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DESENSITIZATION BY GLYCYRRHETINIC ACID OF OTHER STIMULATORY SUBSTANCE-INDUCED INCREASES IN INTRACELLULAR CALCIUM IN A VARIETY OF CELL TYPES

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Abstract—The effects of glycyrrhizin and its aglycone, glycyrrhetinic acid, on the cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) in mastocytoma P-815 cells, DNP-*Ascaris* (antigen) sensitized mast cells, hepatocytes, fibroblasts and endothelial cells were investigated. In these cell types, glycyrrhetinic acid in the concentration range of 20 to 100 μM caused an increase in $[Ca^{2+}]_i$ and inhibited calcium increases induced by an antigen (mast cells), ATP, phenylephrine (hepatocytes) and thrombin (fibroblasts and endothelial cells). Stimulation with phenylephrine, in place of glycyrrhetinic acid, did not inhibit subsequent calcium increases induced by phenylephrine or ATP. On the other hand, glycyrrhizin at concentrations up to 100 μM neither caused an increase in $[Ca^{2+}]_i$ nor inhibited calcium increases induced by other stimulatory substances. These results suggest that the inhibition of the calcium-mediated signal pathway may participate in the cytostatic actions of glycyrrhetinic acid.

Glycyrrhizin, extracted from the roots of licorice (*Glycyrrhiza glabra*), and its aglycone, glycyrrhetinic acid, exhibit various anti-inflammatory [1], anti-allergic [2], anti-gastric ulcer [3], anti-hepatitis [4] and anti-hepatotoxic [5] activities. Biochemical studies on the action of glycyrrhetinic acid have revealed that it has inhibitory effects on the phospholipase A_2-like activity of macrophages [6] and the lipoxygenase activity of mastocytoma P-815 cells [7]. Moreover, it has been shown to block acetylcholine-induced potential amplitudes in diaphragm muscles [8], suggesting that it may affect Ca^{2+} mobilization. We recently demonstrated that glycyrrhetinic acid, but not glycyrrhizin, inhibited histamine release from antigen-stimulated mast cells [9]. Antigen stimulation induced a rapid increase in cytoplasmic Ca^{2+} in mast cells [10], and this elevation of intracellular Ca^{2+} concentration appears to be the primary trigger for initiating histamine release from the cells [11]. Therefore, it is inferred that the site of action of glycyrrhetinic acid in histamine release reaction of mast cells may be a receptor-mediated increase in intracellular Ca^{2+}. Furthermore, glycyrrhetinic acid exerts its inhibitory action on carbon tetrachloride- [12] or galactosamine- [13] induced experimental hapatitis, which are known to be mediated by an increase of intracellular Ca^{2+}. These results suggest that glycyrrhetinic acid may affect calcium metabolism in various cells. Accordingly, we examined the effects of glycyrrhizin and glycyrrhetinic acid on calcium increases induced by various stimulatory substances, such as an antigen, ATP, phenylephrine and the calcium ionophore ionomycin, in mast cells, mastocytoma P-815 cells and hepatocytes.

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MATERIALS AND METHODS

Materials. Glycyrrhetinic acid and glycyrrhizin-NH_4 were supplied by Minophagen (Tokyo, Japan). Fura-2 AM [fura-2 penta(acetoxymethyl)ester] was purchased from Molecular Probes, Inc. (Eugene, OR, U.S.A.). *Bordetella pertussis* (adjuvant) was from the Chiba Serum Institute (Chiba, Japan). Thrombin, collagenase (type IV) and insulin were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ionomycin was from the Calbiochem Co. (La Jolla, CA, U.S.A.). William's medium E was from Flow Laboratories (Irvine, Scotland). Dulbecco's Modified Eagle's Medium (DMEM) was from the Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals were of reagent grade.

*Sensitization to DNP-*Ascaris. DNP-*Ascaris* (conjugated dinitrophenylated *Ascaris* extract) was prepared by the method of Strejan and Campbell [14], and sensitization to DNP-*Ascaris* was achieved according to the method of Tada and Okumura [15]. Male Wistar rats (250–300 g) were immunized by injection, into their forefoot pads, of a total 1 mg of DNP-*Ascaris* mixed with 10^{10} units of *Bordetella pertussis* (adjuvant). Five days later, 0.5 mg of DNP-*Ascaris* alone was injected subcutaneously into their backs. Eight days after the first immunization, the sensitized rats were killed, and mast cells were isolated.

Cells and cell culture. Mastocytoma P-815 cells were maintained in a suspension culture in Fischer's medium supplemented with 5% fetal bovine serum [9]. Cells in the logarithmic phase of growth were

used for the experiments. Mast cells were collected from the peritoneal cavity fluid of male Wistar rats, which had been sensitized to DNP-*Ascaris*, and then separated by Ficoll density gradient centrifugation [16] to 90–93% purity. Parenchymal hepatocytes were isolated from rat liver by the collagenase perfusion method [17]. The hepatocytes were cultivated on collagen-coated coverslips (circular; diameter, 13.2 mm) at a density of 1×10^5 cells/cm^2 in William's medium E, which had been supplemented with 5% calf serum, 10^{-7} M insulin and 10^{-7} M dexamethasone. The cells were incubated for 16–24 hr before the experiments. Collagen was prepared from rat tail tendons [18]. Fibroblasts were isolated from rat dorsal skin by the standard method, and porcine aortic endothelial cells were isolated and grown as described previously [19]. These cells were basically grown in DMEM containing 10% fetal bovine serum and used between passages 4 and 8. For experiments, cells were grown on collagen-coated coverslips (circular; diameter, 13.2 mm) to confluency.

Measurement of the cytosolic free calcium concentration. The cytosolic free calcium concentration ($[Ca^{2+}]_i$) was assayed using the fluorescent calcium indicator, fura-2. Mastocytoma P-815 cells and rat peritoneal mast cells in suspension were centrifuged, washed, and then resuspended in 10 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-buffered Hanks' solution (pH 7.4, 1.2 mM Ca^{2+}) containing 3 μM fura-2 AM for 1 hr at 37°. The concentration of cells during the incubation with fura-2 AM was $1–2 \times 10^6$ cells/mL. After loading, the cells were centrifuged, washed, and then resuspended in the same buffer at a cell density of 3×10^6 cells/mL. Aliquots were placed in quartz cuvettes with a magnetic stirrer and then fluorescence measurements were carried out with a fluorescence spectrophotometer (Jasco, CAF-100). Hepatocytes, fibroblasts and endothelial cells cultured on coverslips were washed with 10 mM Hepes-buffered Hanks' solution (pH 7.4, 1.2 mM Ca^{2+}), and fura-2 AM (final concentration, 10 μM) was added. After loading for 1 hr at 37°, the cells were washed three times with the same buffer. Each coverslip was put in a quartz cuvette, which was placed diagonally in the cuvette holder with the cell monolayer facing the excitation beam. The fura-2 fluorescence was measured with a fluorescence spectrophotometer (Jasco, CAF-100). The measurements and calibration were performed according to Grynkiewicz *et al.* [20].

RESULTS

Effects of glycyrrhetinic acid and glycyrrhizin on $[Ca^{2+}]_i$ in mastocytoma P-815 cells. In the beginning, we investigated the effect of glycyrrhetinic acid alone on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The inset of Fig. 1 depicts a representative tracing of fluorescence change in the suspension of fura-2-loaded mastocytoma P-815 cells. Glycyrrhetinic acid at 25 μM increased $[Ca^{2+}]_i$ from the basal value of 50 nM to a maximum of about 150 nM within 1 min, and the level declined gradually to near the basal level within 5 min. As shown in Fig. 1, the $[Ca^{2+}]_i$ rise

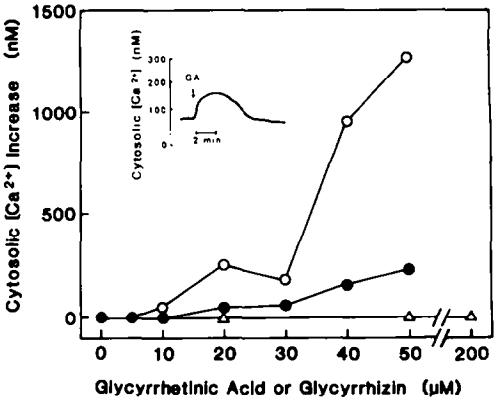

Fig. 1. Concentration-dependent increase in $[Ca^{2+}]_i$ caused by glycyrrhetinic acid and glycyrrhizin in mastocytoma P-815 cells. Cells were loaded with fura-2 AM, and then the peak $[Ca^{2+}]_i$ levels induced by various doses of glycyrrhizin (△) or glycyrrhetinic acid in the presence (●) or absence (○) of 3 mM EGTA were determined as described under Materials and Methods. The inset shows the time course of the changes in $[Ca^{2+}]_i$ in response to 25 μM glycyrrhetinic acid. Glycyrrhetinic acid was added at the time indicated by the arrow. The recording shown is a representative of three independent experiments that yielded similar results.

induced by glycyrrhetinic acid was concentration-dependent in Ca^{2+}-containing medium, but this rise was blocked almost completely in a Ca^{2+}-free medium, suggesting that the $[Ca^{2+}]_i$ changes were due to the influx of extracellular Ca^{2+}. To investigate whether or not the entry of Ca^{2+} is through channels, we examined the effects of Ca^{2+} channel blockers nifedipine, verapamil and La^{3+} on glycyrrhetinic acid-induced $[Ca^{2+}]_i$ increase. No Ca^{2+} channel blockers, however, affected the $[Ca^{2+}]_i$ increase (data not shown). Furthermore, the glycyrrhetinic acid-induced increase in $[Ca^{2+}]_i$ was not reduced significantly in Na^+-depleted choline medium. Another Na^+ substitute, K^+, was also found to be equally unsuccessful in reducing the glycyrrhetinic acid-induced increase in $[Ca^{2+}]_i$ (data not shown). In contrast to the stimulatory effect of glycyrrhetinic acid on $[Ca^{2+}]_i$, glycyrrhizin caused no change in $[Ca^{2+}]_i$ at any concentration up to 200 μM.

Effects of glycyrrhetinic acid and glycyrrhizin on calcium increases induced by calcium-mobilizing agonists in a variety of cell types. We next examined the effects of glycyrrhetinic acid and glycyrrhizin on receptor-mediated mobilization of Ca^{2+}. ATP at 60 μM induced calcium increases via the activation of a P_2-type adenosine receptor in mastocytoma P-815 cells (trace b in Fig. 2A). However, treatment of mastocytoma P-815 cells with 25 μM glycyrrhetinic acid (trace a in Fig. 2A), but not 100 μM glycyrrhizin (Fig. 2B), completely inhibited the subsequent calcium increase induced by ATP. We tested the glycyrrhetinic acid responsiveness of various cells which have Ca^{2+}-mobilizing receptors in terms of inhibition of $[Ca^{2+}]_i$ elevation. Stimulation of mast cells by antigen against DNP-*Ascaris* induced a rapid and transient increase in $[Ca^{2+}]_i$ as shown in trace b in

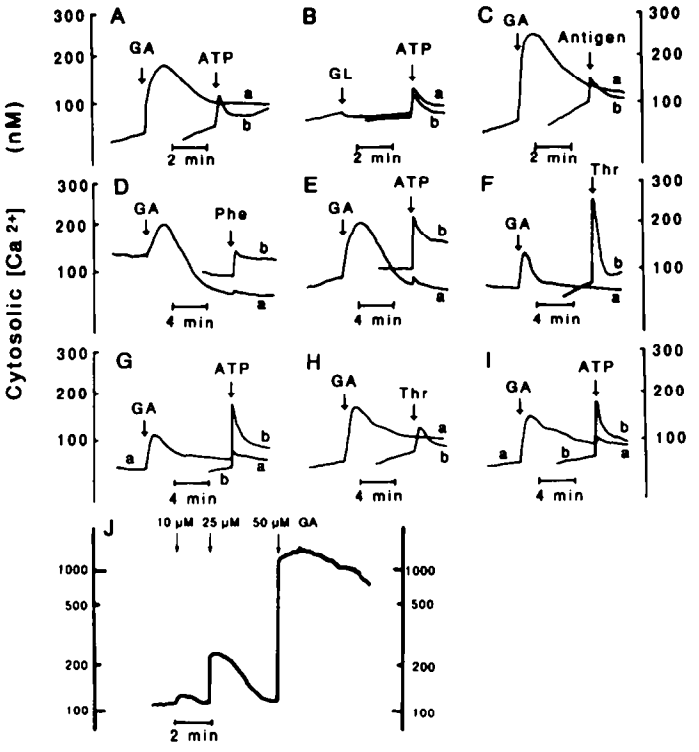

Fig. 2. Effect of glycyrrhetinic acid or glycyrrhizin on calcium increases induced by calcium-mobilizing agonists in mastocytoma P-815 cells, mast cells, hepatocytes, fibroblasts and endothelial cells. Cells were loaded with fura-2 AM, and then $[Ca^{2+}]_i$ was determined as described under Materials and Methods. (A and B) Mastocytoma P-815 cells; 60 μM ATP alone (trace b) or following 25 μM glycyrrhetinic acid (A) or 100 μM glycyrrhizin (B) (trace a) was added to mastocytoma P-815 cells. (C) Mast cells; 10 μg/mL of DNP-*Ascaris* (antigen) alone (trace b) or following 50 μM glycyrrhetinic acid (trace a) was added to mast cells which had been isolated from rats immunized with DNP-*Ascaris*. (D and E) Hepatocytes; 10 μM phenylephrine (D) or 60 μM ATP (E) alone (trace b) or following 50 μM glycyrrhetinic acid (trace a) was added to hepatocytes. (F and G) Fibroblasts; 0.05 units/mL of thrombin (F) or 60 μM ATP (G) alone (trace b) or following 50 μM glycyrrhetinic acid (trace a) was added to fibroblasts. (H and I) Endothelial cells; 0.05 units/mL of thrombin (H) or 60 μM ATP (I) alone (trace b) or following 50 μM glycyrrhetinic acid (trace a) was added to endothelial cells. (J) Mastocytoma P-815 cells; 10, 25 and 50 μM concentrations of glycyrrhetinic acid were added successively to mastocytoma P-815 cells. Abbreviations: GA, glycyrrhetinic acid; GL, glycyrrhizin: Phe, phenylephrine; and Thr, thrombin.

Fig. 2C. As in the case of mastocytoma P-815 cells, glycyrrhetinic acid by itself had the ability to elevate the $[Ca^{2+}]_i$ level and inhibited calcium increases in antigen (DNP-*Ascaris*)-stimulated mast cells at a concentration of 50 μM (trace a in Fig. 2C). In hepatocytes, 10 μM phenylephrine (via activation of α_1-adrenergic receptors) and 60 μM ATP (via activation of P_2-type adenosine receptors) induced a rapid increase in $[Ca^{2+}]_i$, but the level remained elevated over the basal level after the stimulation (trace a in Fig. 2D and 2E). Glycyrrhetinic acid alone at 50 μM also had the ability to elevate the $[Ca^{2+}]_i$ level, while it suppressed the response of phenylephrine or ATP in hepatocytes. We further studied the inhibitory action of glycyrrhetinic acid on thrombin- or ATP-induced elevation of $[Ca^{2+}]_i$ in fibroblasts or endothelial cells. Similar results were obtained for fibroblasts (Fig. 2F and 2G) and endothelial cells (Fig. 2H and 2I), which were stimulated by glycyrrhetinic acid (50 μM) following ATP (60 μM) or thrombin (0.05 units/mL). On the other hand, glycyrrhetinic acid did not desensitize the Ca^{2+}-mobilizing effect of successive challenge with glycyrrhetinic acid itself in mastocytoma cells (Fig. 2J).

Next, we sought to determine whether or not other stimulatory substances in place of glycyrrhetinic acid could desensitize those Ca^{2+}-mobilizing responses. As shown in Fig. 3A, successive stimulation with 10 μM phenylephrine and 50 μM phenylephrine showed that the level of $[Ca^{2+}]_i$ which responded to the second exposure to phenylephrine was slightly lower than that to the first exposure. However, no desensitization was observed with successive stimulation of phenylephrine and ATP (Fig. 3B).

Effect of glycyrrhetinic acid on $[Ca^{2+}]_i$ *increase induced by ionomycin in mastocytoma P-815 cells.* The Ca^{2+} ionophore ionomycin [21] forms a complex with Ca^{2+} by enveloping Ca^{2+} and this complex acts as a vehicle for transporting Ca^{2+} across plasma membranes. Hence, we also examined the effect of glycyrrhetinic acid on the Ca^{2+}-mobilizing activity of ionomycin. As shown in Fig. 4A, 0.01 μM ionomycin

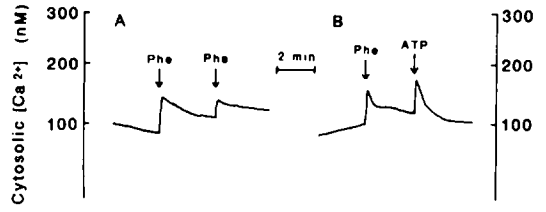

Fig. 3. Effect of phenylephrine (Phe) treatment on the subsequent calcium increases induced by phenylephrine or ATP in hepatocytes. Cells were loaded with fura-2 AM, and then $[Ca^{2+}]_i$ was determined as described under Materials and Methods. The cells were challenged successively with (A) 10 μM phenylephrine and 50 μM phenylephrine, or (B) 10 μM phenylephrine and 60 μM ATP.

induced a rapid increase in $[Ca^{2+}]_i$ and the level remained elevated. If 0.01 μM ionomycin followed the challenge of 25 μM glycyrrhetinic acid, ionomycin-induced Ca^{2+} response was inhibited almost completely. However, the maximal fluorescent intensity of fura-2 in the cells lysed by 5 μg/mL digitonin and the minimal intensity of fura-2 in the presence of 25 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) did not change, indicating that glycyrrhetinic acid does not alter the fura-2 response. Furthermore, 1 μM ionomycin gave the maximal fluorescent intensity of fura-2, demonstrating the saturating response. Glycyrrhetinic acid also suppressed the Ca^{2+} response of 1 μM ionomycin, the suppressed level of $[Ca^{2+}]_i$ being about 500 nM. Even in cells challenged with 10 μM ionomycin, glycyrrhetinic acid suppressed its response, the suppressed level of $[Ca^{2+}]_i$ being also about 500 nM (data not shown). Although the thrombin-induced increase in $[Ca^{2+}]_i$ was higher than that of 25 μM glycyrrhetinic acid, thrombin did not affect the response of either 0.01 μM or 1 μM ionomycin, indicating that the inhibition by glycyrrhetinic acid was not due to $[Ca^{2+}]_i$ elevated by glycyrrhetinic acid. Figure 4B shows the concentration dependence of the effect of ionomycin on $[Ca^{2+}]_i$ in cells pretreated or not pretreated with 25 μM glycyrrhetinic acid. Ionomycin concentration-dependently increased $[Ca^{2+}]_i$, but this increase was strongly suppressed by pretreatment with 25 μM glycyrrhetinic acid.

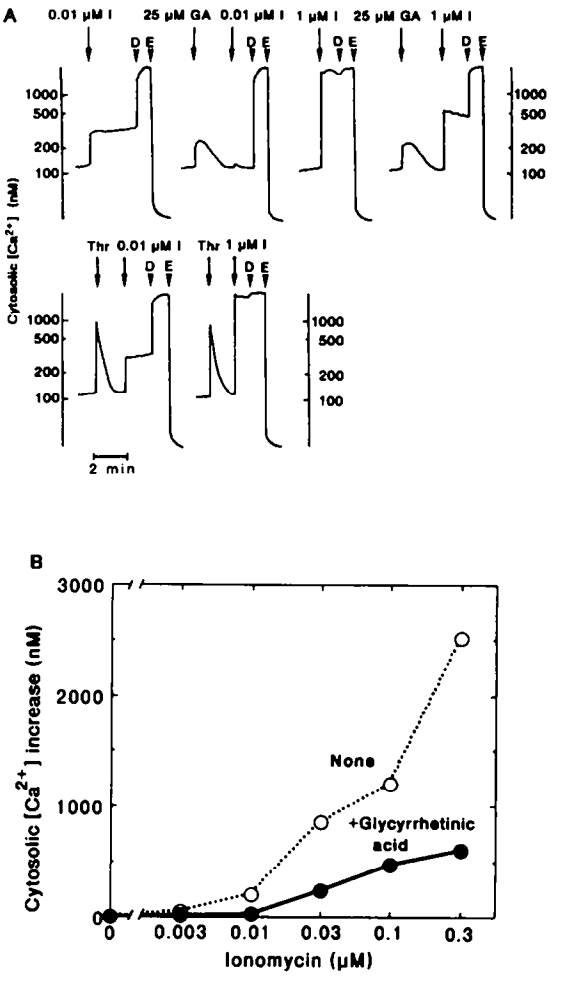

Fig. 4. Effect of glycyrrhetinic acid on the $[Ca^{2+}]_i$ increase induced by ionomycin in mastocytoma P-815 cells. Cells were loaded with fura-2 AM, and then $[Ca^{2+}]_i$ was determined as described under Materials and Methods. (A) After cells were stimulated with vehicle, 25 μM glycyrrhetinic acid or 0.05 units/mL thrombin, they were exposed to 0.01 or 1 μM ionomycin. Maximal and minimal fluorescent intensities of fura-2 were obtained by the addition of 5 μg/mL digitonin and by the subsequent addition of 25 mM EGTA. Arrows mark positions of the addition of various agents. (B) After cells were stimulated with (●) or without (○) 25 μM glycyrrhetinic acid, they were exposed to various concentrations of ionomycin. Abbreviations: GA, glycyrrhetinic acid; I, ionomycin; Thr, thrombin; D, digitonin; and E, EGTA.

DISCUSSION

The present study demonstrates that glycyrrhetinic acid, but not glycyrrhizin, caused a concentration-dependent increase in $[Ca^{2+}]_i$ but induced a decrease in the potency of various stimulants to activate Ca^{2+} mobilization in a variety of cell types. However, the glycyrrhetinic acid-induced change in $[Ca^{2+}]_i$ was a slow response as compared with that induced by calcium-mobilizing agonists (Fig. 2). The response was greatly inhibited when extracellular calcium was removed by EGTA (Fig. 1), indicating that glycyrrhetinic acid increases $[Ca^{2+}]_i$ mainly through calcium influx from the extracellular medium. The exact mechanism of this calcium influx remains unknown; however, voltage-sensitive calcium channels and a Na^+/Ca^{2+} exchanger were not involved in it because this calcium influx was not inhibited by nifedipine, verapamil or La^{3+}, which is a voltage-sensitive calcium channel blocker, or by replacement of Na^+ with K^+ or choline$^+$.

Meanwhile, glycyrrhetinic acid showed an inhibitory effect on the $[Ca^{2+}]_i$ increase induced by a receptor-mediated agonist, such as an antigen [10], phenylephrine [22], ATP [23] and thrombin [24] (Fig. 2). This inhibitory effect was not mediated by

elevation of $[Ca^{2+}]_i$ itself induced by glycyrrhetinic acid, because the increase in $[Ca^{2+}]_i$ induced by phenylephrine in place of glycyrrhetinic acid did not inhibit the subsequent calcium increase induced by phenylephrine or ATP (Fig. 3). Furthermore, glycyrrhetinic acid also inhibited the ionomycin-induced increase in $[Ca^{2+}]_i$, the mechanism of which is quite different from that of receptor-mediated Ca^{2+} mobilization. This effect was not due to the alteration of the fura-2 response by glycyrrhetinic acid, because glycyrrhetinic acid did not affect the maximal or minimal fluorescent intensity of fura-2 obtained from digitonin-lysed cells or cells susbsequently treated with EGTA (Fig. 4A). Increase in $[Ca^{2+}]_i$ could activate calcium pumps leading to the suppression of a subsequent increase in $[Ca^{2+}]_i$. Thrombin stimulated an increase in $[Ca^{2+}]_i$ and this level was higher than that induced by glycyrrhetinic acid. Although thrombin did not suppress the Ca^{2+}-mobilizing activities of either 0.01 μM (low concentration) or 1 μM (high concentration) ionomycin, 25 μM glycyrrhetinic acid strongly suppressed those responses (Fig. 4A). Therefore, the inhibitory effect of glycyrrhetinic acid is not mediated by the activation of calcium pumps. The present data as to this inhibitory effect coincided with a previous finding of the inhibition of the calcium ionophore-induced $[Ca^{2+}]_i$ increase in primary cultured rat hepatocytes measured by atomic absorption spectroscopy [5]. The inhibitory effects of glycyrrhetinic acid on both receptor-mediated Ca^{2+} mobilization and Ca^{2+} ionophore-induced Ca^{2+} influx suggest that the site of action of glycyrrhetinic acid is located on plasma membranes. It has been reported that a Chinese herbal medicine containing glycyrrhizae radix shows an inhibition of Na^+, Ca^{2+} and K^+ currents in snail neurons [25], and that glycyrrhetinic acid blocks potential amplitudes induced by acetylcholine in diaphragm muscle membranes [8]. In addition, glycyrrhetinic acid has been shown to inhibit (Na^+,K^+)-ATPase in kidney basolateral membranes [26], indicating that glycyrrhetinic acid shuts off various ion-mobilizing systems in plasma membranes. From a wide range of inhibitory activities of glycyrrhetinic acid, its target(s) may exist in a certain mechanism participating in various ion-mobilizing systems in plasma membranes. However, molecular characterization of these inhibitory actions of glycyrrhetinic acid should be obtained by further studies using physicochemical methods. Whereas glycyrrhetinic acid itself has Ca^{2+}-mobilizing activity through plasma membranes, it could not evoke histamine release from rat mast cells. Similar results were observed in the activation of adenosine receptors which increased in levels of $[Ca^{2+}]_i$ but failed to stimulate histamine release from RBL-2H3 cells [27]. The exact mechanism of histamine release including increase in $[Ca^{2+}]_i$ remains to be determined. In any case, this inhibitory effect of glycyrrhetinic acid may participate in its anti-inflammatory [1] and anti-hepatotoxic [5] actions, because the calcium ion is involved in the trigger mechanism for antigen-induced histamine release from mast cells [10], and the hepatotoxic effects of carbon tetrachloride [12] and galactosamine [13].

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