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TRANSPORT OF CALCIUM IN THE PERFUSED SUBMANDIBULAR GLAND OF THE CAT

BY S. PORS NIELSEN AND O. H. PETERSEN
From the Institute of Medical Physiology C, University of Copenhagen, Copenhagen, N. Denmark

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SUMMARY

1. In the perfused cat submandibular gland efflux and influx of ^{45}Ca, and concentrations of K, ^{40}Ca and Mg in the effluent from the gland were measured under different experimental conditions.

2. When the standard perfusion fluid was shifted to a high Mg (5 mM) or a low Ca (0·25 mM) solution the efflux of ^{45}Ca from the pre-labelled gland declined. The magnitude and the duration of the effect of the high Mg concentration was more marked at a low external Ca concentration and was abolished by Mersalyl (1 mM). When the standard perfusion fluid was shifted to a Mg-free solution the efflux of ^{45}Ca from the pre-labelled gland increased.

3. After shift of ^{45}Ca containing perfusion fluid from normal to a high Mg (5 mM) solution the influx of ^{45}Ca to the gland increased rapidly.

4. Both acetylcholine (ACh) and adrenaline caused a marked increase in the efflux of ^{45}Ca from the pre-labelled gland. This increase in efflux was also seen under conditions where the gland was unable to secrete, i.e. during perfusion with Ca-free and Na-free tetraethylammonium Locke solutions.

5. Stimulation with ACh failed to reveal any rapidly occurring increase in influx of ^{45}Ca.

6. Stimulation with ACh evoked a small temporary increase in the concentration of ^{40}Ca and Mg in the effluent.

7. It is suggested that Ca uptake by intracellular Ca-accumulating systems of the submandibular gland depends on the external Mg concentration and that ACh and adrenaline cause a release of Ca bound intracellularly.

INTRODUCTION

It is known that salivary glands are unable to secrete for long periods in the absence of external calcium (Douglas & Poisner, 1963), but are able to produce secretory potentials (Petersen, Poulsen & Thorn, 1967). It has been reported that parasympathetic stimulants enhance the uptake of ^{45}Ca in the lacrimal and submandibular glands (Dreisbach, 1964), but the exact role of Ca in the secretory process is unknown. Little is known about the factors which regulate the active extrusion of Ca from the cell interior and the active accumulation of Ca in the organelles of salivary gland cells. The present study on calcium transport in salivary glands was carried out to investigate: (1) the effect of some ionic replacements on ^{45}Ca transport in the resting state, and (2) the effect of stimulation on ^{45}Ca transport. The results obtained showed that Ca transport in the resting state is dependent on the external Mg concentration and that stimulation of the glands with both ACh and adrenaline markedly increased ^{45}Ca efflux from the prelabelled gland. Some of the findings have been briefly reported elsewhere (Nielsen & Petersen, 1971*a*, *b*; Petersen & Nielsen, 1971).

METHODS

Fifty-two cats anaesthetized with chloralose (80 mg/kg I.P.) were used. All experiments were carried out with artificially perfused submandibular glands prepared as previously described (Petersen, 1970). Briefly, all branches from the common carotid artery not supplying the submandibular gland and all the tributaries to the external jugular vein not draining the submandibular gland were ligated. Polyethylene tubing was inserted into the common carotid artery and the external jugular vein, and the gland was perfused with Locke solutions through the catheter inserted into the common carotid artery. A catheter was inserted into the main excretory duct of the submandibular gland for collection of saliva. All experiments were carried out at room temperature (21–25° C).

^{45}Ca efflux experiments. Before the perfusion was started the lingual artery was cannulated and a solution of [^{45}Ca]$CaCl_2$ was infused retrogradely through the lingual artery stump into the artery supplying the submandibular gland. 40 μc ^{45}Ca from an aqueous solution (17 mc/mg Ca) was dissolved in 1 ml. redistilled water and infused at a constant rate in 1 hr. During this period of labelling salivary secretion was evoked every 5 min by applying electrical stimuli at the sectioned lingual nerve for 30 sec (10 V, 10 c/s). After the end of the labelling period the artificial perfusion was started. The experimental set-up is illustrated on Fig. 1. As soon as the perfusion had been established the cat was sacrificed by occlusion of the tracheal cannula. The flow of perfusion fluid through the gland was kept constant (2·5 ml./kg body wt.) throughout the experiment by use of a peristaltic pump (Harvard Multispeed Transmission Peristaltic Pump, 1204). The effluent was collected in tubes placed in a fraction collector (LKB Ultrarac® 7000 Fraction Collector). Samples were taken every minute and in some experiments during stimulation of secretion every 0·2 or 0·3 min. Since during the first 10–15 min in some of the experiments the effluent was slightly contaminated with blood the samples were routinely centrifugated (1900 *g* for 3 min) throughout the experiments,

and the supernatant was separated within 10 min. The gland was usually stimulated by a close arterial injection of 0·06 μmole acetylcholine (ACh). In some experiments adrenaline was used (same molar amount as ACh).

^{45}Ca-influx experiments. In eight short-time experiments collection of samples with short intervals was begun immediately after the beginning of perfusion with Locke solutions containing 3000 c.p.m. ^{45}Ca/ml. The difference of ^{45}Ca concentration between the influent and the effluent was used as a measure of ^{45}Ca influx.

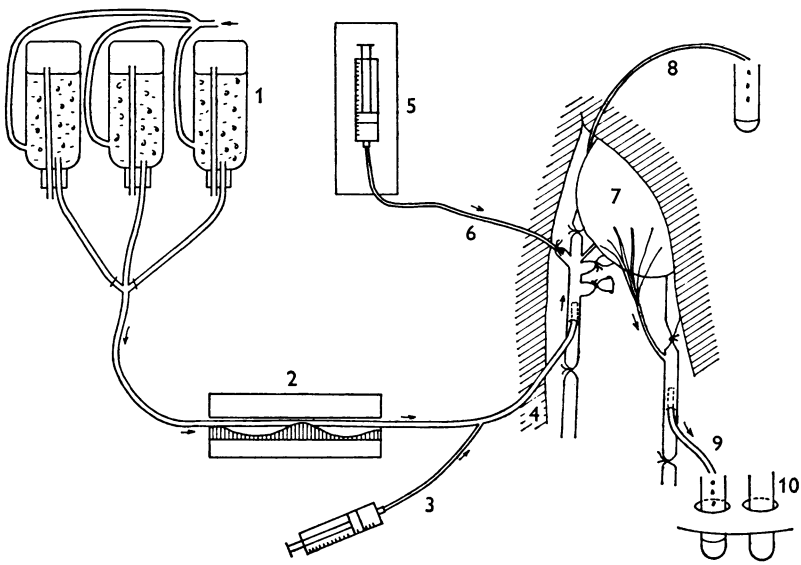

Fig. 1. Schematic illustration of the method of perfusion. 1, Bottles with different Locke solutions; 2, finger pump; 3, side tubing for injection of ACh; 4, main catheter inserted into common carotid artery; 5, infusion pump; 6, catheter inserted into the lingual artery for labelling; 7, submandibular gland; 8, catheter in excretory duct; 9, catheter in the external jugular vein; 10, tubes in fraction collector.

Counting procedure. ^{45}Ca was measured by liquid scintillation counting (Packard Tri-Carb Liquid Scintillation Spectrometer 3375) using the Triton X-100 system (Nadarajah, Leese & Joplin, 1969). The scintillant was composed of two volumes of toluene containing 0·4% (w/v) PPO (2,5 diphenyloxazole), and 0·01% (w/v) POPOP (1·4-bis-2-[5-phenyloxazolyl]-benzene), and one volume of Triton X-100. One ml. of the sample per 8 ml. of scintillant was used. The elements studied did not cause any measurable quenching in the concentrations used. The effect of duration of standing in the counter was negligible. Thus in one experiment (sixty-eight consecutive samples of effluent) the counting rate (S.D.) changed by +0·85 (1·43)% after 12 hr. The counting time of one experiment never exceeded 12 hr.

Other analytical methods. In four experiments the concentration of ^{40}Ca, Mg and K in the effluent was determined. ^{40}Ca and Mg were measured by atomic absorption spectrophotometry (Perkin Elmer Atomic Absorption Spectrophotometer, 290 B) and K was measured by emission flame photometry (Eppendorf). In these experiments stimulation was done by close-arterial infusion of ACh 0·30 μmole/min and the secretory rates in 1 min periods were measured.

Flow experiments. In four experiments perfusion was carried out under a hydrostatic pressure of 80 mm Hg omitting the perfusion pump, and the effect on the flow of high and low concentrations of Mg was tested.

Perfusion fluids. The standard perfusion fluid used contained (mM): NaCl 140; KCl 4·0; Na_2HPO_4 2·4; NaH_2PO_4 0·6; $CaCl_2$ 2·2; $MgCl_2$ 1·0; glucose 5·5. The solution was equilibrated with oxygen for 2 hr before use. In the case of perfusion with a high Mg concentration (5 mM) or a low Mg or Ca concentration (Mg-free, Ca-free or 0·25 mM-Ca) equiosmolality (312 m-osmole/kg) was ensured by corresponding changes in the concentration of NaCl. In some experiments a Na-free tetraethylammonium (TEA) solution was used. This fluid contained (mM): tetraethylammonium chloride 144; $MgCl_2$ 1·0; $CaCl_2$ 2·2; KH_2PO_4 0·6; K_2HPO_4 2·4; glucose 5·5. In some experiments the effect of 1 mM mersalyl (Na salt of O-[N-(3-hydroxymercuri-2-methoxypropyl)carbamyl]phenoxy acetic acid) on ^{45}Ca efflux was studied. Osmolality, Na, K, Ca and Mg concentration of the perfusion fluids were routinely checked.

RESULTS

Transport of ^{45}Ca in the resting state

The effect of different concentrations of Mg and ^{40}Ca in the perfusion fluid on ^{45}Ca efflux was studied in fourteen experiments. A high Mg concentration (5 mM) markedly inhibited ^{45}Ca efflux (Fig. 2). This effect was temporary when the external ^{40}Ca concentration was normal (2·2 mM) but long-lasting when low (0·25 mM). It was completely abolished when mersalyl (1 mM) was added to the perfusion fluid (five experiments). Perfusion with Mg-free Locke solutions increased the ^{45}Ca efflux. Like the effect of 5 mM Mg this effect could be reversed and repeated in the same experiment (Fig. 2).

In four experiments with ^{45}Ca containing perfusion fluids a high Mg concentration (5 mM) increased the difference between the ^{45}Ca concentration of the influent and the effluent when introduced 3–5 min after the beginning of the perfusion (Fig. 3). The flow was always constant when the perfusion pump was used. The increased difference between the ^{45}Ca concentration of the influent and the effluent could have been caused by a vasodilatation and a resultant redistribution of flow with perfusion of non-radioactive parts of the gland. In a test of this possibility we found that no changes of flow were detectable after introduction of high Mg (5 mM) or Mg-free perfusion fluids, when the hydrostatic pressure was used as the driving force (Table 1). A low ^{40}Ca concentration (0·25 mM) depressed ^{45}Ca efflux

^{45}Ca transport after stimulation

Twenty ^{45}Ca efflux experiments with ACh or adrenaline stimulation were carried out. Stimulation of secretion by 0·06 μmole ACh or adrenaline increased the ^{45}Ca efflux rapidly (Fig. 4). Maximum ^{45}Ca efflux was attained 1–2 min after the close arterial injection (Fig. 4), a little later than the peak

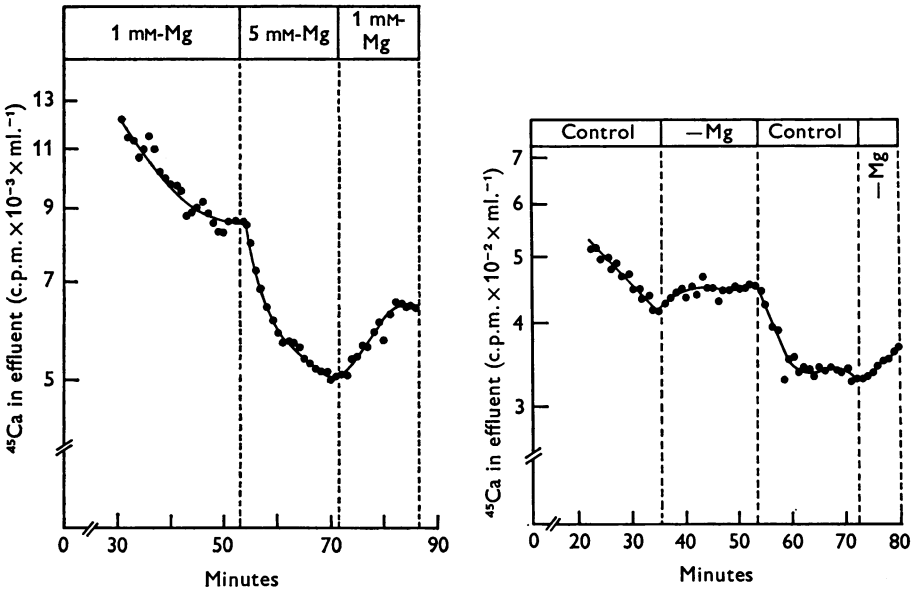

Fig. 2. The effect of a high Mg concentration (5 mM) in the perfusion fluid on ^{45}Ca efflux (^{40}Ca concentration was 0·25 mM) is shown in the left part of the Figure and the effect of omitting Mg from the perfusion fluid on ^{45}Ca efflux in the right part.

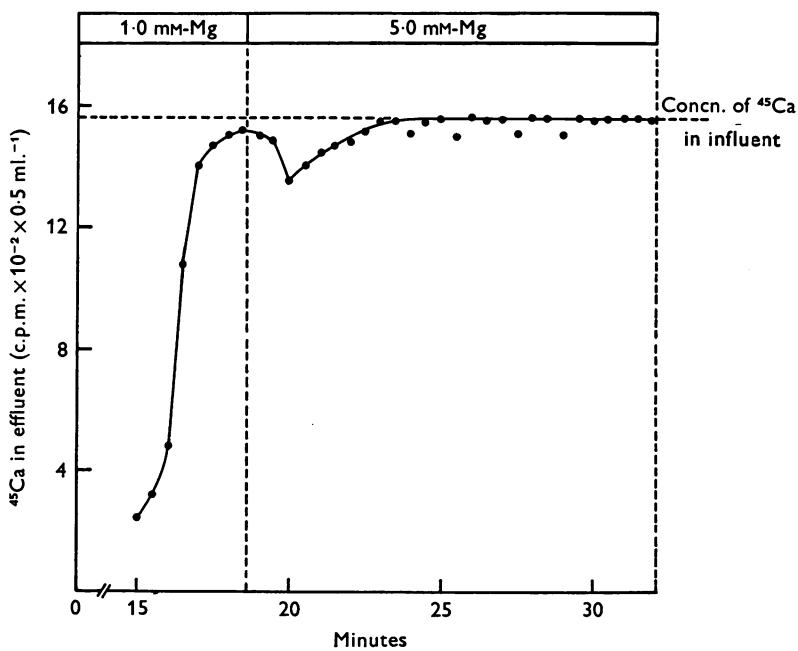

Fig. 3. Short-term 'influx' experiment. Effect of 5 mM-Mg. Perfusion with a non-radioactive Locke solution was begun at zero time. A radioactive perfusion fluid (3200 c.p.m./ml.) was introduced after 15 min and the high Mg solution after another 3½ min.

value for K which was seen after 20–40 sec (Fig. 7). The absolute increase in the concentration of ^{45}Ca in the effluent after stimulation differed from one experiment to the other.

Evidence that the effect did not seem to be caused by diffusion of ^{45}Ca from the saliva was given by the fact that it was observed during perfusion with Ca-free medium, in which situation secretion cannot be

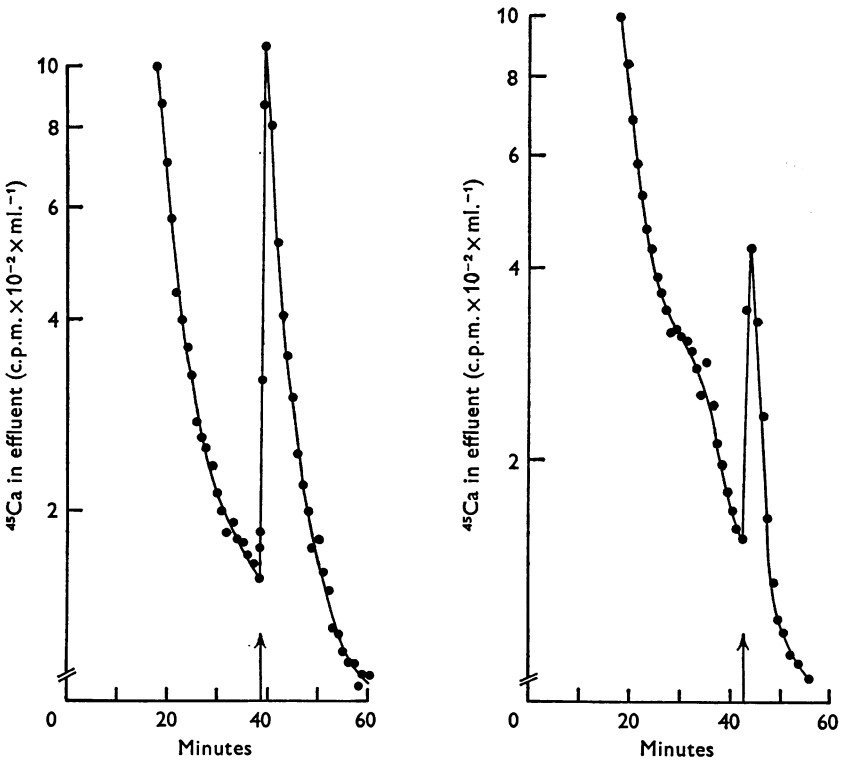

Fig. 4. ^{45}Ca efflux after stimulation (↑) with equimolar (0·06 μmole) amounts of ACh (left) and adrenaline (right).

evoked (Fig. 5). Neither was the effect abolished by Na-free perfusion (Na replaced by TEA), which also inhibits secretion completely.

If the ACh-induced rise in ^{45}Ca efflux was brought about by an increased permeability of the cell membrane one would anticipate an increased influx of ^{45}Ca in the same period. 'Influx' of ^{45}Ca after stimulation with ACh was investigated in four experiments which all failed to show any immediate rise in 'influx' as judged by the difference of ^{45}Ca concentration in the effluent and influent in short-term experiments of the type described above. There seemed, however, to be a delayed, long-lasting rise in 'influx' secondary to the stimulation (Fig. 6).

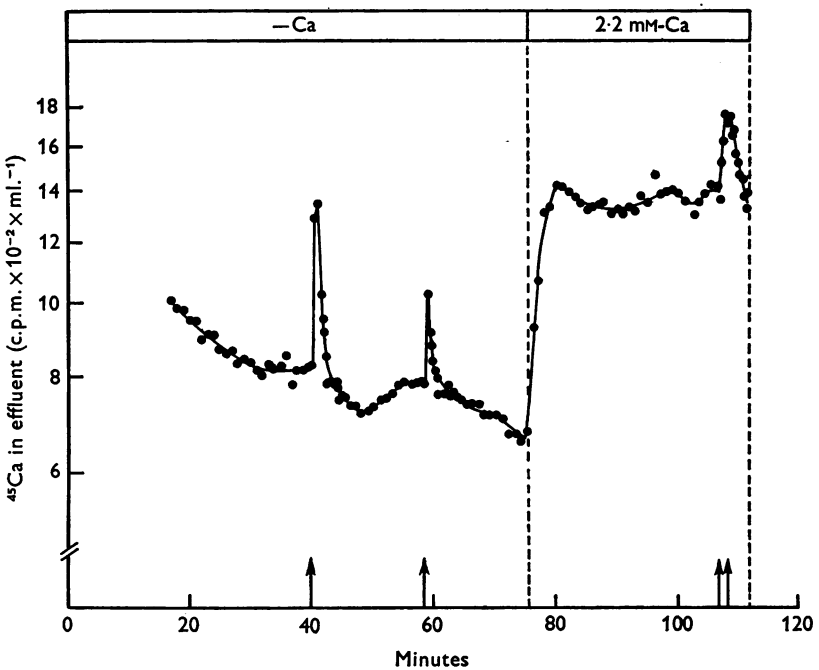

Fig. 5. ^{45}Ca efflux after close-arterial injection of 0·06 μmole ACh (↑) in Ca-free medium. No saliva was produced during Ca-free perfusion. The ability to secrete was restored after shift to a perfusion fluid with normal (2·2 mM) Ca concentration.

TABLE 1. Lack of effect on flow (ml./min) by high and low concentration of Mg in the perfusion fluid. No infusion pump. The driving force was the hydrostatic pressure (80 mm Hg). Collection of eight consecutive 1 min samples

Expt no.	Control (1 mM-Mg)				Mg-free			
1	1·2	1·0	1·2	1·0	1·2	1·0	1·1	1·0
2	1·1	1·0	0·9	1·0	1·0	1·0	0·9	1·0
3	1·2	1·1	1·1	1·0	1·0	0·9	0·9	0·9
4	1·5	1·4	1·3	1·4	1·3	1·3	1·2	1·3
	Control (1 mM-Mg)				High Mg (5 mM-Mg)			
5	1·1	1·0	1·1	1·0	1·3	1·1	1·2	1·1
6	1·3	1·0	1·1	1·0	1·2	1·0	1·2	1·1
7	1·1	1·1	1·2	1·0	1·1	1·1	1·2	1·1
8	0·9	1·0	1·0	1·0	1·1	1·0	1·2	1·0

Concentrations of K, Mg and ^{40}Ca in effluent

Mg-free or high Mg (5 mM) perfusions did not alter the ACh-induced K release or the K concentration in the resting state. Small increments in the concentration of ^{40}Ca and Mg after injection or infusion of ACh were observed (Fig. 7). These minute alterations seemed to occur later than the K release.

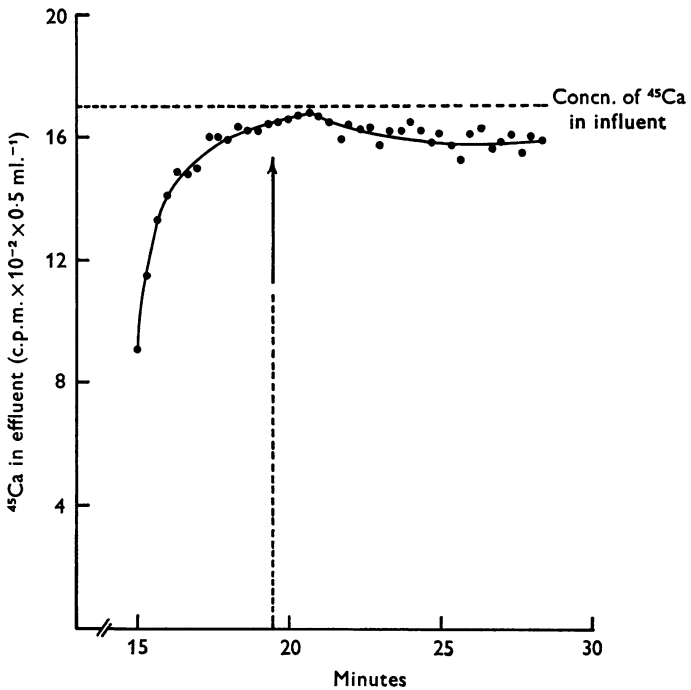

Fig. 6. Short-term 'influx' experiment. Lack of immediate effect of injection of 0·06 μmole ACh (↑). Perfusion with a non-radioactive perfusion fluid was begun at zero time. A radioactive perfusion fluid (3400 c.p.m./ml.) was introduced after 15 min and ACh was injected after another 4½ min (↑).

DISCUSSION

About 80% of the lobular space in the sheep parotid glands consists of acini and 8% is occupied by ducts (Blair-West, Coghlan, Denton, Nelson, Wright & Yamauchi, 1969). Probably only a minor portion of the submandibular gland consists of smooth muscle cells. It is impossible to know how much the smooth muscle cells contribute to the ^{45}Ca efflux in the resting state. The marked changes in ^{45}Ca efflux seen in our experiments after stimulation by ACh and adrenaline are, however, not likely to be explicable by an altered Ca metabolism in smooth muscle cells,

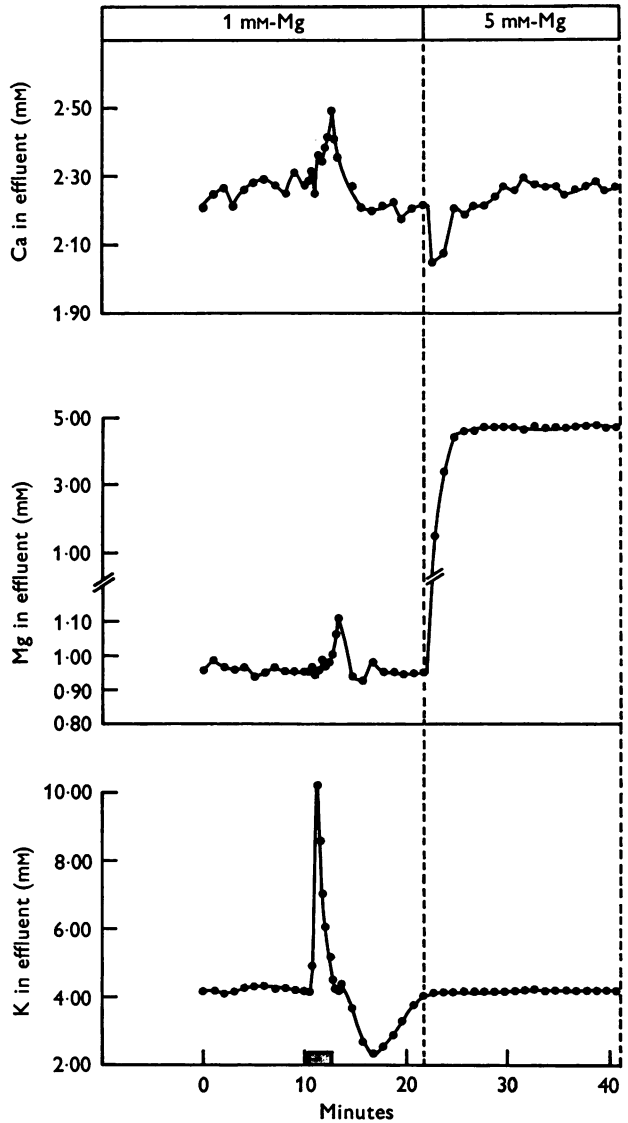

Fig. 7. Effect of infusion of ACh (▮) for 2½ min (0·30 μmole/min) on the concentration of K, Mg and ^{40}Ca in the venous effluent.

since parasympathomimetic drugs or catecholamines cause either no or minute changes in ^{45}Ca fluxes in smooth muscle cells (Lüllmann, 1970). It seems unlikely that a marked proportion of the ^{45}Ca leaving the cells is derived from the glucoprotein cell coat, since in cultures of other epithelial cells pre-labelled with ^{45}Ca cell coat ^{45}Ca is removed by washing with non-radioactive medium within a few minutes (Borle, 1969, 1970).

Ca transport in the resting state. Our results demonstrate that external Mg influences Ca transport in salivary glands noticeably. The enhanced efflux of ^{45}Ca seen after removal of extracellular Mg cannot be explained by a general change in cell membrane permeability since no K release occurs after introduction of Mg-free solution. It is possible that the effect is mediated either by an enhanced permeability of the microsomal membranes to Ca or a decreased active uptake of Ca into the microsomes, since Selinger, Naim & Lasser (1970) have described a powerful Ca-accumulating system dependent on ATP and Mg in microsomes from rat salivary glands. The effect of a high Mg concentration on ^{45}Ca efflux (Fig. 2) could be mediated by an enhanced uptake of Ca into the microsomes. This view was corroborated by the results of the influx experiments. It was impossible to measure true influx of ^{45}Ca with a high time resolution. However, the fact that the initial difference between the concentration of ^{45}Ca in the effluent and the influent in the 'influx' studies was consistently increased by 5 mM-Mg (Fig. 3) suggests that the high external Mg concentration augments Ca influx. Mersalyl is a potent inhibitor of Ca-Mg activated ATPase (Schatzmann, 1970). It increases ^{40}Ca efflux from sarcoplasmatic reticulum of skeletal muscle (Weber, 1971) and inhibits Ca accumulation in microsomes of the parotid gland (Selinger, Naim & Lasser, 1970). The fact that this agent also abolishes the Mg-induced fall in ^{45}Ca efflux in our experiments might suggest that it inhibits a Mg-induced accumulation of Ca in the submandibular gland cells.

Mg is known to interact with a variety of calcium-dependent processes. It is known that Mg is necessary for active Ca uptake by mitochondria in the kidney (Lehninger, 1965) and the liver (Haugaard, Haugaard & Lee, 1969). Also the Ca accumulation in the sarcoplasmatic reticulum of skeletal muscle is Mg dependent (Weber, 1971). Mg depresses the Ca-dependent secretory response to ACh in the salivary gland (Douglas & Poisner, 1963). In a complex cell system like the salivary gland with several intracellular Ca pools (Selinger *et al.* 1970), it is on the basis of our experiments impossible to exclude that the effect of Mg on Ca transport is primarily an effect on the plasma membrane. Little information is available about the effect of Mg on Ca transport across the plasma membrane in cells with small microsomal or mitochondrial Ca pools. However, Schatzmann (1970) reported that the rate of Ca extrusion was diminished in resealed red cells at low concentrations of Mg.

Ca transport in the stimulated state. One question that might be raised in connexion with the very marked increase in the efflux of ^{45}Ca from the submandibular gland after ACh stimulation is whether it could merely be a reflexion of changes in the vascular bed of the gland. ACh is a powerful vasodilator and an increased flow of perfusion fluid through the

gland would *per se* increase the wash-out of ^{45}Ca from the prelabelled gland. However, in the present experiments the glands were perfused at a constant flow. Still a redistribution of flow could occur after ACh stimulation. However, the glands were perfused at flows equal to the maximal flows seen after stimulation during constant pressure perfusion, i.e. under conditions where the resistance of the vascular bed of the gland determines the flow. This should ensure that all vessels were maximally dilated. Thus no change in the distribution of the perfusion fluid flow through the gland should occur after ACh stimulation in our experiments. Also adrenaline which causes marked vasoconstriction in the perfused cat submandibular gland (Petersen, 1970) gave rise to a similar increase in ^{45}Ca efflux as that seen after ACh (Fig. 4). If a redistribution of flow occurred after ACh stimulation we would also expect that the 'influx' curve (Fig. 6) should show a rapid increase in ^{45}Ca 'influx' with the same time course as the rise in efflux seen in the efflux experiments (Fig. 4). Since this was not found it seems justifiable to conclude that the rise in ^{45}Ca efflux seen after stimulation with ACh represents an increased efflux from the cells mediated by an effect of ACh on the cells. Another question is, whether the extra efflux of ^{45}Ca represents a primary loss across the luminal cell membranes with a secondary diffusion back to the venous effluent. This question cannot be answered with certainty. Our finding that a qualitatively normal ACh induced ^{45}Ca release occurs during perfusion with Ca-free and Na-free TEA solutions, conditions where the glands did not secrete, indicates that the ^{45}Ca release phenomenon is independent of the formation of saliva. We know that K is released across both the luminal and contraluminal cell membranes after stimulation (Burgen, 1956). Nielsen & Petersen (1970) have shown that the salivary concentration of Ca is high initially after the onset of chordalinqual stimulation. If the increase in ^{40}Ca concentration seen in the effluent after stimulation (Fig. 7) is the sign of the same phenomenon as the increase in ^{45}Ca efflux after ACh (Fig. 4) it must represent an enhanced Ca transport across the contraluminal cell membrane, since the concentration of Ca in the saliva is much lower than in the perfusion fluid (Nielsen & Petersen, 1970). The lack of any rapidly occurring Ca 'influx' after ACh (Fig. 6) does not support the idea that the ACh mediated ^{45}Ca efflux can be explained by an enhanced permeability of the cell membranes to Ca. A more reasonable explanation for this finding is that the ACh induced ^{45}Ca efflux is due to an enhanced concentration of Ca in the cytosol with a resultant diffusion and/or active extrusion of Ca across the cell membranes. Such an increase in the concentration of Ca in the cytosol could be evoked by a release of Ca bound in the microsomes. The loss of ^{40}Ca from the gland to the perfusion fluid can (Fig. 7) only be

explained by an active extrusion of Ca from the cytoplasm to the extracellular fluid since the concentration of Ca in the cytosol is very much lower than that present in the perfusion fluid. A secondary rise in ^{45}Ca 'influx' as found in our experiments with ACh was anticipated since Ca is lost initially both to the saliva (Nielsen & Petersen, 1970) and to the extracellular fluid, and is in agreement with the findings of Dreisbach (1964). Possibly, cyclic-AMP might mediate a release of Ca from the intracellular Ca pools after stimulation (Nielsen & Petersen, 1971*b*) as has been suggested for the liver (Friedman & Rasmussen, 1970).

The significance of the stimulation-induced Ca-release from intracellular stores is at present unknown. It is possible that separate activator mechanisms exist for fluid secretion and protein secretion. ACh-induced Na entry would stimulate salt and water transport (Petersen, 1971), whereas ACh-induced Ca release might stimulate protein secretion. It has recently been demonstrated that in the exocrine rat pancreas pancreozymin and ACh, both stimulating protein secretion, enhance ^{45}Ca release from the pre-labelled gland, whereas secretin, mainly stimulating bicarbonate and water transport, has no effect on Ca release (Case & Clausen, 1971).

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