EFFECTS OF POLYMER LENGTH AND CYSTEINE RESIDUES ON AIRWAY β-ADRENERGIC RECEPTOR FUNCTION

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Abstract

Tannin, isolated from cotton bracts and implicated in the pathogenesis of byssinosis, inhibits isoproterenol-stimulated cAMP release from airway cells in part by decreasing cell surface β-adrenergic receptor number and uncoupling the β-adrenergic receptor from its stimulatory G-protein (Gs). We have hypothesized that tannin, because of its long polymer length and unusual monoflavanoid composition, interacts with the hydrophobic plasma membrane surface of the β-adrenergic receptor and alters receptor binding and Gs coupling. In these studies, we demonstrate that decreased polymer length blocks tannin's inhibitory effects on chloride secretion, on cell surface β-adrenergic receptor number and on isoproterenol-stimulated cAMP release. We also demonstrate that pretreatment with N-acetylcysteine, which interacts with cysteine residues, inhibits tannin's effects on isoproterenol-stimulated cAMP release. We conclude that polymer length and cysteine residues are essential for tannin's inhibitory effects on the airway epithelium.

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 (Bouhuys, A. 1976). Acute symptoms of chest tightness, wheezing and shortness of breath begin several hours after exposure to cotton dust in susceptible workers and are accompanied by across shift changes in lung function and the development of an acute pulmonary inflammatory reaction in the airways (Kawamoto, et. al., 1987; Rylander, et. al., 1983).

While the etiology of byssinosis is not known, endotoxin and tannin isolated from cotton bracts, the thin, brittle leaves surrounding the cotton boll, have been implicated as important etiologic agents (Rohrbach, 1994; Rylander, 1982). Tannin, isolated from cotton bracts (CBE), inhibits chloride secretion across the airway epithelium, in a site-specific, dose-dependent and reversible manner (Cloutier et.al., 1984; Cloutier and Rohrbach 1986). Tannin also decreases cell surface β-adrenergic receptor number without affecting the dissociation constant (Cloutier, et. al., 1997). When the β-adrenergic receptor is bypassed by forskolin, which acts directly on the catalytic subunit of adenylyl cyclase, tannin noncompetitively and reversibly inhibits adenylyl cyclase activity in a dose-dependent manner (Cloutier and Guernsey 1995). Tannin has no effect on bradykinin binding or on the activity of another surface membrane enzyme, acetylcholine esterase (Hartman et. al., 1993, unpublished observation). Thus, these effects of tannin are both selective and specific for the β-adrenergic receptor and adenylyl cyclase.

Like condensed tannins derived from other woody plants, the tannin present in cotton mill dust is a polymer of monoflavanoid subunits (Rohrbach et. al., 1990). Tannin's effects on the airway epithelium and on platelets, are however, unique. CBE tannin inhibits Cl^- secretion in airway epithelial cells and platelet 5-hydroxytryptamine (5-HT) release while tannin from other sources including tea, or dusts from corn, soybean and grain either increase Cl^- secretion or alter the integrity of the paracellular pathway (Cloutier and Rohrbach 1989, unpublished observation). Structural features of tannin which might contribute to these unique aspects include the polydisperse nature of CBE tannin, the unusually long polymer length (~ 9.4 monomer subunits), the abundance of hydroxyl groups and the unusual monoflavanoid composition (procyanidin and prodelphinidin in a ratio of 2:3) (Chan 1985). How tannin exerts its effects is not known. Cyanidins, in general however, have been shown to react with cysteine residues which are important in the structure and function of the β-adrenergic receptor and may explain how tannin alters signal transduction pathways (Haslam 1989).

We have hypothesized that the tannin polymer, through an affinity for cysteine residues, alters the tertiary configuration of the β-adrenergic receptor and thus affects the binding of b-agonists to the receptor and the coupling between the receptor and its stimulatory G protein. To test this hypothesis, we examined the effects of changes in tannin polymer length and the effects of N-acetylcysteine, which interacts with cysteine residues, on isoproterenol-stimulated cAMP accumulation, on cell surface β-adrenergic receptor number and on chloride secretion. In these experiments, we demonstrate that polymer length is essential for the effects of tannin on isoproterenol-stimulated cAMP accumulation, for changes in cell surface receptor number and for inhibition in chloride secretion and that N-acetylcysteine inhibits the effects of tannin on cAMP accumulation.

Materials and Methods

Bovine tracheas were obtained from a local slaughterhouse and placed in cold Hanks buffered saline solution (HBSS). In some experiments, the posterior membranous portion of the bovine trachea, stripped of the trachealis muscle, was mounted as a flat sheet between two halves of an Ussing chamber as previously described (Cloutier and Rohrbach 1986). Each half chamber was filled with Ringers solution maintained at 37°C, aerated with 95% O_2, 5% CO_2 and recirculated through each half chamber using a bubble lift apparatus. An automatic voltage clamp electrometer and standard techniques were used to measure electrophysiologic parameters. Fluxes were performed in electrically matched,

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paired tissues. ^{36}Cl (4 μCi, ICN, Irvine, CA) was added to either the mucosal bathing solution for m-to-s fluxes or to the submucosal bathing solution for s-to-m fluxes. After a 30 minute equilibration period, samples were taken every l5 min for 75 min. After this control period, tannins of different molecular weights were added to the mucosal bathing solution and sample collection was continued every 15 min for 75 min. Experiments were performed under short-circuit conditions (I_{sc}), interrupted every 15 min for 45 s to record the transepithelial potential difference (Ψ_{ms}) and the tissue resistance (R_t).

Cell suspensions were prepared by scoring, stripping and cutting the bovine tracheal epithelium (BTE) into small pieces using sharp dissection as previously described (Cloutier and Guernsey 1995). 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, Biowhittaker) with 5% fetal calf serum containing dithiothreitol (DTT, 5mM, Sigma Chemical Co., St. Louis, MO), deoxyribonuclease 1 (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 37EC 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) 80μg gentamicin, 2.5 μg fungizone, 100 U penicillin and 100 μg 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.

The effect of tannin on cell surface receptor number was determined using ^{3}H-CGP 12177 (44 Ci/mmol; New England Nuclear, Wilmington, DE). BTE cells in culture (~10^5 cells) were exposed to 25 μg/ml tannin for 30 min and then incubated in 1 ml DMEM containing 25 mM HEPES and 30 μg/ml BSA (Sigma) (pH 7.4) at 4°C for 3 h in the presence of a saturating concentration (1 nM) of ^{3}H-CGP-12177. Cell surface receptor density was calculated from one-point analysis in which 10^{-6} M propranolol was used to assess nonspecific binding. Results from tannin experiments were compared to similar experiments using isoproterenol (10^{-5} M for 3 h), which is known to cause a rapid decrease in cell surface receptor number (Cloutier and Guernsey 1993).

cAMP was measured using a 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 (Lowry et. al., 1951). cAMP levels were calculated as pmol cAMP per mg protein.

In some experiments, cells in culture were incubated for 6 h at 37°C with either 10 mM or 30 mM N-acetylcysteine (Sigma). The N-acetylcysteine was dissolved in cell media (DMEM-F12) and pH adjusted with NaOH before adding to the culture well. cAMP levels were measured under various conditions in the presence and absence of tannin.

Condensed tannins were isolated from the 1985 crop of bracts from Acala SJ-5 cotton grown in Texas utilizing sequential Amicon ultrafiltration and a modification of the procedure of Taylor and associates as previously described (Cloutier and Rohrbach 1986; Taylor et. al. 1971). Stock solutions of high molecular weight tannin (YM10 retentate, molecular weight > 10,000 dalton), "medium" molecular weight (YM10 filtrate, molecular weight 1,000 - 10,000 dalton) and low molecular weight (YM2 retentate, molecular weight 1,000 - 5,000 dalton) were prepared daily, immediately before use, by dissolving the tannin in water. The high molecular weight tannin was prepared at a concentration of 19.2 mg/ml which represented the tannin concentration in the cotton bracts extract used in our original study (Cloutier and Rohrbach 1986). This high molecular weight tannin constituted 54% of the total tannin in the CBE while the YM2 retentate constituted 21% and the YM10 filtrate constituted 46% of the total tannin (Cloutier and Rohrbach 1989). Tannin concentrations are reported as μg/ml.

Data were analyzed using analysis of variance and Student's test or as described (Bruning and Kintz 1977).

Results and Discussion

In Ussing chamber experiments, the high molecular weight tannin (YM10 retentate) decreased Ψ_{ms} and I_{sc} and increased R_t, as previously described (Table 2) (Cloutier and Rohrbach 1986). This high molecular weight tannin also decreased net chloride secretion. In contrast, neither the YM10 filtrate tannin nor the YM2 retentate tannin had any effect on net chloride secretion. Small changes in electrophysiologic properties were observed with the YM2 retentate and matched increases in unidirectional chloride fluxes without change in net flux were observed with the YM10 filtrate (Table 1).

The effects of tannins of different molecular weight on cell surface receptor number are shown in Table 2. High molecular weight tannin (YM10 retentate) and isoproterenol decreased cell surface receptor number. The YM10 filtrate and the YM2 retentate had no effect on cell surface receptor number.

Similarly, the effects of polymer length and molecular weight on isoproterenol-stimulated cAMP accumulation were examined. High molecular weight tannin inhibited isoproterenol-stimulated cAMP accumulation while both lower molecular weight tannins had no effect on isoproterenol-stimulated cAMP accumulation (Figure 1). The effect of increasing concentrations of low molecular weight tannin on isoproterenol-stimulated cAMP was also examined (Figure 2). At concentrations up to 50 μg/ml, low molecular weight tannin had no effect on isoproterenol-

stimulated cAMP accumulation. At concentrations of the YM2 retentate greater than 50 μg/ml, however, increased isoproterenol-stimulated cAMP was observed. This is similar to the stimulatory effect observed with other low molecular weight tannins isolated from tea and from brown cotton bracts extract (Cloutier and Rohrach 1989).

When the β-adrenergic receptor was bypassed by forskolin, high molecular weight tannin inhibited forskolin-stimulated cAMP accumulation while low molecular weight tannin had no effect (Table 3). This effect of forskolin on isoproterenol-stimulated cAMP was dose-dependent.

N-acetylcysteine (NAC) pretreatment had no effect on basal cAMP levels. The increase in basal cAMP levels at 30mM N-acetylcysteine approached but was not statistically significant. N-acetylcysteine also had no effect on isoproterenol-stimulated cAMP accumulation. However, at both 10 mM and 30 mM, N-acetylcysteine blocked the effect of tannin on isoproterenol-stimulated cAMP accumulation and both concentrations were equally effective in inhibiting tannin's effects irrespective of their ability to stimulate basal cAMP accumulation (Figure 3). The mechanism for this inhibition is not known although it is possible that the interaction of NAC with cysteine residues blocks the interaction of tannin with these same cysteine residues. NAC has been shown to activate non-CFTR (cystic fibrosis transmembrane conductance regulator) Cl^- conductance (Kottgen et.al., 1996) which is compatible with our current data.

These data demonstrate that polymer length is essential for the effects of tannin on cell surface receptor number, on chloride secretion, on isoproterenol-stimulated cAMP and on forskolin-stimulated adenylyl cyclase activity. We have previously demonstrated that tannin also decreases $-adrenergic receptor density and uncouples the receptor from Gs and that these effects are also related to polymer length (Cloutier et. al., 1997). The tannin present in cotton mill dust is a polymer of monoflavanoid subunits. Compared to other plant tannins, cotton bract tannin has a longer polymer length (~9.4 monomer units) and an unusual monoflavanoid composition (procyanidin and prodelphinidin in a ratio of 2:3). Cyanidins strongly react with cysteine residues and of the 15 cysteine residues present in the β-adrenergic receptor, four are in the putative extracellular domain (Haslam 1989; Lefkowitz et. al.., 1983). These four cysteines form disulfide bonds which provide stability to the receptor, result in high affinity ligand binding, contribute to the pharmacologic specificity of agonists and antagonists, promote coupling of the β-adrenergic receptor to Gs proteins and play a major role in agonist-induced stimulation of adenylyl cyclase (Lefkowitz et. al., 1983; Noda et. al., 1994; Stadel and Nakada 1991). We have hypothesized that cotton bract tannin interacts with extracellular cysteine residues and this interaction affects ligand binding, uncouples the receptor from Gs and inhibits cAMP accumulation. Results presented here and previously in which polymer length was changed either by oxidation or through ultrafiltration, and results from experiments with N-acetylcysteine support this hypothesis.

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References

Bouhuys, A. 1976. Scheduled asthma in the textile industry. Lung. 154;3-16.

Bruning, J.L. and B.L. Kintz. 1977. Computational Handbook of Statistics. Scott Foresman. Glenview, IL. pp113-116.

Chan, B.G. 1985. From L- phenylalanine to condensed tannins: A brief review of tannin chemistry and biochemistry and a report of its distribution in cotton plant parts. Proceedings of the 9th Cotton Dust Research Conference. P.J. Wakelyn and R.R. Jacobs (Eds). National Cotton Council: Memphis, TN. pp49-52.

Cloutier, M.M. and L. Guernsey. 1993. Tannin inhibits protein kinase C pathway in canine tracheal epithelium. Proceedings of the 17th Cotton Dust Research Conference. L.N. Domelsmith, R.R. Jacobs and P.J. Wakelyn (Eds). National Cotton Council: Memphis, TN. pp332-334.

Cloutier, M.M. and L. Guernsey. 1995. Tannin inhibits adenylate cyclase in airway epithelial cells. Am.J. Physiology: Lung Cell. Mol. Physiol.268;L851-L855.

Cloutier, M.M., K. Lesniak, J.A. Russell and M. Rohrbach. 1984. Effect of cotton bracts extract on canine tracheal epithelium and shunt pathway. Am. Rev.Respir. Dis. 130;1087-1090.

Cloutier, M.M. and M.S. Rohrbach. 1986. Effects of endotoxin and tannin isolated from cotton bracts on the airway epithelium. Am. Rev. Respir. Dis. 134;1158-1162.

Cloutier, M.M. and M.S. Rohrbach. 1989. Effects of tannins from different sources on airway epithelial and platelet function. Lung. 167;117-128.

Cloutier, M.M., C.M. Schramm and L. Guernsey. 1997. Tannin inhibits the cAMP β-adrenergic receptor pathway in bovine tracheal epithelium. Am. J. Physiol: Lung Cell. Mol. Physiol. in press.

Hartman, B.N., M.M. Cloutier and L. Guernsey. 1993. Effect of tannin on bradykinin and epinephrine receptors in bovine tracheal epithelial cells. Proceedings of the 17th

Cotton Dust Research Conference. L.N. Domelsmith, R.R. Jacobs and P.J. Wakelyn (Eds). National Cotton Council: Memphis, TN pp349-351.

Haslam, E. 1989. Plant polyphenols. IN: Plant Polyphenols. R. Hemingway and P. Laks (Ed). Cambridge University Press. Cambridge. pp1-15.

Kawamoto, M.M., D.H. Garabrant, J. Held, J.R. Balmes, J. Patzman, D.V. Dimick, J.A. Simonowitz and L. Bernstein. 1987. Respiratory effects of cotton dust exposure in the cotton garnetting industry. Am. J. Indus. Med. 11;505-515.

Kottgen, M., A.E. Busch, M.J. Hug, R. Greger and K. Kunzelmann. 1996. N-acetyl-L-cysteine and its derivatives activate a Cl^- conductance in epithelial cells. Pflugers Arch.-Eur. J. Physiol. 43l;549-555.

Lefkowitz, R.J., J.M. Stadel and M.G. Caron. 1983. Adenylate cyclase-coupled β-adrenergic receptors: structure and mechanisms of activation and desensitization. Annu. Rev. Biochem. 52;159-186.

Lowry, O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem 193;265-275.

Noda, K., Y. Saad, R.M. Graham and S.S. Karnik. 1994. The high affinity state of the b_2-adrenergic receptor requires unique interaction between conserved and non-conserved extracellular loop cysteines. J. Biol. Chem. 269;6743-6752.

Rohrbach, M.S. 1994. Biological activity of condensed tannins. IN: Organic Dusts: Exposure, Effects and Prevention. R. Rylander and R.R. Jacobs (Ed). Lewis Publishers, Inc. Chelsea. pp69-91.

Rohrbach, M.S., T.J. Kreofsky and B.C. Chan. 1990. Structural determinants of platelet agonist activity of cotton bract condensed tannin.Env. Res. 52;199-209.

Rylander, R. 1982. Bacteria as etiological agents in byssinosis. Eur. J. Respir. Dis. 123;34-36.

Rylander, R., P. Haglind and B.T. Butcher. 1983. Reactions during working shift among cotton mill workers. Chest. 84;403-407.

Stadel, J.M. and M.T. Nakada. 1991. Molecular biology of b-adrenoceptors. IN:b-Adrenoceptors: Molecular Biology, Biochemistry and Pharmacology. R.R.Ruffolo (Ed). S. Karger A.G. Switzerland. pp26-66.

Taylor, G., A.A.E. Massoud. and F. Lucas. 1971. Studies on the aetiology of byssinosis. Br. J. Ind. Med 28;143-151.

Table 1. Effects of Tannins of Different Molecular Weights on Electrophysiologic Properties and Cl^- Fluxes.

	CONTROL	YM2 Retentate	P*
Ψ_{ms} (mV)	14.2 ± 1.4	12.8 ± 1.0	ns
I_{sc} (µA/cm^2)	66.4 ± 4.6	53.5 ± 3.5	< 0.001
R_t (Ωcm^2)	195 ± 14	227 ± 15	< 0.001
Jsm (µEq/cm^2·h)	2.60 ± 0.55	3.01 ± 0.51	ns
Jms (µEq/cm^2·h)	1.43 ± 0.40	1.81 ± 0.26	ns
Jnet (Jsm-Jms)	1.17 ± 0.20	1.21 ± 0.25	ns
	CONTROL	M10 Filtrate	P
Ψ_{ms} (mV)	16.9 ± 1.2	17.8 ± 0.9	< 0.01
I_{sc} (µA/cm^2)	71.7 ± 5.3	71.7 ± 4.7	ns
R_t (Ω·cm^2)	177 ± 9	189 ± 10	ns
Jsm (µEq/cm^2·h)	2.58 ± 0.23	3.09 ± 0.08	< 0.05
Jms (µEq/cm^2·h)	1.68 ± 0.20	2.07 ± 0.08	< 0.05
Jnet (Jsm-Jms)	0.90 ± 0.11	1.02 ± 0.08	ns
	CONTROL	YM10 Retentate	P
Ψms (MV)	16.2 " 1.9	8.9 ±0.18	< 0.001
I_{sc} (µA/cm^2)	87.1 ±9.1	46.5 ± 5.2	< 0.001
R_t (Ωcm^2)	171 ±12	204 ± 18	< 0.05
Jsm (µEq/cm^2·h)	2.83 ±0.55	2.63 ± 0.31	ns
Jms (µEq/cm^2·h)	1.72 ±0.39	2.04 ± 0.17	ns
Jnet (Jsm-Jms)	1.11 ±0.28	0.59 ± 0.18	< 0.05

*Data expressed as the mean ± SEM of l2 different paired tissues for each condition compared to control values. Explanation of abbreviations Ψ_{ms}: transepithelial potential difference I_{sc}, short-circuit current; R_t, tissue resistance.

Table 2. Effect of Tannins of Different Molecular Weights on Cell Surface $-Adrenergic Receptor Number *

	RECEPTOR NUMBER	n	P**
Control	18.0 ±1.8	8	
Isoproterenol (l0^{-5}M)	5.2 ± 1.8	4	< 0.001
YM2 Retentate (25µg/ml)	l6.4 ± 1.9	4	ns
YM10 Filtrate (25µg/ml)	l6.0 ± 1.5	4	ns
YM10 Retentate (25µg/ml)	10.6 ± 0.9	4	< 0.02

*Data expressed as the mean " SEM of n measurements as determined by ^{3}H-CGP-12177 binding.
**Compared to control value

Table 3. Effects of Tannins of Different Molecular Weights on Forskolin - Stimulated cAMP Accumulation*

	cAMP Accumulation (pmol/mg protein)	n	P**
CONTROL	215 ± 37	8	
5µM Forskolin	5254 ± 1290	6	< 0.001^H
YM2 Retentate			
10 µg/ml	5549 ± 1307	8	ns
25 µg/ml	4672 ± 1216	7	ns
YM10 Filtrate			
10 µg/ml	5693 ± 1115	8	ns
25 µg/ml	4532 ± 895	7	ns
YM10 Retentate			
10 µg/ml	2659 ± 8l6	9	< 0.02
25 µg/ml	2968 ± 620	8	< 0.01

*Data expressed as the mean " SEM of "n" observations. Cells were exposed to the tannins for l0 minutes followed by a l0 minute exposure to forskolin.
**Compared to forskolin alone ^{H} Compared to control.

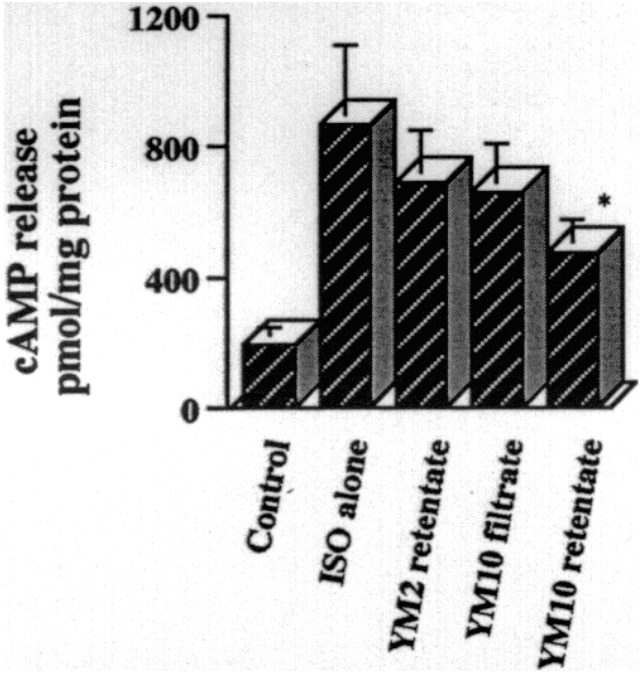

Figure 1. Effect of tannins of different molecular weights on isoproterenol-stimulated cAMP release. Cells in structure were exposed to different tannins (25 µg/ml) for 10 min prior to a 10 min exposure to Isoproterenol (10-5M). Data are expressed as the mean ± SEM of 6 observations. *p<0.05 compared to isoproterenol alone.

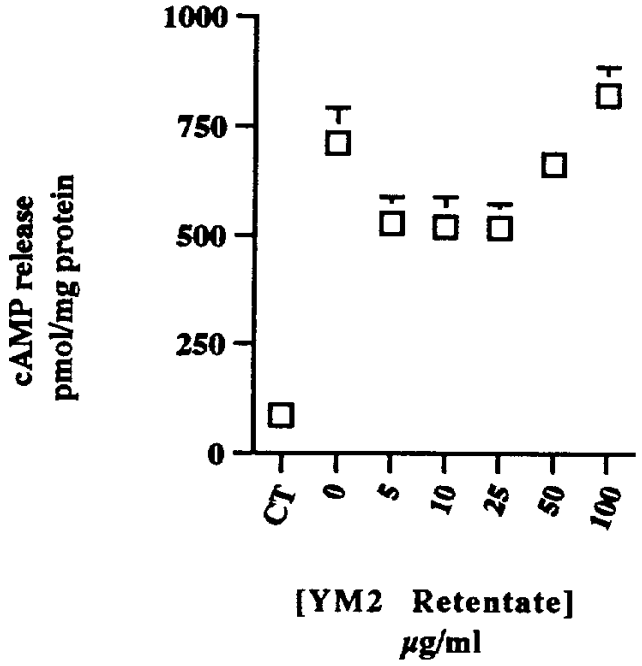

Figure 2. Effect of increasing concentrations of YM2 retentate on isoproterenol-stimulated cAMP release. Cells in culture were pretreated with different concentrations of YM2 retentate for 10 min prior to a 10 min stimulation with 10-5M isoproterenol. Data expressed as the mean ± SEM of 6 observations. Exposure to 50 µg/ml YM10 retentate for 10 min in these same cells followed by isoproterenol resulted in a cAMP release of 342 ± 28 pmol/mg protein.

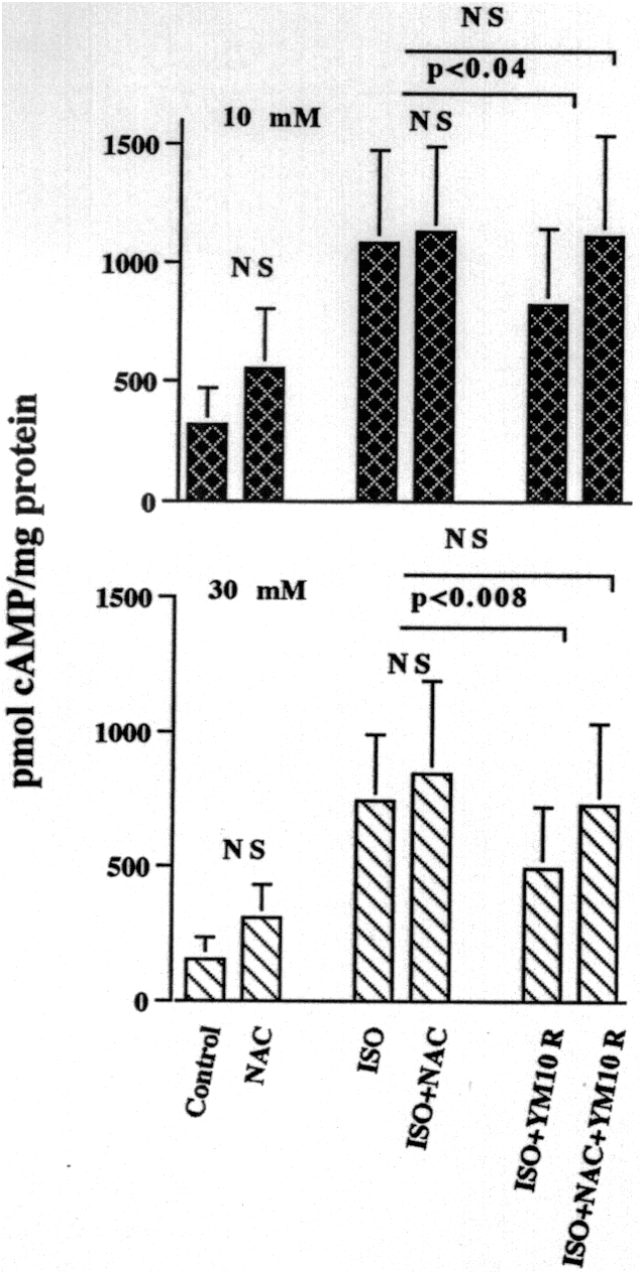

Figure 3: Effect of N-acetlycysteine (NAC) (10 or 30 mM for 6 h) on isoproterenol (ISO)-stimulated (10-5 M) cAMP accumulation in the presence and absence of tannin (25 µg/ml). ISO alone increased cAMP levels compared to control levels (p>0.001) but there was no difference in the degree of stimulation of cAMP between ISO alone and ISO + NAC, N.S. = not significant. Tannin inhibition of ISO-stimulated cAMP was blocked in cells pre-treated with both concentrations of NAC.