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Characteristics of a Pure Endogenous Activator of the Gastric H^+,K^+-ATPase System

EVALUATION OF THE ROLE AS A POSSIBLE INTRACELLULAR REGULATOR*

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An endogenous activator capable of stimulating the gastric H^+,K^+-ATPase activity has been purified to homogeneity from dog and pig gastric cells and found to be a dimer of two identical 40-kDa subunits in the active state. Identical nature of the activator monomers was revealed by the detection of lysine as the sole N-terminal amino acid. The activator from one species can stimulate the H^+,K^+-ATPase from another species and vice versa. Such cross-activation is consistent with the striking similarities in the amino acid composition between the two species, suggesting considerable homology in the activator molecules from different species. The activator exhibited several unique features during modulation of the H^+,K^+-ATPase reaction. It appreciably enhances affinity of the H^+,K^+-ATPase for K^+, known to increase turnover of the enzyme. To complement this K^+ affinity, the activator also enhances ability of the H^+,K^+-ATPase to generate more transition state (E^*·ATP) complex by increasing the entropy of activation ($\Delta S‡$) of the system as revealed from an Arrhenius plot of the data on temperature activation. In addition, the activator shows both positive cooperativity and strong inhibition, depending on its concentration. Thus, up to the ratio of the H^+,K^+-ATPase and activator of about 1:2 (on the protein basis), the activator shows sigmoidal activation (Hill coefficient = 4.5), but beyond such concentration a strong inhibition was observed. Finally, Ca^{2+} at low (2–4 μM) concentration strongly inhibits the activator-stimulated H^+,K^+-ATPase. It is proposed that the activator may be acting as a link in the signal transducing cascade system between the intracellular second messenger (Ca^{2+}) and the physiological response (gastric H^+ transport).

The stomachs of all vertebrate animals so far studied actively secrete hydrochloric acid against a concentration gradient of over a millionfold. The secretory membrane-located H^+,K^+-ATPase in the acid-secreting (parietal) cells has recently been identified (1, 2) as the enzymatic mechanism for the transport of protons.

Although our knowledge on several aspects of gastric acid secretion such as the actions and interactions of secretagogues (3) with the cell surface (basolateral) membrane and the molecular mechanism of the secretory membrane-located H^+,K^+-ATPase function has advanced significantly in recent years, very little is known about the cytosolic biochemical events which underlie secretagogue stimulation. A role of cyclic AMP (4–7), Ca^{2+} (8–10), and protein kinases (11–13) has been implicated. However, the specific metabolic steps involved in the regulation of H^+ transport by these agents are unknown.

Since the hormonal signals (first messengers) from the basolateral side need to be transmitted through the cytoplasm before being transduced at the H^+,K^+-ATPase pump site it is obvious that some cytosolic second messengers such as cyclic AMP and Ca^{2+} may be involved in the total chain of intracellular events. However, no direct effect of either cAMP or Ca^{2+} at near physiological concentration has thus far been demonstrated on the activity of the H^+,K^+-ATPase. It is therefore highly likely that other intracellular regulatory factors are involved in linking the second messenger signals to the final physiological response of gastric H^+ transport.

One such regulator may be the 80-kDa cytosolic activator protein demonstrated to activate the gastric H^+,K^+-ATPase and K^+-*p*-nitrophenyl phosphatase activities *in vitro* (14–18). There are several known unique features of the endogenous activator which implicate the activator as an intracellular regulator. First, the activation of the gastric H^+,K^+-ATPase is a general phenomenon, *i.e.* it occurs in every vertebrate animal studied so far (14, 19). Second, the activator from one species is capable of stimulating the gastric H^+,K^+-ATPase from another species and vice versa (14, 19). Third, activation of the H^+,K^+-ATPase reactions also causes an increase in the rate of H^+ transport. Thus, the H^+,K^+-ATPase-mediated rate of H^+ transport into the interior (lumen) of the gastric microsomal vesicles is increased considerably in the presence of the activator (19). Fourth, the activator stimulation of the H^+,K^+-ATPase can be totally obliterated by micromolar Ca^{2+}, suggesting a critical interplay between this second messenger and the activator in the regulation of the H^+,K^+-ATPase function (14). Finally, the dimeric (two identical 40-kDa subunits) activator molecules are only effective in activating the H^+,K^+-ATPase reaction; the monomers are totally ineffective (19). Such differential effects of the monomeric and dimeric activator may be the mark of a delicate regulation mechanism.

The purpose of the present investigation was twofold: (*a*) to purify the activator from pig gastric cells and compare it with that from dog (19) with a view to finding out the underlying commonalities and unique features and (*b*) to explore further the mechanisms of activator regulation of the

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gastric H⁺,K⁺-ATPase reaction. The data demonstrate that the amino acid composition of the pig and dog endogenous activator are closely similar. The N-terminal amino acid is lysine for both species. The data on the H⁺,K⁺-ATPase activation revealed the following: the affinity of the H⁺,K⁺-ATPase for K⁺ is increased significantly in the presence of the activator. The activator demonstrated sigmoidal activation as well as inhibition, depending on the concentration of the activator; the higher concentrations were inhibitory. The effects of temperature on the activator activation of the H⁺,K⁺-ATPase demonstrated no appreciable changes in the break point (Arrhenius) temperatures compared to the unactivated reaction. The energy of activation (E_a) and the enthalpy of activation ($\Delta H\ddagger$) were appreciably higher above 34 °C in the activated reaction. The effects of increasing concentrations of Ca^{2+} demonstrated that Ca^{2+} at low (4 μM) concentration obliterated the activator-activated portion of the gastric H⁺,K⁺-ATPase reaction. The data have been discussed in terms of the possible role of the endogenous activator as a cytosolic regulator of gastric H⁺ transport.

EXPERIMENTAL PROCEDURES[1]

Isolation of Gastric Microsomes—Fresh pig stomachs were purchased from the local slaughterhouse (Schalar Slaughter House, Bridgewater, NY). The gastric microsomes were isolated following the method (14) published previously.

Purification of the H⁺,K⁺-ATPase and the Endogenous Activator—The H⁺,K⁺-ATPase associated with purified gastric microsomes was further purified to homogeneity with high specific activity. The procedure (20) involves solubilization of the extrinsic proteins in 0.033% (w/v) SDS[2] by sonication followed by equilibrium sucrose density gradient centrifugation. The band (buoyant density = 1.115) contains nearly all of the H⁺,K⁺-ATPase activities associated with the original starting material (gastric microsomes) and shows very high specific activities. The purified H⁺,K⁺-ATPase shows a single major 100-kDa band and a diffuse 85-kDa glycoprotein band on SDS-PAGE. A preliminary account of the purification procedure has been reported (20, 21). The details will be published elsewhere.

The details for the purification of the activating factor are included in the Miniprint.

Pretreatment of the H⁺,K⁺-ATPase Preparation with Activator—The activator needs to be preincubated with gastric microsomes for a brief period (about 1 min) at 37 °C to show consistent activation. However, longer incubation (10–15 min) beyond the 1-min period does not change the extent of activation. For the sake of convenience of the assay, we use a 10-min preincubation period in our routine assay procedures. Briefly, gastric microsomes or the pure H⁺,K⁺-ATPase preparation was first incubated for 10 min at 37 °C without (controls) and with the desired concentrations of the activator. The membranes treated without and with the activator were then transferred to an ice bath (0–4 °C) for subsequent assay of the H⁺,K⁺-ATPase and K⁺-*p*-nitrophenyl phosphatase activities within 1 h.

Assay of the H⁺,K⁺-ATPase and K⁺-p-Nitrophenyl Phosphatase—For the ATPase, the incubation mixture contained, in a total volume of 0.95 ml, 50 μmol of Pipes (pH 6.8), 5 μmol of Mg^{2+}, and 5 μg of the microsomes with and without 20 μmol of K⁺. Following a preincubation period of 10 min at 37 °C the reactions were started with 0.05 ml of 40 mM Tris-ATP (pH 6.8) and incubated for 10 more min. The reactions were stopped by ice-cold trichloroacetic acid and assayed for P_i as previously described (15–17). The assay conditions for the K⁺-*p*-nitrophenyl phosphatase were the same as those for the ATPase except that the pH of incubation was 7.5 (50 μmol of Tris-HCl) and the substrate used was 5 mM *p*-nitrophenyl phosphate (15–17).

SDS-PAGE was run following Fairbanks *et al.* (22) and as described (24). The protein was assayed by the Lowry procedure (23).

Determination of the Amino Acid Composition and the N-terminal Amino Acid—The amino acid compositions of the pig and dog activator were determined as follows. The pure activator (about 5 mg) in 50 mM Tris, 0.2 mM dithiothreitol, and 0.2 mM EDTA (pH 7.4) was dialyzed exhaustively against glass-distilled water and lyophilized. The dry protein was dissolved in 6 N HCl and hydrolyzed in the presence of a trace (10 μl in 1 ml) amount of phenol at 105 °C for 24, 48, and 72 h under vacuum. The hydrolyzed protein was evaporated to dryness, and the amino acid composition was determined by a Beckman System 6300 high performance analyzer. Since tryptophan was destroyed under the condition of acid hydrolysis, its concentration was determined from the absorption spectra of the pure activating factor and the known molar absorption of tryptophan (24). The cystein residue in the activating factor was quantified by oxidation to cysteic acid followed by amino acid analysis (25).

The N-terminal amino acid was determined by the dansylation method (26). The salt-free lyophilized protein was dansylated in 0.5 M $NaHCO_3$ buffer (pH 9.0) containing 1% SDS at 37 °C for 4 h. The labeled protein was dialyzed, lyophilized, and subsequently hydrolyzed in 6 N HCl at 110 °C for 16 h. The dansyl-N-terminal amino acid was identified by TLC in three different solvent systems, such as (*a*) benzene:pyridine:acetic acid :: 16:4:1; (*b*) chloroform:benzyl alcohol:acetic acid :: 70:30:3; and (*c*) *n*-butyl alcohol:pyridine:acetic acid:water :: 30:20:6:24.

RESULTS

Purity of the Activator—The SDS-PAGE (Fig. 4) of the pH precipitate and the peak fractions of the active activator from column 1 (Fig. 1) and column 2 (Fig. 2) show that the activator activity correlates well with a 40-kDa protein band. The material eluted from column 2 and having very high activator activity consists almost entirely of a 40-kDa peptide. However, the molecular weight calibration of the Sephacryl column shows that the active activator has a molecular mass of 80 kDa. Hence, the active activator appears to be a protein of two 40-kDa subunits. This idea of dimeric activator is supported by the fact that treatment of the active activator (80 kDa) with urea (4 M) and subsequent column chromatographic (Fig. 3) separation generated only 40 kDa peptides with total loss of activator activity. In addition, the N-terminal amino acid for both the dog and pig activator has been found to be consisting of lysine alone (see below), suggesting that the two monomers are probably identical. Hence, the active species of the activator molecules appear to be a dimer of two identical subunits.

Chemical Characterization of the Dog and Pig Activator: Amino Acid Composition—The amino acid compositions of the dog and pig activator are shown in Table I. Since the content of acidic amino acids is far in excess of the basic residues in the activator from both species, the activator is a highly acidic protein, with an isoelectric point in the acidic range. This observation is consistent with the fact that considerable enrichment of both the activator activity and associated 40-kDa SDS-PAGE band (Fig. 4) is observed in the pH 4.8 precipitate of the cytosolic fraction (15). It is noteworthy that the amino acid compositions of the dog and pig activator are nearly identical, with the exception that glycine, alanine, and glutamic acid are higher, whereas lysine is lower and methionine is absent in the dog activator.

N-terminal Amino Acids—The N-terminal amino acid for both the dog and pig activator was identified to be only lysine by the dansylation technique.

Some Enzymatic Characteristics of the Pure Endogenous Activator—Fig. 5 shows the effects of pig activator as a function of increasing K⁺ in the assay medium on the activities of the H⁺,K⁺-ATPase and K⁺-*p*-nitrophenyl phosphatase

[1] Portions of this paper (including part of "Experimental Procedures" and Figs. 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-3530, cite the authors, and include a check or money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Pipes, 1,4-piperazineethanesulfonic acid; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

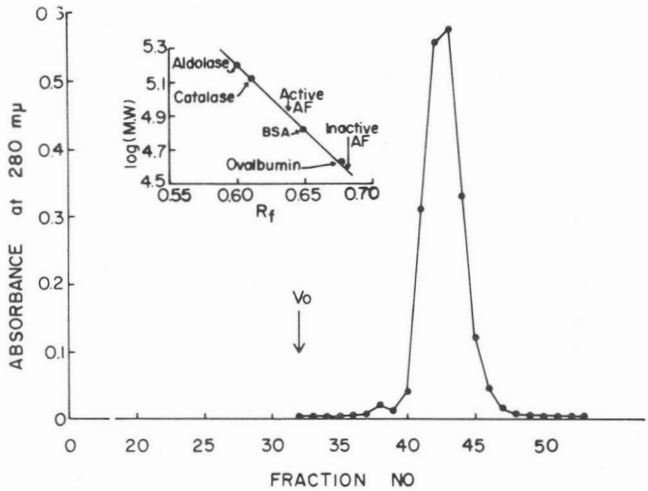

FIG. 3. **Sephacryl S-200 (67 × 1.6 cm) chromatography of the urea-inactivated activator factor preparation.** Pure activator protein (1 mg) was incubated at 37 °C for 5 min without and with 4 M urea in 1 ml of 50 mM Pipes-Tris buffer (pH 6.8) containing 0.2 mM dithiothreitol and 0.2 mM EDTA. Following incubation, 9 ml of ice-cold buffer medium was added. Aliquots containing 2–10 μg of the activator were tested for activity. The activator factor (*AF*) activity was totally abolished following urea treatment. The urea-treated activator was dialyzed, concentrated, and put on the column. The molecular weight calibration of the column showing the position of migration of the active and the inactive activator factor is shown in the *inset*. *BSA*, bovine serum albumin.

TABLE I

Amino acid composition of the activating factor (AF) protein isolated from dog and pig gastric cells

Amino acids	Dog AF	Pig AF
	mol amino acid/mol monomer (40 kDa)	
Nonpolar		
Ala	23	19
Val	22	21
Leu	23	30
Ile	21	20
Pro	19	21
Met	0	2
Phe	14	13
Trp	8	8
Polar (uncharged)		
Gly	36	32
Ser	40	41
Thr	25	23
Cys	1	1
Tyr	12	14
Negatively charged[a]		
Asp	37	39
Glu	34	26
Positive charged		
Lys	7	10
Arg	7	7
His	3	4
Percent nonpolar	39.1	40.4
Total residues	332	331

[a] The values also include the glutamine and asparagine which are converted to the respective glutamic and aspartic acids under conditions of acid hydrolysis of the activator factor. The values for valine, leucine, and isoleucine were derived from 72 h hydrolysis. The serine and threonine content was obtained following extrapolation of the 12, 24, 48, and 72 h hydrolysis data to 0 h.

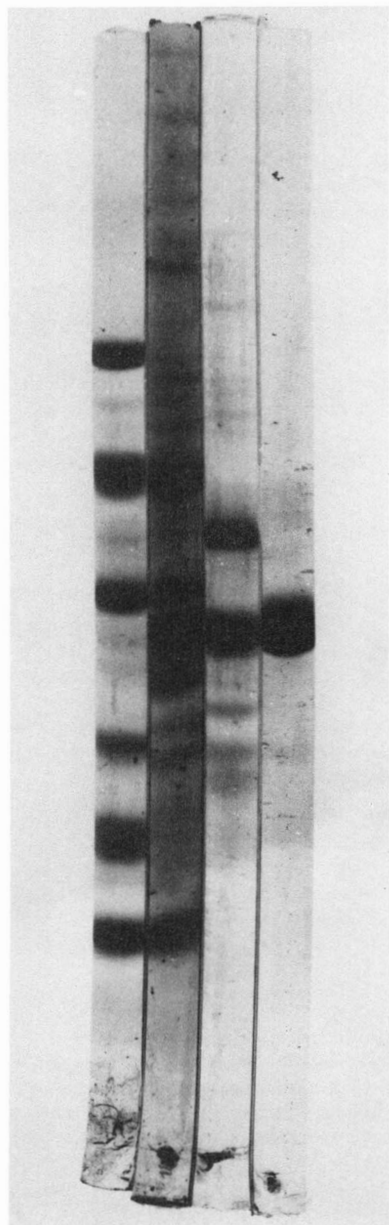

FIG. 4. **SDS-PAGE profiles of various fractions during purification of the activator protein.** The SDS-PAGE was run following the procedure described previously (19). The gels from *left* to *right* are the molecular weight standards (from top, 92.5, 66.2, 45, 31, 21.5, and 14.4 kDa), pH 4.8 precipitate, and the active fractions from column 1 and column 2. Enrichment of the 40-kDa band during purification is clearly evident. The pure activator (active fraction from column 2) is a homogenous band of 40-kDa peptide.

associated with a pure preparation of H^+,K^+-ATPase purified from dog gastric microsomes (20, 21). Both the H^+,K^+-ATPase and K^+-*p*-nitrophenyl phosphatase are appreciably activated by the pure activator. However, there are some noteworthy differences. The affinity to K^+ remains unchanged during activator-induced activation of the K^+-*p*-nitrophenyl phosphatase, whereas a marked increase in K^+ affinity is evident in the activator activation of the H^+,K^+-ATPase (Fig. 5). Such differential effects of the activator on the H^+,K^+-ATPase and K^+-*p*-nitrophenyl phosphatase activities might argue against the concept that the latter reaction is a partial step in the total H^+,K^+-ATPase reaction sequence, as has recently been suggested (27, 28). Similar to the pure H^+,K^+-ATPase preparation, nearly identical results in changing the

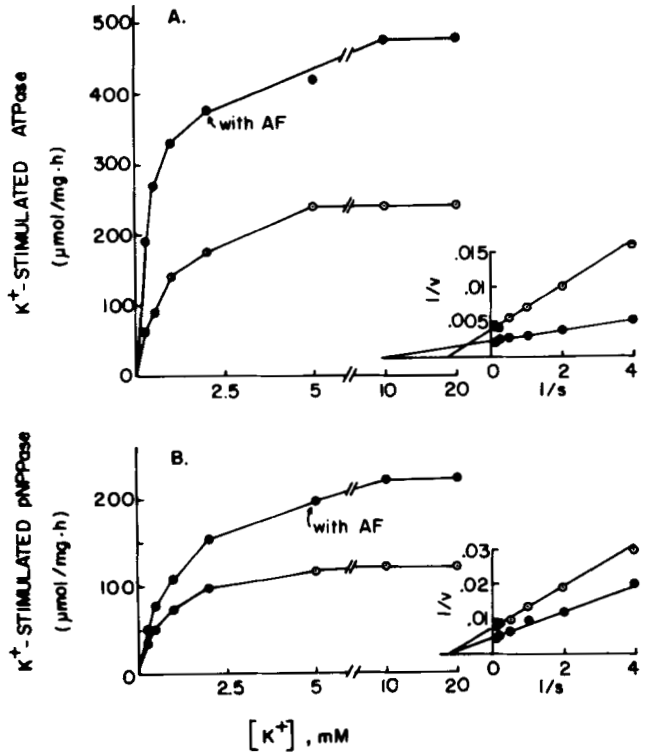

FIG. 5. **Effects of increasing concentrations of K^+ on the activating factor activation of the gastric H^+,K^+-ATPase (*A*) and K^+-*p*-nitrophenyl phosphatase (*pNPPase*) (*B*) activities by the endogenous activator.** The symbols are: O–O, without, and ●——●, with activator for both H^+,K^+-ATPase and K^+-*p*NPPase activities. The insets are the Lineweaver-Burk plot of the data. The pure pig H^+,K^+-ATPase preparation did not have any detectable Mg^{2+}-ATPase activity. The data are typical of three to four separate studies. About 2 μg of the pure pig H^+,K^+-ATPase and for activator activation, 3 μg of the pure dog activator, was used in the assay. The data (not shown) with gastric microsomes as the source of enzyme showed identical patterns with respect to affinity toward K^+. The details of the assay are given under "Experimental Procedures."

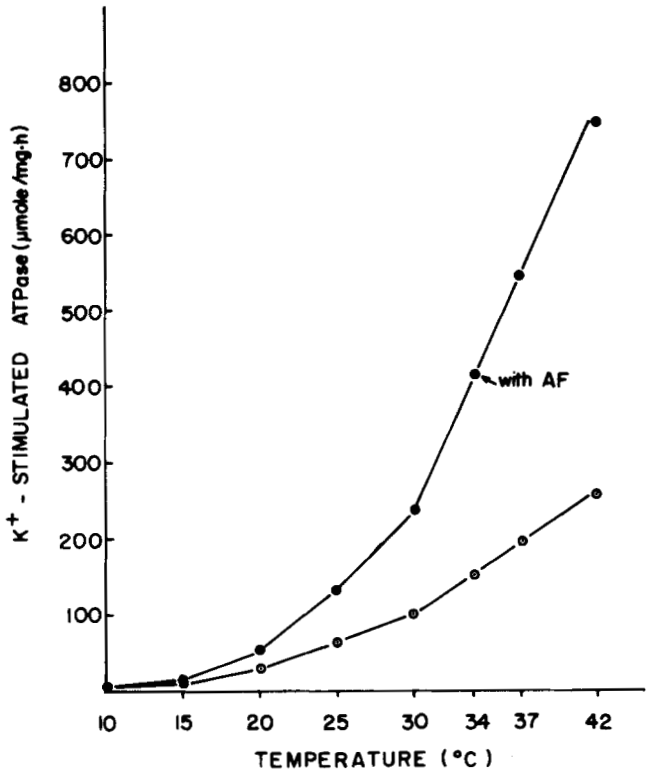

FIG. 6. **Effects of increasing temperatures on the activities of the gastric H^+,K^+-ATPase in the absence and presence of the pure activator.** Symbols are: O–O, without, and ●–●, with activator. The time period of the reactions was varied due to the variation of the reaction rates with temperature, keeping the other conditions such as concentrations of ATP (2 mM), Mg^{2+} (2 mM), K^+ (10 mM), pure H^+,K^+-ATPase (2 μg), and the pure activator protein (3 μg) constant. The assay periods were 60 min for 10 and 15 °C, 30 min for 20 and 25 °C, and 10 min for 30 °C and above. Preincubation of the activator with H^+,K^+-ATPase was done for 10 min at the respective temperatures before assaying the H^+,K^+-ATPase activity. The details of the H^+,K^+-ATPase assay are given under "Experimental Procedures." It is noteworthy that (besides the absolute rates) the temperature activation curve using impure H^+,K^+-ATPase (gastric microsomes) also showed a pattern nearly identical to the data shown above with pure H^+,K^+-ATPase preparation. The data are typical of three separate studies.

K^+ affinity were observed with the impure (gastric microsomes) H^+,K^+-ATPase.

An Arrhenius plot (Fig. 7) of the data (Fig. 6) on the effects of various temperatures on activation of the gastric H^+,K^+-ATPase reaction by the activator revealed some interesting features. The activator does not have any appreciable effects on the two transition temperatures—one around 20 °C (between 20.5 and 22 °C) and the other around 34 °C (between 33.3 and 34.5 °C). Such lack of alteration of breakpoint temperatures by the activator suggests that the activator-induced activation of the H^+,K^+-ATPase does not occur via an activator-membrane (lipid) interaction, but probably occurs as a direct effect on the H^+,K^+-ATPase molecule. However, the energy of activation (E_a) increased considerably above 34 °C with a concomitant increase in the enthalpy ($\Delta H‡$) of activation (calculated from the equation $\Delta H‡ = E_a - RT$) in the presence of the activator (Fig. 7). Since the free energy of activation ($\Delta G‡$) must also decrease above 34 °C when marked activator-induced activation occurs, it appears from the relation $\Delta G‡ = \Delta H‡ - T\Delta S‡$ that, in the process of activator activation of the H^+,K^+-ATPase, the entropy of activation of ($\Delta S‡$) must also increase. Table II gives activation data calculated for 37 °C. The values of $\Delta S‡$ were calculated from the reaction rate constant (k) using the following equation:

$$k = \frac{K_B \cdot T}{h} \cdot e^{-\Delta H‡/RT} \cdot e^{\Delta S‡/R}$$

where K_B is Boltzmann's constant, h is Plank's constant, R is the gas constant, and T is absolute temperature. The k values were obtained from V_{max} at saturating substrate concentration using a molecular weight of 270,000 for the H^+,K^+-ATPase (29). The $\Delta G‡$ values were then calculated as $\Delta G‡ = \Delta H‡ - T\Delta S‡$. The $\Delta G‡$ values are 16.6 and 15.9 for the control (unactivated) and activator-activated reactions, respectively, consistent with the corresponding rate constants (k) of 15.4 and 42.4 s^{-1}. In presence of the activator the $\Delta S‡$ is thus less negative than that of the control reaction, and hence the process of activator activation is entropy-driven. A similar increase in E_a has been reported during activation of the adenylate cyclase by dopamine (30).

Another mode of activator-induced regulation was revealed during our study on the effects of different concentrations of activator on H^+,K^+-ATPase reaction (Fig. 8). The activator molecules demonstrated both "positive" cooperativity and a strong inhibition in their effects on the H^+,K^+-ATPase reaction, depending on concentration of the activator protein. The activation of the H^+,K^+-ATPase at increasing activator shows a sigmoidal activation curve up to a certain concentration beyond which an inhibition is observed. Thus, up to the H^+,K^+-ATPase to activator ratio of 1:1 (on the protein basis),

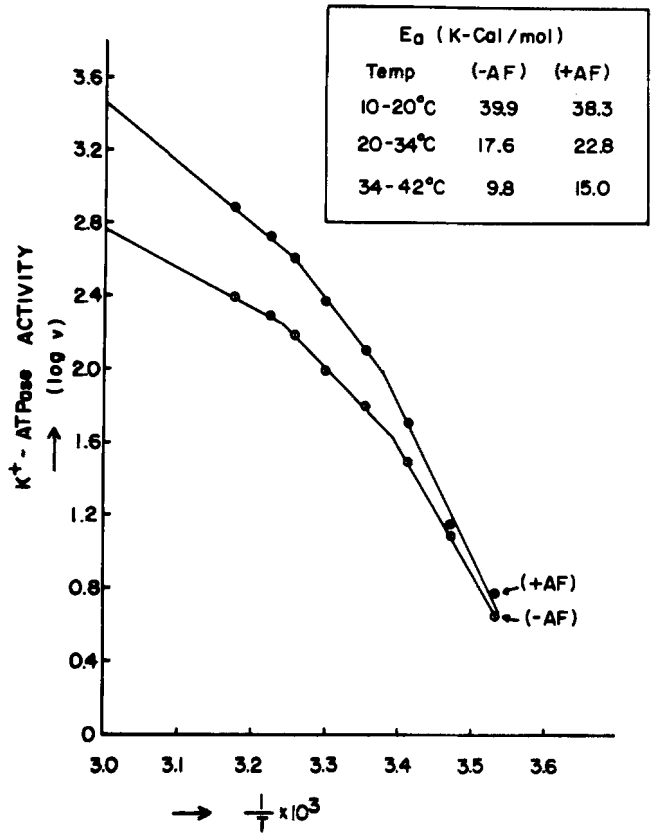

FIG. 7. **Arrhenius plot of the data shown in Fig. 6.** The energy of activation (E_a) values calculated from the slope of the Arrhenius plot is shown in the *inset*. It is noteworthy that the E_a value above 22 °C increases significantly in presence of the activating factor.

TABLE II

Thermodynamic quantities of the activating factor (AF) activated and unactivated gastric H⁺,K⁺-ATPase reactions

Thermodynamic parameters at 37 °C	−AF	+AF
E_a (kcal/mol)	9.8	15.0
H (kcal/mol)	9.3	14.4
G (kcal/mol)	16.6	15.9
S (cal·mol^{-1}·deg^{-1})	−23.5	−4.8
k (rate constant, s^{-1})	15.4	42.4

a sigmoidal activation (Hill coefficient = 4.5) is evident showing an optimal stimulation. However, beyond an ATPase to activator protein ratio of 1:2 the inhibition is apparent, and the activation is totally abolished at an ATPase to activator protein ratio of 1:5. Thus another mode of regulation by the activator—a down-regulation of the H⁺,K⁺-ATPase—also appears possible. Although the mechanisms and significance of the down-regulation are not clear at present, the effect could be of important physiological significance for the intracellular regulation of gastric H⁺ transport. The demonstrated positive cooperativity is a well-known phenomenon exhibited by many allosteric enzymes like the H⁺,K⁺-ATPase.

If the cytosolic activator protein indeed acts as an intracellular regulator of the gastric H⁺,K⁺-ATPase, it must also be responsive to the second messengers such as cAMP and Ca^{2+}. So far we have not found any effect of cAMP. However, Ca^{2+} shows a dramatic inhibition of the activator-stimulated H⁺,K⁺-ATPase activity (Fig. 9). Thus, up to 1 μM, the Ca^{2+} is innocuous for the activator-stimulated H⁺,K⁺-ATPase, but beyond 1 μM, the activity rapidly decreases and becomes abolished at 4 μM Ca^{2+}. It is noteworthy that the activator-

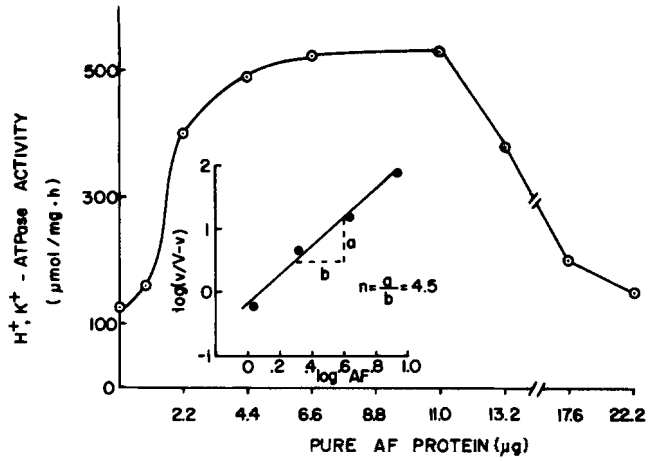

FIG. 8. **Effects of increasing concentration of pure endogenous activator on the gastric H⁺,K⁺-ATPase reaction.** 4 μg of the pure pig gastric H⁺,K⁺-ATPase and the indicated amount of the pure activator were used in each assay. The details of the H⁺,K⁺-ATPase assay are given under "Experimental Procedures." The *inset* shows the Hill plot of the data. Note the inhibition of the H⁺,K⁺-ATPase at higher (over 11 μg of activator/4 μg of the H⁺,K⁺-ATPase) concentrations of the activator. The data are typical of three separate studies. It is noteworthy that, similar to the pure H⁺,K⁺-ATPase, the impure H⁺,K⁺-ATPase (associated with isolated gastric microsomes) also gives identical profiles, with the exception that the absolute specific activities were lower in the latter case.

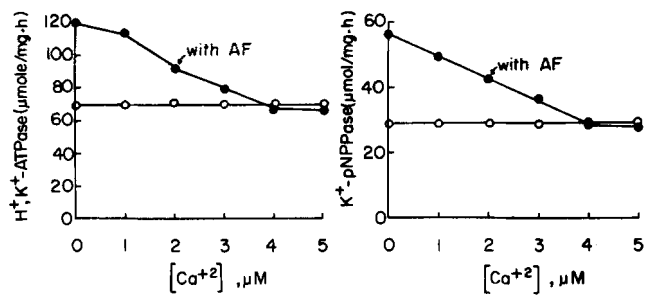

FIG. 9. **Effects of increasing concentrations of Ca^{2+} on the activator activation of the gastric microsomal H⁺,K⁺-ATPase reaction.** About 10 μg of the gradient-purified pig gastric microsomes and 10 μg of the pure dog activator protein were used for the assay. The concentration of free Ca^{2+} was regulated by EGTA according to Pershadsing and McDonald (41) and as reported earlier (23). The details of the H⁺,K⁺-ATPase and K⁺-*p*-nitrophenyl phosphatase assay are given under "Experimental Procedures." The data are typical of four different studies.

stimulated portion of the H⁺,K⁺-ATPase is only affected by such low concentrations of Ca^{2+} without affecting the existing K⁺-stimulated component. Similar general effects are also observed for the K⁺-*p*-nitrophenyl phosphatase reaction. However, unlike the H⁺,K⁺-ATPase, the activator-stimulated K⁺-*p*-nitrophenyl phosphatase shows a linear inhibition by Ca^{2+} from 1–4 μM range (Fig. 9).

DISCUSSION

The data reveal that the cytosolic activator protein is a dimer of two identical 49-kDa subunits. Since the activator from each of such diverse species as frog, rabbit, dog, and pig can stimulate the H⁺,K⁺-ATPase from other species and vice versa (13, 18, 19), it is expected that they will be homologous at least in those regions of the primary structure having direct interaction with the H⁺,K⁺-ATPase. The observed close similarities in the amino acid composition of the activators from pig and dog are consistent with such an idea.

The increase in the affinity of the H⁺,K⁺-ATPase for K⁺

at the high affinity K^+ site indicates that interaction of the activator with some site at the cytosolic domain of the ATPase is capable of generating favorable changes at a distal site (luminal) across the bilayer for efficient K^+ binding. Such activator-induced increase in affinity for K^+ is likely to generate a more efficient K^+/H^+ exchange (19). Ability of the H^+,K^+-ATPase to hydrolyze ATP is also enhanced under these conditions. The evidence discussed below suggests that the generation of the transition state ($E^* \cdot ATP$) is enhanced during activator activation of the H^+,K^+-ATPase reaction.

The Arrhenius plot of the data on the activator activation of the H^+,K^+-ATPase with increasing temperature demonstrates lack of effect of the activator on the transition temperatures. The observation suggests that the activator exerts direct effects on the H^+,K^+-ATPase molecular rather than via some indirect interaction with the membrane lipid components. The mechanistic and energetic consequences of the activator interaction were also revealed upon further analysis of the data.

The energy of activation (E_a) for the H^+,K^+-ATPase reaction calculated from Arrhenius plot was not significantly altered by the presence of the activator below the first transition temperature (20 °C). Above 34 °C, the E_a was significantly higher for the activator-activated reaction than for the unactivated reaction (15.0 and 9.8 kcal/mol, respectively). Consequently, the enthalpy of activation ($\Delta H‡$) is higher for the activator-activated reaction, since $\Delta H‡ = E_a - RT$. However, since activation by the activator occurs at all temperatures that we examined above 20 °C, the free energy of activation ($\Delta G‡$) has to be decreased in the presence of activator. A reduction in $\Delta G‡$ despite an increase in $\Delta H‡$ can only be accomplished by an increase in the entropy of activation ($\Delta S‡$), as demonstrated by the equation $\Delta G‡ = \Delta H‡ - T\Delta S‡$. The process of activation of the H^+,K^+-ATPase by the endogenous activator is thus entropy-driven. A possible interpretation of this observation is that the activator may increase the entropy of the activated transition state in the H^+,K^+-ATPase reaction pathway by organizing the critical reaction groups in favorable orientations. Concomitantly, the freedom of movement of parts of the ATPase molecule may be somewhat restricted, resulting in an increase in $\Delta H‡$. Since the activator enhances affinity of the H^+,K^+-ATPase to K^+, it appears that the activator also modulates the E_1 to E_2 transition.

Some insight into the mode of activator interaction with the gastric H^+,K^+-ATPase is obtained from the studies on ATPase activation with increasing activator concentration. The activator molecules demonstrate strong positive cooperativity (Hill coefficient = 4.5) during H^+,K^+-ATPase activation. The data suggest that cooperative binding of the activator with the H^+,K^+-ATPase oligomer occurs over a small part of the activator concentration range; *i.e.* the bound molecules of the activator interact in some way with the empty sites on the cytosolic domain of the H^+,K^+-ATPase to increase their affinity for the activator molecules. Such positive cooperativity is the mark of a delicate regulation mechanism and is inherent in the living system.

Following the optimal activation due to positive cooperative interaction, further increase in the activator caused marked inhibition in the H^+,K^+-ATPase activity. Such inhibition may reflect another form of endogenous activator regulation of the gastric H^+,K^+-ATPase reaction under physiological conditions. Precise reasons for the observed down-regulation remain unknown.

The preceding information demonstrates that the activator molecules have considerable regulatory influence on the activity of the H^+,K^+-ATPase. However, in order for the activator to act as an intracellular regulator, the activator must not only demonstrate the ability to regulate the H^+,K^+-ATPase but also, in turn, must be capable of being itself regulated by the cytosolic second messengers. Calcium, an important second messenger and critically essential for gastric acid secretion (31–33), inhibits the activator-stimulated H^+,K^+-ATPase reaction at a concentration range (2–4 μM) slightly higher than the normal physiological level (1 μM). Such extreme sensitivity of the activator-stimulated H^+,K^+-ATPase activity to Ca^{2+} not only suggests a precise and delicate regulatory mechanism for maintenance of the Ca^{2+} in different intracellular compartments of the parietal cells, but also emphasizes the role of both Ca^{2+} and the activator as members of a signal transducing cascade system in the process of gastric H^+ transport. Although it is likely that a Ca^{2+}-dependent conformational change may be involved in the activator or H^+,K^+-ATPase molecule or both, precise effects of Ca^{2+} at the molecular level remain to be determined.

Recently, the acid secretion by the isolated rat parietal cells has been demonstrated to be inhibited by a mechanism involving a Ca^{2+}-stimulated, phospholipid-dependent protein kinase C system of the parietal cells (34, 35). Thus, both dibutyryl cAMP and histamine-stimulated acid secretion was found to be strongly inhibited by 12-*O*-tetradecanoylphorbol-13-acetate as well as 1-oleoyl-2-acetylglycerol. It will be of much interest to see whether such Ca^{2+} inhibition (protein kinase C-mediated) is related to the observed (Fig. 9) Ca^{2+} inhibition of the activator-stimulated H^+,K^+-ATPase.

The data presented in this paper offer some important insight into the activator regulation of the gastric H^+,K^+-ATPase reaction. In addition, the study opened new avenues for further evaluation of the role of the endogenous activator as a possible link in an intracellular signal transducing cascade system. In view of recent revelations (36–41) similar cascades may be more like a general principle in biological signal transduction.

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REFERENCES

1. Forte, J. G., Machen, T. E., and Obrink, K. J. (1980) *Annu. Rev. Physiol.* **42,** 111–126
2. Ray, T. K., and Fromm, D. G. (1981) *J. Surg. Res.* **31,** 496–505
3. Soll, A. H. (1982) *Gastroenterology* **198,** 216–223
4. Takahasi, S., Moriwaki, K., Himeno, S., Kuroshima, T., Shinomura, Y., Hamabe, S., Kurokawa, M., Saito, R., Kaitani, T., and Tarui, S. (1983) *Life Sci.* **33,** 1401–1408
5. Machen, T. E., Rutten, M. J., and Ekblad, E. B. M. (1982) *Am. J. Physiol.* **242,** G79–G84
6. Rutten, M. J., and Machen, T. E. (1981) *Gastroenterology* **80,** 928–937
7. Dozois, R. R., Kim, J. K., and Dousa, T. P. (1978) *Am. J. Physiol.* **4,** E546–E551
8. Barglindh, T., Sachs, G., and Takeguchi, N. (1980) *Am. J. Physiol.* **239,** G90–G94
9. Kirkegaard, P., Christiansen, J., Peterson, B., and Skouolsen, P. (1982) *Scand. J. Gastroenterol.* **17,** 533–538
10. Muallem, S., Fimmel, C. J., Pandol, S. J., and Sachs, G. (1986) *J. Biol. Chem.* **261,** 2660–2667
11. Shaltz, L. J., Bools, C., and Reimann, E. M. (1981) *Biochim. Biophys. Acta* **673,** 539–551
12. Chew, C. S. (1985) *J. Biol. Chem.* **260,** 7540–7550
13. Ray, T. K., and Forte, J. G. (1974) *Biochem. Biophys. Res. Commun.* **61,** 1199–1206
14. Ray, T. K. (1978) *FEBS Lett.* **92,** 49–52

15. Ray, T. K., and Nandi, J. (1983) *Biochem. J.* **212,** 887–890
16. Sen, P. C., and Ray, T. K. (1980) *Arch. Biochem. Biophys.* **202,** 8–17
17. Nandi, J., Wright, M. V., and Ray, T. K. (1983) *Biochemistry* **22,** 5814–5821
18. Ray, T. K., and Nandi, J. (1984) in *Gastric H^+ Transport in Epithelia* (Forte, J. G., Warnock, D. G., and Rector, F. C., eds) pp. 161–169, John Wiley & Sons, New York
19. Bandopadhyay, S., and Ray, T. K. (1986) *Prep. Biochem.* **16,** 21–32
20. Nandi, J., and Ray, T. K. (1984) *Proc. N. Y. Acad. Sci.* **435,** 183–186
21. Meng-Ai, Z., Nandi, J., and Ray, T. K. (1984) *Fed. Proc.* **43,** 2054
22. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) *Biochemistry* **10,** 2606–2617
23. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
24. Goodwin, T. W., and Morton, R. A. (1946) *Biochem. J.* **40,** 628–632
25. Hirs, C. H. W. (1967) *Methods Enzymol.* **11,** 59–62
26. Grey, W. R. (1972) *Methods Enzymol.* **25,** 121–138
27. Ray, T. K., and Nandi, J. (1986) *Biochem. J.* **233,** 231–238
28. Ray, T. K., and Nandi, J. (1985) *FEBS Lett.* **185,** 24–28
29. Saccomani, G., Sachs, G., Cuppoletti, J., and Jung, C. Y. (1981) *J. Biol. Chem.* **256,** 7727–7729
30. Hoffman, P., and Tabakoff, B. (1982) *Biochem. Pharmacol.* **31,** 3103–3106
31. Kasbekar, D. K., and Chugani, H. (1976) in *Gastric H^+ Ion Secretion* (Kasbekar, D. K., Sachs, G., and Rehm, W. S., eds) pp. 187–211, Marcel Dekker, New York
32. Schwartz, M., Kashiwa, H. K., Jacobson, A., and Rehm, W. S. (1967) *Am. J. Physiol.* **212,** 241–246
33. Michelangeli, F., and Ruiz, M. C. (1984) in *Gastric H^+ Transport in Epithelia* (Forte, J. G., Warnock, D. G., and Rector, F. C., eds) pp. 149–160, John Wiley & Sons, New York
34. Anderson, N. G., and Hanson, P. J. (1984) *Biochem. Biophys. Res. Commun.* **121,** 566–572
35. Anderson, N. G., and Hanson, P. J. (1985) *Biochem. J.* **232,** 609–611
36. Robinson, G. A., Butcher, R. W., and Sutherland, E. W. (1971) *Cyclic AMP,* Academic Press, New York
37. Brown, J. E., Rubin, L. J., and Ghalayini, A. J. (1984) *Nature* **311,** 160–163
38. Gilman, A. G. G. (1984) *Cell* **36,** 577–579
39. Vandenberg, C. A., and Montal, M. (1984) *Biochemistry* **23,** 2339–2352
40. Stieve, H. (ed) (1986) The Molecular Mechanism of Photoreception, Dahlem Konferenzen, Springer-Verlag, Berlin
41. Pershadsing, H. A., and McDonald, J. M. (1980) *J. Biol. Chem.* **255,** 4087–4093

Supplementary Material to: Characteristics of a Pure Endogenous Activator of the Gastric H^+,K^+-ATPase System: Evaluation of the Role as a Possible Intracellular Regulator

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EXPERIMENTAL PROCEDURE

Purification of the Endogenous Activator: The activator was purified from the cytosolic fraction of the fundic cells of pig gastric mucosa following the method (19) described recently for the purification of dog activator. The purification procedure (at 0-4°C) involves simultaneous concentration and dialysis of the cytosolic fraction under negative pressure (using Micro-ProDicon, 60 kDa cutoff membrane), pH 4.8 precipitation and two consecutive Sephacryl S-200 column chromatography steps using first a long column (88cm x 2.8cm) and then a short column (67 cm x 1.6 cm). The typical yield of the activator from 462 mg of cytosolic protein was 15.9 mg and 9.4 mg in the first and second column with high activator activity. The material from the second column was pure activator showing a single 40 kDa band on SDS-PAGE.

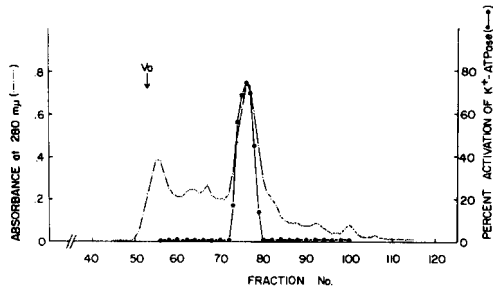

Fig. 1. Chromotographic purification of the pig endogenous activator using long (88 cm x 2.8 cm) sephacryl S-200 column. About 60 mg (2 ml volume) of pH 4.8 precipitate of the dialysed concentrated supernatant (Micro-ProDicon, 60 Kd cutoff membrane) was charged on to the column. The column was eluted with 50 mM Tris (pH 7.4) containing 0.2 mM EDTA and 0.2 DTT at a flow rate of 16.3 ml/hour. Fractions (total 110) of 3.4 ml collected and each was assayed for protein and activator activity. The details of the activator factor assay are given in the text.

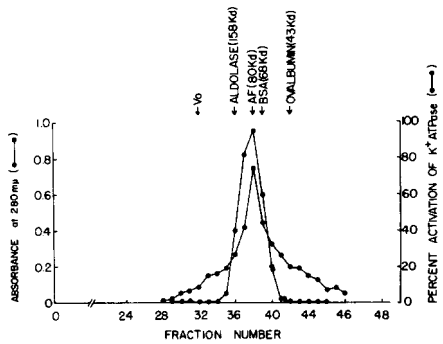

Fig. 2. Chromotographic purification of the pig endogenous activator using short (67 cm x 1.6cm) sephacryl S-200 column. The active fractions from the long columns (Fig. 1) were pooled together, dialysed and concentrated as in Fig. 1 against 2.5 L of 2.5 mM Pipes buffer (pH 7.4) containing 0.2 mM DTT and 0.2 mM EDTA, and charged to the column. About 1 ml (20-30 mg) was put on and eluted with the same buffer as in Fig. 1 above using a flow rate of 24.5 ml per hour. Forty-six fractions (1.7 ml each) were collected. Each fraction was assayed for protein and activator factor activity.