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The calcium dependency of mucus glycoconjugate secretion by canine tracheal explants

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1 Canine tracheal explants, incubated overnight with [^{14}C]-glucosamine, elicited an enhanced secretion of ethanol-precipitated ^{14}C-labelled glycoconjugate when challenged with methacholine, 10 μM.

2 Explants were rendered deficient in total calcium content and unresponsive to methacholine, 10 μM, by incubating them in calcium-free medium for 18 to 22 h; however, the secretory response to the cholinergic agonist was restored with the addition of calcium to the medium.

3 A dose-response relationship resulted when explants were challenged with methacholine in nutrient medium containing varied calcium concentrations (0.45 to 7.2 mM); alterations in the calcium concentration in the absence of methacholine had no significant effect on the basal secretion of ^{14}C-labelled glycoconjugate.

4 The calcium-selective ionophore A23187, 10 μM, stimulated [^{14}C]-glycoconjugate secretion and induced the most significant effect in the presence of nutrient medium containing calcium.

5 Verapamil, 10 μM, a calcium-entry blocker failed to inhibit basal or stimulated [^{14}C]-glycoconjugate secretion; however, the intracellular calcium antagonist TMB-8, 10 to 100 μM, inhibited methacholine-induced [^{14}C]-glycoconjugate secretion in a dose-dependent manner.

6 These data suggest that respiratory mucus secretion is a calcium-dependent process and that intracellular calcium is more vital than extracellular calcium in supporting this phenomenon.

Introduction

Since the proposal by Douglas & Rubin (1961a, b) that a rise in intracellular free ionized calcium (Ca^{2+}) functions as an important trigger for stimulus-secretion coupling, extensive research has been directed towards understanding the complexities of cellular secretion. Evaluation of this hypothesis using secretory tissues of diverse origin, has documented that Ca^{2+} mediates secretion from nearly every tissue examined. Secretion of macromolecules by various exocrine glands including: (a) digestive enzyme secretion by the exocrine pancreas, (b) glycoprotein and enzyme secretion by the salivary glands, and (c) prealbumin secretion by the lacrimal glands, has been shown to be mediated through Ca^{2+} (Rubin, 1982).

The involvement of Ca^{2+} in the secretion of macromolecular components of mucus from secretory cells of the respiratory tract has received relatively little attention in comparison with that of the other aforementioned exocrine systems. Bogart *et al.* (1977) and Conover & Conod (1978) showed an association between Ca^{2+} and respiratory mucus secretion from isolated rabbit trachea and Balfre (1978) indicated that Ca^{2+} may play a role in the release of mucus from hen trachea. More recently Coles *et al.* (1982) presented data which suggested that Ca^{2+} is required for cholinergically-stimulated, but not basal discharge of, radiolabelled mucus glycoconjugate secretion from canine tracheal explants. Furthermore, these authors proposed that intracellular Ca^{2+} may be more important for this process than entry of extracellular Ca^{2+}. With the exception of these few publications, we are unaware of any other published studies which describe the involvement of Ca^{2+} in respiratory mucus secretion.

By manipulating the Ca^{2+} concentrations presented to respiratory tissues and through the use of two Ca^{2+}-antagonists, verapamil, a calcium-entry blocker (Fleckenstein, 1977), and 8-(N, N-diethylamino)-octyl 3, 4, 5-trimethoxybenzoate hydrochloride (TMB-8), an intracellular Ca^{2+}-antagonist (Chiou & Malagodi, 1975), the involvement of Ca^{2+} in canine tracheal secretions has been

examined more completely. It was the goal of the present study to demonstrate conclusively that intracellular Ca^{2+} is more vital than extracellular Ca^{2+} in supporting respiratory mucus secretion.

Methods

Assay of respiratory secretion

The method of Coles & Reid (1981) as modified by Barsigian & Barbieri (1982) was employed for the quantitation of respiratory mucus secretion from canine tracheal explants. Briefly, adult mongrel dogs of either sex, weighing 8 to 13 kg, were anaesthetized by an intravenous injection of pentobarbitone sodium solution, 35 mg kg^{-1}. The trachea was removed under aseptic conditions and immediately placed into sterile, ice cold, Ca^{2+}-free Medium 199 with Earle's salts (Gibco Laboratories, Grand Island, NY). Normal Medium 199 contains 1.8 mM Ca^{2+}; therefore, the use of Ca^{2+}-free Medium 199 allowed the Ca^{2+} concentration to be varied freely. After removing and discarding extraneous tissues, i.e., blood vessels, nerves and fascia, and the membranous portion of the trachea, the tracheal rings were separated and sectioned into full thickness pieces of approximately one-quarter ring in size. Tissues were placed in tared, sterile 25 ml Erlenmyer flasks containing 2 ml of sterile Ca^{2+}-free Medium 199 and the flasks were reweighed; adjustments were made so that each flask contained 100 ± 10 mg of tissue (blotted wet weight).

Following a 2 h wash period, the medium was removed from each flask and replaced with 2 ml of fresh normal or Ca^{2+}-free Medium 199 (depending on the experimental protocol) containing 1.15 μCi ml^{-1} D-[1-^{14}C]-glucosamine hydrochloride. The tissues were incubated under 95% O_2 plus 5% CO_2 at 37°C in a metabolic shaker for 18 to 22 h. Following this period of radiochemical labelling, the medium was aspirated from each flask, discarded and replaced with 2 ml of fresh (non-radioactive) Medium 199. Two sequential 10 min incubations (harvesting periods A and B) were then performed. Samples which served as controls were incubated in Medium 199 alone (Ca^{2+}-free or with Ca^{2+} added, depending on the nature of the experiment) in both periods; drug-treated samples were generally incubated alone during period A and in medium plus drug during period B. In 5 separate experiments (285 explants examined), the total release of ^{14}C-labelled ethanol precipitated radioactivity ([^{14}C]-glycoconjugate) during period A ranged from 1187 to 12644 d.p.m. per 100 mg of tissue, with a mean ± s.e.mean of 4293 ± 264 d.p.m. per 100 mg. To minimize this inherent variability for various experimental comparisons, the data were normalized and the secretory response was defined as the ratio of harvesting period B/period A ^{14}C-labelled ethanol precipitated radioactivity ([^{14}C]-glycoconjugate) (Chakrin *et al.*, 1973). Stimulation of secretion was noted as a statistically significant increase in the ratio of period B/period A [^{14}C]-glycoconjugate for the treatment groups relative to that of the control group. This method of analysis was advantageous since each sample served as its own internal control; variations due to differences in tissue weights and the absolute amount of mucus secreted by each sample were reduced. In one study (Table 3) drugs were added to treatment samples during both periods A and B and secretory responses were compared as d.p.m. per 100 mg of tissue.

The harvesting medium was mixed with absolute ethanol and allowed to react overnight at 4°C. Radiolabelled glycoconjugate was separated from the free [^{14}C]-glucosamine by centrifugation at 3,000 *g* for 20 min and washed 3 times in 75% (v/v) ethanol. The washed precipitates were suspended in 0.5 M sodium hydroxide solution at room temperature for 18 h before preparation for liquid scintillation counting. All counting data were corrected for quenching according to the Channels-Ratio method (Bush, 1963) and converted to d.p.m. An analysis of variance was used to determine quantitative differences in [^{14}C]-glycoconjugate secretion among groups.

Experiments were designed to study the effects of methacholine chloride (Sigma Chemical Co.), ionic calcium (as the chloride, Baker Chemical Co.), and the ionophore A23187 (Calbiochem-Behring Corp.) on respiratory glycoconjugate secretion. Atropine sulphate (Sigma Chemical Co.) was used as a representative muscarinic receptor blocking agent. Verapamil hydrochloride (Knoll Pharmaceutical Co.) and TMB-8 (Aldrich Chemical Co.) were employed as representative calcium-channel and intracellular calcium antagonists, respectively. These agents were added to the incubating tissues 50 min before harvesting and during harvesting periods A and B.

Solutions of methacholine, calcium, atropine, verapamil and TMB-8 in Medium 199 were prepared fresh on the morning of use. A23187 was prepared as a stock solution, 0.5 mg ml^{-1}, in absolute ethanol and stored in the dark at −20°C; an aliquot of the stock solution was diluted with Medium 199 immediately before use. Whenever A23187 was employed, appropriate ethanol-containing (1% v/v) control medium was also used.

Assay for calcium in tracheal explants

Tracheal explants were prepared as previously described and incubated in either normal or Ca^{2+}-free Medium 199 without [^{14}C]-glucosamine under 95%

O_2 plus 5% CO_2 at 37°C in a metabolic shaker for 18 h. Each explant was removed from the medium, rinsed for 10 s in deionized water and assayed for total Ca^{2+} content by the method of Ferko & Bobyock (1980) using atomic absorption spectrophotometry. All samples were read in duplicate, while standard Ca^{2+} solutions were read in triplicate.

Results

Initial experiments established that when canine tracheal explants were incubated in normal Medium 199 (labelling and harvesting periods A and B) the ratio of period B d.p.m./period A d.p.m. was 0.691 ± 0.021 (mean ± s.e.mean) in 38 untreated control tissues from 5 dogs and 0.984 ± 0.096 in 22 explants treated with methacholine, 10 μM (an increase of 42%, $P < 0.01$). The effect of Ca^{2+} on [^{14}C]-glycoconjugate secretion was initially studied by altering the Ca^{2+} concentrations during the labelling and harvesting periods. The responses of control explants incubated in Medium 199 with 0.45 to 3.6 mM Ca^{2+} were not significantly different from each other, and the responses of methacholine-treated tissues were all essentially the same. Moreover, tissue responses in these experiments were not different from those reported above. Only when the tissues were incubated in Ca^{2+}-free Medium 199 during the labelling and harvesting periods could significant changes in the secretory response be observed.

Table 1 shows the combined data from two replicate experiments in which explants were labelled and harvested in either normal (1.8 mM Ca^{2+}) or Ca^{2+}-free Medium 199. The first group of tissues in which Ca^{2+} was present during all incubations, elicited responses comparable to previous studies: control ratio B/A: 0.688 ± 0.021; methacholine-treated ratio B/A: 0.922 ± 0.040; change from control: 34%. After labelling in normal Medium 199 and then harvesting in Ca^{2+}-free medium, the tissue responses were not significantly different from the previous group. Likewise, if the explants were labelled in Ca^{2+}-free medium and Ca^{2+} (1.8 mM) was included in the incubation medium during periods A and B, the tissue responses were again similar. However, when Ca^{2+} was absent from the medium during both the labelling period and the harvesting periods, the stimulatory response to methacholine challenge was abolished; in addition, it was observed that the ratio of period B to A radioactivity in the control tissues of this set was significantly increased over that of other control groups. Further, more [^{14}C]-glycoconjugate was elaborated from the tissues incubated completely in Ca^{2+}-free medium during period A (2,966 ± 166 d.p.m. per 100 mg of tissue) than from tissues incubated with Ca^{2+} (2,403 ± 138 d.p.m. per 100 mg of tissue; means ± s.e.means, $P < 0.05$).

Based on these results, several experiments were performed in which all tissues were incubated during labelling in Ca^{2+}-free Medium 199. Since the secretory response to methacholine challenge could be observed by the addition of Ca^{2+} during the harvesting periods, the use of the Ca^{2+}-free medium during labelling permitted the Ca^{2+} concentrations to be varied at will during periods A and B. Figure 1 shows that the effect of methacholine, 10 μM, in promoting [^{14}C]-glycoconjugate secretion was directly related to the Ca^{2+} concentration (0.45 to 7.2 mM) included

Table 1 Effect of Ca^{2+} on control and methacholine-treated tracheal explants

Calcium chloride (1.8 mM) added during		*Treatment*[a]		*%*
Labelling period	*Harvest periods A & B*	*during harvest period B*	*^{14}C-macromolecular ratio period B/period A*[b]	*change from respective control*
Yes	Yes	None	0.688 ± 0.021 (14)	—
		Methacholine	0.922 ± 0.040 (15)	33.9[c]
Yes	No	None	0.692 ± 0.038 (16)	—
		Methacholine	0.880 ± 0.046 (16)	27.2[c]
No	Yes	None	0.713 ± 0.038 (17)	—
		Methacholine	0.928 ± 0.041 (16)	30.2[c]
No	No	None	0.827 ± 0.036[d] (15)	—
		Methacholine	0.851 ± 0.048 (16)	2.9

[a] No treatment (control) or methacholine, 10 μM.
[b] Values are means ± s.e.mean of the number of explants tested in parentheses.
[c] Significantly different from respective control, $P < 0.005$.
[d] Significantly different from all other controls, $P < 0.05$.

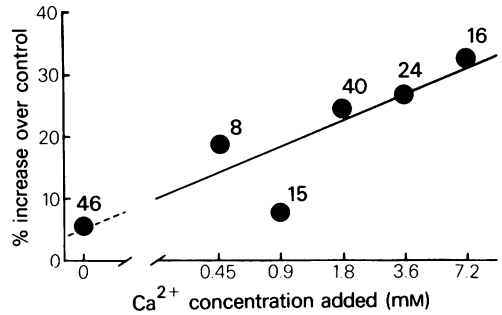

Figure 1 Relationship of Ca^{2+} concentration and the response to methacholine. Each point represents the mean % increase over untreated controls for methacholine-treated tissues at the respective Ca^{2+} concentration; numbers indicate the number of explants tested at each Ca^{2+} concentration. Correlation coefficient for the regression line: 0.722; significantly different from 0, $P < 0.01$.

in the medium during the harvesting periods. These Ca^{2+} concentrations had no observable effect on the secretory response of untreated control tissues. Therefore, a dose-response relationship was established for Ca^{2+} in the presence of methacholine. Higher Ca^{2+} concentrations could not be employed since a precipitate formed in the incubation medium at Ca^{2+} concentrations above 10 mM.

The inhibitory effect on methacholine-induced secretion by labelling and harvesting in Ca^{2+}-free medium may have been due to either depletion of cellular Ca^{2+} required for cholinomimetic-stimulated [^{14}C]-glycoconjugate secretion or non-specific cytotoxicity. Using tissues that were incubated for 18 h in either Ca^{2+}-containing or Ca^{2+}-free Medium 199, it was confirmed that the total explant Ca^{2+} content was significantly reduced (84%) in those tissues incubated in Ca^{2+}-free medium (Table 2). Additionally, in a set of tracheal explants prepared from the same animal and assayed for glycoconjugate secretion, as before methacholine stimulated secretion only in the presence of Ca^{2+} and the basal release of [^{14}C]-glycoconjugate in Ca^{2+}-free medium was significantly greater than that of the Ca^{2+}-containing group.

In further experiments, tissues which failed to respond to methacholine due to incubation in Ca^{2+}-free medium during labelling and harvesting, were made to respond to the secretagogue by addition of Ca^{2+} to the harvesting medium. Table 3 shows data from one representative experiment; six groups of tissues were labelled in Ca^{2+}-free Medium 199 and then treated as either controls or with methacholine, 10 μM. Only the methacholine plus Ca^{2+} treated groups showed a significant stimulation of [^{14}C]-glycoconjugate secretion over its respective control (55.5% increase in period A; 36.9% increase in period B). As noted in previous experiments the inclusion of Ca^{2+} during either period A or B to untreated control tissues significantly reduced the amount of precipitated [^{14}C]-glycoconjugate when compared to the [^{14}C]-glycoconjugate released by control tissues incubated without Ca^{2+}. These data clearly show that the apparent increase in secretion from control tissues incubated in Ca^{2+}-free medium and the failure of methacholine to effect a significant stimulatory response in the absence of Ca^{2+} were not due to a nonspecific cytotoxic action caused by incubation in Ca^{2+}-free medium for over 18 hours.

Douglas (1978) stated that Ca^{2+} was capable of stimulating secretion if the cellular membrane had been rendered leaky to it by prolonged exposure to a Ca^{2+}-free medium. We used the Ca^{2+}-selective

Table 2 Effect of Ca^{2+}-free incubation on total explant Ca^{2+} content and secretory responses

	Labelling medium			
	Calcium-containing (1.8 mM)[a]		*Calcium-free*[a]	
Total explant Ca^{2+} (nmol mg^{-1})	6.28 ± 0.29 ($n = 6$)		1.01 ± 0.06[b] ($n = 5$)	
% change	—		−83.9	
	Drug treatment[c]			
	None	*Methacholine*	*None*	*Methacholine*
Secretory ratio: period B/period A	0.629 ± 0.029 ($n = 9$)	0.897 ± 0.065[d] ($n = 9$)	0.744 ± 0.031 ($n = 8$)	0.753 ± 0.049 ($n = 8$)
% change	—	+42.9	—	+1.2

[a] Values represent the mean ± s.e.mean
[b] Significantly different from Ca^{2+}-containing controls, $P < 0.001$
[c] No treatment (control) or methacholine, 10 μM
[d] Significantly different from control, $P < 0.01$

Table 3 Effect of Ca^{2+} on control and methacholine-treated tracheal explants

Calcium chloride (3.6 mM) added during harvest period		*Treatment*[a] *during harvest*	*^{14}C-macromolecular radioactivity during harvest period*[b]	
A	*B*	*periods A and B*	*A*	*B*
Yes	Yes	None	9559 ± 834[c]	8005 ± 871[d]
		Methacholine	14863 ± 1269[e]	10501 ± 657[f]
No	Yes	None	14473 ± 1247	10645 ± 1255[d]
		Methacholine	16265 ± 1168	14572 ± 1250[f]
No	No	None	14384 ± 1228	17207 ± 1364
		Methacholine	14596 ± 987	15737 ± 1058

[a] No treatment (control) or methacholine, 10 μM
[b] Values are mean d.p.m. per 100 mg tissue ± s.e.mean for 8 explants per group.
[c] Significantly different from Ca^{2+}-free controls, $P < 0.005$.
[d] Significantly different from Ca^{2+}-free controls, $P < 0.001$.
[e] Significantly different from the respective control, $P < 0.001$.
[f] Significantly different from the respective control, $P < 0.01$.

ionophore A23187 to enhance Ca^{2+} transport through membranes of the secretory cells. Figure 2 shows that in the absence of Ca^{2+}, A23187 (10 μM) had a small effect on mucus secretion and methacholine (10 μM) was without effect. However, both A23187 and methacholine in media containing Ca^{2+} significantly stimulated the release of [^{14}C]-glycoconjugate. Atropine (10 μM) which completely blocked the stimulatory effect of methacholine, failed to affect the stimulation of mucus secretion produced by A23187.

These results imply that intracellular stores of Ca^{2+} may constitute the primary Ca^{2+} fraction responsible for canine tracheal mucus secretion. In order to substantiate this, studies were performed using two calcium antagonists, verapamil, a calcium-entry blocker, and TMB-8, an intracellular Ca^{2+} antagonist. Table 4 shows that canine tracheal explants labelled in Ca^{2+}-free medium and harvested with Ca^{2+} during periods A and B were stimulated by methacholine (10 μM), A23187 (10 μM), and potassium chloride (100 mM) (increase over untreated control: 55.4%, 145.4% and 40.9%, respectively). Verapamil, 10 μM, had no significant effect on basal

Table 4 Effect of verapamil on basal and stimulated release of ^{14}C-glycoconjugate

Drug treatment	N[A]	*Mean ratio period B/period A ± s.e.mean*	*% change from respective control*
Untreated control	18	0.687 ± 0.023	—
Verapamil, 10 μM	18	0.714 ± 0.014	+3.9
Methacholine, 10 μM	17	1.068 ± 0.063[B]	—
Methacholine, 10 μM + Verapamil, 10 μM	18	0.929 ± 0.052[E]	−13.1
A23187, 10 μM		1.686 ± 0.140[B]	—
A23187, 10 μM + Verapamil, 10 μM		1.818 ± 0.113[D]	+7.9
KCl, 100 mM	16	0.968 ± 0.033[C]	—
KCl, 100 mM + Verapamil, 10 μM	17	0.890 ± 0.044[E]	−8.1

[A] Number of explants tested in each group. Explants were obtained from 2 dogs (replicate experiments).
[B] Significantly different from untreated control, $P < 0.01$
[C] Significantly different from untreated control, $P < 0.05$
[D] Significantly different from verapamil-treated control, $P < 0.01$
[E] Significantly different from verapamil-treated control, $P < 0.05$

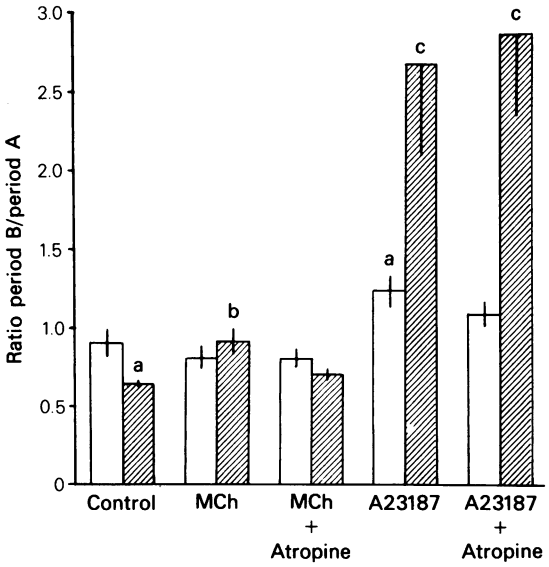

Figure 2 Effect of Ca^{2+}-containing and Ca^{2+}-free medium on the stimulation of [^{14}C]-glycoconjugate secretion elicited by methacholine (MCh), 10 μM, and A23187, 10 μM. Each column represents the mean ± s.e.mean ratio (vertical bars) of period B/period A ^{14}C-macromolecular radioactivity; eight tissues per group. Open columns represent incubation in Ca^{2+}-free Medium 199; shaded columns represent incubation in Medium 199 with 3.6 mM CaCl. [a]Significantly different from Ca^{2+}-free control, $P<0.05$. [b]Significantly different from Ca^{2+}-containing control, $P<0.01$. [c]Significantly different from Ca^{2+}-containing control, $P<0.001$.

or stimulated [^{14}C]-glycoconjugate release. Similar results were obtained in tissues labelled overnight in normal Medium 199.

TMB-8 (10 to 100 μM) inhibited, in a dose-dependent fashion, methacholine-induced [^{14}C]-glycoconjugate secretion from explants labelled with Ca^{2+}, but harvested in Ca^{2+}-free medium (Table 5). Basal responses were not significantly affected by the compound at 10 and 30 μM. However, TMB-8, 100 μM significantly increased basal [^{14}C]-glycoconjugate release.

Discussion

The data clearly demonstrate that Ca^{2+} is required for the secretion of respiratory mucus from cholinergically-stimulated canine tracheal tissues. Methacholine elicited a consistent and significant secretion of ^{14}C-labelled glycoconjugate from tracheal explants as long as Ca^{2+} was present in the incubation medium during either labelling or harvesting (Table 1) and the magnitude of the secretory response was similar in all groups of tissues, i.e., approximately 30% above control. Furthermore, it appears that the source of Ca^{2+} need not be extracellular since methacholine stimulated secretion in Ca^{2+}-free medium following labelling in medium containing Ca^{2+}. These data are in agreement with those presented by Coles *et al.* (1982) who, using histological and autoradiographical techniques, showed that secretion of glycoconjugate was stimulated by methacholine in both Ca^{2+}-containing and Ca^{2+}-free EGTA-containing medium, but not when

Table 5 Effect of TMB-8 on methacholine-induced tracheal explant secretory responses

Drug treatment	N[A]	*Mean ratio period B/period A ± s.e.mean*	*% change from respective control*
None (control)	18	0.629 ± 0.025	—
Methacholine, 10 μM	17	0.832 ± 0.038[B]	32.3
TMB-8, 10 μM	18	0.652 ± 0.021	—
Methacholine, 10 μM + TMB-8, 10 μM	18	0.790 ± 0.019[C]	21.2
TMB-8, 30 μM	18	0.689 ± 0.023	—
Methacholine, 10 μM + TMB-8, 30 μM	18	0.783 ± 0.032	13.6
TMB-8, 100 μM	16	0.746 ± 0.023[B]	—
Methacholine, 10 μM + TMB-8, 100 μM	18	0.820 ± 0.035	9.9

[A]Number of explants in each group. Explants were obtained from 2 dogs (replicate experiments).
[B]Significantly different from control, $P<0.05$.
[C]Significantly different from TMB-8 (10 μM), $P<0.05$.

the trachea first underwent a preincubation wash in Ca^{2+}-free EGTA-containing medium before being stimulated with methacholine under Ca^{2+}-free conditions. These authors suggested that intracellular calcium stores are able to support short periods of methacholine-induced secretion, but that when tissues are exposed to Ca^{2+}-depleting conditions, the tissues may become more dependent on extracellular Ca^{2+}.

In agreement with this hypothesis, we showed that overnight labelling in Ca^{2+}-free Medium 199 reduced the total tracheal explant Ca^{2+} content by 80% (Table 2) and rendered methacholine-induced secretion of [^{14}C]-glycoconjugate dependent upon the media Ca^{2+} concentration used during harvesting (Figure 1). Furthermore, reintroduction of Ca^{2+} into the medium of tissues previously harvested in Ca^{2+}-free Medium 199 resulted in a significant stimulatory response to the cholinomimetic agonist (Table 3). These data strongly imply that Ca^{2+} is the mediator responsible for methacholine-induced [^{14}C]-glycoconjugate secretion from the canine trachea.

In order to demonstrate more clearly that Ca^{2+} was the primary messenger involved in respiratory mucus secretion, A23187 was employed to enhance Ca^{2+} transport into the secretory tissue. In the absence of methacholine, A23187 caused a substantial release of [^{14}C]-glycoconjugate in Ca^{2+}-containing medium, an effect observed previously. Bogart *et al.* (1977) showed subjectively that A23187, 2 mM, promoted mucus secretion from rabbit tracheal explants exposed to the compound in a Ca^{2+}-rich solution; calcium chelation with EGTA abolished the ionophore-induced response. Conover & Conod (1978) measured the release of mucus-like insoluble material from rabbit trachea stimulated by A23187, 20 μM to 20 mM. Results of the present investigation agree with these findings except that the concentrations of A23187 used in their studies were quite high and the responses modest in magnitude. Our results also agree with those of Balfre (1978) who introduced A23187, 2 μM, for 15 min into the lumen of hen trachea and caused an increase in mucin secretion ($51 \pm 12\%$).

The ability of A23187 to elicit a small response in Ca^{2+}-free medium suggests that factors other than Ca^{2+} play a role in the secretion of respiratory mucus. A23187 does not activate muscarinic receptors (Figure 2, absence of effect of atropine), and is not completely selective for Ca^{2+}; other cations have been shown to be transported through membranes by this ionophore (Pfeiffer & Lardy, 1976) which may have influenced mucus secretion. Alternatively, ionophore-induced [^{14}C]-glycoconjugate secretion may have been the result of Ca^{2+} release from intracellular storage sites, e.g., the endoplasmic reticulum, irrespective of the extracellular conditions, as has been demonstrated in other systems (Pressman, 1976). The variability in the secretory response to A23187 in the present and the aforementioned studies may be the result of species differences or differences in experimental methods.

It is interesting that incubation of explants in Ca^{2+}-free Medium 199 during both the labelling and harvesting periods resulted in greater release of [^{14}C]-glycoconjugate than when Ca^{2+} was included during some phase of the assay (as seen in the greater ratio of B/A in the Ca^{2+}-free controls of Table 1 and Figure 2, and the greater ^{14}C-macromolecular radioactivity per 100 mg of Ca^{2+}-free control tissues, Table 3). Balfre (1978) observed a similar effect on isolated hen trachea; a large increase in mucin release, concurrent with an increased potential difference across the tracheal wall, was observed when tissues were incubated in a Ca^{2+}-free bathing fluid. Enhanced cellular permeability to the macromolecular components of respiratory mucus as a result of membrane-bound Ca^{2+} loss may have been responsible. This phenomenon is well documented (see Rubin, 1982) and needs further investigation with respect to tracheal secretion.

As previously mentioned, the results strongly imply that Ca^{2+} bound to cellular membranes or other intracellular sites and released after activation of muscarinic receptors may constitute the primary fraction responsible for canine glycoconjugate secretion. That verapamil, a calcium-entry blocker had no significant effect on [^{14}C]-glycoconjugate secretion induced by methacholine and potassium chloride (Table 4) and that TMB-8, an intracellular Ca^{2+}-antagonist, inhibited methacholine-induced secretion in a dose-dependent manner strengthens the hypothesis that intracellular Ca^{2+} is more vital than extracellular Ca^{2+} in supporting canine respiratory mucus secretion.

Is there clinical relevance to the finding that respiratory mucus secretion is a calcium-dependent process and that intracellular Ca^{2+} stores may be the key to controlling exocrine secretion? The pathophysiological basis of certain diseases with a respiratory component may be abnormal calcium metabolism at the tissue level. For example, in cystic fibrosis (CF) there is exocrine gland dysfunction, leading to, among other symptoms, the elaboration of viscous mucus.

Bogart *et al.* (1977) showed that A23187 produced a CF-like mucociliary disturbance, i.e., ciliary dyskinesia and mucus secretion, in rabbit tracheal explants. These authors also showed that similar responses could be obtained by treating the explants with human CF serum and could be abolished by calcium chelation with EGTA. The studies of Ansah & Katz (1980) on inverted vesicles from erythrocytes of CF patients suggested a Ca^{2+}-transport deficiency

which might cause Ca^{2+} to accumulate within cells. Since this investigation and others have demonstrated the importance of intracellular Ca^{2+} for mucus secretion, a defect in cellular calcium metabolism as a postulated cause of CF should receive further attention. Studies in this laboratory are continuing in this regard.

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