic receptor number at the cell surface and to uncoupling of the ß-adrenergic receptor from its stimulatory G protein. Long-term tannin exposure (24 hr) further desensitized the airway epithelium to isoproterenol and forskolin suggesting downregulation of the ß-adrenergic receptor/cAMP pathway by tannin. These studies provide further evidence for the role of tannin in the occupational lung disease, byssinosis.

Introduction

Inhalation of cotton mill dust by textile workers results in the development of the occupational lung disease, byssinosis, in a portion of the workers (1, 2). While the etiology of byssinosis is not known, endotoxin and tannin, isolated from cotton bracts, have been implicated as important etiologic agents (3, 4). Tannin has pronounced biologic effects on a diverse group of cell types including platelets (5, 6, 7, 8), airway epithelial cells (9, 10), pulmonary arterial endothelial cells (11, 12), T lymphocytes (13) and alveolar macrophages (14, 15).

In airway epithelium mounted in Ussing chambers, cotton dust or an aqueous extract derived from cotton bracts (CBE), in low concentrations, inhibits active ion transport (as measured by short-circuit current) and increases the permeability of the paracellular pathway (16). Tannin, isolated from CBE, accounts for approximately 75% of the decrease in short-circuit current by inhibiting net chloride (Cl^-) secretion (10). This inhibition demonstrates specificity for the apical membrane, is dose dependent and reversible (10). The effect of tannin on a number of signal transduction pathways involved in Cl^- secretion has been investigated. These studies have revealed that tannin has many effects upon pathways for Cl^- secretion. Specifically, tannin decreases intracellular calcium stimulation in response to epinephrine, inhibits protein kinase C activity, and stimulates release of nonmetabolized arachidonic acid from bovine airway cells grown in culture - all of which could contribute to inhibition of Cl^- secretion (17, 18, 19, 20).

The most striking of the effects of tannin on the airway epithelium, however, are effects on the ß-adrenergic receptor/cAMP pathway. Tannin inhibits basal and epinephrine-stimulated intracellular cAMP levels in a dose-dependent manner in part by decreasing ß-adrenergic receptor number (R_o) without affecting the dissociation constant (K_d) (21). When the ß-adrenergic receptor is bypassed by forskolin, which acts directly upon the catalytic subunit of adenylate cyclase, tannin noncompetitively and reversibly inhibits forskolin- stimulated adenylate cyclase activity in a dose dependent manner (18). Thus, tannin decreases intracellular levels of cAMP stimulated by epinephrine by inhibiting ß-agonist binding. While tannin also inhibits bradykinin-stimulated cAMP release (probably through effects on adenylate cyclase), in contrast to epinephrine, tannin has no effect on bradykinin binding to airway epithelial cells and no effect on the activity of another surface membrane enzyme, acetylcholine esterase (Unpublished observation). Thus, tannin selectively and specifically inhibits the ß-adrenergic receptor and adenylate cyclase and inhibits Cl^- secretion. By inhibiting Cl^- secretion, tannin inhibits secondary water transport in the airway, mucus secretion and mucociliary transport and may also modulate airway smoth muscle reactivity to both contracting and relaxing agents (22, 23, 24). These effects could result in the pathologic pulmonary findings in patients with byssinosis. Decreases in mucociliary transport would also result in secretion retention and exposure of the epithelium to other substances in cotton dust, such as endotoxin, for longer than normal periods of time. Thus, tannin may directly and indirectly contribute to the pathogenesis of byssinosis.

Tannin could affect the ß-adrenergic receptor and decrease ß-adrenergic receptor number by uncoupling the receptor from its stimulatory protein (Gs), a process associated with receptor phosphorylation, by sequestration of the receptor in intracellular vesicles and/or in the case of long term exposure, by receptor downregulation. In these experiments we demonstrate that tannin desensitizes the ß-adrenergic receptor to isoproterenol and uncouples the receptor from Gs and suggest the possibility of downregulation after long-term tannin exposure.

Mterials and Methods

Bovine tracheas were obtained from a local slaughterhouse and placed in cold Hanks buffered saline solution (HBSS). Cell suspensions were prepared by scoring, stripping and cutting the bovine tracheal epithelium into small pieces using sharp dissection. Cells were isolated by gently stirring the strips at room temperature for two hours in 50 ml 50% Dulbecco's modified Eagle's medium and 50% Ham's F-12 medium (DMEM-F12) with 5% fetal calf serum containing dithiothreitol (5 mM, Sigma Chemical

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Co., St. Louis, MO), deoxyribonuclease I (100 mg/ml, Sigma) and 0.1% protease, type XIV (Sigma). Cells were centrifuged, resuspended in media and allowed to rest for 1 hr at 37°C to remove any contaminating fibroblasts. Cells were then plated onto collagen-coated plastic culture dishes at 250,000/cm^2 and grown in culture medium consisting of DMEM-F12 supplemented with 5% fetal calf serum and (per ml) 80 mg gentamicin, 2.5 mg fungizone, 100 U penicillin and 100 mg streptomycin. After 3-4 days in culture, the culture medium was replaced with HBSS containing 20 mM HEPES (pH 7.4) and various combinations of different compounds as described below. Tannin incubations occurred at 37°C for 10 minutes.

In other experiments, airway epithelial cells were scraped from the surface of bovine tracheas and placed in DMEM -F12 overnight. The suspension was centrifuged and the pellet was washed twice with HBSS. The pellet was resuspended in lysis buffer (10 mM Tris · HCL, 5 mM $MgCl_2$, 2m M DTT, 10 mM PMSF pH 7.4), homogenized and differential centrifugation was used to isolate a crude membrane preparation consisting of both apical and basolateral membrane fragments.

Cyclic AMP levels were measured using a cyclic AMP radioimmunoassay kit (Amersham, Arlington Hts., IL). The cells were then treated with 1N NaOH to dissolve cellular protein which was measured according to the method of Lowry using bovine serum albumin as the standard. Cyclic AMP levels were calculated as pmol cAMP per mg protein.

Condensed tannins were isolated from the 1985 crop of bracts from Acala SJ-5 cotton grown in Texas by a modification of the procedure of Taylor and associates as previously described (10, 25). Stock solutions of the tannin were prepared daily immediately before use by dissolving the tannin at a concentration of 19.2 mg/ml in water. This represented the tannin concentration in the cotton bracts extract we used in our original study (10). Tannin concentrations are reported as μg/ml.

Binding studies were performed using ^{3}H-dihydroalprenolol (^{3}H-DHA) at concentrations between 10^{-10} and 10^{-7} M. Membrane fragments were separated from buffer by vacuum filtration. The pellet was washed twice with 4 ml of buffer solution at 4°C to optimally separate free and bound ^{3}H-DHA and to reduce nonspecific binding. The radioactivity in the pellet was counted using liquid scintillation spectrophotometry. Non-specific binding was determined by displacement of ^{3}H-DHA binding with 10^{-6} M propranolol. Data were analyzed as a function of free ligand concentration using an iterative nonlinear curve fitting program (Ligand) and by Scatchard and Hill analysis to derive K_d and R_o with the latter expressed in terms of membrane protein content (Lowry). In other experiments, crude plasma membranes (~500 mg of protein) were diluted in 50 mM Tris · HCl (pH 7.5), 120 mM NaCl, 5 mM KCl, 3mM $MgCl_2$ (binding buffer) solution and incubated with ^{3}H-DNA (2.5 nM) and 16 concentrations of (-) isoproterenol at 30°C for 30 minutes in the presence or absence of Gpp (NH)p (50 mM). The binding incubations were terminated by the addition of 4 ml of ice cold buffer solution and poured over Whatman GF/C glass fiber filters under vacuum. The filters were washed once with 4 ml cold buffer solution and counted in a liquid scintillation counter.

Results and Discussion

Using BTE cells in culture, the intracellular cAMP response to a 10 minute exposure to 10^{-5} M isoproterenol was measured in cells prechallenged with tannin (25 μg/ml) for 5-120 min. Isoproterenol-stimulated cAMP release was blunted in cells exposed to tannin for increasing times compatible with desensitization. Inhibition began within 5 min and reached a maximum of approximately 50% at 60 min (Figure 1).

ß-adrenergic receptor density was determined using saturation binding with ^{3}H-DHA and BTE membrane fragments at 30°C. In preliminary experiments we demonstrated that binding equilibrium occurred by 30 min and between 0.1 and 5 nM DHA, specific binding was greater than 75% of total binding. In control cells, the K_d for ^{3}H-DHA was 0.41 nM with an R_o of 252 fmol/mg protein (n=3). In cells incubated with tannin (25 μg/ml) for 30 min, there was no change in K_d (0.26 nM) while R_o decreased to 162 fmol/mg protein - a 35% decrease in receptor number (Figure 2).

If tannin inhibits binding of ß-agonists to their receptors by altering the spatial arrrangement of the receptor, tannin might also affect the coupling between the receptor and its stimulatory G-protein (Gs) (26). ß-adrenergic receptors exist in a high and a low affinity state with the high affinity state being coupled to G-protein regulatory processes. Agonist binding affinity was measured by displacement of 2.5 nM ^{3}H-DHA with 16 concentrations of (-) isoproterenol (0, 10^{-10} - 10^{-3} M) in the absence and presence of the non-hydrolyzable G protein analog, Gpp(NH)p (Figure 3). The curve in the absence of the nucleotide was better fit by a 2 site model with K_ds of 44 nM and 870 nM. Under these conditions, high and low affinity receptors were present in approximately equal numbers. In the presence of Gpp(NH)p, the position of the curve moved to the right indicating a lower apparent affinity of the receptor for the agonist. The curve also steepened suggesting a single homogeneous receptor population. Tannin (25 μg/ml) alone steepened the curve and moved it to the right compatible with an increase in low affinity sites. Gpp(NH)p had no further effect on the shape or position of the displacement curve in the presence of tannin.

In another group of experiments, BTE cells were grown to >90% confluence and exposed to either 5 μg/ml or 25

μg/ml tannin for 24 hrs. The isoproterenol-stimulated and forskolin-stimulated cAMP response was measured. Long term tannin exposure at both concentrations decreased basal levels of cAMP and blunted both isoproterenol-stimulated and forskolin-stimulated cAMP release in a dose-dependent manner (Figure 4). Inhibition of isoproterenol-stimulated cAMP release was ~73% after 24 h of tannin compared to 50% at 60 min (Figure 1) and was greater than the inhibition observed when the receptor was bypassed by forskolin. No morphologic changes were noted in cells exposed to either concentration of tannin for 24 hrs.

These experiments demonstrate that tannin desensitizes BTE cells to isoproterenol, decreases ß-adrenergic receptor density and uncouples the receptor from Gs. These data also demonstrate that long term tannin exposure results in profound changes in the cAMP response to isoproterenol and suggest a further decrease in receptor density with long term tannin exposure. The inhibition in isoproterenol-stimulated cAMP release after tannin exposure is greater than the forskolin-stimulated cAMP release compatible with direct effects upon both the ß-adrenergic receptor and upon adenylate cyclase by long term tannin exposure. The mechanism for the decrease in ß-adrenergic receptor number and receptor desensitization is not known but is under active investigation.

These changes in receptor density after acute and "chronic" exposure to tannin, could contribute to the clinical syndrome of byssinosis. The Monday symptoms which occur with acute exposure to cotton dust and the diminishing across shift symptoms that occur during the remaining weekdays are compatible with rapid desensitization of ß-adrenergic receptors with ß-adrenergic receptor-Gs uncoupling. Long exposure times could result in downregulation which presumably could contribute to an overall reduction in receptor number and responsiveness and the blunting of clinical symptoms later in the week. Partial recovery of receptor number and resensitization during weeknights away from the mill could occur and contribute to some continued symptoms with daily exposure. Complete recovery from downregulation would then occur over the weekend or with prolonged absences. This process would produce acute symptoms on Monday with diminishing symptoms during repeated exposure the remainder of the week and recovery of sensitization during the weekend. Such a mechanism of rapid desensitization followed by downregulation has been proposed for patients with asthma on ß-adrenergic drugs except in asthma the stimulus is rarely continuous (27). Tannin exposure, however, is one of the few instances where exposures are repetitive, frequent and occur over long periods of time.

In summary, tannin desensitizes the airway epithelium to ß-agonists and uncouples the ß-adrenergic receptor from Gs. Long term exposure also downregulates the ß-adrenergic receptor and its cAMP pathway. These pathways are important in regulating Cl^- secretion and water transport in the airways.

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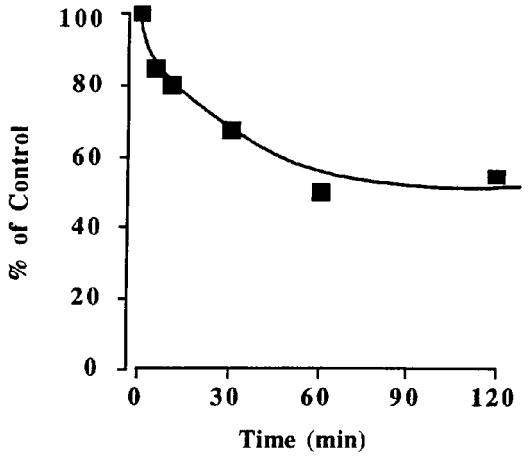

Figure 1. Effect of time of tannin (25 μg/ml) exposure on cAMP response to a 10 min exposure to isoproterenol. Data are expressed as a percent of control (cAMP response to isoproterenol in the absence of tannin).

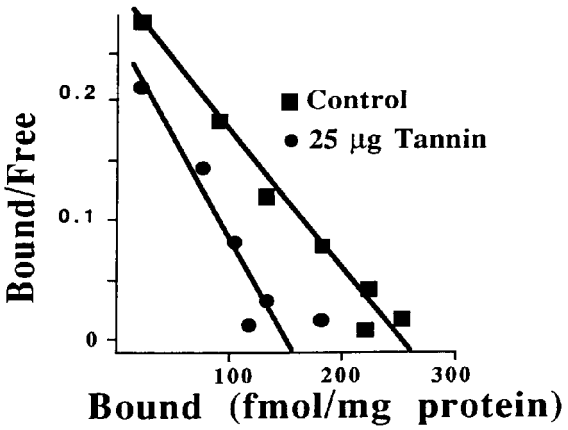

Figure 2. Scatchard analysis of DHA binding in control BTE membraness and in BTE membranes pretreated with tannin (25 μg/ml). Kd was unchanged at 0.41 and 0.26 nM while Ro decreased from 252 to 162 fmol/mg protein.

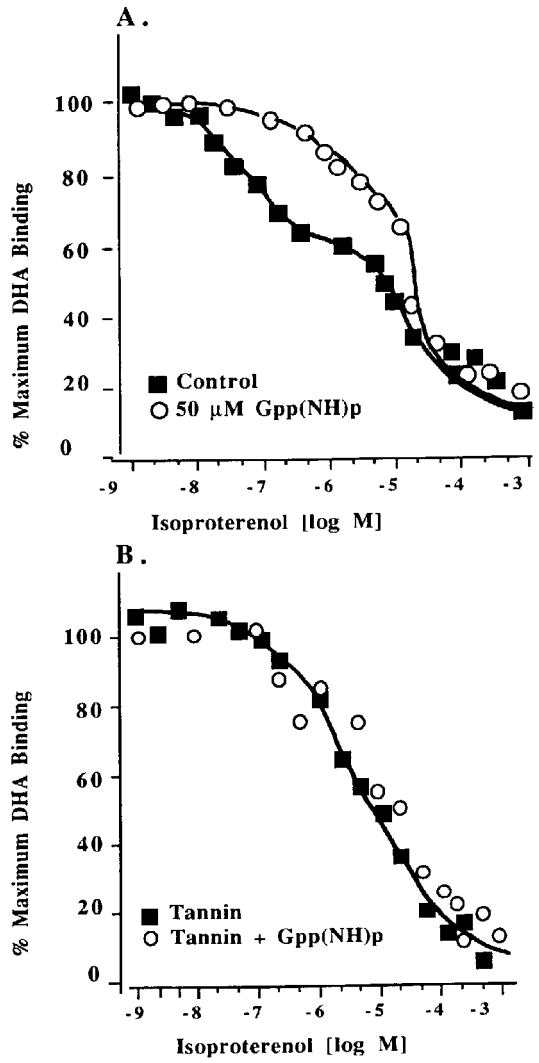

Figure 3. Effect of Gpp(NH)p on DHA displacement by isoproterenol. Experiments were performed in the absence (A) and presence (B) of tannin.

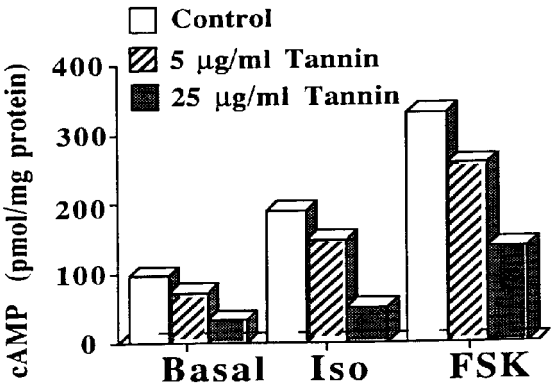

Figure 4. Effect of long-term (24 h) tannin exposure on the cAMP response to isoproterenol and forskolin.