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INDUCTION OF A CHLORIDE CONDUCTANCE IN GASTRIC VESICLES BY LIMITED TRYPSIN OR CHYMOTRYPSIN DIGESTION OR AGEING

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Summary

Transport activity of the hog gastric ($H^+ + K^+$)-ATPase system was measured either as the formation of a proton gradient using the dye probe acridine orange or as the formation of a proton diffusion potential using the cyanine dye 3,3′-diethyloxdicarbocyanine iodide in the presence of the protonophore tetrachlorosalicylanilide. The development of these gradients has been compared in K^+ media in the presence of either Cl^- or SO_4^{2-} as the anionic species. This comparison of proton diffusion potential formation to proton gradient formation has been used to demonstrate that a Cl^- conductance in this vesicular system results from limited enzymic digestion with either trypsin or α-chymotrypsin and from the ageing process itself. The possible significance of this finding is discussed.

Introduction

We have shown previously that purified gastric membrane fractions containing a K^+-ATPase are capable of transporting H^+ inward and K^+ outward upon the addition of ATP [1—3]. Various attempts to show that the primary transport process (i.e. H^+/K^+ antiport) generated electrical signals were unsuccessful [4,5]. From this we concluded that the pump mechanism was not detectably electrogenic [5]. This was in accord with the prior observations of others [6]. pH changes due to application of ion gradients to vesicles in the absence of ATP allows assessment of passive conductances, and by these tech-

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Abbreviations: DOCC, 3,3′-diethyloxodicarbocyanine iodide; TCS, tetrachlorosalicylanilide.

niques it was shown that freshly prepared vesicles had a low H^+ conductance and negligible K^+ and Cl^- conductance [3].

We have reported [5] that a response is observed with the cyanine dye, 3,3'-diethyloxodicarbocyanine iodide (DOCC), when ATP is added to gastric vesicles in the presence, but not in the absence of an artificial H^+ conductor such as tetrachlorosalicylanilide (TCS). We concluded that this probe detected a diffusion potential produced by the proton ion conductor in the presence of a proton gradient. This finding provides a method of assessing the presence of other conductances such as K^+ or Cl^-. Thus, in addition to the protonophore-induced proton conductance, the development of the cyanine dye signal is sensitive to associated conductances such as K^+ or Cl^- or any antiport pathway which can reduce the proton gradient upon which the secondary diffusion potential to which DOCC responds is dependent. To distinguish these pathways, acridine orange, a dye probe of pH gradient that does not depend on an artificial H^+ conductance for signal generation, was used to monitor the effects of various treatments on the formation of the proton ion gradient.

Accordingly, in this paper we describe the change in the development of acridine orange and DOCC signal activity due to ageing, trypsin or chymotrypsin action on gastric vesicles. These experiments suggest that a Cl^- conductance is the major modification produced as a result of these experiments.

Methods and Materials

Preparation of vesicles

Gastric vesicles were prepared from hog gastric mucosa as previously described [4]. For measurements of transport activity, the membrane material which floats on top of the 7% Ficoll/0.25 M sucrose (GFS preparation) was incubated in a suspension with a final concentration of 150 mM KCl, 2 mM $MgCl_2$, 2 mM piperazine-*N*,*N'*-bis(2-ethanesulfonic acid) (Pipes)/Tris (pH 7.4) and 0.3 mg · ml^{-1} membrane protein for 2 h at 23°C. For half the experiments, 75 mM K_2SO_4 and 2 mM $MgSO_4$ were substituted for 150 mM KCl and 2 mM $MgCl_2$.

Trypsin treatment

Trypsin (TPCK, 222 μg/mg) from Worthington was suspended in 20 mM Tris-HCl, pH 7.4, at 1.0 mg · ml^{-1}. An aliquot from this stock was diluted to 0.05 mg · ml^{-1} in 20 mM Tris-HCl, pH 7.4. Trypsin solutions were kept ice-cold until use and were used within 4 h of dilution. For transport experiments, limited digestion of GFS preparation protein was initated by addition of 0.22 μg trypsin to 105 μg of GFS material in 0.35 ml which had been previously incubated in either KCl or K_2SO_4 media (protein/trypsin = 480). After 20 min at 23°C, the digestion was stopped by the addition of 6.6 mg of soybean trypsin inhibitor protein (trypsin inhibitor/trypsin = 30; stock solution was prepared as 5.0 mg · ml^{-1} in 20 mM Tris-HCl, pH 7.4). Transport activity was measured immediately after the addition of trypsin inhibitor protein. For measurements of ATPase activity, the GFS preparation suspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, was treated in the identical manner as for the transport experiments. After addition of trypsin inhibitor, the membranes were

diluted to 0.1 mg · ml^{-1} in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, for use in the assay.

Chymotrypsin treatment

α-Chymotrypsin Sigma (Type I-S) was suspended in 20 mM Tris-HCl, pH 7.4, at 1.0 mg · ml^{-1}. An aliquot from this stock was diluted to 0.2 mg · ml^{-1} in 20 mM Tris-HCl, pH 7.4. The enzyme solutions were kept ice-cold until use which was within 4 h of preparation. For transport experiments, limited digestion of the protein from the GFS preparation was initiated by addition of 1.75 μg α-chymotrypsin to 105 μg of protein from the GFS preparation previously incubated in either KCl or K_2SO_4 media (protein/chymotrypsin = 60). The incubation period was 20 min at 23°C. Transport activity was initiated after a 20 min incubation by addition of 0.8 mM MgATP. For measurements of ATPase activity, GFS material suspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, was treated in the identical manner as for the transport experiments. At the end of the 20 min incubation period however, further digestion was inhibited by addition of 0.4 mM phenylmethylsulfonyl fluoride. After addition of phenylmethylsulfonyl fluoride the membrane protein was diluted to 0.1 mg · ml^{-1} in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, for use in the assay.

Ageing

The GFS membrane suspension, as the original gradient fraction, was kept refrigerated at 4°C for the desired time intervals.

Transport assays

Measurement of pH gradient. Following the appropriate treatment, 5 μM acridine orange was added to the vesicle suspension. This material was placed in the Aminco DW-2 split-beam spectrophotometer set at reference 446 nm and sample 496 nm. Transport was initiated by the addition of 0.8 mM MgATP, pH 7.4.

Measurement of H^+ diffusion potential. Following the appropriate treatment, 4 μM 3,3′-diethyloxodicarbocyanine iodide and 6 μM tetrachlorosalicylanilide (TCS) were added to the vesicle suspension. This material was placed in the DW-2 spectrophotometer set at reference 636 nm; sample 588 nm. The H^+ induced diffusion potential formation resulting from activation of the H^+/K^+ exchange pump by the addition of 0.8 mM MgATP, pH 7.4, was observed as the change in absorbance of the cyanine dye as a function of time.

ATPase activity

Conditions for the ATPase reaction have been described previously [1]. Release of inorganic phosphate was measured by the method of Yoda and Hokin [7]. Protein concentration was determined by the method of Lowry et al. [8].

Chemicals

Trypsin and trypsin inhibitor protein were purchased from Worthington. α-Chymotrypsin was purchased from Sigma. The disodium salt of ATP was purchased from Sigma. All chemicals were of reagent grade.

Results

Chymotrypsin digestion of the GFS system

Addition of ATP to the K^+-loaded GFS membrane system has been shown to energize a H^+ inward/K^+ outward neutral exchange pump [4,5]. Formation of this proton gradient can be monitored optically as the loss of absorbance of acridine orange at 496 nm [5,9], and this dye has been used to monitor passive H^+ pathways (i.e. K^+/H^+ antiport, H^+ conductance) [12].

Traces a and c of Fig. 1a represent the acridine orange-monitored proton gradient formation in KCl medium. The comparison of these two curves indicates that the gastric vesicle population maintains the ability to produce a proton gradient after chymotrypsin digestion. The faster dissipation of the acridine orange signal as a result of chymotrypsin digestion (trace a) is consistent with a more rapid loss of the proton gradient in KCl media. Fig. 1a (trace b) represents the superimposed signals for both control and chymotrypsin-digested material in a K_2SO_4 medium. As in KCl medium the vesicles' ability to achieve a proton gradient is not impaired. Additionally, the substitution of SO_4^{2-} for Cl^- masks the chymotrypsin-induced H^+ leak which is observed in the KCl medium. The results from this anion substitution is consistent with a lack of sensitivity of the transport ATPase in the functionally vesicular

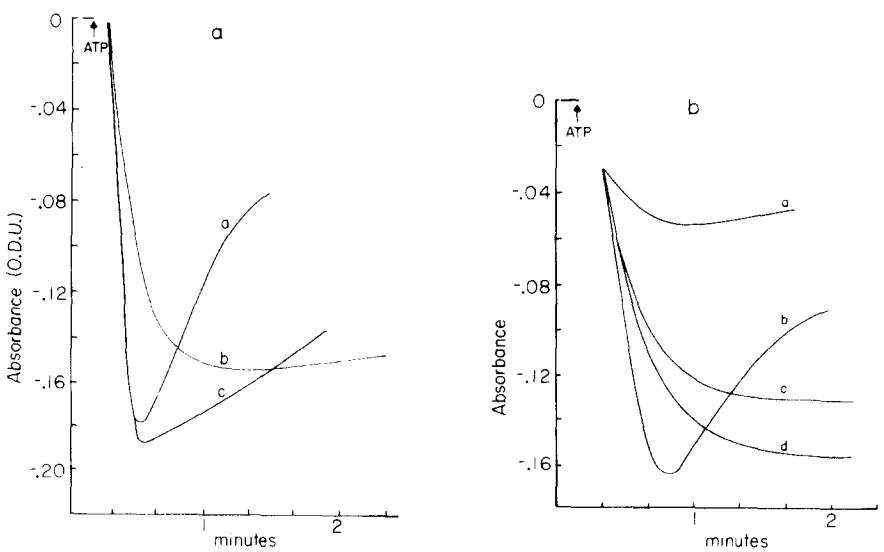

Fig. 1. (a) Sensitivity of the acridine orange response to limited chymotrypsin digestion. Hog GFS (0.3 mg · ml^{-1}) incubated for 2 h at room temperature in either KCl or K_2SO_4 medium (see Methods and Materials) were exposed to chymotrypsin (protein/chymotrypsin = 60) for 20 min at room temperature. 5 μM acridine orange was added 1 min before completion of the digestive procedure. At 20 min, 0.8 mM MgATP was added to the partially digested suspension and the absorbance change at 496—446 nm followed. Curve a, chymotrypsin-treated GFS preloaded in KCl; curve b, GFS preloaded in K_2SO_4 and chymotrypsin-treated GFS preloaded in K_2SO_4; curve c, GFS control in KCl medium. (b) Sensitivity of the DOCC signal in the presence of TCS to limited chymotrypsin digestion. This experiment was identical to (a) up to the point of dye addition. 4 μM DOCC + 6 μM TCS was added 1 min before the completion of the digestive procedure. At 20 min 0.8 mM MgATP was added to the partially digested suspension and the absorbance change at 588—636 nm followed. Curve a, chymotrypsin-treated GFS incubated in KCl medium; curve b, control GFS incubated in KCl medium; curve c, chymotrypsin-treated GFS incubated in K_2SO_4 medium; curve d, control GFS incubated in K_2SO_4 medium.

fraction to this level of chymotrypsin digestion, and the induction of a H^+ leak. H^+ efflux could be via a H^+ conductance coupled to a Cl^- conductance, or by an HCl symport. Both the lack of activation of basal K^+-ATPase (Table I) and the maintenance of initial rate or proton gradient formation with the concomitant increased leak rate observed in Fig. 1a (trace a) are inconsistent with a K^+/H^+ antiport leak.

To test the possibility that a conductance change is responsible for the gradient dissipation noted in Fig. 1a (trace a), the chymotrypsin-digested material was tested in a system which is responsive to changes in Cl^- or K^+ conductance pathways. Fig. 1b shows the absorbance changes produced by DOCC in the presence of the protonophore tetrachlorosalicylanilide. The addition of TCS to the reaction media allows the activity of the neutral H^+/K^+ pump to be followed as a proton-dependent diffusion potential. The comparison of this signal to the acridine orange-dependent signal thus discriminates between Cl^- conductance modification and neutral leak pathways produced by the digestive process.

The comparison of traces a and b in Fig. 1b indicates a dramatic decrease in diffusion potential formation after limited chymotrypsin digestion in KCl media. This diffusion potential loss is noted under conditions which minimize the effects of a change in proton conductance (i.e. TCS is present in both control and chymotrypsin-digested material).

It is possible to lower the anionic conductance pathways by substitution of SO_4^{2-} for Cl^-.; Traces c and d show the effects of chymotrypsin digestion on diffusion potential formation in K_2SO_4 medium. In this case, the diffusion potential loss resulting from the digestive procedure is reduced to less than 13%.

As seen in Table I, the measurement of ATPase activity in aged vesicles at 37°C indicates that chymotrypsin digestion produced a partial inactivation of each component of the ATPase reaction. The ionophoretic stimulation is most sensitive, with a loss of 48% activity while basal K^+ is only 28% inhibited. While it is conceivable that this loss of enzymatic activity will reduce the pump rate and subsequent proton gradient formation, the stability of the potential dependent signal in SO_4^{2-} is not apparently related to inactivation of the ATPase complex. Moreover, maintenance of the potential signal in SO_4^{2-} solutions com-

TABLE I

COMPONENTS OF GASTRIC ATPase ACTIVITY

The ATPase activity of each component is expressed in $\mu mol \cdot mg^{-1} \cdot h^{-1}$. Fresh and aged vesicle activities are measured on the same preparation. For comparison of the activities of ATPase, the proteolytic digestions are normalized to control values within the aged vesicle preparation. Both assay procedures were performed on the aged material.

	Fresh vesicles	Aged vesicles	Trypsin	Chymotrypsin
Mg^{2+}	5.4	6.8	5.2	2.9
$Mg^{2+} + K^+$	24.4	55.6	31.0	41.0
$Mg^{2+} + K^+$ + nigericin	110.0	103.0	41.0	65.7

pared to the dramatic loss in Cl^- solutions implies a stronger correlation between the increasing anionic conductance than the decreasing ATPase activity.

Trypsin digestion of the GFS system

Table I indicates that limited digestion of the GFS system by trypsin severely inhibits all components of the transport ATPase. Basal K^+ stimulation is reduced 48% while K^+-nigericin stimulation is reduced by 79%. As in the experiments involving chymotrypsin digestion, this inhibition of ATPase activity is tested in vesicles in which basal K^+ levels have been maximized by ageing.

Fig. 2a shows the effects of trypsin digestion on the acridine orange-monitored, H^+ gradient-dependent signal. Comparison of the treated to untreated material (trace a vs. trace b) indicates both a reduction of magnitude and a slight increase in the leak rate of the proton gradient after partial trypsin digestion. In experiments in which the incubation medium was K_2SO_4, the inhibition of the proton gradient was less dramatic. Trace c indicates gradient formation after trypsin digestion is slightly inhibited. The stabilities of the gradients before and after digestion are approximately the same in K_2SO_4 medium.

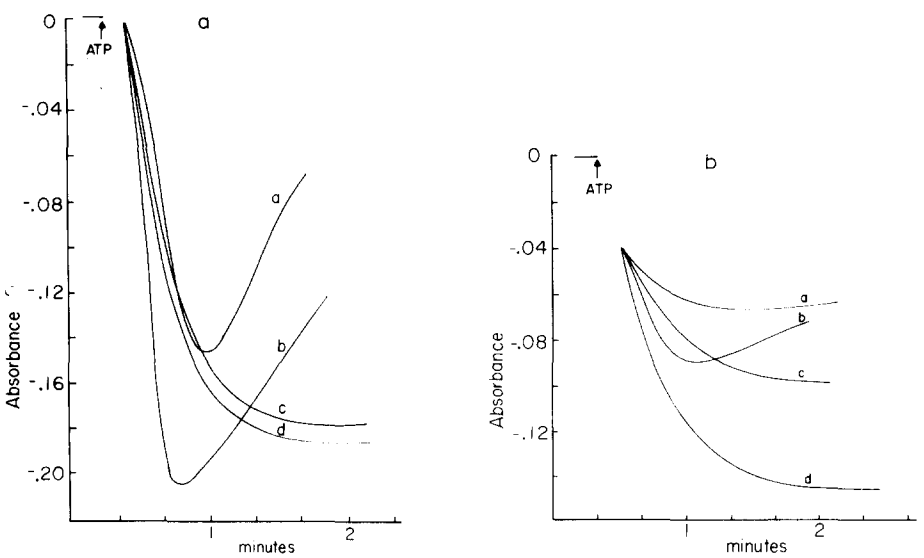

Fig. 2. (a) Sensitivity of the acridine orange response to limited trypsin digestion. Hog GFS vesicles (0.3 mg · ml^{-1}) incubated for 2 h at room temperature in either KCl or K_2SO_4 medium (see Methods and Materials) were exposed to trypsin (protein/trypsin = 480) for 20 min at room temperature. After 20 min the digestion was inhibited by addition of 30-fold excess (trypsin inhibitor/trypsin) trypsin inhibitor protein. 1 min after the addition of 5 μM acridine orange 0.8 mM MgATP was added to the partially digested suspension and the absorption change at 496—446 nm followed. Curve a, trypsin-treated GFS preloaded in KCl medium; curve b, control GFS preloaded in KCl medium; curve c, trypsin-treated GFS preloaded in K_2SO_4 medium; curve d, control GFS preloaded in K_2SO_4 medium. (b) Sensitivity of the DOCC signal in the presence of TCS, to limited trypsin digestion. This experiment was identical to (a) up to the point of dye addition. At this point, 4 μM DOCC + 6 μM TCS was substituted and following the addition of 0.8 mM MgATP, the absorption changes were monitored at 588—636 nm. Curve a, trypsin-treated GFS preloaded in KCl medium; curve b, control GFS preloaded in KCl medium; curve c, trypsin-treated GFS preloaded in K_2SO_4 medium; curve d, control GFS preloaded in K_2SO_4 medium.

In Fig. 2b comparison of the proton-dependent diffusion potential in KCl medium (trace b) to that in K_2SO_4 medium (trace d) indicates a discrepancy of magnitude which can be accounted for by a partial anion shunt conductance present in the material used in the trypsin digestion experiment. Trypsin treatment of this material in KCl media (traces a and b) clearly reduces the ability of these vesicles to generate a potential. Fig. 2b (traces c and d) shows similar experiments in a K_2SO_4 medium. In contrast to the results from ageing and chymotrypsin digestion, the potential-dependent signal in K_2SO_4 is sensitive to trypsin digestion. This is evidenced by the discrepancy between traces c and d in Fig. 2b. While the traces indicate that the potential formation after trypsin digestion are likely to be influenced by contributions of ATPase inhibition, as well as the anionic and cationic conductance pathways, experiments suggest that in general, the formation of a H^+-dependent potential is enhanced by substitution of the less permeant SO_4^{2-} for Cl^-.

Ageing of the GFS system

The effects of ageing on the total activity of the transport ATPase is shown in Table I. The comparison of vesicles aged 12 h (referred to as fresh vesicles) to those aged 88 h indicates a loss of total ATPase activity of only 6%. Table I indicates, that while the sum of the basal K^+ and nigericin-stimulated K^+ activation remain essentially constant, an age-dependent increase in basal K^+ stimula-

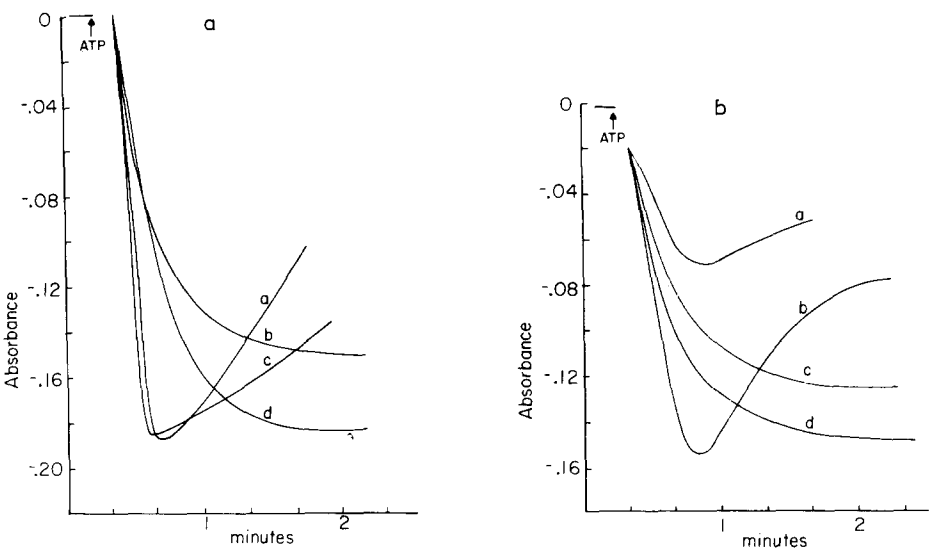

Fig. 3. (a) Sensitivity of the acridine orange response to ageing. The GFS vesicles were aged at 4°C for either 12 or 88 h. At the completion of this time, the hog GFS vesicles (0.3 mg · ml^{-1}) were incubated for 2 h at room temperature in either KCl or K_2SO_4 medium (see Methods and Materials). Following this incubation, 5 μM acridine orange was added to a 350 μl aliquot of the preloaded suspension. After addition of 0.8 mM MgATP, absorption was monitored at 496—446 nm. Curve a, GFS vesicles aged 88 h preloaded in KCl medium; curve b, GFS vesicles aged 12 h preloaded in K_2SO_4 medium; curve c, GFS vesicles aged 12 h preloaded in KCl medium; curve d, GFS vesicles aged 88 h preloaded in K_2SO_4 medium. (b) Sensitivity of the DOCC response in the presence of TCS to ageing. This experiment was conducted exactly as (a) except that 4 μM DOCC and 6 μM TCS were substituted for acridine orange and the absorption change after addition of 0.8 mM MgATP was monitored at 588—636 nm. Curve a, GFS vesicles aged 88 h, preloaded in KCl medium; curve b, GFS vesicles aged 12 h preloaded in KCl medium; curve c, GFS vesicles aged 88 h preloaded in K_2SO_4 medium; curve d, GFS vesicles aged 12 h preloaded in K_2SO_4 medium.

tion is matched by a similar decline in ionophoretic stimulation. Since the ATPase activity is measured under non-equilibrated conditions, this age-dependent increase in K^+ stimulation can be interpreted as an increased permeability of the vesicles to KCl. While K^+-ATPase activity itself is quite stable, the permeability of the vesicles may be less so.

The effects of ageing on the ability of the vesicles to produce and maintain a proton gradient are shown in Fig. 3a. In KCl medium (traces a and c) it is apparent that the magnitude of the proton gradient is unchanged with ageing. The aged vesicles, however, show an increased proton leak rate when the dissipation of the acridine orange signal of the two traces is compared. This proton leak can be eliminated in either case by SO_4^{2-} substitution for Cl^-. Experiments on the same material incubated in K_2SO_4 medium (traces b and d) demonstrate the stability of the proton gradient in the presence of the less permeant anion. The increased absorbance signal shown in curve d indicates the generation of a larger proton gradient in the older vesicles. This difference reflects a limitation of internal K^+ in the fresher vesicles which results from the inability of K_2SO_4 to equilibrate within 2 h at 23°C.

The contribution of the anion conductance pathway to the increased proton leak is again tested by the potential-dependent signal response of DOCC in the presence of the protonophore TCS. The comparison of traces a and b in Fig. 3b indicates that the capacity of the vesicles to generate a potential in KCl medium is severely inhibited by the ageing process. The substitution of SO_4^{2-} for Cl^- (Fig. 3b, traces c and d) eliminates the age-dependent loss of the potential signal. In this case, a small increase in the potential signal in SO_4^{2-} medium compares to a 61% signal loss in Cl^- medium.

Discussion

The GFS preparation contains a 10^5 molecular weight group of peptides which has been shown to contain a neutral exchange (H^+_{in}/K^+_{out}) pump [4]. Under control conditions (i.e. untreated fresh preparation) the ATP-induced energization of this pump produces a proton gradient whose magnitude has been previously demonstrated to be affected by the pump requirements for internal K^+ and the amount of ATP present [3]. Maintenance of the H^+ gradients across the membrane structure can also be regulated by other cation antiport or anion symport systems which could react to the gradient formed by the primary pump process.

In the experiments involving ageing the K^+-stimulated ATPase activity is only slightly affected. While the trypsin-treated enzyme is more severely inhibited, this inhibition correlates with a lessening of signal activity. Accordingly, the simplest interpretation of the effect is that, rather than the primary pump mechanism, associated ion transport pathways have been affected.

Since transport of H^+ by the protonophore is electrogenic, it is likely that either a K^+ conductance or Cl^- conductance may account for the decrease in the H^+ gradient formed. The substitution of Cl^- by SO_4^{2-}, a less permeant anion, increased the magnitude of the responses as well as reduced the leak rate to less than those of the control in KCl medium. This can be taken as indicating that a Cl^- conductance is induced by either limited trypsin or α-chymotrypsin digestion or by ageing.

It is also possible however, that a symport or cotransport of HCl is occurring. This possibility was tested by observing the absorbance signals of acridine orange when a proton gradient is placed across the gastric vesicle membrane by ATP energization [5,9,12]. The magnitude of the proton gradient is relatively unaffected by ageing or chymotrypsin treatment. In both, the increased leak rate is compatible with a small increase in H^+ conductance which when added to an increased Cl^- conductance results in a small, but measurable leak of protons. This relative insensitivity of the proton gradient formation compared to the formation of a TCS-dependent potential is also supported by the observations of trypsin-treated material. The lack of recovery of potential-dependent signal activity in the K_2SO_4 medium after trypsin treatment is probably due to the level of inhibition of ATPase activity resulting from this treatment. While the pH gradient dependent signal (acridine orange) in the K_2SO_4 medium appears to indicate a small loss of proton gradient, this repeats a pattern observed in similar experiments (not reported here) which is a decrease in the rate of formation of the proton gradient and a subsequent net decrease in magnitude.

In this paper, we have shown that a Cl^- conductance develops in gastric vesicles when those vesicles are aged or subjected to limited digestion with trypsin or α-chymotrypsin. The previously reported findings that valinomycin was able to stimulate ATPase activity and proton uptake was interpreted as being due to entry of a valinomycin-K^+ associated with Cl^- [3,4]. This could be as a complex, or through two pathways, one for valinomycin-K^+ and the other for Cl^-. If the latter, then this Cl^- conductance appears to be minimal in the absence of ATP and also to be minimal for a Cl^--dependent H^+ leak, even in the presence of TCS in fresh vesicles. It is possible however that this Cl^- conductance is coupled in some way to pump activity.

Alternatively, if a Cl^- conductance exists only following degradation of vesicle structure, the physiological significance of this conductance is questionable. It is however, of interest that an anion-specific conductance appears since it may be difficult to postulate an adventitial anion carrier or channel. Indeed, the most plausible model to date for 'active' Cl^- secretion by the stomach suggests that it is driven by an electrogenic basal-laterally located ($Na^+ + K^+$)-ATPase coupled on the same membrane to a NaCl neutral cotransport path, with a Cl^- conductance on the apical membrane and a Na^+ conductance in the shunt pathway [10,11]. It could be, therefore, that the modifications employed here expose the Cl^- conductance that, due to preparative procedure, is inhibited in the fresh vesicle preparation. Since, in this work we have not modified anionic conductance by the use of anionic ionophores, we can draw no conclusions as to the level of H^+ conductance as changed by the treatment of the vesicles by proteases or ageing.

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