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The effect of the PKC inhibitor GF109203X on the release of Ca^{2+} from internal stores and Ca^{2+} entry in DDT_1 MF-2 cells

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1 The effects of the specific protein kinase C (PKC) inhibitor, GF109203X, were measured on the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), and on histamine H_1 receptor- and thapsigargin-mediated increases in $[Ca^{2+}]_i$ in DDT_1 MF-2 smooth muscle cells.

2 After pretreatment of cells with GF109203X (5 μM, 45 min), the histamine (100 μM)-induced initial rise in $[Ca^{2+}]_i$, representing Ca^{2+} mobilization from internal stores, was inhibited (by $59 \pm 7\%$). The slowly declining phase of the histamine induced Ca^{2+} response, reflecting Ca^{2+} entry, was enhanced ($83 \pm 26\%$) in the presence of the PKC inhibitor.

3 The histamine induced release of Ca^{2+} from internal stores, measured after blocking Ca^{2+} entry with $LaCl_3$ was inhibited by GF109203X in a concentration-dependent manner (IC_{50}: 3.1 ± 1.1 μM).

4 Histamine-induced formation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) was not changed in the presence of GF109203X.

5 The PKC activating phorbol ester, phorbol 12-myristate 13-acetate (PMA, 1 μM), strongly reduced histamine-induced $Ins(1,4,5)P_3$ formation ($58 \pm 16\%$). This effect was reversed by GF109203X (5 μM). Furthermore, PMA diminished histamine evoked Ca^{2+} release ($50 \pm 6\%$) and blocked Ca^{2+} entry completely.

6 The rise in $[Ca^{2+}]_i$ caused by blocking endoplasmic reticulum Ca^{2+}-ATPase with thapsigargin (1 μM), was strongly reduced ($57 \pm 3\%$) after pretreatment of cells with GF109203X. Downregulation of PKC by long-term pretreatment of cells with PMA (1 μM, 48 h) did not abolish this effect of GF109203X ($48 \pm 3\%$ inhibition).

7 In permeabilized DDT_1 MF-2 cells preloaded with $^{45}Ca^{2+}$ in the presence of GF109203X, the amount of $^{45}Ca^{2+}$ released by $Ins(1,4,5)P_3$ (10 μM) was markedly reduced ($42 \pm 9\%$). GF109203X did not release Ca^{2+} itself and did not impair $Ins(1,4,5)P_3$ receptor function.

8 Uptake of $^{45}Ca^{2+}$ by intact cells, representing Ca^{2+} entry, was enhanced by GF109203X ($65 \pm 11\%$), by histamine ($24 \pm 6\%$) and also by thapsigargin ($121 \pm 10\%$). The GF109203X- and the thapsigargin-induced uptake of $^{45}Ca^{2+}$ were not additive.

9 These data suggest that GF109203X reduces the filling-state of intracellular $Ins(1,4,5)P_3$ sensitive Ca^{2+} stores by inhibiting the Ca^{2+} uptake into these stores, thereby promoting store-dependent (capacitive) Ca^{2+} entry.

Keywords: GF109203X; Ca^{2+} entry; Ca^{2+} release; $Ins(1,4,5)P_3$; PKC; PMA; histamine; thapsigargin; DDT_1 MF-2 cell

Introduction

Ligand induced receptor stimulation is often associated with the activation of phospholipase C (PLC), via heterotrimeric regulatory G-proteins, leading to the hydrolysis of phosphatidyl inositol(4,5)bisphosphate and the formation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and diacylglycerol (DAG). Interaction of $Ins(1,4,5)P_3$ with its receptor on the endoplasmic reticulum causes the release of Ca^{2+} (Streb *et al.*, 1983; Berridge, 1993), whereas DAG is supposed to activate protein kinase C (PKC) (Nishizuka, 1988).

Several studies suggest that DAG-activated PKC is involved in the agonist mediated stimulation of PLC. For example, activation of PKC by phorbol esters resulted in reduction of histamine H_1 receptor mediated formation of inositol phosphates (Jones *et al.*, 1990). Inhibition of PKC was shown to enhance the histamine and bradykinin induced formation of $Ins(1,4,5)P_3$ in bovine adrenal chromaffin cells (Boarder & Challis, 1992). In contrast, an inhibitory feedback loop causing homologous desensitization of histamine evoked Ca^{2+} release was shown to be PKC-independent in HeLa cells (Smit *et al.*, 1992). Recently, a positive feedback loop mediated by activation of PKC and modulating histamine and metacholine evoked Ca^{2+} metabolism was suggested in bovine tracheal cells (Hoiting *et al.*, 1995). PKC was also shown to affect Ca^{2+} entry from the extracellular space (Rane & Dunlap, 1986; Tornquist & Tashijian, 1990; Kostyuk *et al.*, 1992).

In DDT_1 MF-2 smooth muscle cells, stimulation of histamine H_1 receptors leads to the formation of $Ins(1,4,5)P_3$ accompanied by an increase in $[Ca^{2+}]_i$ caused by its release from internal stores and by Ca^{2+} entry (Molleman *et al.*, 1991; Sipma *et al.*, 1995). It has been found that the PKC inhibitor Ro-31-8220 (Wilkinson *et al.*, 1993) does not affect the histamine induced rise in $[Ca^{2+}]_i$ in DDT_1 MF-2 cells (Dickenson & Hill, 1993). As shown by us the selective PKC inhibitor, GF109203X (Toullec *et al.*, 1991), strongly inhibited the histamine induced Ca^{2+}-response. The aim of this study is to investigate the effects of GF109203X on Ca^{2+} handling and its mechanism of action.

Methods

Cell culture

DDT_1 MF-2 cells, derived from a Syrian hamster vas deferens were cultured in Dulbecco's modified essential medium sup-

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plemented with 7 mM $NaHCO_3$, 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulphonic acid (HEPES) at pH 7.2 (DMEM) and 10% foetal calf serum at 37°C in 5% CO_2 (Hoiting *et al.*, 1990).

Measurement of Ins(1,4,5)P_3

DDT_1 MF-2 cells were grown in monolayers in 9.6 cm^2 plastic wells as described previously (Sipma *et al.*, 1995). The medium was replaced by 2 ml DMEM at 20°C, 30 min before the start of the experiment by adding agonists. After the medium had been removed, reactions were stopped with 400 μM 5% trichloroacetic acid (TCA) and placed on ice for at least 45 min. Samples were washed 3 times with 800 μl water saturated diethylether and neutralised with KOH (25 μl, 0.2 M).

Mass measurements of Ins(1,4,5)P_3 were performed as described earlier, using a standard curve of Ins(1,4,5)P_3 in ether extracted TCA solution (Molleman *et al.*, 1991). In brief, samples were assayed in 25 mM Tris/HCl (pH = 9.0), 1 mM EDTA, 1 mg bovine serum albumin, [^{3}H]-Ins(1,4,5)P_3 (3,3 Ci $mmol^{-1}$, 2000 c.p.m./assay) and about 1 mg binding protein for 15 min. The binding protein was isolated from fresh beef liver (Chivers *et al.*, 1989). Bound and free radioactivity were separated by centrifugation. The radioactivity in the pellet was determined by liquid scintillation counting.

Measurements of intracellular calcium

Intracellular Ca^{2+} concentrations were measured as described earlier (Hoiting *et al.*, 1990). Cells (10^6 cells ml^{-1}), suspended in DMEM containing 10% foetal calf serum were loaded with Indo-1/AM (1.5 μM) for 45 min at 37°C. The cells were collected by centrifugation (5 min, 1000 × *g*) and washed twice before the fluorescence measurement with a buffer solution containing (mM): NaCl 145, KCl 5, $MgSO_4$ 0.5, $CaCl_2$ 1, D-glucose 10 and HEPES 10 (pH 7.4) (Hesketh *et al.*, 1983). Calcium-free solution contained Mg^{2+} (6.2 mM) to prevent membrane leakage and EGTA (0.1 mM) to remove extracellular Ca^{2+} (Den Hertog, 1981). Indo-1 fluorescence of the cells (excitation: 325 nm, emission 400 nm and 480 nm) was measured at 22°C. The cell suspension was continuously magnetically stirred. The internal calcium concentration was calculated (Hesketh *et al.*, 1983) by use of 0.015% of Triton X-100 as a permeabilizing agent.

$^{45}Ca^{2+}$ efflux measurements

The cells were plated on poly-L-lysine (0.01 mg ml^{-1}) coated wells (10^6 cells/well) 15 h before the start of the experiment. The experiments were carried out at 22°C, following the same procedure as described previously (Missiaen *et al.*, 1990; Van der Zee *et al.*, 1995). In brief, cells were equilibrated for 1 h with a modified Krebs solution containing (in mM): NaCl 135, KCl 5.9, $CaCl_2$ 1.5, $MgCl_2$ 1.2, glucose 11.5 and HEPES 11.6 (adjusted to pH 7.2). After this, the cells were permeabilized by incubation for 10 min with saponin (40 μg ml^{-1}) in a solution containing (in mM): KCl 100, imidazole 30, $MgCl_2$ 2, ATP 1, and EGTA 1 (pH 7.0) and subsequently loaded for 5 min with $^{45}Ca^{2+}$ by exposure to 500 μl solution containing 10.5 μCi ml^{-1} $^{45}CaCl_2$ (specific activity: 19.3 Ci g^{-1}) with a final composition (in mM): KCl 100, imidazole 30, $MgCl_2$ 5, ATP 5, EGTA 0.44 and NaN_3 5 and $CaCl_2$ 0.12 (pH 7.0); the free Ca^{2+} concentration of this solution was 0.15 μM. The efflux was performed by adding 1 ml of a solution containing (in mM) KCl 100, imidazole 30, $MgCl_2$ 2, ATP 1, EGTA 1 and NaN_3 5 (pH 7.0) to the cells and replacing it every 2 min during 30 min. The $^{45}Ca^{2+}$ present in each of the efflux samples and the remaining $^{45}Ca^{2+}$ in the cells at the end of the efflux procedure was measured by liquid scintillation counting. The time course of the tracer wash-out was calculated by summing in retrograde order the amount of tracer remaining at the cells at the end of the efflux and the amount of tracer collected during the successive time invervals. This time course became mono-exponential after 8–10 min. The $^{45}Ca^{2+}$ release was represented as the fractional loss of $^{45}Ca^{2+}$ per minute, representing the amount of $^{45}Ca^{2+}$ leaving the cell, normalized to the amount of labelled $^{45}Ca^{2+}$ present in the cell at that time.

$^{45}Ca^{2+}$ uptake by intact cells

$^{45}Ca^{2+}$ uptake was measured essentially according to Yano *et al.* (1983). DDT_1 MF-2 cells were grown in 9.6 cm^2 plastic dishes. Culture medium was replaced by a buffer solution, at 22°C, containing (in mM): NaCl 145, KCl 5, $MgSO_4$ 0.5, $CaCl_2$ 0.5, glucose 10 and HEPES 10 (adjusted to pH 7.4), 45 min before the addition of $^{45}Ca^{2+}$. During this period some cell preparations were exposed to GF109203X (5 μM). $^{45}Ca^{2+}$ uptake was started by removing the solution and replacing it by the same buffer (1 ml) supplemented with agonists (histamine, uridine 5'-triphosphate (UTP), thapsigargin), GF109203X (when indicated) and $^{45}Ca^{2+}$ (10 μCi). Uptake of $^{45}Ca^{2+}$ was stopped after 5 min by aspirating the solution and addition of 1 ml ice-cold buffer supplemented with 20 mM EGTA in the absence of $CaCl_2$. After this procedure, cells were washed quickly 3 times with buffer without $CaCl_2$ and containing 2 mM EGTA. Cells were lysed in the presence of NaOH (1 ml, 0.2 M) and radioactivity was measured by liquid scintillation counting. Data were corrected for nonspecific binding as determined by adding buffer with $^{45}Ca^{2+}$ and immediately terminating uptake.

Data analysis

Data are presented as means ± s.e.mean. Data were considered significantly different from control values when $P < 0.05$ by use of Student's unpaired *t* test. A sigma plot logistic curve fit program (Jandel Scientific, U.S.A.) was used to determine EC_{50} values and to analyze binding parameters obtained from the Ins(1,4,5)P_3 radioligand binding assay.

Chemicals

GF109203X (3-[1-[3-(Dimethylamino)propyl]-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrolyl-2,5-dione) was from Boehringer (Germany). Indo-1/AM was from Molecular Probes (U.S.A.). Thapsigargin, H7 (1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride), H9, staurosporine, ATP, UTP and inositol 1,4,5-trisphosphate sodium salt were purchased from Sigma (U.S.A.). Phorbol 12-myristate 13-acetate (PMA) was from Gibco BRL (U.S.A.). Histamine dihydrochloride was from Fluka (Switzerland). $^{45}CaCl_2$ was obtained from Amersham International (U.K.). D-[2-^{3}H]-inositol 1,4,5-trisphosphate was from Du Pont-New England Nuclear (U.S.A.). Lanthanum chloride, HEPES and all other chemicals were from Merck (Germany).

Results

GF109203X and the histamine induced PLC pathway

Stimulation of histamine H_1 receptors induces a rapid rise in $[Ca^{2+}]_i$, mainly caused by release of Ca^{2+} from internal stores, followed by a slowly declining phase due to Ca^{2+} entry across the plasma membrane (Figure 1a, Den Hertog *et al.*, 1992). We observed that pretreatment of cells with the specific PKC inhibitor, GF109203X (5 μM, 45 min), inhibited the histamine-induced maximal increase in $[Ca^{2+}]_i$, but enhanced the slowly declining phase of the Ca^{2+} response (Figure 1b, Table 1). This latter component reflects Ca^{2+} entry, since addition of $LaCl_3$ (50 μM, Den Hertog *et al.*, 1992) to the cells, rapidly reduced $[Ca^{2+}]_i$ to (below) the basal level (Figure 1c).

These results show an inhibitory effect of GF109203X on histamine induced Ca^{2+} release and promotion of Ca^{2+} entry. The effects of GF109203X were compared with those of a

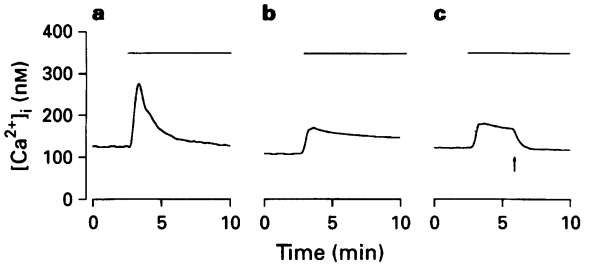

Figure 1 The effect of GF109203X on the histamine H_1-receptor-mediated increase in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}. Histamine (100 μM)-induced increases in $[Ca^{2+}]_i$ were measured in non-pretreated DDT_1 MF-2 cells (a), in cells pretreated with the PKC inhibitor GF109203X (b, 5 μM, 45 min) and in GF109203X-pretreated cells where $LaCl_3$ (50 μM, arrow) was added during the plateau-phase of the histamine response (c). Horizontal bars indicate the presence of histamine. Each tracing is representative of at least 6 experiments.

Table 1 The effect of GF109203X and PMA on the histamine-induced increase in $[Ca^{2+}]_i$ in DDT_1 MF-2 cells

Pretreatment	*Basal* $[Ca^{2+}]_i$	*Histamine-induced increase in* $[Ca^{2+}]_i$ (nM) *Maximal*	*After 5 min*
None	131±5	154±17	23±3
PMA	129±4	50±6[b]	0±0[b]
GF109203X	102±4[a]	63±11[b]	42±6[b]

Preincubations with GF109203X (5 μM, 45 min) or phorbol 13-myristate 12-acetate (PMA, 0.5 μM, 5 min) were performed before stimulation with histamine (100 μM). Basal $[Ca^{2+}]_i$, the maximal increase in $[Ca^{2+}]_i$ above the basal level and the increase in $[Ca^{2+}]_i$ after 5 min of histamine application are shown. [a]Different from non pretreated basal $[Ca^{2+}]_i$, $P<0.05$; [b]different from the histamine-induced increase in $[Ca^{2+}]_i$ in non-pretreated cells, $P<0.01$. Data are expressed as mean±s.e.mean of at least 6 experiments.

PKC activating phorbol ester, PMA. After exposure of DDT_1 MF-2 cells to PMA (1 μM, 5 min), both the transient and the slowly declining phase of the histamine-induced Ca^{2+} response were strongly reduced (Figure 2b, Table 1). PMA also did not change basal $[Ca^{2+}]_i$ immediately after addition (not shown). The histamine-evoked release of Ca^{2+} from internal stores, as measured in the absence of extracellular Ca^{2+} was likewise inhibited by PMA (Figure 2c,d, Table 2).

Inhibition of histamine evoked Ca^{2+} release in the presence of GF109203X might be due to reduced $Ins(1,4,5)P_3$ formation. The unstimulated $Ins(1,4,5)P_3$ level was slightly enhanced in GF109203X-pretreated cells. However, the histamine (100 μM, 1 min) induced maximal $Ins(1,4,5)P_3$ level (Sipma *et al.*, 1995) was not affected by pretreatment of cells with GF109203X (Figure 3). In the presence of PMA (1 μM, 5 min), histamine (100 μM) did not significantly increase the $Ins(1,4,5)P_3$ level. This inhibition of histamine-induced $Ins(1,4,5)P_3$ formation in the presence of PMA was counteracted by GF109203X pretreatment.

GF109203X and Ca^{2+} mobilization

The effects of GF109203X on $[Ca^{2+}]_i$ were studied in more detail. GF109203X (5 μM) elicited a very slow increase in $[Ca^{2+}]_i$ (9±2 nM, in 10 min, $n=4$) when extracellular Ca^{2+} was available and after blocking Ca^{2+} entry with 50 μM $LaCl_3$ (in 10 min: 9±2 nM*, 7±2 nM*, respectively; *$P<0.05$, paired t test). Untreated basal $[Ca^{2+}]_i$ did not change in 10 min (not shown). These results suggest that GF109203X provoked Ca^{2+} release from internal stores. Therefore, we investigated the effect of GF109203X on the release of Ca^{2+} from

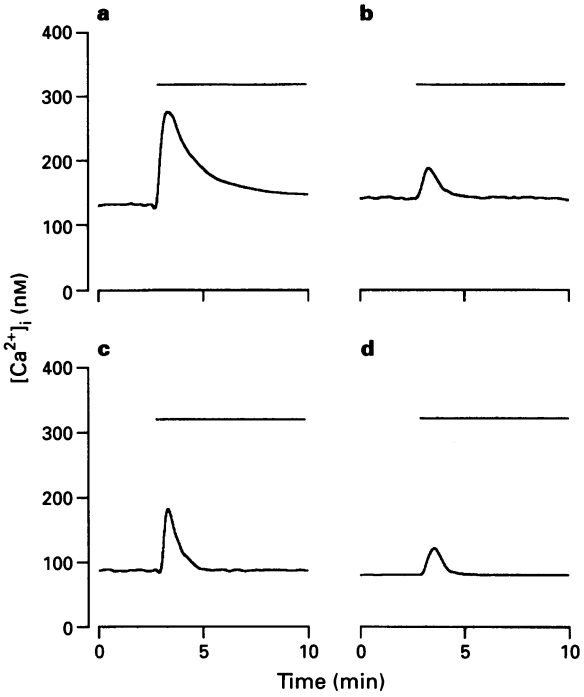

Figure 2 The effect of phorbol 13-myristate 12-acetate (PMA) on histamine H_1 receptor-mediated increase in $[Ca^{2+}]_i$. The histamine (100 μM)-induced increase in $[Ca^{2+}]_i$ was measured in DDT_1 MF-2 cells (a), in the absence of extracellular Ca (c), in cells pretreated with PMA (0.5 μM, 5 min, b), also in the absence of extracellular Ca^{2+} (d). Horizontal bars indicate the presence of histamine. Each tracing is representative of at least 4 experiments.

Table 2 The effect of GF109203X and PMA on the histamine-induced increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} in DDT_1 MF-2 cells

Pretreatment	*Basal* $[Ca^{2+}]_i$	*Histamine-induced increase in* $[Ca^{2+}]_i$ (nM)
None	77±5	105±5
PMA	80±6	53±6[a]
GF109203X	82±4	40±7[b]

Preincubations with GF109203X (5 μM, 45 min) and phorbol 13-myristate 12-acetate (PMA, 0.5 μM, 5 min) were performed before stimulation with histamine (100 μM). Basal $[Ca^{2+}]_i$ and the maximal increase in $[Ca^{2+}]_i$ above the basal level during histamine stimulation are shown. [a]Different from histamine-induced increase in $[Ca^{2+}]_i$ in non-pretreated cells, $P<0.01$, Data are expressed as mean±s.e.mean of at least 4 experiments.

$Ins(1,4,5)P_3$-sensitive stores. Pretreatment of cells with GF109203X (5 μM, 45 min) caused a strong reduction of the histamine-mediated and $Ins(1,4,5)P_3$-induced Ca^{2+} release, observed after blocking Ca^{2+} entry with $LaCl_3$ (50 μM, Figure 4). The histamine-induced increase in $[Ca^{2+}]_i$ was 93±13 nM and with GF109203X: 38±7 nM ($P<0.01$, $n=6$). This effect was dependent on the concentration of GF109203X (Figure 5). Cells had to be pretreated with GF109203X (5 μM) for at least 15 min before the effect on Ca^{2+} release became apparent (not shown). GF109203X also reduced the histamine (100 μM) evoked Ca^{2+} release observed in the absence of extracellular Ca^{2+} (Table 2). The effect of the PKC inhibitor on the filling-state of intracellular Ca^{2+} stores was further investigated by emptying the stores with the inhibitor of endoplasmic pump Ca^{2+}-ATPase, thapsigargin (Thastrup *et al.*, 1990), in the presence of $LaCl_3$ to prevent Ca^{2+} entry. As expected, thapsigargin (1 μM) caused a transient increase in $[Ca^{2+}]_i$ (Figure

6a, Table 3). GF109203X (5 μM, 45 min) diminished the rise in $[Ca^{2+}]_i$ induced by thapsigargin (Figure 6b, Table 3).

In order to investigate whether the action of GF109203X was due to inhibition of PKC, cells were exposed to PMA (1 μM) for 48 h, known to cause downregulation of PMA-sensitive PKC isoenzymes. After this pretreatment with PMA, GF109202X still affected Ca^{2+} release elicited by thapsigargin (Table 3), strongly suggesting a PKC-indpendent action of GF109203X. Remarkably, prolonged pretreatment of cells with PMA (1 μM, 48 h) caused a solid increase in the resting Ins(1,4,5)P_3 level and completely inhibited histamine (100 μM,

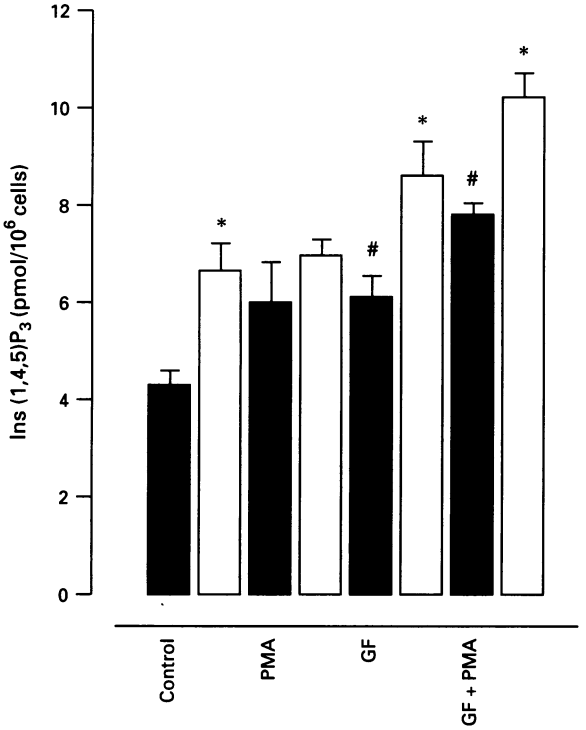

Figure 3 Histamine H_1 receptor-mediated changes in Ins(1,4,5)P_3 formation in the presence of GF109203X and/or phorbol 13-myristate 12-acetate (PMA). Maximal histamine (100 μM, 1 min)-induced Ins(1,4,5)P_3 formation was measured in non-pretreated cells and in cells pretreated with GF109203X (GF, 5 μM, 45 min) or PMA (1 μM, 5 min) or both GF109103X and PMA. Solid columns represent basal Ins(1,4,5)P_3 values, whereas open columns indicate the histamine-evoked level of Ins(1,4,5)P_3. # Different from unstimulated level in non-pretreated cells, $P<0.05$. *Different from respective unstimulated level, $P<0.05$. Data are expressed as mean±s.e.mean of 8 experiments.

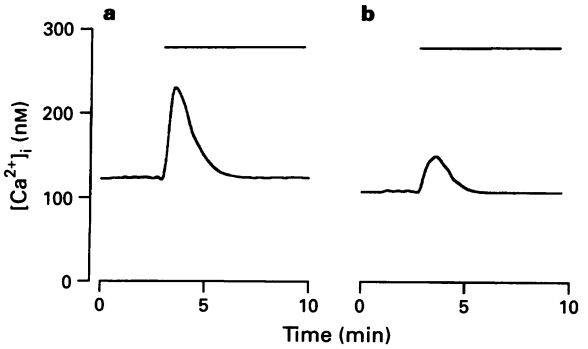

Figure 4 Histamine H_1 receptor-mediated release of $[Ca^{2+}]_i$ in the presence of GF109203X. The histamine (100 μM) induced increase in $[Ca^{2+}]_i$ after block of Ca^{2+} entry with $LaCl_3$ (50 μM) was measured in non-pretreated DDT_1 MF-2 cells (a) and in cells pretreated with GF109203X (b, 5 μM, 45 min). Horizontal bars indicate the presence of histamine. Each tracing is representative of 6 experiments.

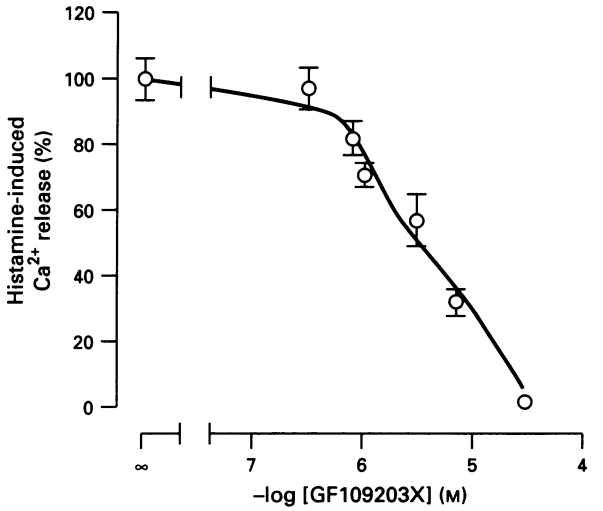

Figure 5 Concentration-dependent inhibition of histamine-induced Ca^{2+} release by GF109203X. The histamine (100 μM)-induced maximal increase in $[Ca^{2+}]_i$ after block of Ca^{2+} entry with $LaCl_3$ (50 μM) was measured in the presence of different concentrations of GF109203X. Each symbol represents mean of 4–6 experiments; vertical lines show s.e.mean.

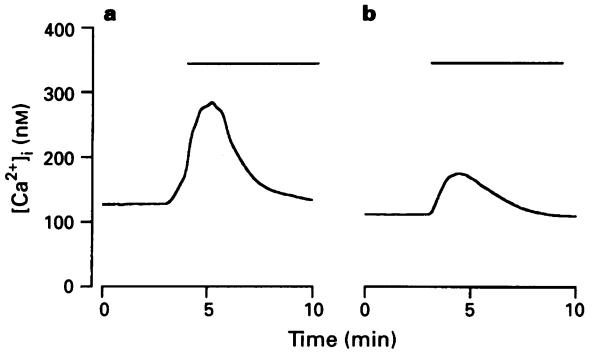

Figure 6 Thapsigargin-induced release of $[Ca^{2+}]_i$ in the presence of GF109203X. The thapsigargin (1 μM)-induced increases in $[Ca^{2+}]_i$ after block of Ca^{2+} entry with $LaCl_3$ (50 μM) were measured in non-pretreated DDT_1 MF-2 cells (a) and in cells pretreated with GF109203X (b, 5 μM, 45 min). Horizontal bars indicate the presence of thapsigargin. Each tracing is representative of 6 experiments.

Table 3 The effect of GF109203X on the thapsigargin-induced increase in $[Ca^{2+}]_i$ in the presence of $LaCl_3$ in DDT_1 MF-2 cells

	Thapsigargin-induced increase in $[Ca^{2+}]_i$ (nM)	
Pre-treatment	*Control*	*48 h PMA pretreatment*
$LaCl_3$	157±29	142±10
$LaCl_3$ + GF109203X	68±5[a]	73±4[a]

Preincubation with GF109203X (5 μM) was performed at 45 min before stimulation with thapsigargin (1 μM). Ca^{2+} entry from the extracellular environment was inhibited by pretreatment of cells with $LaCl_3$ (50 μM, 5 min). This experiment was also carried out on cells pretreated with phorbol 13-myristate 12-acetate (1 μM) for 48 h. Maximal changes in $[Ca^{2+}]_i$ after application of thapsigargin are shown. Basal values of $[Ca^{2+}]_i$ after PMA pretreatment were: control 133±9 nM; GF109203X pretreated 105±5 nM. [a]Different from thagsigargin-induced increase in $[Ca^{2+}]_i$ in non-pretreated cells, $P<0.01$, Data are expressed as mean±s.e.man of at least 6 experiments.

1 min) induced Ins(1,4,5)P_3 formation (unstimulated level: 4.3±0.3 pmol/10^6 cells; histamine: 6.6±0.6 pmol/10^6 cells*; PMA 48 h: 11.6±0.6 pmol/10^6 cells; PMA+histamine: 11.5±0.9 pmol/10^6 cells; *$P<0.05$, $n=6$). In accord with these results, histamine-evoked Ca^{2+} release was completely inhibited in cells pretreated with PMA (1 μM, 48 h, not shown).

GF109203X and Ca^{2+} store filling

In view of the results obtained so far, the effect of GF109203X was examined in permeabilized DDT_1 MF-2 cells. GF109203X, like thapsigargin, may reduce the filling of intracellular Ca^{2+} stores by inhibition of Ca^{2+} pumping or alternatively, this compound may stimulate Ca^{2+} mobilization from these stores. The amount of $^{45}Ca^{2+}$ released by a maximal effective concentration of Ins(1,4,5)P_3 (10 μM, van der Zee *et al.*, 1995) was reduced when GF109203X (5 μM) was present during the Ca^{2+}-ATPase-dependent $^{45}Ca^{2+}$ loading period (Figure 7, 58±9% of control release, $n=8$). If stores were allowed to accumulate $^{45}Ca^{2+}$ in the absence of GF109203X, the ability of Ins(1,4,5)P_3 (10 μM) to release $^{45}Ca^{2+}$ was not affected by GF109203X (added 4 min before Ins(1,4,5)P_3, 93±6% of control value, $n=8$), which was also found with a half-maximal effective concentration of Ins(1,4,5)P_3 (3 μM, 102±5% of control value, $n=4$). GF109203X itself (added at $t=10$ min, Figure 7) did not cause $^{45}Ca^{2+}$ release.

GF10920X and Ca^{2+} entry

The results obtained after stimulation of GF109203X-pretreated cells with histamine in the presence of extracellular Ca^{2+} (Figure 1, Table 1) suggest that this inhibitor affects Ca^{2+} entry across the plasma membrane. Moreover, if GF109203X decreases the filling-state of intracellular Ca^{2+} stores, store-dependent (Putney, 1986) Ca^{2+} entry might be stimulated. Therefore, we determined the uptake of extracellular $^{45}Ca^{2+}$ by non-permeabilized vital cells. Enhanced $^{45}Ca^{2+}$ accumulation was observed after pretreatment of cells with GF109203X (5 μM, 45 min, Table 4). Remarkably, histamine (100 μM) only marginally stimulated $^{45}Ca^{2+}$ uptake, also in comparison with the P_{2U}-purinoceptor agonist UTP. Histamine did not enhance $^{45}Ca^{2+}$ accumulation induced by GF109203X. Thapsigargin (1 μM), however, induced a substantial $^{45}Ca^{2+}$ uptake that was not additive to the GF109203X elicited Ca^{2+} entry. The less selective PKC inhibitor, staurosporine, which was also shown to affect the filling-state of intracellular Ca^{2+} stores in DDT_1 MF-2 cells (Himpens *et al.*, 1993), likewise caused considerable $^{45}Ca^{2+}$ accumulation. In contrast, the PKC inhibitors Ro-31-8220, H7 and H9 only slightly increased $^{45}Ca^{2+}$ accumulation in DDT_1 MF-2 cells (Table 4).

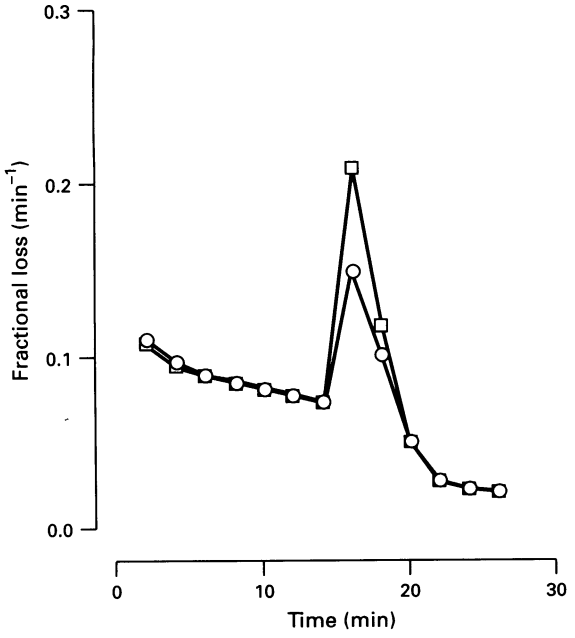

Figure 7 Effect of GF109203X on the fractional Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ loss from permeabilized DDT_1 MF-2 cells. Addition of Ins(1,4,5)P_3 (10 μM) for 4 min at $t=14$ min caused a transient increase in $^{45}Ca^{2+}$ loss (□). When GF109203X (5 μM) was added to the cells during the $^{45}Ca^{2+}$ loading period, the effect of Ins(1,4,5)P_3 on $^{45}Ca^{2+}$ efflux was reduced (○). Data shown represent a typical experiment out of a series of 8.

Discussion

The histamine H_1-receptor induced rise in $[Ca^{2+}]_i$ is caused by Ca^{2+} mobilization from intracellular stores via stimulation of Ins(1,4,5)P_3-sensitive receptors and by Ca^{2+} entry from the extracellular environment, respectively (Molleman *et al.*, 1991; Sipma *et al.*, 1995). The Ca^{2+} release mediated by histamine H_1 receptors was inhibited by GF109203X as represented by the lower Ca^{2+} response, also observed after preventing Ca^{2+} entry with $LaCl_3$ or by omitting extracellular Ca^{2+}. However, the slowly declining component of the histamine-induced Ca^{2+} response was enhanced by GF109203X, which is indicative of enhanced Ca^{2+} entry.

The histamine-induced formation of Ins(1,4,5)P_3 was not affected by GF109203X and is therefore not involved in the modulation of histamine induced Ca^{2+} release and Ca^{2+} entry by GF109203X. Interestingly, pretreatment of bovine adrenal chromaffin cells with the PKC inhibitor Ro-31-8220 was shown to augment histamine- and bradykinin-induced Ins(1,4,5)P_3 formation, suggesting a PKC-mediated negative feedback loop affecting PLC activity in these cells (Boarder & Challiss, 1992). In agreement with the results obtained with GF109203X, the less specific PKC inhibitor, staurosporine, did not affect histamine induced Ins(1,4,5)P_3 formation in DDT_1 MF-2 cells (van der Zee, 1994). Thus, histamine-induced PLC activation is apparently insensitive to histamine-activated PKC in DDT_1 MF-2 cells. Activation of PKC induced by acute phorbol ester treatment of cells strongly reduced the hista-

Table 4 $^{45}Ca^{2+}$ uptake in DDT_1 MF-2 cells

Treatment	*$^{45}Ca^{2+}$ entry (% of non-pretreated)*
None	100±6
GF109203X	167±8[b]
Histamine	124±6[a]
Histamine+GF109203X	155±12[b]
Thapsigargin	221±10[b]
Thapsigargin+GF109203X	238±12[b]
Staurosporine	207±13[b]
Ro-31-8220	135±19[a]
H7	136±13[a]
H9	118±9
UTP	356±18[b]

Ca^{2+} entry was determined by measuring uptake $^{45}Ca^{2+}$ in non-pretreated DDT_1 MF-2 cells and in cells pretreated (45 min) with GF109203X (5 μM), staurosporine (100 μM), Ro-31-8220 (10 μM), H7 (100 μM) or H9 (100 μM). Agonists (histamine 100 μM, thapsigargin 1 μM, UTP 100 μM) were added simultaneously with extracellular $^{45}Ca^{2+}$. After 5 min the $^{45}Ca^{2+}$ content in the cells was determined. The uptake is expressed as percentage of the uptake in non-pretreated cells, which was taken as 100%. Different from basal uptake of $^{45}Ca^{2+}$, [a]$P<0.05$; [b]$P<0.01$. Data are expressed as mean±s.e.mean of 6–16 experiments.

mine-induced formation of Ins(1,4,5)P_3 and the concomitant Ca^{2+} release. Since GF109203X readily reversed the action of PMA on histamine-induced Ins(1,4,5)P_3 formation, the action of PMA on Ins(1,4,5)P_3, at least, can be attributed to PKC activation. The effects of PMA on histamine evoked Ins(1,4,5)P_3 formation and Ca^{2+} release were probably caused by activation of PKC that is not inducible by histamine.

Pretreatment of cells with GF109203X reduced the release of Ca^{2+} elicited by histamine but not the formation of Ins(1,4,5)P_3. Therefore, this PKC inhibitor might affect the filling-state of intracellular Ins(1,4,5)P_3-sensitive stores. In agreement with this hypothesis, GF109203X itself acutely increased $[Ca^{2+}]_i$ and strongly inhibited the rise in $[Ca^{2+}]_i$ induced by thapsigargin, known to inhibit Ca^{2+}-ATPase pumps of intracellular stores (Thastrup *et al.*, 1990; Bian *et al.*, 1991). The experiments on permeabilized cells showed that GF109203X reduced the amount of Ins(1,4,5)P_3-releasable $^{45}Ca^{2+}$, but did not release stored $^{45}Ca^{2+}$. Furthermore, GF109203X did not affect Ins(1,4,5)P_3-evoked Ca^{2+} release when added after the $^{45}Ca^{2+}$ loading period, which shows that GF109203X did not impair Ins(1,4,5)P_3-receptor function e.g. by competing with Ins(1,4,5)P_3 for binding at the Ins(1,4,5)P_3 receptor. Since Ca^{2+} uptake is thought to be mediated by Ca^{2+}-ATPase-pumps (MacLennan *et al.*, 1985; Gunteski-Hamblin *et al.*, 1988), GF109203X most likely inhibits this mechanism in DDT_1 MF-2 cells. It was noticed that GF109203X itself slightly increased the resting level of Ins(1,4,5)P_3, which might explain the reduced filling of Ins(1,4,5)P_3-sensitive Ca^{2+} stores. However, this hypothesis should imply an acute effect of GF109203X on $^{45}Ca^{2+}$ release, followed by reduced Ins(1,4,5)P_3-induced Ca^{2+} release, which was not the case. Since thapsigargin also induces an enhanced Ins(1,4,5)P_3 level in DDT_1 MF-2 cells (Sipma *et al.*, personal observation), the effect of GF109203X on the Ins(1,4,5)P_3 level is most likely due to the increase in $[Ca^{2+}]_i$.

GF109203X reduces the filling-state of intracellular Ca^{2+} stores. This implies that store-dependent Ca^{2+} entry might be facilitated, as demonstrated by an enhancement of the slowly declining component of the histamine evoked Ca^{2+} response. In accord with this, $^{45}Ca^{2+}$ uptake was promoted by GF109203X in intact cells. Thapsigargin likewise induced considerable $^{45}Ca^{2+}$ uptake, via store dependent Ca^{2+} entry (Putney, 1986). Interestingly, the effect of GF109203X on $^{45}Ca^{2+}$ accumulation was not additive to that of thapsigargin, suggesting a common mechanism. The P_{2U}-purinoceptor agonist, UTP, enhanced $^{45}Ca^{2+}$ uptake to a much larger extent than histamine. This confirms an earlier observation that the sustained phase of the P_{2U} purinoceptor-mediated Ca^{2+} response, representing Ca^{2+} entry, is more pronounced than that observed with histamine (Den Hertog *et al.*, 1992; Sipma *et al.*, 1995).

The effects of GF109203X on the histamine-induced increase in $[Ca^{2+}]_i$ under conditions in which Ca^{2+} entry is allowed, are sufficiently explained by the reduced filling-state of intracellular Ins(1,4,5)P_3-sensitive stores, the inhibition of reuptake of released Ca^{2+} and the stimulation of store-dependent Ca^{2+} entry. The PKC mediated inhibition of histamine-induced Ca^{2+} entry, as observed in the presence of PMA is at least partly attributed to reduced stimulation of store-dependent Ca^{2+} entry, in view of the reduced Ins(1,4,5)P_3 formation and subsequent Ca^{2+} release.

The effects of GF109203X on $[Ca^{2+}]_i$ might be due to inhibition of histamine-activated PKC as suggested recently for bovine tracheal cells (Hoiting *et al.*, 1995). However, several studies, including one on DDT_1 MF-2 cells (Himpens *et al.*, 1993), have described effects of staurosporine on $[Ca^{2+}]_i$ that were independent of PKC activity (Nigam *et al.*, 1992; Wong *et al.*, 1992; Turkson *et al.*, 1994). Since GF109203X-induced inhibition of Ca^{2+} release elicited by thapsigargin was also observed after prolonged exposure of cells to PMA, a treatment known to downregulate PKC, the effect of GF109203X on Ca^{2+} uptake into the stores is most likely not related to PKC activity. Prolonged pretreatment with PMA completely inhibited histamine-induced Ins(1,4,5)P_3 formation and the histamine evoked increase in $[Ca^{2+}]_i$. Therefore, the downregulation of PKC could not be assessed by analyzing the effect of acute phorbol ester treatment on a functional histamine-induced response under these conditions. However, pretreatment of DDT_1 MF-2 cells with 100 nM phorbol ester for 24 h caused an almost complete loss of the α-isoform of PKC as measured with an antibody selective for the α-isoform (B.B. Fredholm *et al.*, personal communication). Besides the lack of effect of phorbol ester pretreatment on the action of GF109203X, the PKC inhibitors, Ro-31-8220 (Dickenson & Hill, 1993; Sipma *et al.*, personal observation), H-7 and H-9 (Himpens *et al.*, 1993) did not affect $[Ca^{2+}]_i$ in DDT_1 MF-2 cells. These inhibitors did not or only modestly enhance $^{45}Ca^{2+}$ accumulation in intact cells. It is noticed that phorbol esters do not cause activation or downregulation of all PKC isoenzymes. The presence of the PKC isozymes α, ε and ζ was shown in DDT_1 MF-2 cells (Assender *et al.*, 1994). The ζ isoenzyme lacks the diacylglycerol binding site and is therefore not sensitive to phorbol esters (Lee & Severson, 1994). GF109203X may inhibit this ζ isoenzyme activity. GF109203X was found to inhibit ζ activity at much higher concentrations than necessary for inhibition of other isoenzymes (Martiny-Baron *et al.*, 1993). This observation might correspond with the inhibition of histamine induced Ca^{2+} release by GF109203X with an IC_{50} value of about 3 μM, which is a higher value than that found for inhibition of other PKC-mediated effects (Toullec *et al.*, 1993). Therefore, if the effects of GF109203X are related to inhibition of PKC, only the ζ isozyme might be involved.

In conclusion, GF109203X reduces the filling-state of intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores by inhibiting Ca^{2+} uptake, thereby activating the mechanism of store-dependent Ca^{2+} entry in DDT_1 MF-2 cells.

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