Biochimica et Biophysica Acta 817 (1985) 147–153
Elsevier

BBA 72645

Thiocyanate and nitrite inhibit proton translocation in gastric musosa

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(Received January 29th, 1985)

Key words: Proton translocation; Cross-over point; Secretion inhibitor; Acid secretion; (Frog gastric mucosa)

Isolated frog gastric mucosa was used to study the separation of formation of protons (or their precursors) from proton translocation by using various inhibitors. Both thiocyanate (SCN^-) and nitrite (NO_2^-) inhibit the acid secretion in spontanously secreting mucosa. The inhibition is reversed when the inhibitor is removed such that the excess acid secreted above baseline in the 'off'-period compensates for the amount inhibited in the 'on'-period. Both agents also inhibit the effect on acid secretion of pulse stimulation with histamine though to a lesser extent. Upon removal of the inhibitor, the total amount of acid secreted in excess of basal is equal to that observed with histamine alone. Likewise, metiamide, an H_2-antagonist, also inhibits acid secretion with or without histamine. However, in contrast to SCN^- and NO_2^-, removal of this inhibitor is without effect on the acid-secretion rate. These results indicate that both SCN^- and NO_2^- inhibit the proton translocation rather than the formation of protons or their precursors as is the case with metiamide.

A recent study conducted in this laboratory [1] showed that a pulse of high stimulator level results in prolonged secretion, i.e., beyond the length of time the stimulator is present. In addition, it was noted that the total amount of acid secreted depends only on the exposure (integral of stimulus) and not upon the shapes of either stimulatory or secretory curves. This indicated that a biochemical species once formed under the influence of secretagogue must be released or metabolized exactly at the rate of proton secretion regardless of any limitations set upon the rate of secretion, such as saturation. For this biochemical species, we have used the operational term 'acid' both in the previous study [1] as well as throughout this article. To understand the invariant relationship between secretagogue exposure and total acid secreted, the existance of a pool where 'acid' is sequestered and from which it can be released, is implied. Otherwise, no explanation can be given to the invariance. The hypothesis that the formation of 'acid' and translocation of protons are separate processes is the consequence of the existence of such a sequestered pool of 'acid'.

In an attempt to test this hypothesis of separate formation of 'acid' and translocation of protons we will implement the method of identification of the 'cross-over point' used in enzymatic reactions in which an agent is employed to block a certain enzymatic step thereby causing the accumulation of the substrate of that step [2]. If the formation of 'acid' and translocation of protons are not separate processes then the addition and continuous presence of any inhibitor would cause a permanent suppression of proton secretion and upon removal of the inhibitor the proton secretion rate would never exceed the rate observed before inhibition;

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that is, in the post-inhibitory period there is a net decrease in total amount of protons secreted. This has practical therapeutic consequences since some inhibitors such as H_2-antagonists (cimetidine) would essentially lower the amount of protons secreted.

If, on the other hand, the formation of 'acid' and the translocation of protons are separate processes, some inhibitors may identify this cross-over point between formation of 'acid' and translocation of protons by decreasing the proton secretion rate only during their presence in the system but upon their removal accumulated 'acid' has to be released with a consequent overshoot of the proton secretion rate such that there is no loss of total amount of protons secreted.

Thiocyanate (SCN^-) is a powerful inhibitor of gastric acid secretion [3]. However, the mechanism by which the inhibition occurs remains obscure. Recently, Hersey et al. [4] reviewed various hypotheses for the mechanism of action. They concluded that the inhibition is due to an increase in the rate of proton-gradient dissipation. Berglindh [5] suggests that while the protons are formed, SCN^- causes the protons to immediately 'reunite' with the base. A hypothesis suggested by Rehm et al. [6] and Gutknecht and Walter [7] is that while the protons are formed they are not transported into the lumen but rather combine with SCN^- in the membrane and diffuse back into the cell as HSCN. This hypothesis is also partly supported by Reenstra and Forte [8] who suggest that SCN^- increase the passive transport of protons, possibly as a back-flux of HSCN. Durbin [9] also subscribed to the back-flux theory based on ^{36}Cl studies. Nandi and Ray [10], on the other hand, suggest that SCN^- induces a conformational aberration in the $(H^+ + K^+)$-ATPase system such that the protons generated cannot be transported out of the membrane. They suggest the same mechanism for nitrite (NO_2^-) and cyanate (CNO^-). Thus, all of the above investigators [4–10] agree that protons are indeed formed, since only if protons are formed can they back-flux, be dissipated or be prevented from exiting.

This study was conducted to further investigate the separation of 'acid' formation from proton translocation by using the following inhibitors: metiamide, SCN^- and NO_2^-. A flow-through system and continuous proton secretion recording technique that has recently been developed in our laboratory [1] was used. The proton production activity is analysed in terms of total amount of protons secreted which is a unique function of exposure.

Materials and Methods

Bullfrogs (*Rana catesbiana*) from Lemberger Laboratories were used in all experiments. Histamine and cyclic AMP were purchased from Sigma. Metiamide was a gift from Smith, Kline and French. All other reagents were analytical grade.

Stomachs were removed from double-pithed frogs, and the mucosa, after being freed from the outer smooth muscle layer by blunt dissection, was mounted between lucite chambers with a support of nylon mesh on the secretory side. To enable rapid changes of nutrient solution, a flow-through system was used: the gassed solution was pumped through the nutrient chamber at a rate of 1 ml/min and was mixed in the chamber by a small magnetic stir-bar. The nutrient solution contained (in mM): NaCl, 79; $NaHCO_3$, 18; KCl, 4; $CaCl_2$, 1.8; $MgCl_2$, 0.8, and sodium hydroxybutyrate, 10. The solution was gassed with 95% O_2 and 5% CO_2. The secretory solution was 120 mM NaCl and oxygenated with 100% O_2. Acid secreted into the secretory solution was monitored by a pH-stat by keeping the pH at 7.0 with isotonic 15 mM NaOH. An autoburette (Radiometer, Copenhagen) was connected via a counter and a Binary Coded Decimal (BCD) interface (Hewlett-Packard) to a HP-85 computer (Hewlett-Packard). After mounting, the mucosae were allowed to recover for 2 h in order to establish a solid baseline of acid secretion (for details see Ref. 1).

Inhibition of acid secretion was done at different concentrations (0.5–5 mM for NaSCN, 1 and 3 mM for $NaNO_2$ and 0.1 mM for metiamide) and kept from 15 to 60 min. The concentrations were chosen to reflect the inhibition of secretion alone and not the inhibition of respiration (mitochondria) that occurs at higher concentrations [4]. Inhibition was also done in the presence of a stimulator. In these instances, the inhibitor and stimulator were given simultaneously, the inhibitor as a 30-min step and the histamine or cyclic AMP as a pulse.

The pulse here means that the tissue was exposed to the stimulator only for a short period since the stimulator is continously washed out by the flow-through system with a half-life of approx. 2 min [1]. (The exposure in mM · min is equal to the initial concentration in mM times the duration in min, i.e. the integral of an exponential with a half-life of 2 min. Thus, the exposure to a pulse amounts to 3 min · concentration, while the exposure to a step is $(t + 3)$ min · concentration.) Both, inhibitor and stimulator were added by introducing 30 μl of test substance (1/100th of the nutrient chamber volume) into the nutrient chamber followed by fresh nutrient solution containing appropriate concentration of the inhibitor but no stimulator. The step-inhibition was terminated by changing to plain nutrient solution.

Data analysis. Data stored on magnetic tapes of the HP-85 computer were prepared for analyses by subtracting exponentially declining baseline estimated with the help of both graphic and numeric outputs. The spontaneous secretion at the beginning (i.e., baseline) of the experiment had a mean value of 1.08 μequiv. H^+/cm^2 per h $\pm$ S.E. = 0.08 ($n = 72$). Then, the times of, as well as the values of the trough and the peak of the acid secretion curve were estimated by search for a group of five contiguous points yielding the lowest and highest mean value. Total amount of acid secreted was determined by direct summation of acid-secretion rates recorded every minute (for details see Ref. 1).

A logarithmic function was used to express the relationship between total amount of acid inhibited and exposure, the time integral of the inhibition curve. This function is identical with that expressing the relationship between total amount of acid secreted and the exposure to secretagogue [1].

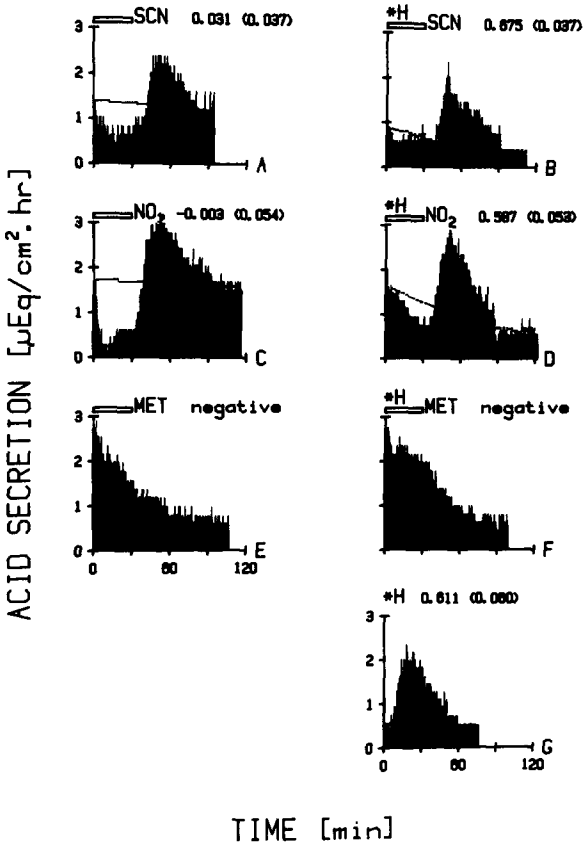

Fig. 1. The effects of inhibitors on acid-secretion rates and amount of acid secreted in frog gastric mucosa. The graphs presented in this figure are representative samples with the amount of acid secreted in μequiv. H^+/cm^2 given as the mean and (S.E.) of 3–4 experiments. The line across the graphs is the exponentially declining basal acid-secretion rate. The inhibitors were administered to the nutrient side at 1 ml/min for 30 min as indicated by the bar. Histamine was administered as a 1 μM pulse at time zero indicated by H and *. For explanation of pulse see Material and Methods. Panels A, C and E are spontanously secreting mucosa. Panels B, D, F and G are stimulated with 1 μM histamine. Panels A and B: 3 mM SCN^-; panels C and D: 3 mM NO_2^-; panels E and F: 0.1 mM metiamide; panel G: 1 μM histamine.

Results

Inhibition of acid secretion by SCN^- and NO_2^-

Inhibition of basal acid secretion rate. Administration of SCN^- or NO_2^- to a spontaneously secretion gastric mucosa results in a decline in acid-secretion rate to a new steady state. This inhibition is reversed when the inhibitor is removed such that the rate of acid secretion rises above baseline and then declines back to the baseline (Fig. 1A and C).

The area in Fig. 1 corresponds to the total amount of acid secreted in μequiv. H^+/cm^2 from the time of inhibition until the return of secretion rate to basal levels. Since the acid-secretion rate is monitored every minute, direct summation of the secretion rate over an interval yields the total

amount of acid secreted during that interval. After addition of SCN^- (or NO_2^-), acid secretion is suppressed below the basal secretion rate (interpolated exponentially in the figure). This area is by definition negative and implies the acid not secreted as compared with the amount of acid that would have been secreted if it were not inhibited. On the other hand, in the later period (after the removal of the inhibitor), the area is positive, i.e., more acid is secreted compared with the amount of acid that would have been secreted were it not (previously) inhibited. In the case of SCN^- and NO_2^- inhibition, the two areas (the negative and the positive) are sufficiently close to each other that they cancel each other, producing total acid secreted in excess of the basal amount to be insignificantly different from zero (Fig. 1A and C). Thus, the total amount of acid secreted above baseline from the time of inhibition until the return to baseline is zero or, simply stated, the amount inhibited in the 'on'-period all comes out during the 'off'-period.

In the range of concentrations (0.5–5 mM SCN) and duration (15–60 min), both the amount of acid inhibited and amount of rebound acid are functions of the exposure (concentration · duration) of SCN (Fig. 2). The amount of inhibited acid as a function of exposure was fitted to a logarithmic function. In other words, the amount of acid inhibited (or rebound) is independent of the manner in which the inhibitor is administered. There is no significant difference between amount of acid inhibited and the amount of rebound acid except at 5 mM SCN^- for 60 min. This deviation probably reflects impairment on the mitochondrial level and not a direct effect on acid secretion.

Inhibition of stimulated acid-secretion rate. When a pulse of 1 μM histamine is administered at the same time as SCN^- or NO_2^-, the inhibition is less pronounced. Upon removal of the inhibitor, the secretion rate overshoots the baseline, reaches a maximum and returns to baseline (Fig. 1B and D). However, when total amount of acid secreted above the baseline is calculated in these experiments, the acid released in the 'off'-period more than compensates for the amount inhibited during the 'on'-period such that the total amount secreted is equal to the amount seen with the stimulator alone (Fig. 1G).

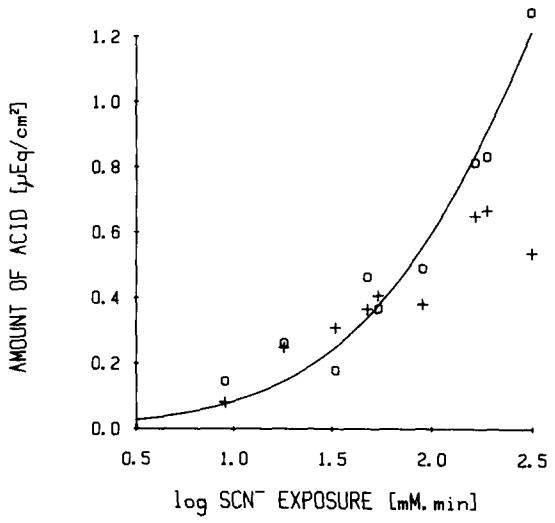

Fig. 2. Amount of acid inhibited as a function of SCN^- exposure. The mucosae were treated with SCN^- (0.5–5 mM) for different lengths of time (15–60 min) as in Fig. 1. The total amounts of inhibited (○) and rebound (+) acid in absolute values are plotted as a function of SCN^- exposure (concentrations · duration). Each data point is the average of 3–5 experiments. The data for the amount of inhibited acid are fitted to a logarithmic function. (For explanation see Materials and Methods.)

Similiar patterns were observed when 10 mM cyclic AMP was given as a pulse together with 3 mM SCN. The amount of acid secreted for 10 mM cyclic AMP in the presence of SCN was 0.334 ± 0.062 μequiv. H^+/cm^2 ($n = 3$) as compared with 0.284 ± 0.055 ($n = 5$) for cyclic AMP alone.

Inhibition of acid secretion by H_2-antagonist

Administration of metiamide (H_2-antagonist) to a spontaneously secreting mucosa also results in decline in acid-secretion rate to a new steady state (Fig. 1E). However, in contrast to SCN^- or NO_2^-, when this inhibitor is removed the acid-secretion rate remains at this lower steady-state level. Thus, the deviation from baseline acid secretion during a particular time period is negative. Simultaneous addition of a pulse of 1 μM histamine and inhibitor gives the same response as with inhibitor alone, i.e., negative excess acid secreted (Fig. 1F).

Discussion

The use of flow-through system in conjunction with an on-line microprocessor makes it possible

to study the dynamics of acid secretion in details that was not feasible in earlier studies. A flow-through system in which the tissue is constantly supplied with fresh solution, has the added advantage that any metabolites formed in the course of the experiment are removed from rather than being accumulated in the nutrient solution. This approach allows for a closer identification of the mechanism of action of stimulators as well as inhibitors.

Previous work from this laboratory [1] indicated that the reason for the unusual dynamics of proton secretion, namely, prolonged secretion after the removal of a high concentration of secretagogue, is due to the existence of a storage pool of 'acid'. Those data could be explained only if a concept of previously produced intermediate precursor of acid (or acid alone) was considered: then, a limited secretion rate would allow for the eventual quantitative release of the stored 'acid'. The inhibition experiments here were designed precisely to further test this concept.

The major new observation here is the rebound of proton secretion after the removal of inhibitors such as SCN^- and NO_2^- and that this rebound strictly compensates for the inhibition of proton secretion in the presence of the inhibitor both during basal and stimulated (histamine and cAMP) secretion. Moreover, it was observed that both the amount of acid inhibited and the amount of rebound acid were increasing functions of the exposure to the inhibitor, analogous to the relationship observed between amount of acid secreted and secretagogue exposure [1].

In addition, two cross-over points were identified. Metiamide blocks the action of histamine completely. The place of the action is before the adenylate cyclase and, as a result, 'acid' cannot be formed. On the other hand, SCN^- and NO_2^- both interact with the biochemical pathway after adenylate cyclase since 'acid' is formed in their presence (under the action of both histamine and cyclic AMP). The existence of these two cross-over points supports our original observation [1] that once the stimulus signal reaches beyond a certain step in the biochemical pathway, it cannot be stopped, except temporarily and what 'acid' was produced under the stimulator action must eventually be released as protons.

These observations allow for only a limited number of interpretations. Only such an interpretation that can simultaneously explain both the prolonged secretion after a pulse of high concentration of a stimulator (the observation reported in Ref. 1) and the delayed proton secretion caused by post-adenylate cyclase inhibitors upon their removal (as reported here) is pertinent to our consideration.

A comprehensive hypothesis that takes both phenomena into account, i.e., prolonged and delayed proton secretion, is that of an enzyme that catalyzes the release of 'acid' from the sequestered 'acid' pool. The content of the pool can be increased by secretagogue stimulation since as an enzymatic process it is saturable. At high stimulation level, an excess accumulation of 'acid' would occur in the sequestered pool and only the absence (removal) of further stimulation can eventually decrease the size of the pool. The same enzyme can also form an enzyme-inhibitor-complex: the inhibitor would decrease the level of free enzyme (alternatively expressed as an increase of K_m) and would in the presence of basal stimulation lead to accumulation of 'acid' in the sequestered pool. However, upon removal of the inhibitor the pre-inhibition concentration of free enzyme becomes available (K_m reverts to normal) resulting in the release of excess accumulated 'acid' observed as a rebound with an eventual return to basal secretion rate. The conservation of the amount of protons released during inhibition and post-inhibition periods is inherent in this model.

The only existing hypothesis that is compatible with the above interpretation of both inhibition by SCN^- and NO_2^- and of prolonged proton secretion after removal of stimulator is the one suggested by Nandi and Ray [10]. According to their hypothesis, SCN^- and NO_2^- induce a conformational aberration in the $(H^+ + K^+)$-ATPase preventing formed protons to be transported out of the membrane.

None of the remaining hypotheses, viz., that of 'proton gradient dissipation' [4], that of the inability of keeping acid and base separated [5] or that of 'back-flux' of protons which delivers the protons back to the general proton pool in the cytosol [6–9] can be competitive with the above model because neither by their nature can explain the

prolonged proton secretion due to high pulse stimulation. However, if one was forced to consider these hypotheses, one finds that they do not explain the rebound phenomenon. This is not surprising since at the time of the formulation of these hypotheses the rebound phenomenon was unknown. Furthermore if one wanted to explain the rebound phenomenon by backflux, the hypothesis would have to include a sequestered pool since in its present form [6–9] where the protons are delivered back to the general proton pool in the cytosol the backflux leads to pre-inhibition conditions, i.e., no excess of accumulated 'protons'. This alternative is operationally not distinguishable from direct competitive inhibition of an enzyme by SCN^- and NO_2^-. However, because of the high dissociation constant of HSCN [11] and the consequent necessity of identification of the sequestered pool with HSCN and because it does not explain prolonged secretion due to high pulse stimulation, this alternative is less likely. Instead, it is highly probable that direct enzyme inhibition is the cause of the temporary inhibition of acid secretion by SCN^- and NO_2^-. Planar anions, such as SCN^- and NO_2^-, are known to form so called 'dead-end' complexes with other enzymes [12,13].

If one was to speculate over the nature of the sequestered 'acid' pool, there seem to be only two kinds of substances which could be sequestered: either it is a precursor of protons or it is protons themselves. In either case, precursor or protons are naturally lost from the sequestered pool by the process of acid secretion.

The conjecture that an accumulation of activator (coenzyme) of an enzyme is responsible for the prolonged and/or delayed proton secretion, is not feasible for the reason that activators by definition are not modified by their action on the enzyme. For an activator to be identical with the sequestered pool, the activator cannot be metabolized and the emptying of the sequestered activator pool would have to proceed such that the inhibition of the rate of disappearance of activator from the sequestered pool is proportional to the inhibition of acid secretion. This would be a highly esoteric process.

The possibility that the precursor of protons is identical with ATP would require that ATP is sequestered and not communicating with the general pool of ATP. However, there is no support for such an idea, since SCN^- has little effect on either the ATP hydrolysis [8], or ATP regeneration (oxygen consumption) [4]. Consequently, no substantial amounts of ATP would be accumulated. It has furthermore been shown that cellular ATP levels are unaffected during SCN^- inhibition [14]. The idea that the ATP would be accumulated in a very small compartment not detectable in whole cell measurement is disqualified on the following grounds. In the case of stimulation with 1 μM histamine pulse in the presence of 1 mM SCN^- for 30 min, the amount of ATP would be 500 nmol/cm^2 (assuming $H^+ : ATP = 1:1$ [8]) or 100 nmol/mg protein (bullfrog mucosa has 5 mg protein/cm^2). Since the total amount of ATP + ADP + AMP is of the order of 10 nmol/mg protein [15], such an accumulation is obviously impossible.

The general concensus is that protons are formed even in the presence of SCN^- [4–10]. Thus, to propose that the sequestered pool of 'acid' is identical with protons is in keeping with that idea. The lowering by SCN^- of intracellular pH during histamine stimulation from 8.0 to 7.1 as reported by Hersey [16] gives further credence to the sequestered pool being protons. Hersey does in fact in this study suggest that the SCN^- inhibition of acid involves a process whereby protons are accumulated within a compartment that does not communicate with the glandular lumen. And while the explanation forwarded by Rehm et al. [6] of the formation of HSCN could fit the hypothesis of a sequestered pool it is contrary to the lowering of intracellular pH [16] thus bringing us back to the sequestered pool being protons themselves. The decrease in aminopyrine uptake by SCN^- in rabbit gastric glands as reported by Berglindh [5] would suggest that the 'acid' pool in the cell is inaccessible to the aminopyrine that accumulates in the intraglandular space. The inhibitory effect of weak bases on the inhibition by SCN^- has been suggested to be due to a simple competition between the weak base and SCN^- for protons [6,7]. Another possible explanation is that these weak bases compete with SCN^- for the low-affinity K^+ site on the $(K^+ + H^+)$-ATPase that has been suggested to be responsible for the vectorial translocation of formed protons out of the membrane [10].

In summary, the data do indicate that both

SCN^- and NO_2^- inhibit the translocation of protons out of the membrane, thus confirming the conclusion [1] of separation of the processes of formation of 'acid' and translocation of protons. However, the data do not support the back-flux theory since it would indeed be fortuitous if exactly the same amount of acid was secreted in the 'off'-period as was inhibited during the 'on'-period.

Finally, even if an alternative mechanism could be accepted to explain the inhibitory effects of SCN^- and NO_2^-, such an alternative also has to be able to explain the effect of stimulator on acid secretion (viz., the accumulation of 'acid') in the absence of any inhibitors. Thus, at this stage the only comprehensive model that can account for both phenomena is the sequestered 'acid' pool and the subsequent release from that pool.

Acknowledgements

This study was supported by a grant from the National Institutes of Health AM-21448 (E.B.M.E.). We are grateful for the skillful technical assistance supplied by Ms. Lily Hu. We are also grateful for the fruitful discussions with and helpful suggestions by the late Dr. Richard P. Durbin, and for laboratory space provided by Dr. Clifford W. Deveney.

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