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Cyclic nucleotides fail to affect mucus glycoconjugate secretion from canine tracheal explants

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Canine tracheal explants, incubated overnight with [^{3}H]glucosamine, elicited an enhanced secretion of ethanol-precipitated [^{3}H]labelled glycoconjugate when challenged with methacholine, 10 μM. Neither the β-adrenoceptor agonist isoprenaline, 10 μM, nor the phosphodiesterase inhibitor theophylline, 10 mM, had any significant effect on glycoconjugate secretion. Dibutyryl cyclic AMP, 1 mM, and dibutyryl cyclic GMP, 1 mM, alone or in combination with theophylline, 10 mM, were devoid of activity on unstimulated or methacholine-stimulated tracheal explants. The calcium ionophore A23187, 10 μM, stimulated [^{3}H]glycoconjugate secretion from each of the tissues tested; however, the cyclic nucleotides failed to modify this response. These data indicate that the cyclic nucleotides play little, if any, role in mucus glycoconjugate secretion by the canine trachea.

It is currently believed that cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) serve as modulators of calcium-mediated secretion and that may even directly control secretion in certain tissues (for review, see Rubin 1982). Investigations into the role of cAMP in the secretion of respiratory mucus have focused principally on the secretion of electrolytes and water. β-Adrenoceptor agonists (Al-Bazzaz et al 1977; Marin & Zaremba 1978; Davis et al 1979), theophylline (Al-Bazzaz et al 1977), and dibutyryl cAMP (Al-Bazzaz 1981) have all been reported to increase the secretion of chloride ions towards the luminal mucosa of canine trachea in-vitro.

The role of cyclic nucleotides in the secretion of the macromolecular components of tracheal mucus is poorly characterized. Phipps et al (1980) showed that terbutaline enhanced the release of radiolabelled mucus from feline isolated trachea and Liedtke et al (1982) described the stimulant activity of β-adrenoceptor agonists, the phosphodiesterase inhibitor isobutylmethylxanthine, and 8-bromo-cAMP on mucus glycoprotein secretion from cat trachea in-vitro. Shelhamer et al (1980) showed that 8-bromo-cGMP was an effective mucus secretogogue in human bronchial tissues in-vitro, but that dibutyryl cAMP had no effect. The role of cyclic nucleotides as modulators of stimulated tracheal mucus secretion has not been reported, nor has their influence on basal mucus glycoconjugate secretion from canine tracheal explants.

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Methods

The method used for the quantitation of canine tracheal mucus secretion was a modification of the organ culture technique of Coles & Reid (1981). Details of the procedure used have been described previously (Barsigian & Barbieri 1982; Barbieri et al 1984). Briefly, the tracheae of adult male mongrel dogs (9–11 kg), given a fatal intraperitoneal injection of pentobarbitone sodium solution, 50 mg kg^{-1}, were removed, sectioned, and tracheal explants were incubated overnight in serum-free Medium 199 with Earle's salts (Gibco Laboratories) containing D-[6-^{3}H]glucosamine hydrochloride (specific activity, 38 Ci mmol^{-1}), 3·6 μCi ml^{-1} (Amersham Corporation) under 95% O_2: 5% CO_2 at 37 °C in a metabolic shaker.

The following day the tissues were washed with fresh Medium 199 and incubated for two sequential periods (Harvesting Periods A and B) of either 10 or 30 min duration. Samples which served as controls were incubated in Medium 199 alone in both periods; drug-treated samples were incubated alone during

Period A and in medium with drug during Period B. Following each Harvesting Period the medium was collected and the ^{3}H-labelled mucus precipitated with 75% (v/v) ethanol and prepared for liquid scintillation counting.

The secretory response was defined as the ratio of Harvesting Period B/Period A ^{3}H-labelled ethanol precipitated radioactivity ([^{3}H]glycoconjugate). Alteration of secretion was noted as a statistically significant change in the ratio of Period B/Period A [^{3}H]glycoconjugate for the treatment groups relative to that of the control group or to each other. An analysis of variance followed by the Scheffe's test for multiple comparisons was applied to all experiments to determine quantitative differences in [^{3}H]glycoconjugate secretion among groups.

Experiments were designed to study the effects of dibutyryl cAMP (sodium salt, Sigma Chemical Co.), dibutyryl cGMP (sodium salt, Sigma), isoprenaline (Sigma) and theophylline (Merck Sharpe and Dohme) on basal respiratory glycoconjugate secretion. In addition these compounds were tested on tissues treated with methacholine chloride, 10 μM (Sigma), or the calcium ionophore A23187 (2-[(3β,9α,11β-trimethyl)-8-(2-pyrrolecarboxymethyl)-1,7-dioxaspiro[6,6]undecyl-2β-methyl]-5-methylaminobenzoxazole-4-carboxylic acid) 10 μM (Calbiochem-Behring Corp.), two agents shown to enhance radiolabelled glycoconjugate secretion from canine tracheal explants (Barbieri et al 1984).

Results

In preliminary experiments, methacholine, 10 μM, stimulated the secretion of ^{3}H-labelled glycoconjugate when incubated with the explants for 10 min; the ratio of Period B/Period A was 0·818 ± 0·011 (mean ± s.e.m.) in 84 untreated control tissues from 13 dogs and 1·090 ± 0·024 in 84 explants exposed to methacholine (an increase of 33%; $P < 0·001$). Using Harvesting Periods of 30 min duration, the B/A ratio was 0·781 ± 0·014 (mean ± s.e.m.) in 30 control explants and 0·995 ± 0·028 in 32 methacholine-treated tissues (change from control 22%; $P < 0·001$). Although a 10 min incubation period was more appropriate for methacholine stimulation than a 30 min incubation, all drugs were tested using both protocols, since the effects of cyclic nucleotides in other secretory systems have been reported to require up to 30 min to become fully expressed (Heisler 1974).

Data from two replicate experiments designed to examine the effects of drugs which would stimulate endogenous cyclic nucleotide activity are summarized in Table 1. At incubations of 10 and 30 min, neither the β-adrenoceptor agonist isoprenaline, 10 μM, nor the phosphodiesterase inhibitor theophylline, 10 mM, had any significant effect on basal glycoconjugate secretion. Furthermore, theophylline, 10 mM, did not alter the secretory response to methacholine challenge.

Table 1. Effect of various drugs on the secretion of [^{3}H]glycoconjugate.

Drug treatment	n[a]	Mean ratio Period B/ Period A ± s.e.m.	Percent change from control
10 min incubations[b]			
Control	12	0·806 ± 0·013	—
Methacholine, 10 μM	12	1·054 ± 0·044[d]	+30·8
Isoprenaline, 10 μM	10	0·828 ± 0·070	+ 2·7
Theophylline, 10 mM	12	0·872 ± 0·030	+ 8·2
Methacholine, 10 μM + Theophylline, 10 mM	12	1·053 ± 0·055[d]	+30·6
Isoprenaline, 10 μM + Theophylline, 10 mM	6	0·871 ± 0·040	+ 8·1
30 min incubations[b]			
Control	8	0·814 ± 0·029	—
Methacholine, 10 μM	10	0·961 ± 0·060[c]	+18·1
Isoprenaline, 10 μM	6	0·858 ± 0·079	+ 5·4
Theophylline, 10 mM	11	0·872 ± 0·036	+ 7·1
Methacholine, 10 μM + Theophylline, 10 mM	10	0·942 ± 0·026	+15·7
Isoprenaline, 10 μM + Theophylline, 10 mM		Not Done	

[a] Number of explants in each treatment group.
[b] Explants were obtained from two dogs (replicate experiments)
[c] Significantly different from control at $P < 0·05$.
[d] Significantly different from control at $P < 0·01$.

The direct activity of the dibutyryl derivatives of cAMP and cGMP was examined on basal (Table 2) and methacholine-stimulated (Table 3) tracheal explants. At 1 mM, neither cyclic nucleotide had any significant effect on basal or methacholine-induced [^{3}H]glycoconjugate secretion from explants treated for 10 or 30 min. Concentrations of these compounds below 1 mM were also without effect. Furthermore, the addition of theophylline, 10 mM, to the samples challenged with the

Table 2. Effect of dibutyryl cAMP and dibutyryl cGMP on [^{3}H]glycoconjugate secretion.

Drug treatment	n[a]	Mean ratio Period B/ Period A ±s.e.m.	Percent change from control
10 min incubations[b]			
Control	21	0·846 ± 0·018	—
Methacholine, 10 μM	20	1·205 ± 0·054[c]	+42·4
Dibutyryl c AMP, 1 mM	21	0·843 ± 0·020	− 0·4
Dibutyryl c GMP, 1 mM	21	0·803 ± 0·015	− 5·1
30 min incubations[b]			
Control	15	0·797 ± 0·019	—
Methacholine, 10 μM	17	0·974 ± 0·038[c]	+22·2
Dibutyryl c AMP, 1 mM	17	0·856 ± 0·020	+ 7·4
Dibutyryl c GMP, 1 mM	16	0·840 ± 0·019	+ 5·4

[a] Number of explants in each treatment group.
[b] Explants were obtained from three dogs (replicate experiments).
[c] Significantly different from control at $P < 0·01$.

Table 3. Effect of dibutyryl cAMP and dibutyryl cGMP on methacholine-stimulated [^{3}H]glycoconjugate secretion.

Drug treatment	n[a]	Mean ratio Period B/ Period A ± s.e.m.	Percent change from control
10 min incubations[b]			
Control	14	0·836 ± 0·022	—
Methacholine, 10 μM	13	1·096 ± 0·045[d]	+31·1
Methacholine, 10 μM +Dibutyryl cAMP, 1 mM	13	1·066 ± 0·054[d]	+27·5
Methacholine, 10 μM +Dibutyryl cGMP, 1 mM	14	1·033 ± 0·034[d]	+23·6
30 min incubations[c]			
Control	21	0·779 ± 0·018	—
Methacholine, 10 μM	21	0·960 ± 0·032[d]	+23·2
Methacholine, 10 μM +Dibutyryl cAMP, 1 mM	20	0·989 ± 0·032[d]	+27·0
Methacholine, 10 μM +Dibutyryl cGMP, 1 mM	21	0·976 ± 0·037[d]	+25·3

[a] Number of explants in each treatment group.
[b] Explants were obtained from two dogs (replicate experiments).
[c] Explants were obtained from three dogs (replicate experiments).
[d] Significantly different from control at $P < 0·01$.

Table 4. Effect of dibutyryl cAMP and dibutyryl cGMP on A23187-stimulated [^{3}H]glycoconjugate secretion.

Drug treatment	n[a]	Mean ratio Period B/ Period A ± s.e.m.	Percent change from control
10 min incubations[b]			
Control	21	0·846 ± 0·018	—
A23187, 10 μM	20	2·555 ± 0·360[d]	+202·0
A23187, 10 μM +Dibutyryl c AMP, 1 mM	21	2·454 ± 0·283[d]	+190·1
A23187, 10 μM +Dibutyryl c GMP, 1 mM	20	2·519 ± 0·318[d]	+197·8
30 min incubations[c]			
Control	7	0·778 ± 0·024	—
A23187, 10 μM	7	1·932 ± 0·224[d]	+148·3
A23187, 10 μM +Dibutyryl c AMP, 1 mM	7	1·896 ± 0·173[d]	+143·7
A23187, 10 μM +Dibutyryl c GMP, 1 mM	7	1·884 ± 0·187[d]	+142·2

[a] Number of explants in each treatment group.
[b] Explants were obtained from three dogs (replicate experiments).
[c] Explants were obtained from one dog.
[d] Significantly different from control at $P < 0·01$.

combination of methacholine and cyclic nucleotides did not alter the previously observed secretory responses.

Finally, the influence of dibutyryl cAMP and dibutyryl cGMP on secretion stimulated by the calcium ionophore A23187 was examined. In these experiments A23187, 10 μM, elicited a 200% and 145% increase in secretion of [^{3}H]glycoconjugate ($P < 0·01$) at 10 and 30 min, respectively (Table 4). The cyclic nucleotide derivatives did not significantly alter these responses.

Discussion

During the past decade, considerable attention has been directed toward establishing an interaction between calcium and cyclic nucleotides in stimulus-secretion coupling. Goldberg et al (1974) introduced the terms monodirectional and bidirectional to describe the control of cellular function. According to these authors, monodirectional control involves the mediation of cellular function by a single stimulant, i.e., when the stimulant is present, the cell is activated to elicit its characteristic response and when the stimulant is absent the cell remains inactive. Bidirectional control was proposed as involving two stimulants with opposing actions which integrate, in a dynamic way, the control of cellular function. Berridge (1975) proposed that calcium and cAMP are the primary mediators of cellular activity and viewed monodirectional cells as being provoked into activity by the action of single stimulant, with Ca^{2+} serving as the second messenger. Secondary stimulants, if involved, were proposed as complementing the activity of the primary stimulant via a mechanism in which cAMP extended the activity resulting from the rise in cellular Ca^{2+} provoked by the primary stimulant. For bidirectional cells, Ca^{2+} was proposed as mediating the stimulatory effect of the primary stimulant with the secondary stimulant serving to lessen the magnitude of the calcium signal via cAMP-mediated mechanisms (Berridge 1975).

The studies of Coles et al (1982) and Barbieri et al (1984) clearly establish a role for intracellular calcium in mediating cholinergic stimulation of mucus secretion from the canine trachea. In the present investigation, the absence of any noticeable effects of isoprenaline, theophylline, and the dibutyryl cyclic nucleotide derivatives on basal, methacholine- or A23187-induced secretion of [^{3}H]glycoconjugate from canine tracheal explants strongly suggests that the secretory cells of this tissue are monodirectionally controlled, i.e., cholinoceptor activation serves as the primary stimulant with intracellular calcium mediating the effect. This concept is in agreement with the anatomical demonstration of cholinergic innervation to the canine tracheal submucosal glands but the absence of any adrenergic innervation (Wardell et al 1970). The difference in the present data and the observed stimulant effects of cAMP in the cat (Liedtke et al 1982) and cGMP in man (Shelhamer et al 1980) is probably due to species variability. Our observations do not preclude the existence of other physiological mediators, e.g., prostanoids, purines, peptides or electrolytes, and the actions of these putative physiologic agonists on tracheal mucus secretion should be studied.

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Induction of drug metabolizing enzymes in the liver of rats infested with *Fasciola hepatica*

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Adult, male rats were infected with 20 metacercariae of *Fasciola hepatica* given orally, other rats were left untreated. Five weeks after infestation, some animals received phenobarbitone, 3-methylcholanthrene, β-naphthoflavone or Arochlor 1254, to induce liver drug metabolizing enzymes. Fascioliasis provoked decreases in aminopyrine *N*-demethylase, aniline hydroxylase, the mutagenic activity of cyclophosphamide and cytochrome P-450 concentration in untreated or phenobarbitone or Arochlor pretreated rats. In contrast, cytochrome b5, NADPH cytochrome c reductase, ethyoxycoumarin *O*-deethylase and the enzymatic activation of ethidium bromide were not affected by fascioliasis whatever pretreatment was given. Fascioliasis decreased liver drug metabolizing enzymes which were specifically induced by both phenobarbitone and Arochlor, this could be due to either the specific action of toxic excretions of flukes or to the particular localization of tissue damage within the liver lobule.

Hepatic microsomal cytochrome P-450 concentration and drug metabolizing enzymes fell 3 to 8 weeks after rats had been given orally 20 metacercariae of *Fasciola hepatica* (Galtier et al 1983).

The existence of several isozymes of rat liver cytochrome P-450 differing in substrate selectivity and immunological and physicochemical properties is now well established, the forms of cytochrome P-450 induced by phenobarbitone being shown not to be identical with those induced by 3-methylcholanthrene or β-naphthoflavone (Lu 1979).

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We have investigated the effects on fluke infected rats of phenobarbitone, Arochlor 1254 (ARO), 3-methylcholanthrene or β-naphthoflavone with the aims of determining the inducibility of mixed function oxidases compared with the response in normal, uninfected rats and of characterizing the microsomal cytochrome P-450 species which are particularly inhibited five weeks after infection.

Materials and methods

Phenobarbitone, 3-methylcholanthrene (Serva, Heidelberg, GFR), Arochlor 1254 (Alltech, Eke, Belgium), β-naphthoflavone (Ega-Chemie, Steinheim, GFR), ethoxycoumarin (Boehringer-Manneim, Meylan, France), ethidium bromide and cyclophosphamide (Sigma, St Louis, USA) were used as received.

Treatment of animals. Male, Sprague Dawley rats, about 150 g, were randomly distributed into control or infected groups of 8 rats housed in cages of 4 animals. Food (UAR alimentation, Villemoisson, France) and drinking water were freely available. Each rat to be infected received by gastric tube 20 metacercariae of *Fasciola hepatica* suspended in a 1% polysorbate aqueous solution. The development of the condition was observed clinically and by means of biochemical studies of blood samples. Parallel studies were made on uninfected controls, which received no treatment, since