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INHIBITION OF ANAPHYLACTIC HISTAMINE RELEASE FROM HETEROLOGOUSLY SENSITIZED MAST CELLS: DIFFERENTIAL EFFECTS OF DRUGS WHICH INTERFERE WITH CALCIUM INFLUX

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Abstract. Drug effects were studied on anaphylactic histamine release from rat mast cells sensitized *in vitro* with mouse IgE antibody. When histamine release was elicited by adding Ca^{++} at various times after antigen-stimulation of sensitized cells in Ca^{++}-free medium, the drugs to be tested were added shortly before each Ca^{++} addition. Quercetin was effective only when added before or immediately after antigen. Theophylline and disodium cromoglycate (DSCG) were active irrespective of the time interval between antigen and Ca^{++} addition. Verapamil was more effective when added before or simultaneously with antigen than when added later. When tested in the two-stage experiments, quercetin showed inhibition only in Stage 1 and verapamil was inhibitive primarily in Stage 1, while theophylline and DSCG were only inhibitive in Stage 2. It seems that quercetin selectively and verapamil primarily act to block calcium-gate opening resulting from antigen-antibody interaction on the mast cell membrane, while theophylline and DSCG selectively inhibit the passage of calcium through open calcium channels.

Key words : histamine release, quercetin, disodium cromoglycate, theophylline, verapamil.

Histamine release from rat mast cells induced by antigen-antibody interaction or the presence of such substances as dextran requires Ca^{++} in the reaction medium (1, 2). There is a close correlation between the stimulus-induced increase in the amount of ^{45}Ca uptake by mast cells and the percentage of histamine released (3). Calcium appears to enter mast cells through calcium channels opened by various stimuli. It seems that the initial stimuli trigger some processes which eventually lead to calcium channel activation. Increased methylation of phospholipids (4) and uncoupling of Ca^{++}-ATPase which is tightly coupled to the calcium entry pathway in the resting state (5) may be involved in such a transduction mechanism.

Disodium cromoglycate (DSCG) (6-10), theophylline (11, 12) and quercetin (5, 13) inhibit histamine release from mast cells induced by immunologic and other stimuli. The effect of these drugs is probably caused by blocking calcium influx into these cells (3, 5, 13, 14). It has not been determined exactly how these inhibitors act on the calcium influx mechanism in mast cells. Verapamil and D-600,

calcium blockers, inhibit catecholamine secretion from the adrenal medulla (15, 16) and vasopressin secretion from the hypophysis (17, 18), which are both known to require extracellular Ca^{++}. However, the possible effects of these calcium blockers on histamine release have not been studied in detail.

From rat peritoneal mast cells sensitized *in vitro* with mouse IgE antibody, a marked and constant degree of anaphylactic histamine release which depends on extracellular Ca^{++} is elicited in the presence of phosphatidylserine (PS) (19). The decay of responsiveness to Ca^{++} of the sensitized cells stimulated by antigen in a Ca^{++}-free medium is fairly slow. In view of these characteristics, heterologously induced anaphylactic histamine release seems suitable for observing the modes of action of drugs which may interfere with calcium influx into mast cells.

Therefore, I decided to study the effects of DSCG, theophylline, quercetin and verapamil on this histamine release reaction in an attempt to elucidate the mechanisms of the action of these drugs and the release process *per se*.

MATERIALS AND METHODS

Chemicals. Ovalbumin (5 x crystalline, OA) was purchased from ICN Pharmaceuticals (Cleveland, O.) ; bovine serum albumin (fraction V) from Armour Pharmaceutical Co. (Kankakee, Ill.); DSCG from Fujisawa Pharmaceutical Co. (Osaka, Japan); and PS, theophylline and quercetin from Nakarai Chemicals (Kyoto, Japan). Verapamil was a gift from Eisai Co. (Tokyo, Japan).

Antigen-induced histamine release from sensitized mast cells. As previously described (19), rat peritoneal mast cells were collected from male Sprague-Dawley rats and sensitized *in vitro* with mouse reaginic anti-OA serum which had been collected from male BALB/c mice. Mixed cell suspensions containing mast cells were used without further purification. After being washed twice with a large volume of Hanks' balanced salt solution containing 0.05 % bovine serum albumin (HBSS) (19), the sensitized mast cells were subjected to histamine releasing stimuli as previously described (19). Usually, OA (the final concentration of 20 μg/ml) was added to the cell suspensions 3 min after PS (the final concentration of 30 μg/ml) at 37℃ and the incubation was continued for a further 5 min. The final reaction volume was 1.0 ml. After the reaction was stopped by cooling, each cell suspension was centrifuged. Both the supernatant and the precipitate were assayed for histamine content. PS, OA and the drugs to be tested were suspended (PS and quercetin) or dissolved in HBSS at ten times the final concentrations and 0.1 ml of each was added to the cell suspensions. In order to study the response of mast cells suspended in Ca^{++}-free medium, Ca^{++}-free HBSS (HBSS without added Ca^{++}) was used as the washing and suspending medium. Unless otherwise indicated, PS, OA and Ca^{++} were added to the cell suspensions in that sequence. The final concentration of Ca^{++} was made equal to that in ordinary HBSS (1.26 mM). As previously reported (19), the intensity of antigen-independent (PS + Ca^{++})-induced histamine release varies depending on both mast cell and antiserum pools. Therefore, unless otherwise specified, all experiments were done using such mast cell and antiserum pools as to give as low a degree of (PS + Ca^{++})-induced release as possible.

Two-stage experiments. PS was added to the sensitized mast cells suspended in Ca^{++}-free HBSS at 37℃. OA was added 3 min after PS and the incubation was continued for a further 45 sec (Stage 1). Immediately after incubation, 5 ml of ice cold Ca^{++}-free HBSS was added to each cell suspension and the resulting mixture was centrifuged at 150 x *g* for 3 min at 0℃.

The precipitated cells were washed once with 3 ml of ice cold Ca^{++}-free HBSS and resuspended in the same medium. The cell suspensions were placed in a 37℃ bath and PS was added. Ca^{++} was added 3 min after PS and the incubation was continued for a further 5 min at the same temperature (Stage 2). The final reaction volume in each stage was 1.0 ml. The addition of drugs to be tested was made shortly before OA (drug effect in Stage 1) or Ca^{++} (drug effect in Stage 2) and the influence on the histamine release occurring during Stage 2 was investigated. Even without added Ca^{++}, OA induces a considerable degree of histamine release from sensitized mast cells in this heterologous system (19). Therefore parallel experiments were also conducted in which the influence of each drug on the (PS + OA)-induced histamine release occurring during the first stage procedure was studied. In these experiments, aliquots of the same pool of cells were subjected to only the first stage of the treatment. After cooling and centrifuging each reaction mixture, both the supernatant and precipitate were assayed for histamine content.

Non-antigen-induced histamine release. The procedures were the same as described above except that OA was not added. Unless otherwise indicated, both the sequence and interval of the addition of PS, Ca^{++} and the drugs to be tested were the same as specified for the antigen-induced release.

Histamine assay. The histamine content of each sample was determined fluorometrically as described previously (19). The histamine release value was expressed as the percentage of the released histamine to the total histamine content, *i.e.*, the histamine in the supernatant and precipitate. None of the drugs tested except quercetin interfered with histamine assay to any significant extent. Interference by quercetin was compensated for using appropriate standards. The histamine release value in the entire two-stage procedure was expressed as the percentage of histamine present in the final supernatant to the total histamine which remained at the end of the second stage. No correction was made for histamine released during the first stage of the procedure.

RESULTS

General aspects of drug action. Drug effects were studied on the histamine release induced by OA in the presence of PS from the sensitized cells suspended in ordinary HBSS. DSCG added 10 sec before OA showed dose-dependent inhibition in the range of 0.002-0.05 mM, and the effect reached a plateau at 0.1-0.2 mM. Theophylline added 10 min before OA also showed dose-dependent inhibition in the range of 0.05-1 mM. The inhibitory effect of quercetin added 1 min before OA increased with increase in its concentration from 0.013 to 0.2 mM.

The effect of DSCG (0.1 mM) completely disappeared when the cells were preincubated with the drug for more than 8 min before OA addition. When the cells which had developed tachyphylaxis to DSCG were washed twice with fresh HBSS their sensitivity to newly added DSCG was restored to a considerable degree. Both theophylline (1 mM) and quercetin (0.1 mM) showed inhibition when the cells were preincubated with them for only 10 sec before OA addition. Any longer period of preincubation neither enhanced nor reduced the effect of these two drugs.

On the (PS + OA + Ca^{++})-induced histamine release from the cells suspend-

ed in Ca^{++}-free HBSS, verapamil added 1 min before the simultaneous addition of OA and Ca^{++} had a dose-dependent inhibitory effect in the range of 0.05-0.1 mM, and marked inhibition was observed at 0.1 mM. In ordinary HBSS, only slight inhibition was obtained even at 0.1 mM. A period of only 10 sec of preincubation with verapamil was almost enough to inhibit histamine release, and further prolongation of the preincubation period resulted in only a slight increase in the inhibitory effect. In concentrations over 0.1 mM, verapmil itself released histamine from sensitized mast cells. Histamine release induced by 0.2 mM verapamil amounted to 57.4 $\pm$ 5.4% (mean $\pm$ S.E.M., n=3) of the total.

Effects of drugs on histamine release induced by Ca^{++} added at various times after antigen stimulation in Ca^{++}-free medium. As previously reported (19), the decay in the responsiveness to Ca^{++} of the sensitized cells stimulated by antigen in Ca^{++}-free HBSS containing PS was relatively slow. The maximum release was induced by Ca^{++} added within 1 min after antigen (Fig. 1). The drugs to be tested were added shortly before each Ca^{++} addition, and the effects on such Ca^{++}-provoked histamine release were studied.

Quercetin added before or immediately after OA addition produced inhibition of the histamine release (Fig. 1-A), but the effect rapidly declined with the delay of drug addition. Quercetin added 30-60 sec after OA was almost ineffective, and the addition of the drug later than 60 sec had no effect.

Both theophylline and DSCG inhibited histamine release irrespective of the time interval between OA and Ca^{++} addition (Fig. 1-B & 1-C). In these cases, the time vs. release curve in the presence of the drug shifted downward in parallel with the control. When DSCG was added constantly 2 min after OA, the inhibitory effect of DSCG diminished with the delay of Ca^{++} addition, *i.e.*, the development of tachyphylaxis to DSCG was observed (Fig. 1-C).

The inhibition was marked when verapamil was introduced before or simultaneously with OA (Fig. 1-D). Although less marked, a constant degree of inhibition was observed when verapamil was added later than 30 sec after OA.

Drug effects observed in the two-stage experiments. The sensitized mast cells which had been stimulated with OA in Ca^{++}-free HBSS containing PS and then washed at low temperature released histamine in response to added Ca^{++} in the presence of PS (Tables 1, 2, 3 & 4).

When quercetin was added in Stage 1, the anaphylactic histamine release occurring during Stage 2 procedure was markedly inhibited (Table 1). Although quercetin present only during Stage 2 also significantly inhibited the total release during Stage 2, the effect was far less marked than when it was added in Stage 1. Taking into account the inhibitory effect on the antigen-independent release, quercetin added in Stage 2 was practically ineffective in inhibiting net histamine release due to antigen stimulation. Quercetin had a significant inhibitory effect on the (PS + OA)-induced release which occurred during the first stage of the procedure.

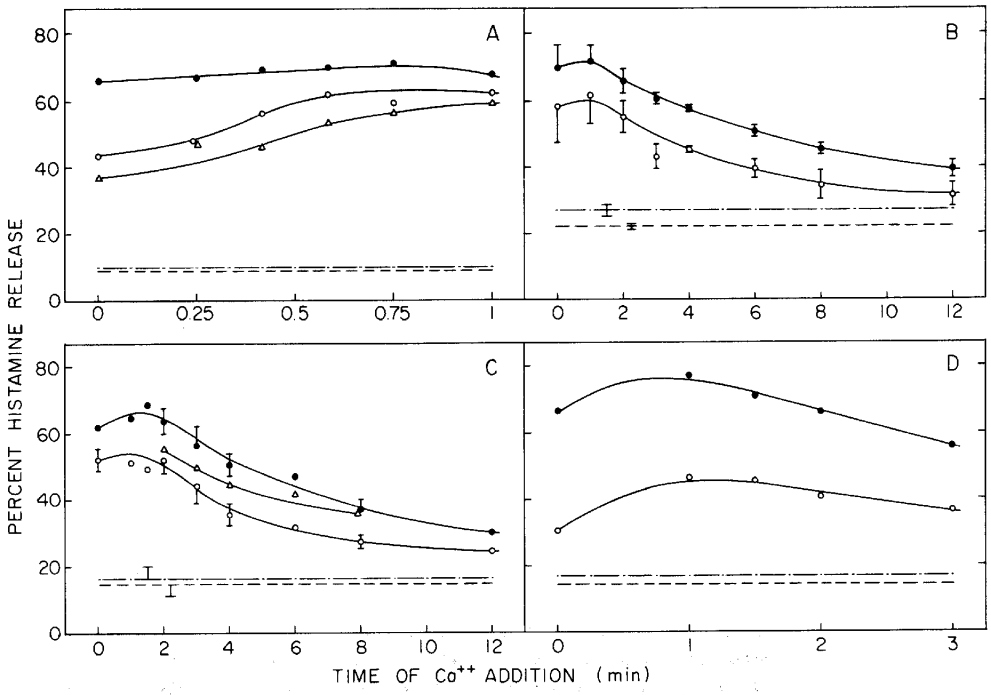

Fig. 1. Differential effects of Ca^{++}-influx inhibitors on histamine release from sensitized mast cells induced by Ca^{++} added at various times after antigen stimulation in Ca^{++}-free HBSS. (PS + OA + Ca^{++})-induced histamine release in the absence (●) and presence (○ and △) of inhibitors. (PS + OA)-induced release in the absence of inhibitors in this series of experiments was 19.2 ± 1.0 % of the total (mean ± S.E.M., n = 9). (PS + Ca^{++})-induced release in the presence (— — —) and absence (— · —) of inhibitors. *A*, quercetin (0.2 mM) was added 10 or 30 sec prior to Ca^{++}. (PS + OA + Ca^{++})-induced release in the absence (●) and presence (○: added 10 sec prior to Ca^{++}; △: added 30 sec prior to Ca^{++}) of quercetin. Results from a single duplicate experiment. *B*, theophylline (1 mM) was added 3 min prior to Ca^{++}. All values are the means ± S.E.M. of three duplicate experiments on different pools of cells. *C*, DSCG (0.1 mM) was added 10 sec prior to each Ca^{++} addition (○). A parallel single experiment was conducted, in which DSCG was added constantly 2 min after OA (△). All other values are the means of two or three duplicate experiments and the vertical bars represent S.E.M. *D*, verapamil (0.1 mM) was added 1 min prior to Ca^{++}. All values are the means ± S.E.M. of three duplicate experiments.

Both theophylline and DSCG showed significant inhibition of the anaphylactic release occurring during Stage 2 only when present during Stage 2 (Tables 2 & 3). Theophylline and DSCG slightly enhanced the (PS + OA)-induced release.

When present in either stage, verapamil markedly inhibited the anaphylactic release occurring during Stage 2. However the effect of its presence in Stage 1 was slightly more marked than its presence in Stage 2 (Table 4). The influence of verapamil on antigen-induced histamine release in the absence of added Ca^{++} was

TABLE 1. EFFECT OF QUERCETIN ON HISTAMINE RELEASE FROM SENSITIZED MAST CELLS STUDIED BY THE TWO-STAGE PROCEDURE[a]

Exp.	Treatment		% Histamine release	
	Stage 1	Stage 2	In the presence of OA	In the absence of OA
(a)	None	None	53.6 ± 3.4	21.2 ± 5.1
(b)	Quercetin[b]	None	19.5 ± 0.8[d]	4.3 ± 0.4
(c)	None	Quercetin	32.8 ± 2.9[e]	6.1 ± 0.4
(d)[c]	None	——	21.9 ± 1.3	7.4 ± 0.5
(e)[c]	Quercetin	——	14.1 ± 0.6[f]	4.7 ± 0.3[f]

a, Quercetin (0.1 mM) was added 30 sec prior to OA or Ca^{++}. All values are the means ± S.E.M. of three duplicate experiments on different pools of cells.

b, Because quercetin was almost in suspension, some of the quercetin added in Stage 1 could not be removed by washing.

c, This pair of experiments was included to study the effect of quercetin on the (PS + OA)-induced histamine release occurring during the first stage procedure.

d, $P < 0.001$ as compared with the value in (a) by student's *t*-test.

e, $P < 0.01$ as compared with the value in (a).

f, $P < 0.01$ as compared with the value in (d).

TABLE 2. EFFECT OF THEOPHYLLINE ON HISTAMINE RELEASE FROM SENSITIZED MAST CELLS STUDIED BY THE TWO-STAGE PROCEDURE[a]

Exp.	Treatment		% Histamine release	
	Stage 1	Stage 2	In the presence of OA	In the absence of OA
(a)	None	None	38.1 ± 4.4	14.6 ± 1.7
(b)	Theophylline	None	35.7 ± 4.5	13.4 ± 2.3
(c)	None	Theophylline	22.6 ± 4.3[b]	9.8 ± 1.5
(d)	None	——	19.1 ± 4.9	7.9 ± 1.3
(e)	Theophylline	——	22.1 ± 4.9	8.1 ± 1.5

a, Theophylline (1 mM) was added 2 min prior to OA or Ca^{++}. All values are the means ± S.E.M. of four duplicate experiments. Other procedures were the same as in Table 1.

b, $P < 0.05$ as compared with the value in (a).

TABLE 3. EFFECT OF DSCG ON HISTAMINE RELEASE FROM SENSITIZED MAST CELLS STUDIED BY THE TWO-STAGE PROCEDURE[a]

Exp.	Treatment		% Histamine release	
	Stage 1	Stage 2	In the presence of OA	In the absence of OA
(a)	None	None	41.7 ± 4.1	10.9 ± 2.4
(b)	DSCG	None	37.2 ± 2.6	12.5 ± 3.3
(c)	None	DSCG	27.5 ± 1.7[b]	9.3 ± 1.4
(d)	None	——	19.2 ± 4.9	8.2 ± 1.1
(e)	DSCG	——	21.4 ± 6.0	8.0 ± 1.1

a, DSCG (0.1 mM) was added 20 sec prior to OA or Ca^{++}. All values are the means ± S.E.M. of four duplicate experiments. Other procedures were the same as in Table 1.

b, $P < 0.02$ as compared with the value in (a).

TABLE 4. EFFECT OF VERAPAMIL ON HISTAMINE RELEASE FROM SENSITIZED MAST CELLS STUDIED BY THE TWO-STAGE PROCEDURE[a]

Exp.	Treatment		% Histamine release	
	Stage 1	Stage 2	In the presence of OA	In the absence of OA
(a)	None	None	38.2 ± 5.6	7.7 ± 0.1
(b)	Verapamil	None	9.8 ± 1.6[b]	7.6 ± 0.5
(c)	None	Verapamil	19.5 ± 3.0[c]	10.2 ± 0.3[d]
(d)	None	——	17.8 ± 1.9	6.6 ± 0.6
(e)	Verapamil	——	14.4 ± 0.6	13.2 ± 0.6[e]

a, Verapamil (0.1 mM) was added 1 min prior to OA or Ca^{++}. All values are the means ± S.E.M. of three duplicate experiments. Other procedures were the same as in Table 1.

b, $P < 0.01$ as compared with the value in (a).

c, $P < 0.05$ as compared with the value in (a).

d, $P < 0.005$ as compared with the value in (a).

e, $P < 0.005$ as compared with the value in (d).

obscured due to its enhancement of the release induced by PS alone.

Drug effects on antigen-independent (PS + Ca^{++})-induced histamine release. When the (PS + Ca^{++})-induced histamine release was slight, all the test drugs added shortly before Ca^{++} exerted some degree of inhibitory effect on this release (Fig. 1 and Table 1). When marked (PS + Ca^{++})-induced histamine release was produced by appropriate antiserum and mast cell pools, theophylline and DSCG, added either prior to PS or between PS and Ca^{++}, had significant inhibitory effects on such release (Fig. 2).

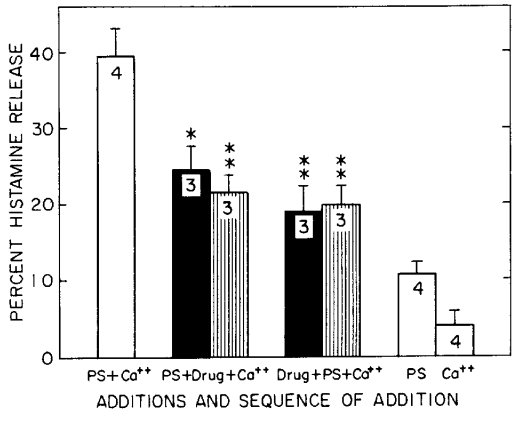

Fig. 2. Effect of theophylline and DSCG on (PS + Ca^{++})-induced histamine release from sensitized peritoneal mast cells. Ca^{++} was added 3 min after PS to the sensitized cells suspended in Ca^{++}-free HBSS. Theophylline (1 mM; filled columns) was added 30 sec prior to Ca^{++} or PS, while DSCG (0.1 mM; hatched columns) was added 10 sec before. Neither drug had any significant effect on histamine release induced by PS or Ca^{++} added alone. The figures at the top of columns indicate the number of duplicate experiments on different pools of cells and the vertical bars represent S.E.M. Significant difference from the control value in the presence of PS and Ca^{++} indicated by asterisk; *$P < 0.05$, **$P < 0.02$.

DISCUSSION

DSCG, theophylline and quercetin, known inhibitors of anapnylactic histamine release, had similar inhibitory effects on the heterologous passive anaphylaxis presented here to those observed in other experimental systems (5, 8, 11-13, 20). Therefore, this system can be used for the screening and analysis of action mechanisms of anti-allergic drugs.

Relatively high concentrations of verapamil or D-600 are required to inhibit secretory phenomena which depend on extracellular Ca^{++} (15, 17). In spite of the rather abrupt dose-response relationship, the fact that fairly high concentrations of verapamil inhibited histamine release in the present experiments suggests the relevance of calcium channel activation in mast cells to the heterologously induced histamine release. The fact that the inhibition was pronounced only when the cells were incubated with verapamil in the absence of Ca^{++} suggests that in this inhibition there exists some component of the competition between the drug and Ca^{++} at calcium entry pathways. The histamine release induced by high concentrations of verapamil in the absence of antigen appears to be due to cell injury.

When sensitized mast cells suspended in a Ca^{++}-free medium are stimulated by antigen and left at 37℃, the responsiveness of the cells to Ca^{++} rapidly declines with time (21). However, it has been suggested that in the heterologous passive anaphylaxis the process leading to calcium-gate opening persists until approximately 1 min after antigen stimulation (19). Quercetin, theophylline, DSCG and verapamil added shortly before each Ca^{++} addition showed differential patterns of inhibition of such histamine release, depending on the time interval between OA and drug addition. These results indicate that the process of antigen-induced calcium influx into mast cells can actually be divided into two steps. Each of these steps could be the site of action of drugs which interfere with calcium influx into these cells; the first step may be named "the process of calcium-gate opening". All possible chain reactions occurring between the antigen binding to IgE on the cell surface and the activation of calcium channels (the step of transduction) and the channel activation *per se* are arbitrarily included in this process. The second step may be termed "the passage of calcium through open calcium channels".

Considering that quercetin was effective only for a short time after antigen addition, it seems that quercetin selectively acts in the first step, *i.e.*, blocks the process of calcium-gate opening. This brief period was approximately equal to the time required for producing the maximum release. This further supports the above view that this process is transient ending within a short period after antigen stimulation even in the presence of antigen.

The time/release curve was shifted downward parallel to the control by theophylline or DSCG. Verapamil was more effective when added close in time to antigen addition than when added later. These results suggest that both theophylline and DSCG selectively block the passage of calcium through open calcium channels in the mast cell membrane and that verapamil blocks both the process of calcium-

gate opening and the passage of calcium. This is also supported by the fact that in the Ca^{++}-free medium the tachyphylaxis to DSCG similar to that observed in the Ca^{++}-containing medium developed with the increase in the time interval between DSCG and Ca^{++} addition in the presence of antigen.

Since almost no decay in the responsiveness to Ca^{++} of the antigen-stimulated sensitized cells occurs at 0-2℃, it is possible to arbitrarily divide the entire reaction of histamine release from basophils and mast cells into two stages (22, 23). Because the calcium-gate opening is a brief process (see above), calcium influx mechanism in this heterologous system can be clearly divided into two stages, that is, the calcium-gate opening and the passage of calcium through open channels. The following results from two-stage experiments, in which antigen was removed after full activation of calcium channels, reinforced the view presented above concerning the mechanism of action of each tested drug: Quercetin added in Stage 1, theophylline and DSCG added in Stage 2 and verapamil added in Stage 1 or Stage 2, all produced inhibition of the anaphylactic histamine release occurring during Stage 2.

There is a close correlation between the inhibitory effects of flavonoid compounds including quercetin on Ca^{++}-ATPase and on anaphylactic histamine release from mast cells (5). Therefore, it has been suggested that quercetin inhibits the ligand-induced uncoupling from calcium entry pathway of Ca^{++}-ATPase which is tightly coupled to the former in the resting state (5). This view is compatible with the opinion that the effect of quercetin may be attributable to its selective blockage of the process of calcium-gate opening.

It has been suggested that DSCG is active in a water-lattice form. DSCG in this form selectively combines with divalent cations such as Ca^{++} (24). In the present study, DSCG may have inhibited the passage of calcium through open channels by existing in the aforementioned form in juxtaposition to the surface of mast cell membranes. A specific protein of mast cells is phosphorylated when they are recovering from compound 48/80-induced histamine release (25). It has recently been found that DSCG causes phosphorylation of the same protein in normal rat mast cells (26). In view of the results obtained in the present experiments, phosphorylation of the specific protein may be related to a kind of sieving mechanism which directly controls the amount of calcium passing through open channels.

Verapamil does not inhibit the activity of Ca^{++}-ATPase (27, 28). D-600 shows both direct antagonism of calcium influx and membrane stabilizing action in the parotid gland (29). Such an observation is consistent with the present results. The blockage of the passage of calcium through open channels by verapamil may be due to competitive antagonism of calcium influx. The inhibition of the calcium-gate opening by verapamil may be a result of membrane stabilizing action similar to that of papaverine, the mother compound of verapamil.

The (PS + OA)-induced histamine release from the sensitized cells suspended in the Ca^{++}-free medium probably occurs by utilizing intracellular calcium (19).

Both quercetin and verapamil substantially inhibited this release. This suggests that the aforementioned transduction mechanism is linked to the mobilization of calcium from intracellular reservoirs as well as to the calcium channel activation in the cell membrane.

All of the test drugs inhibited the antigen-independent (PS + Ca^{++})-induced histamine release. Consequently, it is suggested that calcium influx into mast cells is pivotal in the (PS + Ca^{++})-induced histamine release, although the pattern of calcium entry in this reaction may be different from the pattern discussed for the antigen-induced release.

The present results clearly show that there are considerable differences in the mode of action between the test drugs believed to inhibit Ca^{++}-influx into mast cells. However, it remains to be determined how the action mechanisms discussed above are related to the other well-established effects of the test drugs, *e.g.* the inhibition of phosphodiesterase by theophylline.

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