

Research Article

Ya-hom, a Thai herbal remedy, has opposing effects on histaminergic and cholinergically mediated acid secretory related responses in gastric glands isolated from rabbits

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ABSTRACT

Ya-hom, a Thai traditional medicine, is used as a treatment for gastric discomfort. We previously found that Ya-hom inhibits both histamine and carbachol-stimulated acid secretion in gastric fistula rats. Herein, we used isolated gastric glands and highly enriched parietal cells from rabbit to determine whether or not Ya-hom acts directly on the acid-secreting parietal cell. Ya-hom inhibited carbachol-stimulated [¹⁴C] aminopyrine (AP) accumulation in rabbit gastric glands and prevented carbachol-induced elevation of [Ca²⁺]_i in parietal cells. In contrast, Ya-hom potentiated histamine-stimulated AP accumulation and increased the stimulatory effect of the specific adenylyl cyclase activator, forskolin, in the presence of the histamine H₂ receptor antagonist, cimetidine. Ya-hom had no effect on downstream cAMP-dependent signaling events including phosphorylation of LASP1 (LIM and SH3 domain protein 1), and F-actin redistribution. Because the cholinergic signaling pathway is mediated via calcium-dependent mechanisms, we compared effects of Ya-hom on carbachol-induced elevation of [Ca²⁺]_i with those of thapsigargin, a specific endoplasmic reticulum Ca²⁺ pump inhibitor that also potentiates histamine-stimulated AP accumulation. Both agents similarly potentiated histamine-stimulated AP accumulation and their actions were not additive. Furthermore, Ya-hom inhibited thapsigargin-induced elevation of [Ca²⁺]_i. Based on these findings, we postulate that Ya-hom induces a slow release of calcium from intracellular stores thereby suppressing the cholinergic response and potentiating the cAMP signaling pathway linked to the histamine response. Since Ya-hom directly inhibits the stimulatory effect of carbachol on acid secretion, the gastric action of this herbal remedy may target vagally-dependent HCl secretion.

Keywords:

Ya-hom, Gastric acid secretion, Gastric glands, Parietal cells, Intracellular calcium, Histamine, Carbachol

1. INTRODUCTION

Ya-hom is a folk formula that is used to treat fainting, nausea, vomiting and gastric discomfort. This traditional remedy comprises of various types of herb as in Table 1. Most scientific reports have focused on effects of specific Ya-hom components on the cardiovascular system. To date, only a few reports have addressed effects of certain of these components on the gastrointestinal system where *G. glabra*¹ and *M. arvensis*² has been

shown to inhibit gastric acid secretion, *G. glabra*³, *A. lappa*⁴, and *C. cassia*⁵ to have antiulcer activity, and *G. glabra*⁶, *M. officinalis*⁷, *A. lappa*⁸, *S. aromaticum*^{9,10} and *A. gramineus*¹¹ to have spasmolytic activity. Currently, there is lack of knowledge concerning the overall effect of Ya-hom on gastrointestinal function. Since stomach discomfort can be caused by increased gastric acid secretion, we previously analyzed the effect of Ya-hom on gastric secretion to verify its benefit as a potential acid secretory suppressant and, hence, a beneficial

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Table 1. The composition of Ya-hom.

Scientific name	Family	g in 100 g of Ya-hom
<i>Agastache rugosa</i> (Fisch. & C.A.Mey.) Kuntze	Lamiaceae	7.1
<i>Acorus gramineus</i> Aiton	Acoraceae	3.5
<i>Lysimachia foenum-graecum</i> Hance	Primulaceae	3.3
<i>Citrus nobilis</i> Lour.	Rutaceae	7.1
<i>Magnolia officinalis</i> Rehder & E.H.Wilson	Magnoliaceae	11.8
<i>Cinnamomum cassia</i> (L.) J.Presl	Lauraceae	7.1
<i>Mentha arvensis</i> L.	Lamiaceae	3.5
<i>Asarum sieboldii</i> Miq.	Aristolochiaceae	2.3
<i>Ligusticum striatum</i> DC.	Apiaceae	9.3
<i>Glycyrrhiza glabra</i> L.	Fabaceae	4.8
<i>Syzygium aromaticum</i> (L.) Merr. & L.M.Perry	Myrtaceae	7.1
<i>Aucklandia lappa</i> DC.	Asteraceae	7.1
<i>Aquilaria malaccensis</i> Lam.	Thymelaeaceae	7.1
<i>Atractylodes ovata</i> (Thunb.) DC.	Asteraceae	9.3
<i>Angelica anomala</i> Avé-Lall.	Apiaceae	3.5
Menthol	-	4.7
Borneo camphor	-	1.4

treatment for gastric discomfort. Using a rat gastric fistula model, we have demonstrated that the water extract of Ya-hom inhibits both carbachol and histamine-stimulated gastric acid, pepsin and soluble mucus secretion but increases visible mucus secretion¹². The water extract of Ya-hom also inhibits histamine and cholinergic agonist-stimulated gastric acid secretion in stomachs isolated from mice¹³.

In the present study, we examined the effects of the water-soluble extract of Ya-hom on cellular acid secretory responses and subcellular signaling pathways. The *in vitro* investigations were performed using rabbit-derived tissues, including isolated gastric glands, acutely isolated highly enriched parietal cells and primary culture of parietal cells.

We selected the rabbit model for several reasons. Firstly, the rabbit (*Oryctolagus cuniculus*) has proven to be an enormously useful model for the analysis of cellular and subcellular mechanisms of gastric acid secretion. Furthermore, this species has a close phylogenetic proximity to humans and, now that the genome has been sequenced, is expected to become an important translational model, facilitating efforts to bridge the gap between small rodent models and larger primates¹⁴. Most notably, with regard to the present study, rabbits display *in vivo* acid secretory responses that are similar to humans¹⁵.

To assess cellular acid secretory responses, we used a well-characterized gastric gland model¹⁶⁻²⁰, a semi-intact gastric mucosal isolate composed of acid-secreting parietal cells, pepsinogen secreting chief cells, mucous neck cells along with a few histamine-containing enterochromaffin-like (ECL) cells¹⁶. In glands, cell-cell contacts and polarities are maintained and parietal cell

secretions accumulate within a sealed off luminal space. Neural and hormonal influences are absent. Parietal cell acid secretion is assessed indirectly by measuring accumulation of ¹⁴C-aminopyrine (AP) within the gland lumens. AP is a weak base with a pKa of 5. It traverses cellular membranes freely when unprotonated but becomes cell impermeant upon protonation. Thus, AP is specifically trapped when it enters highly acidic compartments uniquely associated with HCl secreting parietal cells¹⁶. Traditionally, AP accumulation in gastric glands is expressed as the ratio of ¹⁴C-labelled AP trapped within acidic intra-versus extra-glandular compartments. Thiocyanate (SCN), which has been shown to completely block specific AP accumulation, is used to correct for non-specific trapping of AP in pelleted glands¹⁷ and was similarly used in the present study. Because glands contain histamine-secreting ECL cells, agonists such as acetylcholine and analogs such as carbachol and bethanechol have the capacity to elicit the release histamine from these cells thereby confounding direct effects of these agents. To avoid such influences, histamine H₂ receptor antagonists such as cimetidine, ranitidine, etc. can be added to glandular preparations prior to cholinergic stimulation.

Following the successful isolation and characterization of gastric glands, additional models were developed to expand the understanding of acid secretory mechanisms to the level of the parietal cell itself. These models included acutely isolated, highly enriched parietal cells and primary cultures of parietal cell isolates. Both models have been shown to respond appropriately to secretagogue stimulation and have proved to be a proper model for analysis of specific parietal cell signaling events²¹⁻²⁶ as shown in the present

study. In the present study, these models were similarly employed.

2. MATERIALS AND METHODS

2.1. Materials and animals

Ya-hom was obtained from Five Pagodas Pharmacy Co. Ltd., Thailand. The water-soluble form was prepared by boiling Ya-hom in water for 15 min at a ratio of 1 kg:10 L. After filtration through cotton and muslin cloth, the clarified substrate was lyophilized and stored at -20°C. Ya-hom extract was analyzed for total sugar content, uronic acid and phenolics. Total neutral sugar content was determined by the reaction with phenol in the presence of sulfuric acid using glucose as a standard. The total uronic acid content was colorimetrically determined by the m-hydroxydiphenyl assay using galacturonic acid as a standard. Total phenolics were determined using Folin and Ciocalteu Reagent and calculated as gallic acid equivalents. Analyses revealed that the water-soluble extract contained phenolic acid (equivalent to gallic acid, 10.57 mg/g dry extract), polysaccharide (5.9% w/w) and uronic acid (0.55% w/w).

Dimethylamine- ^{14}C -aminopyrine was purchased from NEN/Dupont, Wilmington, DE. Liquid scintillation, ScintisafeTM 30% was purchased from Fisher Scientific, Pittsburgh, PA. ECL detection was from Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England. Oregon Green phalloidin was from Molecular Probes. Fura-2, thapsigargin and ionomycin were purchased from Calbiochem, La Jolla, CA. Optiprep was from GibcoBRL, Grand Island, NY. Matrigel was from Collaborative Biomedical Products, Bedford, MA. All other chemicals were obtained from Sigma Chemical, St. Louis, MO. Male New Zealand White rabbits with 4-5 kg of body weight were used for all experiments. All animal procedures were reviewed and approved by the Animal Care and Use Committee at the Medical College of Georgia, protocol no. 05-08-167.

2.2. Isolation of rabbit gastric glands

Gastric glands were used to assess acid secretory responses using ^{14}C -aminopyrine accumulation measurements and to assess effects of Ya-hom on cAMP-dependent morphological changes using a fluorescently-labelled (Oregon Green) phalloidin, a bicyclic peptide that specifically binds to F- but not G-actin. Glands were also used, along with isolated parietal cells, to analyze effects of Ya-Hom on LASP1 phosphorylation as explained below.

Isolated gastric glands were prepared and as previously described¹⁸⁻²⁰. In brief, stomach of deeply anesthetized rabbits were perfused with phosphate-

buffered saline (PBS; in mM: 149.6 NaCl, 3 K₂HPO₄ and 0.64 NaH₂PO₄, pH 7.4), prewarmed to 37°C and gassed with 100% oxygen. The mucosa was stripped from gastric corpus muscle layers, finely minced and incubated with medium A (in mM: 114.4 NaCl, 5.4 KCl, 5 Na₂HPO₄, 1 NaH₂PO₄, 1.2 MgSO₄, 15 HEPES, 1 CaCl₂, and 10 mg/L phenol red, pH 7.4) containing 1 mg/ml collagenase (type I) at 37°C for 25-30 min. The resulting digest was filtered through a nylon mesh, rinsed three times with medium B (medium A plus 2 mg/ml glucose, 2 mg/ml bovine serum albumin (fraction V), 0.11 mg/ml sodium pyruvate and 1 mM DTT) and resuspended 1 ml of 10 min gravity settling gastric gland in medium B to a final volume of 30 ml. Dry weights of the gastric gland suspensions were calculated as previously described¹⁸⁻²⁰.

2.3. Measurement of ^{14}C -aminopyrine (AP) accumulation in gastric glands

One ml aliquots of suspended gastric glands containing 0.1 μCi dimethylamine- ^{14}C -aminopyrine (specific activity 100-115 mCi/mmol) were diluted with 1 ml medium B and gently agitated at 37°C for 30 min before adding carbachol or histamine or forskolin. Carbachol and histamine were dissolved in medium A. Forskolin, a specific adenylyl cyclase activator, thapsigargin, an intracellular Ca²⁺-ATPase inhibitor, and ionomycin, a nonspecific calcium ionophore were dissolved in DMSO (final concentration, 0.1%) and a similar amount of DMSO vehicle was added to control flasks). Cimetidine (H₂ receptor antagonist, 10 μM) or Ya-hom were added at the beginning of incubation unless otherwise indicated. Glands isolated from a single rabbit were used for each separate experiment and all assays were performed in duplicate. At indicated times following addition of experimental agents, 1-ml aliquots of gland suspensions were withdrawn and immediately centrifuged (10,000 rpm, 10 sec). Supernatants were rapidly withdrawn, pellets were rinsed once with medium that did not contain ^{14}C -aminopyrine then dissolved in 200 μl of conc. HNO₃ (50°C, 10 min). Aliquots of supernatants and acid extracts were counted in a liquid scintillation spectrometer. Results were expressed as the ratio of intraglandular (acid space) to extra glandular ^{14}C -aminopyrine concentration and calculated with corrections for ^{14}C -aminopyrine trapped in the pellet as previously described^{20,27}.

2.4. F-actin localization in gastric glands with fluorescently tagged phalloidin

Isolated gastric glands were incubated in medium B with and without Ya-hom (10 mg/ml, 37°C, 30 min) then stimulated with forskolin (3 μM) for 15 min. At the conclusion of incubation, gastric glands were rinsed once with PBS, resuspended in paraformaldehyde solu-

tion (7% paraformaldehyde, 0.25 M sucrose and 0.05 M cacodylate buffer, pH 7.0) and settled at room temperature for 15 min. The fixed gastric glands were rinsed three times with PBS and stored in PBS containing 0.05% sodium azide at 4°C. For fluorescent staining, glands were washed with PBS, permeabilized with 0.2% Triton X-100 in PBS for 15 min then fixed on slides. F-actin labeled with Orange Green Phalloidin (1:400 dilution) was analyzed by confocal microscopy as previously described²⁸.

2.5. Isolation and purification of rabbit parietal cells

Parietal cells, enriched to 80-90% purity, were used to assess changes in $[Ca^{2+}]_i$ at the single cell level and to define specific effects of Ya-Hom on cAMP-dependent phosphorylation of LASP1 in the parietal cell. Cells were isolated from rabbit gastric mucosae using sequential pronase/collagenase digestion then enriched to 80-90% purity using Optiprep step gradients as previously described¹⁹. Primary cultures of enriched parietal cells were maintained on Matrigel-coated glass-bottomed 35-mm dishes in a complete medium [1:1 mixture of Ham's F-12 and DMEM, pH 7.4; BSA (2 mg/ml), epidermal growth factor (50 ng/ml), hydrocortisone (10 nM), insulin (5 µg/ml), transferrin (5 µg/ml), sodium selenite (5 ng/ml), Novobiocin (50 µg/ml) and gentamicin sulfate (100 µg/ml)]²².

2.6. Measurement of intracellular free calcium ($[Ca^{2+}]_i$) responses in primary cultures of parietal cells

Changes in $[Ca^{2+}]_i$ were measured with fura-2 using a dual-wavelength excitation ratio technique as previously described^{23,24}. Parietal cells in primary culture were loaded with fura-2 by incubating in culture medium [1:1 mixture of Ham's F-12 and DMEM, pH 7.4, without bicarbonate; BSA (2 mg/ml)] containing the cell-permeant form (fura-2 AM, 4 µM) for 30 min in a humidified 37°C air incubator. Cells were then rinsed twice with culture medium and placed in the incubator for 15 min before addition of various agents. Ya-hom (10 mg/ml) or DMSO vehicle were added 5-20 min prior to carbachol (10 µM) or thapsigargin (3 µM). In experiments with thapsigargin and ionomycin, the latter agent (10 µM) was added after the thapsigargin-induced rise in $[Ca^{2+}]_i$ was observed. Images acquired at 340/380 nm excitation, 510 nm emission wavelengths were digitized and analyzed with Openlab software (Agilent). Calcium binding to fura-2 increases the fluorescent signal, the intensity of which is traditionally displayed as a range of pseudo-color shades with blue representing low $[Ca^{2+}]_i$ and yellow to red representing high $[Ca^{2+}]_i$ ²⁹. This format was used in the present study as well.

2.7. LASP1 phosphorylation assay

LASP1 is a highly conserved, actin-associated phosphoprotein that has been linked to acid secretory regulation in a knockout mouse model³⁰ and shown to undergo increased phosphorylation in parietal cells following histamine stimulation^{25,26,28,30}. Increased LASP1 phosphorylation is mediated via the cAMP signaling pathway²⁵. Alterations in LASP1 phosphorylation are readily detected in the form of an apparent molecular weight (Mr) "band shift" on Western blots using a specific monoclonal LASP1 antibody (8C6) that we engineered²⁵ and which now commercially available from several companies including, VWR, Fisher, Merck and Sigma. The 8C6 antibody recognizes both phosphorylated and non-phosphorylated forms of LASP1²⁵.

To detect changes in LASP1 phosphorylation, isolated gastric glands or parietal cells were incubated in medium B with and without Ya-hom (10 mg/ml, 37°C, 30 min) then stimulated with forskolin for 15 min. Glands or cells were centrifuged and supernates discarded. Resultant pellets were washed with cold PBS and heated in SDS/BME solution (0.3% SDS and 1% β-mercaptoethanol, 100°C, 3 min). Dissolved protein was subjected to SDS-PAGE followed by Western blot analysis on nitrocellulose using the LASP1 monoclonal antibody, 8C6²⁵, and ECL detection. As discussed above, the phosphorylated form of LASP1 is readily detected on Western blot based on an upward Mr "band shift"²⁵.

2.8. Statistical analyses

Parametric data were expressed as means±SE. Multiple comparisons were analyzed by ANOVA and Tukey-Kramer pos-hoc test with the use of a computer program Instat 2.03. Differences were considered statistically significant when the *P* value was <0.05.

3. RESULTS

3.1. Effect of Ya-hom on carbachol-stimulated AP accumulation in gastric glands

Lower concentrations of Ya-hom (10, 15 mg/ml) had no significant effect on basal AP accumulation in gastric glands (24.6±1.1, 29.9±1.6, and 27.4±1.7 respectively). However, 20 mg/ml Ya-hom did elicit a small, but significant increase in AP accumulation (36.3±1.7).

As previously shown³¹, carbachol stimulates gastric gland AP accumulation in a biphasic, time-dependent manner that peaks at 15 min in response to a 10 µM dose (Figure 1). Ya-hom (10 mg/ml) suppressed carbachol-stimulated AP accumulation at all time points sampled; however, this inhibition reached statistical significance only at 15 and 30 min. Maximal inhibition occurred 15 min after carbachol addition (53.1±13.0 vs 103.3±17.9; Figure 1) and was dose-dependent (Figure 2). When gastric glands were preincubated with Ya-hom

(30 min, 5 mg/ml and higher concentrations), carbachol-stimulated AP accumulation was significantly inhibited. This inhibitory effect was most pronounced when Ya-hom was added 30 min prior to carbachol ($51.1 \pm 2.7\%$ of the response to $10 \mu\text{M}$ carbachol); however, Ya-hom also significantly inhibited the cholinergic response when added simultaneously with or 5 min after carbachol ($71.2 \pm 6.1\%$ and $78.2 \pm 5.9\%$ of the response to $10 \mu\text{M}$ carbachol, respectively) (Figure 3).

3.2. Effect of Ya-hom on the calcium signaling pathway in parietal cells

As previously reported²⁶, we confirmed that carbachol ($10 \mu\text{M}$) increases intracellular free calcium in parietal cells in primary culture. Figure 4 depicts a representative experiment in which parietal cell responses to carbachol and effects of Ya-hom can be observed. Overall, 92.1% of observed parietal cells (representing a total of 38 cells sampled) responded to stimulation with $10 \mu\text{M}$ carbachol. Ya-hom preincubation for 20 min led to a small increase in $[\text{Ca}^{2+}]_i$ but no further increase was elicited by carbachol in any of sampled cells ($n=38$). Approximately 20% of parietal cells that were incubated with Ya-hom for less than 15 min responded to carbachol with a small increase in intracellular free calcium which was consistently lower than responsive control cells (data not shown).

3.3. Effect of Ya-hom on histamine-stimulated AP accumulation in gastric glands

Ya-hom significantly potentiated the stimulatory effect of histamine ($1 \mu\text{M}$) on AP accumulation in a dose-dependent manner (5, 10 and 20 mg/ml ; Figure 5). At the highest concentration tested (20 mg/ml), Ya-hom potentiation of the AP response was $200.5 \pm 58.9\%$ greater than the response to $1 \mu\text{M}$ histamine alone. In the presence of the histamine H_2 receptor antagonist, cimetidine ($10 \mu\text{M}$), 10 mg/ml Ya-hom also potentiated the stimulatory effect of the adenylyl cyclase activator, forskolin, on gastric gland AP accumulation (Figure 6). Furthermore, as shown in Figure 6, preincubation of glands with Ya-hom for 30 min induced a leftward shift in the forskolin dose response curve ($0.1\text{--}10 \mu\text{M}$).

3.4. Effect of Ya-hom on forskolin-stimulated LASP1 phosphorylation

Because Ya-hom potentiated forskolin-stimulation when tested across a wide range of forskolin concentrations and had no stimulatory effect on AP accumulation ratios in the presence of the H_2 -receptor antagonist, cimetidine, we concluded that this action was not associated with Ya-hom-induced elevation of $[\text{cAMP}]_i$. To determine if Ya-hom might modulate other known downstream events within the cAMP signaling

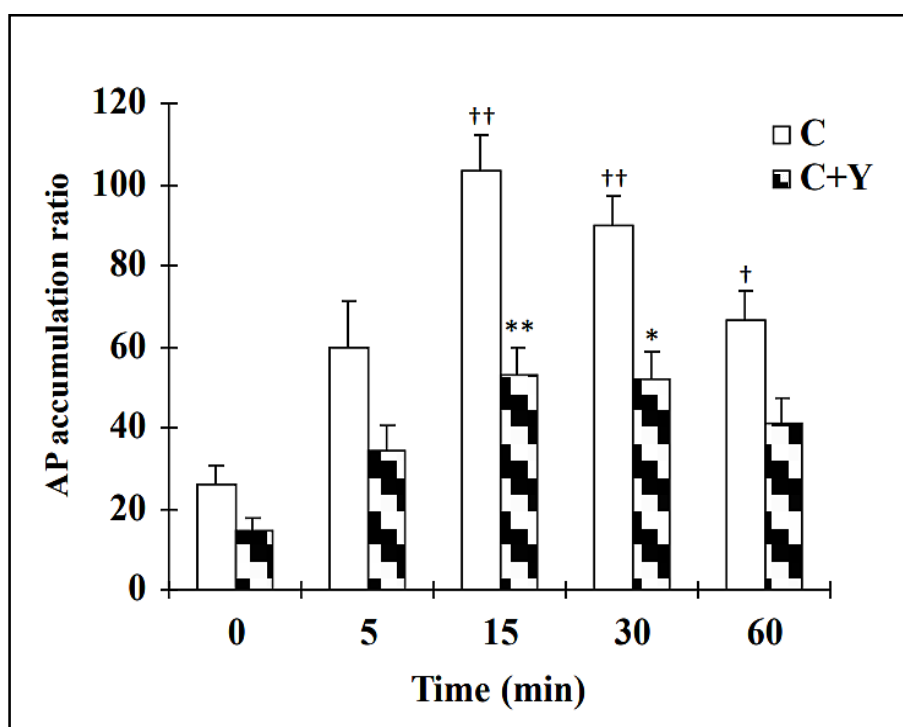


Figure 1. Time course of the effect of Ya-hom on carbachol-stimulated gastric gland AP accumulation. Isolated gastric glands were incubated (37°C , 30 min) in the presence of the H_2 receptor antagonist, cimetidine ($10 \mu\text{M}$) with or without Ya-hom (Y, 10 mg/ml , filled bars). Glands were removed for assay at t_0 . Carbachol (C, $10 \mu\text{M}$) was then added and samples taken for measurement of AP accumulation at indicated time points. Values were expressed as means \pm SEMs of 4 gland preparations isolated from 4 different rabbits. $\dagger P < 0.05$ vs carbachol+cimetidine at time 0, $\dagger\dagger P < 0.001$ vs carbachol+cimetidine at time 0, $*$ $P < 0.05$ vs carbachol+cimetidine at 30 min; $** P < 0.001$ vs carbachol+cimetidine at 15 min.

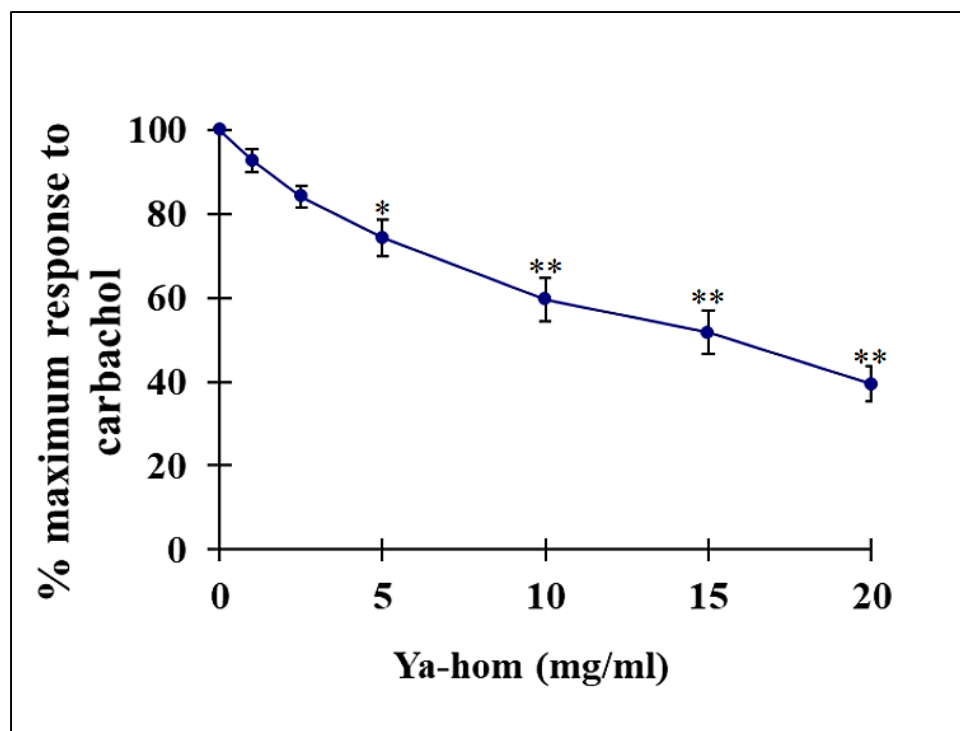


Figure 2. Dose response curve of Ya-hom inhibition of carbachol-stimulated gastric gland AP accumulation. As in figure 1, glands were preincubated with cimetidine (10 μ M) in the absence (control) or presence of Ya-hom (1, 2.5, 5, 10, 15 and 20 mg/ml) at 37°C for 30 min before adding carbachol (10 μ M). AP accumulation ratios are expressed as % response to 10 μ M carbachol (58.1 \pm 14.4) and are means \pm SEMs of 5 gland preparations from 5 different rabbits. * P <0.01 vs control, ** P <0.001 vs control.

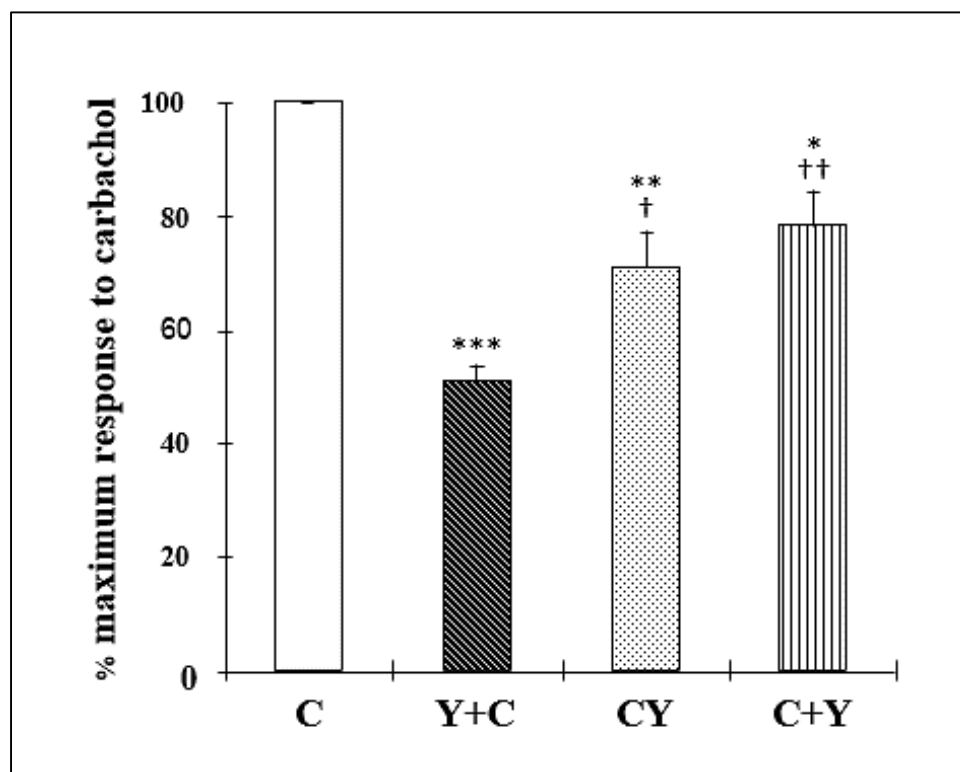


Figure 3. Effect of sequential addition of Ya-hom and carbachol on gastric gland AP accumulation. Cimetidine (10 μ M) was added to all flasks 30 min prior to addition of carbachol (10 μ M) and Ya-hom (10 mg). Samples were collected 15 min after carbachol addition. C, carbachol; (Y+C) 30 min preincubation with Ya-hom; (CY) Simultaneous addition of Ya-hom and carbachol; (C+Y) Ya-hom added 5 min after carbachol. AP accumulation ratios are expressed as % response to 10 μ M carbachol (C) and expressed as means \pm SEMs of 4 gland preparations from 4 different rabbits. * P <0.01 vs C, ** P <0.01 vs C, *** P <0.001 vs C. † P <0.5 vs Y+C, †† P <0.01 vs Y+C.

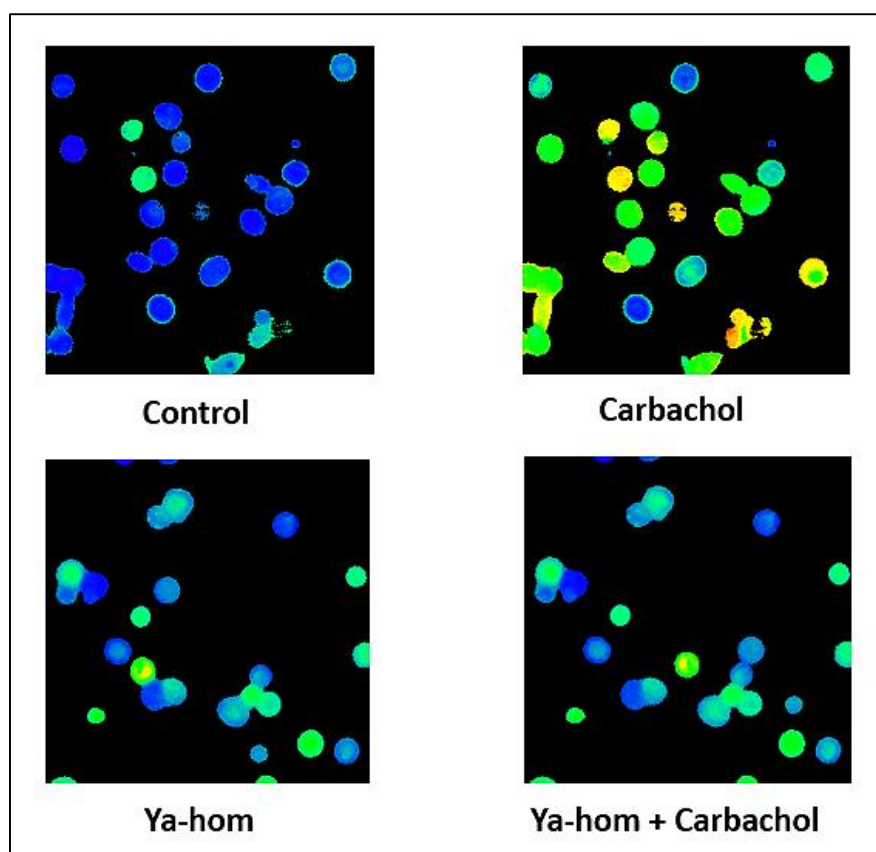


Figure 4. Ya-hom inhibits carbachol-stimulated increases in intracellular free calcium in parietal cells in primary culture. Cells were loaded with the fluorescent $[Ca^{2+}]_i$ indicator, fura2, as described in Methods. Carbachol (10 μ M) addition led to an increase in $[Ca^{2+}]_i$ in 92.1% of control parietal cells (38 cells). Preincubation of Ya-hom (10 mg/ml) for 20 min inhibited the response of all observed parietal cells (38 cells) to carbachol stimulation. Similar results were observed in 3 independent experiments.

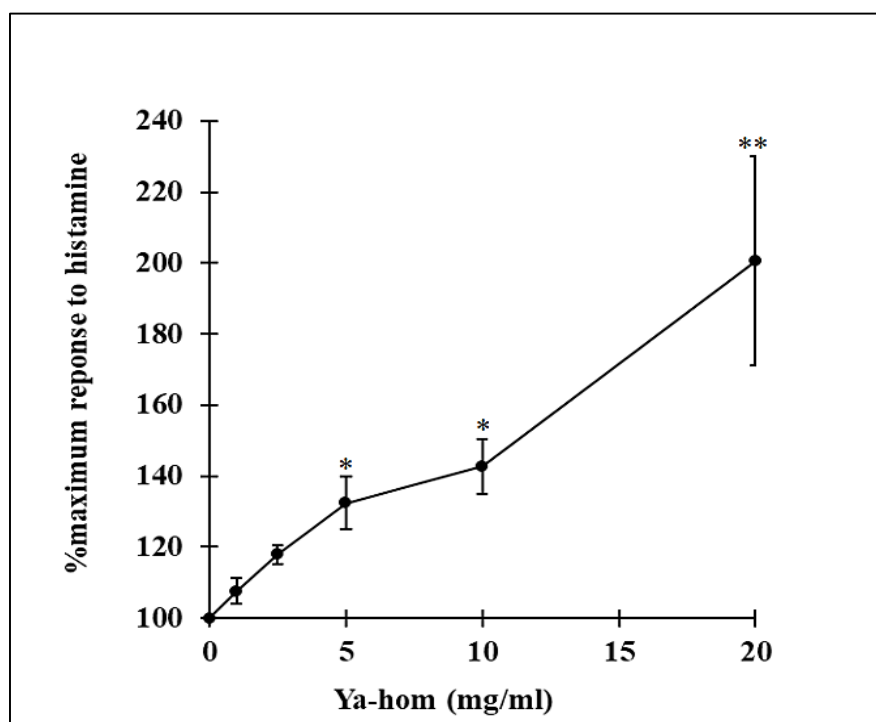


Figure 5. Dose-dependent effects of Ya-hom potentiation of histamine-stimulated gastric gland AP accumulation. Isolated gastric glands were incubated in the absence (control) and in the presence of Ya-hom (1, 2.5, 5, 10 and 20 mg/ml) at 37°C for 30 min before adding histamine (1 μ M). AP accumulation ratios were calculated as % response to 1 μ M histamine (128.9 ± 11.2) and expressed as means \pm SEMs of 4 gland preparations isolated from 4 different rabbits. * $P < 0.05$ vs control, ** $P < 0.001$ vs control.

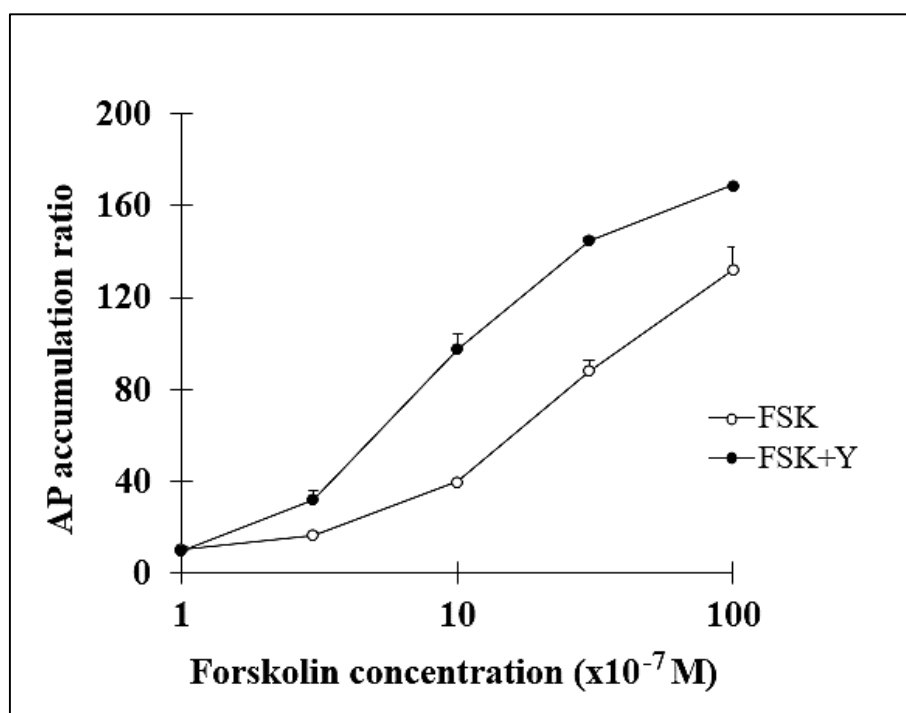


Figure 6. Effect of Ya-hom on dose-dependent forskolin-stimulated AP accumulation. Isolated gastric glands were preincubated with the H_2 receptor antagonist, cimetidine (10 μ M, 37°C, 30 min), along with Ya-hom where indicated (Y, 10 mg/ml, closed circles). Forskolin (FSK) was then added at indicated concentrations. AP accumulation ratios were determined 15 min after adding forskolin and expressed as means \pm SEMs of 3 gland preparations from 3 different rabbits. * $P < 0.001$ vs forskolin alone.

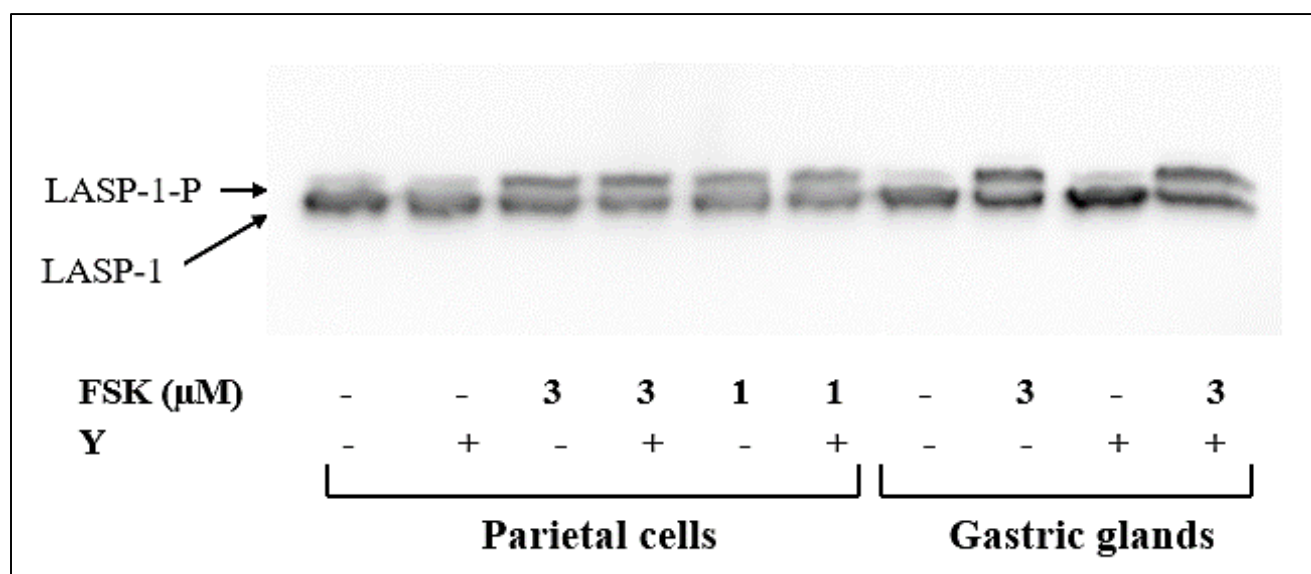


Figure 7. Western blot of parietal cell and isolated gastric gland extracts showing that Ya-hom has no effect on basal or forskolin-stimulated LASP1 phosphorylation. Parietal cells or gastric glands from two different rabbits were incubated with Ya-hom (YH, 10 mg/ml) for 30 min prior to forskolin (FSK) stimulation. Forskolin is a specific activator of adenylyl cyclase known to increase [cAMP]_i and activate PKA. In parietal cells, both forskolin and histamine are known to elevate [cAMP]_i and this increase in cAMP levels has been linked to increased phosphorylation of the actin-associated protein, LASP1. Increased phosphorylation of Lasp1 can be identified based on an apparent Mr “band shift” (arrow).

pathway, we examined its effects on LASP1 phosphorylation as well as F-actin redistribution. LASP1 is a previously identified substrate for cAMP-dependent protein kinase (PKA), and the phosphorylation of this protein is correlated with histamine-stimulated, cAMP-dependent activation of acid secretion²⁸. The specific

adenylyl cyclase activator, forskolin, similarly increases intracellular cAMP, activates PKA and increases LASP1 phosphorylation in parietal cells²⁵. When LASP1 is phosphorylated, the response can be readily detected (using a previously characterized LASP1 mab, 8C6) as an apparent Mr band shift on Western blot²⁸. These

findings are recapitulated in Figure 7 wherein incubation of highly enriched parietal cells or gastric glands with forskolin can be seen to induce a dose-dependent Mr band shift in LASP1 immunoreactivity. Incubation of parietal cells or gastric glands with Ya-hom for 30 min prior to forskolin stimulation did not alter LASP1 phosphorylation (Figure 7).

3.5. Effect of Ya-hom on forskolin-stimulated parietal cell F-actin distribution.

Stimulation of parietal cells with cAMP-dependent agonists is also known to elicit dramatic morphological changes associated with F-actin redistribution in parietal cells³². To determine if Ya-hom alters F-actin distribution, control and forskolin-stimulated parietal cells were visualized by immunofluorescent staining with Oregon green-labelled phalloidin. As shown in Figure 8, Ya-hom had no detectable effect on F-actin distribution in either control or forskolin-stimulated parietal cells.

3.6. Effect of Ya-hom on the parietal cell response to thapsigargin and ionomycin.

Since we found no evidence for upstream or known downstream effects of Ya-hom on the cAMP signaling pathway, we tested for effects on the calcium signaling pathway that might explain the potentiating effect of Ya-hom. Thapsigargin, which is an inhibitor of ATPase-dependent uptake of Ca^{2+} into intracellular stores, was previously shown to elevate parietal cell $[\text{Ca}^{2+}]_i$ and to potentiate histamine and forskolin stimulating HCl secretion³³. In the present study, Ya-hom was found to elicit a small increase in $[\text{Ca}^{2+}]_i$, thus, it was possible that Ya-hom induces a slow release of Ca^{2+} from intracellular stores and this Ca^{2+} release is responsible for the potentiation of cAMP-induced secretory response. If this were the case, then Ya-hom would be expected to suppress thapsigargin-induced release of Ca^{2+} from intracellular stores by depleting these stores. As expected, both thapsigargin (3 μM) and ionomycin (10 μM), a Ca^{2+} ionophore, increased intracellular free calcium levels in fura-2 loaded parietal cells (Figure 9). When cells were preincubated with Ya-hom (10 mg/ml, 20 min), the thapsigargin-induced elevation in $[\text{Ca}^{2+}]_i$ was indeed significantly suppressed. In contrast, Ya-hom had no significant effect on ionomycin-induced elevation of $[\text{Ca}^{2+}]_i$. Notably, Ya-hom and thapsigargin had a similar potentiating effect on histamine-stimulated AP accumulation (Figure 10). In addition, the effects of Ya-hom and thapsigargin on histamine-stimulated AP accumulation were not additive, suggesting the possibility that both agents were affecting intracellular calcium storage mechanisms. Potential sites of Ya-hom action are summarized in Figure 11.

4. DISCUSSION

Previously, a water-based extract of Ya-hom was found to inhibit both histamine- and carbachol-stimulated secretion of gastric acid, pepsin and soluble mucus but to potentiate visible mucus secretion in gastric fistula rats. Maximal inhibition of histamine and carbachol-stimulated gastric acid secretion was detected at 4 g/kg Ya-hom (45.5% and 53.8% respectively). Further increases in Ya-hom (8 mg/kg) did not alter the inhibitory effect¹². In isolated mouse whole stomach, Ya-hom, at a concentration of 10 mg/ml, also inhibited gastric acid secretion stimulated by histamine and cholinergic agonist, bethanechol¹³. In the present study, we expanded our investigations to analyze the effects of Ya-hom at the level of the parietal cell. Using a similar water-soluble extract, we found that Ya-hom inhibited carbachol-stimulated AP accumulation in gastric glands with a maximum inhibition of 60% at a dose of 20 mg/ml, a finding that is similar to previous observations in gastric fistula rats¹². Alterations in the sequential addition of Ya-hom and carbachol revealed that preexposure to Ya-hom is required to elicit maximal inhibition.

Ya-hom alone, at doses of 10 and 15 mg/ml appeared to elicit small increases in basal AP accumulation in isolated gastric glands; however, this effect was significant only at the highest dose tested (20 mg/ml). We hypothesized that Ya-hom might initiate the release of small amount(s) of histamine from ECL cells within gastric glands, thereby leading to the observed increase in "basal" AP accumulation. This hypothesis is supported by observations that there was a small decrease in AP accumulation when Ya-hom was added along with the specific H_2 receptor antagonist, cimetidine (Figure 1). However, because the apparent effects of Ya-hom on basal AP accumulation are quite small, we cannot yet conclude that Ya-hom induces the release of endogenous histamine from ECL cells, nor can the effect of Ya-hom on other endogenous mediators be excluded.

In direct contrast to the inhibitory effect of Ya-hom on carbachol, this agent enhanced the stimulatory effect of histamine on AP accumulation. Ya-hom also potentiated forskolin-stimulated AP accumulation in the presence of histamine H_2 receptor antagonist, cimetidine, an observation that supports an interaction between Ya-hom and downstream events within the cAMP signaling pathway.

Although Ya-hom had no effect on forskolin-stimulated LASP1 phosphorylation, an effect on PKA-dependent phosphorylation events cannot be entirely ruled out, as a modulatory action on other, as yet unidentified, PKA substrates is possible. The site of Ya-hom action may also be further downstream of H_2 receptor- and PKA-dependent activation events. However, based on our observations that Ya-hom does not alter F-actin

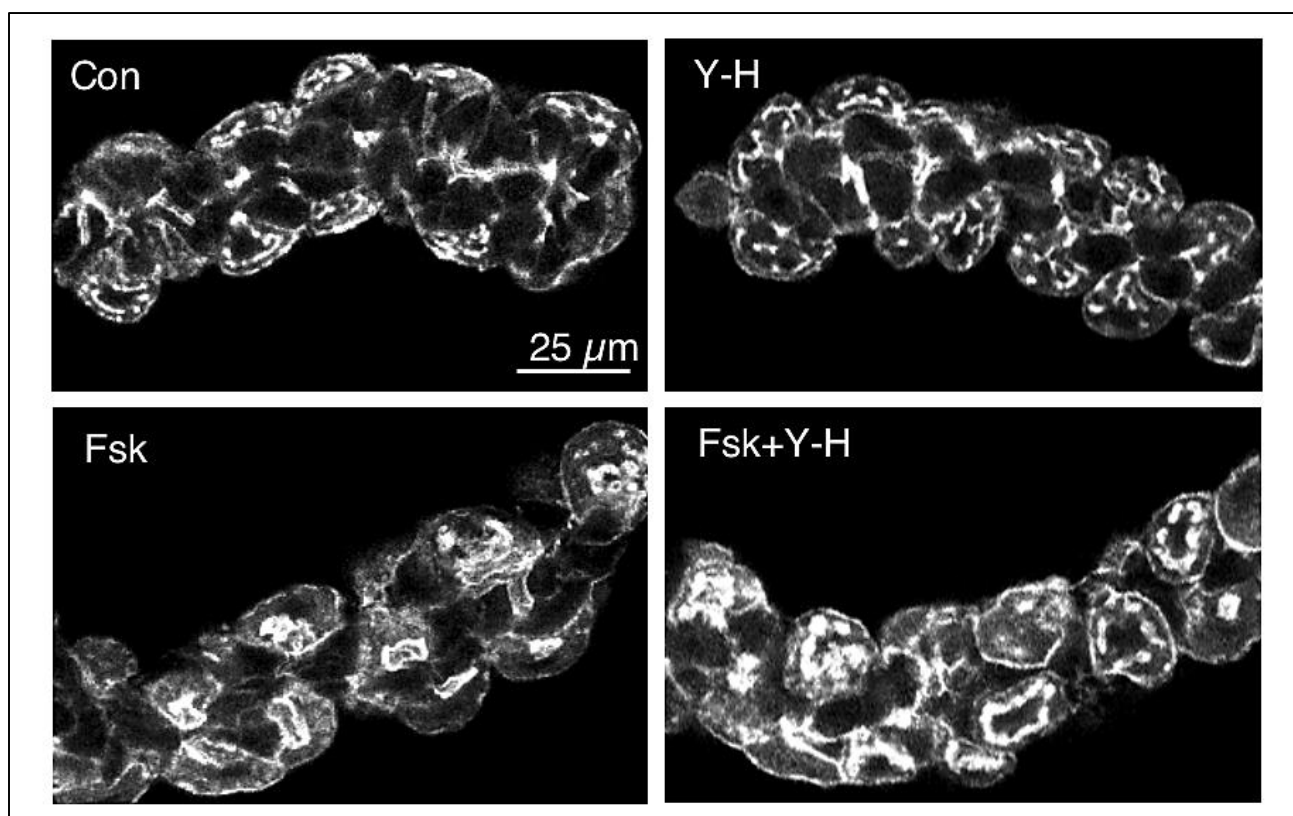


Figure 8. Ya-hom has no effect on basal or forskolin-stimulated F-actin distribution. Gastric glands were preincubated with and without Ya-hom (Y-H) for 30 min. A portion were stimulated with forskolin (Fsk, 10 μ M, 15 min). Con, DMSO vehicle. F-actin distribution were analyzed in paraformaldehyde-fixed Oregon Green phalloidin-stained glands by confocal microscopy.

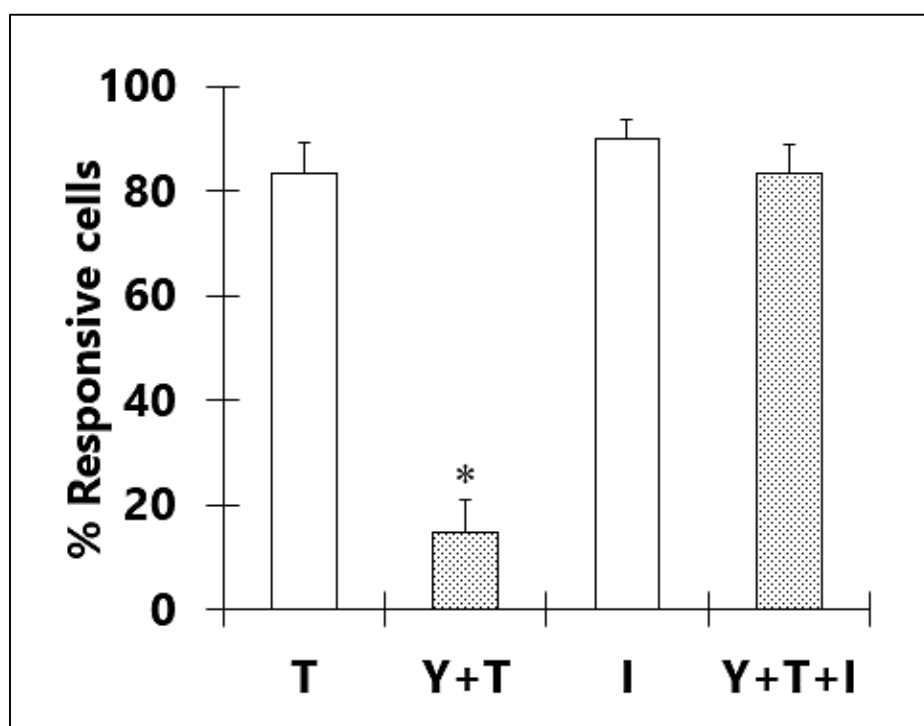


Figure 9. Effect of Ya-hom on thapsigargin and ionomycin-induced an increase in intracellular free calcium. Fura2-loaded parietal cells were preincubated with and without Ya-hom (Y, 10 mg/ml) for 20 min prior to thapsigargin (T, 3 μ M) addition. Ionomycin (I, 10 μ M) was added immediately after thapsigargin's effect had been observed (Y+T+I). Values are calculated as % responsive cell of total observed cells and expressed as means \pm SEMs of 6 independent experiments. * $P < 0.001$ vs thapsigargin alone.

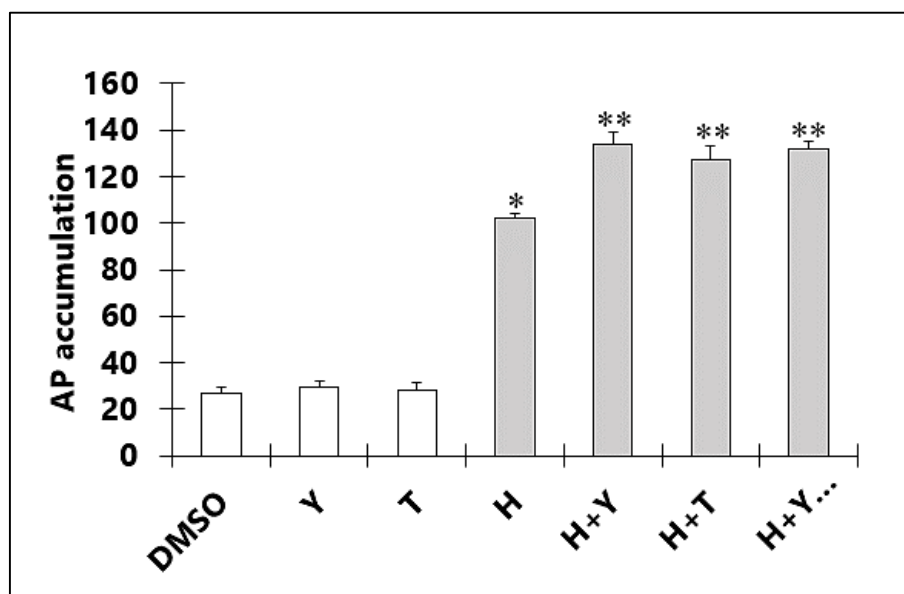


Figure 10. Effects of Ya-hom and thapsigargin on histamine-stimulated gastric gland AP accumulation. Isolated gastric glands were incubated with and without of Ya-hom (Y, 10 mg/ml, 37°C, 30 min). As thapsigargin was dissolved in dimethylsulfoxide (DMSO, final concentration 0.1%), a similar concentration was added to all other flasks upon addition of thapsigargin (T, 3 μ M) in order to control for possible non-specific effects of this vehicle. Where indicated, glands were stimulated with histamine (H, 1 μ M, closed bars) for 30 min. AP accumulation ratios were expressed as means \pm SEMs of 4 independent experiments. * $P < 0.05$ vs DMSO control, ** $P < 0.001$ vs DMSO control.

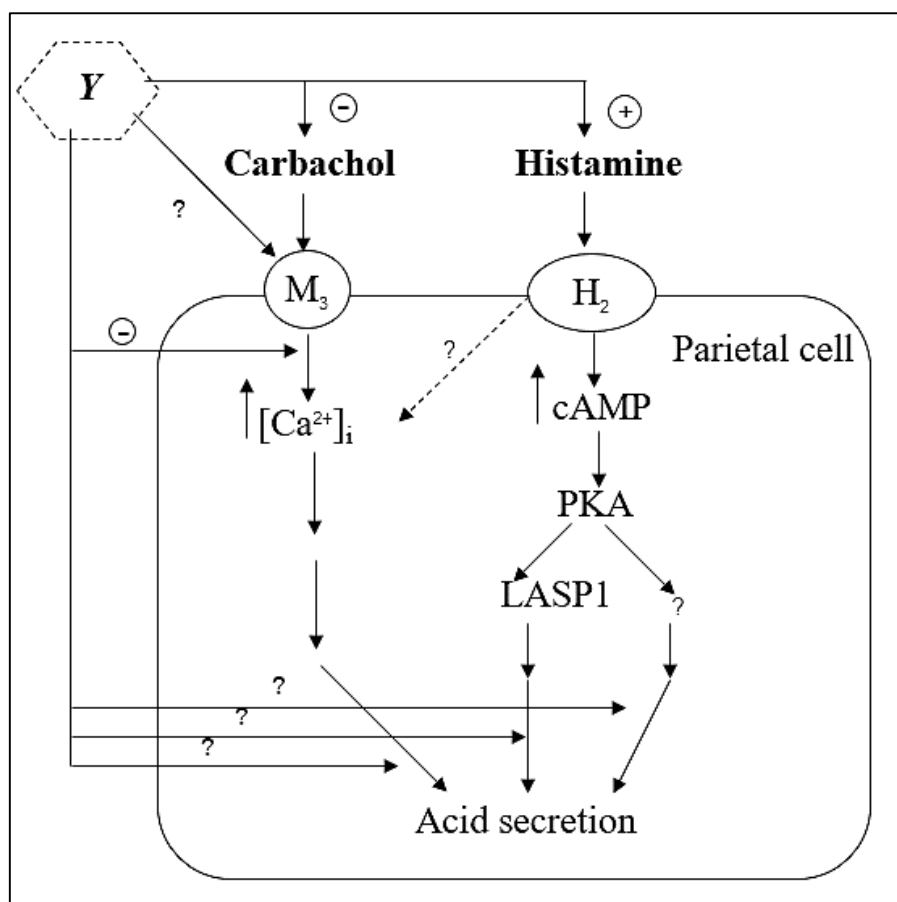


Figure 11. Schematic diagram depicting potential sites of Ya-hom actions on cAMP and Ca^{2+} -dependent signaling pathways within the gastric parietal cell.

We hypothesize that Ya-hom modulates calcium release from intracellular storage sites, thereby suppressing the cholinergic response. However, this does not exclude the other possible mechanisms. Ya-hom may interact directly with the M_3 muscarinic receptor. It may also act on sites parallel to or beyond PKA-dependent LASP1 phosphorylation or on other unknown calcium-regulated signaling events to potentiate acid secretion stimulated by histamine.

distribution in control or forskolin-stimulated parietal cells, it is unlikely that Ya-hom directly affects the morphological changes that occur within the parietal cell following stimulation.

Several Ya-hom ingredients including *G. glabra*³⁴, *A. lappa*³⁵, *S. aromaticum*⁹ and *A. gramineus*¹¹, have been found to decrease histamine-induced contraction of tracheal and ileal smooth muscle. Those reports do not contradict present findings because histamine effects on smooth muscle are mediated via H₁ receptor-linked mechanisms whereas gastric parietal cell HCl secretion is regulated by the histamine H₂ receptor subtype³⁶. This is an important distinction because both cholinergic M₃ receptor³⁷ and histamine H₁ receptor³⁶ subtype-linked activations are mediated via intracellular calcium signaling events rather than the cAMP-PKA pathway. Thus, Ya-hom may inhibit H₁-receptor associated smooth muscle contraction by mechanisms similar to those associated with inhibition of cholinergic M₃ receptor-mediated gastric acid secretion.

It is not surprising that Ya-hom could inhibit the effect of carbachol in our study as previously shown to inhibit carbachol stimulation in gastric fistula rats and to inhibit bethanechol stimulated HCl secretion in isolated mouse whole stomach. The effects of Ya-hom on cholinergic functions has not been studied in other organs; however, some Ya-hom ingredients including *G. glabra*^{6,34}, *A. lappa*^{8,35}, *S. aromaticum*^{9,10} and *A. gramineus*¹¹, are known to relax tracheal and ileal smooth muscle by antagonizing acetylcholine. Since the muscarinic M₃ receptor subtype plays an important role in regulating tracheal and ileal smooth muscle contraction as well as parietal cell acid secretion^{37,38}, it is possible that Ya-hom's cholinergic antagonism involves effects on an M₃ receptor subtype or its signal transduction pathway. The finding that Ya-hom not only inhibits carbachol-stimulated AP accumulation but also carbachol-induced elevation of [Ca²⁺]_i, suggests the possibility that Ya-hom interacts directly with parietal cell M₃ muscarinic receptors. However, we believe this scenario to be unlikely because 1) Ya-hom required a pre-incubation period to exert a maximal inhibitory effect on carbachol-induced elevation in [Ca²⁺]_i and 2) an inhibitory action on the M₃ receptor does not explain Ya-hom-induced potentiation of the histamine-induced acid secretory response. Thus, we hypothesize that the site of the modulatory effects of Ya-hom action is intracellular, rather than at the membrane receptor level.

Because there is evidence that the well-documented synergistic interactions of histamine and carbachol on parietal cell HCl secretion involves the release of calcium from intracellular storage sites³⁹ and present observations that pre-incubation with Ya-hom induces a small increase in [Ca²⁺]_i. We hypothesized that the potentiating effect of Ya-hom on the histamine response might similarly involve alterations in intra-

cellular calcium distribution. To investigate this possibility, we used two agents that are known to elevate [Ca²⁺]_i by different mechanisms, thapsigargin and ionomycin. We found that preincubation of parietal cells with Ya-hom for at least 20 min led to inhibition of the thapsigargin-induced rise in [Ca²⁺]_i. In contrast, shorter exposure times did not suppress this response (data not shown). Because thapsigargin is known to be an intracellular calcium storage pool Ca²⁺-ATPase inhibitor, our findings suggest that Ya-hom may induce a slow, time-dependent release of calcium from the same thapsigargin-regulated intracellular calcium storage site(s). In contrast, Ya-hom had no effect on the parietal cell calcium response to ionomycin, a calcium ionophore. These data suggest that Ya-hom does not directly affect the influx of extracellular calcium. Based on these findings, we postulate that Ya-hom may suppress cholinergic stimulation of parietal cell HCl secretion by modifying the release of calcium from intracellular storage sites.

It has been shown that thapsigargin-induced elevation in intracellular free calcium is not associated with an increase in acid secretion³³. The present data confirm this observation and further show that Ya-hom similarly has no independent effect on basal AP accumulation in the presence of H₂ receptor antagonists. In contrast, both Ya-hom and thapsigargin potentiated histamine-stimulated AP accumulation to the same extent, with no further increase when they were added simultaneously. These combined data suggest that Ya-hom and thapsigargin may work through similar mechanisms. Thapsigargin can stimulate acid secretion when added together with phorbol ester, a protein kinase C (PKC) activator³³, and there is evidence to suggest that calcium-independent PKC isoforms are involved in mediating the acid secretory response to carbachol⁴⁰. Thus, the ability of Ya-hom to enhance cAMP-dependent secretagogue effects may also involve the activation of calcium-independent signal transduction pathways. Alternatively, a low-level release of calcium from agonist-sensitive intracellular stores may be sufficient to explain the ability of Ya-hom to potentiate histamine and forskolin-stimulated acid secretory responses.

5. CONCLUSION

Our findings suggest that Ya-hom attenuates carbachol-stimulated gastric gland AP accumulation by slowly releasing calcium from agonist-sensitive intracellular pool(s), thereby suppressing the cholinergic response. In contrast, a Ya-Hom induced rise in intracellular free calcium may potentiate histamine and forskolin-stimulated gastric gland acid secretory responses. The synergistic interaction between Ya-hom and histamine in isolated gastric glands is in direct contrast to the inhibitory effects of this agent on

histamine-stimulated acid secretion in gastric fistula rats and isolated mouse whole stomach. These conflicting observations may be explained by additional actions of Ya-hom on vagally-mediated acetylcholine release and/or other enteric mediators. Ya-hom might also induce the release of gastric inhibitory hormones such as somatostatin, thereby causing the inhibition of gastric secretion. Our findings suggest the possibility that combined use of Ya-hom with H₂-receptor antagonist drugs will be more beneficial for inhibiting gastric secretion than a Ya-hom-antimuscarinic drug combination. The mechanisms of Ya-hom inhibition of histamine action *in vivo* as compared to histamine stimulation of parietal cells *in vitro* requires further investigation to elucidate the various pathways that may be affected by Ya-hom exposure.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Ethics approval

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