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Mechanism of inhibition of IgE-dependent histamine release from rat mast cells by Xestobergsterol A from the Okinawan marine sponge *Xestospongia bergquistia*

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Abstract. Histamine release from rat peritoneal mast cells induced by anti-IgE was essentially complete within 4–5 min. Xestobergsterol A and B, which are constituents of the Okinawan marine sponge *Xestospongia bergquistia* Fromont, dose-dependently inhibited anti-IgE-induced histamine release from rat mast cells. The IC_{50} values of xestobergsterol A and B for histamine release in mast cells activated by anti-IgE were 0.07 and 0.11 μM, respectively. Anti-IgE stimulated PI-PLC activity in a mast cell membrane preparation. Xestobergsterol A dose-dependently inhibited the generation of IP3 and membrane-bound PI-PLC activity. Moreover, xestobergsterol A inhibited Ca^{2+}-mobilization from intracellular Ca^{2+}-stores as well as histamine release in mast cells activated by anti-IgE. On the other hand, xestobergsterol B did not inhibit the membrane-bound and cytosolic PI-PLC activity, IP3 generation or the initial rise in $[Ca^{2+}]_i$ in mast cells activated by anti-IgE. These results suggest that the mechanism of inhibition by xestobergsterol A of the initial rise in $[Ca^{2+}]_i$, of the generation of IP3, and of histamine release induced by anti-IgE, was through the inhibition of PI-PLC activity.
Key words. Xestobergsterol A; histamine release; PI-PLC; IP3; signal transduction.

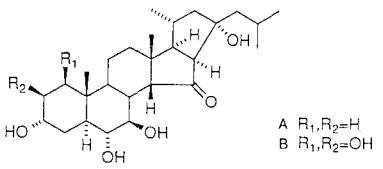
Mast cells release a variety of inflammatory mediators through the reaction of cell-bound IgE antibodies with multivalent antigen[1]. The biochemical events involved in the triggering of histamine release suggest that the activation of various membrane-associated enzymes is involved in the transduction of triggering signals for mediator release[2–5]. Since mobilization of intracellular calcium[6,7] and enhancement of the hydrolysis of phosphoinositides[8] are early events in IgE-dependent mediator release, an important role of phospholipase C (PLC) in mediator release is implied. Recently, it was reported that binding of epidermal growth factor and platelet-derived growth factor to their receptors resulted in the activation of protein tyrosine kinase (PTK) activity of the receptors, which in turn activated PLC through tyrosine phosphorylation[9]. Moreover, Benhanmon et al. showed that $FC_\varepsilon R1$ signalling in RBL-2H3 cells was accompanied by protein tyrosine phosphorylation[10].

Marine organisms have been the source of a lot of steroids, with many different pharmacologically active groups. Recently, we reported that xestobergsterol A and B, two unique pentacyclic steroids from the Okinawan marine sponge *Xestospongia bergquistia* Fromont, strongly inhibited the histamine release from rat mast cells induced by anti-IgE[11]. However, the mechanism of this effect is still unknown. This study was undertaken to investigate the mechanism of action of xestobergsterol A on IgE-dependent histamine release from rat isolated peritoneal mast cells and, in particular, to examine the role of PLC activity in the secretory process.

Material and methods

Rat peritoneal mast cells were obtained from male Wistar rats (200–300 g), and mast cells purified using the method of Németh and Rőhlich[12,13]. Viability of the cells was >98% as assessed by trypan blue exclusion. Passively sensitized rat mast cells were prepared as described previously[14].

Assay of histamine. Purified mast cells from normal rats were incubated for passive sensitization with sensitized rat sera for 1 h at 4 °C[14]. The mast cell suspensions in Tyrode-Hepes solution (pH 7.4) were incubated in duplicate at 37 °C with or without xestobergsterol A and B for 5 min before the addition of an appropriate concentration of anti-IgE. Tyrode-Hepes solution contained 124 mM NaCl, 4.0 mM KCl, 1.0 mM $CaCl_2$, 10 mM $NaHCO_3$, 5.6 mM glucose, 0.64 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 5 mM 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (Hepes), 50 mg/ml of phosphatidylserine and 50 mg of BSA/l. BSA was omitted from the solution in experiments on histamine release. At intervals, aliquots of the solution were withdrawn and the reaction was stopped by the addition of 1 ml of Tris-EDTA buffer. The Tris-EDTA buffer contained 25 mM Tris, 120 mM NaCl, 5 mM KCl, 1 mM EDTA and 200 mg human serum albumin per liter. Then, the cells were separated from the buffer containing released histamine by centrifugation at 130 × g for 10 min at 4 °C. Residual histamine remaining in the cells was released by disrupting the cells with 100% trichloric acid (TCA) (final concentration 10% TCA), and centrifugation at 1500 × g for 15 min at 4 °C. Histamine content



Structure of Xestobergsterol A and B

was determined fluorometrically[15]. The amount of histamine released was calculated as a percentage of the total histamine present in the control suspension.

Determination of IP3. Measurement of IP3 was carried out using a commercially available kit (Amersham) and the manufacturer's protocol. Briefly, purified mast cells were extracted with chloroform/methanol (1/2) on ice for 10 min. Methanol fractions containing phosphorylated inositols were lyophilized and mixed with bovine adrenal IP3-binding proteins in the presence of a limiting amount of tracer D-myo-[^{3}H]inositol 1,4,5-triphosphate. The mixtures were centrifuged at 2000 × g for 10 min and radioactivity bound to IP3-binding protein was measured in a β-scintillation counter.

Analysis of phospholipase C activity. Phosphoinositide-specific phospholipase C activity in vitro was measured as previously described[16]. 1×10^7 cells were disrupted by sonication in 1 ml lysis buffer (25 mM Tris, pH 7.5/ 25 mM sucrose/1 mM EGTA/5 mM $MgCl_2$). After centrifugation at 10,000 × g for 30 min, the supernatant (=cytosol) was removed and the pellet was used as membrane fraction after sonication in 0.2 ml lysis buffer. Quantities of 1–20 µg membrane or 20 µl cytosol were incubated with 0.2 mM [^{3}H] phosphatidylinositol-4,5-bisphosphate (5 µCi/mol; Amersham; Arlington Heights, IL) in 20 mM sodium phosphate (pH 6.8), 40 mM KCl, 1 mM sodium pyrophosphate, 0.65% octylglucoside, 0.4 mM EGTA, 0.8 mM $CaCl_2$, aprotinin (10 µg/ml) and leupeptin (10 µg/ml). The mixture was incubated for 15 min at 37 °C and the reaction stopped by adding 100 µl of 1% bovine serum albumin, followed by 500 µl of 10% TCA. The precipitate was removed by centrifugation and the radioactivity present in 0.5 ml of the supernatant was determined. The counts from a control incubation with no enzyme were subtracted from all values, and the quantity of IP3 present in the supernatant was calculated from the net increase in radioactivity in the supernatant. Under the conditions of the assay, the reaction rate was linear with incubation time and amount of protein added. Sensitized mast cells were preincubated with xestobergsterol A and B for 5 min and then challenged with anti-IgE (200 µg/ml) in the presence of xestobergsterol A and B. The reaction was stopped by the addition of 1% BSA and 10% TCA after appropriate intervals.

Measurement of intracellular calcium concentration. The determination of intracellular calcium concentration in mast cells was carried out as described by Magistris et al.[17]. Fluorescence was recorded using a fluorimeter (SLM 8000) with a temperature-controlled cuvette and magnetically driven stirrer. Purified mast cells (2×10^6 cells/ml) were incubated at 37 °C for 45 min with 3 µg/ml indo-1 acetoxymethyl ester (indo-1) in Tyrode-Hepes solution. The cell suspension washed one time with buffer A (HBSS, 0.1% BSA and 1 mM NaPO). Samples of the cell suspension (2×10^6 cells/ml) were placed in the cuvette described above. Then, all reagents were added with a microsyringe directly into the cuvette, without interrupting the recording. The cell suspension was incubated at 37 °C for 5 min with xestobergsterol A and B, and challenged with anti-IgE (200 µg/ml). Fluorescence excitation and emission wavelengths were 350 and 400 nm, respectively.

L-α-phosphatidylserine and indo-1 AM were purchased from Sigma Chemical Company, St. Louis, Mo., USA and indo-1 was obtained from Wako Pure Chemical Industries, Osaka, Japan. *Bordetella pertussis* vaccine was purchased from the Chiba Serum Institute, Chiba, Japan.

Results

Anti-IgE at its optimal concentration of 200 µg/ml induced a time-dependent release of histamine from rat mast cells. The histamine release was essentially complete with 4–5 min, and 58.5 ± 2.3% of total histamine was released after 5 min. When added to the reaction medium, xestobergsterol A strongly and dose-dependently inhibited histamine release from rat mast cells induced by anti-IgE. At 0.001, 0.01 and 0.1 µM, the inhibition by xestobergsterol A of histamine release

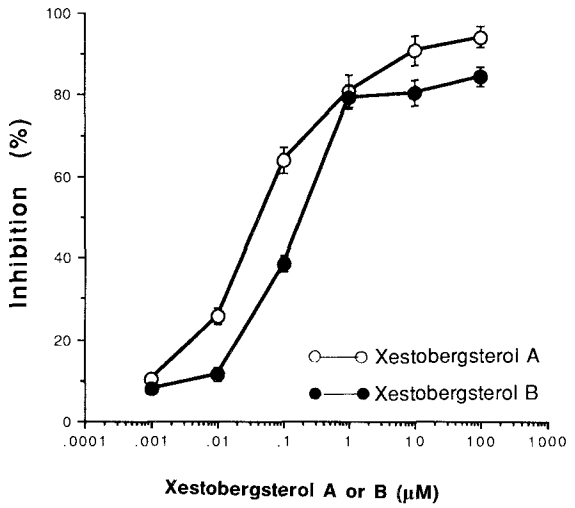


Figure 1. Concentration response curve for the inhibition of anti-IgE-induced histamine release by xestobergsterol A and B. Spontaneous histamine release from mast cells was 5.2 ± 0.23%, and this value was subtracted from each experimental value. Each point represents the mean of 5 experiments and vertical bars indicate SEM.

from rat mast cells at 5 min after challenge with anti-IgE was 11.2 ± 1.2, 26.2 ± 1.5 and 65.0 ± 2.3%, respectively (fig. 1). At 100 μM, 95.0 ± 2.5% inhibition was achieved. Xestobergsterol B also strongly and dose-dependently inhibited histamine release from rat mast cells induced by anti-IgE. At 100 μM, the inhibition by xestobergsterol B of histamine release from rat mast cells at 5 min after challenge with anti-IgE was 85.0 ± 2.8% (fig. 1). The IC_{50} values of xestobergsterol A and B on histamine release in rat mast cells activated by anti-IgE were 0.07 and 0.11 μM, respectively.

Because IgE-mediated mast cell activation is associated with PI-PLC-mediated production of the second messengers, inositol 1,4,5-triphosphate(IP3) and 1,2-diacylglycerol(1,2-DAG), we examined the effect of xestobergsterol A and B on this signalling pathway. Passively sensitized rat mast cells were challenged with anti-IgE (200 μg/ml), and kinetics of IP3 generation was analyzed (fig. 2). Anti-IgE induced a rapid increase in IP3 production in mast cells, with its production in 15 s being followed by a decrease with 1 min. Xestobergsterol A strongly and dose-dependently inhibited the generation of IP3 (fig. 3). At 0.01, 0.1 and 1 μM, the inhibition by xestobergsterol A of generation of IP3 at 15 s after challenge with anti-IgE was 23.8 ± 1.8, 65.0 ± 2.3 and 75.2 ± 2.8%, respectively.

In order to address the problem that xestobergsterol A could result from either direct or indirect inhibition of PI-PLC activity, passively sensitized mast cells were preincubated for 5 min with various concentration of xestobergsterol A, and PI-PLC activity of cell lysates was determined by using phosphatidylinositol 4,5-bisphosphate(PIP2) as a substrate. Treatment of rat mast

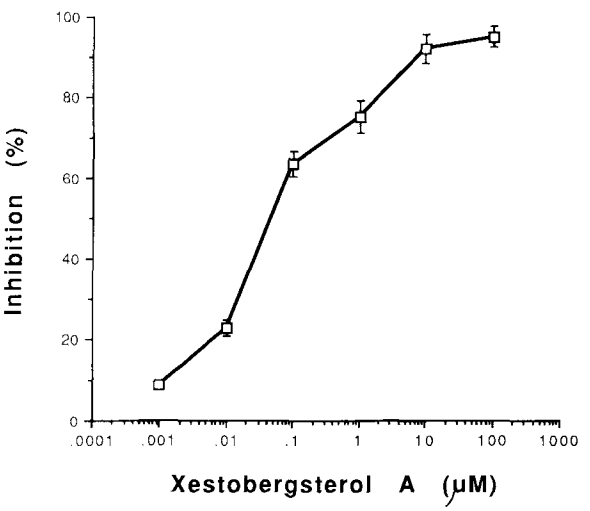


Figure 3. Concentration response curve for the inhibition of anti-IgE-induced generation of IP3 by xestobergsterol A. The cell was challenged with 200 μg/ml of anti-IgE and incubated for 15 s. The generation of IP3 in control mast cells was 0.3 pmol/10^7 cells, and was subtracted from experimental values. Each point represents the mean of 5 experiments and vertical bars indicate SEM.

cells with xestobergsterol A affected the ability of the membrane fraction to hydrolyze the substrate to form IP3. As shown in figure 4, the formation of [^{3}H] inositol 1,4,5-triphosphate from labelled phosphatidylinositol 4,5-bisphosphate was inhibited at a xestobergsterol A concentration of 10 and 100 μM. This result was apparent with both membrane-bound and cytosolic PI-PLC activity. Thus the inhibitory effect of xestobergsterol A on inositol phospholipid turnover in IgE-stimulated mast cells is due to direct inhibition of PI-PLC activity. Purified passively sensitized mast cells were challenged

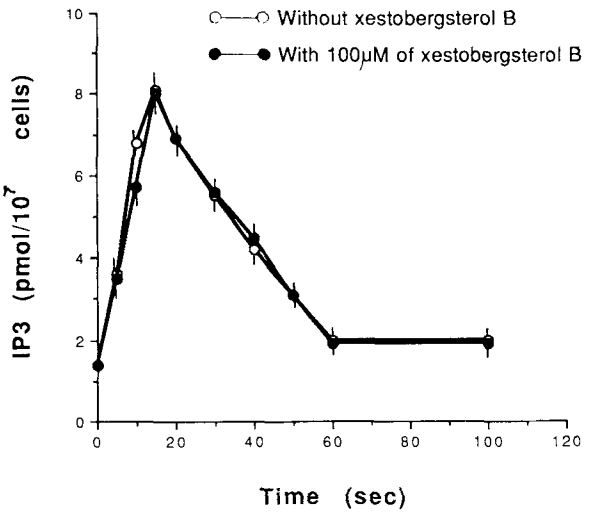


Figure 2. Kinetics of anti-IgE-induced generation of IP3 and effect of xestobergsterol B on the generation of IP3 induced by anti-IgE (200 μg/ml). The generation of IP3 in control mast cells was 0.3 pmol/10^7 cells and was subtracted from experimental values. Each point represents the mean of 5 experiments and vertical bars indicate SEM.

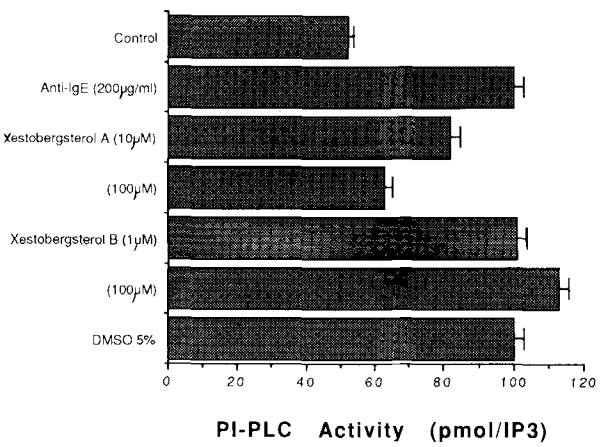


Figure 4. Effect of xestobergsterol A and B on the enzymatic activity of PI-PLC. Passively sensitized mast cells (1×10^7 cells/ml) were treated for 5 min with xestobergsterol A and B, and 5% DMSO. The cells were disrupted by sonication and pellet and cytosol fractions were assessed for PLC activity by the procedures described in 'Materials and methods'. Abscissa represents pmoles IP3 formed by hydrolysis of ^{3}H-phosphatidylinositol 4,5-bisphosphate. Each point represents the mean of 5 experiments and vertical bars indicate SEM.

with 200 µg/ml of anti-IgE. Anti-IgE induced a rapid initial rise in $[Ca^{2+}]_i$ within several seconds which was followed by a further slower increase of $[Ca^{2+}]_i$ (second rise) reaching a maximum at 1 min after challenge with anti-IgE. The increase occurred in two steps; a rapid initial rise caused by Ca^{2+}-mobilization from the intracellular Ca^{2+}-store, and a second sustained rise caused by an influx of extracellular calcium. The effect of xestobergsterol A on the initial rise in $[Ca^{2+}]_i$ in the mast cells activated by anti-IgE was examined. Xestobergsterol A inhibited the initial rise and it completely prevented the second rise. In the presence of 0.01, 0.1 and 1 µM of xestobergsterol A, the inhibitory effect was 11.8 ± 1.5, 30.5 ± 2.5 and 59.5 ± 3.0%, respectively (fig. 5). At 100 µM 82 ± 2.8% of inhibition was achieved (fig. 6).

In contrast, xestobergsterol B (1–100 µM) failed to affect membrane-bound and cytosolic PI-PLC activity, IP3 generation and the initial rise in $[Ca^{2+}]_i$ in rat mast cells activated by anti-IgE (figs 2, 6).

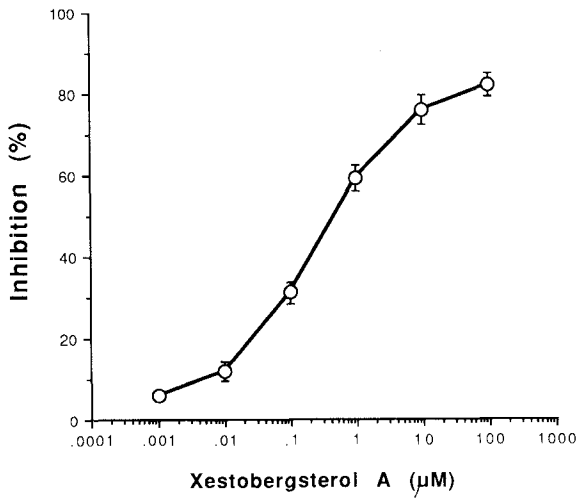


Figure 5. Concentration response curve for the inhibition of xestobergsterol A of the increase in $[Ca^{2+}]_i$ in passively sensitized mast cells induced by anti-IgE. Cells were preincubated for 5 min in the presence of xestobergsterol A and then challenged with 200 µg/ml of anti-IgE. Each point represents the mean of 5 experiments and vertical bars indicate SEM.

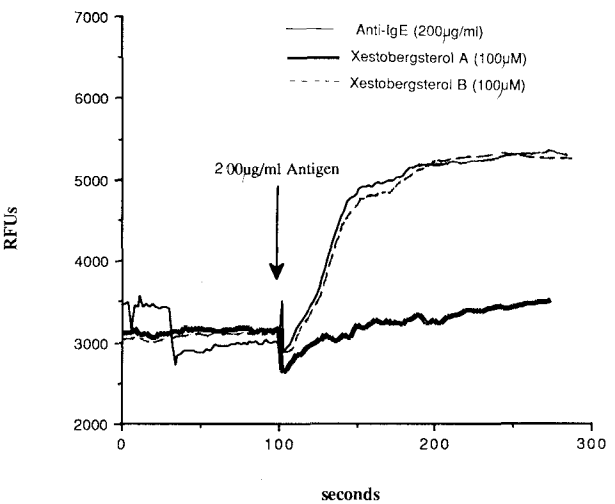


Figure 6. Effect of xestobergsterol A and B on intracellular Ca^{2+} of passively sensitized mast cells induced by anti-IgE. After the cells (2×10^6 cells/ml) were preincubated for 5 min at 37 °C with or without xestobergsterol A and B and then were challenged with 200 µg/ml of anti-IgE. Changes in indo-1 fluorescence (400 nm) were monitored over time.

Discussion

These experiments were undertaken to clarify the effect of xestobergsterol A on anti-IgE-induced histamine release from rat peritoneal mast cells. Xestobergsterol A and B are extracts from the Okinawan marine sponge *Xestospongia bergquistia* Fromont and are two unique pentacyclic steroids. Xestobergsterol A and B strongly and dose-dependently inhibited the histamine release from rat mast cells induced by anti-IgE.

Cross-linking of cell-bound IgE antibodies on mast cells by antigen induces activation of various membrane-associated enzymes, which leads to the release of vasoactive amine[18,19]. The cellular events that take place following stimulation of mast cells include breakdown of inositolphospholipids, transient increase of the intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$, and increase in cyclic nucleotides. Furthermore, the possible involvement of other biochemical processes, such as phosphatidylinositol (PI) turnover, protein kinase C (PKC) and GTP-binding proteins in mediator release have been explored[19].

Agonists stimulate a rapid PLC-mediated hydrolysis of inositol 1,4-bisphospate (IP2) with generation of two second messengers, 1,2-DAG and IP3. The former has been shown to activate PKC by increasing its affinity for Ca^{2+}, whereas the latter has been shown to mobilize intracellular calcium stores in a number of tissues[20]. IP3 regulates the release of Ca^{2+} from endoplasmic reticulum, an event which initiates the cellular activation process[21].

Aggregation of the high affinity receptor for IgE ($FC_\varepsilon R1$) on the surface of mast cells results in the rapid hydrolysis of membrane inositol phospholipids by PLC. We previously showed that 2-nitro-4-carboxyphenyl-N,N-diphenylcarbanate (NCDC), a PLC inhibitor, inhibits histamine release, the initial rise in $[Ca^{2+}]_i$ and the generation of IP3 in mast cells activated by anti-IgE and that these inhibitory effects showed a close quantitative correlation. It is suggested that the rapid generation of IP3 may initiate the release of stored Ca^{2+} that leads to IgE-dependent histamine release. Rapid increase in the generation of IP3 was observed within 15 s after addition of anti-IgE. Xestobergsterol A dose-dependently inhibited the generation of IP3. The initial rise in $[Ca^{2+}]_i$ and the generation of IP3 in mast cells activated by anti-IgE showed a close quantitative correlation.

It is well established that cross-linking of $FC_{\varepsilon}R1$ on mast cells induces the activation of PLC[2], an increase in intracellular Ca^{2+} [5], activation of PKC[19] and histamine release. The present study demonstrated that xestobergsterol A inhibited not only the anti-IgE-induced initial rise in $[Ca^{2+}]_i$ and the generation of IP3, but also PI-PLC activity. These results suggest that xestobergsterol A is involved directly in anti-IgE-induced activation of PI-PLC.

The mechanisms involved in the transduction of receptor-mediated signals to PLC remain elusive. It was suggested that a GTP-binding protein (G-protein) may be involved in this process[22]. However, Saito et al. have shown that pretreatment of peritoneal mouse mast cells with pertussis toxin or with cholera toxin failed to affect IgE-dependent histamine release[23,24]. From these results, they suggested that a G-protein is not involved in the transduction of IgE-mediated triggering signals to PLC in mast cells. However, the role of G-proteins in IgE-dependent mediator release is not known. Recently, Kawakami et al.[25] showed that activation of PTK(s) is an early event upstream of the activation of PLC, and is involved in transduction of IgE-dependent triggering signals to mediator release. Since genistein, a PTK inhibitor, does not affect PI-PLC in the lysate of resting or activated mast cells, one may speculate that PTK is not involved in antigen-induced activity of PLC. From these results, it is suggested that xestobergsterol A is not involved in anti-IgE-induced activation of PTK. It has been reported that binding of ligand to EGF receptors induces activation of PLC through tyrosine phosphorylation of the enzyme[26]. A second type of receptor-mediated activation of PLC is catalyzed by receptors with intrinsic protein tyrosine kinase activity such as the EGF and PDGF receptors. Recent studies have demonstrated that PLCγ-1 is translocated to the membrane following activation of these receptors[27]. This problem requires further investigation.

On the other hand, xestobergsterol B failed to affect the PI-PLC activity, IP3 generation and the initial rise in $[Ca^{2+}]_i$ in mast cells activated by anti-IgE. Comparison of physicochemical data of xestobergsterol A with those of B revealed that the only difference was that B has hydroxyl groups at the C1 and C2 positions. Comparative data regarding these two analogues could provide important insights into structure-activity relationships.

The inhibitory effect of xestobergsterol A and B on histamine release, generation of IP3 and Ca^{2+}-mobilization was not due to its cytotoxic action. The effect of xestobergsterol A and B disappeared entirely after the cells had been washed. The purity of xestobergsterol A and B was above 99%; hence, it is not possible that another non-specific substance was included. Therefore the effect of xestobergsterol A and B is specific.

These results suggest that the inhibitory mechanism of the effect of xestobergsterol A on the initial rise in $[Ca^{2+}]_i$, the generation of IP3, and histamine release induced by anti-IgE was due to the inhibition of PI-PLC activity. We suggest that the activity of PI-PLC is involved in an early stage of the biochemical process of IgE-dependent histamine release.

Abbreviations: IP2, inositol 1,4-bisphosphate; IP3, inositol 1,4,5,-triphosphate; PIP2, Phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PI, phosphatidylinositol; PKC, protein kinase C. PTK, protein tyrosin kinase; 1,2-DAG, 1,2-diacylglycerol; G-protein, GTP-binding protein.

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