Volume 162, number 2 FEBS 0895 October 1983

ATP-dependent calcium sequestration and calcium/ATP stoichiometry in isolated microsomes from guinea pig parotid glands

Andreas Immelmann and Hans-Dieter Söling

Abteilung für Klinische Biochemie, Zentrum Innere Medizin der Universität Göttingen, Humboldtallee 1, D-3400 Göttingen, FRG

Received 18 August 1983

ATP-dependent calcium uptake was studied in isolated guinea pig parotid gland microsomes. The apparent K_m for free Ca^{2+} was 0.41 μM, the apparent K_m for $ATP \cdot Mg^{2-}$ 0.23 mM. The pH optimum was 6.8–7.0. Subfractionation of the microsomes revealed that the highest specific uptake activity resided in a rather dense fraction of the endoplasmic reticulum. The calcium uptake/ATPase stoichiometry was determined in the absence of exogenous magnesium in the submicrosomal fractions. It ranged from 1–2. It is concluded that in vivo the stoichiometry is the same as in sarcoplasmic reticulum, namely 2.

Parotid gland *Microsome* *Calcium uptake*

1. INTRODUCTION

Microsomes from exocrine and endocrine secretory organs have been shown to possess the capacity for ATP-dependent accumulation of calcium [1–7]. However, the physiological role of this process has been questioned as the apparent K_m for calcium has been found usually to be $\geqslant 10^{-6}$ M. In [5], a K_m for calcium uptake by isolated submandibular microsomes of 25 μM was reported.

Except for a report in which the calcium/ATP stoichiometry for isolated rat pancreatic microsomes has been indirectly estimated [2], no data concerning this stoichiometry are available for mammalian secretory organs. The interference of measurements of this stoichiometry with the high activity of Mg^{2+}-ATPase present in the microsomes and contaminating plasma membranes has contributed to this.

Here, we have examined the kinetic parameters of the ATP-driven calcium uptake in isolated guinea pig parotid gland microsomes and determined the calcium/ATP stoichiometry in microsomal subfractions after removal of most of the contaminating plasma membrane material.

2. MATERIALS AND METHODS

Fed male guinea pigs (180–250 g) of the Pirbright-White strain (Lippische Versuchstierzucht, Extertal) were used. The animals were anesthetized by intraperitoneal injection of 90 mg nembutal/kg. The abdomen and thorax were opened and the animals exsanguinated. The parotid glands were removed and suspended in medium A (NaCl 120 mM; KCl 5 mM; $MgCl_2$ 1.2 mM; $CaCl_2$ 3 mM; 3-hydroxybutyrate 5 mM; Tris–HCl 20 mM, pH 7.4) at 37°C. Parotid gland lobules were subsequently prepared under a stereomicroscope and incubated in medium A for 30 min at 37°C in the presence of O_2. The lobules were then weighed and homogenized with a 10-fold (v/w) volume of medium B (sucrose 0.3 M; ascorbic acid 2 mM; Hepes 20 mM, pH 7.2) in a glass/Teflon Potter-homogenizer. The homogenate was spun for 10 min at 1000 × g_{max} and the sediment discarded. The supernatant was centri-

Published by Elsevier Science Publishers B.V.

fuged for 15 min at 12000 × g_{max} which removed almost completely mitochondria and some of the heavy microsomes. The supernatant was spun for 70 min at 50000 × g_{max}. The sediment representing the microsomal fraction was suspended in medium C (sucrose 0.3 M; histidine–HCl 10 mM, pH 6.8) (usually in 1 ml for lobules from 2 animals). Protein was 1.5–3 mg/ml. All steps were carried out in the cold.

2.1. *Measurement of ATP-driven calcium uptake*

The standard incubation contained (final conc.): KCl 120 mM; histidine–HCl 50 mM; $MgCl_2$ 5 mM; sodium azide 10 mM; ATP 5 mM; $^{45}CaCl_2$ 0.1 mM (specific act. 3 mCi/mmol). The final pH was 6.8 at 37°C. The reaction was started by addition of microsomes (final conc. 100 μg protein/ml). At the indicated time point 200 μl aliquots were removed and pipetted onto cellulose acetate filters (type SM 66, 0.2 μm pore size, Sartorius, Göttingen) pre-soaked in 0.5% albumin solution. The medium was removed by suction and unspecifically adsorbed ^{45}Ca removed by washing with 2.5 ml 100 mM $CaCl_2$. The filters were transferred into glass counting vials, dried and dissolved with 4 ml dioxan. Thereafter, 0.6 ml water and 5 ml Unisolve I scintillator were added and the samples counted in a Mark III liquid scintillation spectrometer. The calcium concentration in the incubation medium was also controlled by atomic absorption spectroscopy.

When the concentration of free calcium was varied this was done by adding varying concentrations of EGTA. To calculate the free calcium concentration the association constants of calcium as well as of magnesium with EGTA, ATP and oxalate were considered. The association constants with EGTA were from [8], those with ATP from [9] and those with oxalate from [10]. On the basis of these constants the free calcium concentration was calculated on a Wang 2000 desk computer using an iterative calculation program developed in our laboratory by Dr J. Kleineke.

2.2. *Subfractionation of microsomes*

Microsomes were isolated and suspended as given above. The suspension (1.2 ml) was layered on top of a discontinuous sucrose gradient consisting of 1.2 ml each of 43% (w/w), 38% and 34% sucrose. It was centrifuged in a SW 65 swing-out rotor (Beckman Instruments) for 120 min at 370000 × g_{max}. Membrane fractions were isolated from the sample/34% interphase (IF 1), from the 34%/38% interface (IF 2), and from the 38%/43% interphase (IF 3). In addition the pellet fraction (PF) was collected. All fractions were diluted with 5 ml medium C and spun again for 60 min at 100000 × g_{max}. The pellets were resuspended in 0.2–0.5 ml medium C.

2.3. *Determination of ATPase and marker enzyme activities*

ATPase activity was determined as in [11] after determination of the specific activity of [γ-^{32}P]ATP used. 5'-Nucleotidase was determined as in [12], alkaline phosphodiesterase by a modification of the method in [13]. The incubation mix contained (final concentrations): Tris–HCl 60 mM, pH 9.0; $CaCl_2$ 12.5 mM; thymidine-5'-monophospho-nitrophenylester 2.5 mM. The test was started by addition of an aliquot of the membrane fraction. Thiamine pyrophosphatase was determined as in [14]. RNA was measured according to [15].

2.4. *Materials*

Biochemicals and auxiliary enzymes came from the Boehringer Mannheim Corp. (Mannheim-Waldhof); bovine serum albumin and 3-hydroxybutyrate were purchased from Behringwerke (Marburg) and Biomol (Ilvesheim), respectively. All other chemicals came from E. Merck AG (Darmstadt). $^{45}CaCl_2$ and [γ-^{32}P]ATP were from Amersham-Buchler Co. (Braunschweig) and scintillation cocktail Unisolve I from Zinsser (Frankfurt).

3. RESULTS

3.1. *Calcium uptake plus and minus oxalate*

Oxalate supported calcium uptake was almost linear with time over the 20 min observation time, whereas a significant calcium uptake in the absence of oxalate occurred only during the first 2 min (fig.1). The oxalate supported calcium uptake rate calculated from the first 10 min was 29.4 ± 12.5 nmol.mg protein^{-1}.min^{-1} at 37°C (mean ± SEM from 6 different microsomal preparations).

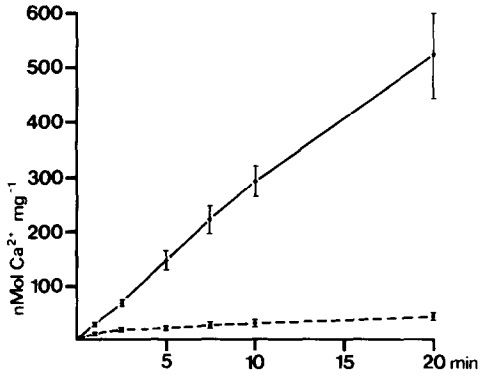

Fig.1. Oxalate-dependent and oxalate-independent (···) calcium uptake by isolated guinea pig parotid gland microsomes. The uptake was measured at 37°C, pH 6.8 and 0.1 mM calcium. Further details in section 2.

3.2. *Dependence of calcium uptake on pH and ATP*

The pH dependency is given in fig.2. The optimum is around pH 7.0 in contrast to most plasma membrane transport systems which show an optimum at pH >8. The dependence on the concentration of ATP at saturating calcium concentration is given in fig.3. The reaction follows a hyperbolic relationship and the apparent K_m for ATP is 0.23 ± 0.016 mM (mean ± SEM from 5 different microsomal preparations).

3.3. *Dependence of calcium uptake on the free calcium concentration*

The concentration of free calcium was varied by changing the amount of EGTA added at a fixed total calcium concentration. The uptake velocity was hyperbolically related to the concentration of free calcium (fig.4), and the apparent K_m for free

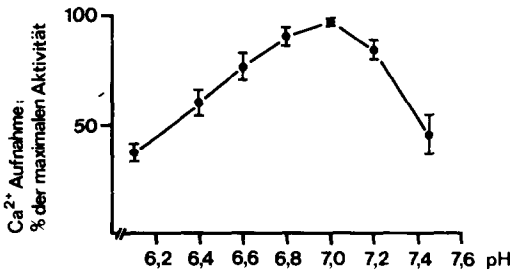

Fig.2. pH-Dependence of oxalate supported calcium uptake by isolated guinea pig parotid gland microsomes.

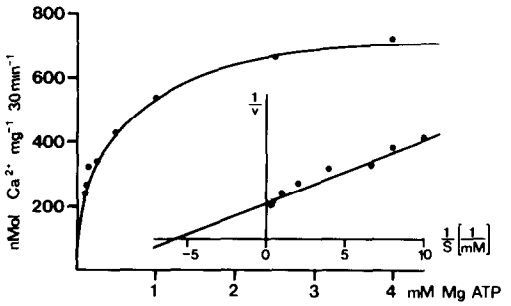

Fig.3. Dependence of calcium uptake velocity by isolated guinea pig parotid gland microsomes on the concentration of ATP·Mg^{2-}. The calcium uptake was measured in the presence of oxalate.

calcium as calculated from the double-reciprocal plot was 4.1 ± 0.6 × 10^{-7} M (mean ± SEM from 5 different microsomal preparations). This value is in reasonable agreement with the value of 2 × 10^{-6} M observed with cat pancreatic microsomes [7].

3.4. *Determination of the calcium/ATP stoichiometry*

To this end microsomes were incubated in parallel for the measurement of calcium uptake and for the determination of ATP-hydrolysis. The incubation conditions were as in fig.1 except that ATP was 0.2 mM. In each experiment one sample contained ^{45}Ca, the other sample [γ-^{32}P]ATP. To measure this stoichiometry the calcium-dependent ATPase activity had to be discriminated from the calcium-independent (basal) ATPase activity. This 'basal' activity was determined as the activity re-

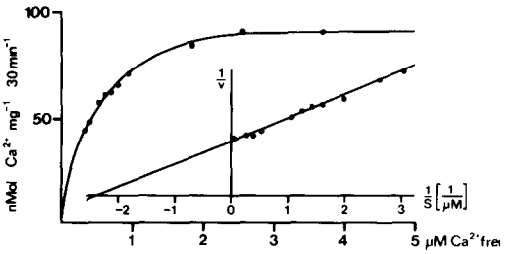

Fig.4. Dependence of oxalate supported calcium uptake by isolated guinea pig parotid gland microsomes on free [Ca^{2+}]. The measurements were performed at 37°C and pH 6.8. The free [Ca^{2+}] was varied with EGTA as in section 2.

maining in the presence of EGTA. As this 'basal' activity was considerably higher than the calcium-specific ATPase activity (calculated as the difference between total ATPase activity and 'basal' ATPase) calcium uptake and ATPase activity were measured without added magnesium. The omission of external magnesium reduced the calcium uptake rate by about 80%. Addition of EDTA to remove endogenous magnesium led to a complete loss of calcium accumulating activity (not shown).

In the absence of external magnesium the 'basal' ATPase activity dropped to about 30% of the calcium-ATPase activity. But even under these conditions the calcium/ATP stoichiometry for calcium uptake was of the order of 1/15, indicating a considerable contamination of our preparation with unsealed or leaky membrane material. As we suspected that this could be at least in part due to contamination with plasma membrane material we decided to subfractionate the parotid gland microsomes. Calcium uptake and ATPase activities were again determined without addition of magnesium to the medium. The results are summarized in table 1. It is obvious that fraction IF 1 contains predominantly plasma membranes as indicated by the high specific activities of 5′-nucleotidase and alkaline phosphodiesterase. It contains also the bulk of the Golgi membranes as indicated by the relatively high specific activity of thiamine pyrophosphatase. Our initial suspicion that a considerable part of the measured 'basal' and 'calcium-dependent' ATPase activities resulted from contamination with plasma membranes was confirmed by the data: IF 1 contains by far the highest specific activities for both ATPases. Nevertheless, the calcium uptake was minimal as indicated by the low calcium/ATPase ratio.

Assuming that the activities of 5′-nucleotidase and alkaline phosphodiesterase in fractions IF 2, IF 3, and PF result from a residual contamination with plasma membranes, the measured calcium ATPase values can be corrected for the contamination with plasma membrane calcium ATPase. The corrected values are given in table 2. Fractions IF 3

Table 1

Activities of marker enzymes, RNA, basal and calcium-dependent ATPases, and calcium uptake in whole guinea pig parotid gland microsomes and in microsomal subfractions

Measured parameter	Microsomes	IF 1	IF 2	IF 3	PF
5′-Nucleotidase	100	236	28	16	13
Alkaline phosphodiesterase	157	340	89	50	24
Thiamine pyrophosphatase	22	42	17	15	17
RNA	104	39	104	131	246
Calcium-ATPase	552	1058	273	174	97
Basal ATPase	197	182	33	32	23
Calcium uptake	35	21	66	112	63

The results are expressed as follows: 5′-nucleotidase, μg P_i.mg protein^{-1}.30 min^{-1}; alkaline phosphodiesterase, mU/mg protein; thiamine pyrophosphatase, μg P_i.mg protein^{-1}.60 min^{-1}; RNA, μg/mg protein; calcium-dependent and basal ATPase, nmol P_i.mg protein^{-1}.10 min^{-1}; calcium uptake, nmol.mg protein^{-1}.10 min^{-1}. For further details see section 2. Mean values from 5 expt

Table 2

Calculation of the calcium uptake/calcium-ATPase ratio in submicrosomal fractions after correction for contamination with plasma membrane calcium-ATPase

	IF 2	IF 3	PF
Contamination with plasma membrane (5′-nucleotidase) (percent of specific activity in IF 1)	11.8	6.6	5.3
Contamination with plasma membrane (alkaline phosphodiesterase) (percent of specific activity in IF 1)	26.0	14.8	7.1
Contamination with plasma membrane calculated as mean value from 5′-nucleotidase and alkaline phosphodiesterase contamination (percent of specific activity in IF 1)	18.9	10.7	6.2
Calcium-ATPase corrected for contamination with plasma membrane calcium-ATPase (specific activity)	74	61	31
Calcium uptake / Corrected calcium-ATPase	0.89	1.83	2.02

Contamination was calculated from the data presented in table 1. For simplicity IF 1 was taken as representing plasma membranes

and PF containing the highest specific RNA content and the lowest specific activities of plasma membrane enzymes exhibit calcium/ATP stoichiometries from 1 (IF 2) to 2 (IF 3 and PF).

Calcium transport into rat parotid microsomes was inhibited by calmodulin antagonists like chlorpromazine and trifluoperazine in [5]. We have obtained similar results: guinea pig parotid gland microsomal calcium uptake was completely abolished by 50–100 μM trifluoperazine (unpublished). However, this cannot be taken as evidence for a calmodulin dependence of microsomal calcium uptake for 2 reasons:

(1) Pre-washing of parotid gland microsomes with up to 10 mM EGTA which should remove most of microsome bound calmodulin, did not significantly affect microsomal calcium uptake;
(2) The inhibition of microsomal calcium uptake by trifluoperazine could not be overcome by even extremely high (200 μg/ml) concentrations of exogenous calmodulin (unpublished).

4. DISCUSSION

In agreement with reports employing rat pancreatic [2] or parotid gland [1,3,4] microsomes, guinea pig parotid gland microsomes possess also an ATP-driven calcium uptake system. Considering the higher temperature used in our experiments the maximum capacity for calcium uptake was in the same range as that observed with rat parotid microsomes [3].

Due to the extremely high Mg-ATPase activity in the plasma membranes and microsomes of parotid glands, the establishment of a calcium/ATP stoichiometry for microsomal calcium uptake had not been successful. However, as shown here, a careful removal of most of the contaminating plasma membrane material and a drastic lowering of the magnesium concentration reduced the unspecific ATPase activity to values which made the measurement of such a stoichiometry possible. According to table 2, one to two calcium ions are translocated per ATP split. A ratio of close to one for rat pancreatic microsomes was indirectly calculated in [2].

Assuming that even under our conditions some of the vesicles had been leaky it seems likely that under in vivo conditions microsomal calcium uptake proceeds with a calcium/ATP stoichiometry of 2 as described for sarcoplasmic reticulum from skeletal muscle [16].

The subfractionation of parotid gland microsomes revealed that the highest calcium uptake capacity resided in a rather dense membrane fraction (heavy endoplasmic reticulum). However, in accordance with data obtained with rat submandibular gland microsomes [5] the maximum calcium uptake capacity did not comigrate with that fraction which exhibited the highest RNA content (fraction PF).

ACKNOWLEDGEMENTS

This work was supported by a grant (grant I/38071) from the Stiftung Volkswagenwerke.

REFERENCES

[1] Selinger, Z., Naim, E. and Lasser, M. (1970) Biochim. Biophys. Acta 203, 326–334.
[2] Ponnapa, B.C., Williams, J.A. and Dormer, J.L. (1981) Am. J. Physiol. 240, 122–129.
[3] Bonis, D. and Rossignol, B. (1982) FEBS Lett. 137, 63–66.
[4] Kanagasuntheram, P. and Teo, T.S. (1982) Biochem. J. 208, 789–794.
[5] Terman, B.I. and Gunter, T.E. (1983) Biochim. Biophys. Acta 730, 151–160.
[6] Preisler, M. and Williams, J.A. (1983) J. Membr. Biol. 73, 137–143.
[7] Kribben, A., Tyrakowskim, T. and Schulz, I. (1982) Am. J. Physiol. 244G, 480–490.
[8] Portzehl, H., Caldwell, P.C. and Rüegg, J.C. (1963) Biochim. Biophys. Acta 79, 581–593.
[9] Alberty, R.A. (1967) J. Biol. Chem. 243, 1337–1343.
[10] Martell, A.E. and Smith, R.M. (1974) Critical Stability Constants, Plenum, London, New York.
[11] Seals, J.R., McDonald, J.M., Bruns, D. and Jarret, L. (1978) Anal. Biochem. 90, 785–795.
[12] Gerlach, U. and Hiby, W. (1974) in: Methoden der Enzymatischen Analyse (Bergmeyer, H.V. ed) vol.2, pp.903–908, Chemie-Verlag, Weinheim.
[14] Bischoff, D., Thran-Thi, T. and Decker, K.F. (1975) Eur. J. Biochem. 51, 353–361.
[14] Morre, D.J. (1971) Methods Enzymol. 22, 138–139.
[15] Fleck, A. and Munro, H.N. (1962) Biochim. Biophys. Acta 55, 571–583.
[16] Hasselbach, W. and Makinose, M. (1961) Biochem. Z. 333, 518–528.