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INHIBITORY MECHANISM OF TRITOQUALINE ON HISTAMINE RELEASE FROM MAST CELLS

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Abstract—Tritoqualine (TRQ, (+)-(*R**)-7-amino-4,5,6-triethoxy-3-[(*R**)-5,6,7,8-tetrahydro-4-methoxy-6-methyl-1,3-dioxolo[4,5-g]isoquinolin-5-yl]phthalide) strongly inhibited the increased metabolism of [^{3}H]arachidonic acid-labeled phospholipid and $^{45}Ca^{2+}$ influx in mast cells stimulated by compound 48/80 (compd 48/80), Concanavalin A (Con A) plus phosphatidylserine (PS), or 2,4-dinitrophenyl-coupled-ascaris extracts (DNP-asc). However, TRQ did not disturb the binding of ^{14}C-labeled compd 48/80 to the mast cell membrane. The activity of calmodulin purified from mastocytoma P-815 cells was inhibited by TRQ at IC_{50} 1.0 μM. From these results, it is concluded that the inhibitory mechanism of TRQ on stimulus-induced histamine release from mast cells may be mediated at least partially by the inhibition of Ca^{2+} influx and calmodulin activity.

Tritoqualine (TRQ, Fig. 1) is a compound which has been proven to be effective in clinical patients with allergic disorders such as pollinosis, asthma and urticaria [1, 2]; its effectiveness on patients with chronic hepatitis also was reported by Ishii *et al.* [3, 4]. Though the pharmacological mechanism of TRQ as an antiallergic agent is ascribed to its inhibitory activity on histidine decarboxylase (HDC) [5, 6], Umezu *et al.* reported that TRQ does not inhibit HDC but shows a marked inhibitory effect on the induction by several stimuli of the histamine release from rat peritoneal mast cells [7].

Accumulated evidence indicates that the increase in phospholipid metabolism associated with a rise in Ca^{2+} influx is necessary in order to trigger histamine release from mast cells by a variety of stimuli [8, 9]. In addition, more recent observation indicates that calmodulin activation relating to Ca^{2+} metabolism is also involved in the regulation of metabolism of arachidonic acid [10]. To clarify the mode of action of TRQ in histamine release from stimulated mast cells, we investigated the effect of TRQ on metabolism of phospholipid and Ca^{2+} influx accompanied by histamine release from mast cells.

EXPERIMENTAL PROCEDURES

Preparation of mast cells. Mast cells were collected from peritoneal cavity fluid of male Wistar rats weighing 300–350 g and were concentrated by Ficoll density gradient centrifugation to 90–93% purity. The mast cells were suspended in Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 11.9 mM $NaHCO_3$ and 5.6 mM glucose) containing 0.1% gelatin at pH 7.4 (Tyrode–gelatin solution). The number of cells was determined with a Coulter counter (model Z, Coulter Electronics, Hialeah, FL, U.S.A.). Immunized mast cells were prepared from the rats by treatment with or without 0.4 ml of 2,4-dinitrophenyl-coupled-ascaris (DNP-asc) antiserum 48 hr before decapitation.

Histamine release assay. Mast cells (1.9×10^5 cells), suspended in 1 ml of Tyrode–gelatin solution, were incubated at 37° for various times with or without a stimulus: compound 48/80 (compd 48/80, 0.4 μg), 2,4-dinitrophenyl-coupled-ascaris extracts (DNP-asc, 400 μg), or Concanavalin A (Con A, 50 μg) plus phosphatidylserine (PS, 10 μg). Each reaction was terminated by cooling, and the mixture was immediately centrifuged at 280 *g* for 1 min at 25°. Released histamine in the supernatant fraction was assayed fluorometrically by the method of Shore *et al.* [11].

Assay of [^{3}H]arachidonic acid release. Mast cells (2×10^5 cells) were suspended in a plastic dish (2.5 cm in diameter, Falcon) containing 2.5 ml of RPMI 1640 medium with 5 μCi of [^{3}H]arachidonic acid and were incubated at 37° for 4 hr in 5% CO_2-95% air with 100% humidity. During the incubation, [^{3}H]arachidonic acid-labeled mast cells adhered to the plastic wall. After removing the medium by

TRQ (Tritoqualine)

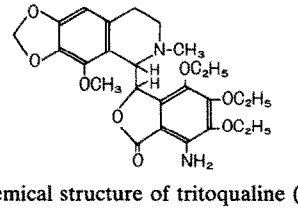

Fig. 1. Chemical structure of tritoqualine (TRQ).

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suction, the labeled mast cells were rinsed two times with cold Tyrode–gelatin solution (2 ml) and resuspended in fresh Tyrode–gelatin solution containing 10 μM TRQ. After preincubation at 37° for 30 min, bovine serum albumin (final concentration 0.1%) was added. The release of [^{3}H]arachidonic acid from labeled mast cells was initiated by the addition of 0.4 μg of compd 48/80 and was stopped time-dependently by diluting the mixture with 2 ml of the same cold solution. The cells were rinsed immediately with 2 ml of cold Tyrode–gelatin solution two times to remove the unbound radioactivity. The radioactivity in adhered mast cells was determined in 8 ml of Triton–toluene scintillant (3:1) containing 0.5% of 2,5-diphenyloxazole (PPO), after dissolving the cells with 1 N NaOH. The released radioactive arachidonic acid was calculated by subtracting the residual [^{3}H]-count from that of control cells that had not been incubated.

Metabolism of [^{3}H]arachidonic acid-derived phospholipid fractions in mast cells prelabeled with [^{3}H]arachidonic acid. Mast cells (2×10^5 cells) were preincubated with 2 μCi of [^{3}H]arachidonic acid for 20 min at 37° to label intracellular arachidonic acid pools. Aliquots of the prelabeled cell suspension were then transferred to incubation tubes with, and without, 10 μM TRQ in Tyrode–gelatin solution and preincubated at 37° for 15 min. The reaction was initiated by adding one of the following stimuli: compd 48/80 (0.4 μg), DNP-asc (400 μg) plus PS (10 μg), or Con A (50 μg) plus PS (10 μg). PS was added to the cell suspension 5 min prior to the addition of DNP-asc or Con A. The reaction was terminated by the addition of an ice-cold lipid extraction mixture of $CHCl_3$, methanol and conc. HCl (100:50:1, by vol.) containing 2,6-ditertial methyl-4-methylphenol to prevent oxidation of lipids. The lipid extraction was performed by shaking vigorously for 3 min. The lower organic phase was separated by centrifugation at 1000 *g* for 5 min at 4°, and the aqueous phase was washed once with 0.8 ml of chloroform ($CHCl_3$). The combined low phase was dried at room temperature under N_2 and dissolved in a solution of 0.1 ml of $CHCl_3$ and methanol (2:1, v/v). The phospholipids were separated by two-dimensional thin-layer chromatography on silica gel plates (Kieselgel F1, 500 LS 254, Schleicher & Schull, West Germany) in a solvent of $CHCl_3$, methanol, ammonium hydroxide and water (65:35:3:2, by vol.) for the first dimension, and then in a solvent of $CHCl_3$, acetone, methanol, acetic acid and water (10:4:2:2:1, by vol.) for the second dimension. After being stained with 0.8% (w/w) I_2 in methanol, the lipid-containing areas were scraped into counting vials and measured for radioactivity in 8 ml of scintillant which was a mixture of toluene (800 ml), Triton (200 ml) and water (45 ml) containing 0.5% PPO.

Separation of [^{3}H]arachidonic acid-derived prostaglandins in mast cells by thin-layer chromatography. The cell suspension was extracted after adjusting the pH to 3.5 to 4.0 with acetic acid. $CHCl_3$ (10 ml) was used twice to extract [^{3}H]prostaglandins from 1 ml of incubation medium. The $CHCl_3$ layers were added together and evaporated to dryness. The residue was then dissolved in a solvent of $CHCl_3$, heptane, acetic acid and ethanol (100:100:2:30, by vol.) and evaporated again under nitrogen to dryness. Samples were dissolved in 50 μl of benzene and were spotted on thin-layer chromatography plates (60F245, Merck, U.S.A.). Radioactive and nonradioactive prostaglandins (PG) $F_{2\alpha}$, E_2, D_2, A_2, thromboxane B_2 and 6-keto-$PGF_{1\alpha}$ were prepared in benzene and used as standards. The plate was developed in a solvent of hexane, ethylacetate, acetic acid and water (30:54:12:60, by vol.). The regions of the plates with the cold prostaglandin and arachidonic acid were sprayed with 10% phosphomolybdic acid. The plate was then placed in a hot air oven for 10 min to react the acid spray with the standards. Regions corresponding to the chemical standards were scraped into scintillation vials, and the radioactivity was determined.

Measurement of $^{45}Ca^{2+}$ influx. Mast cells were prepared according to the above procedure. Mast cells sensitized by DNP-asc antibodies were collected from rats injected with 0.4 ml of DNP-asc antiserum 48 hr before decapitation. Prepared mast cells were washed three times with 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) medium and preincubated at 37° for 5 min in the same medium. Cells were incubated with or without TRQ (10 μM) for 20 min and then were prelabeled with $^{45}Ca^{2+}$ by incubating with 2 μCi of $^{45}Ca^{2+}$. As a stimulus, compd 48/80 (0.5 μg), DNP-asc (400 μg) or Con A (50 μg) plus PS (10 μg) was added to the medium 15 min after addition of $^{45}Ca^{2+}$. After 15 sec of incubation at 37°, aliquots (0.1 ml) were filtered using HAWP 0.32 μm Millipore filters. The filters were immediately rinsed three times with 2 ml of either 0.25 M sucrose prepared from ion-free distilled water to remove unbound free $^{45}Ca^{2+}$ or 0.9% NaCl containing 1 mM EDTA and 1 mM $LaCl_2$ to remove surface-bound calcium [12]. After drying, the filters were placed in counting vials including 2 ml of methylcellosolve, and $^{45}Ca^{2+}$ radioactivity was determined in 10 ml of 0.5% PPO in toluene.

Assay of calmodulin. Calmodulin from mastocytoma P-815 cells was purified through a flufenazine-affinity column according to the method of Moore and Dedman [13] and was assayed by measuring the activity ratio of c-AMP phosphodiesterase (PDase) from beef brain in the presence and absence of TRQ.

Preparation of ^{14}C-labeled compd 48/80 and inhibitory effect of TRQ on binding to mast cells. [^{14}C]Compd 48/80 (tridecamer), which is an active fraction of compd 48/80 that induces Ca^{2+}-dependent histamine release from mast cells, was prepared according to the method of Koibuchi *et al.* [14]. After treatment of mast cells (5×10^5 cells) suspended in Tyrode–gelatin solution (0.2 ml) with TRQ (10 μM) or vehicle, [^{14}C]compd 48/80 (tridecamer, 0.25 μg/50 μl) was added to the suspension mixture and kept for 15 min at 0°. The reaction mixture was mixed with 20% Ficoll solution (1 ml) and centrifuged for 30 sec at 15,000 rpm. Radioactivity in the precipitated cell fraction was counted using a Triton–toluene scintillator.

Preparation of DNP-asc antibody. 2,4-Dinitrophenyl-coupled ascaris extract (DNP-asc) and rat DNP-asc antibody were prepared by the procedure of Azuma *et al.* [15]. *In vivo* sensitization of mast

cells was conducted by intraperitoneal injection of rat DNP-asc antibody to male Wistar rats.

Chemicals. TRQ, which was synthesized by Mitsubishi Chemical Ind. Ltd., was dissolved in 0.1 N HCl at concentrations of 1–2 mM and was added in the incubation mixture to a final concentration of 10–20 μM. RPMI 1640 medium was purchased from the Nissei Seiyaku Co. (Tokyo, Japan). [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (80–120 Ci/mmole) and $^{45}CaCl_2$ (10–40 mCi/mg Ca^{2+}) were purchased from Amersham-Searle (Amersham, England). Arachidonic acid, PGE_2, PGD_2, $PGF_{2\alpha}$, thromboxane B_2 and 6-keto $PGF_{1\alpha}$ were purchased from the Funakoshi Pharm. Co. (Tokyo, Japan). Other chemicals were all analytical grade preparations obtained commercially.

RESULTS

Effect of TRQ on the metabolism of [^{3}H]arachidonic acid in rat peritoneal mast cells stimulated by histamine releasing agents. Histamine release from rat peritoneal mast cells stimulated by compd 48/80 was strongly inhibited by TRQ at 10 μM (Fig. 2A, and Ref. 7). As reported by Sullivan and Parker [9], compd 48/80 time-dependently increased the incorporation of [^{3}H]arachidonic acid into membraneous phospholipids such as phosphatidylcholine (PC: Fig. 2B), phosphatidylinositol (PI: Fig. 2C) and phosphatidic acid (PA: Fig. 2D) of mast cells. Prior treatment of mast cells with 10 μM TRQ almost completely suppressed the increase in these [^{3}H]arachidonic acid-labeled phospholipids in compd 48/80-stimulated mast cells. Our previous studies showed that DNP-asc antigen or Con A plus PS-induced histamine release is suppressed by TRQ [7]. Figure 3 demonstrates the inhibitory effect of TRQ on the increase in [^{3}H]arachidonic acid-labeled phospholipids in the mast cells stimulated by these histamine releasing agents (Fig. 3). In these experiments, histamine release by these stimuli was inhibited to below 41% compared to the vehicle-treated mast cells. To examine further the role of TRQ on phospholipid metabolism, we investigated the release of [^{3}H]arachidonic acid from mast cells and the metabolism of [^{3}H]arachidonic acid into prostaglandins via the cyclooxygenase pathway. Compd 48/80 time-dependently increased the release of radioactivity from [^{3}H]arachidonic acid-prelabeled mast cells. TRQ inhibited strongly the [^{3}H]arachidonic acid release from mast cells at a concentration of 10 μM (Fig. 4). Though some of the released arachidonic acid served for the synthesis of prostaglandins, TRQ at 10 μM significantly suppressed these metabolic changes of [^{3}H]arachidonic acid (Table 1).

Effect of TRQ on $^{45}Ca^{2+}$ movement and calmodulin activity in mast cells. As shown in Fig. 5, intracellular $^{45}Ca^{2+}$ amounted to about one-fifteenth of total $^{45}Ca^{2+}$ in mast cells when pre-equilibrated with $^{45}Ca^{2+}$ (0.9 mM) for 15 min. TRQ did not disturb the exchange of $^{45}Ca^{2+}$ with the total $^{45}Ca^{2+}$ pool (extracellular plus intracellular pools) at a concentration of 10 μM. A noticeable increase in intracellular and extracellular $^{45}Ca^{2+}$ influx was observed by the treatment with compd 48/80; however, TRQ inhibited the increase in intracellular $^{45}Ca^{2+}$ (Fig. 5A), but extracellular $^{45}Ca^{2+}$ was not changed in TRQ-treated mast cells (Fig. 5B) that were stimulated by compd 48/80. TRQ also inhibited the intracellular $^{45}Ca^{2+}$ uptake in mast cells stimulated by DNP-asc antigen or Con A plus PS (Fig. 5A).

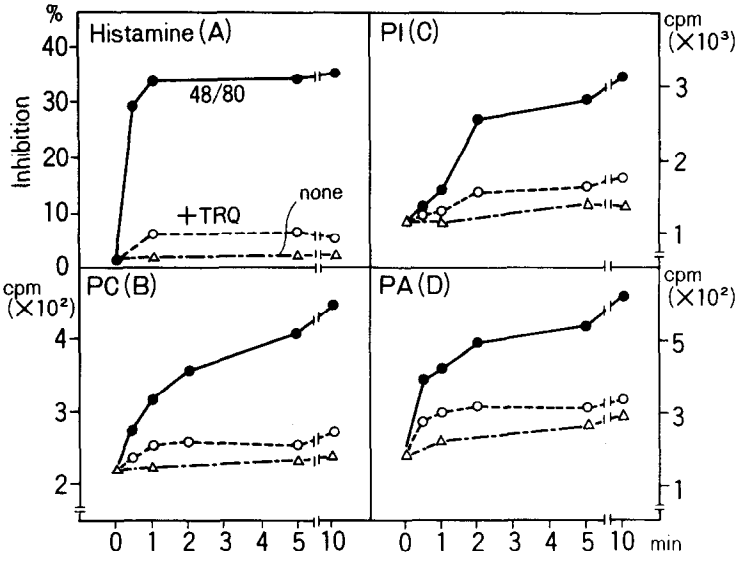

Fig. 2. Effect of TRQ on the metabolism of phospholipid in mast cells stimulated by compd 48/80. After being treated with TRQ (10 μM) (○) and vehicle (●), mast cells were stimulated by compd 48/80 (0.4 μg); (△) represents non-stimulated mast cells. (A) Histamine release from mast cells was expressed as a percent of total histamine in mast cells. In (B), (C) and (D), the time course changes in the amounts of phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidic acid (PA) are expressed.

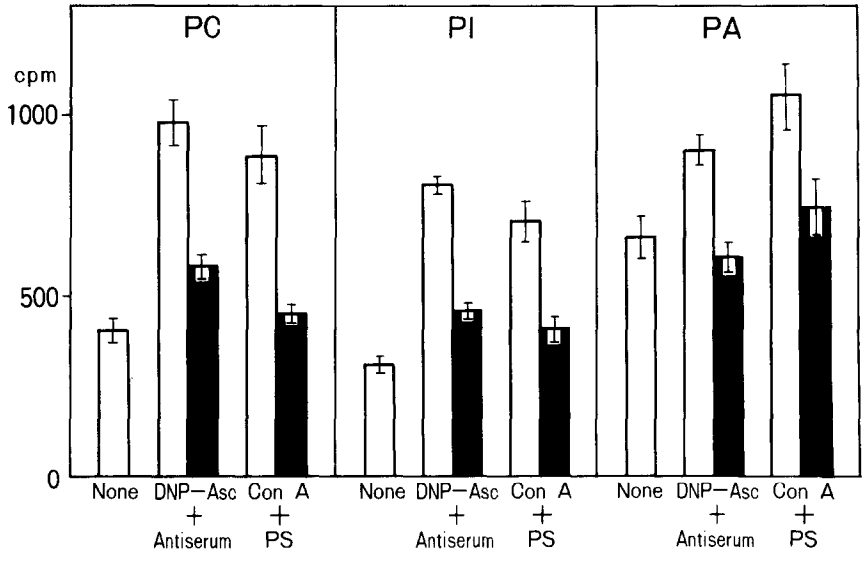

Fig. 3. Effect of TRQ on the metabolism of ^{3}H-labeled phospholipid in mast cells stimulated by DNP-asc plus PS or Con A plus PS. After being treated with TRQ (10 μM) or vehicle, mast cells were stimulated by DNP-asc + PS or Con A + PS, and incubation was continued for 15 min. White and black columns show the radioactivity in PC, PI or PA extracted from vehicle or TRQ-treated mast cells respectively. Each column represents the mean value ± S.E. of three determinations.

As previously reported, during the inhibition of Con A-induced histamine release by W-7, a calmodulin inhibitor, the histamine releasing response is thought to be regulated by calmodulin [16]. The inhibitory effect of TRQ on calmodulin prepared from mastocytoma P-815 cells was measured by the ratio of beef brain c-AMP phosphodiesterase activities in the presence and absence of TRQ (Fig. 6). TRQ dose-dependently inhibited the calmodulin activity, and the IC_{50} for calmodulin was approximately 1 μM.

Effect of TRQ on ^{14}C-labeled compd 48/80 (tridecamer) binding to the mast cells. TRQ at a concentration of 10 μM did not disturb the binding of ^{14}C-labeled compd 48/80 to the mast cells. The binding of ^{14}C-labeled compd 48/80 (tridecamer) to the mast cells treated with vehicle and 10 μM TRQ was 6.18 and 6.13 nmoles/10^5 cells respectively.

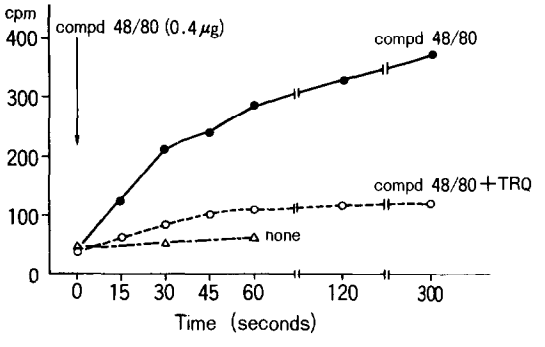

Fig. 4. Effect of TRQ on time-dependent [^{3}H]arachidonic acid release from mast cells stimulated by compd 48/80. Key: (●) vehicle-treated mast cells, (○) TRQ (10 μM)-treated mast cells, and (△) compd 48/80-nontreated mast cells. Released [^{3}H]arachidonic acid was calculated by subtracting the residual [^{3}H]arachidonic acid in the mast cells from total radioactivity added before the stimulation

DISCUSSION

The present study shows that TRQ effectively inhibited the early changes in phospholipid turnover and $^{45}Ca^{2+}$ uptake in compd 48/80-stimulated mast cells without affecting the binding activity of ^{14}C-labeled compd 48/80. This suggests that the inhibitory effect of TRQ on histamine release occurred at a step in the reactions subsequent to the binding of compd 48/80 to mast cells. The details of the sequential response that induces histamine release after compd 48/80 stimulation in mast cells have not been elucidated sufficiently. Recently, Koibuchi *et al.* [14] showed that compd 48/80 consists of several oligomeres that exhibit different modes of activation in response to extracellular Ca^{2+} for histamine release. Among them, a tridecamer of compd 48/80 used in the present experiments is a unique oligomer that induces a Ca^{2+}-dependent histamine release after Ca^{2+}-independent binding to mast cells. This characteristic of a tridecamer in the histamine releasing reaction is comparable to that of antigen-induced mast cell degradation. Both binding and the subsequent releasing activity of a tridecamer were partially suppressed by prior treatment of mast cells with DNP-asc antiserum, indicating partial sharing of binding sites of a tridecamer with an IgE antiserum [14]. Therefore, the inhibitory effect of TRQ on DNP-asc-induced histamine release cannot be ascribed to the binding of IgE to a membrane receptor but to the subsequent releasing mechanism.

Kennerly *et al.* [17] first reported that an early change in ^{32}P-labeled-PA, PI and PC fractions occurs in ^{32}P-labeled mast cells when stimulated by compd

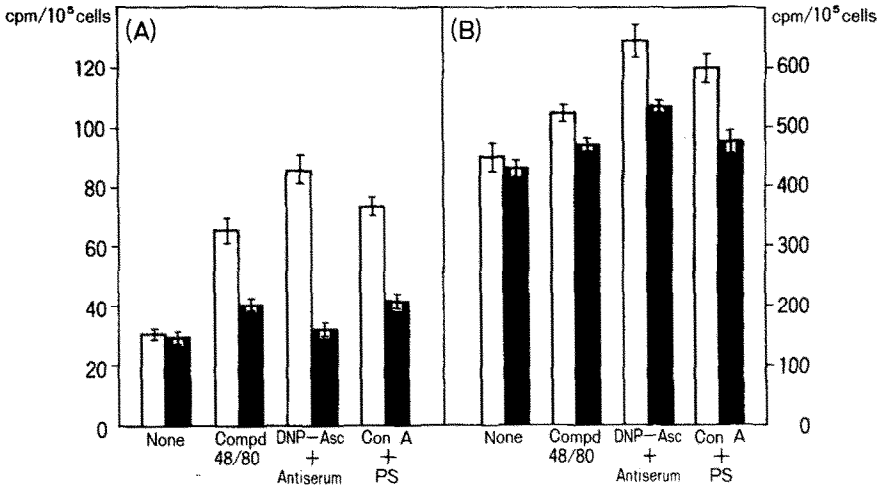

Fig. 5. Effect of TRQ on $^{45}Ca^{2+}$ influx in the mast cells. Mast cells were stimulated by compd 48/80, DNP-asc plus PS, or Con A plus PS. Intracellular and total $^{45}Ca^{2+}$ were measured as described in the experimental procedure. (A) intracellular $^{45}Ca^{2+}$. (B) total $^{45}Ca^{2+}$ pool (extracellular plus intracellular pools). White and black columns express vehicle-treated and TRQ (10 μM)-treated mast cells respectively. Each column represents the mean ± S.E. of triplicate measurements.

48/80 or Con A plus PS. Accumulation of [^{3}H]arachidonic acid into the phospholipid fraction in stimulated mast cells was inhibited almost completely by TRQ (Figs. 2 and 3). Accordingly, the subsequent release of arachidonic acid (Fig. 4) and the ensuing increase in prostaglandin products (Table 1) in compd 48/80-stimulated mast cells were also diminished by TRQ. Goth and Knoohuizen [18] suggested that the metabolites of arachidonic acid play an important role in histamine release from mast cells. They found also that compd 48/80-induced histamine release, as in the case of antigen-induced release, was inhibited by eicosa-5,8,11,14-tetraynoic acid, a blocker of both lipoxygenase and cyclooxygenase pathways of arachidonic acid. Thus, the inhibitory site of TRQ may be derived from the suppression of the phospholipid metabolism accompanied by the production of trigger materials for a histamine release.

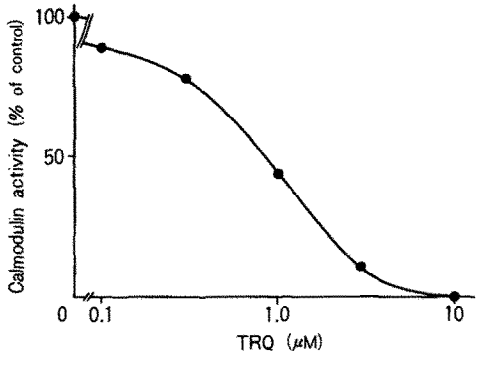

Fig. 6. Inhibitory effect of TRQ on calmodulin activity. Calmodulin was prepared from mastocytoma P-815 cells, and calmodulin activity was measured as described in Experimental Procedures.

In addition, Ca^{2+} is required as a mediator in stimulus-secretion coupling in mast cells [8], for example, Ca^{2+} is involved in the regulation of metabolism of phospholipids by modulating the activities of phospholipase A_2 (PLase A_2) in rabbit platelets [19] and phospholipase C (PLase C) from rat liver [20]. A possible requirement of Ca^{2+} for lipoxygenase activity has been reported in basophil leukemia cells [21]; also nordihydroguiaretic acid (NDGA), an inhibitor of Ca^{2+} influx and of lipoxygenase in neutrophils and mast cells, inhibits histamine release induced by compd 48/80 [22, 23] or by melittin, a potent PLase A_2 activator [22]. The inhibition of Ca^{2+} influx into compd 48/80-stimulated mast cells by TRQ treatment, therefore, may be the essential mechanism of the inhibitory effect of TRQ on histamine release. Douglas and Nemeth [16] demonstrated the possible role of calmodulin on the histamine releasing mechanism in the compd 48/80-stimulated mast cells. In addition, Suzuki *et al.* [24] showed the inhibitory action of calmodulin inhibitor on histamine release. TRQ inhibited mastocytoma

Table 1. Effect of TRQ on the biosynthesis of [^{3}H]arachidonic acid-derived prostaglandins in the mast cells stimulated by compd 48/80

	Radioactivity (cpm/10^5 cells/2 min)		
	None	48/80	48/80 + TRQ
PGD_2	110.4 ± 7.97	404.1 ± 60.7	219.3 ± 13.1
PGE_2	65.8 ± 13.9	184.1 ± 41.3	92.4 ± 10.5
$PGF^{2\alpha}$	60.4 ± 16.7	188.0 ± 73.4	78.8 ± 18.1

Methods are described in Experimental Procedures. Each value represents the mean ± S.E. of three measurements.

P-815 cell-derived calmodulin activity in a range of concentrations as low as 1 μM (Fig. 6), which concentration was very close to the IC_{50} against histamine release activity induced by compd 48/80 [7]. This differs from W-7, which required about a 100 times higher concentration for histamine release inhibition than the IC_{50} for calmodulin, suggesting the high penetrance of TRQ into mast cells. Though TRQ seems to show an inhibitory effect on histamine release from mast cells stimulated by compd 48/80 by an inhibition of calmodulin, Gietzen *et al.* [25] previously reported that compd 48/80 itself inhibits activities of the calmodulin-dependent enzymes erythrocyte Ca^{2+}-transporting ATPase and rat brain phosphodiesterase in *in vitro* experiments. There seems, therefore, to be a contradiction in the explanation for the inhibition of histamine release by calmodulin inhibition. It has not been demonstrated, however, whether or not compd 48/80 may be effective in the suppression of intracellular calmodulin activity in intact mast cells. In fact, Suzuki *et al.* [24] found biphasic activity of calmodulin inhibitor W-7, showing that W-7 not only inhibited the release of histamine from mast cells induced by Con A or A23187 at about 30 μM but also stimulated it at concentrations above 100 μM. This suggests an unresolved response of compd 48/80 as a calmodulin inhibitor in mast cells; this point needs to be explored further.

Hirata *et al.* reported that the binding of Con A to the receptor in the membrane surface of mast cells induces the activation of methyltransferase, followed by an increase in PC synthesis and the activation of phospholipase A to degrade the PC formed in association with the increased influx of Ca^{2+}, resulting in the increased synthesis of various metabolites of the lipoxygenase and cyclooxygenase pathways [26]. The activation of methyltransferase, however, is not assured in mast cells stimulated by compd 48/80 [26]. Considering that TRQ inhibits both changes in histamine release [7] and phospholipid metabolism (Fig. 2) induced not only by Con A plus PS and DNP-asc but also by compd 48/80, the inhibitory site of TRQ presumably is associated with a reaction common to both, except for the inhibition of methyltransferase.

Thus, the pharmacological effect of TRQ to inhibit Ca^{2+} influx or calmodulin-dependent enzyme activity may play an important role in inhibiting histamine release from mast cells by some stimuli.

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