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ELECTROPHORESIS OF ISOLATED SECRETORY GRANULES FROM GUINEA-PIG SUBMAXILLARY GLAND

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1 The nature of the surface charge on secretory granules from the submaxillary gland of the guinea-pig has been studied by electrophoresis of the isolated granules. The granules suspended in buffered sucrose solutions move to the anode.

2 Divalent ions reduce the anodal movements of the granules. Calcium and magnesium neutralize the net surface charge of the granules. This result supports the hypothesis that the electrostatic surface charge which can be reduced by calcium ions may be a major factor in controlling the fusion of secretory granules to the cell membrane, before release by exocytosis.

3 The mechanisms which couple membrane events with enzyme or hormone release from secretory glands are considered.

Introduction

Calcium ions have been shown to play an essential role in coupling changes in membrane permeability produced by acetylcholine and noradrenaline with the secretion of saliva (Douglas & Poisner, 1963). Both transmitters fail to evoke salivary secretion in the absence of calcium (see Douglas, 1968; Rasmussen, 1970). Two basic hypotheses have been evolved to explain the mechanism by which the membrane signal is coupled to the secretory response by calcium ions.

Control by contractile proteins

Because calcium ions are known to initiate contraction in skeletal muscle, it has been proposed that similar contractile protein filaments (microtubules and microfilaments) regulate the terminal intracellular movement of secretory granules. Calcium ions could cause such filaments to contract, thereby moving granules to the luminal cell membrane, producing fusion of granules with the cell membranes and initiating secretion (Lacy, Howell, Young & Fink, 1968).

Control based on electrochemical property of membranes

The surface of the secretory granules and the inner side of the luminal cell membranes are assumed to carry the same net charge, so that the granules, which move randomly in the cytoplasm, are prevented from fusing with the luminal membrane. Calcium ions could cause secretion by neutralizing the net surface charge so that successful collisions between the granules and the cell membrane can occur, resulting in an increased number of exocytotic events (see Banks, 1966; Matthews, 1970).

In this study we have tried to test the feasibility of the second hypothesis. According to this hypothesis, the granules should carry a net negative surface charge that should be neutralized by the addition of calcium ions. Banks (1966) in a preliminary study showed that granules from the adrenal medulla are negatively charged and that calcium ions reduce their mobility in an electrical field. In a very recent report the electrokinetic properties of isolated chromaffin granules have been analysed by microscopic particle electrophoresis; the membrane of chromaffin granules carries a net negative surface charge which is neutralized by divalent cations (Matthews, Evans & Dean, 1972). Similar tentative findings have been reported for granules isolated from the neurohypophysis (see Poisner & Douglas, 1968). Experiments were designed to study the net surface charge of secretory granules isolated from the guinea-pig submaxillary gland. The results suggest that an electrochemical mechanism could be involved in coupling physiological stimuli and salivary secretion.

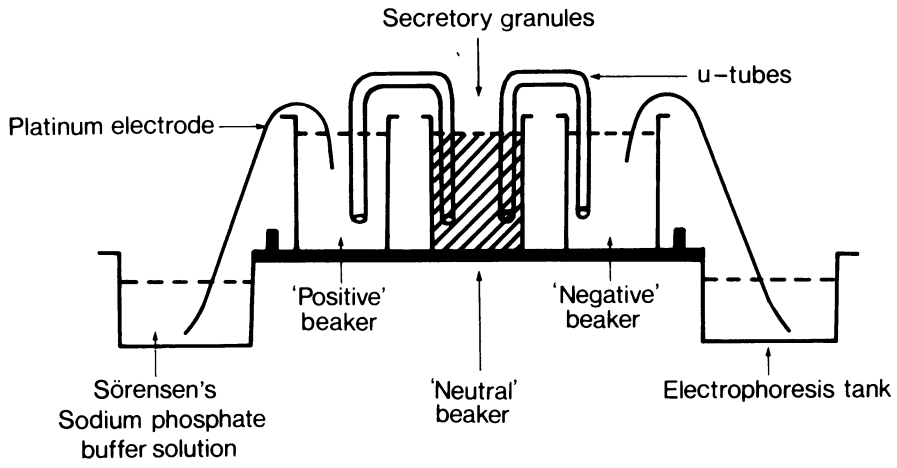

Fig. 1 Diagram of cell arrangement for particle electrophoresis. Glass beakers were placed on a perspex platform. Buffered sucrose (0.7 M; 22 ml) was added to the positive and negative beakers. Isolated secretory granules (2 ml) were suspended in 20 ml buffered sucrose solution and added to the centre beaker. The beakers were connected by U-tubes which were filled with the buffered sucrose solution, care being taken to avoid bubbles. Electrical continuity between the electrophoresis tank and the beakers was obtained with platinum electrodes encased in PVC tubing. The two troughs of the electrophoresis tank contained sodium phosphate buffer (100 mM; pH 7.0) in all the experiments.

Methods

Preparation of granules

In each experiment, submaxillary glands were removed from six to eight guinea-pigs, homogenized, and the secretory granules isolated on sucrose-density gradients; the procedure used was similar to that described in detail previously (Bhoola & Heap, 1970).

Electrophoresis

The apparatus used in the electrophoresis of the secretory granules is shown in Figure 1. The apparatus consisted of three small beakers (30 cm^3, capacity) connected in series by two glass siphons (internal diameter, 4.1 mm; external diameter, 6.1 mm; internal length of siphons, 9.0 cm). The granules were placed in the central beaker and a potential applied by placing the tips of platinum electrodes (encased in PVC tubing) in the outer beakers and in Sörensen's sodium phosphate buffer (100 mM, pH 7.0) contained in a Shandon electrophoresis tank. The electrophoresis was carried out at 4° C. The experimental conditions were such as to minimize passive movement, sedimentation and rupture of the granules.

The secretory granules were suspended in buffered 0.7 M sucrose. Three buffers (100 mM, pH 7.0) morpholinopropane sulphonic acid–KOH (MOPS), sodium phosphate and tris-(hydroxymethyl) methylamine-HCl (TRIS) were used (see results section). The specific details of the electrophoresis are given in the figure legends. In the experiments with calcium and magnesium, each ion was added to all three beakers. In order to avoid siphoning, sample aliquots were taken simultaneously from the three beakers. Granule movement was determined by measurement of the optical density of each sample at 540 nm and 700 nm, before and after addition of a lytic amount of Triton-X100 (10%). The difference in light scattering recorded before and after Triton-X100 provided a measure of the number of intact granules. Phase-contrast microscopy was used to confirm the presence of intact granules in the samples and to determine the concentration of Triton-X100 that was sufficient to rupture rapidly and effectively the secretory granules.

Results

Electrophoresis was carried out in seven different experiments with sodium phosphate buffer (100 mM, pH 7.0). In each case the granules moved to the anode (the positive beaker), suggesting that they possess a net negative surface charge. Initially there was a lag while the granules moved

into the dead space of the U-tubes; only subsequently did migration occur into the positive beaker. Migration increased steadily with time (see Table 1). The time course is not linear because there is a slow rupture of granules, so that the resultant increase in light scattering in the positive beaker is always less than would be predicted. The optical density readings from the negative beaker showed that no net movement of granules had occurred to the cathode.

In order to examine the effect of siphoning in one experiment, after the potential had been applied for 1 h, siphoning was induced with resulted in the granules moving into the negative beaker. With continued electrophoresis the negatively charged granules re-migrated to the anode (Figure 2). This experiment demonstrated clearly that the consistent migration of the granules to the anode was not a siphoning artefact. In control experiments the effect of passive movement of granules through the U-tubes was also tested by applying no potential to the granule suspension; the granules showed no net movement to either pole.

The action of calcium ions on the net surface charge of the granules could not be ascertained with the sodium phosphate buffer because of the reduction in the solubility of calcium in phosphate solutions. At first TRIS (pH 7.0) which does not chelate calcium ions (N. Good, personal communication) was used. In two control experiments the granules showed slight movement to the cathode; such a change in the direction of movement could only be explained by assuming that the large positive TRIS cation binds to the granule, which results in a reversal of the net surface charge on the granule membrane. Because calcium ions would not be expected to affect the mobility of the positively charged granules this buffer was considered unsatisfactory. The next buffer used was MOPS (100 mM, pH 7.0) which is also known not to chelate calcium ions (N. Good, personal communication). Electrophoresis performed in the MOPS buffer confirmed the finding that the granules were negatively charged (Table 2). The effect of calcium ions on the migration of granules was therefore tested with MOPS as buffer. At 0.04 mM calcium, granule migration was not affected, while at 4 mM it was completely inhi-

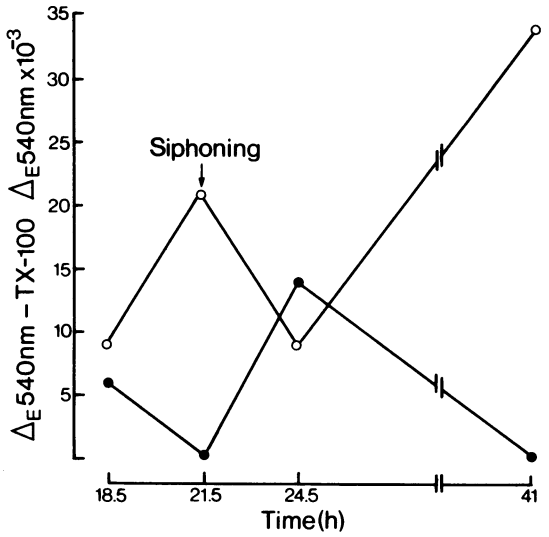

Fig. 2 Migration to the anode of isolated secretory granules suspended in sodium phosphate buffer (pH 7.0; 100 mM). After 21.5 h of particle electrophoresis, siphoning was produced and measurements 3 h later confirmed the presence of the granules in the negative beaker. Continued electrophoresis reversed the effect of siphoning so that the granules in the negative beaker migrated to the centre beaker in the direction of the positive role. Particle electrophoresis carried out at 6 mA (•), cathode; (○), anode.

Table 1 Migration of secretory granules in sodium phosphate buffer

Wavelength (nm)	*Migration time (h)*	*Optical density*	
		Positive electrode	*Negative electrode*
700-TX700	16	0.018	0.002
	42	0.023†	0
540-TX540	18.5	0.009*	0
	21.5	0.021*	0
	42	0.034*	0
		0.043†	0

Optical density measurements carried out at 700 nm and 540 nm before and after Triton-X100. Accumulated data of three separate experiments.
*† Indicate values obtained in the same experiment.

bited (Table 3). Calcium ions stopped granule migration to the anode; in addition, unlike TRIS buffer, there was no reversal in the direction of migration. The concentration of calcium in the isolated secretory granule fraction, in the absence of added calcium, was 100-150 μM. Magnesium (4 mM) also inhibited granule migration. But 4 mM cyclic 3′,5′-adenosine monophosphate (cAMP) did not prevent granule movement.

Discussion

This study suggests that there is a net negative charge on the surface of secretory granules isolated from the guinea-pig submaxillary gland and suspended in sodium phosphate or MOPS buffer at pH 7.0. Such a net surface charge could influence the movement of the secretory granule from its site of formation in the Golgi apparatus to its site of release at the luminal membrane, particularly if potential gradients were established between the basal and luminal membranes in the resting and in the secreting cell.

The first postulate of an electro-chemical model, requiring that secretory granules must possess a net negative surface charge, is fulfilled in the study. This finding agrees with reports that hormone-containing granules from the adrenal medulla and the neurohypophysis have a net negative surface charge (see Banks, 1966; Poisner & Douglas, 1968; Matthews *et al.*, 1972). In the presence of a sufficient concentration of calcium ions, the net surface charge is neutralized and the granules would cease to be repelled from the luminal (secretory) cell membrane. The neutralization of the surface charge of granules by divalent cations satisfies the second criterion of the model. The localized release of calcium associated with the luminal membrane could produce threshold neutralization of the surface charge on the granular membrane and the events terminating in exocytosis could then cascade, resulting in secretion.

Dibutyryl cyclic adenosine monophosphate stimulates enzyme secretion from the guinea-pig submaxillary gland *in vitro* (Bhoola & Lemon, 1972). However, cAMP did not prevent the migration of the granules (see Table 3). This suggests that if exocytosis is controlled by calcium and if a purely electrochemical model for the fusion of granules with the secretory membrane is valid, then the effects of cAMP must be on that part of the secretory process prior to exocytosis. It is possible that cAMP releases calcium from intracellular calcium pools to initiate enzyme secretion (see Nielsen & Petersen, 1972). Such a cAMP-mediated effect on calcium stores could only apply when secretion is induced by noradrenaline but not by acetylcholine, because adenylate cyclase of guinea-pig submaxillary gland is stimulated by noradrenaline but not by acetylcholine (Bhoola & Lemon, 1973).

Much of the evidence for a microtubular mediated model is based on the effects of the antimitotic drug colchicine. Colchicine disrupts the mitotic spindle and other microtubular systems (Borisy & Taylor, 1967). Cochicine has been shown to inhibit the secretion of granules (see Gillespie, Levine & Malawista, 1968; Poisner & Bernstein, 1971) and so, by analogy, a microtubular mediated secretion has been suggested for many secretory glands. Although both acetylcholine and potassium evoke secretion from the adrenal medulla, colchicine blocks only the acetylcholine- and not the potassium-induced secretory response (Douglas & Sorimachi, 1972). Such evidence calls into serious doubt the view that microtubules or microfilaments regulate exocytosis. Matthews (1970, 1971) has calculated, with

Table 2 Migration of secretory granules in morpholinopropane sulphonic acid-KOH (MOPS) buffer

		Optical density	
	Migration time (h)	*Positive electrode*	*Negative electrode*
I.	1.5	0.000	0.002
	18.5	0.062	0
II.	4	0.007	0
	18.25	0.230	0.016

Optical density measurements carried out at 540 nm before and after Triton-X100 (540-TX540). I and II represent accumulated data of two separate experiments.

Table 3 Effect of calcium and cyclic 3′,5′-AMP (cAMP) on migration of secretory granules in morpholinopropane sulphonic acid-KOH (MOPS) buffer

		Optical density	
	Migration time (h)	*Positive electrode*	*Negative electrode*
I. $CaCl_2$ 0.04 mM	18	0.046	0
II. $CaCl_2$ 0.1 mM	17	0.032	0
III. $CaCl_2$ 4 mM	17	0.006	0.004
IV. cAMP 4 mM	17	0.062	0

Optical density measurements carried out at 540 mM before and after Triton-X100 (540-TX540). Each set of values obtained in separate experiments.

certain assumptions, that the electrochemical model for granular secretion is thermodynamically feasible. So it may be appropriate to reconsider the purely electrochemical model for the secretion of granules.

The present study, together with the recently reported experiments on the isolated chromaffin granules, strongly suggests that the electrokinetic properties regulate the intracellular fusion of granules with the secretory membrane. However, there are still steps in the terminal process of secretion which require elucidation: for example, what control system directs the granules to the secretory (luminal) cell membrane, so that secretion can occur into the ducts in the exocrine and into capillary in the endocrine gland; and what is the relationship between the mechanism which controls the intracellular movement of granules and the requirement for metabolic energy in the secretory process.

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