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STIMULATION OF INOSITOL PHOSPHATE PRODUCTION AND GTPase ACTIVITY BY COMPOUND 48/80 IN RAT PERITONEAL MAST CELLS

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Summary: Phospholipase C activity, GTPase activity and cytosolic-free calcium concentration in mast cells were stimulated by compound 48/80. Accumulation of inositol phosphates in rat mast cells was stimulated by guanosine 5'-[γ-thio]-triphosphate. Guanosine 5'-[γ-thio]triphosphate, however, exhibited no effect upon the purified phospholipase C activity and upon phospholipase C in the mast cell homogenate. The stimulatory effect of compound 48/80 upon phospholipase C activity of intact mast cells was observed to have been well correlated with that on GTPase activity of mast cell homogenate. Compound 48/80 exhibited no effect upon the binding of radioactive guanosine 5'-[γ-thio]triphosphate to mast cell homogenate. Phospholipase C activity was verified by the above results to become affected by compound 48/80 through guanine nucleotide-binding regulatory protein.

The condensation product of N-methoxyphenylamine with formaldehyde produces a potent histamine-liberating agent called compound 48/80(1). This compound is employed as a classic mast cell secretagogue which releases histamine and 5-hydroxytryptamine(5-HT) (2,3). Compound 48/80 has been previously indicated by Ortner and Chignell to be bound to mast cell membrane proteins and could cause non-covalent cross-linkage of membrane proteins(4). Mast cells can be isolated in a rather pure form(5), respond to various agents such as concanavalin A, compound 48/80 and antigen (6). The above characteristics make mast cells a suitable candidate for studying the ligand-receptor interaction and subsequent mediator release.

The initial step of increased inositol phospholipids turnover following receptor stimulation is well documented to be a consequence of the hydrolysis of

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Abbreviations used: DSBB. Dulbecco's sodium bicarbonate buffer; GTPγS. guanosine 5'-[γ-thio]triphosphate; IP_1. IP_2. IP_3. inositolmono-, -bis-, -triphosphate; TCA. trichloroacetic acid; G-protein, guanine nucleotide-binding regulatory protein; 5-HT. 5-hydroxytryptamine.

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inositol phospholipids by phospholipase C (PLC) (7-11). The stimulation of receptor is also reported to increase the intracellular concentration of calcium. The stimulative effect can be both a result of calcium influx and a release of calcium from the internal stores. For example, 5-HT stimulates inositol phosphate production in a cell-free system from blowfly salivary glands(12). GTPγS increases inositol phosphate production in a cell-free system. GTP is indicated by these results to be involved in mediating the effects of calcium mobilizing hormones upon phosphoinositide breakdown(12). The basic cycle of GTP binding and hydrolysis has been indicated in the mediation of transmembrane signalling by hormones(13,14).

Compound 48/80 is a mast cell secretagogue which produces a PI breakdown and an accumulation of phosphatidic acid in rat mast cells which occurs concomitantly with stimulation of histamine release (15). Compound 48/80 has, however, been reported as a potent inhibitor of phospholipase C (16). The membrane bound PLC from NG108-15 cells has been purified in our previous study (11). The mast cell is therefore now used here as a model so as to investigate the effect of compound 40/80 upon GTPase, cytosolic-free calcium([Ca^{2+}]i) and purified PLC.

Compound 48/80 is reported here to cause the release of histamine and [^{3}H]5-HT, increase of [Ca^{2+}]i and stimulation of inositol phosphates(IPs) accumulation in intact mast cells. Compound 48/80 has no effect upon both crude and purified PLC activity, whereas it stimulates the GTPase activity without affecting the binding ability of GTPγS on GTPase. From these results, it is suggested that compound 48/80 may indirectly activate PLC activity on a step involved in the activation of GTPase.

MATERIALS AND METHODS

Materials: Compound 48/80, guanosine 5'-[γ-thio]triphosphate(GTPγS) were purchased from Sigma Chemical Co.(St. Louis, MO). 2-[^{3}H]myo-inositol(15 Ci/mmol) was provided by American Radiolabeled Chemicals. Inc.(St.Louis.MO). Aquasol-2 came from Du Pont Co.(Wilminston. DE). [γ-^{32}P] guanosine 5'-triphosphate (10 Ci/mmol) [^{3}H]-5'-hydroxytryptamine(15 Ci/mmol) and [^{35}S]-GTPγS (Specific activity 1180 Ci/mmol) were provided by Du Pont Co.(Wilmington).

Isolation of mast cells: Mast cells were obtained from the peritoneal cavity of Sprague-Dawley male rats(200-250 g) and purified by using a Ficoll gradient method according to the procedure of Cooper and Stanworth(5).The cells were finally suspended in Dulbecco's sodium bicarbonate buffer(DSBB)(pH=7.3) containing 137 mM NaCl, 2.7 mM KCl, 1mM $MgCl_2$, 0.9 mM $CaCl_2$, 23.1 mM $NaHCO_3$ and 100 mM glucose.

Determination of compound 48/80-induced release of [^{3}H]-5HT: 7.2 x 10^5 mast cells were placed in a 5 ml plastic test tube and suspended in 0.5 ml DSBB. The mast cells were incubated with 0.1 μM 5-HT in the presence of 0.24 μCi [^{3}H]5-HT(specific activity 26.5 Ci/mM) at 37℃ for 5 minutes. Following completion of incubation, the sample was centrifuged at 750 x g for one minute. The supernatant was discarded and the cell pellet was resuspended in 0.5 ml

ice-cold DSBB by mixing with a vortex Genie mixer. This procedure was repeated once. The mast cell suspension was suspended in 0.12 ml DSBB and 0.01 ml aliquots were added to each of 12 tubes containing 0.23 ml DSBB. Each tube then received either 0.01 ml DSBB(as control) or various doses of compound 48/80 from(1 μg/ml to 10 μg/ml). The samples were incubated at 37℃ for 30 minutes in a shaking water bath with a shaking speed of 100 oscillations/min. At the end of the incubation, the medium and mast cells were separated by centrifugation as described. The [^{3}H]5-HT was extracted from the cell pellet with 1 ml ethanol. 10 ml Omni-Szintisol(Merk) scintillation cocktail was added to each vial containing the [^{3}H]5-HT extract. The amount of radioactivity was determined using a Beckman LS 3801 scintillation spectrometer. [^{3}H]5-HT released in the presqnce or absence of compound 48/80 was calculated by subtracting the amount of [^{3}H]5-HT in mast cells incubated with compound 48/80 from those incubated without compound 48/80.

Determination of compound 48/80 induced release of endogenous histamine: 3.6 x 10^5 mast cells were suspended in 0.24 ml DSBB in the presence or absence of 0.01 ml compound 48/80 (final concentration from 1 μg/ml to 10 μg/ml). The samples were incubated for two minutes and centrifuged. Histamine in the resulting supernatant and pellet was fluorometrically measured according to the method described by Shore et al.(17) The percentage release of histamine was calculated as reported by Ishizuka et al.(15)

Determination of the effect of compound 48/80 and GTPγS upon the accumulation of inositol phosphates: Mast cells were prelabeled with tritiated myo-inositol (20 μCi/ml) in DSBB containing 190 μM of unlabeled myo-inositol for 30 minutes. At the end of prelabeling the cells were washed and resuspended in DSBB containing 25 mM LiCl. Drugs at the desired concentration were added. Incubations were terminated after 10 minutes with one ml of ice-cold TCA. Inositol phosphates were separated and quantitated according to the method described by Berridge et al.(8)

Preparation of [^{3}H]-myoinositol labeled phosphotidylinositol (PI) and polyphosphatidylinositol (PIP.PIP_2): 100 μCi of [^{3}H]-myoinositol was added to mast cells(5 x 10^7 cells) suspended in DSBB. After labeling for one hour with [^{3}H]-myoinositol, the cells were separted from the labeling medium by centrifigation at 500 x g for 5 minutes. The cells were then washed twice with ice cold DSBB. The radiolabeled phospholipids in the cells were extracted and separated using the methods of Hauser et al.(9) and Pappu and Hauser,respectively.(10) PI,PIP and PIP_2 were purified by thin layer chromatography and recovered according to the method of Chiang, et al.(25) The purified PI, PIP and PIP_2 were used separately as a substrate for determining the catalytic activity of purified PLC.

PLC assay: For the measurement of the effect of compound 48/80 on PLC activity, two enzyme sources were used. Purified PLC enzyme which was previously obtained from NG108-15 cells' membrane(11) and a crude PLC enzyme from the homogenate of the Mast cells(6.2 x 10^5 cells) were used. The substrate was prepared in the presence of 0.1% sodium deoxycholate. The PLC activity in the presence or absence of compound 48/80 was performed in a reaction mixture containing 80 μM pig liver PI, 12000 dpm of [^{3}H]-PI (specific activity, 140 dpm/nmol), 0.1 % sodium deoxycholate, 3 mM $CaCl_2$ and 50 mM Hepes, pH 7.0 and appropriate amounts of enzyme in a total volume of 200 μl. After 20 minutes at 37℃ the reaction was stopped with 0.5 ml of chloroform:methanol:concentrated HCl (100:100:0.6) followed by 0.3 ml of 1 N HCl containing 5 mM EGTA. Following extraction, a 400 μl portion of the aqueous phase was removed for liquid scintillation counting.

[Ca^{2+}]i determination: [Ca^{2+}]i was measured by the fura-2 acetoxymethyl ester method following incubation at 37℃ for 45 minutes. After loading, cells were washed three times with DSBB. The assay mixture contained 120 mM NaCl, 5 mM KCl, 10 mM D-glucose, 2.0 mM $CaCl_2$, 1.5 mM $MgCl_2$ and 25 mM Hepes pH 7.4 and various amounts of compound 48/80 in a total volume of 3 ml. The fluoresence of the cell suspensions was measured in a cuvette using a dual-wavelength fluorometer (SPEX, CM system).[Ca^{2+}]i was calculated using the ratio of the fluoresence at 340 nm to that at 380 nm as described by Grynkiewicz et al.(18) Rmax was obtained by adding 60 μM digitonin to the cuvette at the end of the assays. Excess EGTA was subsequently added to obtain Rmin. A Kd of 224 nM(18) for fura 2/Ca^{2+} equilibrium was used.

GTPase assays: The effect of compound 48/80 upon GTPase activity was performed with the following method. Mast cells (5.6 x 10^6 cells) were homogenized with DSBB. The assay buffer contained 25 μl (15 μg protein) of mast cell homogenate, 10 mM EDTA, 0.2% bovine serum albumine, 0.5 mM ascorbic acid and 1 mM adenyl 5-(imidodiphosphate) mixed with [γ-^{32}P]GTP (specific activity 40,000 CPM/pmol) and different concentrations of compound 48/80 in a total volume of 0.1 ml. The reaction was stopped by the addition of 10 μl of cold 50% trichloroacetic acid (TCA) kept chilled on ice. The mixture was centrifuged for 25 minutes at 800 x g and then 90 μl of the supernatant was removed and assayed for ^{32}P-labeled inorganic phosphate(Pi). ^{32}Pi release was quantitated by the ammoniumolybdate extraction method(19). Nonspecific GTP hydrolysis was determined by the addition of 250 μM nonradioactive GTP.

[^{35}S]GTPγS binding: The method described by Northrup et al. was basically followed.(20) Binding of the [^{35}S]GTPγS bound to crude homogenate of mast cells was determined by incubating 25 μl (15 μg protein) of mast cell homogenate for one hour at 30°C with a reaction mixture(500 μl) containing 50 mM Tris-HCl at pH 7.7, 5 mM $MgCl_2$ 0.05% BSA, 1 mM EDTA, 0.5 mM CHAPS, 0.1M NaCl, 1 mM dithiothreitol and [^{35}S]GTPγS (40000 dpm/pmol).Following the incubation, the radioactivity of each sample was measured after filtration through the nitrocellulose filters.

Protein determination: Proteins were determined according to the method of Lowry et al.(21)

Statistics: One way analysis of variance was used for analysis of all of the data, which was followed by Student-Newman-Keuls test between groups to determine significance.

RESULTS

The release of histamine and [^{3}H]-5HT from mast cells increase ($p<0.01$) in a dose-dependent manner (Fig.1). The potency of compound 48/80 to release histamine (EC50=1.0 μg) is higher than that to release 5-HT(EC50 >1μg). Compound

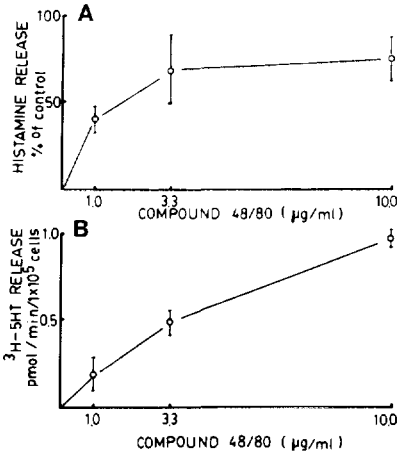

FIG. 1.Compound 48/80-induced release of histamine and [^{3}H]-5HT.
In [^{3}H]-5HT release study, mast cells were labelled with [^{3}H]-5HT. Estimations of the release of [^{3}H]-5HT and histamine have been described under "materials and methods". Mast cells were incubated with various concentrations of compound 48/80 for two minutes to stimulate histamine (Panel A) and [^{3}H]-5HT (Panel B) release. Each point is the means ± SEM of three separate experiments.

TABLE 1

EFFECT OF COMPOUND 48/80 AND GTPγS UPON THE ACCUMULATION OF INOSITOL PHOSPHATES

Incubation	IP_1	IP_2	IP_3
	DPM/10 min/mg protein		
Control	8648 ± 220	1385 ± 122	689 ± 84
Compound 48/80 (1.0 μg/ml)	10486 ± 248[a]	1408 ± 146	780 ± 101
Compound 48/80 (3.3 μg/ml)	18894 ± 301[b]	1895 ± 150[a]	1004 ± 119[a]
Compound 48/80 (10.0 μg/ml)	20496 ± 284[b]	1966 ± 124[b]	1201 ± 138[b]
GTPγS (10 μM)	11804 ± 233	1296 ± 117	880 ± 99
GTPγS (33 μM)	13649 ± 240[a]	1538 ± 110	1049 ± 144[a]
GTPγS (100 μM)	15469 ± 304[b]	1684 ± 202[a]	1201 ± 133[b]

Mast cells were prelabelled with tritiated myo-inositol and then incubated. IPs were separated according to the method described in "materials and methods".Data are means ± SEM from three experiments, each with triplicate samples. [a] $p<0.01$. [b] $p<0.001$ as compared with control.

48/80 significantly stimulated ($P<0.01$) the accumulation of IPs (Table 1). The GTPγS (33.3 μM), a potent activator of G-protein(22), causes at least 121% and 152% increase in the respective accumulation of IP_1 and IP_3.

Increasing the GTPγS concentration to 100 μM, GTPγS stimulates the accumulation of IPs. Both the accumulation effect of compound 48/80 and GTPγS

TABLE 2

EFFECT OF COMPOUND 48/80 AND GTPγS UPON CELL HOMOGENATE AND PURIFIED PHOSPHOLIPASE C

Incubation	PLC activity (nmol/min/mg protein)	
	Cell homogenate	purified enzyme
Control	11.3 ± 0.5	3896 ± 210
Compound 48/80 (3.3 μg/ml)	12.1 ± 0.8	4088 ± 301
GTPγS (33.3 μM)	11.6 ± 0.6	3729 ± 244

Mast cells (6.2×10^5 cells) were homogenized with glass to glass homogenizer. The cell homogenates were employed for determining the PLC activity. Purified PLC enzyme obtained from NG108-15 cell's membrane(25) was utilized. PLC activities were determined according to the method described in "materials and methods". The substrate employed here was PI. Data are means ± SEM from three experiments, each with duplicate samples. No significant difference between control and drug-treated groups were observed.

on IPs are concentration-dependent between 1 and 10 μg for compound 48/80 and between 10 μM and 100 μM for GTPγS. By using the method of Hauser et al (9) and Pappu and Hauser (10), the PI, PIP and PIP_2 were isolated and purified from rat peritoneal mast cells. (Figure 2). By using these purified inositol phospholipids as a substrate to test the catalytic activity of purified PLC, the result indicates that purified PLC catalyzed all the inositol phospholipids (Table 3). Both crude and purified PLC activity were not affected by compound 48/80 or by GTPγS even if the concentration of compound 48/80 increased to 10 μg/ml or the concentration of GTPγS increased to 100 μM.

Mast cells (10^7 cell/ml) were loaded with 10 μM fura-2 acetoxymethyl ester at 37°C for 45 minutes. Cells were washed three times with DSBB. Under these conditions, the basal $[Ca^{2+}]i$ was steadily maintained in the 110-140 nM range. The marked change in $[Ca^{2+}]i$ is illustrated in figure 3. which has been elicited upon the addition of 1 to 10 μg/ml compound 48/80. The stimulant effect of compound 48/80 was concentration-dependent between 1 and 10 μg as indicated in curve B, C and D (Fig.3). [γ-^{32}P]GTP hydrolysis(Figure 4) in mast cell homogenate was stimulated by compound 48/80 in a concentration-dependent manner but compound 48/80 had no effect on the binding of GTPγS to mast cell homogenate.

DISCUSSION

Compound 48/80 has indicated by observed results to have secretagogue effect upon mast cells and it causes the release of histamine and of reuptaken 5-HT.

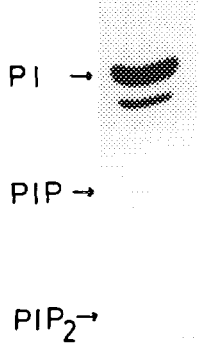

FIG. 2. Purified PI, PIP and PIP_2 The arrows point to the purified PI, PIP and PIP_2.

TABLE 3

EFFECT OF COMPOUND 48/80 UPON PURIFIED PHOSPHOLIPASE C CATALYTIC ACTIVITY IN THE PRESENCE OF DIFFERENT INOSITOL PHOSPHOLIPIDS

Incubation	IP_1	IP_2	IP_3
	Radioactivity (CPM)		
Control	1320 ± 210	1101 ± 228	1349 ± 198
Compound 48/80 (10 μg/ml)	1244 ± 188	1189 ± 202	1299 ± 223

PI, PIP and PIP_2 recovered from thin layer chromatograph plate were directly used for testing the catalytic activity of purified PLC. The assay system contained five thousand CPM of each substrate in 0.1% sodium deoxycholate. 3 mM $CaCl_2$. 50 mM Hepes, pH 7.0, and 0.1 μg of purified PLC in a total volume of 200 μl. Following 20 minutes at 37℃, the reaction was stopped and the metabolites extracted. Radioactivity of the metabolites were determined as described in "materials and methods". Data are means ± SEM from three separate experiments, each with duplicate samples.

These results is similar to that previously observed by Paton and Befus et al. (1.23) Figure 1. The potency of compound 48/80 to induce histamine release has been compared here with its pontency to elicit [^{3}H]-5HT release; The former is higher than the latter.

A rise in [Ca^{2+}]i is generally believed to be one of crucial steps in the sequence of secretory cell activation (24). Ishizuka and Nozawa reported

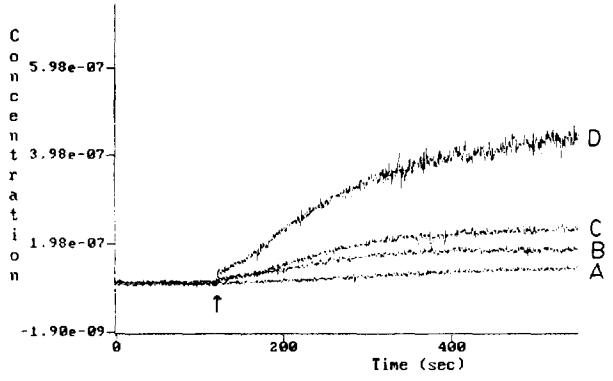

FIG. 3. The effect of compound 48/80 upon intracellular calcium in rat peritoneal mast cells.
Purified mast cells were prepared from rat peritoneal cavity and loaded with fura-2 as described under "materials and methods". Cells were maintained at 37℃ in DSBB, with gentle stirring, during the assay. Fluoresence emission was measured at 340 nm and 380 nm. The additions of different concentrations of compound 48/80(1.0, 3.3 and 10.0μg/ml) are indicated (↑) in the tracings. This tracing is representative one of four similar experiments. A indicates baseline; B, C and D indicate the addition of 1.0 μg/ml, 3.3 μg/ml and 10.0 μg/ml of compound 48/80, respectively.

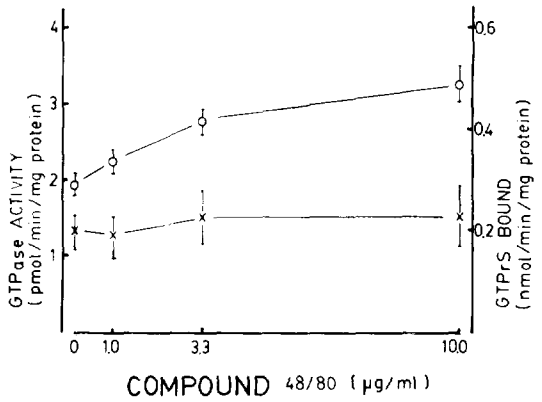

FIG. 4. Effect of compound 48/80 upon GTPase activity and GTPγS-binding. As indicated, mast cell homogenates were incubated with different concentrations of compound 48/80. o---o indicate GTPase activity. x---x indicate GTPγS-binding. Without compound 48/80 represents the basal levels of GTPase activity and GTPγS-binding.

that the $^{45}Ca^{2+}$ uptake of mast cell, upon activation by compound 48/80, was markedly increased up to 30 seconds and then recovered to the initial unactivated level.(24) We use Fura-2 to measure the $[Ca^{2+}]i$ of mast cells. Compound 48/80 increased the $[Ca^{2+}]i$ of mast cells and sustained for more than 9 minutes without returning to the initial unactivated level.

The PIP degradation in mast cells, upon stimulation by antigen, is inferred by the results of Ishizuka et al. to possibly be an initial step in the histamine release process (25). The accumulation of PA in rat mast cells has also been demonstrated by them to have occurred concomitantly with the stimulation of histamine release by compound 48/80 (15). PLC has been reported to localize in both the supernatant fraction of sonicated human platelets and the particulate fraction.(26) We used purified PLC from the membrane of NG108-15 cells, mast cell and mast cell homogenate to determine PLC activity. Our results indicate that PLC activity was indeed stimulated by compound 48/80 in intact mast cells (Table 1), but not in mast cell homogenates, nor in purified PLC.(Table 2). The PLC enzyme we purified catalyzed all three inositol phospholipids (namely PI, PIP and PIP_2).

Polyphosphoinositide phosphodiesterase of human neutrophil plasma membranes can be simply activated by adding GTP analogues in the presence of various concentrations of calcium that pertain in unstimulated cells (27). The GTP analog, GTPγS(33 μM), a potent activator of G-protein (22) caused a 121% and 152% increase in the respective accumulation of IP_1 and IP_3 in intact mast cells

but not in mast cell homogenates or in purified PLC. GTPγS is inferred by this result to be unable to directly stimulate PLC. It also suggests that a G-protein must be indispensibly associated with PLC.

Guanine nucleotide-binding regulatory proteins (G-proteins) mediate many receptor-coupled signal transduction events. Many species of G-proteins including Gs, Gi, transducin and Go have been well reported to posses GTPase activities (28 30). GTPase assumedly function as molecular switches that alternate between GTP- and GDP-bound conformational states (31). The stimulatory effect of compound 48/80 upon PLC activity of intact mast cells correlates well with (r=0.95) the stimulatory effect of compound 48/80 upon GTPase activity of mast cell homogenate.

The increased GTPase activity, in the presence of compound 48/80, generates more GDP for exchange of GTP. The increased rate of exchange of GTP for GDP then activated the PLC activity. The effect of compound 48/80 upon GTPase suggested that GTPase has potential in functioning as a target for useful drugs. Though adenyl cyclase is the only effector target of the G-proteins so far tested (32), results obtained here involving GTPase provide evidence that PLC activity was affected by compound 48/80 through G-protein activation.

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