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Homologous Desensitization of Histamine-Mediated Signal Transduction System in C6 Glioma Cells

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Abstract

Molecular events involved in the homologous desensitization of histamine-mediated signal transduction system in glioma cells are not well understood. The aim of this study was designed to gain further insight into possible events in the process using the C6 glioma cells. Incubation of histamine caused increases in inositol phosphate (IP_1) formation and intracellular free-calcium concentration $[Ca^{2+}]_i$ in C6 glioma cells *via* the activation of a G-protein-coupled phospholipase C (PI-PLC). Histamine also caused an increase in extracellular release of arachidonic acid (AA) and formation of glycerophosphoinositol (GPI). These effects are likely to be mediated through the activation of receptor-coupled phospholipase A2 (PLA_2). Pretreatment of C6 cells with histamine, from 0.1 μM to 1 mM concentrations, for 10 to 60 min significantly reduced the histamine-induced IP_1 production, $[Ca^{2+}]_i$ accumulation, AA release and GPI formation, despite repeated wash of the cells with buffer solution. Staurosporine (10 nM), a protein kinase C (PKC) inhibitor, reversed almost completely IP_1 production, or partially for $[Ca^{2+}]_i$, GPI formation and AA release of this homologous desensitization effect of histamine. Pretreatment of C6 cells with phorbol 12-myristate 13-acetate (PMA), a PKC activator, at 0.1 nM to 0.1 μM for 2 to 15 min caused a reduction of histamine-induced IP_1 formation and $[Ca^{2+}]_i$ accumulation, but enhanced histamine-induced AA release and GPI formation. Ten nM staurosporine completely reversed the effect of PMA on histamine-induced IP_1 formation and partially on $[Ca^{2+}]_i$ accumulation. However, staurosporine potentiated the effect of PMA on histamine-induced AA release and GPI formation, but the effect could be blocked by H7, a calcium-dependent PKC inhibitor. Our results indicate that activation of PKC by histamine in the signal transduction system is involved in the histamine-induced homologous desensitization event. Since PMA pretreatment could not mimic histamine-induced homologues desensitization event in AA release and GPI formation, it is likely due to the dual actions of this protein kinase activator: on calcium independently, and also on calcium dependent *via* influx of calcium ion through the plasma membrane. The calcium flux effect of PMA is related to the difference between PMA and histamine on the effects of AA release and GPI formation *via* activation on PLA_2. The results of this study provided strong evidence that PKC is involved in this homologous desensitization caused by continuous histamine receptor activation.

Key Words: histamine, homologous desensitization, protein kinase C, signal transduction system

Introduction

In our previous report, we found that incubation of C6 glioma cells with histamine caused an increase in inositol phosphate (IP_1) formation and free $[Ca^{2+}]_i$ accumulation in the cells *via* the activation

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of a G-protein-coupled phospholipase C (PI-PLC). Histamine also caused an increase in extracellular release of arachidonic acid (AA) and intracellular formation of glycerophospho-inositol (GPI). These effects are likely mediated through the activation of receptor-coupled phospholipase A_2 (PLA_2) (30).

There are two types of desensitization: homologous and heterologous desensitization in agonist-activated G-protein-coupled receptors (GPCRs). When an agonist is in high concentration or interacts with its site for a longer period of time, and the resulting receptor to decrease its response to a signaling molecule, this is called homologous desensitization. However, if repeated stimulation of a receptor by one agonist results in desensitivity to a range of other agonists when they interact with their receptors, it is called heterologous desensitization (7). The molecular mechanisms of causing desensitization are not limited to the receptor level only, and/or can occur at post-receptor levels (13, 27), and the causing mechanisms can vary from some possible factors, such as cell lines, organs or species used in the studies in different receptor systems (21, 22).

In the signal transduction pathway, homogenous desensitization is a common phenomenon in many receptor activation-mediated cellular responses. After a prolong action of a neurotransmitter or mediator with its membrane receptor, the cellular responses induced by these stimulators are significantly decreased (12). However, the molecular events involved in this phenomenon are not completely understood. The protein phosphorylation by receptor kinases or protein kinases in the signal transduction pathway to interact with their substrate proteins such as receptor molecule, G-protein has been proposed as the cause of this phenomenon (19). However, supporting experimental data are lacking.

Clinically, continuous stimulation of cells with agonists generally results in a state of desensitization (also referred to as adaptation, refractoriness or down-regulation) such that the effect that follows continued or subsequent exposure to the same concentration of drug is diminished. This phenomenon, called tachyphylaxis, occurs rapidly and is therapeutically important; an example is attenuated response to the repeated use of beta adrenergic receptor agonists as brochodilators for the treatment of asthma (1).

In this study, C6 glioma cells were used, and histamine-induced second messengers, IP_1 formation, $[Ca^{2+}]_i$ accumulation, GPI production and AA and its metabolites released, were measured as the parameters for the activation of this signal transduction. Desensitization phenomenon conditions were made under the use of these cells pre-treated with varying concentrations of histamine, or a PKC activator, PMA (phorbol 12 –myristate 13 acetate), for a certain period of time, to investigate if histamine-induced desensitization event could be observed.

If this phenomenon could be observed, a specific PKC inhibitor, staurosporine or H7 (10), was used in pretreatment to test if the inhibitor had any effect on this event. It is known that histamine-induced second-messenger production is *via* receptor activation, which is coupled to G-protein molecules, and then through the activation of PI-PLC enzyme to generate diacylglycerol (DG) and IP_3 production. With the increase of DG and increase of $[Ca^{2+}]_i$, DG and $[Ca^{2+}]_i$ can activate PKC. Phosphorylation by this kinase on certain substrate proteins, such as its receptor, or G-protein molecule, can cause a reduction of IP_1 formation, $[Ca^{2+}]_i$, can alter the changes on GPI production or AA release. With the results of this observation, the involvement of PKC on homologous desensitization can be suggested.

Materials and Methods

The radioisotopes used in this study, [1,2-^{3}H]L-myoinositol (45.8 Ci/mmole), [^{3}H]arachidonic acid (100 Ci/mmole) and [^{3}H]mepyramine (24.7 Ci/mmole) were purchased from New England Nuclear (NEN) Co. (Boston, MA, USA). Protein kinases of staurosporine (Calbiochem, San Diego, CA., USA) and 1-(5-isoquinolinylsulfonyl)-1-methylpiperazine (H7: Seikagaku America Corp. St Petersburg, FL., USA) were used. Other chemicals, histamine HCl, chlorpheniramine, 3, 4, 5-tri-methoxy-benzoic acid 8-(diethyl amino) octylester (TMB-8), lanthanum chloride, sodium fluoride, aluminium chloride, fura-2/AM, nifedipine and verapamil were purchased from Sigma Chemical Co. (St. Louis, MO, USA); SK&F 96365 was obtained from Biomol. Res. Lab. Inc. (Plymouth Meeting, PA, USA). Ready Safe Liquid Scintillation Cocktail was obtained from Beckman Co. (Fullerton, CA, USA) for sample counting use in the Beckman LS-3801 scintillation counter. A Cation Counter, Spex Model CMIT 111 (Rayonics Scientific Inc., St Laurent, QC, Canada) was also used in this study.

Cell Culture

The C6 glioma cell line was a gift from Professor Ted-Cheng Chou at the Institute of Neuroscience, National Yang-Ming University. These cells were cultured in Ham's F-10 medium (JRH Bio-Sciences, Inc. Lenexa, KS, USA) with 10% fetal bovine serum (Hyclone Co., Logan, UT, USA). Cells in T-75 flasks were kept at 37°C in a 5% CO_2 and 95% O_2 incubator. The culture medium was changed every other day. This cell line has been used widely since its establishment in 1968 (3).

C6 glioma cells were seeded on 3 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 the unlabeled portion was washed out three times with a washing buffer containing 116 mM NaCl, 26.2 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2.5 mM KCl, 1.5 mM $MgSO_4$, 2.5 mM $CaCl_2$ and 20 mM glucose.

Phosphatidylinositol (PI) Turnover Analysis (6, 25)

Labeled [^{3}H]myo-inositol (1 Ci/ml) was added into each flask for 24-h incubation in a F10 culture medium without serum. At the end of incubation, this culture medium was removed and a washing buffer was used to wash these cells three times. Buffer solution (2.475 ml) was added into the incubation flask, and the cells were incubated at 37°C for 40 min. Ten mM LiCl was then added to prevent breakdown of inositol phosphates into inositol. After that, a stimulator such as histamine was added into the flask for a period of time such as 60 min. At the end of the incubation, an ice-cold washing buffer was used to wash the cells twice to end the reaction in the flask. The glass plates were removed for counting and were kept in the scintillation counting vials. For further extraction of inositol phosphates, the method of Pearce *et al*. (26) was used.

Analysis of GPI and PI (25)

The PI products were analyzed with anion-exchange chromatography. Half 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 the resin. Ten ml double-distilled water was added to wash out free form of PI. Then 6 ml 60 mM sodium formate/5 ml sodium tetraborate was added to wash out the [^{3}H]glycerol-phosphoinositol ([^{3}H]GPI), and the first 2 ml was collected in a scintillation vial. Finally, 2 ml 0.2 M ammonium formate/0.1 M formic acid elution solution was added to wash out the [^{3}H]IP_1. This liquid was collected in scintillation vials, then mixed well with 6 ml of scintillation fluid for scintillation counting.

Arachidonic Acid (AA) Metabolism Study (16)

C6 glioma cells were plated at a concentration of 5×10^5 cells in a 35-mm incubation plate, kept at 37°C in a 5% CO_2 and 95% O_2 incubator for 2 days until cells reached confluent state (about 1.5×10^6 cells). The cultured medium was changed to F-10 culture medium that contained [^{3}H]arachidonic acid (0.15 μCi/ml) without serum and kept at in a 37°C incubator for 24 h in order to label the cells with the radioisotope materials. Before the reaction was started, the radioisotope material was removed and washed 5 times with a washing buffer containing NaCl (118 mM), KCl (4.7 mM), glucose (10 mM), HEPES (20 mM), pH 7.4, at 37°C. Following this, 2.475 ml buffer solution (the composition is shown above), plus 25 μl test sample at the selected concentration was added to start the reaction at 37°C. At the end of the reaction time, 1 ml of cell culture medium was removed and centrifuged at 200 × g for 10 min to precipitate the cells. After centrifugation, 1 ml supernatant was removed, and 3 ml scintillation counter fluid was added and mixed prior to counting with a scintillation counter.

Intracellular Calcium Determination (11)

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 at pH7.4) were centrifuged at 750 × g for 3 min, and the cells were washed twice with the 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 min before centrifugation. The supernatant was then removed, and cells were washed with the loading buffer twice in order to remove the remaining fura-2/AM. The cells were suspended with the loading buffer at a concentration of 5×10^6 cells/ml and kept in a 15 ml plastic centrifuge tube covered with aluminum foil to prevent light penetration. The assay was performed in the dark. At the end of the experiment, 0.3% digitonin was added to combine fura-2 with Ca^{2+} to obtain Rmax, and ethylene diamine tretraacetic acid (EGTA) was added to a final concentration of 0.1 M to compete with fura-2 for Ca^{2+} to obtain Rmin. Intracellular calcium concentration was obtained by using the method of Grynkiewicz *et al*. (11).

[^{3}H]Mepyramine Binding Assay (14)

Cell membranes (about 150 μg of membrane protein in 0.4 ml), prepared from C6 glioma cells by the method of Smit *et al*. (29), were incubated with 12 nM [^{3}H]mepyramine at 25°C for 60 min in one set with the presence of 1 mM chlorpheniramine as a nonspecific binding, whereas another set without the presence of chlorpheniramine as a total binding. At the end of incubation, 5 ml cold washing buffer was added to each tube to stop the reaction. The whole

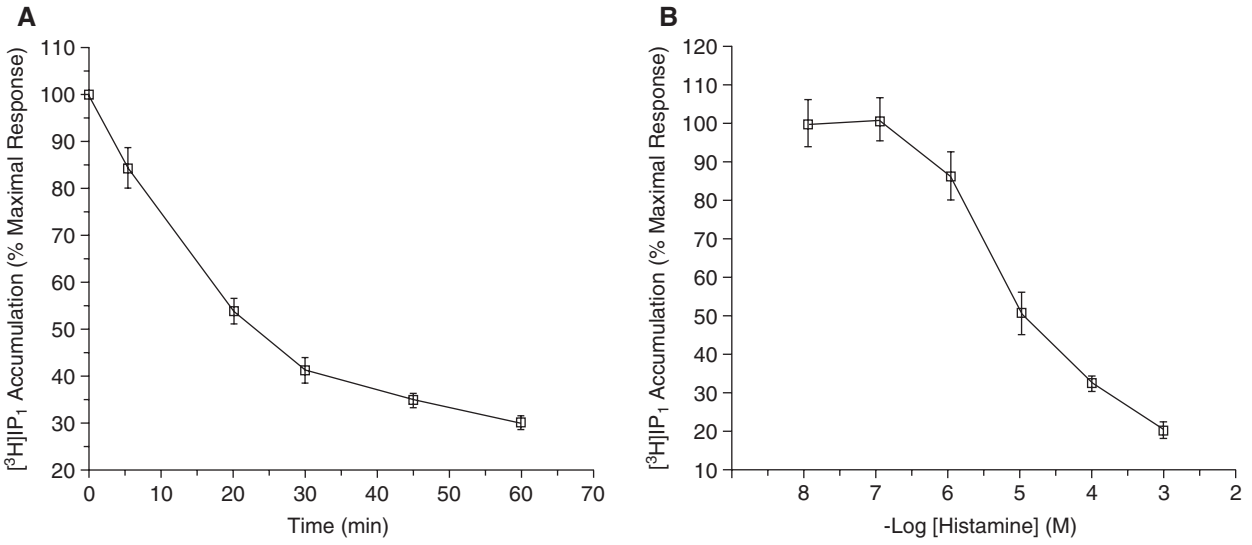

Fig. 1. Time- and concentration-dependence of histamine-induced desensitization of histamine-stimulated IP_1 accumulation. A. Time-dependence of histamine-induced desensitization of histamine-mediated IP_1 accumulation. Cells were preincubated with 100 μM histamine for the indicated periods of time during labeling with [^{3}H]inositol. Cells were washed and incubated with 100 μM histamine for a further 60 min in the presence of 10 mM LiCl. Results are expressed as percentage of the histamine-stimulated response (46,170 ± 2,450 dpm/10^6 cells). Values are means ± S.E.M. from three independent experiments performed in triplicates. B. Concentration-dependence of histamine-induced desensitization of histamine-stimulated accumulation of IP_1 accumulation. Cells were preincubated with the indicated concentrations of histamine for the last 60 min in [^{3}H]inositol-labeling medium for 24 h. Then cells were washed and incubated for a further 60 min with 100 μM histamine in the presence of 10 mM LiCl. Results are expressed as percentage of the histamine-stimulated response (48,273 ± 2,599 dpm/10^6 cells). Values presented are means ± S.E.M. from three independent experiments performed in tripriclates.

volume of the reaction mixture was poured onto a GG/B filter paper pre-soaked with 1 μM mepyramine solution under the vacuum pump and then washed twice with 4 ml washing buffer. The filter papers kept in counting vials were dried for 1 h at 30°C before adding 3 ml of Ready Safe scintillation counter fluid. The vials were vortexed vigorously for 3 to 5 min, and were kept for 30 min before counting in a beta counter.

Statistical Analysis

Student's *t*-test was used for statistical analysis; the significant difference was set as $P < 0.05$ between two groups in the comparison. Each experiment was carried out in triplicates, and each test was repeated a minimum of three times.

Results

Homologous Desensitization Caused by Prolongation Histamine Treatment

On histamine-induced IP_1 formation, C6 glioma cells were labeled with [^{3}H]myoinositol for 24 h; before the end of this labeling step, 100 μM histamine was added as an agonist pre-treatment step for 5, 20, 30, 45 and 60 min. These treated cells were then washed three times with a washing buffer. In the presence of 10 mM LiCl, these cells were incubated with 100 μM histamine for an additional 60 min. A significant inhibitory effect of histamine-induced IP_1 formation was observed. This inhibitory effect was a time dependent event over 60 min of pretreatment period. The inhibitory effect reached a steady state and maximal inhibition was about 70%; the $T_{1/2}$ value was calculated as 15 min (Fig. 1A).

These cells were then pretreated for 60 min with varying concentrations of histamine. Homologous desensitization was found to be a concentration-dependent effect; the maximal inhibition under 1 mM histamine pretreatment was 80% with respect to the response obtained in the presence of 100 μM histamine for an incubation period of 60 min. The IC_{50} value was calculated as 10 μM (Fig. 1B).

On histamine-induced GPI accumulation, when C6 glioma cells were pretreated for 60 min with varying concentrations of histamine, homologous desensitization was also found to be a concentration-dependent effect with respect to the response of 100 μM histamine without histamine pretreatment; the inhibition of 100 μM histamine pretreatment was 32% and the IC_{50} value was calculated as 1.26 μM (Fig. 2A).

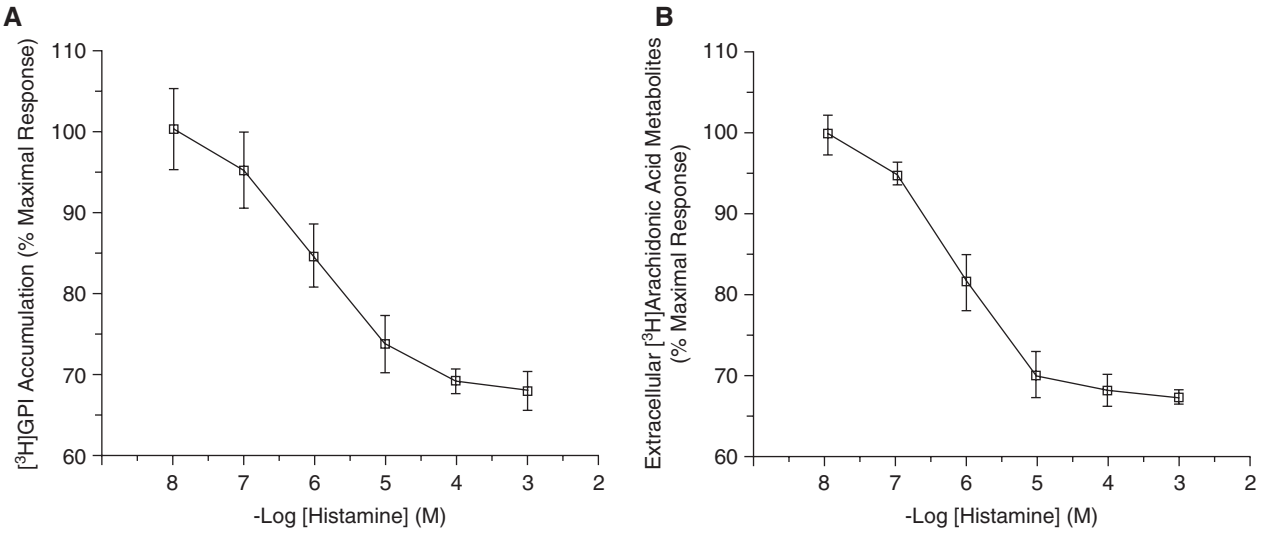

Fig. 2. Concentration-dependence of histamine-induced desensitization of histamine-stimulated accumulation of GPI and arachidonic acid (AA) metabolites released in C6 glioma cells. A. Concentration-dependence of histamine induced desensitization of histamine-stimulated accumulation of GPI in C6 glioma cells. Cells were preincubated with the indicated concentrations of histamine for the last 60 min in [^{3}H]inositol-labeling medium for 24 h. Then pretreated cells were washed and incubated for a further 60 min with 100 μM histamine in the presence of 10 mM LiCl. Results are expressed as percentage of histamine-stimulated response (1,929 ± 97 dpm/10^6 cells). Results presented are means ± S.E.M. from three independent experiments performed in triplicates. B. Concentration-dependence of histamine medicated desensitization of histamine-stimulated arachidonic acid (AA) metabolites release in C6 glioma cells. Cells were preincubated with the indicated concentrations of histamine for the last 60 min in [^{3}H]AA-labeled medium for 24 h. Then pretreated cells were washed and incubated with 100 μM histamine for a further 60 min. Results are expressed as percentage of histamine-induced response (1,750 ± 70 dpm/10^6 cells). Values shown are the means ± S.E.M. from three independent experiments performed in triplicates.

On histamine-induced AA release, when C6 glioma cells were pretreated for 60 min with varying concentrations of histamine, homologous desensitization was found to be a concentration-dependent effect, with respect to the response of 100 μM histamine under without histamine pretreatment; the inhibition of 100 μM histamine pretreatment was 32%, the IC_{50} value was calculated as 1 μM (Fig. 2B).

On histamine-induced free calcium ion $[Ca^{2+}]_i$ increase, when C6 glioma cell were pretreated for 60 min with 100 μM of histamine, homologous desensitization was found, and inhibition with respect to the response of 100 μM histamine under without histamine pretreatment was 76% (Fig. 3).

Effects of PKC Inhibitor Staurosporine on Histamine-Induced Homologous Desensitization Responses

Before C6 glioma cells were pretreated with 100 μM histamine for 60 min, the cells were incubated with 10 nM staurosporine for 70 min. After washing, 100 μM of histamine was added to these cells and incubated for 60 min. There was homologous desensitization effect of histamine on itself induced GPI accumulation, and AA release was completely

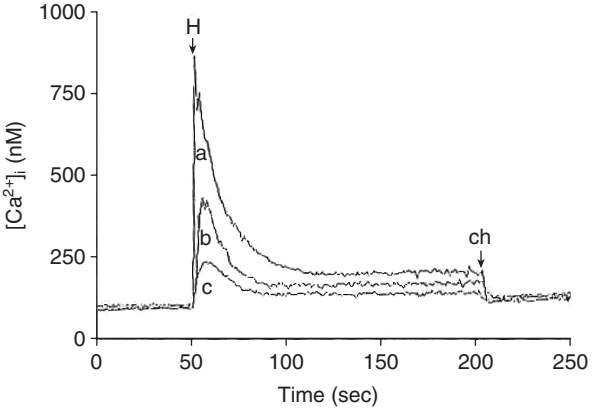

Fig. 3. Reversing effect of staurosporine on histamine-induced desensitization of histamine-stimulated elevation of $[Ca^{2+}]_i$. Assay conditions are detailed in Materials and Methods. Cells, preincubated with (trace b) or without (trace c) 10 nM staurosporine for 60 min, were incubated with 100 μM histamine for 60 min before stimulation of 100 μM histamine (trace a). Tracings shown are from a typical experiment repeated four times with nearly identical results. H = histamine, ch = 100 μM chlorpheniramine.

reversed (Table 1), whereas histamine-induced IP_1 accumulation was reversed by 24%, and itself induced $[Ca^{2+}]_i$ release was reversed by 40% (Fig. 3).

Table 1. Effects of staurosporine on histamine-induced desensitization of histamine-mediated [^{3}H]IP$_1$ accumulation, [^{3}H]GPI production and [^{3}H]AA release in C6 glioma cells

Treatment	IP$_1$	GPI	AA
		% of basal level	
Vehicle (V)	100 ± 17	100 ± 15	100 ± 15
Histamine (H)	1200 ± 34	205 ± 8.5	154 ± 8.0
H-Pretreated + H	343 ± 25	146 ± 8.0	111 ± 7.0
Staurosporine (S) + H-Pretreated + H	531 ± 34*	240 ± 9.0*	163 ± 7.5*

Cells were preincubated with 10 nM staurosporine and 100 μM histamine for the last 120 min, and 60 min, respectively in the labeling medium. The pretreated cells were washed and stimulated with vehicle or 100 μM histamine in the presence or absence of 10 mM LiCl. Each value is expressed as percentage of controls 94,136 ± 143 dpm/10^6 cells for IP$_1$ assay, 957 ± 80 dpm/10^6 cells for GPI assay, and 1,040 ± 81 dpm/10^6 cells for AA assay and represents means ± S.E.M. of three separate experiments. V = vehicle, S = 10 nM staurosporine, H = 100 μM histamine. Asterisk (*) indicates significant effect of staurosporine on histamine-induced desensitization when compared with the correspondent control (based on Student's *t*-test. $P < 0.05$).

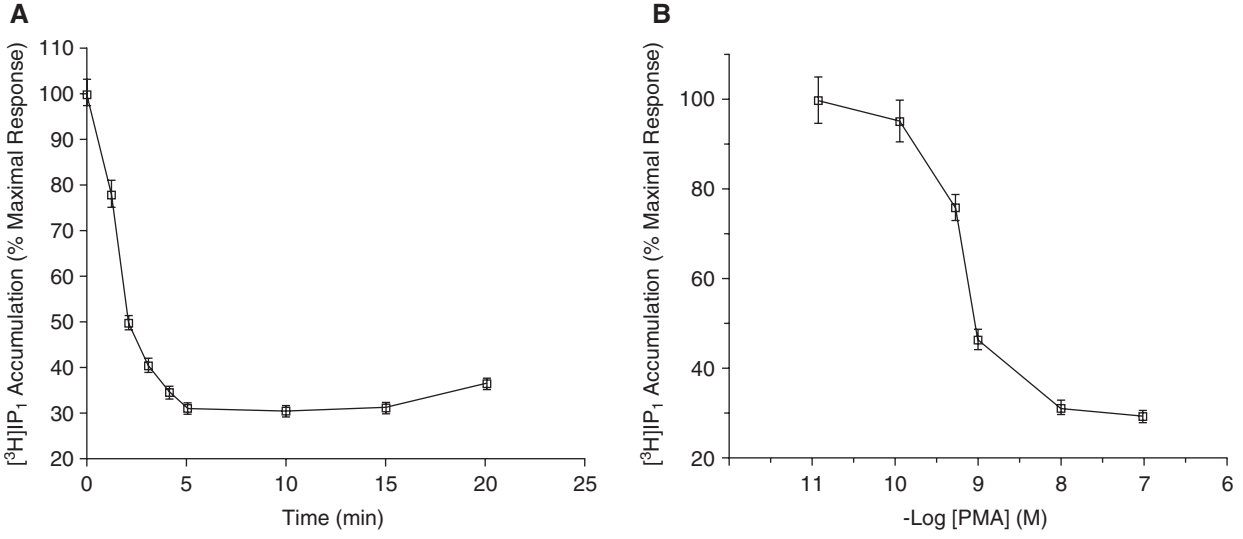

Fig. 4. Time- and concentration-dependence of PMA-induced inhibition of histamine-mediated IP$_1$ accumulation. A. Time-dependence of PMA-induced inhibition of histamine-mediated IP$_1$ accumulation. Cells were preincubated with 10 nM PMA for the indicated periods of time during labeling with [^{3}H]inositol. Then pretreated cells were washed and incubated for a further 60 min with 100 μM histamine in the presence of 10 mM LiCl. Results are expressed as percentage of the histamine-stimulated response (47,378 ± 2,181 dpm/10^6 cells). Results are means ± S.E.M. from three independent experiments performed in triplicates. B. Concentration-dependence of PMA-induced inhibition of histamine stimulated accumulation of IP$_1$. Cells were preincubated with the indicated concentrations of PMA for 10 min. Then pretreated cells were washed and incubated for a further 60 min with 100 μM histamine in the presence of 10 mM LiCl. Results are expressed as percentage of the histamine-stimulated response (48,375 ± 2,481 dpm/10^6 cells). Results are means ± S.E.M. from three independent experiments performed in triplicates.

Effects of PMA Pretreatment on Histamine-Induced Cellular Responses in C6 Glioma Cells.

Since PKC inhibitor staurosporine could inhibit histamine-induced homologous desensitization, the protein kinase activator, PMA, was used to see if it could cause desensitization on histamine responses. On histamine-induced IP$_1$ accumulation, before C6 glioma cells were pretreated with 100 μM histamine for 60 min, the cells were incubated with 10 nM PMA for different periods of time in the presence of 10 mM LiCl, and the cells were incubated with 10 μM histamine for 60 min. PMA had a significantly inhibitory effect on histamine-induced IP$_1$ accumulation. The inhibitory effect of PMA on this event could be found within a few minutes of pretreatment; after 5 min pretreatment, the inhibitory effect reached a steady state, and its $T_{1/2}$ was calculated as 1.5 min (Fig. 4A). Under varying concentrations of PMA pretreated for 10 min, PMA caused a concentration-dependent effect

Table 2. Time course of the reversing effect of staurosporine on PMA-induced inhibition of histamine-stimulated IP_1 accumulation in C6 glioma cells

Treatment	$[^3H]IP_1$ Accumulation[#]					
Period (min)	0	5	10	25	60	90
Staurosporine	30 ± 2	45 ± 3	58 ± 4	78 ± 6	90 ± 10	80 ± 7

Cells were preincubated with 10 nM staurosporine for the indicated periods of time prior to the addition of 10 nM PMA for the last 10 min during labeling with [^{3}H]inositiol. Cells were then washed and incubated for a further 60 min with 100 μM histamine in the presence of 10 mM LiCl. Results are expressed as percentage of the histamine-stimulated response (44,7378 ± 2,454 dpm/10^6 cells) in unpretreated cells (*vs.* histamine only as 100%). Values are means ± S.E.M. from three independent experiments performed in triplicates. [#]In the presence of 0.1 mM histamine only = 100%.

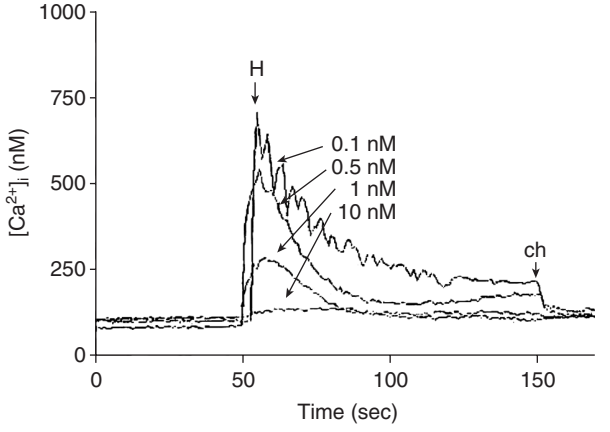

Fig. 5. Inhibitory effects of PMA on histamine-induced elevation of $[Ca^{2+}]_i$. Assay conditions are detailed in the Materials and Methods. When pretreated, Fura-2-loaded cells were incubated with the indicated concentration of PMA for 3 min followed by addition of histamine (100 μM). Tracings shown are from a typical experiment repeated four times with nearly identical results. H = histamine, ch = 100 μM chlorpheniramine.

on histamine-induced IP_1 accumulation response. In the presence of 10 nM PMA, the effect reached a steady state, and its IC_{50} value was calculated as 6.3×10^{-10} M (Fig. 4B).

On histamine-induced $[Ca^{2+}]_i$ increase, under varying concentrations of PMA pretreatment for 3 min, 100 μM histamine was added to the C6 glioma cells. PMA caused a significant inhibitory effect on transient and sustained $[Ca^{2+}]_i$ increase. At 10 nM PMA pretreatment, it could completely inhibit histamine-induced $[Ca^{2+}]_i$ increase, and its IC_{50} values was calculated as 6.3×10^{-10} M (Fig. 5).

Effects of Staurosporine on PMA Caused Inhibition on Histamine-Induced Cellular Response in C6 Glioma Cells

To study histamine-induced IP_1 formation, 10 nM staurosporine was used to pretreat cells at varying time periods, and 10 nM PMA was then added to C6 glioma cells for 10 min before washing. Under 10 mM LiCl treatment and in the presence of 100 μM histamine for 60 min, the inhibitory effect of PMA on histamine-induced IP_1 formation could be reversed by 10 nM staurosporine at different pretreatment time periods. After 60 min pretreatment with staurosporine, the inhibitory effect on histamine-induced IP_1 formation could be reversed by 10 nM staurosporine treatment in the range of 90 ± 10% (Table 2). On histamine induced $[Ca^{2+}]_i$ increase, 10 nM PMA pretreatment caused an inhibitory effect by 90% on 100 mM histamine-induced cellular $[Ca^{2+}]_i$ accumulation was reversed by 10 nM staurosporine pretreatment for 10 min. However, this reversing effect on either transient or sustained phases of $[Ca^{2+}]_i$ caused by histamine was only to the extent of 44% inhibition (Fig. 6).

Effects of Staurosporine and H7 on the Enhancement Action of PMA on Histamine-Induced GPI Accumulation and AA Release

When 10 nM PMA was added to C6 glioma cells for 10 min, it could cause 19% increase in AA release over the basal level, and 60% increase in GPI accumulation (Table 3). In the presence of 10 nM PMA for 10 min and the addition of 100 μM histamine for 60 min, the productions of AA and GPI were increased by 20% to 30% with respective to that of histamine only. These productions were potentiated by 10 nM strausporine, but the effect was reversed by H7. From this observation, PMA apparently did not have an inhibitory effect on histamine-induced AA release or GPI accumulation, but had a significantly additive effect on both parameters. Besides, the enhancement effect of PMA on histamine-induced AA release or PGI accumulation could be further potentiated by pretreatment of stauroporine, but the effect was reversed by H7, a calcium-dependent protein kinase inhibitor (Table 3).

Table 3. Effects of protein kinase C inhibitors staurosporine and H7 on the enhancement action of PMA on histamine-induced AA and GPI production in C6 glioma cells

Treatment	% of Basal Level	
	AA	GPI
Vehicle	100 ± 6.7	100 ± 6.7
PMA (P)	119 ± 10	162 ± 11.0[a]
Histamine (H)	162 ± 10[a]	215 ± 13.2[a]
PMA + Histamine	194 ± 11.5[a]	281 ± 14.82[a]
P + H + Staurosporine (S)	325 ± 25.7[b]	480 ± 26.6[b]
P + H + S + U (H7)	190 ± 12.0	285 ± 15.0

Cells were preincubated with 10 nM PMA for the last 10 min in the labeling medium. Pretreated or unpretreated cells were washed and stimulated with vehicle or 100 μM histamine in the presence (for GPI assay) or absence (for AA assay) of 10 mM LiCl for a further 60 min. Basal value (100%) was 882 ± 70 dpm/10^6 cells for GPI, and was 1,120 ± 75 dpm/10^6 cells for AA. Each value shown is the means ± S.E.M. of three separate experiments. Vehicle, *P* = 10 nM PMA, His = 100 μM histamine. For pretreatment, 50 μM H7 (added 40 min before addition of histamine); 10 nM staurosporine (added 70 min before addition of histamine). Significant effects: a (*vs*. that of vehicle), and b (*vs*. that of the combination of PMA + staurosporine and histamine) (based on Student's *t*-test. $P < 0.05$).

Table 4. Effects of PMA on the binding of [^{3}H]mepyramine to H1 receptor in crude plasma membrane of C6 glioma cells

Treatment	[^{3}H] Mepyramine Binding		
	Total Binding	Non-Specific Binding	% Specific Binding
Without PMA Treatment	3277 ± 185	784.5 ± 37	76 – 78
With PMA Treatment	3207 ± 184	784.0 ± 35	77 – 78

Crude plasma membrane isolated from C6 glioma cells, which had been pretreated with or without 10 nM PMA for 5 min, was incubated with or without 100 μM chlorpheniramine in the presence of 12 nM [^{3}H]mepyramine for binding assay as described in Materials and Methods.

Effect of PMA on [^{3}H]Mepyramine Binding to Histamine H1 Receptor of Crude Plasma Membrane of C6 Glioma Cells

When crude plasma membranes isolated from C6 glioma cells, which had been pretreated with or without 10 nM PMA for 5 min, was incubated with or without 100 μM chlorpheniramine in the presence of 12 nM [^{3}H]mepyramine for binding assay, there was no difference in the specific binding to histamine H1 receptor sites (Table 4). From the results described above (Table 3, Figs. 4, 5 and 6), PMA could cause desensitization of histamine-induced physiological responses; however, the numbers of histamine H1 receptor were still the same between PMA-treated and untreated conditions.

Discussion

The waning responses to cell-surface receptor activation during persistent stimulation with agonists (desensitization) are a feature common to many forms of trans-membrane signaling. Up to date, there are reports regarding regulatory processes that modulate the extensive group of receptors that are linked to PI-PLC to the production of inositol 1,4, 5-triphosphate and 1,2-diacylglycerol (12). A major mechanism underlying desensitization is the involvement of phosphorylation events to occur on the substrate proteins of the receptor molecule, G-protein, PI-PLC or other related regulatory proteins. In the past decades, GPCR phosphorylation by second messenger-dependent protein kinases such as protein kinase A (PKA) and PKC has been regarded as the principal mechanism of GPCR desensitization (4).

In this study, when C6 glioma cells pre-exposed to histamine for a period of time even at 10 μM, or at higher concentrations such as 100 μM, for 10 min or longer, the histamine-induced productions of IP_1 formation, $[Ca^{2+}]_i$ accumulation, AA release or GPI production were found to be significantly decreased, and it was a desensitization event. One of the possible mechanisms proposed was due to the activation of the secondary messenger kinase, PKC, and the phosphorylation of substrates in the signal transduction pathway, such as receptor molecule, G-protein or

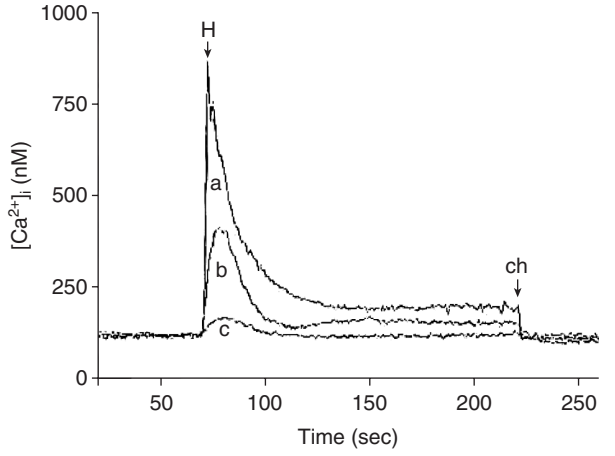

Fig. 6. Reversing effect of staurosporine on PMA-induce inhibition of histamine-stimulated elevation of $[Ca^{2+}]_i$. Assay conditions are detailed in Materials and Methods. Cells preincubated with (trace b) or without (trace c) 10 nM staurosporine for 60 min were incubated with 10 nM PMA for 3 min before stimulation of 100 μM histamine, and unpretreated cells were stimulated with 100 μM histamine (trace a). Tracings shown are from a typical experiment repeated four times with nearly identical results. H = histamine, ch = chlorpheniramine (100 μM).

PI-PLC that might have resulted in the observed desensitization and decreases in these productions.

The involvement of PKC in such an event was supported by studies on the use of PKC inhibitor staurosporine. At the lower concentration of 10 nM staurosporine exposure for 60 min, the homologous desensitization effect by histamine could be significantly reversed or altered. To obtain further support of such a possibility, PMA, a PKC activator, was used to replace the role of DG for the activation of PKC. When C6 glioma cells were pre-exposed with 10 nM PMA for 3 min or more, histamine-induced cellular response decreased significantly. The desensitization response was dependent on the exposure time and concentrations of PMA. The PMA induced histamine desensitization could be reversed by the pretreatment of PKC inhibitor staurosporine on IP_1 formation and calcium level change.

Our results revealed that activation of PKC by histamine in the signal transduction system is involved in the histamine-induced homologous desensitization event on IP_1 production, $[Ca^{2+}]_i$ accumulation, AA release and GPI formation. However, PMA pretreatment could mimic parameters of IP_1 formation and calcium ion accumulation, but not histamine-induced homologues desensitization event in AA release and GPI formation. The discrepancy is likely due to the dual actions of this protein kinase activator, and of a calcium flux activator through the plasma membrane (2, 18).

Several studies have shown that PMA could affect cell functions by mechanism(s) other than calcium-independent PKC activation. PMA might have altered the phospholipid bilayer structure of cellular membranes *via* calcium influx to activate a calcium-dependent PKC, making the substrate more available for PLA_2, which releases AA and its metabolites (8). It has been suggested that PKC and PLA_2 share similar regulatory sequences which could explain a direct stimulation of PLA_2 by PMA through the action of calcium ion (23). From the observations reported in this study, PMA apparently did not have inhibitory effects on histamine-induced AA release or GPI accumulation, but PMA did show a significant additive effect on both parameters. Besides, the enhancement effect of PMA on histamine-induced AA release or PGI accumulation could be further potentiated by the pretreatment of stauroporine, but reversed by H7 (10, 17), a calcium-dependent protein kinase inhibitor (Table 3).

In 1986, Benovic *et al*. (5) reported that a novel protein kinase, not a second messenger-dependent protein kinase, with the ability to phosphorylate the agonist-occupied receptor, was a landmark in GPCR biology. It was soon found to be just one of a family of kinases, and was then termed as G-protein coupled receptor kinases (GRKs). The GRKs have since been shown to play an important role in the agonist-induced phosphorylation and desensitization of many GPCR responses (28). However, GRK phosphorylation of GPCRs was insufficient by itself to produce extensive desensitization of the receptor response (30). Accordingly, another family of regulatory proteins was identified called arrestins (20) with the ability to bind with high affinity to the agonist-occupied, GRK-phosphorylated GPCR, uncoupling it from G-protein activation and thus inducing desensitization of the receptor-generated response.

According to the report of McCreath *et al*. (21), agonist-induced desensitization of H1 receptor-mediated inositol phospholipid hydrolysis in human umbilical vein endothelial cells was found to be insensitive to protein kinase inhibitors such as staurosporine, Ro31-8220 or KN62. The involvement of protein kinase C activation was less possible for the desensitization of histamine H1-receptor-mediated [3H]-inositol phosphate formation. The results of McCreath *et al*. are different from ours, and whether the discrepancies are related to cell lines used that are coupled with different subtypes of PKC (9, 24), or are associated with different receptor density is unclear. Any links to G-protein-coupled receptor kinases (GRKs) and related phosphorylation events are for worthy of testing.

In other reports, regulation of human H1 hista-

mine receptor by G protein-coupled receptor kinase 2 was studied by Iwata *et al*. (15). In the study, overexpression of G protein-coupled receptor kinase, which can promote histamine H1 receptor phosphorylation in intact HEK293 cells, completely inhibited inositol phosphate production stimulated by histamine H1 receptor activation were reported. These findings demonstrate the GRK2 is the principal kinase mediating H1 histamine receptor desensitization in these cells. In HEK 293 cells, one might consider the function of endogenous H1 histamine receptor and the role of endogenous GRKs being quite different from that of the normal expression of GRKs (22).

Besides, long-term exposure of G-protein-coupled receptors to an agonist can result in reduced responses because of down-regulation involving loss of receptors from the cell surface. This can potentially be due to internalization of receptors and increase receptor breakdown, or due to reduction in the rate of receptor synthesis. However, down-regulation usually occurs following exposure to an agonist for periods longer than 2 h (13). For testing the possibility of receptor internalization or increase receptor breakdown in this study, the effect of PMA pretreatment on the binding of [^{3}H]mepyramine to H1 receptor in crude plasma membrane of C6 glioma cells was studied and no differences were found. The data indicated no evidence to prove the existence of such a possibility in the short-term desensitization effect. Hence, it seems likely that the majority of the reduction in responsiveness observed following prior histamine exposure (for less than 2 h) in C6 glioma cells was due to desensitization, but not down-regulation.

In conclusion, our results indicate that activation of PKC by histamine in signal transduction system is involved in the histamine-induced homologous desensitization event in shorter exposure time of less than 2 h. Phosphorylation of substrates at the post-receptor level such as PI-PLC and/or PLA_2 by PKC is linked to this homologous desensitization.

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