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Effects of 2-Azafluorenones on Phosphatidyl-Inositol Specific Phospholipase C Activation in C6 Glioma Cells

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Abstract

Little information is known about the effects of 2-azafluorenones on the cellular signal transduction pathway. In this study, C6 glioma cells were used to test the effect of 2-azafluorenone and its related compounds on histamine-induced phophatidyl inositol (PI) turnover, because of some similarities in chemical structure between these compounds and histaminergic receptor antagonists, such as chlorpheniramine. To investigate the mode of action, studies of the effects of these compounds on PI turnover induced by AlF_4^-, Adenosine 5' triphosphate (ATP), bradykinin and A23187 were carried out. Histamine increased [^{3}H]inositol 4-phosphate (IP_1) formation *via* the activation of phophatidyl inositol specific phospholipase C (PI-PLC) to break down the labeled precursor substrate. This effect could be blocked by chlorpheniramine, 2-azafluorenone, and 4-methyl-2-azafluorenone in a dose and time-dependent manner; this inhibitory effect was also found in chemically related compounds of 2-azafluorenones such as 6-chloro-2-azafluorenone and 6-methoxy-2-azafluorenone. The AlF_4^-- induced [^{3}H]IP_1 accumulation was *via* an activation of G-protein coupled with PI-PLC to break down the labeled PI substrate. These effects could be inhibited by 2-azafluorenone and 4-methyl-2-azafluorenone. A23187 induced IP_1 accumulation *via* an activation on PI-PLC to break down the labeled PI in a G-protein-independent manner. The effect could also be inhibited by 2-azafluorenone and 4-methyl-2-azafluorenone. ATP- and bradykinin-induced IP_1 formation occur *via* an activation of their individual receptor sites, which are different from the receptor sites of histamine. These effects could also be inhibited by 2-azafluorenone and 4-methyl-2-azaflureonone. Depletion of calcium ion by using a calcium free buffer, with or without the addition of TMB-8 (an intracellular calcium antagonist) decreased histamine-induced [^{3}H]IP_1 formation. Even in these conditions histamine-induced response was also inhibited by 2-azafluorenone and 4-methyl-2-azafluorenone. In summary, the inhibitory effect of 2-azafluorenone and its related compound 4-methyl-2-azafluorenone on PI-PLC is likely the cause of reduction in histamine-induced [^{3}H]IP_1 formation. Based on these results, the receptor site, its coupled G-proteins, and calcium ion are not the targets for their action.

Key Words: 2-azafluorenone, PI-PLC, histamine receptor, PI turnover, ATP, bradykinin

Introduction

Azafluorenone derivatives have been reported to possess aldose reductase inhibitor properties (5). These compounds are also found to be structurally related to a significant class of alkaloids such as onychines (16). A convenient method for the synthesis of 2-azafluorenone and other tricyclic pyridines by intramolecular cyclization of 4-aryl-pridines was reported by Shiao *et al*. (14). In addition, Hundsdorf

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et al. (5) reported a new and general synthesis of substituted 2-azafluorenones for drug development purposes.

Recently, Prachayasittikul *et al*. (11, 12) obtained bioactive azafluorenone alkaloids from the *Polyalthia debilis* (Pierre) Finet and Gagnep (Annonaceae) plants. They reported that the bioactive extracts of these plants have potential antimicrobial, antimalarial and cytotoxic activities. These findings suggest potential uses of *P. debilis* in medicinal applications.

In this study, we used C6 glioma cells to test the effects of 2-azafluorenone and its related compounds on histamine-induced phosphatidylinositol (PI) turnover, because of similarities in the chemical structures of these compounds (15) and histamine H1 receptor antagonists such as chlorpheniramine to seek further biological activities of these compounds, in addition to the above mentioned ones.

Materials and Methods

The radioisotope-labeled compound, [1, 2-^{3}H] L-myo-inositol (45.8 Ci/mmole), used in this study was purchased from New England Nuclear Co. (Boston, MA, USA). 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 use in Beckman LS-3801 model for sample counting. Cation Counter (from Spex Model CMIT 111, Edison, NJ, USA) was used in this study.

Cell Culture

The C6 glioma cell line was a gift from Professor T. T. Chou at the Institute of Neuroscience, National Yang-Ming University. These cells were cultured in Ham's F-10 medium (J.R. H. Bio-Science, Shawnee Mission, KS, USA) with 10% fetal bovine serum (Hyclone Co. Logan, UT, USA). Cells in T-75 flasks were kept at 37°C, 5% CO_2 and 95% O_2 in an incubator. The culture medium was changed every other day. This cell line has been used widely for decades since its establishment in 1968 (1). Although it has been cultured extensively *in vitro*, its tumorigenicity remains quite stable (17).

Methods

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 a humidified incubator at 37°C and 5% CO_2 until cell growth reached confluency. Before the experiment, the medium was changed to F-10 culture medium without addition of bovine serum. Labeled materials were added to each flask for the stated period of time, and then the unlabeled portion was washed out by washing with TM buffer (50 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 7.5) three times.

Phosphatidylinositol (PI) Turnover Analysis (2, 10)

Labeled [^{3}H] myo-inositol (1 μCi/ml) was added into each flask for incubation for 24 h, and the culture medium was then removed. The flask was washed three times with TM buffer. 10 mM LiCl was then added to prevent the break down 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 buffer was used to wash twice to end the reaction in the flask. The glass plates were removed for counting and kept in the scintillation counting vial. Subsequently, extraction of phosphor inositol metabolites was carried out. An amount of 2.4 ml chloroform/methanol mixture (1:2) (v/v) was added to the scintillation vials, which contained the C6 glioma cells. At the end of the PI turnover reaction, the vials were vortexed vigorously for 30 sec in order to extract [^{3}H]IP_1 from the cells. Chloroform (0.8 ml) and 0.8 ml of double-distilled water were added and stirred thoroughly for 15 sec. The scintillation vials were kept and shaken in a sonicator for 15 min to remove the cells from the glass plates. After centrifugation at 500 g for 10 min to separate the solution into a top aqueous layer and a bottom chloroform layer, 200 μl was removed from the chloroform layer to scintillation vials in which 3 ml liquid scintillation cocktail was added. After mixing, the vials were counted to determine [^{3}H] inositol incorporation.

Analysis of GPI and PI (10)

The PI was analyzed by anion-exchange chromatography. Half milliliter 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. Ten ml of double-distilled water was added to wash out free PI. Then 6 ml 60 mM sodium formate and 5 ml sodium tetraborate were added to wash 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 solution was added to wash out

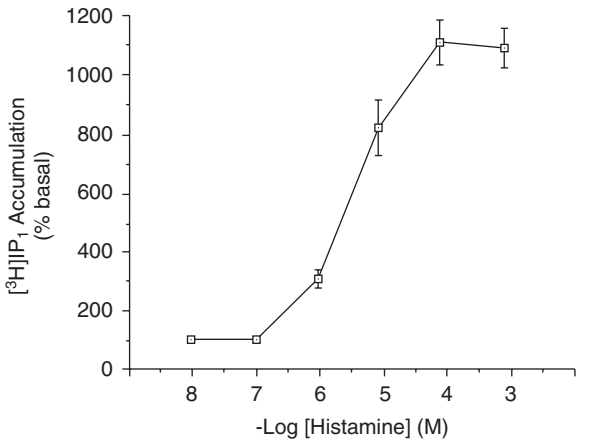

Fig. 1. Concentration-dependence of histamine-stimulated accumulation of [^{3}H]inositol 4-phosphate in C6 glioma cells. [^{3}H]Inositol labeled cells were incubated with 10 mM LiCl, The cultures were then exposed to various concentrations of histamine for 60 min. The basal activity measured in the absence of histamine was 3216.7 ± 52.4 dpm/10^7 cells. The data presented are the means ± S.E.M. from five independent experiments, which were performed in triplicates.

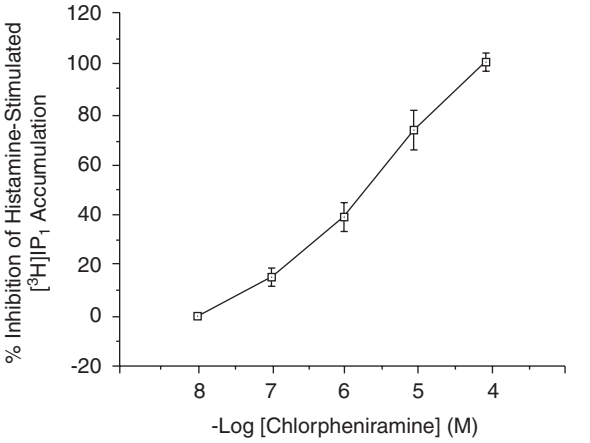

Fig. 2. Inhibitory effect of chlorpheniramine, a histamine H1 antagonist, on histamine-stimulated accumulation of [^{3}H]IP$_1$ accumulation. [^{3}H]Inositol labeled cells were preincubated with various concentrations of chlorpheniramine for 10 min, and then incubated a further 60 min with 100 μM histamine in the presence of 10 mM LiCl. Results are expressed as percentage of the histamine-stimulated response (41919 ± 1612 dpm/10^7 cells) in an un-pretreated culture. The data presented are the means ± S.E.M. from three independent experiments performed in triplicates.

the [^{3}H]IP$_1$ which was collected in scintillation vials, then mixed well with 6 ml of scintillation fluid for scintillation counting.

Statistical Analysis

In this study, 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 at least three times.

Results

Histamine-Induced [^{3}H]IP$_1$ Accumulation

After incubation of varying concentrations of histamine with the C6 glioma cells at 37°C in the presence of 10 mM LiCl, there was a concentration-dependent accumulation of [^{3}H]IP$_1$ formation, which reached a maximal response at 100 μM (Fig. 1). The maximal amount of [^{3}H]IP$_1$ accumulation was about 11-fold over the basal level and had an EC50 of 6 μM. This histamine-induced [^{3}H]IP$_1$ accumulation could be blocked by the histamine H1 antagonist chlorpheniramine. Pre-treatment with varying concentrations of chlorpheniramine (from 10^{-8} to 10^{-4} M) for 10 min resulted in a concentration dependent blocking effect on histamine-induced [^{3}H]IP$_1$ accumulation. The IC50 value of chlorpheniramine was 5 μM. (Fig. 2).

Effects of 2-Azafluorenone and Its Derivatives on Histamine-Induced [^{3}H]IP$_1$ Accumulation

The structure of 2-azafluorenones has some similarities to the antihistaminergic drug chlorpheniramine. Pre-incubation with 100 μM of 2-azafluorenone (F1) or its derivatives, 4-methyl-2-azafluorenone (F2), 6-chloro-2-azafluorenone (F3) and 6-methoxy-2-azafluorenone (F4) for 10 min, could block 100 μM histamine-induced [^{3}H]IP$_1$ accumulation in a 60 min incubation period. This resulted in inhibitory effect in the range of 44 to 55% (Fig. 3).

The compounds 2-azafluorenone and 4-methly 2-azafluorenone had a concentration dependent inhibitory effect on 100 μM histamine-induced [^{3}H]IP$_1$ accumulation. The maximal inhibition of both compounds was found at 1 mM. The IC50 values were 100 μM and 150 μM for 2-azafluorenone (Fig. 4A) and 4-methyl 2-azafluorenone (Fig. 4B), respectively.

The presence of 100 μM 2-azafluorenone caused an inhibitory effect on 100 μM histamine-induced [^{3}H]IP$_1$ accumulation at different incubation time points. At 37°C and in the presence of 1 mM LiCl, histamine induced [^{3}H]IP$_1$ accumulation demonstrated a time dependent increase. Maximal increase was seen at 40 min; a half maximal level was at 17 min. An inhibitory effect of 2-azafluorenone on 100 μM histamine-induced [^{3}H]IP$_1$ accumulation was

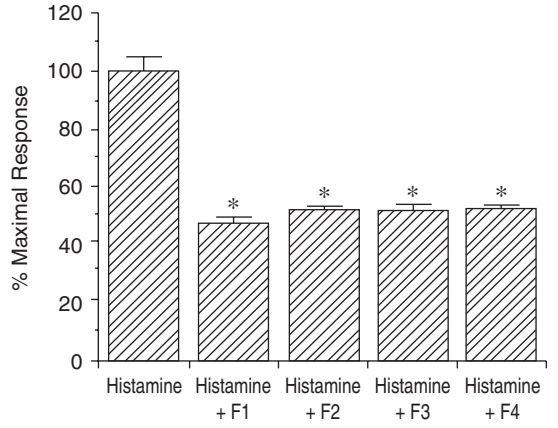

Fig. 3. Inhibitory effect of 2-azafluorenone on histamine-stimulated [^{3}H]IP$_1$ accumulation. [^{3}H] Inositol-labeled cells were preincubated with 100 µM of various compounds from F1 to F4 for 10 min, and then incubated for a further 60 min with 100 µM histamine in the presence of 10 mM LiCl. Results are expressed as percentage of the histamine-stimulated response (41215.7 ± 1321 dpm/10^7 cells) in un-pretreated cultures. Results are means ± S.E.M. from three independent experiments. F1, 2-azafluorenone; F2, 4-methyl 2-azafuorenone; F3, 6-chloro 2-azafluorenone and F4, 6-methoxy 2-azafluorenone. *Significant effect of 2-azafluorenone treatment on histamine response when compared to the value of histamine only (based on Student's *t*-test; $P < 0.05$).

observed. Inhibition ranging from 40 to 55% was seen at 20 to 60 min incubation period (Fig. 5).

Effects of 2-Azafluorenone and 4-Methyl 2-Azaflurenone on ATP- or Bradykinin-Induced IP$_1$ Accumulation

2-Azafluorenone or 4-methyl 2-azaflurenone was found to inhibit ATP- or bradykinin-induced IP$_1$ accumulation. Pre-incubation with 100 µM of 2-azafluorenone or 4-methyl 2-azafluorenone, followed by a further 60 min incubation period with 1 mM ATP or 10 mM bradykinin (BK) in the presence of 10 mM LiCl resulted in an inhibitory effect in the range of 45 to 55% (Fig. 6).

Effects of 2-Azafluorenone and 4-Methyl 2-Azafluorenone on AlF_4^--Induced [^{3}H]IP$_1$ Accumulation

Pre-incubation with 100 mM of 2-azafluorenone or 4-methyl 2-azafluorenone for 10 min, and followed by incubation for a further 60 min with 100 mM AlF_4^- in the presence of 10 mM LiCl, resulted in a 45 to 55% inhibition of [^{3}H]IP$_1$ accumulation (Fig. 7A).

Effects of 2-Azafluorenone and 4-Methyl 2-Azafluorenone on A23187-Induced [^{3}H]IP$_1$ Accumulation

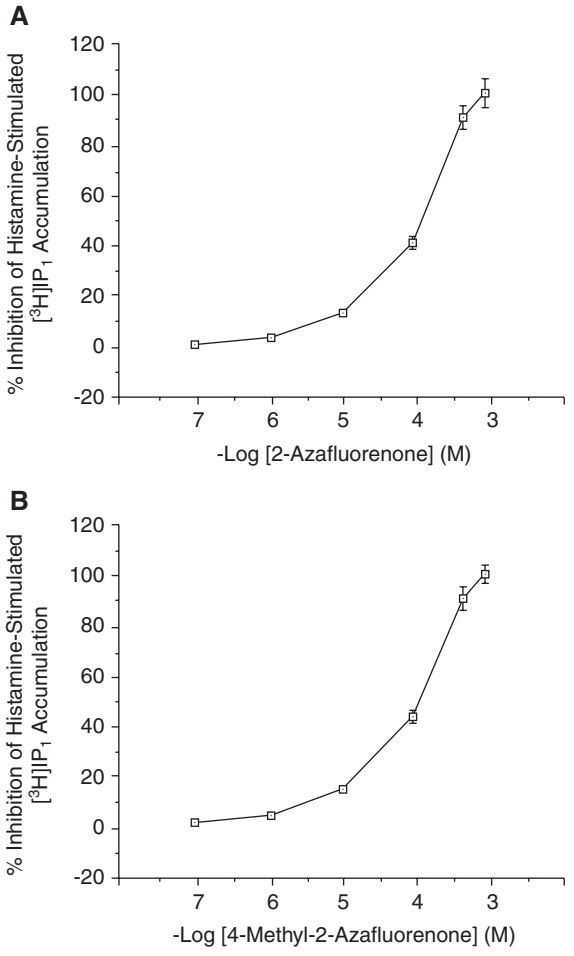

Fig. 4. Inhibitory effect of 2-azafluorenone on histamine-stimulated accumulation of [^{3}H]IP$_1$: effects of 2-azafluorenone (A) and 4-methyl 2-azafluorenone (B). [^{3}H] Inositol-labeled cells were preincubated with 100 µM of various concentrations of 2-azafluorenone for 10 min, and then incubated a further 60 min with 100 µM histamine in the presence of 10 mM LiCl. Results are expressed as a percentage of the histamine-stimulated response (41719 ± 1612 dpm/10^7 cells) in an un-pretreated culture. The data presented are the means ± S.E.M. from three independent experiments performed in triplicates.

Pre-incubation with 100 µM 2-azafluorenone (F1) or 4-methyl 2-azafluorenone (F2) for 10 min, and followed by addition of 100 µM of A23187 and incubation for an additional 60 min, F1 and F2 caused an inhibitory effect on A23187-induced [^{3}H]IP$_1$ accumulation by 43% and 50%, respectively (Fig. 7B).

Effects of TMB-8, 2-Azafluorenone, 4-Methyl 2-Azafluorenone, with or without Calcium Ion in the Incubation Buffer on Histamine-Induced [^{3}H]IP$_1$ Accumulation

In the incubation medium without calcium, 100

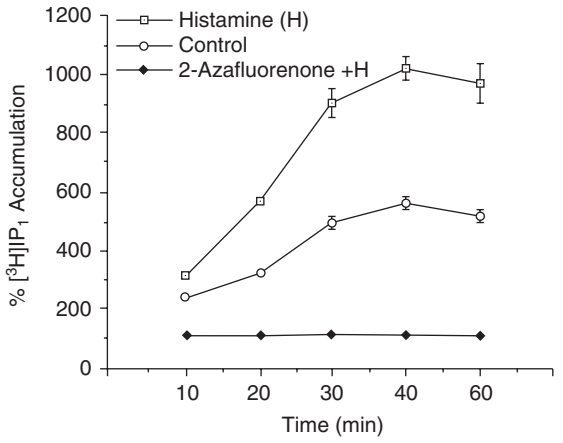

Fig. 5. Time course of histamine-stimulated $[^3H]IP_1$ acuumulation in C6 glioma cells in the presence of 2-azafluorenone. $[^3H]$ Inositol-labeled cells were incubated for various periods with a vehicle or 100 μM histamine in the presence or absence of 100 μM 2-azafluorenone in a medium containing 10 mM LiCl. The data are expressed as a percentage of $[^3H]IP_1$ accumulation at zero time (expressed as 100%). Basel level of the assay was 3199.6 ± 41.7 dpm/10^7 cells in the absence of histamine. The data presented are the means ± S.E.M. from three independent experiments, which were performed in triplicates.

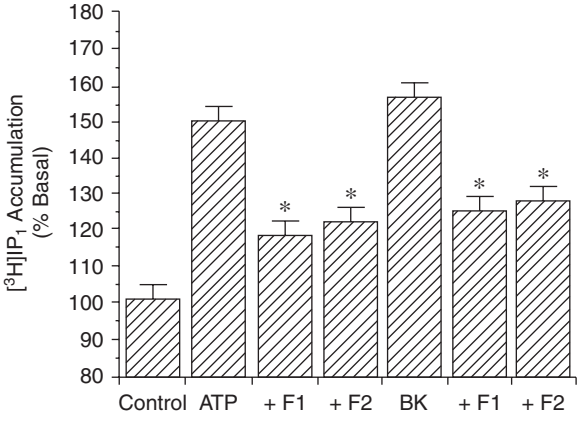

Fig. 6. Effect of 2-azafluorenone (F1) and 4-methyl 2-azafuorenone (F2) on ATP or bradykinin (BK)-stimulated $[^3H]IP_1$ accumulation. $[^3H]$ Inositol-labeled cells were preincubated with 100 μM of 2-azafluorenone (F1) or 4-methyl 2-azafluorenone (F2) for 10 min, and then incubated a further 60 min. with 1 mM ATP and 10 μM bradykinin in the presence of 10 mM LiCl. Results are expressed as percentage of basal response (1334.7 ± 122 dpm/10^7 cells) in the absence of ATP and bradykinin (BK). The data presented are the means ± S.E.M. from three independent experiments, which were performed in triplicate. *Significant effect of 2-azafluorenone (F1) and 4-methyl 2-azaflorenone (F2) treatment on ATP or bradykinin response when these values are compared to the corresponding controls (based on Student's *t*-test; $P < 0.05$).

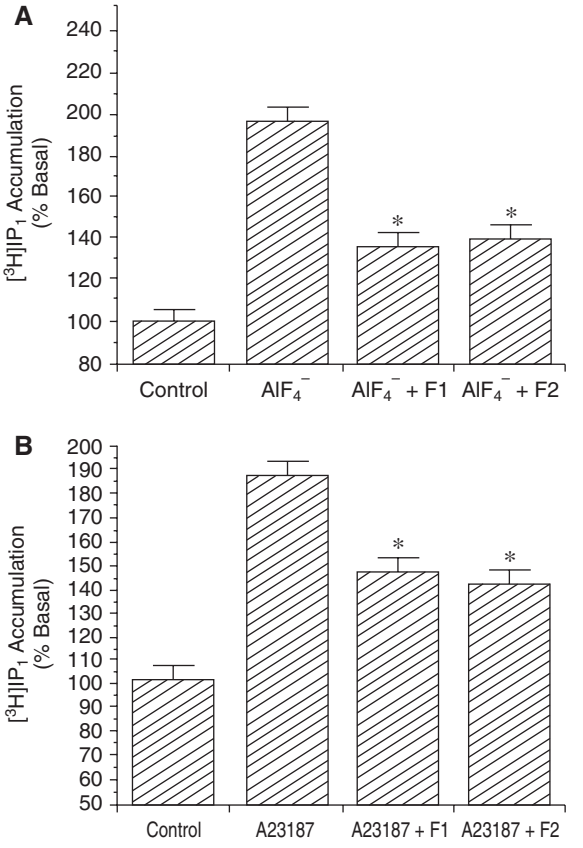

Fig. 7. Effect of 2-azafluorenone (F1) and 4-methyl 2-azafluorenone (F2) on AlF_4^--stimulated $[^3H]IP_1$ accumulation (A), and on A23187-stimulated $[^3H]IP_1$ accumulation (B). $[^3H]$ Inositol-labeled cells were preincubated with 100 μM of 2-azafluorenone (F1) or 4-methyl 2-azafluorenone (F2) for 10 min, and then incubated a further 60 min. with 10 μM AlF_4^- or 1 μM A23187 in the presence of 10 mM LiCl. Results are expressed as a percentage of basal response (1495 ± 102 dpm/10^7 cells) in the absence of AlF_4^-. The data presented are the means ± S.E.M. from three independent experiments, which were performed in triplicates. *Significant effect of 2-azafluorenone (F1) and 4-methyl 2-azafluorenone (F2) treatment on AlF_4^- response, when compared to value of AlF_4^- or A23187 only (based on Student's *t*-test; $P < 0.05$).

mM histamine-induced IP_1 formation was only about 45 to 50% of that in medium with calcium. Preincubation with 100 μM of 2-azafluorenone (F1) or 4-methly 2-azafuorenone (F2) for 10 min, followed by addition of 100 μM histamine to induce $[^3H]IP_1$ formation from $[^3H]$ inositol in the presence of 100 μM LiCl for 60 min with or without calcium in the incubation buffer, resulted in a significant inhibitory effect on $[^3H]IP_1$ accumulation. In the medium without calcium, addition of 50 μM TMB-8 (an intracellular calcium antagonist) caused an 80% inhibition of histamine-induced $[^3H]IP_1$ accumulation with respect

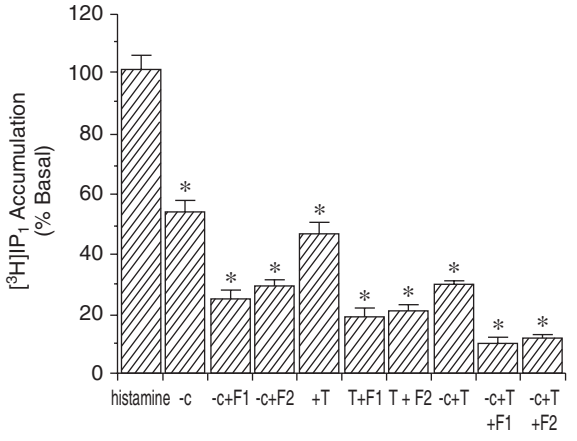

Fig. 8. Effects of 2-azafluorenone (F1), 4-methyl 2-azafluorenone (F2) and TMB-8 (T) on histamine-stimulated [^{3}H]IP$_1$ accumulation in a buffer with or without (-c) Ca^{2+}. [^{3}H]Inositol-labeled cells were preincubated with 100 μM of 2-azafluorenone (F1), 4-methyl 2-azafluorenone (F2) or 50 μM TMB-8 for 10 min and then incubated for a further 60 min with 100 μM histamine in the presence of 10 mM LiCl in a buffer with or without 2.54 mM Ca^{2+}. Results are expressed as a percentage of histamine response (42272 ± 1262 dpm/10^7 cells). The data presented are the means ± S.E.M. from three independent experiments, which were performed in triplicates. *Significant effects of 2-azafluorenone (F1), 4-methyl 2-azafluorenone (F2), TMB-8 (T) or Ca^{2+} without (-c) in a buffer treatment on histamine response when these values are compared to the corresponding controls (based on Student's *t*-test; $P < 0.05$).

to medium without TMB-8. Further addition of 100 μM F1 or F2 in the presence of TMB-8 could cause an additional 12% inhibition of histamine induced [^{3}H]IP$_1$ accumulation (Fig. 8).

Discussion

Some receptor activation can cause membrane phosphatidyl-inositol break-down to generate metabolites of diacylglycerol (DG) and inositol 1, 4, 5-triphosphate (IP3) *via* a link to the pathway of enzyme PI-PLC. Further action of these products may result in an increase in $[Ca^{2+}]_i$ release or protein kinase C activation (2). The sequences of this chain reaction play a very important role for cellular functions, such as cell proliferation, secretion, apoptosis and muscle contraction. It has been reported that PI-PLC is composed of five isomers: α, β, γ, δ, and ε, that are expressed in different tissues. In rat brains, δ and ε subtypes are the main isomers of PI-PLC (13). Under physiological conditions, calcium ion, AA, DG and glutathione are the main activators; whereas protein kinase C, Mg^{2+}, nicotinamide adenine dinucleotide (NAD) and zinc are the main inhibitors. They play an important role in normal physiological functions (3).

Histamine-induced [^{3}H]IP$_1$ accumulation is a concentration and time dependent phenomenon, and this reaction can be blocked by histamine H1 receptor antagonist of chlorpheniramine with an IC50 of 3.5×10^{-6} M. However, [^{3}H]IP$_1$ accumulation and this biological response can be blocked by 2-azafuorenone and 4-methyl 2-azafluoreone with IC50 of 5.3×10^{-5} M and 7.2×10^{-5} M, respectively. In order to investigate the mode of action of these compounds, further studies were carried out.

Other cellular substances, such as ATP or bradykinin were used to activate PI-PLC and this resulted in an increase of [^{3}H]IP$_1$ accumulation. This increase in [^{3}H]IP$_1$ accumulation could be blocked by 2-azafluorenone (F1) and 4-methyl 2-azafluorenone (F2). Also, F1 and F2 could block AlF_4^--induced [^{3}H]IP$_1$ accumulation. Based on the above observation, the F1 and F2 effects on [^{3}H]IP$_1$ production were beyond the receptor level, but on the G-protein and at the PI-PLC enzyme level.

Calcium ionophore A23187 can induce an influx of Ca^{2+} into cells to cause the activation of PI-PLC and result in an increase of [^{3}H]IP$_1$ accumulation. But this effect does not occur *via* the activation of G-protein, since A23187-induced [^{3}H]IP$_1$ accumulation could be blocked by 100 μM 2-azafluourenone and 4-methyl 2-azafluorenone. The results suggest that the inhibition of [^{3}H]IP$_1$ accumulation induced by these compounds was not on the G-protein site, but on the PI-PLC molecule itself.

Intracellular calcium ion concentration is regulated by the calcium channel gates. Calcium channels can be divided into at least 3 types: (a) voltage-sensitive calcium channels with 4 subtypes: T, N, L and P; (b) receptor-operated calcium ion channels *via* the activation of different receptor subtypes; (c) second messenger operated calcium channels to open intracellular calcium storage sites (8, 9).

However, increased by calcium-free medium, histamine-induced [^{3}H]IP$_1$ accumulation was 52% increased compared to that observed under calcium-containing conditions. But in the addition of 100 μM 2-azafluorenone, it could further decrease [^{3}H]IP$_1$ accumulation even under calcium-free conditions. The results indicate that the effects of these compounds on [^{3}H]IP$_1$ accumulation do not require the presence of calcium ion. Besides, under calcium-free conditions and in the presence of TMB-8, addition of 2-azafluorenone (100 μM) could further decrease [^{3}H]IP$_1$ accumulation. This result suggests that the effect of 2-azafluorenone was not at the level of calcium channel gates. Here, TMB-8 acted as an intracellular calcium antagonist (4, 7).

The action of signal transduction pathways is

dependent on various components including lipid signaling molecules, such as phosphoinositides and related enzymes. Evidence supports the hypothesis that inositol lipid cycle is involved in astrocytes activation during neuro-degeneration and in tumor progression. Recently, Lo Vasco *et al*. (6) reported that stimulation with lipopolysaccharide could cause an activation of expression of PI-PLC isoenzymes in cultured astrocytes. In this study the functional data do not distinguish among the PI-PLC isoforms expressed in the C6 glioma cells; however, enzyme inhibitors such as 2-azafluorenones, that act on PI-PLC component are worthy of further investigation for drug development.

In summary, 2-azafluorenones inhibit [^{3}H]IP_1 accumulation through activation by histamine, AlF_4^-, ATP, bradykinin and A23187 (a calcium ionophore). From the observation on the mode of action, the blocking effects of these compounds are not on receptor, G-proteins or calcium channel molecules, but at the enzyme level of PI-PLC. Inhibition of PI-PLC activation results in a decrease of [^{3}H]IP_1 accumulation. Further studies may reveal that this effect is specific on PI-PLC but not on other enzymes that have been taken up.

Acknowledgments

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References

1. Benda, P., Lightbody, J., Sato, G., Levine, L. and Sweet, W. Differentiated rat glial cell strain in tissue culture. *Science* 161: 370-371, 1968.
2. Berridge, M.J., Downes, R.M.C., Downes, C.P., Heslop, J.P. and Irvine, R.F. Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J*. 212: 473-482, 1983.
3. Crooke, S.T. and Bennett, C.F. Mammalian phosphoinositide specific phospholipase C isoenzymes. *Cell Calcium* 10: 309-323, 1989.
4. Himmel, H.M. and Ravens, U. TMB-8 as a pharmacologic tool in guinea pig myocardial tissues: effects of TMB-8 on force of contraction and on action potential parameters in atrial and papillary muscles. *J. Pharmacol. Exp. Ther.* 255: 293-299, 1990.
5. Hundsdorf, H., Blyumin, E.V. and Neunhoeffer, H. A new and general synthesis of substituted 2-azafluorenones. *Synthesis* 2002: 2532-2536, 2002.
6. Lo Vasco, V.R., Fabrozo, C., Fumagalli, L. and Cocco, L. Expression of phosphoinositide-specific phospholipase C isoenzymes in cultured astrocytes activated after stimulation with lipopolysaccharide. *J. Cell. Biochem*. 109: 1006-1012, 2010.
7. Magagoli, M.H. and Chiou, C.Y. Pharmacological evaluation of a new Ca^{2+} antagonist, 8-(N, N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8): studies in smooth muscle. *Eur. J. Pharmacol*. 27: 25-33, 1974.
8. Meldolesi, J. and Possan, T. Pathways of Ca^{2+} influx at the plasma membrane: voltage-, receptor- and second messenger-operated channels. *Exp. Cell Res*. 171: 217-283, 1987.
9. Nowycky, M.C., Fox, A.P. and Tsien, R.W. Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature* 316: 440-443, 1985.
10. Pearce, B., Cambray-Deakin, M., Morrow, C., Grimble, J. and Murphy, S. Activation of muscarinic and α1-adrenergic receptors on astrocytes results in the accumulation of inositol phosphates. *J. Neurochem*. 45: 1534-1540, 1985.
11. Prachayasittikul, S., Buttrawong, K., Worachartcheewan, A., Ruchirawat, S. and Prachayasittikul, V. Isolation of bioactive metabolites from root extracts of *Polyalthia Debilis*. *Eur. J. Sci. Res*. 38: 596-603, 2009.
12. Prachayasittikul, S., Manam, P., Chinworrungsee, M., Isarankura-Na-Ayudhya, C., Ruchirawat, S. and Prachayasittikul, V. Bioactive azafluorenone alkaloids from *Polyathia debilis* (Pierre) Finet & Gagnep. *Molecules* 14: 4414-4424, 2009.
13. Rhee, G.R., Shu, P.G., Ryu, S.H. and Lee, S.Y. Studies of inositol phospholipid-specific phospholipase C. *Science* 244: 546-550, 1989.
14. Shiao, M.J., Perng, C.Y. and Shen, C.C. Studies of intramolecular cyclization of 4-arylpyridines. II. Syntheses of condensed tricyclic pyridines. *J. Chinese Chem. Soc*. 38: 47-50, 1991.
15. Shiao, M.J., Peng, C.J., Wang, J.S. and Ma, Y.T. Synthesis of several 2-azafluorenones and 5H[1] benzopyrano [3,4-c] pyridine-5-ones. *J. Chinese Chem. Soc*. 39: 173-176, 1992.
16. Xie, N. and Yang, N.Y. A new azafluorenone alkaloid from *Alphonsea Monogyma*. *Chinese Chem. Lett.* 10: 671-672, 1999.
17. Zheng, X., Shen, G., Yang, X. and Liu, W. Most C6 cells are cancer stem cells: Evidence from clonal and population analyses. *Cancer Res*. 67: 3691-3697, 2007.