INHIBITION BY COLCHICINE OF CARBAMYLCHOLINE INDUCED GLYCOPROTEIN SECRETION BY THE SUBMAXILLARY GLAND. A POSSIBLE MECHANISM OF CHOLINERGIC INDUCED PROTEIN SECRETION.

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1. Introduction

During recent years, our knowledge about protein secretion by exocrine glands and the pathway of intracellular transport of these proteins increased considerably, thanks, chiefly to studies on the parotid [1] and the pancreas [2–7]. These proteins are synthesized on ribosomes attached to the membrane of the rough endoplasmic reticulum (RER), segregated within the cisternae of the RER, then transferred via the Golgi apparatus and packaged into condensing vacuoles, which are transformed into zymogen granules. Finally the proteins are discharged from these granules into the acinar lumina. Palade's studies indicated 2 energy requiring locks in the course of this intracellular transport: the first lock connects the cisternae of RER and the condensing vacuoles whereas the second lock concerns the discharge of zymogen granules. The latter process is regulated by cholinergic agonists [6] in the pancreas. However, overall mucin secretion by submaxillary glands is induced by cholinergic *and* adrenergic transmitters (mainly norepinephrine). These stimulations are calcium dependent [8], this cation plays an active role in general secretory mechanisms : Douglas and Rubin [9] showed that the acetylcholine induced secretion of catecholamines, in the adrenal medulla, was also Ca^{2+} dependent. After a period of perfusion with Ca^{2+} free medium, when Ca^{2+} was added to the perfusate itself, a discharge of catecholamines was evoked.

Moreover, microtubules seem to be involved in secretory mechanisms as reported by Lacy et al. [10]. In this latter work, insulin secretion in response to glucose is inhibited by colchicine which has been shown to bind to microtubule protein subunits [11, 12]. Calcium might be acting on the equilibrium between 2 microtubular forms [13].

In the present experiments we have studied the mucin secretion of rat submaxillary glands. As rat mucin contains 50% of sugars [14], it is therefore easy to follow this secretion by labeling the macromolecule with ^{14}C-glucose. Stimulation of mucin secretion is obtained by carbamylcholine. This effect is suppressed by colchicine. Glycogen degradation observed in the presence of the cholinergic agonist is inhibited by tetracaïne.

A possible mechanism of cholinergic agonist induced secretion is proposed.

2. Materials and methods

Uniformly labeled ^{14}C-glucose (150 mCi/mmole) was obtained from CEA, Saclay (France). Carbamylcholine was purchased from Mann Research, New York (USA) and colchicine from Calibiochem, Los Angeles (USA). Tetracaïne, Codex (France), phentolamine (α-blocker) and propranolol (β-blocker) from CIBA (Switzerland).

Male albino rats (6–8 wk, 150–200 g) were killed by decapitation. The submaxillary glands were immediately removed and cut into small pieces (2–3 mm in size).

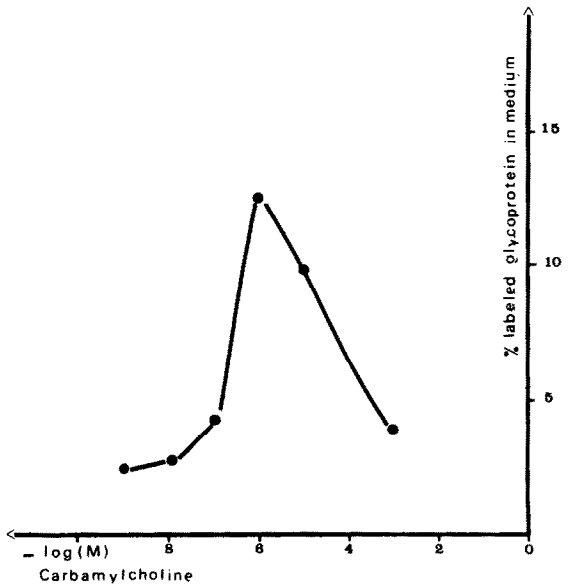

Fig. 1. Percentage of glycoproteins secretion from submaxillary glands as a function of carbamylcholine concentrations. Incubation time : 90 min.

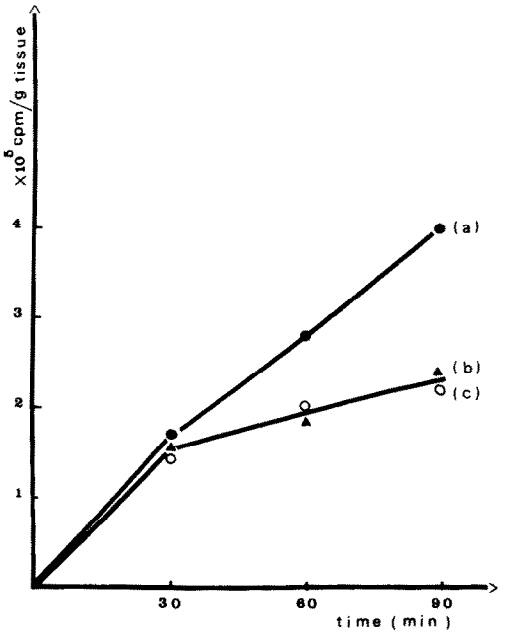

Fig. 2. TCA soluble fraction of the submaxillary glands. Time course labeling during incubation. (a) : Control; (b) : 10^{-6} M carbamylcholine; (c) 10^{-6} M carbamylcholine + 10^{-5} M colchicine.

2.1. *Incubation procedures*

The incubations were performed in a Warburg incubator. Every incubation vessel contained 150–200 mg glands in 7 ml of Krebs–Ringer bicarbonate buffer at 37°, equilibrated with 95% O_2, 5% CO_2 gas and containing 0.55 mM glucose.

Incubations were performed according to 2 procedures : 1) Secretion was investigated as the active protein synthesis and intracellular transport processes take place. The submaxillary glands were incubated with ^{14}C-glucose for different times ranging from 30–90 min. Carbamylcholine, colchicine, tetracaïne and α- and β-blockers when present were added at the onset of the incubation period. 2) When glycoprotein discharge was investigated, glands were incubated during a 1 hr pulse with ^{14}C-glucose, washed with Krebs–Ringer medium and incubated again in a ^{14}C-glucose free medium during various times. In this case carbamylcholine and colchicine, when present, were added at the onset of the second incubation period.

2.2. *Fractionation*

After incubation the gland fragments were isolated by centrifugation, dried and weighed. They were homogenized with an Ultraturax homogenizer in 5 ml cold water. The homogenate was centrifuged at 50,000 *g* for 30 min. The pellet was discarded. The glycoproteins of the supernatant and the incubation medium were precipitated with 20% trichloracetic acid (TCA), 0.1% phosphotungstic acid. The precipitate was decontaminated according to the procedure previously described [15]. The radioactivity of the samples was counted on a Nuclear Chicago model C 115 low background counter. The secretion is indicated by expressing the amount of labeled glycoproteins present in the incubation medium as a percentage of the sum of labeled glycoproteins in tissue plus medium.

3. Results

Fig. 1 shows that carbamylcholine (10^{-6} M) evokes a 4-fold increase of protein output. This stimulating effect declines with higher concentrations of the agonist. Whether this decrease is due to an inhibition by an excess of the agonist at the receptor level or to

Table 1
Phentolamine and propranolol effects on 10^{-6} M carbamylcholine induced metabolic and secretory responses of surviving rat submaxillary glands.

	Control	Carb 10^{-6} M	Carb 10^{-6} M phentolamine 10^{-4} M	Carb 10^{-6} M propranolol 10^{-4} M	Carb 10^{-6} M phentolamine 10^{-4} M propranolol 10^{-4} M
Total homogenate Radioactivity (cpm/g tissue)	800,000	430,000	600,000	300,000	550,000
Protein Radioactivity (cpm/g tissue)	285,000	120,000	220,000	80,000	190,000
Secretion (%)	2.6	7.4	9.7	10.2	5.3

The incubation is performed according to the first procedure. Incubation time : 90 min.

an unphysiological increase of membrane lipid turnover as established previously [16] is presently unknown.

Table 1 demonstrates the carbamylcholine effects with or without α- and β-blockers which inhibit the effects of adrenergic transmitters. Carbamylcholine can evoke a discharge of noradrenaline [17] from sympathic nerve endings but the results reported illustrate that the cholinergic agonist has an effect by itself (on all parameters: total homogenate radioactivity, protein radioactivity and secretion).

In fig. 2 the variations of radioactivity of TCA soluble fractions is shown at different times with or without carbamylcholine and colchicine. The TCA soluble fraction contains mainly the metabolic intermediates and glycogen. The radioactivity of this fraction increases linearly during 90 min (a). In the presence of carbamylcholine (b) or carbamylcholine plus colchicine (c), the slope decreases after 30 min incubation. This phenomenon might be explained by a glycogen degradation (see below).

Fig. 3 shows the discharge of labeled mucins during pulse experiments. Carbamylcholine stimulates the secretory response but colchicine depresses it by about 30%. Jamieson and Palade [6] made a similar observation while studying pancreatic secretion.

Table 2 illustrates the effects of carbamylcholine in the presence or in the absence of either colchicine or tetracaïne on the labeling of the total homogenate and glycoprotein and on the secretion. Colchicine inhibits the carbamylcholine induced secretion but does not suppress its effect on the total homogenate and protein radioactivity (identical results are obtained for 60 min incubation period). On the other hand tetracaïne suppresses the carbamylcholine effect on total homogenate radioactivity, and increases protein radioactivity and secretion.

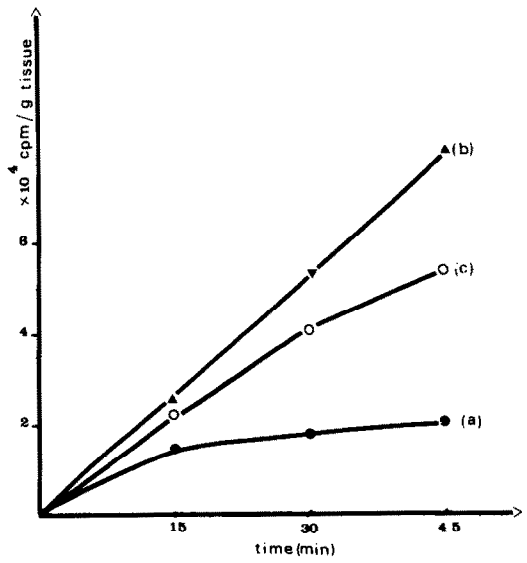

Fig. 3. Discharge of labeled mucins in following pulse labeling experiments. (a) : Control; (b): 10^{-6} M carbamylcholine; (c) : 10^{-6} M carbamylcholine + 10^{-5} M colchicine.

Table 2
Tetracaine and colchicine effects on carbamylcholine 10^{-6} M induced metabolic and secretory responses of surviving rat submaxillary glands.

	Exp.	Control	Carb 10^{-6} M	Carb 10^{-6} M colchicine 10^{-5} M	Carb 10^{-6} M tetracaïne 10^{-4} M
Total homogenate					
Radioactivity (cpm/g tissue)	1	940,000	567,000	505,000	–
	2	950,000	542,000	–	962,000
Protein					
Radioactivity (cpm/g tissue)	1	317,000	128,000	110,000	–
	2	344,000	131,000	–	418,000
Secretion (%)	1	3.5	10	4	–
	2	3	8	–	12

The incubation is performed according to the first procedure. Incubation time : 90 min.

4. Discussion

The highest stimulation of mucin secretion is obtained *in vitro* for a concentration of 10^{-6} M carbamylcholine. Experiments with α- and β-blockers indicate that this effect is due to the cholinergic agonist. Colchicine inhibits the carbamylcholine induced secretion. In the experiments on mucin discharge (fig. 3), the inhibition is only about 30%. However, in the experiments in the presence of ongoing protein synthesis and intracellular transport (table 2), this inhibition reaches about 90%. It may be assumed that in the first case microtubular subunits have already assembled during the first incubation period into microtubules (linked to the secretion granules and the plasma membranes) which, hence forward, are insensitive to colchicine. In the second case colchicine, added at the onset of the incubation, blocks microtubule formation [11, 12] and inhibits the secretion. This interpretation agrees with the hypotheses of Gillespie [13] and of Rasmussen [22].

Carbamylcholine (10^{-6} M) decreases TCA soluble radioactivity. The results displayed in table 2 illustrate that this effect is strictly due to carbamylcholine. It was reported by Kasai and Changeux [18] that the permeability to neutral permeants remains unchanged in the presence of carbamylcholine. Hence decrease of TCA soluble radioactivity could be caused by glycogen degradation which is Ca^{2+} and cyclic AMP dependent. Cholinergic agonists enhance Ca^{2+} uptake in rat submaxillary and parotid glands as it has been shown by Dreisbach [19, 20]. In our experiments, tetracaïne, an inhibitor of Ca^{2+} uptake [21] suppresses the carbamylcholine dependent decrease of total homogenate and protein radioactivity. Hence in our case, the increase in Ca^{2+} due to carbamylcholine could activate the phosphorylase kinase [23, 24] and trigger the degradation of glycogen. Colchicine, which is acting at the microtubular level, has no effect on this phenomenon. The decrease observed in the protein radioactivity could thus be caused by a dilution of metabolic intermediates due to glycogen degradation.

Jamieson and Palade [6] have shown that the last step of protein intracellular transport is regulated by secretogogues (carbamylcholine) and that their effect is blocked by inhibitors of oxydative phosphorylation. It may be postulated that this energy-requiring step consists in a conformational change of microtubules (connected with secretion granules and the plasma membrane) due to their phosphorylation. Phosphorylation of structural elements of the intracellular transport system involved in neurotransmitters release has already been proposed by Rasmussen [22]. Moreover,

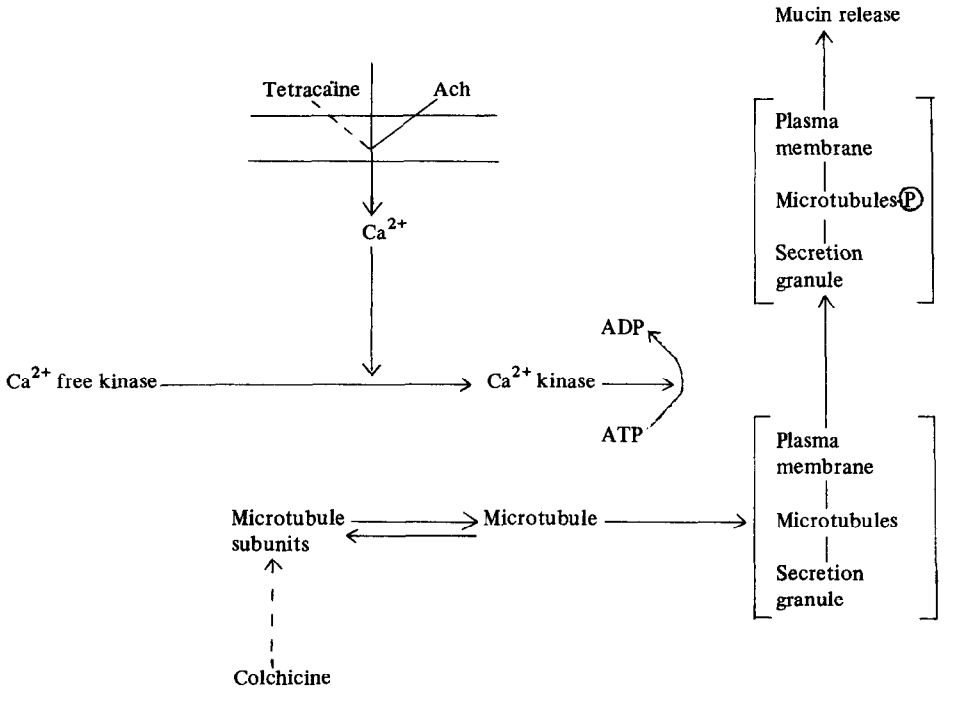

Fig. 4. Proposed mechanism of cholinergic agonist induced secretion.

it has been recently reported that cyclic AMP dependent kinase is located in the synaptic membrane [25].

An overall outline of adrenergic and cholinergic stimulations on the mucin secreting salivary glands may be summarized as follows. In adrenergic induced secretion, adenylcyclase is activated; we may assume that microtubule phosphorylating kinase is cyclic AMP dependent. Cholinergic agonists are not likely to act by such a mechanism, as it was observed on various tissues (brain, heart) that these substances enhance cyclic GMP formation but curtail cyclic AMP synthesis [26, 27]. Rasmussen and Tennenhouse (quoted by Palade [6]) also found "a relative insensitivity of the adenyl cyclase system in the pig exocrine pancreas" to secretogogues which nevertheless enhance secretion in this organ. In this connection, the role of calcium in cholinergic agonist induced secretion must be underlined: by analogy with the "direct" activation of phosphorylase kinase by Ca^{2+} ions [23, 24] it may be postulated that Ca^{2+} ions are the "second messenger" of cholinergic action on secretory mechanisms, as schematized in fig. 4, activating directly the microtubule phosphorylating kinase as well as the glycogen breakdown*.

In the presence of α- and β-blockers, tetracaïne which inhibits Ca^{2+} uptake [21] suppresses carbamylcholine induced secretion by about 50%.

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* Preliminary results which are not reported in the present work indicate that dibutyrylcyclic GMP is inactive on mucin secretion and metabolic events. Whereas dibutyrylcyclic AMP stimulates both glycoprotein biosynthesis and secretion.

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