FUNCTIONAL DIFFERENCES BETWEEN K^+-ATPase RICH MEMBRANES ISOLATED FROM RESTING OR STIMULATED RABBIT FUNDIC MUCOSA

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1. Introduction

Gastric HCl is generated at the apical pole of the oxyntic cell [1]. When the cell is stimulated to secrete, extensive morphological transformation occurs. The identifiable apical membrane surface area increases several fold and concurrently a drastic decrease in the density of cytoplasmic tubular and vesicular structures takes place [2,3].

Several years ago, a microsomal preparation capable of H^+ uptake in the presence of K^+, Mg^{2+} and ATP and rich in a K^+–Mg^{2+}-dependent ATPase was isolated from fundic mucosa homogenates [4,5]. Further studies have localized the enzyme to the secretory caniculi of the oxyntic cell [6] and have shown that H^+-accumulation in isolated gastric microsomes is the result of a K^+–H^+ exchange pump mechanism requiring intravesicular K^+ [7]. It seems possible, then, that the microsomes are merely membrane sacs derived from the apical surface so that their intravesicular space corresponds to the canalicular and luminal space of the cell and/or to the intravesicular space of the tubulovesicular structures characteristic of the non-stimulated cell [8]. However, a serious objection to this proposition could be raised in that gastric microsomes require an exogenous K^+-permeability pathway, often provided by the addition of valinomycin to the medium, to achieve and sustain a considerable pH gradient.

We have demonstrated that stimulation of the oxyntic cell results in a 50% decrease in the yield of gastric microsomes with the concomitant appearance in the cell homogenates of a new type of membrane, rich in the K^+-ATPase enzyme but larger and denser than the microsomes [9]. These membranes cosediment with the nuclear and mitochondrial material from which they could be separated, yielding preparations of high K^+-ATPase activity. The analogy between the morphological transformations and the 'flow' of the K^+-ATPase between membranes of different properties (or alternatively the transformation of the membranes that incorporate the K^+-ATPase) led us to propose that the gastric microsomes are directly derived from the tubulovesicular structures while the new membranes are fragments of the expanded apical membrane of the stimulated cell [9]. We report now the expression in these stimulation-associated (s.a.) membranes of high K^+-permeability mechanism that eliminates the need of an exogenous K^+ ionophore to attain efficient H^+-uptake.

Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenyl hydrazone; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris, tris (hydroxymethyl) aminomethane

2. Methods

Stimulation or inhibition of gastric secretion was induced and monitored in paired young New Zealand rabbits by the methods in [10]. The animals were killed by an air embolism, the stomachs were quickly removed and the fundic mucosa was stripped from the muscle coat. The mucosa was minced and homogenized by 15 passes in a Potter-Elvehjem homogenizer in 40 vol. 120 mM mannitol, 40 mM sucrose, 5 mM Pipes, 1 mM EDTA (pH 6.7). The resulting homogenates were fractionated by successive centrifugations into: P_1, the debris and nuclear pellet obtained at 1000 × *g* for 10 min; P_2, the mitochondrial pellet obtained at 9500 × *g* for 10 min; P_3, the microsomal pellet obtained at 100 000 × *g* for 55 min and a final

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supernatant. (After sedimentation of P_1 the pH was adjusted to 7.7 with a 1 M Tris-base solution.) The P_2 pellet from stimulated tissue was further fractionated in a 11–16% Ficoll-400 step gradient (built in 300 mM sucrose–1 mM Tris–HCl (pH 7.7)) centrifuged for 8 h at 120 000 × *g* in a Beckman SW27 rotor. The membranes equilibrating on top of the 16% Ficoll layer (s.a. membranes) were collected and washed free of polymer by dilution in 300 mM sucrose and recentrifugation. Gastric microsomes were obtained from the microsomal pellet from resting tissue homogenates by fractionation in the Ficoll step gradient and recovery from the top of the 11% polymer layer. Alternatively they were obtained, similarly to the microsomes from hog stomach, in a 27–33% sucrose step gradient [11]. All operations were done at 0°C. Protein was determined by the Bradford assay [11]. (K^+–H^+)-ATPase was assayed as in [13] but all the solutions included 0.1 mM ouabain.

Known pH gradients across the vesicle membranes were generated by the succinate–Tris method in [14], and were used to determine the dependence of fluorescence quenching of acridine orange on the ΔpH. A full description of the methods has been given in [11]. Dye quenching was then used to monitor the actual ΔpH-values obtained in the vesicles in the ATP-driven acidification experiments.

Reswelling rates of vesicles suspended in a hypertonic salt medium were measured by the change in the 90° light scattering using a 400 nm incident beam. The rationale for the use of this method to estimate vesicle permeability to solutes has been discussed [15]. All fluorescence and light scattering measurements were performed using a Perkin Elmer-MPF44A spectrofluorimeter.

3. Results

Generation of pH gradients across sealed membrane vesicles by the succinate–Tris method [14] provided a simple way to assess the existence of tightly sealed vesicles within the membrane suspensions. The results (fig.1) suggested that the sealed volumes and degree of dye binding displayed by the s.a. membrane preparations were comparable to those of microsomal preparations. We proceeded therefore to test the ATP-driven H^+-accumulation capability of the s.a. vesicles. For all the systems compared, the dependence of dye uptake on K^+, permeable anions (such as Cl^- or NO_3^-), Mg^{2+} and ATP was confirmed by ion replacement (Na^+ for K^+, maleate for Cl^- or NO_3^-) or omission from the medium (Mg^{2+} and ATP). In the gastric microsomes, in addition, an exogenous K^+-ionophore (i.e., valinomycin) had to be present in the medium to achieve a considerable intravesicular acidification (fig.2A). In the s.a. vesicles intravesicular acidification was spontaneous and independent of valinomycin (fig.2B). Preincubation in KCl increased the initial rate of H^+ uptake for the microsomes in valinomycin-free medium but had no effect in the s.a. vesicles. Using the data of fig.1 as an experimental calibration system, the apparent maximal ΔpH-values achieved were calculated to be ~4.5–5.0 pH units for both the microsomes and the s.a. vesicles. Interestingly, if the actual composition of the cell cytoplasm is approximated by decreasing $[Cl^-]$ to 60 mM a better uptake is observed for the s.a. membranes. In gastric microsomes reduction of $[Cl^-]$ decreased the rate of H^+ uptake and the final ΔpH-values achieved [11]. Addition of CCCP, a H^+ ionophore, resulted in gradient dissipation in the new vesicles only if valinomycin was present in the medium. Nigericin, an ionophore able to transport H^+ and K^+, in contrast, quickly dissipated the gradients in all cases. Table 1 summarizes the dependence of ATPase activity on KCl and

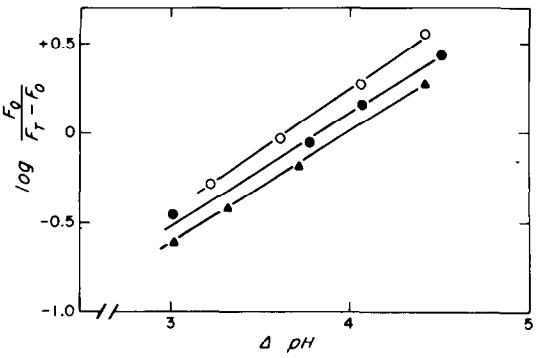

Fig.1. The experimental relationship between the extent of fluorescence quenching and the ΔpH-values generated artificially in the vesicles by the succinate–Tris method. Membrane suspensions were incubated at 36°C in 150 mM KCl, 1 mM $MgSO_4$, 10 mM Na-succinate, 5 μM acridine orange (pH 4.3) for 1 min (enough time to achieve pH equilibration). The pH of the extracellular medium was then quickly raised (t = 0) by addition of a small volume of Tris base. F_T is the total fluorescence measured after dissipation of the ΔpH and F_O is the fluorescence measured at t = 0. Experiments were carried using 20 μg protein/ml of pig (○) or rabbit (●) gastric microsomes or stimulation-associated membranes (▲).

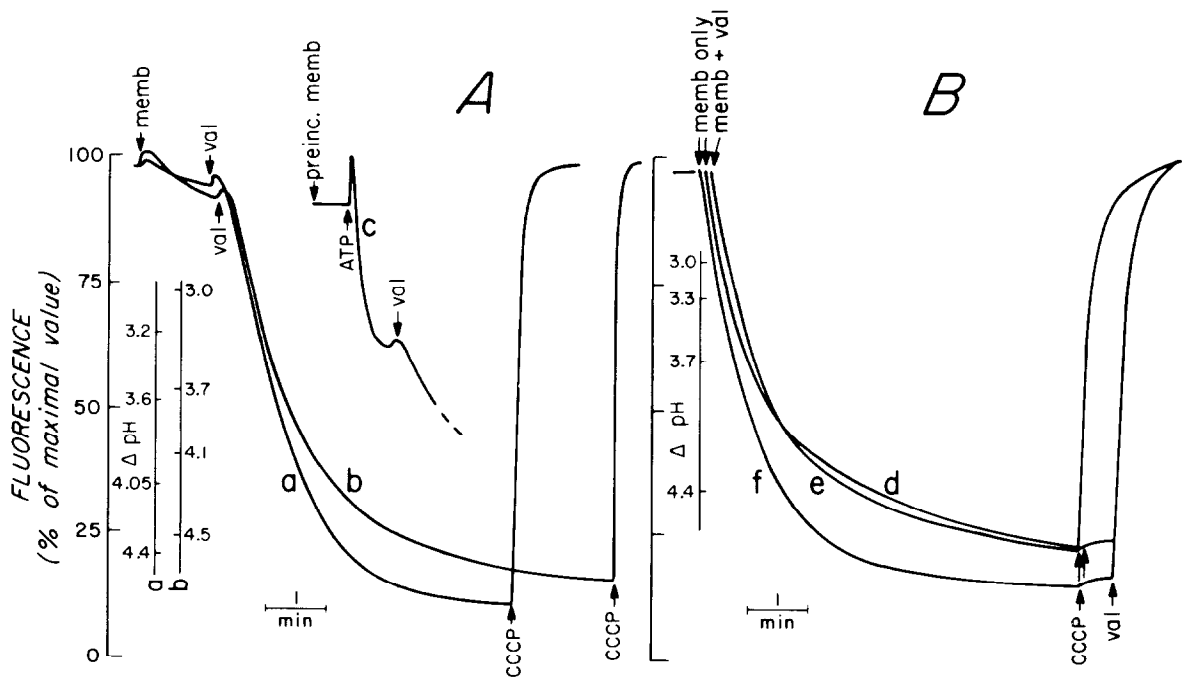

Fig.2. ATP-driven intravesicular acidification in gastric microsomes and s.a. membranes. Experiments were started by addition of a small aliquot of concentrated membrane suspension to the reaction solution (normally 150 mM KCl, 1 mM $MgSO_4$, 1 mM ATP, 10 mM Pipes, 5 μM acridine orange (pH 7.0)) to 20 μg protein/ml a final conc. In the preincubation experiments the membranes were incubated for 1 h at 25°C in ATP free medium and the reactions were started by addition of ATP. On the left (A) the results obtained for pig (a), rabbit (b) or preincubated rabbit microsomes (c) are displayed. Rabbit microsomes isolated in either Ficoll or sucrose gradients yielded identical results. The right side (B) presents the results for s.a. membranes in the absence (d) or presence (e) of valinomycin and a reduced (60 mM) Cl^- concentration (f). To achieve this concentration, 90 mM Cl^- was replaced by 45 mM maleate and 45 mM sucrose. Additions of 5 μM valinomycin (val.) or 10 μM CCCP are indicated by arrows at 36°C. The results of fig.1 have been included as inset calibration scales.

Table 1
ATPase activities for the various gastric membrane preparations

Membrane prep.	Basal ATPase	K^+-Stimulated ATPase	
	Sucrose, 30 mM	150 mM KCl	150 mM KCl + 2.5 μM Val.
Microsomes			
pig	3.3	21.2	44.4
rabbit	20.2	36.4	59.8
s.a. Membranes	12.5	49.4	47.1

ATPase activities are given in μmol P_i mg protein^{-1} . h^{-1}. Membranes (~20 μg protein) were diluted in 1 ml 1 mM $MgSO_4$, 10 mM Pipes (pH 7.0), 0.1 mM ouabain and either sucrose (300 mM) or KCl (150 mM). The samples were warmed to 36°C and the reaction was started by addition of 25 μl 40 mM ATP (pH 7.0) and carried out for 8 min. Basal ATPase activity is defined as that occurring in the absence of K^+ (i.e., 300 mM sucrose); K^+-ATPase activities were obtained by subtracting the basal activity from that measured in the respective KCl media

valinomycin for all the preparations studied. The observed effect of valinomycin on these activities are qualitatively consistent with the observations made in the ATP-driven acidification experiments.

The effect of valinomycin in the microsomal system clearly exemplifies the requirement for intravesicular K^+ in order to achieve efficient H^+ uptake. In the new membrane vesicles, due to the insensitivity of H^+ uptake to K^+-preincubation or to valinomycin, the need for internalized K^+ remained untested. Therefore, permeability properties were assessed by measuring the reswelling rates following exposure of the vesicles to a pulse of hypertonic salt (fig.3). In the case of the s.a. vesicles reswelling is a very fast process independent of valinomycin characterized by $t_{1/2}$ ~30 s (fig.3A). No differences could be measured for the rates of flux of KCl and KNO_3, but when K^+ was replaced by Na^+ or choline, reswelling became much slower. In contrast, microsomes exposed to K^+-salts reswelled slowly in the absence of valinomycin and

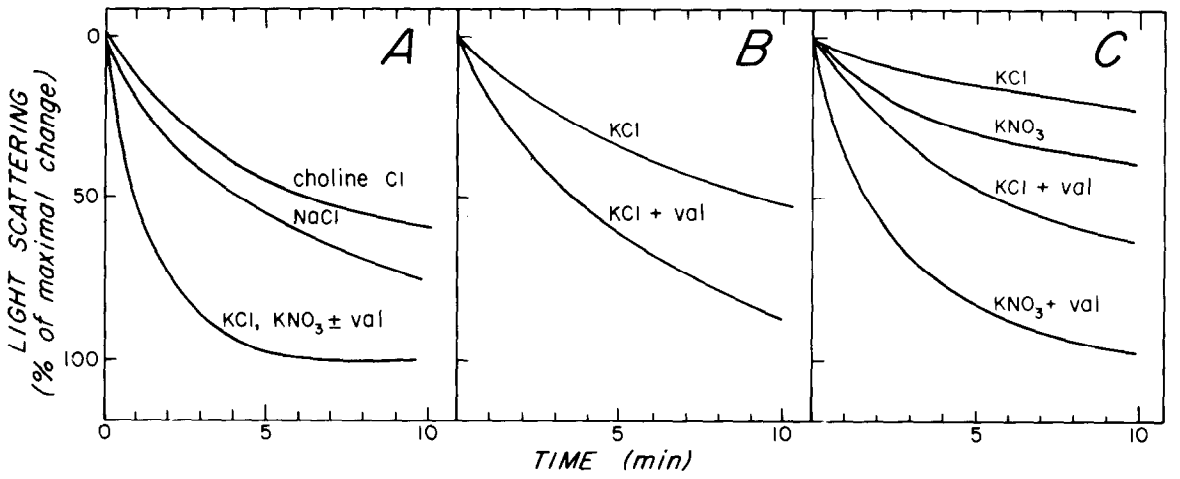

Fig.3. Light scattering changes for microsomal and s.a. membrane suspensions exposed to hypertonic salt solutions. Suspensions of s.a. membranes (A) and rabbit (B) or pig (C) gastric microsomes in 50 mM sucrose, 1 mM $MgSO_4$, 1 mM Pipes (pH 7.0) with or without 5 μM valinomycin, were manually admixed (1:1) with a 250 mM sucrose, 1 mM $MgSO_4$, 10 mM Pipes (pH 7.0) and 200 mM of salt. Light scattering at right angles to the incident beam was monitored using a Perkin Elmer MPF44A spectrofluorometer with excitation and emission monochromators set at 400 nm. Experiments were carried out at 25°C and at 30 μg protein/ml final conc.

reswelling in KNO_3 seemed to be considerably faster than in KCl. Valinomycin substantially increased the reswelling rates (fig.3B,C).

4. Discussion

On the basis of these comparative experiments, it can be concluded that a measurable amount of the s.a. membranes exist as tightly sealed vesicles in a configuration that, as for the case of the gastric microsomes, allows acidification of the intravesicular space through a H^+ pump mechanism. Unlike the classical microsomes, however, the s.a. vesicles are able to generate large H^+ gradients when the medium is complemented only with chemicals normally found in the cell cytoplasm. Their functional properties are, thus, compatible with those outlined for the stimulated oxyntic cell reinforcing our early proposition that the s.a. membranes are formed by vesiculation of the apical membrane physiologically rendered to its stimulated state [9]. A gap of 1–2 orders of magnitude between the H^+ gradients generated across the apical membrane in intact tissue [16] and the gradients measured in this work for the vesicles remains unexplained. However, since the presence of vesicles sealed in the reverse configuration and/or of sealed vesicles unrelated to the pump system will result in overestimations of the sealed space actually available for the ATP-driven acidification, the estimated ΔpH-values represent only lower limits. The enhanced H^+-uptake observed when the Cl^- concentration is lowered to about that of the cell cytoplasma concentration raises the possibility that the difference in environments for H^+-translocation 'in vivo' and 'in vitro' contributes to the gap in ΔpH.

The functional properties displayed by the s.a. vesicles could be explained on the basis of the known H^+–K^+ exchange properties of the ATPase pump by the co-existence of an endogenous K^+ permeability pathway. A comparison of the rate of vesicle reswelling, measured at 25°C, with the rates of the ATP-driven H^+ uptake that were measured at 36°C, suggests that the rates of KCl influx are greater than the rates at which the ATPase exchange pump can recycle internalized K^+. This notion is also consistent with the insensitivity of H^+ uptake to preincubation in KCl. A test of the relative accuracy of our estimation of salt influx by the reswelling rates is provided by their comparison with direct tracer measurements in pig gastric microsomes. Thus, $t_{1/2}$ for KCl equilibration of ~30 min and 7 min in the absence or presence of valinomycin, respectively, can be inferred from fig.3. Similar values have been obtained for the equilibration of $K^{36}Cl$ [11] or $^{85}RbCl$ [17] and the relative differences between Cl^- and NO_3^- have also been

documented [13]. The nature of the mechanism responsible for the high K^+ permeabilities exhibited by the s.a. vesicles is a matter for future study. At least one of the properties observed, the valinomycin dependence of CCCP-gradient dissipation, suggests that this pathway is in fact an electroneutral K-salt translocating system. The details surrounding this possibility are currently being investigated in our laboratory.

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