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Gastric H^+ secretion: histamine (cAMP-mediated) activation of protein phosphorylation

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Activation of H^+ secretion by the gastric parietal cell involves major changes in morphology, metabolic activity and ion pathways of the secretory membrane. These changes are elicited by histamine binding to the H_2 receptor, raising cAMP levels and presumably activating cAMP-dependent protein kinase. Concomitantly, the intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$, increases. Studies were performed to determine whether cAMP-mediated protein phosphorylation accompanies histamine activation of H^+ secretion and to catalogue the major protein species serving as substrates for cAMP – dependent protein kinase in the parietal cell. 80% pure rabbit parietal cells, prepared by Nycodenz bouyant density centrifugation, were used. To investigate only cAMP-mediated effects, histamine-dependent changes in $[Ca^{2+}]_i$ in these cells were abolished by depleting intracellular Ca^{2+} stores and performing experiments under Ca^{2+}-free conditions. Acid secretion and steady-state levels of protein phosphorylation were then measured in unstimulated (cimetidine-treated) and histamine-stimulated cells. In intact parietal cells, concommitant with histamine stimulation of H^+ secretion, increases in the level of protein phosphorylation were observed. Significantly changing phosphoproteins found in supernatant fractions showed apparent subunit sizes of approx. 148, 130, 47 and 43 kDa, and in microsomal fractions included those at approx. 130, 51 and 47 kDa. In parietal cell homogenates, using [γ-^{32}P]ATP, cAMP elicited significant phosphorylation of eight supernatant proteins and twelve microsomal proteins, which included the histamine-dependent phosphoproteins found in the intact parietal cell, except for the 51 kDa microsomal protein. As a working hypothesis, these proteins are involved in stimulus-secretion coupling in the parietal cell.

Introduction

The gastric parietal cell, responsible for secretion of acid by the stomach, is an excellent model system for the study of stimulus-secretion coupling. Activation of acid secretion by this cell requires occupancy of a histamine (H_2) receptor, a cholinergic (muscarinic) receptor or a gastrin receptor present on the basal-lateral membrane. Irrespective of secretagogue used, upon stimula-

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IBMX, isobutylmethylxanthine; DCCD, *N,N'*-dicyclohexylcarbodiimide; TCA, trichloracetic acid; DMSO, dimethylsulfoxide; Bt_2cAMP, dibutyryl cAMP.

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tion, the parietal cell undergoes large (i) morphological changes (ii) changes in the properties of the apical membrane and (iii) metabolic changes. The abundant mitochondria, randomly distributed in the resting cell become clustered in the stimulated cell. Cytosolic membrane vesicles (tubulovesicles) which contain the gastric H^+/K^+-ATPase, the H^+ pump, disappear following stimulation and numerous long microvilli appear lining the secretory canaliculus [1]. Cl^- [2] or K^+ and Cl^- [3] conductances in the pump-containing membranes increase, with a resultant increase in HCl production at the apical membrane of the parietal cell. Associated with these changes is an increase in oxygen consumption [4].

In the parietal cell, it is well established that histamine activates adenylate cyclase, resulting in increased intracellular levels of cyclic AMP [5,6]. Gastrin and cholinergic agonists have been shown to cause an increase in the Ca^{2+} permeability of the basal-lateral membrane [7,8], leading to increased concentration of intracellular free Ca^{2+}, ($[Ca^{2+}]_i$) [8,9]. Chew [10,11] and Negulescu and Machen [12] have recently shown that not only carbachol and gastrin, but also histamine, result in increases in $[Ca^{2+}]_i$ from intracellular stores. With carbachol and gastrin, this Ca^{2+} release is mediated by inositol 1,4,5-trisphosphate [11]. Since cyclic AMP-dependent protein kinases, both type I and type II, have been identified in the parietal cell [13,14], it is presumed that the intracellular mechanism of action of cAMP is to activate this cAMP-dependent protein kinase, with the consequent phosphorylation of target proteins.

Changes in the phosphorylation state of proteins is a general mechanism by which neurotransmitters, hormones and other signals exert their effects in a vast variety of tissues [15,16]. Determination of the targets of cAMP-mediated protein phosphorylation is essential for the understanding of stimulus-secretion coupling. Recently, in other tissues, some specific targets or substrates of the cAMP-dependent protein kinase have been identified to be, in some cases, cytoskeleton-associated proteins [17,18] and in other cases, ion channels [19,20]. Such targets are of particular relevance to the parietal cell, since stimulation of acid secretion results in large and rapid morphological rearrangements, which are thought to involve the cytoskeleton and in changes in the ionic conductance properties of the apical membrane.

In the work presented, a partially purified parietal cell preparation [21] was used to examine the substrates of cAMP-dependent protein kinase. Such targets for this enzyme were observed and defined in supernatant and microsomal fractions of homogenates of parietal cells, following cAMP addition and in histamine-stimulated intact parietal cells, after $[Ca^{2+}]_i$ changes had been abolished. The more highly purified parietal cells were used for the study to minimize interference from other cell types, particularly peptic cells. Histamine alone was used as the secretagogue since it is parietal cell specific [22] and is the major effector of intracellular cAMP elevation. Since histamine is now also known to cause an increase in $[Ca^{2+}]_i$, to investigate only cAMP-mediated effects, $[Ca^{2+}]_i$ changes were abolished by depleting intracellular Ca^{2+} stores and performing experiments under Ca^{2+}-free conditions after loading the cells with the Ca^{2+} chelators quin2/AM [23] or fura-2/AM [24]. Under these conditions, parietal cells continued to respond to histamine and histamine-dependent (cAMP-mediated and Ca^{2+}-independent) changes in the level of phosphorylation of target parietal cell proteins were observed and defined. Preliminary accounts of some of these data have appeared in abstract form [25,26].

Materials and Methods

Cell preparation and measurement of acid secretion. Partially purified and functional parietal cells from rabbit gastric mucosa were prepared as described by Berglindh [21]. Briefly, gastric mucosa was treated with pronase, followed by collagenase. The resulting crude cell preparation consisting of 35–50% parietal cells was purified by bouyant density gradient centrifugation using Nycodenz. Parietal cells were enriched at the 1.078 g/ml interface to about 80% by this procedure and were 95% viable as measured by Trypan blue exclusion (Berglindh, unpublished observations). Acid secretion was monitored by the uptake of the weak base [*dimethylamine*-^{14}C]aminopyrine using standard incubation medium, as described by Berglindh et al. [4]. Routinely, aminopyrine ratios

were measured in these parietal cells after 30 min incubation with and without a variety of secretagogues to monitor the responsiveness and viability of the cells (see Table II, Results). This was done just prior to and immediately following the phosphorylation experiments, to ensure that the cells maintained their viability and ability to secrete acid. No deterioration of secretory responses was observed. The viability of the cells was also measured fluorimetrically by quantitating ethidium bromide (25 μM) uptake as described by Gomperts [27]. Maximum ethidium bromide uptake (0% viability) was measured after addition of large doses of digitonin and observing no further fluorescence change. Unstimulated (cimetidine-treated) and histamine-stimulated cells were found to be 90% viable after 30 min incubation.

Standard incubation medium comprised (mM): Na^+, 143.4; K^+, 5.4; Mg^{2+}, 1.2; Ca^{2+}, 1.0; Cl^-, 139.8; SO_4^{2-}, 1.2; HPO_4^{2-}, 5.0 and $H_2PO_4^-$, 1.0. The medium also contained 2 mg/ml glucose, 2 mg/ml rabbit albumin, 10 mg/l Phenol red and the pH was 7.4.

Depletion of intracellular Ca^{2+} stores and measurement of $[Ca^{2+}]_i$. Parietal cells were washed three times in Ca^{2+}-free and P_i-free medium (pH 7.4) containing 0.2 mM EGTA prior to the experiment. The medium contained 132.5 mM Na^+, 5.4 mM K^+, 142.5 mM Cl^-, 1.2 mM Mg^{2+}, 1.2 mM SO_4^{2-}, 11.4 mM Hepes, 5.5 mM Tris (pH 7.4), 2 mg/ml glucose and 2 mg/ml rabbit albumin. The cells were then incubated in this medium either for 30 min at 37°C with 50 μM quin2/AM or for 10 min with 2 μM fura-2/AM and 150 μM Ca^{2+}. The medium containing quin2/AM or fura-2/AM was removed and the cells were washed twice prior to measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured as previously described [9,28] using a SPEX dual excitation, dual emission cation measurement instrument. For quin2, excitation and emission wavelengths were 339 and 492 nm with slit-widths of 4 and 10 mm, respectively. For fura-2, excitation and emission wavelengths were 340/380 and 510 nm, respectively, with 2 mm slit-widths. Parietal cells resuspended in fresh medium were transferred to a thermostatically controlled cuvette with a stirring system. $[Ca^{2+}]_i$ was measured in resting cells. The effects of 10^{-4} M histamine and 10^{-5} M carbachol were measured, followed by the addition of 1 mM Ca^{2+} to the medium. At the end of the experiment, the Ca^{2+} signal was calibrated by adding digitonin (20 μg/ml cells) to permeabilize the cells and to determine F_{max}. Then 2 mM Mn^{2+} was added to completely quench the signal. Control experiments were performed using cells washed and incubated as described above, but in medium containing 1 mM Ca^{2+} throughout. The effect of 10^{-4} M cimetidine and 10^{-4} M atropine on the histamine and carbachol responses in control cells was also investigated.

Measurement of $[^{32}P]P_i$ uptake into cells and cellular protein. Prior to performing phosphorylation experiments, the time required to load parietal cells with $[^{32}P]P_i$ to a steady state was investigated. Unstimulated parietal cells, after treatment to deplete intracellular Ca^{2+} stores as described above were incubated at 37°C with 10 μCi $[^{32}P]P_i$/ml cells. At various times, 500 μl aliquots of cells plus medium were rapidly spun down and washed two times with ice-cold medium. 1.5 ml of ice-cold 5% TCA was then added to the cells to precipitate the cellular proteins. After spinning at 3000 rpm for 5 min, the TCA supernatant was separated from the protein pellet. 1 ml of the TCA supernatant was then added to 1 ml of cold acidified ammonium molybdate (4% $(NH_4)_6Mo_7O_{24}$, w/v, four parts: 60% PCA 1 part). After mixing, 2.5 ml of cold isobutanol/benzene (1 : 1, v/v) was added and the mixture was vortexed for 30 s. Following a 5 min 3000 rpm centrifugation, 200 μl of the isobutanol/benzene layer was counted in ACS scintillation fluid for ^{32}P radioactivity. The protein pellets were washed two times with ice-cold 5% TCA, extracted with ethanol/diisopropyl ether (1 : 1, v/v), then dissolved in 1 M NaOH at 50°C and counted in Dimilume 30 (Packard) scintillation fluid. Protein determinations were carried out on parallel samples using the method of Lowry et al. [29].

$[^{32}P]P_i$ loading and cell incubation. Parietal cells were first depleted of intracellular Ca^{2+} as described above by washing in Ca^{2+} and P_i-free medium containing 0.2 mM EGTA, followed by incubation with quin2/AM or fura-2/AM. After removing the medium containing quin2/AM or fura-2/AM, the cells were incubated for 30 min at 37°C with $[^{32}P]P_i$ (10–50 μCi/ml cell suspension)

to load the cells with the isotope. The experiment was started by adding either 10^{-4} M cimetidine or 10^{-4} M histamine to obtain control (unstimulated) and stimulated cells, respectively. Cimetidine was added to block the H_2 receptor. In some experiments, 1.3 mM Ca^{2+} was added immediately following cimetidine or histamine. 1.0 ml aliquots were taken at 15 min, rapidly centrifuged in an Eppendorf centrifuge and the supernatant was discarded. The cell pellet was immediately frozen in a solid CO_2/methanol mixture and kept at −80 °C until further processing. In parallel experiments, acid secretion was monitored by adding [^{14}C]aminopyrine instead of [^{32}P]P_i to the cell suspension and measuring aminopyrine ratios at 15 and 30 min after addition of cimetidine or histamine. In some cases, the experiment was performed in the absence of added isotope and cell pellets were frozen and kept at −80 °C for labelling with [γ-^{32}P]ATP as will be described.

Processing of cell pellets and preparation of samples for SDS-gel electrophoresis. The frozen cell pellets were homogenized by sonication in an ice bath twice for 10 s in ice-cold medium containing 50 mM sodium phosphate (pH 7.4), 20 mM NaF, 2 mM sodium pyrophosphate, 2 mM EDTA and 0.2 mM EGTA (homogenization medium) using a probe sonicator. The samples were then separated into a supernatant fraction and a microsomal fraction. The sonicate was first centrifuged for 2 min at 12000 × *g* and the pellet was discarded. The supernatant was then centrifuged for 15 min at 100000 × *g*, yielding a supernatant fraction and a crude microsomal fraction (pellet). Proteins in the samples were then precipitated twice with ice-cold 5% TCA (w/v) and 5 mM phosphate (final concentrations) and the precipitates were collected by a 5 min centrifugation in an Eppendorf centrifuge. The precipitates were then resuspended by sonication in chloroform/methanol (1 : 1, v/v) or ethanol/dipropyl ether (1 : 1, v/v) to remove phospholipids, spun down and dried under nitrogen. The protein pellet was dissolved in 2% SDS in 0.5 M Tris-Cl buffer (pH 6.8). The protein concentration of the samples was determined using the method of Lowry et al. [29], with bovine serum albumin as standard.

SDS-polyacrylamide gel electrophoresis and autoradiography. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [30] using 10% and 3.5% acrylamide in the separating and stacking gels, respectively. Samples were mixed with application buffer and heated in a water bath to 100 °C for 1 min prior to application. Equal amounts of protein were applied to each well and this varied between 25 and 100 μg protein per well depending on the experiment. High- and low-molecular-weight standards (Bio-Rad) were run on each gel. The gels were stained with Coomassie blue and after destaining, were treated with 5% TCA at 90 °C for 20 min to reduce background radioactivity [31]. The gels were photographed, dried and then autoradiographed at −80 °C in Dupont cassettes containing Cronex Hi-Plus intensifying screens. Cronex 4 X-ray film was used and developed in a standard X-ray processor after appropriate exposure times (3–14 days). Autoradiographs were scanned using a laser densitometer (LKB). Linearity of the [^{32}P]P_i autoradiography was checked by running gels with varied amounts of [^{32}P]P_i-labelled cell proteins (10, 25, 50 and 100 μg) and comparing the band intensity for several proteins. The Coomassie blue-stained protein pattern of control (unstimulated) and stimulated cells were identical. Therefore, ^{32}P-labelled proteins were quantitated by measuring the ^{32}P-protein peak area using the scan of autoradiograms. Results are expressed as ^{32}P-protein peak area after stimulation relative to control (unstimulated) ^{32}P-protein peak. Statistical significance of the difference between control (unstimulated) and stimulated ^{32}P-protein peak areas was performed using the students *t*-test.

[^{32}P]ATP labelling of homogenates. Frozen unstimulated (cimetidine-treated) cell pellets were homogenized in 500 μl medium by sonication. The sonication medium contained 50 mM sodium phosphate (pH 7.4), 0.2 mM EGTA, 0.2 mM EDTA, 10^{-4} M IBMX, 10^{-4} M ouabain, 10^{-4} M oligomycin and 10 μM *N,N'*-dicyclohexylcarbodiimide (DCCD). Homogenates were separated into supernatant and microsomal fractions as described above. Phosphorylation reactions were initiated by adding Mg/ATP (2 mM and 1 mM) preadjusted to pH 7.4 with Tris base containing 10 μCi[γ-^{32}P]ATP with or without 10^{-5} M cAMP. Reaction mixtures were incubated for 3 min at

37 °C. The reactions were stopped by the addition of 500 μl 10% TCA and 10 mM P_i. The fractions were then prepared for SDS-gel electrophoresis and autoradiography as described. The presence of 20 mM NaF in the medium had no measurable effect on the results obtained.

Materials. [*dimethylamine*-^{14}C]Aminopyrine (117 mCi/mmol) was obtained from Amersham. [^{32}P]P_i as H_3PO_4 (285 Ci/mg) and adenosine 5′-[^{32}P]triphosphate, tetratriethylammonium salt (4286 Ci/mmol) were all from ICN. Pronase was from Merck and Nycodenz was from Accurate Chemical. Collagenase, histamine, 8-bromo-cyclic AMP, dibutyryl cyclic AMP, rabbit albumin, cyclic AMP (sodium salt), oligomycin, ouabain, DCCD, ethidium bromide and digitonin were obtained from Sigma; forskolin was from Calbiochem. Molecular weight standards for SDS-gel electrophoresis were from Bio-Rad. Quin2/AM was a gift from S. Muallem and fura-2/AM was from Molecular Probes. All other chemicals were of reagent grade. Quin2/AM (50 mM) and fura-2/AM (2 mM) were made up in dimethylsulfoxide (DMSO). DMSO alone at the final concentration used had no effect on the parameters measured.

Results

Effect of cyclic AMP in homogenates

Unstimulated (cimetidine-treated) parietal cells were homogenized and supernatant and microsomal fractions were prepared, as described in the Materials and Methods. The effect of 10^{-5} M cAMP on the phosphorylation of proteins by [γ-^{32}P]ATP was then investigated. cAMP had no obvious effect on the protein pattern, being apparently identical in the presence and absence of cAMP (data not shown). However, cAMP stimulated phosphorylation of proteins with a range of apparent molecular weights between 148 and 26 kDa in both the supernatant (Fig. 1A) and microsomal (Fig. 1B) fractions. Table I shows the mean

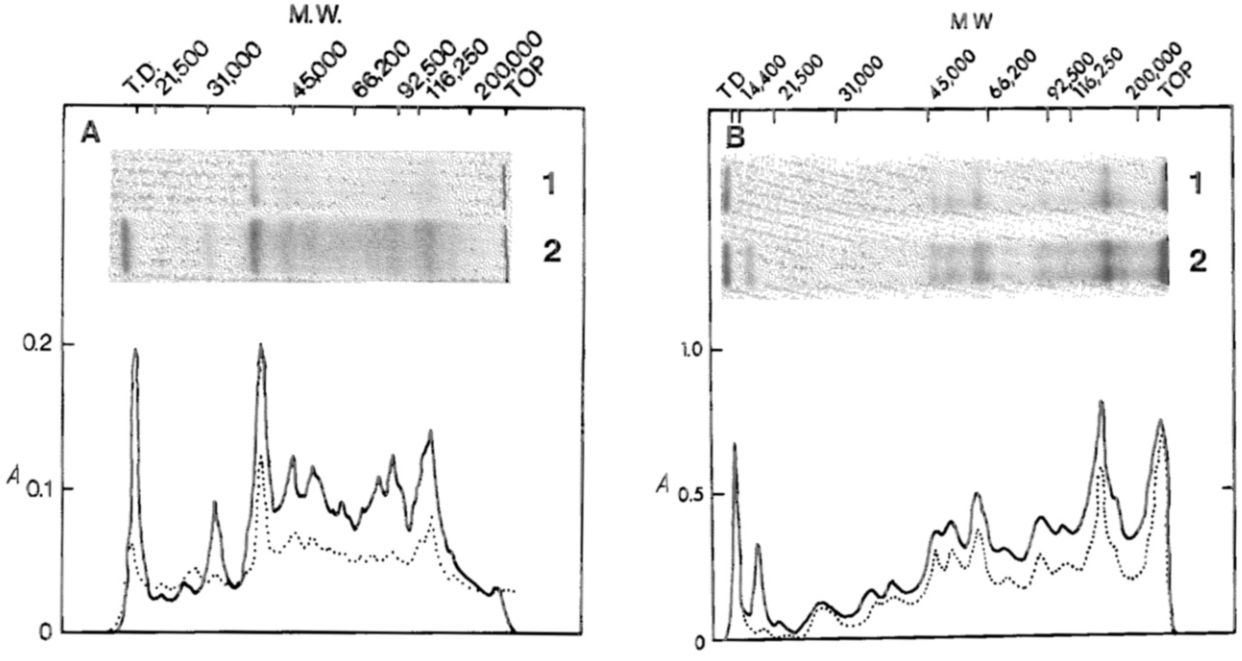

Fig. 1. Phosphorylation of parietal cell homogenates by endogenous cAMP-dependent protein kinase. (A) Supernatant proteins (100 μg) of unstimulated cells were incubated at 37 °C with [γ-^{32}P]ATP in the absence and presence of 10^{-5} M cAMP. Coomassie blue-stained electrophoretograms were autoradiographed and scanned. Lane 1, autoradiogram in the absence of cAMP (·····); lane 2, autoradiogram in the presence of cAMP (———). The scans of the autoradiograms are also shown. (B) Microsomal proteins (100 μg) of unstimulated cells were treated in the same manner as the supernatant proteins shown in A. Lane 1, autoradiogram in the absence of cAMP (·····); lane 2, autoradiogram in the presence of cAMP (———). In both supernatant and microsomal fractions, a number of proteins increased their state of phosphorylation upon treatment with cAMP (see Table I). MW, molecular weight.

TABLE I

APPARENT MOLECULAR MASSES OF AND INCREASES IN PHOSPHOPROTEINS IN PARIETAL CELL HOMOGENATI FRACTIONS IN THE PRESENCE OF [^{32}P]ATP AND IN THE PRESENCE OR ABSENCE OF cAMP

Differentially labelled phosphoproteins in supernatant and microsomal fractions from parietal cell homogenates using endogenou cAMP-dependent protein kinase, [γ-^{32}P]ATP, 10^{-5} M cAMP and 10^{-4} M IBMX. Results are expressed as the ^{32}P-protein peak are in the presence of cAMP relative to control without cAMP. Data are expressed as mean ± S.E. n, number of experiments. n.s., no significant = $P > 0.05$.

Supernatant				Microsomes			
molecular mass (kDa)	(n)	^{32}P-protein peak area +cAMP/−cAMP	P (<)	molecular mass (kDa)	(n)	^{32}P-protein peak area +cAMP/−cAMP	P (<)
147±2	(5)	1.64±0.28	n.s.	148±1	(5)	1.29±0.11	n.s.
131±1	(5)	1.68±0.21	0.05	130±0.2	(5)	1.25±0.07	0.05
97±2	(5)	2.33±0.20	0.01	99±1	(5)	1.36±0.04	0.01
84±2	(4)	2.19±0.33	0.05	85±1	(5)	1.37±0.07	0.01
				70±1	(4)	1.35±0.09	0.05
64±1	(5)	1.88±0.30	0.05	60±0.5	(5)	1.22±0.06	0.05
56±1	(5)	1.98±0.19	0.01	52±0.6	(5)	1.18±0.06	0.05
47±0.4	(5)	1.85±0.33	0.05	47±0.5	(5)	1.22±0.07	0.05
42±0.4	(5)	1.77±0.26	0.05				
				39±0.3	(5)	1.24±0.08	0.05
33±0.2	(5)	3.05±0.40	0.01	35±0.4	(5)	1.42±0.14	0.05
30±1	(4)	1.27±0.08	n.s.	29±0.5	(3)	1.17±0.12	n.s.
27±1	(4)	1.12±0.12	n.s.	27±0.3	(4)	1.43±0.12	0.05
				22±0.5	(4)	2.28±0.30	0.05
				20±0.6	(5)	3.74±0.34	0.002

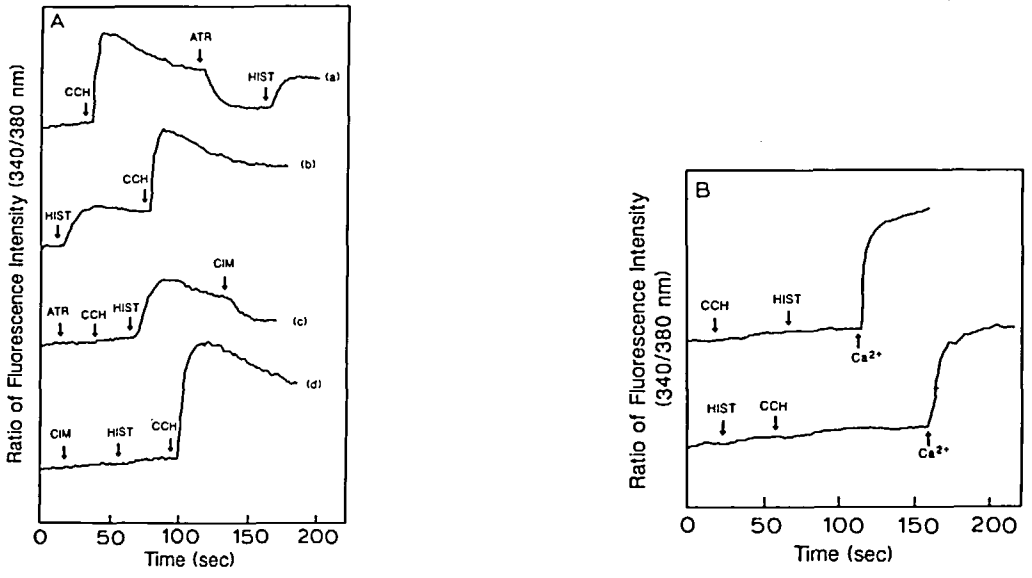

Fig. 2. Intracellular free [Ca^{2+}] responses. (A) Control cells: parietal cells were washed and loaded with fura-2/AM in medium containing 1 mM Ca^{2+}. $[Ca^{2+}]_i$ changes were measured using fura-2/AM (as described in Materials and Methods) in response to carbachol (CCH) (10^{-5} M) and histamine (HIST) (10^{-4} M). Atropine (ATR) (10^{-4} M) blocked only the carbachol response; cimetidine (CIM) (10^{-4} M) blocked only the histamine response. (B) Ca^{2+}-depleted cells: parietal cells were washed and loaded with fura-2/AM in Ca^{2+}/P_i-free medium containing 0.2 mM EGTA. $[Ca^{2+}]_i$ changes were monitored using fura-2/AM as described in Materials and Methods. Neither carbachol (10^{-5} M) nor histamine (10^{-4} M) alone or together elicited any change in $[Ca^{2+}]_i$. Thus, under these conditions, histamine-dependent (and carbachol-dependent) $[Ca^{2+}]_i$ changes were abolished. Addition of 1 mM Ca^{2+} to the medium resulted in an increase in $[Ca^{2+}]_i$ to control levels in the presence of both secretagogues.

apparent molecular weights of detectable phosphoproteins and their peak area in the presence of cAMP relative to control, which was in the absence of cAMP, resulting from endogenous cAMP-dependent protein kinase activity in several experiments. cAMP elicited significant phosphorylation of eight and twelve phosphoproteins in the supernatant and microsomal fractions, respectively. cAMP-induced increases in phosphorylation level varied, with the most striking increases occurring in 97, 84 and 33 kDa supernatant proteins and in 22 and 20 kDa microsomal proteins. Smaller but significant increases were also observed in several proteins including 131, 47 and 42 kDa supernatant proteins and 130 and 47 kDa microsomal proteins. In both fractions, two or three phosphoproteins did not significantly change their level of phosphorylation.

Therefore, cAMP-dependent protein kinase was present and capable of phosphorylating endogenous substrates in both fractions. The next important question was of whether similar proteins also showed increased phosphorylation levels in the intact parietal cell, following histamine stimulation.

Intracellular free [Ca^{2+}]responses

Since histamine stimulation of acid secretion results not only in increased intracellular cAMP, but also in increased intracellular free Ca^{2+} from intracellular stores and from the medium [8–12], to investigate only cAMP-mediated phosphorylation of target substrates in intact parietal cells, the cells were first depleted of intracellular Ca^{2+} stores by washing in Ca^{2+}-free medium containing EGTA (0.2 mM) and loading with fura-2/AM or quin2/AM as described in the Materials and Methods. $[Ca^{2+}]_i$ changes in parietal cells in response to histamine (10^{-4} M) and carbachol (10^{-5} M) were investigated in these conditions and compared to control responses of cells kept in Ca^{2+}-containing medium throughout. Fig. 2A shows typical $[Ca^{2+}]_i$ responses using fura-2/AM in control cells. Thus, as shown in scan (a) carbachol caused a rapid increase in $[Ca^{2+}]_i$ followed by a decrease to a plateau level. Addition of atropine (10^{-4} M) following carbachol reduced $[Ca^{2+}]_i$ to resting (unstimulated) levels and histamine could still elicit a response. Scan (b) shows a typical response to histamine. $[Ca^{2+}]_i$ increased to about one third that observed with carbachol, and subsequent addition of carbachol elicited a further increase in $[Ca^{2+}]_i$. The carbachol response was abolished by prior addition of atropine (scan c) with persistence of the histamine response. Scan (c) also shows that addition of cimetidine (10^{-4} M) after histamine resulted in a distinct decrease in $[Ca^{2+}]_i$. Addition of cimetidine before histamine abolished the histamine response, but not the carbachol response (scan d). Mean values for $[Ca^{2+}]_i$ were: resting (unstimulated) cells, 119 ± 16 nM ($n = 6$); histamine-treated cells, 207 ± 39 nM (3) and carbachol-treated cells (peak response), 321 ± 73 nM (4). These values are similar to those measured by Chew and Brown [11] in purified parietal cell suspensions. The histamine and carbachol responses are lower than those measured by Negulescu and Machen [12] on single responding parietal cells.

In contrast, as shown in Fig. 2B, with cells treated to deplete intracellular Ca^{2+}, neither histamine nor carbachol alone or together caused any significant increase in $[Ca^{2+}]_i$. Subsequent addition of 1 mM Ca^{2+} to the medium resulted in an increase in $[Ca^{2+}]_i$. Mean values of $[Ca^{2+}]_i$ in these experiments were: resting (unstimulated) cells, 57 ± 4 nM (5); histamine treated cells, 71 ± 6 nM (5); carbachol-treated cells (peak response), 90 ± 8 nM (5). Upon Ca^{2+} addition to the medium in the presence of histamine and carbachol, $[Ca^{2+}]_i$ increased to 324 ± 68 nM (4), a value similar to that measured in control cells indicating that the cells were not damaged or leaky to Ca^{2+}. Thus, under the conditions used, intracellular stores of Ca^{2+} were depleted and, thus, the histamine-dependent increase in $[Ca^{2+}]_i$ was abolished. As will be shown below the acid secretory response to histamine was maintained to a large extent (60%) in these Ca^{2+}-depleted cells as compared to that measured in control cells.

[^{32}P]P_i uptake and utilization

To investigate histamine-dependent phosphorylation changes in the intact parietal cell, the cells required loading with [^{32}P]P_i to a steady-state level. Fig. 3 shows that [^{32}P]P_i uptake into parietal cells was rapid in the first 15 min and then levelled off. The inset indicates that the [^{32}P]P_i taken up

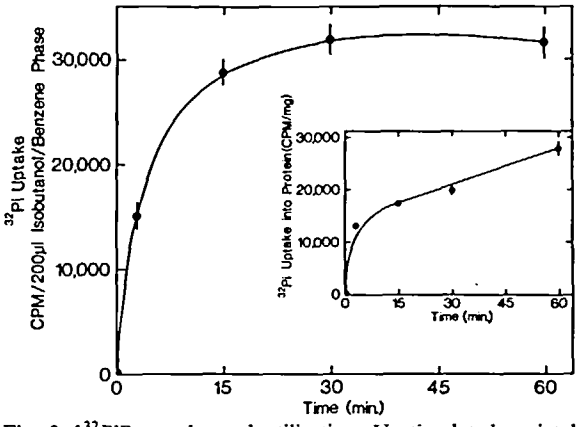

Fig. 3. [^{32}P]P_i uptake and utilization. Unstimulated parietal cells were incubated with 10 µCi/ml cells [^{32}P]P_i. [^{32}P]P_i content of the cells was determined from the radioactive phosphomolybdate complex extracted into isobutanol/benzene at the indicated times as described in the Materials and Methods. Inset: TCA precipitates of the cells described above were further extracted with ethanol/diisopropyl ether as described in Materials and Methods at the indicated times. Steady-state levels of labelling of [^{32}P]P_i-pools and ^{32}P-protein occurred within 30 min, which was, therefore, used in subsequent experiments.

by the cells was incorporated into protein (defined as TCA-insoluble, organic solvent-insoluble material). Thus, protein phosphorylation also occurred rapidly in the first 15 min and then levelled off within 30 min. Therefore, in subsequent phosphorylation experiments, a 30 min loading time with [^{32}P]P_i was used.

Secretory responses

Routinely, the responsiveness of the parietal cells to a variety of secretagogues was measured in standard incubation medium which contains 1 mM Ca^{2+}. The results are shown in Table II (A). Without any additions, or in the presence of cimetidine, the cells accumulated aminopyrine to a ratio of 75. Upon addition of histamine, acid secretion increased 6-fold to a ratio of 467. Similarly, with 8-bromo-cAMP, Bt_2cAMP and forskolin, 6–7-fold increases in secretion were measured. These responses were stable and similar even when remeasured several hours later (data not shown).

To investigate only cAMP-mediated effects, phosphorylation experiments were carried out under Ca^{2+}-free and P_i-free conditions, with intracellular Ca^{2+} stores depleted as described. Under these conditions, the histamine-mediated increase in $[Ca^{2+}]_i$ was abolished (see Fig. 2B). The histamine-stimulated acid secretory response of the cells under these Ca^{2+}-depleted conditions was measured and is shown in Table II (B). As observed in standard incubation medium, histamine resulted in a 6-fold increase in the aminopyrine ratio above the unstimulated level. Addition of Ca^{2+} had no significant effect on the aminopyrine ratios. No difference was observed between cells

TABLE II

PARIETAL CELL ACID SECRETORY RESPONSES

Purified parietal cell acid secretory responses (aminopyrine ratios) in (A) standard medium and in (B) medium used for phosphorylation experiments. Purity of the cells was 78±2% (22). The responses in standard medium (A) were measured after 30 min incubation and showed 6–7-fold increases with histamine, 8-Br-cAMP, Bt_2cAMP and forskolin. The parietal cells in (B) were first washed in Ca^{2+}/P_i-free medium plus EGTA and then incubated with quin2/AM or fura-2/AM as described in the Materials and Methods to deplete intracellular Ca^{2+} stores, followed by 30 min incubation at 37°C to parallel the [^{32}P]P_i loading procedure. Agents were then added as indicated and aminopyrine ratios were measured 15 min and 30 min later. Histamine in the absence or presence of added Ca^{2+}, resulted in a 6-fold increase in the aminopyrine ratio. Ca^{2+} had no significant effect ($P > 0.05$). Data are expressed as mean±S.E. (No. of experiments).

Conditions	Aminopyrine ratio	
	5 min	30 min
(A) In standard medium		
Basal (no addition)	–	75±12 (24)
Cimetidine (10^{-4} M)	–	75±13 (24)
Histamine (10^{-4} M)	–	467±37 (25)
8 Br-cAMP (10^{-3} M)	–	499±37 (25)
Bt_2cAMP (10^{-3} M)	–	413±47 (21)
Forskolin (10^{-5} M)	–	511±53 (21)
(B) In medium used for phosphorylation experiments		
Cimetidine (10^{-4} M)	41± 5 (12)	43± 9 (13) *
Histamine (10^{-4} M)	275±32 (12)	274±29 (13) **
Cimetidine (10^{-4} M) Ca^{2+} (1.3 mM)	55±11 (5)	64±14 (6) *
Histamine (10^{-4} M) Ca^{2+} (1.3 mM)	313±47 (5)	308±43 (6) ***

* Not significantly different ($P > 0.05$) vs. that measured in standard medium shown in (A); ** significantly different ($P < 0.001$) vs. that measured in standard medium shown in (A); *** significantly different ($P < 0.02$) from that measured in standard medium shown in (A).

TABLE III

APPARENT MOLECULAR MASSES OF AND INCREASES IN PHOSPHOPROTEINS IN INTACT $[Ca^{2+}]_i$-DEPLETED PARIETAL CELLS STIMULATED WITH HISTAMINE

Detectable phosphoproteins in supernatant and microsomal fractions of $[Ca^{2+}]_i$-depleted $[^{32}P]P_i$ loaded intact parietal cells as a result of 10^{-4} M histamine stimulation. Results are expressed as the ^{32}P-protein peak area in the presence of histamine relative to control (with cimetidine). Data are expressed as mean ± S.E. *n*, number of experiments. n.s., not significant (= $P > 0.05$).

Supernatant				Microsomes			
molecular mass (kDa)	(*n*)	^{32}P-protein peak area +histamine/ +cimetidine	*P* (<)	molecular mass (kDa)	(*n*)	^{32}P-protein peak area +histamine/ +cimetidine	*P* (<)
148±2 *	(5)	1.33±0.05	0.01	148±2	(3)	1.12±0.08	n.s.
130±1 *	(6)	1.29±0.05	0.01	129±1 *	(3)	1.34±0.06	0.05
97±1	(7)	1.08±0.04	n.s.	103±3	(3)	1.19±0.05	n.s.
				93±1	(3)	1.20±0.12	n.s.
83±1	(5)	1.06±0.04	n.s.	85±1	(3)	1.05±0.05	n.s.
				78±2	(3)	1.13±0.07	n.s.
66±1	(6)	1.08±0.08	n.s.	70±2	(3)	1.15±0.08	n.s.
56±1	(7)	1.07±0.04	n.s.	61±2	(3)	1.08±0.04	n.s.
				51±1 *	(3)	1.43±0.06	0.02
47±0.2 *	(7)	1.50±0.04	0.001	47±0.5 *	(3)	1.38±0.08	0.05
43±1 *	(7)	1.58±0.09	0.002				
35±1	(4)	1.03±0.02	n.s.				
29±0.6	(6)	1.06±0.06	n.s.				
24±0.3	(5)	1.07±0.07	n.s.	25±0.3	(3)	0	n.s.

* Significantly changing phosphoproteins.

loaded with 50 μM quin2/AM or 2 μM fura-2/AM. Therefore, the results were combined. However, when absolute aminopyrine ratios were compared, the histamine response, but not the basal response, in Ca^{2+}-depleted cells was significantly decreased (by 40%) as compared to control cells. Nevertheless, 60% of the histamine-stimulated secretory response was maintained in Ca^{2+}-depleted cells.

Protein phosphorylation in the intact cell

Fig. 4 shows Coomassie blue-stained SDS-gels of the supernatant (Fig. 4A) and microsomal (Fig. 4B) proteins from unstimulated (cimetidine-treated) and histamine-stimulated, $[Ca^{2+}]_i$-depleted parietal cells as well as autoradiographs of the separated ^{32}P-proteins. There were no observable differences in the protein patterns between unstimulated and stimulated cells. Under the conditions used, the H^+/K^+-ATPase of 95–100 kDa located in the crude microsomal fraction is evident, but not highly enriched. Radioactive phosphate incorporation into protein was evident in both unstimulated and stimulated cells, but was increased in stimulated cells. As shown in the scans of the autoradiograms and summarized in Table III, four supernatant proteins of 148, 130, 47 and 43 kDa exhibited significantly increased steady-state ^{32}P-labelling as a result of histamine treatment, while seven other phosphoproteins did not change significantly. In the microsomal fraction, 130, 51 and 47 kDa proteins became more highly phosphorylated, with seven detectable phosphoproteins remaining unchanged. Proteins of similar molecular weight (except for the 148 kDa protein) were observed to be significantly phosphorylated by cAMP-dependent protein kinase in homogenates (see Table I).

In both supernatant and microsomal fractions, seven phosphoproteins did not significantly alter their phosphorylation state following histamine stimulation, indicating that the specific activity of $[^{32}P]$ATP did not change during the experiment. This view is also supported by the finding that

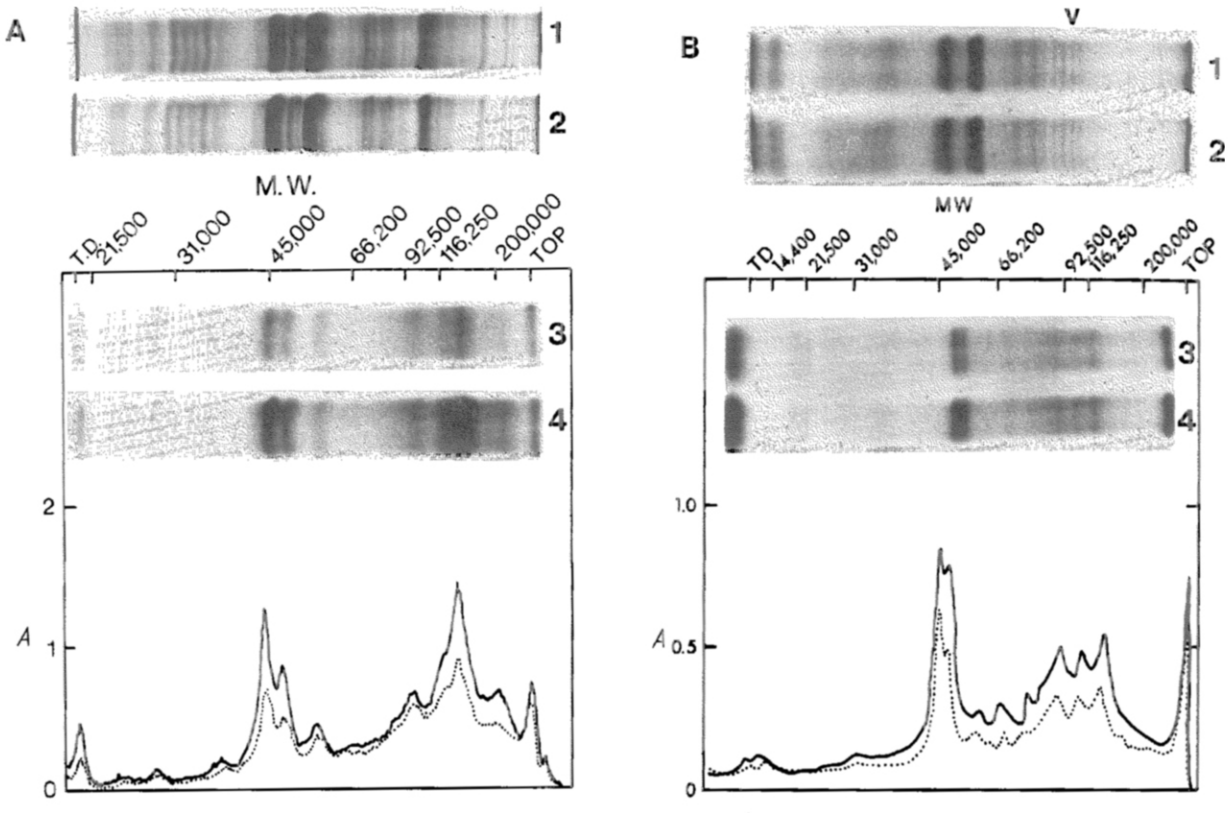

Fig. 4. Phosphoproteins of intact parietal cells. Parietal cells with depleted $[Ca^{2+}]_i$ (as described in Materials and Methods) were loaded with $[^{32}P]P_i$ for 30 min and then incubated for an additional 15 min with either 10^{-4} M cimetidine or 10^{-4} M histamine to obtain unstimulated and stimulated cells, respectively. Supernatant (A) and microsomal (B) fractions were obtained, processed and separated by SDS-PAGE as described in Materials and Methods. Lanes 1 and 2 show Coomassie blue-stained protein patterns of unstimulated and stimulated cells, respectively, and show no differences. The arrowhead indicates the presumed position of H^+/K^+-ATPase in the microsomal (B) fraction. Lanes 3 and 4 show autoradiograms of lanes 1 and 2. Also shown are the scans of the autoradiograms of unstimulated (·····) and stimulated (———) cells. In both fractions, histamine stimulation resulted in an increase in the phosphorylation state of several proteins (see Table III). Supernatant changing phosphoproteins were approx. 148, 130, 47 and 43 kDa proteins. Microsomal changing phosphoproteins were approx. 130, 51 and 47 kDa proteins. Similar proteins (except for the 51 kDa microsomal protein) were observed to change in parietal cell homogenate fractions as a result of cAMP treatment (see Table I).

different proteins increased their phosphorylation level by differing amounts.

Effect of medium Ca^{2+}

The changes in protein phosphorylation following histamine stimulation observed in intact cells (Fig. 4) were obtained in cells with depleted $[Ca^{2+}]_i$. The effect of Ca^{2+} (1.3 mM) addition to the medium immediately following cimetidine or histamine, was examined after the parietal cells were loaded with $[^{32}P]P_i$ under Ca^{2+}-depleted conditions. Fig. 5A and B show the effect of Ca^{2+} addition to the medium on the ^{32}P-protein pattern of supernatant and microsomal fractions, respectively, from stimulated parietal cells. In both fractions, a lower steady-state level of protein phosphorylation was observed. The effect was more pronounced on the proteins which showed histamine-dependent increases in phosphorylation. Thus, when the ^{32}P-protein patterns of unstimulated and stimulated cells were compared (Fig. 5C and D), only increases in the phosphorylation levels of the 47 and 130 kDa supernatant proteins were still evident. In three experiments, similar

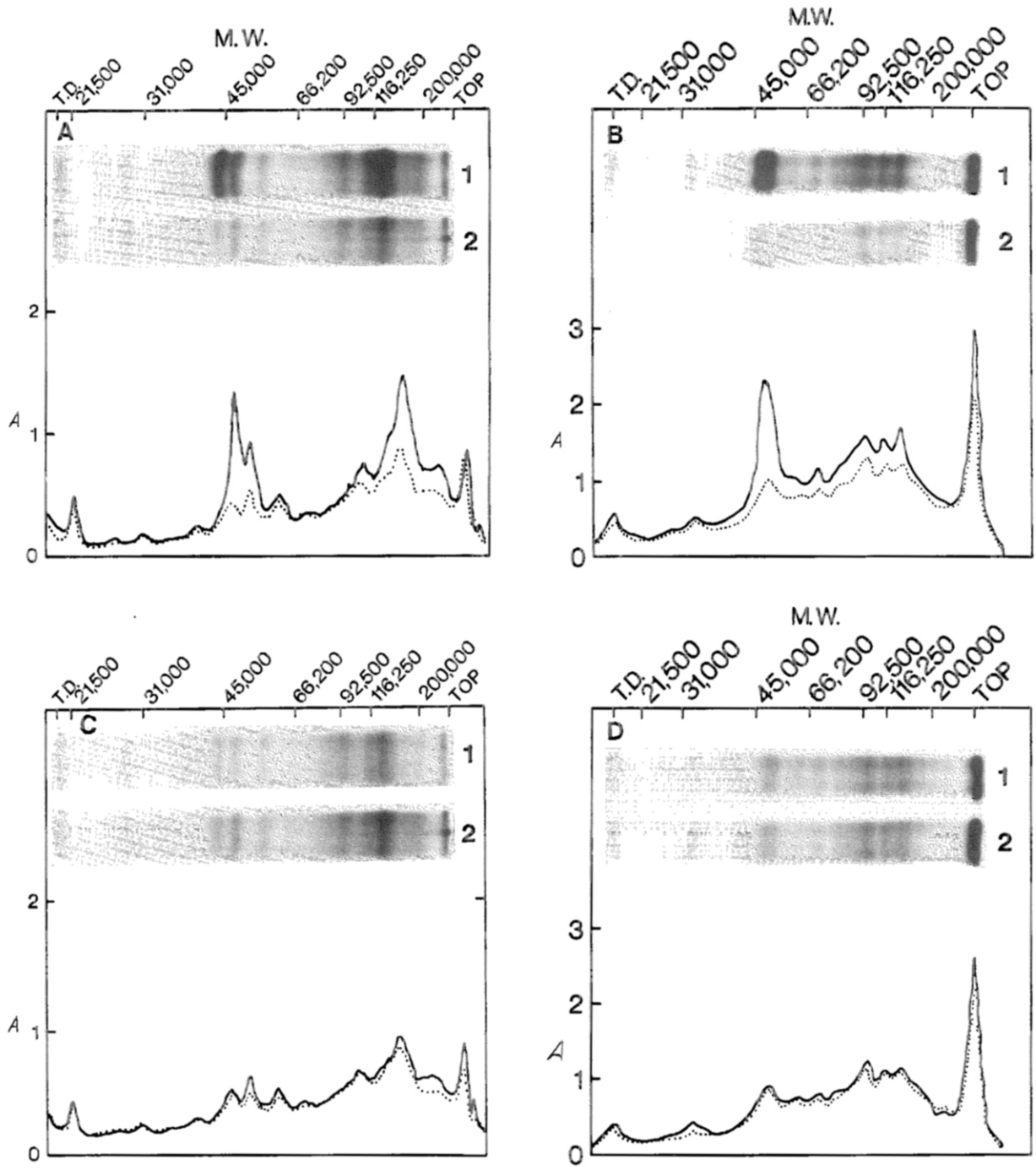

Fig. 5. Effects of medium Ca^{2+}. Unstimulated $[Ca^{2+}]_i$ depleted parietal cells were loaded with [^{32}P]P_i as described in Materials and Methods. The cells were then treated with cimetidine unstimulated cells) or stimulated with 10^{-4} M histamine for 15 min. Ca^{2+} (1.3 mM) or medium was added immediately following the addition of cimetidine or histamine. The effect of this Ca^{2+} addition on the phosphoprotein pattern of supernatant (A) and microsomal (B) fractions from histamine-stimulated cells is shown. Lane 1, autoradiogram of cells in the absence of Ca^{2+} (———); lane 2, autoradiogram of cells in the presence of Ca^{2+} (·····). In both fractions, a marked decrease in the phosphorylation state of the proteins occurred. Effects were also observed in unstimulated cells. When the phosphoprotein patterns of unstimulated (lane 1, ·····) and stimulated (lane 2, ———) cells in the presence of Ca^{2+} were compared in supernatant (C) and microsomal (D) fractions, increased phosphorylation levels only in the 47 and 130 kDa supernatant proteins were still evident. In three experiments, similar results were obtained.

results were obtained. Under similar conditions, Ca^{2+} addition immediately following cimetidine or histamine had no significant effect ($P > 0.05$) on acid secretion as measured with the aminopyrine ratio method (see Table IIB).

Discussion

Activation of acid secretion by histamine binding to the parietal cell H_2 receptor leads to a characteristic morphological change [1], an increase in O_2 consumption [4] and a change in apical membrane permeability [2,3]. With histamine treatment, concerted regulation of these cell processes is thought to occur via cAMP as second messenger, which initiates phosphorylation of key cellular regulatory proteins. Although it has long been known that increased $[Ca^{2+}]_i$ occurs with acetylcholine and gastrin [8,9], it is only recently that similar but smaller changes in $[Ca^{2+}]_i$ have also been observed following histamine treatment [10–12]. In whole animals, the H_2 antagonist, cimetidine abolished pentagastrin and carbachol-stimulated acid secretion, while the stimulated morphology was unaffected [32]. Therefore, it appears that the presence of cAMP is essential for the full expression of the stimulated state of acid secretion, although it is only with histamine treatment that intracellular cAMP actually increases [5,6]. Thus cAMP plays a central and vital role in the stimulation of acid secretion irrespective of secretagogue used.

The parietal cell proteins which change their state of phosphorylation as a result of histamine treatment in the absence of $[Ca^{2+}]_i$ changes and, thus, attributable to cAMP alone, are therefore likely to serve important regulatory roles in the cell systems involved in the control of acid secretion. Of interest are peripheral membrane proteins (including cytoskeletal elements), soluble proteins (including enzymes involved in oxidative metabolism and glycolysis) and integral membrane proteins (including proteins involved in ion transport or in linkage of cytoskeletal elements).

In the present survey of parietal cell phosphoproteins, a partially purified parietal cell preparation (80%) was employed. The contaminating cells consisted of a low percentage of peptic cells and an unidentified small cell type. Whereas peptic cells as well as parietal cells both exhibit secretor responses with cAMP analogues, only parietal cell respond to histamine in the rabbit [22]. Thus, th use of histamine, rather than cAMP analogues ensured that any observed changing phos phoproteins in intact cells were derived only fron parietal cells.

As a first step towards identification of thos phosphoproteins involved in the cAMP-mediate regulation of gastric acid secretion, the presence o parietal cell substrates for endogenous cAMP-de pendent protein kinase was investigated. A num ber of putative substrates of cAMP-dependen protein kinase were found to be present in th gastric parietal cell, both in supernatant and mi crosomal fractions (see Table I). cAMP elicite large increases in the phosphorylation of thre supernatant proteins (97, 84 and 33 kDa) and tw microsomal proteins (22 and 20 kDa). Smaller bu significant increases were also observed in severa proteins including the 131, 47 and 42 kDa super natant proteins and the 130 and 47 kDa micro somal proteins. cAMP-dependent protein phos phorylation of eight supernatant proteins from parietal cell (75% pure) homogenates has been reported [33]. Six of these proteins (140, 100, 86, 62, 58 and 48 kDa) were of similar apparent molecular weight as those found in the present work. The magnitude of the changes was not reported.

It was, then, of prime importance to examine whether these putative substrates were also substrates for the cAMP-dependent protein kinase in the functional intact parietal cell upon stimulation with histamine. Since histamine, the established elevator of intracellular cAMP in gastric parietal cells [5,6], has also recently been shown to cause an increase in $[Ca^{2+}]_i$ [10–12], in order to investigate cAMP-mediated effects alone, $[Ca^{2+}]_i$ was depleted in the cells prior to the phosphorylation experiments such that histamine-stimulated changes in $[Ca^{2+}]_i$ were abolished (see Fig. 2B). These Ca^{2+}-depleted cells were then preloaded with cimetidine (unstimulated cells) or stimulated with histamine. In parallel to the phosphorylation experiments, under exactly similar conditions, histamine resulted in a 6-fold increase in acid secretion with maintenance of 60% of the absolute aminopyrine ratio with histamine as compared to

control parietal cells which were not Ca^{2+}-depleted. If various pools of inorganic and organic phosphate exist in the parietal cell, it would be difficult to ensure that all such pools were labelled to the same extent in unstimulated and stimulated cells. However, in this study, a number of phosphoproteins in intact parietal cells showed insignificant changes in phosphorylation upon stimulation (see Table III), indicating that the specific activity of cellular ATP was similar whether or not the cells were stimulated.

The concept of unstimulated and stimulated cells is a physiological definition based on relative rates of acid secretion, the rate of metabolism and the quantitative assessment of attendent cell morphology. Even in 'unstimulated cells', some level of acid secretion is always present [4], which is unaffected by cimetidine or atropine. Morphological analysis shows that a remnant of the apical canalicular membrane persists in unstimulated cells [1]. There is also a low but finite amount of cAMP present in unstimulated cells [5]. Therefore, it was not surprising that background labelling of phosphoproteins was always evident in unstimulated cells. Nevertheless, upon stimulation of the cells with histamine, under $[Ca^{2+}]_i$-depleted conditions, concomitant with the 6-fold increase in acid secretion, four supernatant proteins (148, 130, 47 and 43 kDa) and three microsomal proteins (129, 51 and 47 kDa) significantly increased their state of phosphorylation. These changes must be occurring only in parietal cells, since histamine alone, known to be parietal cell-specific [22], was used as secretagogue. Additionally, these changes occur solely as a result of the activation of parietal cell cAMP-dependent protein kinase by cAMP, since $[Ca^{2+}]_i$ did not change in these experiments (see Fig. 2B). Except for the 51 kDa microsomal protein, these changing phosphoproteins were also observed to be phosphorylated by cAMP-dependent protein kinase in homogenates. The other cAMP-dependent changes in protein phosphorylation observed in parietal cell homogenate fractions may arise from (i) cell types other than the parietal cell, since the cells were only 80% pure; (ii) changes in conformation of parietal cell substrate proteins following homogenization; and/or (iii) from changes in availability of parietal cell substrate proteins after homogenization. The molecular weights of pepsinogen and pepsin have been reported to be 38 and 35.5 kDa [34]. Thus, in intact cell experiments the changing phosphoproteins do not correspond to pepsinogen or pepsin.

When $[Ca^{2+}]_i$ was increased to normal levels by adding 1.3 mM Ca^{2+} to the medium immediately following cimetidine or histamine, a reduction in the steady-state level of phosphorylation was observed at 15 min. This effect was more pronounced on the histamine-dependent phosphoproteins, such that when comparing phosphoprotein patterns of unstimulated and stimulated cells, increased phosphorylation of only the 47 and 130 kDa supernatant proteins was still evident. These results suggest that increases in $[Ca^{2+}]_i$ may promote endogenous phosphatase activity. In contrast, Ca^{2+} addition had no effect on acid secretion as measured by the aminopyrine ratio at 15 min. Nevertheless, the Ca^{2+}-induced reduction in both the steady-state level of protein phosphorylation and the histamine-dependent changes in protein phosphorylation observed at 15 min without a concomitant effect on the steady-state aminopyrine ratio does not negate a functional role of these phosphoproteins in acid secretion. Phosphorylation of these proteins may serve as a trigger for the cellular events required for stimulation of secretion and/or may affect the rate at which the steady-state level is achieved. Oddsdottir et al. [35] have recently reported that a 42 kDa parietal cell protein showed histamine-dependent increased phosphorylation at 1 min, with the maximum observed at 5 min and which was then undetectable at 15 min. These experiments, performed without depleting $[Ca^{2+}]_i$ in the presence of medium Ca^{2+} are in agreement with our finding that the histamine-dependent increased phosphorylation of a 42 kDa protein under $[Ca^{2+}]_i$-depleted conditions, is undetectable at 15 min in the presence of Ca^{2+}. Thus, it seems that in the absence of $[Ca^{2+}]_i$ changes, some cAMP-mediated protein phosphorylation changes are maintained as well as magnified. The findings of Oddsdottir et al. [35] also support the view that some phosphoprotein changes may serve as triggers for secretion. It is interesting that the morphological change in parietal cells following histamine and IBMX addition has been reported to be maximal 5 min after secretagogue addition,

while the maximum aminopyrine ratio was not achieved until 20 min after stimulation [36]. Additionally, histamine-dependent peak changes in $[Ca^{2+}]_i$ occur within 25–30 s, while the peak increase in intracellular cAMP occurs at 10 min [5]. Detailed investigation of the role of Ca^{2+} in the time course of the histamine-stimulated protein phosphorylation, morphological and acid secretory changes are in progress as a first step towards understanding the physiological role of Ca^{2+} in the histamine response in parietal cells.

Histamine-related increased phosphorylation levels of: (i) 80 and 120 kDa proteins associated with an 18% Ficoll density gradient-purified membrane fraction isolated from gastric glands [37] and (ii) 27 and 40 kDa parietal cell proteins associated with a particulate fraction isolated from purified parietal cells [38] have been reported recently. We did not detect significant changes in the phosphorylation of proteins of similar molecular weights. Several reasons could explain these differences. Urushidani et al. [37] used histamine with IBMX as secretagogue in gastric glands. Although histamine alone is parietal cell-specific, peptic cells respond to cAMP analogues [22] and, thus, it is possible that IBMX affects peptic cells. Since gastric glands contain about 50% parietal cells with peptic and endocrine cells making up the other 50%, the 80 and/or 120 kDa changing phosphoproteins detected by Urushidani et al. [37] may have derived from a cell type other than the parietal cell. Additionally, $[Ca^{2+}]_i$ was not depleted, which would explain why phosphoprotein changes similar to those observed in our work were not observed. Chew and Brown [38] reported that the acute addition of EGTA or BAPTA (1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetracetic acid) plus 50 μM quin2/AM after $[^{32}P]P_i$ loading had no effect on the 27 and 40 kDa changing phosphoproteins observed on two-dimensional electrophoresis after histamine stimulation of parietal cells. This is not surprising, since, it has been shown that under similar conditions, $[Ca^{2+}]_i$ changes still occur [10–12], because the intracellular stores of Ca^{2+} are maintained. In our work, intracellular Ca^{2+} stores were depleted by extensive washing in Ca^{2+}-free medium containing EGTA, as confirmed by our measurements which showed that $[Ca^{2+}]_i$ changes due to histamine (and carbachol) were abolished. It is also possible that differences in sample preparation as well as the subsequent analysis by SDS-PAGE could explain why we did not observe changes in phosphoproteins of similar molecular weights. Experiments using two-dimensional electrophoresis may reveal other changing phosphoproteins, not detected in the present work.

Having identified four supernatant and three microsomal proteins which showed histamine-dependent (cAMP-mediated, Ca^{2+}-independent) increases in phosphorylation, it is now possible to approach identification of these proteins as well as investigation of their functional role(s) in the generation and maintenance of the stimulated state of acid secretion. Preliminary data examining parietal cell phosphoproteins associated with a Triton X-100-insoluble fraction (known to consist of a residue enriched in cytoskeletal proteins [39,40]) suggest on the basis of similar molecular weights, that two of the histamine-dependent changing phosphoproteins (130 and 47 kDa) may be cytoskeleton-associated [26]. Work is now in progress to show specifically that these are the changing phosphoproteins. Possible roles of such cytoskeletal proteins might be to link the cytoskeleton to the membrane or to modify the stability of microfilaments. A 130 kDa phosphoprotein (vinculin) has been described in stress fiber attachment points in chick myotubes [41]. Vinculin has also been shown to be phosphorylated by cAMP-dependent protein kinase as well as by C-kinase [42]. Possible similarity between vinculin and the 130 kDa parietal cell phosphoprotein is at present being investigated, since vinculin is found in parietal cells [43].

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