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INHIBITION OF HUMAN PLATELET SECRETION AND OF Ca^{2+}, CALMODULIN-DEPENDENT PROTEIN PHOSPHORYLATION BY THE ANTIALLERGIC AGENT GMCHA

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Abstract—Calcium ion-dependent interaction with purified calmodulin (CaM), of a potent inhibitor of histamine release from mast cells, *trans*-4-guanidinomethylcyclohexanecarboxylic acid *p*-tert-butylphenylester (GMCHA), was investigated using 5-(dimethylamino)-1-naphthalenesulfonyl-calmodulin (dansyl-CaM). GMCHA undergoes a fluorescence increase with the Ca^{2+}–dansyl-CaM complex but there is no significant change in the dansyl-CaM fluorescence with GMCHA, up to a 10 μM in the absence of calcium ion. This suggests that binding of GMCHA to CaM is Ca^{2+}-dependent on the apparent K_d is approximately 1 μM. GMCHA suppressed the fluorescence of the hydrophobic probe 8-anilino-1-naphthalenesulfonate (ANS), in the presence of the Ca^{2+}–CaM complex, with an IC_{50} value of 3 μM.

[^{3}H]*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) bound to the purified CaM was displaced, in a concentration dependent manner by GMCHA, the K_i value of GMCHA against the binding of W-7 to CaM was 2.3 μM, and there was a selective inhibition of the Ca^{2+}–CaM induced activation of enzymes such as myosin light chain kinase. Increasing the CaM concentration in the presence of Ca^{2+} overcame the GMCHA-induced inhibition of myosin light chain kinase activation, with a K_i value of 2.7 μM. GMCHA at these concentrations is effective in inhibiting the release of histamine from mast cells. Moreover, this compound suppresses platelet secretion and relaxes vascular strips, at concentrations similar to those seen with the CaM interacting action and characteristic of CaM antagonists such as W-7.

GMCHA also inhibits the Ca^{2+}, CaM-dependent myosin light chain phosphorylation of human platelets. These results suggest that GMCHA, a potent inhibitor of histamine release from mast cells, suppresses platelet secretion, relaxes vascular smooth muscle and inhibits Ca^{2+}, CaM-dependent protein phosphorylation, all at similar concentrations.

In cases of bronchial asthma and rhinitis, the binding of antigen to IgE on the surface of mast cells causes a release of histamine, prostaglandins and other compounds. Although the critical role for calcium ions in the stimulus–secretion coupling is established, the molecular mechanism whereby calcium induces the exocytotic response is unknown [1]. Of all the known intracellular calcium binding proteins, only calmodulin (CaM)§ is ubiquitously distributed in eukaryotic cells and may be a universal intracellular receptor for calcium ions and the major physiological mediator of a wide range of cellular responses evoked by the cation [2]. Numerous antiallergic agents prevent the release of histamine from mast cells. Disodium cromoglycate inhibits the antigen–antibody-mediated influx of calcium ion into mast cells [3] and modulates the phosphorylation of mast-cell proteins [4, 5]. Several other antiallergy compounds seem to act in a similar manner [6] and all may have no significant effects on the functions of calmodulin [7]. *Trans*-4-guanidinomethylcyclohexanecarboxylic acid *p*-tert-butylphenylester (GMCHA) is a potent inhibitor of histamine release from mesenteric rat mast cells, as induced by antigen–antibody action, compound 48/80, polymyxin and neurotensin [8]. This compound exerts a dose-dependent inhibition of histamine release from isolated rat mast cells, without affecting the uptake of calcium ion [9]. We now report that GMCHA inhibits human platelet secretion, interacts with the Ca^{2+}–CaM complex and inhibits Ca^{2+}, CaM-dependent protein phosphorylation, all at similar concentrations. Using this agent we obtained pharmacological evidence for the role of Ca^{2+}, CaM-dependent protein phosphorylation in degranulation of mast cells and in platelet secretion.

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§ Abbreviations used: CaM, calmodulin; GMCHA, *trans*-4-guanidinomethylcyclohexane-carboxylic acid *p*-tert-butylphenylester; dansyl-CaM, 5-(dimethylamino)-1-naphthalenesulfonyl-calmodulin; ANS, 8-anilino-1-naphthalenesulfonate; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; dansyl chloride, 5-(dimethylamino-1-naphthalenesulfonyl chloride; Da, dalton.

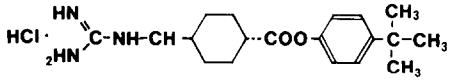

Fig. 1. Chemical structure of *trans*-4-guanidinomethyl-cyclohexanecarboxylic acid *p*-tert-butylphenylester (GMCHA).

MATERIALS AND METHODS

Materials. Bovine brain CaM was purified and checked for homogeneity, as described [10]. Myosin light chain kinase from chicken gizzard was purified as described [11, 12]. Myosin light chain was prepared from chicken gizzard by the method of Perrie and Perry [13]. CaM-deficient Ca^{2+}-dependent cyclic nucleotide phosphodiesterase was purified from bovine brain, as described [14]. 5-(Dimethylamino)-1-naphthalenesulfonylchloride (dansyl chloride) and 8-anilino-1-naphthalenesulfonate (ANS) were purchased from Wako Pure Chemical Industries, Ltd. Adenosine 5′-[γ-^{32}P]triphosphate and cyclic [^{3}H]guanosine 3′,5′-monophosphate (GMP) were obtained from Amersham International plc. [^{3}H]*N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7) was synthesized as described [15]. The chemical structure of *trans*-4-guanidinomethyl-cyclohexanecarboxylic acid *p*-tert-butylphenylester (GMCHA) is shown in Fig. 1.

Procedures. Myosin light chain kinase activity was assayed as reported [12, 16]. CaM was assayed for its ability to activate a fixed amount of CaM-deficient phosphodiesterase, under standard conditions [17]. The equilibrium binding procedure followed that described by Hummel and Dreyer [18]. The displacement of [^{3}H]W-7 from purified CaM in the presence of calcium ion by various concentrations of GMCHA was measured, as described [17]. All fluorescence measurements were made using an Aminco-Bowman spectrofluorometer with the thermostat set at 25°. Total dilution never exceeded 5%, and relative fluorescence values were uniformly corrected for dilution. Fluorescence intensity was determined with excitation at 350 nm, and emission intensity was monitored at 500 nm. ATP secretion and serotonin release from human platelets and protein phosphorylation in intact platelets were assayed as reported [19]. Effect of GMCHA on vascular strips was examined, as described [20].

RESULTS

Interaction of GMCHA with calmodulin

Binding of GMCHA to CaM was investigated by measuring the effect of GMCHA on the intensity of the dansyl-CaM fluorescence [21]. Titration of dansyl-CaM with GMCHA in the presence of Ca^{2+} led to a significant increase in the fluorescence of dansyl-CaM, from 0.1 μM to 10 μM of GMCHA, with an apparent K_d value of approximately 1.0 μM (Fig. 2). In the absence of calcium ion, there was no significant change in the fluorescence intensity of dansyl-CaM with GMCHA, up to 10 μM. These results suggest that the interaction between CaM and GMCHA is Ca^{2+}-dependent.

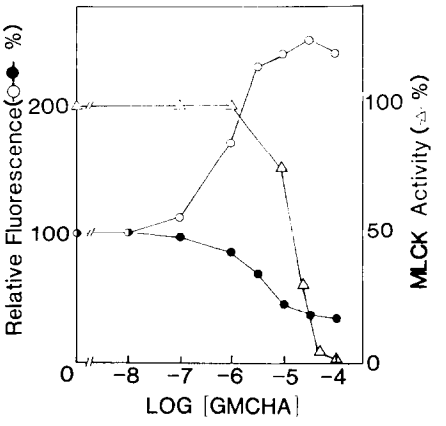

Fig. 2. Interaction of GMCHA with CaM monitored by fluorescence measurement and myosin light chain kinase inhibition. Titration of dansyl-CaM (1.2 μM) with GMCHA (—○—), effect of GMCHA on 4 μM ANS fluorescence with Ca^{2+}–CaM (2 μM) complex (—●—) and inhibiton of Ca^{2+}–CaM induced activation of myosin light chain kinase (—△—). Preparation of dansyl-CaM and fluorescence measurements were as described in Materials and Methods. Myosin light chain kinase activity was assayed as reported [11].

It has been reported that the binding of Ca^{2+} to CaM results in exposure of a hydrophobic domain and that this domain may serve as the interface for the Ca^{2+}-dependent binding to hydrophobic probes such as 8-anilino-1-naphthalenesulfonate (ANS) as well as enzymes and CaM antagonists [22, 23]. The effects of GMCHA on the fluorescence of ANS in the presence of CaM and calcium ion were investigated to determine the affinity of this compound for the hydrophobic region of CaM.

As shown in Fig. 2, GMCHA suppressed the fluorescence of the ANS–Ca^{2+}–CaM complex, in a concentration dependent fashion, and the IC_{50} value of GMCHA was 3.0 μM. As the CaM antagonists such as W-7 inhibit histamine release from mast cells [24] and also platelet secretion [25], and [^{3}H]W-7 has been demonstrated to bind to CaM in a Ca^{2+}-dependent manner [17], we investigated the relationship between GMCHA binding sites and W-7 binding sites on CaM. The [^{3}H]W-7 binding to CaM was inhibited by GMCHA, in a concentration-dependent fashion (Fig. 3). The stoichiometry of interaction between GMCHA and [^{3}H]W-7–CaM complex in the presence of Ca^{2+} was determined from a Dixon plot [26] and the K_i value of this compound against the binding of W-7 was 2.3 μM.

The specificity and potency of GMCHA as a CaM antagonist were examined by analysis of its inhibitory effect on myosin light chain kinase (Fig. 2). Increasing the concentration of GMCHA progressively inhibited the activation of myosin kinase. The concentration of GMCHA which inhibited the activation of myosin light chain kinase by 50% was 15 μM. On the other hand, GMCHA inhibited less effectively the catalytic fragment of this protein kinase produced by trypsin treatment [16], in the absence of CaM and with an IC_{50} value of 150 μM. We then determined whether the GMCHA-induced inhibition of myosin

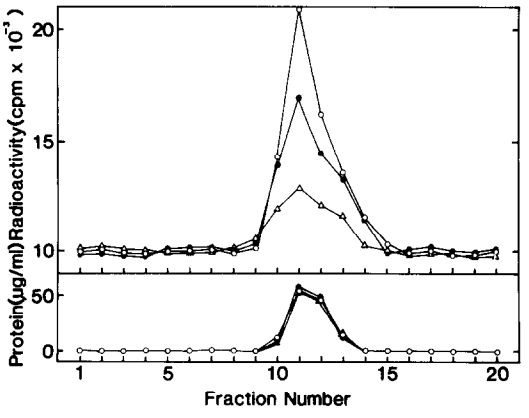

Fig. 3. Effect of GMCHA on W-7 binding to CaM. Elution profile for measurement of W-7 binding to CaM. Sephadex G-50 (0.9 × 27.0 cm) was pre-equilibrated with buffer containing 20 mM Tris–HCl, pH = 7.5, 20 mM imidazole, 3 mM magnesium acetate, 0.5 μM [^{3}H]W-7, 100 μM $CaCl_2$ and various concentrations of GMCHA (—○—), none; —●—, 2 μM; —△—, 5 μM). Purified bovine brain CaM (200 μg) was used for each.

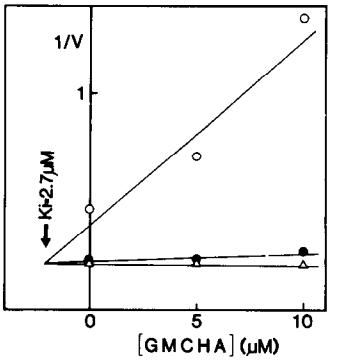

Fig. 4. Kinetic analysis of GMCHA-induced inhibition of activation of myosin light chain kinase was carried out using Dixon plots [26]. Myosin light chain kinase activity was measured in the presence of 0.1 mM $CaCl_2$ and 10 ng/ml (—○—), 1 μg/ml (—●—) or 30 μg/ml (—△—) CaM.

light chain kinase activation by Ca^{2+}-CaM could be overcome by increasing the concentration of CaM. Kinetic analysis of the GMCHA-induced inhibition of activation of myosin kinase revealed an inhibition of this activity, in a competitive fashion with CaM and with a K_i value of 2.7 μM (Fig. 4). The results (Table 1) indicate that GMCHA interacts with the Ca^{2+}–CaM complex, at concentrations similar to those which inhibit Ca^{2+}–CaM dependent enzymes such as myosin light chain kinase (Fig. 4) and suppresses histamine release from mast cells [9].

Inhibition of platelet secretion

The effect of GMCHA on human platelet ATP secretion and aggregation induced by thrombin (0.1 U/ml) was also investigated. GMCHA produced a dose-dependent inhibition of ATP secretion and platelet aggregation by 0.1 U/ml thrombin, the IC_{50} values being 3.1 μM and 6.8 μM, respectively (Fig. 5A). [^{14}C]Serotonin secretion of human platelets by collagen (1 μg/ml) and thrombin (0.07 U/ml) was also suppressed by GMCHA, the IC_{50} value being 4.8 μM and 2.6 μM respectively (Fig. 6).

^{32}P-labelled platelets were activated by thrombin (0.1 U/ml) in the presence (3, 10, 30 μM) or absence of GMCHA. When the stimulus-induced phosphorylation of platelet proteins was determined by autoradiography following SDS–polyacrylamide gel electrophoresis, the 20 kDa myosin light chain was phosphorylated, time dependently. GMCHA inhibited the phosphorylation of myosin light chain and the IC_{50} value at 30 sec after the addition of thrombin was 5.0 μM (Fig. 7).

If GMCHA does have inhibitory effects on mast cell degranulation and platelet secretion through Ca^{2+}-dependent interactions with CaM, then this compound should be a potent vascular relaxant. In fact, GMCHA produced a significant relaxation of vascular strips which contracted in the presence of KCl in a concentration of 20 mM and the ED_{50} (molar concentration) in rabbit mesenteric arteries was 3.0 μM (Fig. 5B).

Table 1. Effect of GMCHA on the intracellular calcium, calmodulin-dependent messenger system and various Ca^{2+}-dependent cell functions

	ED_{50} or K_i* (μM)	N†
Change in fluorescence of dansyl-CaM	1.0	3
Change in fluorescence of ANS with Ca^{2+}–CaM complex	3.0	3
Inhibition of [^{3}H]W-7 binding to Ca^{2+}–CaM complex	2.3*	3
Inhibition of myosin light chain kinase	2.7*	3
Inhibition of platelet myosin light chain phosphorylation	5.0	3
Inhibition of ATP release from platelets	3.1	3
Inhibition of platelet serotonin secretion induced by thrombin	2.6	3
Inhibition of platelet serotonin secretion induced by collagen	4.8	3
Relaxation of vascular strips	3.0	3
Inhibition of histamine release from mast cell	6.1†	

* K_i values determined by Dixon plot [26].
† N is the number of experiments.
‡ This data is from Akagi *et al.* [9].

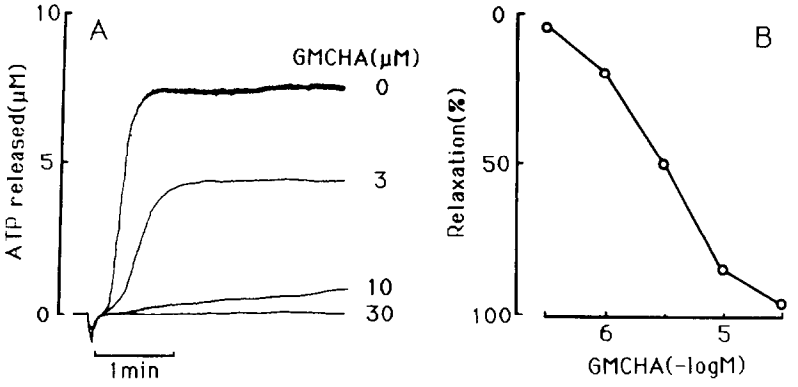

Fig. 5. Effect of GMCHA on ATP release from platelets (A) and contraction of vascular strips (B). (A) Secretion of ATP was measured by the firefly luminescence assay method [25]. Washed human platelets were activated by 0.1 U/ml thrombin (arrow). The final concentration of GMCHA is shown in μM by each curve. (B) Dose–response curve for the vascular relaxing effects of GMCHA in K^+-contracted stripes of rabbit mesenteric artery. Experimental procedures were as described in Materials and Methods.

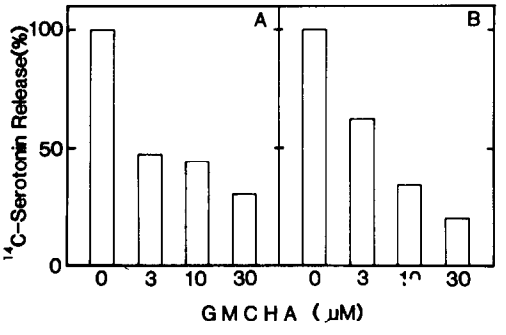

Fig. 6. Effect of GMCHA on [^{14}C]serotonin release from human platelets stimulated by (A) thrombin (0.07 U/ml) or (B) collagen (1 μg/ml). Control release was 69% of the total amount of radioactivity originally contained in platelets.

Table 1 shows the effective concentrations of GMCHA in interaction with the Ca^{2+}–CaM complex, inhibition of Ca^{2+}, CaM-dependent enzyme, suppression of Ca^{2+}-dependent secretion of mast cells and platelets, and relaxation of vascular strips. The affinities of this compound for CaM, determined by several procedures, are similar to the concentrations which inhibit release reactions from mast cells and platelets. These results suggest that GMCHA inhibits these Ca^{2+}-dependent cell functions through inhibitory effects on Ca^{2+}, CaM-dependent protein phosphorylation.

DISCUSSION

We obtained evidence that GMCHA, a novel inhibitor of histamine release from mast cells [8],

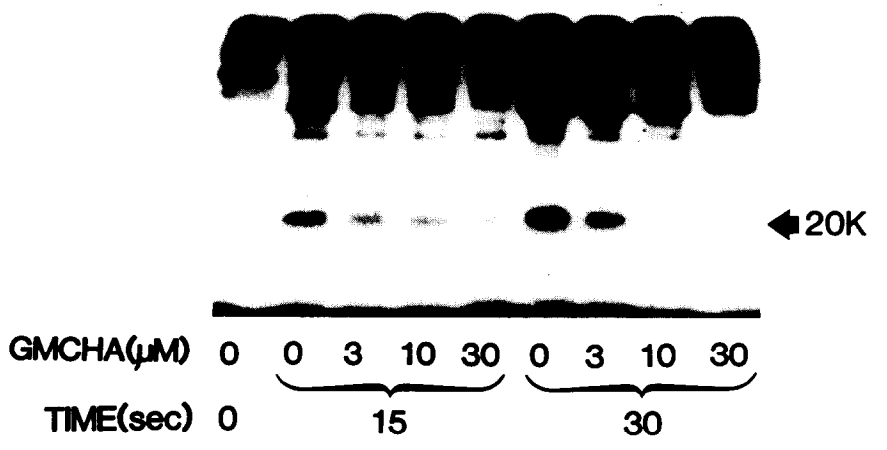

Fig. 7. Autoradiography of the thrombin-induced protein phosphorylation of human platelets following SDS–polyacrylamide gel electrophoresis at 0, 15 and 30 sec after addition of thrombin (0.1 U/ml), in the absence or presence of 3 μM, 10 μM or 30 μM GMCHA. Protein phosphorylation analysis was done as reported [18]. We confirmed that the 20 kDa protein phosphorylated was myosin light chain, by urea–glycerol gel electrophoresis, as reported [33] and immunoblotting with antibody against purified myosin light chain from chicken gizzard.

interacts with the Ca^{2+}–CaM complex, as determined using various procedures. We found that the affinity of GMCHA for Ca^{2+}–CaM is high and the dissociation constants are approximately 1–3 μM. These concentrations are similar to those which inhibit histamine release from mast cells [9], platelet secretion, and relax vascular strips.

The results suggest that GMCHA suppresses histamine release from mast cells induced by antigen–antibody action, compound 48/80, polymyxin and neurotensin [8], through interactions with the Ca^{2+}–CaM complex and by inhibiting Ca^{2+}, CaM-dependent protein phosphorylation but without affecting the Ca^{2+} uptake [9]. No significant change in cAMP contents in rat mast cells were noted with 50 μM GMCHA [9]. Calmodulin mediates the control of a large number of key enzymes, including myosin light chain kinase [2]. The calcium-dependent protein phosphorylation of mast cells has been demonstrated [27]. It may be of general importance that stimulation of mast cells with the ionophore A23187 or compound 48/80 leads to a rapid, Ca^{2+}-dependent phosphorylation of proteins with molecular weights of 68,000, 59,000 and 42,000. This Ca^{2+}-dependent protein phosphorylation accompanies or precedes histamine secretion from mast cells. Moreover, calmodulin antagonists such as neuroleptic drugs and W-7 [24] were reported to inhibit mast cell secretion elicited by antigen, by compound 48/80, and by the calcium ionophore A23187. It should be noted that these calmodulin antagonists have no effect on ^{45}Ca uptake in response to A23187, an event similar to that seen with GMCHA. In view of the data presented in this paper, calmodulin might facilitate exocytosis and hence be implicated in the mechanism of GMCHA action on platelet and mast cell secretion [9].

Calmodulin has also been more directly implicated in a number of secretory processes, including the release of ATP and serotonin from platelets [25], of neurotransmitters from synaptosomes [28], of insulin from β-cells of the islets of Langerhans [29] and of fluid and electrolytes from the small intestine [30]. GMCHA which we found to be a novel CaM antagonist inhibited ATP and serotonin secretion from human platelets as well as histamine release from mast cells [9]. The possible effects of Ca^{2+}–CaM on the structural elements of the secretory cell cytoskeleton should be considered. The cytoskeleton seems to be involved in cell motility, including intracytoplasmic movements of secretory granules and various endocytotic and exocytotic vesicles [31]. The contractile response requires the phosphorylation of myosin by myosin light chain kinase, which is regulated by the Ca^{2+}–CaM complex in non-muscle cells and in smooth muscle [32]. In fact, GMCHA relaxed vascular smooth muscle and suppressed platelet secretion, in association with the inhibition of myosin light chain phosphorylation.

Thus, GMCHA is a novel, potent inhibitor of histamine release from mast cells [9] and a selective inhibitor of Ca^{2+}, CaM-dependent protein phosphorylation. Its effects on platelet secretion are inhibitory and it is a potent vascular smooth muscle relaxant. This compound with a unique molecular mechanism differing from that of antiallergic drugs heretofore available should be useful for extended studies related to allergy.

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