

Original Article

Investigation on signal transduction pathways after H₁ receptor activated by histamine in C6 glioma cells: Involvement of phosphatidylinositol and arachidonic acid metabolisms

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Abstract

Background: Information related to histamine-induced cellular responses in C6 glioma cells through second messenger pathways has not been fully studied, especially the involvement of arachidonic acid (AA) metabolism. In addition, specific labeled ligand binding to histamine receptor sites still needs to be clarified.

Methods: Labeled mepyramine ligand was used to study its binding sites; [³H] inositol was used to detect inositol 4-phosphate (IP₁) formation, and fura-2/AM was used to detect intracellular free calcium ion ([Ca²⁺]_i) level activated by the phosphatidylinositol-phospholipase C (PI-PLC) pathway. Also, labeled AA was used to detect the metabolism of AA and its metabolites release via the activation of phospholipase A2 in the presence of histamine.

Results: C6 glioma cells incubated with histamine in the presence of 10 mM LiCl for 60 minutes induced an increase of IP₁ and glycerophosphoric-inositol (GPI) accumulation. In addition, histamine caused an increase of extracellular AA with its metabolite release, eliciting a transient and sustained increase of free [Ca²⁺]_i. The sustained increase of [Ca²⁺]_i was almost or completely blocked by La³⁺ and excess ethylene diamine tetraacetic acid. The calcium ion influx associated with the sustained phase required the presence of histamine on the receptor sites, and could be blocked by a H₁ antagonist, chlorpheniramine.

Conclusion: C6 glioma cells possess histamine H₁ receptors that have affinity towards [³H]mepyramine binding, and are coupled to PI-PLC to generate inositol phosphates and to increase [Ca²⁺]_i, and they are coupled to phospholipase A2 (PLA2) to generate GPI and AA with its metabolite release. The transient increase in [Ca²⁺]_i can be attributed to Ca²⁺ release from intracellular stores, whereas the sustained increase in [Ca²⁺]_i is due to influx of extracellular calcium ions. The sustained increase in [Ca²⁺]_i plays a role in the activation of histamine receptor-coupled PLA2.

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1. Introduction

The C6 glioma cell line was established around 1968,¹ and it has been used for physiologic and pharmacologic studies because this cell line, having a glial cell origin, contains many

types of receptors such as adrenergic, serotonergic, muscarinic, adenosine 5'-triphosphate (ATP), bradykinin, and endothelin.^{2–4} For the histaminergic system, Peakman and Hill⁵ reported that histamine caused an increase in [³H] phosphatidylinositol (PI) turnover and inositol 4-phosphate (IP₁) accumulation; however, an apparent lack of endogenous histamine H₁ receptors in C6 cells was reported by Liu and others⁶ and Fujimoto and colleagues.⁷ Therefore, it was interesting to know if histamine-mediated signal transduction system can still be detected in this cell line or not.

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Previous studies have shown that histamine H₁ receptor stimulation resulted in a pronounced release of arachidonic acid (AA) in smooth muscle cells of the DDT1 MF2 cell line.⁸ In addition, reports showed that dysregulation of AA metabolism plays an important role in influencing critical aspects of tumorigenesis.⁹ Furthermore, some evidence suggested a crucial role of prostaglandin E₂, a metabolic product of AA, in the control of growth, survival, and angiogenic potential of tumor cells.¹⁰ We then decided to use C6 glioma cancer cells to see if there is a link between AA metabolism and histamine interacting with its receptor site.

Little information was found about histamine-treated cells possibly having an alternative pathway of AA metabolism, which is closely linked to its inflammatory role, so it is worth conducting more studies on this issue. At the beginning of this study, we observed a more than 11-fold increase on IP₁ accumulation with the use of [³H] labeled inositol under the maximal stimulation of histamine (at 100 μM), which could be blocked by histamine H₁ receptor antagonist chlorpheniramine. This is evidence of the presence of histamine H₁ receptor expressed endogenously in these glioma cells.⁵ In addition, from the result of the [³H] mepyramine binding to H₁ receptor in cell membranes isolated from C6 glioma cells, the data revealed a significant specific binding.

The presence of histamine H₁ receptors on glial cells was firstly indicated by studies of the binding of [³H] histamine and [³H] mepyramine to glial cells in explant cultures from rat brain.¹¹ [³H] mepyramine binding studies had since revealed the presence of H₁ receptors on rat astrocytes *in vitro*,¹² and activation of these receptors had been shown to stimulate phosphoinositide hydrolysis.¹³

Since histamine plays an important physiologic role in central¹⁴ and peripheral systems,¹⁵ and is involved in inflammatory or allergic syndromes of many reactions, there is a need for more detailed studies of its second messengers in the cells. We used C6 glioma cells to study the involvement of these candidates, including calcium movement, AA metabolism, and other phospholipid metabolism in the signal transduction system. If this cell line is a good model for such an investigation, further studies on down-regulation, desensitization by histamine or other mediators can be performed, including studies that examine the drug effect on the histaminergic receptor system.

2. Methods

2.1. Materials

The radioisotopes used in this study, [1,2-³H] L-myo-inositol (45.8 Ci/mmol), [³H] AA (100 Ci/mmol), [³H]mepyramine (24.7 Ci/mmol) were purchased from New England Nuclear (NEN) Co. (Boston, MA, USA). Other chemicals, histamine HCl, chlorpheniramine, 3, 4, 5-tri-methoxy-benzoic acid 8-(diethyl amino) octyl ester (TMB-8), lanthanum chloride, sodium fluoride, aluminium chloride, fura-2/AM, nifedipine, verapamil were purchased from Sigma Chemical Co. (St. Louis, MO, USA); SK&F 96365 was obtained from BIOMOL

Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Ready Safe Liquid Scintillation Cocktail was obtained from Beckman Co. (Fullerton, CA, USA) for Beckman LS-3801 model sample counting use. Additionally, a Cation Counter (Spex Model CMIT 111; SPEX Industries, Edison, NJ, USA) was used in this study.

2.2. Cell culture

The C6 glioma cell line was a gift from Professor Teh-Cheng Chou at the Institute of Neuroscience at the National Yang-Ming University (Taipei, Taiwan). These cells were cultured in Ham's F-10 medium (JRH Biosciences, Inc., Lenexa, KS, USA) with 10% fetal bovine serum (Hyclone Co., Logan, UT, USA). Cells in T-75 flasks (BD Biosciences, Franklin Lakes, NJ, USA) were kept at 37 °C, 5% CO₂ and 95% O₂ incubator. The culture medium was changed every other day. This cell line has been used widely for decades since its establishment in 1968.¹

2.3. Methods for cell growth in experiments

C6 glioma cells were seeded on three glass cover slips (10 × 40 mm) and kept in a 60-mm culture flask, and then the flask was kept in an incubator until cell growth reached a confluent state. Before the experiment, the medium was changed to F-10 culture medium without addition of 10% fetal bovine serum. Labeled materials were added to each flask for a period of time, and then washed out unlabeled portion with washing buffer [containing NaCl (116 mM), NaHCO₃ (26.2 mM), NaH₂PO₄ (1 mM), KCl (2.5 mM), MgSO₄ (1.5 mM), CaCl₂ (2.5 mM), and glucose (20 mM)] three times.

2.4. PI turnover study^{5,16}

Labeled [³H] myo-inositol (1 μCi/ml) was added into each flask for a 24-hour incubation in a F10 culture medium without 10% fetal bovine serum. At the end of incubation, this culture medium was removed and a washing buffer was used to wash these cells three times. A 2.475 ml of buffer solution was added into incubation flask and maintained/kept at 37 °C for 40 minutes. Then, 10 mM LiCl was added to prevent the breakdown of inositol phosphated into inositol. After that, a stimulator such as histamine was added into the flask for 60 minutes. At the end of incubation, an ice cold washing buffer was used to wash twice to end the reaction in the flask. The glass plates were removed for counting and kept in a scintillation counting vial. For further extraction of inositol phosphates, the method of Pearce and colleagues¹⁷ was used.

2.5. Analysis of GPI and PI⁵

The PI products were analyzed with anion-exchange chromatography. 0.5 ml of AGI × 8 resin (100–200 mesh, formate form) was kept in a Pasteur pipette to form a column. Diluted

aqueous extraction material was added (6.4 ml) into the column, and the PI was combined with resin. Then, 10 ml of double-distilled water was added to wash out the free form of PI. Then, 6 ml of 60 mM sodium formate/5 ml sodium tetraborate was added to wash the [^3H] glycerol-phosphoinositol ([^3H] GPI) out, and the first 2 ml was collected in a scintillation vial. Finally, 2 ml of 0.2 M ammonium formate/0.1 M formic acid solution were added to wash out the [^3H]IP $_1$. The fluid was collected in scintillation vials, then mixed well with 6 ml of scintillation fluid for scintillation vial counting.

2.6. AA metabolism study¹⁸

C6 glioma cells were plated at a concentration of 5×10^5 cells on a 35-mm incubation plate, kept at 37 °C and supplied with a 5% CO $_2$ and 95% O $_2$ incubator for 2 days until the cells reached confluent state (about 1.5×10^6 cells). Then the cultured medium was changed to medium containing [^3H] AA (0.15 $\mu\text{Ci/ml}$) of F-10 cultured medium (not containing bovine serum albumin) and kept at 37 °C incubator for 24 hours to label the cells with radioisotope material. Before the reaction was started, the radioisotope material was removed and washed five times with washing buffer containing NaCl (118 mM); KCl (4.7 mM); glucose (10 mM); [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (20 mM) pH7.4 at 37 °C. Following this, 2.475 ml buffer solution (composition shown before) plus 25 μl of test sample at the selected concentration was added to start the reaction at 37 °C. At the end of reaction time, 1 ml of cell culture medium was removed, placed in a centrifuge tube and centrifuged at $200 \times g$ for 10 minutes to precipitate the cells. After centrifugation, 1 ml of supernatant was removed and 3 ml of scintillation counter fluid was added and mixed prior to counting with scintillation counter.

2.7. Intracellular calcium determination¹⁹

C6 glioma cells in 10 ml of loading buffer (containing 150 mM NaCl, 5 mM KCl, 2.2 mM CaCl $_2$, 1 mM MgCl $_2$, 5 mM glucose, and 10 mM HEPES buffer at pH7.4) were kept in centrifuge tubes and centrifuged at $750 \times g$ for 3 minutes, and the pellet was washed twice with washing buffer. The cells (1×10^7 cells/ml) were removed and 5 μM fura-2/AM was added and then kept at 37 °C for 45 minutes before centrifugation. The supernatant was then removed, and cells were washed with loading buffer twice to remove the remaining fura-2/AM. The remaining cells were suspended with loading buffer at a concentration of 5×10^6 cells/ml and kept in a 15-ml plastic centrifuge tube. It was covered with aluminum foil to prevent light penetration. The tube was in a dark condition during the assay period. At the end of experiment, 0.3% digitonin was added to make fura-2 combined with Ca $^{2+}$ to obtain R $_{\text{max}}$, and ethylene diamine tetraacetic acid (EGTA) was added to a final concentration of 0.1 M to complete with fura-2 for Ca $^{2+}$ to obtain R $_{\text{min}}$. Intracellular calcium concentration was obtained by using the method of Grynkiewicz and colleagues.¹⁶

2.8. [^3H] mepyramine binding assay¹²

Cell membranes (about 150 μg of membrane protein in 0.4 ml) prepared from C6 glioma cells, using the method of Smit and colleagues,²¹ were incubated with 12 nM [^3H] mepyramine at 25 °C for 60 minutes: one set with the presence of 1 mM chlorpheniramine as a nonspecific binding and in another set without the presence of chlorpheniramine as a total binding. At the end of incubation, 5 ml of cold washing buffer was added to each tube to stop the reaction. Then the whole volume of the reaction mixture was poured through the GG/B filter paper (the filter papers were soaked with 1 μM mepyramine solution prior to use) under the vacuum pump and then washed twice with 4 ml washing buffer. The filter papers kept in counting vials were dried for 1 hour at 30 °C before adding 3 ml of Ready Safe scintillation counter fluid (Beckman Co., Fullerton, CA, USA). Then, these vials were vortexed vigorously for 3–5 minutes, and then we let them stand for 30 minutes stately before being put in a beta counter for counting.

2.9. Data analysis

In this study, a two-sample *t* test was used for statistical analysis; the significant difference was set as $p < 0.05$ between the two groups in the comparison. Each experiment was carried out in triplicate, and each test was repeated at minimum three times.

3. Results

3.1. Binding of [^3H] mepyramine to H $_1$ receptor in membrane preparation of C6 glioma cells

Cell membranes isolated from C6 glioma cells were incubated with or without 100 μM chlorpheniramine in the presence of 12 nM [^3H] mepyramine for binding assay. A 77% specific binding (against 23% nonspecific binding) of labeled ligand to H $_1$ receptor sites (i.e., the percentage of [(3250 dpm/tube of total binding minus 773 dpm/tube of nonspecific binding)/3250 dpm/tube of total binding, $n = 4$] under this condition was observed (Table 1).

3.2. Histamine-induced IP $_1$ accumulation in C6 glioma cells

At 37 °C and in the presence of 10 mM LiCl, histamine at 100 μM caused [^3H] IP $_1$ accumulation, and this response was a time-dependent phenomenon. The maximal response of histamine at this concentration was found at 60 minutes and maintained at plateau level for about 10 minutes, and then the response went down (Fig. 1A). From the concentration response study, histamine at 100 μM caused a maximal response of 11.8-fold increase of IP $_1$ accumulation with respect to the basal level (Fig. 1B). Based on the experimental data, the EC $_{50}$ (effective concentration in 50% response) was calculated as 4.1 μM .

Table 1
Specifically bound [^3H] Mepyramine to Histamine H_1 receptor sites in C6 Glioma cells.

Total binding ^a (dpm/tube)	Nonspecific binding ^b (dpm/tube)	Specific binding (%)
[^3H] mepyramine ($n = 4$) 3250 ± 182	773 ± 38	77 ± 3

Binding assay was performed on C6 glioma cells using 12 nM [^3H] mepyramine at 25 °C for 60 minutes, as described in the Materials and Methods sections. Values represent mean \pm SEM.

^a For the total binding, 12 nM [^3H] mepyramine only with cell membranes.

^b For the nonspecific binding, 12 nM [^3H] mepyramine plus 1 mM chlorpheniramine with cell membranes.

3.3. Histamine-induced GPI accumulation in C6 glioma cells

At 37 °C and in the presence of 10 mM LiCl, histamine at 100 μM caused [^3H]GPI accumulation in a time dependent-manner. The maximal response was found at 60 minutes and stayed at this plateau level up to 80 minutes (Fig. 2A). Also, this accumulation response of GPI in the presence of histamine was a concentration-dependent phenomenon. Based on the data obtained, there was a 2.2-fold increase at maximal response with respect to basal level, and the EC50 value was calculated as 2.8 μM (Fig. 2B).

3.4. Histamine-induced AA and its metabolite accumulation in C6 glioma cells

At 37 °C and in the presence of 100 μM of histamine, there was a time-dependent increase of AA and its metabolite accumulation (Fig. 3A). Histamine-induced maximal response of AA accumulation at 60 minutes. This was a 1.6-fold increase over the basal level (Fig. 3B). The EC50 value was calculated as 2.8 μM .

3.5. Histamine-induced intracellular calcium increase in C6 glioma cells

At 37 °C and in the presence of varying concentrations of histamine, there was a concentration-dependent increase of a [Ca^{2+}]_i. The increase was observed in two phases. The first phase of increase was transient and happened within 1–2 seconds, and the second phase of increase was sustained, lasting from 2 seconds to more than 1 minute. For transient phase, a maximal response was seen at 100 μM of histamine (in-site free calcium concentration of the cells was 730 ± 80 nM). The EC50 was 4.9 μM . For the sustained phase, the maximal calcium increase was observed at 3.3 μM of histamine. The calculated EC50 was 0.63 μM . The maximal responses lasted more than 30 minutes (data not shown). If 100 μM of histamine antagonist chlorpheniramine was added, the sustained level of calcium concentration was found decreased to basal level (Fig. 4A). In addition, all the histamine-induced biologic response observed in this study could be completely blocked by chlorpheniramine (Fig. 4B and Table 2).

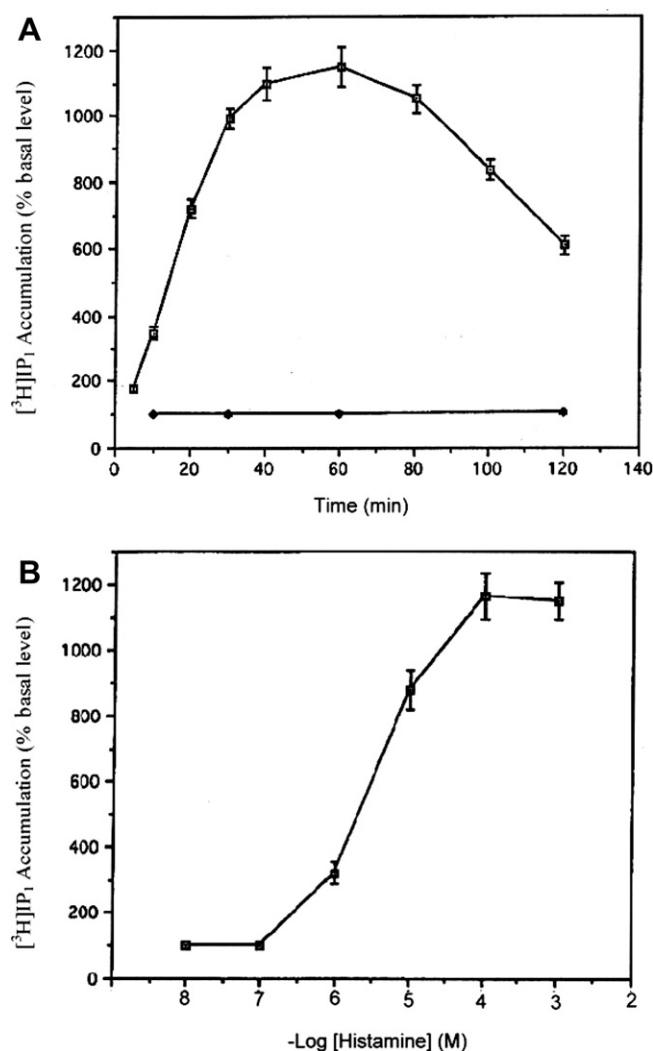


Fig. 1. (A) Time course of histamine-stimulated [^3H] IP_1 accumulation in C6 glioma cells. [^3H] Inositol-labeled cells were incubated for the indicated period of time with vehicle (H_2O) (\blacklozenge) or 100 μM histamine (\square) in the presence of 10 mM LiCl; (B) concentration-dependence of histamine-stimulated accumulation of IP_1 in C6 glioma cells. [^3H] Inositol-labeled cells were washed, and exposed to the indicated concentrations of histamine in the presence of 10 mM LiCl for 60 minutes. Results are expressed as percentage of basal level (4108 ± 160 dpm/ 10^6 cells). Values are mean \pm SEM from three independent experiments performed in triplicate.

3.6. Mechanistic studies of histamine-induced intracellular cellular responses

3.6.1. Effect of calcium chelator EGTA on histamine-induced intracellular calcium increase

In the presence of 2 mM of EGTA, the transient phase of intracellular calcium increase was inhibited by 20% only, whereas sustained phase of intracellular calcium increase was inhibited by 95% (Fig. 5A). Under these conditions, GPI accumulation was inhibited by 80% (Table 3). It is clear that 80% or more of the transient phase of calcium increases observed in the transient phase were from cellular storage sites, whereas intracellular calcium increases seen in the sustained phase were derived mainly from extracellular sources. The influx of calcium into the cells caused the activation of

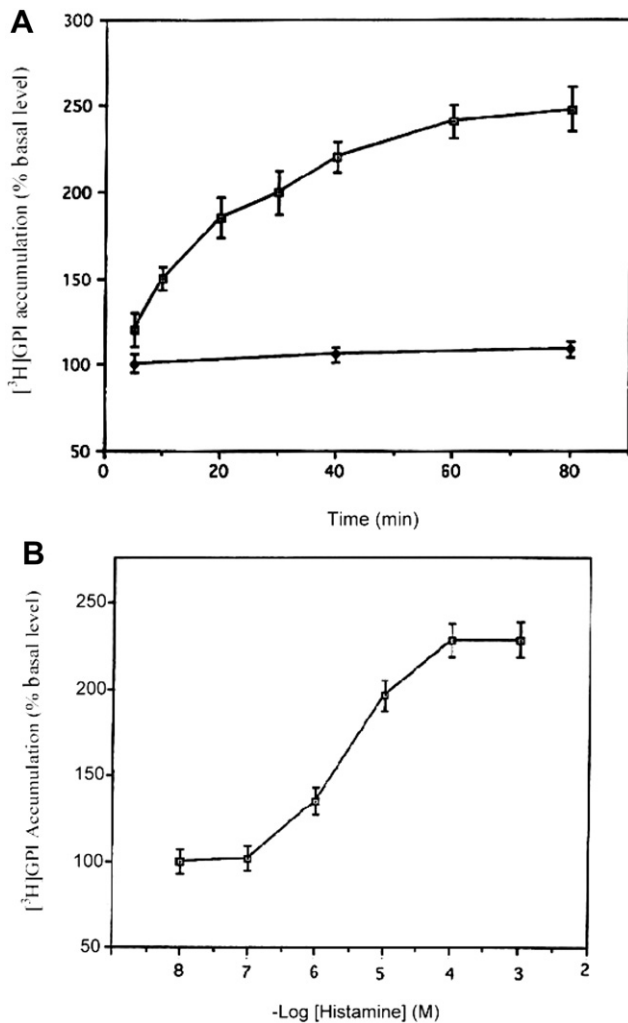


Fig. 2. (A) Time course of histamine-stimulated [³H] GPI accumulation in C6 glioma cells. [³H] Inositol-labeled cells were washed and incubated for the indicated period of time with either vehicle (◆) or 100 μ M histamine (◻) in the presence of 10 mM LiCl; (B) concentration-dependence of histamine-stimulated accumulation of GPI in C6 glioma cells. [³H] Inositol-labeled cells were washed and incubated with the indicated concentrations of histamine in the presence of 10 mM LiCl for 60 minutes. Results are expressed as percentage of basal level (884 \pm 69 dpm/ 10^6 cells). Results are mean \pm SEM from three independent experiments performed in triplicate.

PLA2 (phospholipase A2) and resulted in AA and GPI accumulation.

3.6.2. Effect of calcium channel blocker La^{3+} on histamine-induced intracellular calcium response

The addition of 1 mM La^{3+} completely blocked a histamine-induced sustained calcium increase (Fig. 5B). This is evidence that the sustained phase of intracellular free calcium ion increase is a result of calcium influx from extracellular sources.

3.6.3. Effect of receptor-mediated calcium channel blocker (RMCE) on histamine-induced calcium response

In the presence of 10 μ M SKF 96365, a receptor-mediated calcium entry inhibitor,²² histamine-induced transient phase

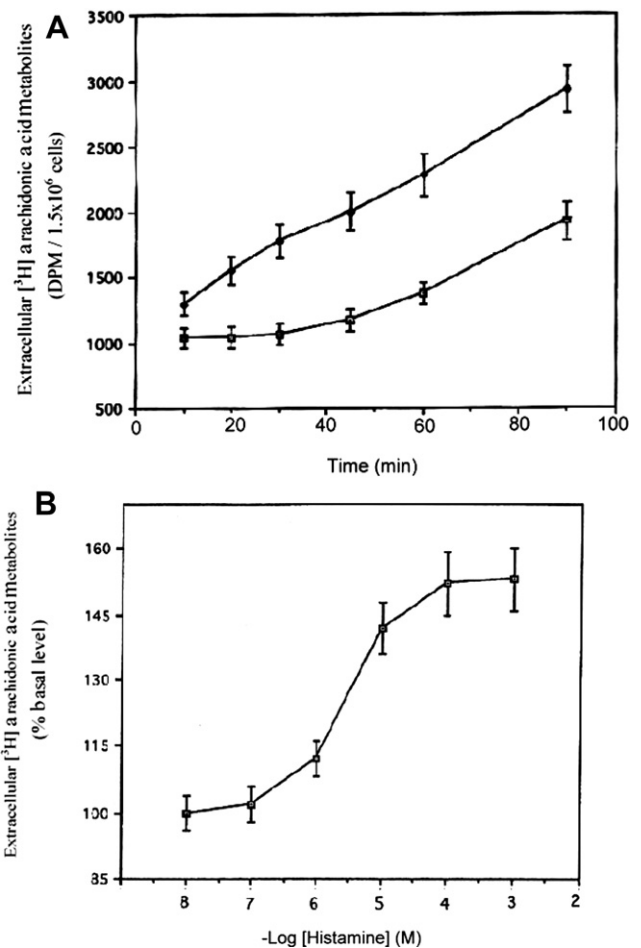


Fig. 3. (A) Time course of histamine-stimulated the release of arachidonic acid (AA) and its metabolites into extracellular medium in C6 glioma cells. [³H] AA-labeled cells were washed and incubated with 100 μ M histamine (◆), or vehicle (◻) for the indicated period of time; (B) concentration-dependence of histamine-stimulated release of AA metabolites in C6 glioma cells. [³H] AA-labeled cells were washed and exposed to the indicated concentrations of histamine for 60 minutes. Results are expressed as percentage of basal level (1105 \pm 60 dpm/ 10^6 cells). Values are the mean \pm SEM from three independent experiments performed in triplicate.

of calcium increase was inhibited by about 20%, whereas sustained calcium increases were inhibited by 67% (Fig. 6A). However, L-type calcium channel blockers such as nifedipine or verapamil did not block sustained calcium phase. Higher KCl concentrations did not cause intracellular calcium increase (data not shown). This observation demonstrated that histamine-induced sustained calcium increase was not due to the opening of the voltage-dependent calcium channel, but rather the opening of receptor-mediated calcium channels.

3.6.4. Effects of intracellular calcium antagonist TMB-8 on histamine-induced calcium response

Under the presence of 100 μ M of TMB-8, an intracellular calcium antagonist and a calcium influx blocker, histamine-induced transient-phase calcium increases were inhibited by 83%, and histamine-induced sustained-phase calcium increases were inhibited by 80% (Fig. 6B).

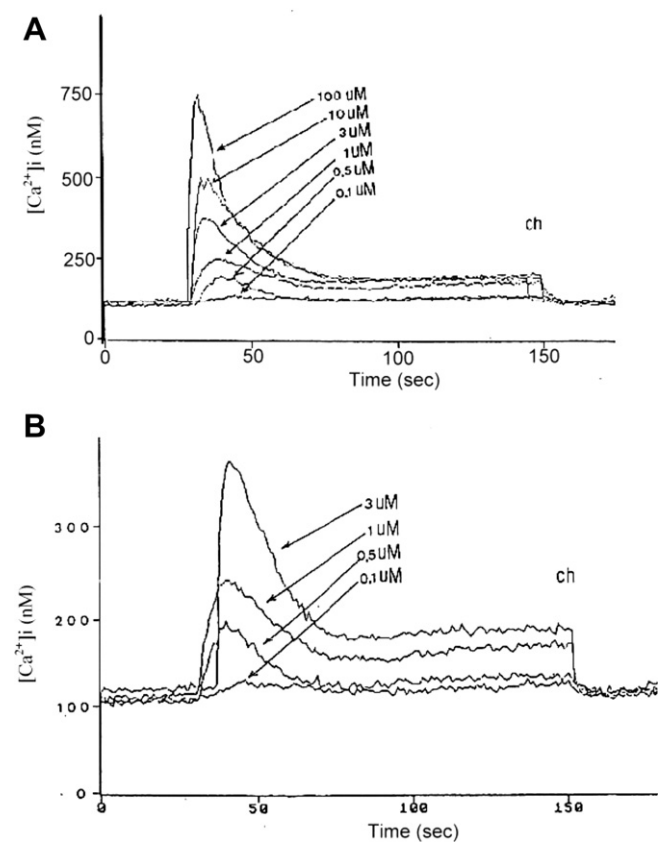


Fig. 4. (A) Concentration-response relationship of histamine-induced peak; and (B) sustained elevation of $[Ca^{2+}]_i$. Assay conditions are detailed in the Materials and Methods section. Fura-2-loaded cells were challenged with the indicated concentrations of histamine. $[Ca^{2+}]_i$ = intracellular free calcium ion.

4. Discussion

From the $[^3H]$ mepyramine binding data, the C6 glioma cell contains endogenous H_1 receptors. Under 12 nM of labeled mepyramine (a concentration close to the K_d (dissociation

Table 2
Effects of H_1 receptor antagonist on Histamine-induced IP_1 accumulation, GPI accumulation, and AA release and elevation of $[Ca^{2+}]_i$.

Treatment	IP_1	Peak $[Ca^{2+}]_i$	GPI	AA
Basal level (%)				
H	1185 ± 71	729 ± 57	200 ± 14	186 ± 7
H + Chor (C)	100 ± 5 ^a	100 ± 3 ^a	100 ± 5 ^a	100 ± 3 ^a

$[^3H]$ Inositol-labeled (for IP_1 and GPI assay) or $[^3H]$ AA-labeled (for AA assay) cells were washed and pretreated with 100 uM chlorpheniramine (Chor) or vehicle for 5 minutes prior to stimulation of 100 uM histamine for 60 minutes. And fura-2-loaded cells were washed and pretreated with vehicle or 100 uM chlorpheniramine for 1 minutes prior to stimulation of 100 uM histamine. Results are expressed as percentage of basal level. Values are mean ± SEM from three independent experiments performed in triplicate or $n = 4$ experiments for calcium assay.
 $[Ca^{2+}]_i$ = intracellular free calcium; AA = arachidonic acid and its metabolites; C = chlorpheniramine; GPI = glycerophosphoric-inositol; H = histamine; IP_1 = inositol 4-phosphate; Peak = transient or peak increase in $[Ca^{2+}]_i$.
^a Significant difference ($p < 0.05$) from values in histamine only; in each assay, basal level = 100%.

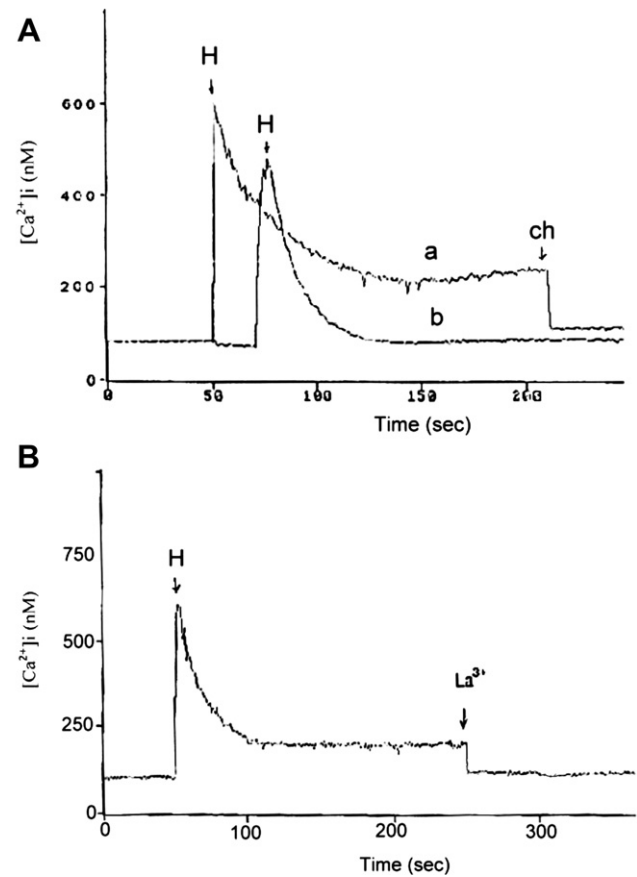


Fig. 5. (A) Exposure of cells to EGTA before challenging with histamine. Assay conditions are detailed in the Materials and Methods section. EGTA (2 mM final concentration) was added to bathing solution followed by histamine (100 uM). H = histamine, ch = chlorpheniramine (100 uM), a trace: without EGTA, b trace: with EGTA; (B) inhibitory effect of La^{3+} on histamine-induced sustained elevation of $[Ca^{2+}]_i$. Assay conditions are detailed in the Materials and Methods section. Fura-2-loaded cells were challenged with 100 uM histamine. Subsequently, La^{3+} (1 mM final concentration) was added. EGTA = ethylene diamine tetraacetic acid.

Table 3
Effects of EGTA and SK&F 96365 on Histamine-induced IP_1 accumulation, GPI accumulation, and elevation of $[Ca^{2+}]_i$ in C6 Glioma cells.

Treatment	IP_1 accumulation	GPI accumulation	Transient $[Ca^{2+}]_i$	Sustained $[Ca^{2+}]_i$
Basal level (%)				
With vehicle (H_2O) only	1164 ± 100	207 ± 10	750 ± 51	200 ± 9
With 2 mM EGTA	1168 ± 112	41 ± 5 ^a	600 ± 20 ^a	10 ± 2 ^a
With 10 uM SK&F 96365	1175 ± 100	68 ± 8 ^a	600 ± 25 ^a	60 ± 5 ^a

$[^3H]$ Inositol-labeled cells (for GPI and IP_1) were washed and pretreated with vehicle (H_2O), 2 mM EGTA or 10 uM of SK&F 96365 for 5 minutes prior to stimulation of 100 uM histamine for 60 minutes in the presence of 10 LiCl. And fura-2-loaded cells (for transient and sustained $[Ca^{2+}]_i$) were washed and pretreated with vehicle (H_2O), 2mM EGTA or 10 uM of SK&F 96365 for 5 minutes prior to stimulation of 100 uM of histamine. Results are expressed as percentage of basal level. Values are mean ± SEM from three independent experiments.
 $[Ca^{2+}]_i$ = intracellular free calcium ion; EGTA = ethylene diamine tetraacetic acid; GPI = glycerophosphoric-inositol; IP_1 = inositol 4-phosphate.
^a Significant difference ($p < 0.05$) from the values of vehicle treatment.

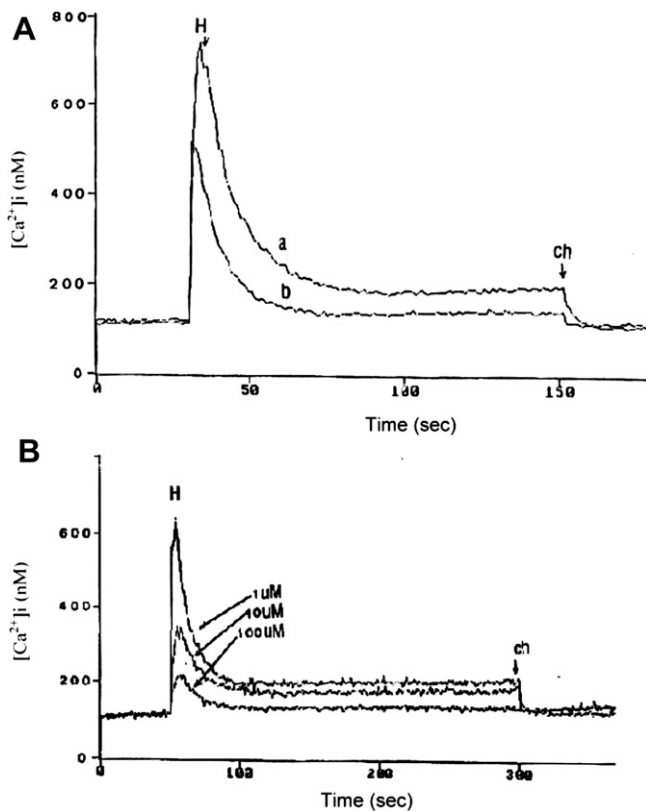


Fig. 6. (A) Exposure of cells to SK&F 96365 before challenging with histamine. Assay conditions are detailed in the Materials and Methods section. SK&F 96365 (10 μ M final concentration) was added to bathing solution followed by histamine (100 μ M), a trace: without SK&F 96365, b trace: with SK&F 96365; (B) inhibitory effect of 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8) on histamine-induced elevation of $[Ca^{2+}]_i$. Assay conditions are detailed in the Materials and Methods section. When pretreated, fura-2-loaded cells were incubated with the indicated concentration of TMB-8 for 3 minutes followed by addition of histamine (100 μ M). H = histamine, ch = chlorpheniramine (100 μ M).

equilibrium constant) value chosen for its binding to H_1 receptors in the primary astrocyte cell study,¹² a 77% specific binding (standing for H_1 receptor sites) was observed, and a 23% nonspecific binding was found under this assay condition. This result revealed H_1 receptors exist in the cells, and these receptors can be detected by histamine H_1 receptor-specific ligand.

The lack of significant [3H]mepyramine binding to C6 glioma cells reported in previous studies suggested a low density of endogenous H_1 receptors in these cells.^{7,11} However, under proper assay conditions, the specific binding sites still could be found. These endogenous H_1 receptors are very efficient in coupling to related secondary messengers via G proteins to perform different cellular responses.²⁰

Stimulation of histamine H_1 receptor by histamine can activate PI-PLC to increase IP_1 accumulation and intracellular $[Ca^{2+}]_i$ via a pertussis toxin-insensitive G protein.^{22,23} Our data confirmed the existence of H_1 receptors in C6 glioma cells, which are coupled to PI turnover, as reported by Peakman and Hill⁵ in the use of [3H] inositol as a precursor in their PI turnover study.

Histamine-induced IP_1 accumulation in C6 glioma cells with an EC_{50} of 4.2 μ M caused more than an 11-fold increase over the basal level at maximal response of 100 μ M histamine. The EC_{50} value obtained in this study is comparable with that of related glial cells and other cells used in the previous studies, such as U373 MG astrocytoma (EC_{50} = 5.4 μ M),²⁴ DDT1 MF-2 (EC_{50} = 10 μ M),²⁵ astrocyte-enriched primary culture (EC_{50} = 1.7 μ M),¹³ oligodendrogloma (EC_{50} = 1.6 μ M),²⁶ and C6 glioma cells (EC_{50} = 24 μ M⁵).

Histamine-induced transient $[Ca^{2+}]_i$ increase, with an EC_{50} of 4.9 μ M, was higher than its sustained $[Ca^{2+}]_i$ increase, with EC_{50} of 0.63 μ M. The sensitivity of histamine-induced sustained calcium phase increase is different from that of transient calcium phase increase. Similar phenomenon has been found in human neuroblastoma cells²⁷ and parotid acinar cells.²⁸

In the presence of 2 mM EGTA, a 20% inhibition of histamine-induced transient $[Ca^{2+}]_i$ increase was observed, whereas a 95% inhibition of histamine-induced sustained $[Ca^{2+}]_i$ increase was found when compared with the data of the medium without EGTA. These results revealed that about 80% of histamine-induced transient Ca^{2+} increase is from intracellular storage sites, and its sustained phase calcium ion increase is from extracellular calcium ion influx, since this effect could be blocked in the presence of 1 mM La^{3+} . In the presence of 10 μ M of SK&F 96365,⁹ histamine-induced transient calcium ion increase could be reduced by 20%, whereas sustained calcium ion increase was reduced by 70%. In the presence of 100 μ M 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8), both transient calcium and sustained phase of calcium level increases were reduced by 83 and 80%, respectively. This is consistent with reports that TMB-8 is a calcium influx blocker and an intracellular calcium antagonist.²⁹

In this study, higher extracellular potassium levels were unable to cause an increase in intracellular calcium. In addition, L-type voltage dependent calcium channel blockers nifedipine and verapamil were unable to reduce sustained calcium ion increase by histamine (data not shown). This is evidence that sustained calcium increase induced by histamine was from H_1 receptor-mediated calcium channels, rather than from voltage-dependent calcium channels.

Histamine-induced GPI accumulation and AA with its metabolite release had an EC_{50} value of 2.8 μ M. It is an indication that both GPI and AA, with its metabolite accumulation, are mediated by the PI hydrolysis pathway via a receptor-coupled G protein system (likely a pertussis toxin-sensitive G protein) to activate PLA2. However, histamine-induced IP_1 accumulation, and its EC_{50} value was 4.2 μ M (versus 2.8 μ M obtained in GPI accumulation), indicates that it may couple to a different G-protein (likely a pertussis toxin-insensitive G protein) to activate phosphatidylinositol-phospholipase C (PI-PLC). In the literature, histamine H_1 receptor activation was found by Murayama and colleagues³⁰ to be mediated via a pertussis toxin-sensitive G-protein and subsequently activate PLA2 in rabbit platelets. Furthermore, Brooks and coworkers³¹ proposed that the activation of PLA2 is coupled to influx of external calcium but not to mobilization of intracellular calcium in C62B glioma cells. Here, influx of

external calcium caused by histamine is likely through activation of Gq/11 protein in PI-PLC pathway.

From the clinical point of view, our data results revealed that the chemical mediator of histamine can interact with its receptors in glioma cells and increase the AA metabolisms and the prostaglandin levels in these cells. Hence, it is well documented that abnormal metabolisms of arachidonic acid and its metabolites are intimately linked to cancer biology.³² Further studies on this aspect are needed.

In conclusion, in C6 glioma cells, histamine interacted with histamine H₁ receptors and caused the activation of PI-PLC, resulting in IP₁ accumulation and intracellular calcium ion increase. Also, it activated PLA2 and resulted in GPI accumulation and AA release. Many physiologic events can happen after the increase of [Ca²⁺]_i, inositol phosphates and AA with metabolite release. Since this cell line uses many secondary messengers of histamine to express the cell responses, it is worthwhile to further study receptor desensitization and/or down-regulation after prolonged activation of H₁ receptors. In addition, studies on which chemicals that can activate or inhibit this signal transduction pathway, and they may become some useful therapeutic agents.

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