Studies on the Role of Calcium Ions in the Stimulation by Adrenaline of Amylase Release from Rat Parotid

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1. Mitochondrial and microsomal fractions were prepared from rat parotid glands. Both fractions were able to take up ^{45}Ca . The mitochondrial ^{45}Ca -uptake system could be driven by ATP (energy-coupled Ca^{2+} uptake) or by ADP+succinate (respiration-coupled Ca^{2+} uptake). Energy-coupled Ca^{2+} uptake was blocked by oligomycin but not by carbonyl cyanide *m*-chlorophenylhydrazone; respiration-coupled Ca^{2+} uptake was blocked by carbonyl cyanide *m*-chlorophenylhydrazone but not by oligomycin. Microsomal Ca^{2+} uptake was dependent on the presence of ATP; the ATP-dependent Ca^{2+} uptake was not affected by oligomycin or carbonyl cyanide *m*-chlorophenylhydrazone. Ca^{2+} uptake by both fractions was inhibited by Ni^{2+} . 2. Incubation of parotid pieces with adrenaline increased the rate of release of amylase and the uptake of ^{45}Ca . The adrenaline-stimulated release of amylase was not dependent on the presence of extracellular Ca^{2+} . 3. The effect of adrenaline on the subcellular distribution of ^{45}Ca in parotid pieces incubated with ^{45}Ca was studied. In parotid tissue incubated with ^{45}Ca , both mitochondrial and microsomal fractions contained ^{45}Ca . Incubation with adrenaline increased the amount of ^{45}Ca incorporated into the mitochondrial fraction but not the microsomal fraction. In parotid tissue preloaded with ^{45}Ca subsequent incubation with adrenaline caused a decrease in the amount of ^{45}Ca found in both the mitochondrial and microsomal fractions. 4. From these data we conclude that the regulation of the cytosolic Ca^{2+} concentration in the parotid may involve both mitochondrial and microsomal Ca^{2+} -uptake systems. We suggest that the action of adrenaline on the parotid may be to increase the movement of Ca^{2+} to the cytosol by increasing the flux of Ca^{2+} across mitochondrial, microsomal and plasma membranes.

Studies with various secretory cell types have led to the view that exocytosis of secretory products may be triggered by a rise in the cytosolic concentration of Ca^{2+} (Malaisse *et al.*, 1971; Douglas, 1968; Rubin, 1970). In many of these cell types, e.g. the pancreatic β -cell (Hales & Milner, 1968), a requirement for extracellular Ca^{2+} has been demonstrated for secretion to be stimulated. This suggests that a major action of stimulants could be on the movement of Ca^{2+} across the plasma membrane to increase the cytosolic concentration either by facilitating Ca^{2+} influx or by inhibiting Ca^{2+} efflux. In other secretory systems, however, it has been difficult to detect a requirement for extracellular Ca^{2+} , e.g. for dibutyryl 3':5'-cyclic AMP-stimulated amylase release from the parotid (Selinger & Naim, 1970), and this has been attributed to the relatively high tissue content of Ca^{2+} . This suggests that mobilization of Ca^{2+} from intracellular stores may also play an important role in initiating secretion. The uptake and release of Ca^{2+} by subcellular components may participate in the regulation of the cytosolic Ca^{2+} concentration in stimulus-secretion

coupling as in excitation-contraction coupling in muscle.

In these studies we have examined the Ca^{2+} -accumulating properties of two subcellular fractions isolated from rat parotid glands, a mitochondrial fraction and a microsomal fraction, and have investigated whether stimulation of release of amylase by parotid is accompanied by changed Ca^{2+} handling by these fractions. We have also studied the effects of Ni^{2+} , a general inhibitor of secretory processes (Dormer *et al.*, 1974), on Ca^{2+} metabolism by the parotid.

Experimental

Materials

All radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. $^{45}\text{CaCl}_2$ was obtained at a specific radioactivity of 15 mCi/mg. [^3H]Inulin was dissolved in water at a specific radioactivity of 20 μCi /mg. Millipore filters (IEA HAWP

00010, 0.45 μm pore-size) were obtained from Millipore Corp., Bedford, Mass., U.S.A. Other reagents were from B.D.H. Ltd., Poole, Dorset, U.K.

Methods

Perfusion of parotid pieces. Parotid glands were obtained from male albino rats (200–300g), which had been starved overnight, and were cut into pieces weighing 8–15mg. Two pieces were placed in a perfusion chamber and perfused with bicarbonate-buffered salt solution (Krebs & Henseleit, 1932) as described by Robberecht & Christophe (1971). The activity of amylase release into the medium was assayed by the method of Bernfeld (1955).

Preparation of subcellular fractions of parotid. Parotid glands obtained from overnight-starved male albino rats were collected in ice-cold sucrose medium (0.25M-sucrose–10mM-Tris–HCl–1mM-EDTA, pH7.4). All subsequent procedures were carried out at 0–4°C. The glands were cut into small pieces and homogenized in sucrose medium (10%, w/v) with a loose-fitting Teflon/glass homogenizer, mechanically driven at approx. 1000rev./min for 1min. The homogenate was filtered through plastic mesh (1mm pore size), diluted with an equal volume of sucrose medium and then centrifuged at 250g for 5min in an MSE bench centrifuge. The supernatant was removed and centrifuged at 10000g for 10min in an MSE High Speed 18 centrifuge to give a mitochondrial pellet. The supernatant was then further centrifuged at 100000g for 60min in an MSE Superspeed 65 centrifuge to give a microsomal pellet. The mitochondrial and microsomal pellets were both washed in sucrose medium and the final pellets resuspended at a concentration of approx. 3mg of protein/ml.

Measurement of ^{45}Ca accumulation by mitochondrial and microsomal fractions of parotid. Mitochondrial and microsomal fractions were prepared as described above and resuspended in a medium consisting of 0.25M-sucrose–10mM-imidazole–HCl, pH7.4. The basic incubation medium for measuring ^{45}Ca accumulation consisted of 500 μl of sucrose–imidazole medium containing $^{45}\text{CaCl}_2$ (0.2 $\mu\text{Ci/ml}$, at a total calcium concentration of 0.1mM). The incubations at 37°C were started by the addition of a suitable portion of the fraction, containing approx. 100 μg of protein, and stopped by filtration through a Millipore filter (0.45 μm pore size). The filters were then washed with 5ml of medium, dried and counted in toluene containing 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (5g/l) in a Packard Tri-Carb liquid-scintillation spectrometer.

Measurement of intracellular, extracellular and ^{45}Ca space in the parotid. Parotid pieces prepared as described above were transferred to 10ml conical flasks (two pieces per flask) containing 1.5ml of Krebs

bicarbonate medium and preincubated for 30min at 37°C. For measurement of intracellular and extracellular spaces, media were changed for fresh medium containing [^3H]inulin (0.2 $\mu\text{Ci/ml}$) and incubated for 30min at 37°C. The media were then removed and assayed for amylase activity. The pieces were blotted thoroughly, weighed on a torsion balance and then dried overnight in a vacuum desiccator over P_2O_5 and weighed again. The dried tissue was then dissolved in Nuclear-Chicago tissue solubilizer (400 μl) at 50°C and radioactivity counted in methoxyethanol-toluene (2:3, v/v) containing 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (6g/l) and naphthalene (80g/l) in a Nuclear-Chicago Isocap scintillation counter. Samples of the media were also counted for radioactivity in the same system. For measurement of ^{45}Ca spaces the same procedure was used except that pieces were incubated in medium containing [^3H]inulin (2 $\mu\text{Ci/ml}$) and $^{45}\text{CaCl}_2$ (0.2 $\mu\text{Ci/ml}$). Dissolved tissue and media were assayed for ^3H and ^{45}Ca simultaneously. Corrections for quenching were made using external standardization.

Measurement of the subcellular distribution of ^{45}Ca in the parotid incubated in ^{45}Ca . Two different procedures were used; in the first, parotid pieces were incubated in Krebs bicarbonate medium containing 0.5mM- Ca^{2+} for 30min at 37°C. The medium was then changed for fresh medium containing $^{45}\text{Ca}^{2+}$ (2 $\mu\text{Ci/ml}$) and incubated at 37°C in the presence or absence of adrenaline (10 μM). In the second procedure, parotid pieces were initially incubated in Krebs bicarbonate medium containing $^{45}\text{Ca}^{2+}$ (2 $\mu\text{Ci/ml}$) for 45min; after brief washing the media were changed for fresh medium containing either 0 or 0.5mM- Ca^{2+} (non-radioactive) and incubated at 37°C in the presence or absence of adrenaline (10 μM). After incubation, the medium was removed, the flask put on ice and 5ml of ice-cold sucrose–Tris–EDTA medium added. Tissue homogenization and isolation of fractions were then performed as described above and the final pellets suspended in 250 μl of the sucrose–Tris–EDTA medium. Samples of the pellets were counted in Packard Instagel on a Packard Tri-Carb liquid-scintillation spectrometer. The amount of protein in each pellet was measured by the method of Lowry *et al.* (1951).

All results quoted in the text are the mean $\pm$ S.E.M. for the number of determinations given in parentheses.

Results

Amylase release

Fig. 1(a) shows that parotid glands perfused *in vitro* respond to adrenaline (10 μM) with a prompt increase in the rate of release of amylase; on removal of the stimulus the rate of amylase release

Fig. 1. Effect of extracellular Ca^{2+} concentration on adrenaline-stimulated amylase release from perfused parotid. Parotid glands were perfused as described in the text with Krebs bicarbonate medium containing 5 mM- β -hydroxybutyrate and the following additions were made at the times given. (a) 0–20 min, no addition; 20–40 min, 10 μM -adrenaline; 40–60 min, no addition. (b) 0–60 min, no Ca^{2+} + 0.2 mM-EGTA; 60–80 min, no Ca^{2+} + 0.2 mM EGTA + 10 μM -adrenaline; 80–100 min, 2.6 mM- Ca^{2+} + 10 μM adrenaline; 100–130 min, no additions. Amylase in the perfusates was measured as described in the text.

Fig. 2. Respiratory control of parotid mitochondria

Mitochondria (0.6 mg of protein) were incubated in 0.8 ml of medium consisting of 0.25 M-sucrose, 10 mM-Tris-HCl (pH 7.4), 0.4 mM-EGTA, 140 mM-KCl, 25 mM-phosphate and 1% (w/v) bovine serum albumin, at 30°C. Where indicated 5 mM-sodium succinate, 2.5 mM-sodium glutamate + 2.5 mM-sodium malate, 0.2 mM-ADP or 50 μM -2,4-dinitrophenol were added. O_2 consumption was measured as described in the text. Dnp-OH, 2,4-dinitrophenol.

returned to the basal value within 10 min. Fig. 1(b) shows that the stimulation of amylase release by adrenaline did not require the presence of extracellular Ca^{2+} . In this experiment the parotid was pre-perfused with Ca^{2+} -free medium containing 0.2 mM-EGTA [ethanedioxybis(ethylamine)tetraacetic acid] for 1 h before addition of adrenaline. Even after more severe conditions of Ca^{2+} depletion, i.e. pre-perfusion for 3 h with Ca^{2+} -free medium containing 2 mM-EGTA, the secretory response to adrenaline, although impaired, was not abolished (results not shown).

Characterization of subcellular fractions

The method used for the preparation of mitochondrial and microsomal fractions from the parotid is based on those of Feinstein & Schramm (1970) for mitochondria and Selinger *et al.* (1970) for microsomal fractions.

The functional integrity of the mitochondria was assessed by conventional methods. Rates of O_2 uptake measured with a Clark oxygen electrode are shown in Fig. 2. The rates of O_2 uptake in the presence of succinate and glutamate plus malate as substrate are similar to those reported by Feinstein & Schramm (1970). Fig. 2 shows that the mitochondria were tightly coupled and that respiration was stimulated by the uncoupler 2,4-dinitrophenol. Respiratory control ratios of 8.5 for succinate-linked respiration and of 6.3 for glutamate-malate-linked respiration were found.

No attempt was made to separate the zymogen granules which sedimented with mitochondria under these conditions. Experiments were performed to

assess the contribution of zymogen granules to the Ca^{2+} -accumulating properties of the mitochondrial fraction. A zymogen-granule-enriched fraction from rat parotid was prepared by the method of Feinstein & Schramm (1970). The contamination of this fraction by mitochondria was assessed by measurement of cytochrome oxidase; the specific activity of cytochrome oxidase in the granule fraction was 33% of that in the mitochondria, and the rate of Ca^{2+} accumulation by the granule fraction was decreased to approximately the same extent as the decrease in mitochondrial content. Moreover this residual Ca^{2+} accumulation by the granule fraction showed the major characteristic of mitochondrial Ca^{2+} uptake, i.e. it was increased by respiratory substrates and this increase was abolished by uncoupling agent. Thus it seems probable that the Ca^{2+} uptake in the mitochondrial fraction is into the mitochondria rather than the zymogen granules.

The contamination of the microsomal fraction by mitochondria was assessed by the mitochondrial marker glutamate dehydrogenase; the activity of glutamate dehydrogenase was 39 units/g of protein in the mitochondrial fraction and 0.9 unit/g of protein in the microsomal fraction. Thus the microsomal fraction contains insufficient mitochondria to account for rates of Ca^{2+} uptake by this fraction. Moreover the characteristics of Ca^{2+} uptake by the two systems are dissimilar (see below).

Ca^{2+} uptake by mitochondrial and microsomal fractions

The time-course studies of ^{45}Ca uptake by mitochondrial and microsomal fractions showed that

uptake was virtually complete in 15 min (Fig. 3). Control experiments showed that in the mitochondrial fraction, uptake under these conditions ceased when virtually all the ^{45}Ca was taken up from the medium.

Fig. 3. Time-course of ^{45}Ca uptake by isolated parotid mitochondria and microsomal fractions

Mitochondrial and microsomal fractions were prepared from rat parotids as described under 'Methods', and incubated in sucrose-imidazole medium containing ^{45}Ca (0.1 mM; 0.2 $\mu\text{Ci}/\text{ml}$) in the presence or absence of 4 mM-Mg-ATP. The incubations were terminated by filtration on Millipore membranes and the ^{45}Ca taken up by the fractions was determined by counting the radioactivity retained by the filters. $\circ$, Mitochondria, no additions; $\bullet$, mitochondria + 4 mM-Mg-ATP; $\triangle$, microsomes, no additions; $\blacktriangle$, microsomes + 4 mM-Mg-ATP. Vertical bars represent ± 1 S.E.M.

Table 1. ^{45}Ca uptake by mitochondrial and microsomal fractions from the parotid

Mitochondrial and microsomal fractions were prepared from rat parotids as described under 'Methods', and incubated for 6 min at 37°C in sucrose-imidazole medium containing ^{45}Ca (0.1 mM; 0.2 $\mu\text{Ci}/\text{ml}$) and the additions shown. The incubations were terminated by filtration on Millipore membranes, and the ^{45}Ca taken up by the fractions was determined by counting the radioactivity retained by the filters. Results are given as the mean $\pm$ S.E.M. for 10 observations. Levels of significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for differences from control with same addition (same line). † $P < 0.05$; †† $P < 0.001$ for difference from control sample with same energy source (same column).

		Uptake of ^{45}Ca (nmol/min per mg of protein)		
Fraction	Additions	Control	+Mg-ATP (4 mM)	+Mg-ADP (4 mM)+ succinate (5 mM)
Mitochondrial	None	0.70 $\pm$ 0.15	24.48 $\pm$ 1.02***	18.72 $\pm$ 3.4***
	Oligomycin (2 $\mu\text{g}/\text{ml}$)	0.77 $\pm$ 0.12	0.60 $\pm$ 0.14††	12.42 $\pm$ 1.8***
	Carbonyl cyanide <i>m</i> -chlorophenyl-hydrazone (40 ng/ml)	0.80 $\pm$ 0.16	10.77 $\pm$ 1.16***††	0.66 $\pm$ 0.26††
	Ni^{2+} (1 mM)	0.54 $\pm$ 0.12	1.05 $\pm$ 0.05***††	—
Microsomal	None	1.73 $\pm$ 0.09	5.41 $\pm$ 0.81**	1.71 $\pm$ 0.26
	Oligomycin (2 $\mu\text{g}/\text{ml}$)	1.98 $\pm$ 0.19	3.31 $\pm$ 0.56*††	1.66 $\pm$ 0.10
	Carbonyl cyanide <i>m</i> -chlorophenyl-hydrazone (40 ng/ml)	1.83 $\pm$ 0.09	4.14 $\pm$ 0.63**	1.76 $\pm$ 0.02
	Ni^{2+} (1 mM)	1.30 $\pm$ 0.08	3.30 $\pm$ 0.44***†	—

Table 2. *Effect of adrenaline on incorporation of ⁴⁵Ca into mitochondria and microsomal fractions by parotid incubated with ⁴⁵Ca*

Parotid pieces were preincubated for 30 min in bicarbonate medium and then incubated for 20 min in medium containing ⁴⁵Ca (0.5 mM; 2 μ Ci/ml) in the presence or absence of adrenaline (10 μ M). After incubation the tissue was homogenized, and mitochondrial and microsomal fractions were obtained and analysed for ⁴⁵Ca as described under 'Methods'. Results are given as the mean $\pm$ S.E.M. for 18 observations. **P* < 0.001 for difference from control.

Fraction	Incubation condition	⁴⁵ Ca in pellet (nmol/mg of protein)
Mitochondrial	Control	0.51 $\pm$ 0.03
	Adrenaline (10 μ M)	0.70 $\pm$ 0.04*
Microsomal	Control	0.73 $\pm$ 0.06
	Adrenaline (10 μ M)	0.82 $\pm$ 0.08

In the microsomal fraction uptake was arrested when the ATP had been used up by the action of ATPase (adenosine triphosphatase) in this fraction. An incubation time of 6 min was used in subsequent studies of ⁴⁵Ca accumulation by these fractions. Table 1 summarizes data that show that the ⁴⁵Ca-accumulating properties of these two fractions show marked differences. The mitochondrial ⁴⁵Ca uptake is stimulated by ATP or by ADP plus succinate. ATP-driven ⁴⁵Ca uptake is totally abolished by oligomycin and partially decreased by carbonyl cyanide *m*-chlorophenylhydrazone, this latter effect perhaps being due to activation of ATPase activity by the uncoupler. The ⁴⁵Ca uptake stimulated by ADP+succinate, on the other hand, is not affected by oligomycin but is totally blocked by carbonyl cyanide *m*-chlorophenylhydrazone. Uptake of ⁴⁵Ca by the

Table 3. *Effect of Ni²⁺ on incorporation of ⁴⁵Ca in mitochondria and microsomal fractions of parotid incubated with ⁴⁵Ca in the presence or absence of adrenaline*

Parotid pieces were preincubated for 90 min in bicarbonate medium and then incubated for 20 min in medium containing ⁴⁵Ca (0.5 mM, 2 μ Ci/ml) in the presence or absence of adrenaline (10 μ M). Ni²⁺ was added at the concentrations shown in the table to both preincubation and incubation media. After incubation the tissue was homogenized, and mitochondrial and microsomal fractions were obtained and analysed for ⁴⁵Ca as described under 'Methods'. Results are given as the mean $\pm$ S.E.M. Numbers in parentheses denote the number of observations contributing to means. Levels of significance: **P* < 0.01; ***P* < 0.001 (for difference from control at same Ni²⁺ concentrations); †*P* < 0.02; ††*P* < 0.01; †††*P* < 0.001 [for difference from sample without Ni²⁺ (same line)].

Fraction	Incubation condition	⁴⁵ Ca in pellet (nmol/mg of protein)				
		[Ni ²⁺] (mM)	...	0	0.5	2.0
Mitochondrial	Control			0.31±0.01	0.21±0.03††	0.13±0.01†††
	Adrenaline (10μM)			0.47±0.03**	0.39±0.04*	0.26±0.05††
Microsomal	Control			0.88±0.07	0.57±0.10†	0.30±0.04†††
	Adrenaline (10μM)			0.84±0.09 (15)	0.65±0.14 (10)	0.31±0.06†† (5)

Table 4. *Effect of adrenaline on retention of ⁴⁵Ca by mitochondria and the microsomal fraction from parotid previously incubated with ⁴⁵Ca*

Parotid pieces were preincubated in bicarbonate medium containing ⁴⁵Ca (0.5 mM; 2 μ Ci/ml) for 40 min; the pieces were washed and then transferred to bicarbonate medium containing 0 or 0.5 mM-Ca²⁺ (non-radioactive) and incubated for 20 min in the presence or absence of adrenaline (10 μ M). After incubation the pieces were homogenized, and the mitochondrial and microsomal fractions were prepared and analysed for ⁴⁵Ca as described under 'Methods'. Results are given as the mean $\pm$ S.E.M. for 12 observations. Levels of significance: **P* < 0.05; ***P* < 0.02; ****P* < 0.01 for difference from control; †*P* < 0.001 for difference from sample incubated in the absence of extracellular Ca²⁺.

		⁴⁵ Ca in pellet (nmol/mg of protein)			
Fraction	Incubation condition	Extracellular [Ca ²⁺] (mM)	...	0	0.5
Mitochondrial	Control			0.12 ± 0.01	0.50 ± 0.03†
	Adrenaline (10 μM)			0.09 ± 0.01*	0.38 ± 0.02***†
Microsomal	Control			0.13 ± 0.02	0.39 ± 0.02†
	Adrenaline (10 μM)			0.07 ± 0.01**	0.29 ± 0.02***†

microsomal fraction, however, shows only ATP-dependence. Succinate plus ADP does not stimulate ^{45}Ca uptake, and oligomycin and carbonyl cyanide *m*-chlorophenylhydrazone have little effect on the ATP-stimulated uptake, indicating that these fractions contain different ^{45}Ca -uptake systems. In both fractions, the uptake of ^{45}Ca is inhibited by Ni^{2+} .

Effects of adrenaline on ^{45}Ca distribution in the parotid

In the first series of experiments parotid tissue was incubated, with or without adrenaline, in medium containing ^{45}Ca . Mitochondrial and microsomal fractions from the tissue were then prepared and their ^{45}Ca contents determined. Control experiments were carried out in which ^{45}Ca was added to a homogenate of non-incubated parotid before isolating the fractions; the amount of ^{45}Ca taken up by the fractions during the isolation procedure was found to be 0.27 ± 0.05 (8) nmol of Ca/mg of protein for mitochondria and 0.09 ± 0.01 (8) nmol of Ca/mg of protein for the microsomal fraction. The addition of Ruthenium Red, which blocks ^{45}Ca uptake by mitochondria, to the homogenate did not affect the ^{45}Ca retrieved in this fraction.

The amount of ^{45}Ca recovered in the mitochondrial fraction increased linearly with the length of incubation (results not shown) and was markedly increased by adrenaline (Table 2). No significant effect of adrenaline on incorporation of ^{45}Ca into the microsomal fraction was observed. The amount of ^{45}Ca incorporated into both fractions was decreased by Ni^{2+} in the presence or absence of adrenaline (Table 3). In a second series of experiment, parotid tissue was preloaded with ^{45}Ca and then incubated in the presence or absence of adrenaline, with either 0 or 0.5 mM- Ca^{2+} (non-radioactive) in the medium. The results of these experiments are given in Table 4. The presence of 0.5 mM- Ca^{2+} in the incubation medium increased 3–4-fold the retention of ^{45}Ca in both subcellular fractions. Adrenaline caused a marked lowering of the ^{45}Ca retained in both fractions whether or not Ca^{2+} was present during the incubation.

Accumulation of ^{45}Ca by the parotid

The total water space of parotid tissue was found to be 0.72 ± 0.02 (6) ml/g wet wt. of tissue. From measurements of the distribution of [^{14}C]sorbitol or [^3H]inulin the intracellular volume was determined as 0.47 ml/g wet wt. of tissue. The ^{45}Ca content of tissue incubated for 0.5 h with 0.5 mM- ^{45}Ca was 0.46 ± 0.01 (32) μmol of ^{45}Ca /g wet wt. of tissue. Of this, ^{45}Ca in extracellular water accounts for 0.15 ± 0.01 μmol /g wet wt. of tissue. In parotid tissue incubated with ^{45}Ca and adrenaline under the same conditions the ^{45}Ca content was increased to $0.51 \pm$

0.01 (11) μmol /g; of this, the extracellular water (which was increased by adrenaline to 0.39 ± 0.05 (14) mg/g wet wt. of tissue without change in the total water content) accounted for 0.14 ± 0.004 (14) μmol /g wet wt. of tissue. The tissue ^{45}Ca in excess of the extracellular space will include ^{45}Ca in intracellular organelles but may also include ^{45}Ca bound to extracellular structures. (The mitochondrial and microsomal fractions we have examined account for only 7 nmol/g in the absence and 9 nmol/g in the presence of adrenaline, assuming the yield of mitochondria and microsomal fraction to be 100%.) Incubation of parotid tissue with 2 mM- Ni^{2+} resulted in a decrease in ^{45}Ca content to 0.31 ± 0.01 (11) μmol /g wet wt. of tissue.

Discussion

Although early studies sought to implicate extracellular Ca^{2+} in the initiation of amylase release by parotid (Selinger & Naim, 1970), later observations suggested that this gland, unlike the islets of Langerhans, is not critically dependent on the presence of extracellular Ca^{2+} for release to be stimulated (Batzri & Selinger, 1973). Our data show that the stimulation of amylase release by adrenaline is not abolished in medium from which Ca^{2+} has been omitted and to which EGTA has been added. In contrast with the lack of dependence on extracellular Ca^{2+} of adrenaline-stimulated amylase release, the release of amylase induced by a high extracellular K^{+} concentration is, however, markedly decreased in the absence of extracellular Ca^{2+} (R. L. Dormer, unpublished work). Adrenaline-stimulated amylase release is, however, inhibited by Ni^{2+} , which suggests that Ni^{2+} not only inhibits the uptake of Ca^{2+} into the cell but also interferes with stimulus–secretion coupling in a manner which may be independent of Ca^{2+} (Dormer *et al.*, 1974).

It seems likely, then, that the cytosolic concentration of Ca^{2+} in the parotid may be principally regulated by intracellular uptake and release mechanisms as suggested for kidney cells by Borle (1972). In muscle cells the Ca^{2+} pump of the sarcoplasmic reticulum plays a major role in the maintenance of the low Ca^{2+} concentration in the cytosol; in liver cells an important role for the accumulation of Ca^{2+} by mitochondria in modifying the internal cellular ionic environment has been postulated (Lehninger, 1970). Borle (1973a) has presented evidence of a model of cellular calcium homeostasis in which mitochondria play a determinant role.

In secretory cells, there is little direct evidence as to the mechanisms controlling the cytosolic Ca^{2+} concentration. Selinger *et al.* (1970) demonstrated that microsomal fractions from parotid and submaxillary glands show ATP-dependent Ca^{2+} uptake and presented evidence that this Ca^{2+} pump was

distinct from mitochondrial Ca²⁺ uptake. In pancreatic β -cells Malaisse (1973) has presented evidence that effects of glucose on Ca²⁺ metabolism may be exerted at the cell membrane, whereas effects of theophylline, an inhibitor of cyclic AMP phospho-diesterase, may be on the translocation of Ca²⁺ from some intracellular organelle(s). The results presented here indicate that both the mitochondrial and microsomal fractions may be of importance in Ca²⁺ metabolism of the parotid. First, studies with isolated mitochondrial and microsomal fractions show that both fractions possess the ability to accumulate ⁴⁵Ca. The mitochondrial accumulation can be supported either by ATP or by respiration (ADP+succinate) and appears similar to the systems established for liver mitochondria (reviewed by Lehninger *et al.*, 1967). The observations of Feinstein & Schramm (1970) that parotid mitochondrial respiration is inhibited by Ca²⁺ may explain why respiration-linked Ca²⁺ uptake by these mitochondria was lower than the ATP-linked uptake. ATP-supported ⁴⁵Ca uptake is inhibited by a mitochondrial ATPase inhibitor, whereas respiration-driven ⁴⁵Ca uptake is inhibited by an uncoupler of oxidative phosphorylation. The microsomal ⁴⁵Ca-uptake system, however, is solely ATP-dependent. Secondly, on incubation of parotid tissue with ⁴⁵Ca, appreciable radioactivity is subsequently found in both these fractions. Thirdly, we have obtained evidence that the stimulation of amylase release by adrenaline is associated with changes in the handling of ⁴⁵Ca by these fractions. We suggest the following interpretation of the observed changes.

The absence of a requirement for extracellular Ca²⁺ in the stimulation of amylase release by adrenaline suggests that the transfer of Ca²⁺ into the cell is not the sole parameter affected by adrenaline. The interaction of adrenaline with the cell appears to increase not only the transfer of Ca²⁺ across the plasma membrane from the extracellular medium to the cytosol, but also from the mitochondrial and microsomal compartment to the cytosol. The resulting increase in the cytosolic Ca²⁺ concentration triggers secretion and also increases the rate of uptake of Ca²⁺ into subcellular compartments. The results of these changes on the tissue distribution of ⁴⁵Ca will depend on the precise experimental conditions. Thus tissue preincubated with ⁴⁵Ca will accumulate ⁴⁵Ca in the mitochondrial and microsomal fractions. Increased efflux of ⁴⁵Ca from these fractions in response to adrenaline will be manifested as a decreased ⁴⁵Ca content on subsequent isolation. However, when ⁴⁵Ca and adrenaline are present together, the increased cytosolic concentration of ⁴⁵Ca resulting from increased influx of ⁴⁵Ca across the plasma membrane will increase the mitochondrial uptake of ⁴⁵Ca, although net transfer of Ca²⁺ will be from mitochondria to cytosol, hence the increased

mitochondrial ⁴⁵Ca content found. The lack of effect of adrenaline on the microsomal ⁴⁵Ca content under these conditions suggests that microsomal Ca²⁺ uptake is unaffected by changes in the cytosolic Ca²⁺ concentration or, alternatively, that increased uptake of ⁴⁵Ca is balanced out by the increased rate of efflux. In essence, then, we believe that the simplest explanation consistent with our data is that the action of adrenaline is exerted at the loci of plasma membrane, mitochondria membrane and microsomal membrane to increase in each case the rate of translocation of Ca²⁺ to the cytosol.

The main conclusion we draw from these studies is that changes of Ca²⁺ handling by parotid tissue occur on stimulation of amylase release with adrenaline. We have no direct evidence for the mechanism of these effects. In view of the finding that the stimulation of amylase release by adrenaline is mediated by β -adrenergic receptors (Batzri *et al.*, 1971) it is possible that cyclic AMP could itself influence the intracellular distribution of Ca²⁺. However, Harfield & Tenenhouse (1973) have shown that the adrenaline-induced rise in parotid cyclic AMP was greatly decreased by the omission of extracellular Ca²⁺, whereas, in agreement with our findings, adrenaline-stimulated amylase release was not inhibited; they suggest that this nucleotide may not be an essential intermediate in the adrenaline-stimulated secretion process.

The secretory activity of a number of cell types has been shown to be inhibited by Ni²⁺, and it was suggested that Ni²⁺ was acting to interfere with Ca²⁺-dependent processes involved in exocytosis (Dormer *et al.*, 1974). In the present study we find that Ni²⁺ does indeed block Ca²⁺ uptake by the mitochondrial and microsomal fractions from the parotid. It is not known, however, whether this action is the basis for Ni²⁺ inhibition of release; it seems likely that Ni²⁺ may interfere with the action of Ca²⁺ on the release process in addition to its effect on Ca²⁺ translocation. The nature of these action(s) of Ca²⁺ has not yet been defined.

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