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THE INHIBITION OF SUBSTANCE P-INDUCED HISTAMINE RELEASE FROM MAST CELLS BY 6, 7-DIHYDRO-6, 8, 8, 10-TETRAMETHYL-8H-PYRANO-[3, 2-G] CHROMONE-2-CARBOXYLIC ACID (EAA)

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Abstract. In the presence of extracellular Ca^{2+}, 6, 7-dihydro-6, 8, 8, 10-tetramethyl-8H-pyrano [3, 2-g] chromone-2-carboxylic acid (EAA) had an inhibitory effect on the substance P-induced histamine release from rat peritoneal mast cells. Not only Ca^{2+} but also Mg^{2+}, Sr^{2+} and Ba^{2+} were effective in enhancing the activity of EAA. Marked tachyphylaxis to EAA developed irrespective of the presence or absence of extracellular Ca^{2+}. Cross-tachyphylaxis was observed between EAA and disodium cromoglycate (DSCG). These results indicate that the mode of action of EAA is similar, but not identical, with that of DSCG.

Key words.: 6, 7-dihydro-6, 8, 8, 10-tetramethyl-8H-pyrano[3, 2-g]chromone-2-carboxylic acid (EAA), disodium cromoglycate, histamine release, alkaline-earth metal ions, substance P.

A novel compound, 6, 7-dihydro-6, 8, 8, 10-tetramethyl-8H-pyrano[3, 2-g]-chromone-2-carboxylic acid (EAA; Fig. 1) (1), has a more potent inhibitory effect on the type I allergic reaction than disodium cromoglycate (DSCG) (2) which is used in the treatment of bronchial asthma. Unlike DSCG this compound is effective by oral administration as well as by parenteral injection. It inhibits not only the IgE-mediated histamine release from rat peritoneal mast cells, but also

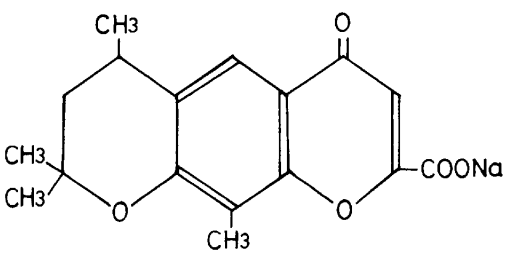

Fig. 1. Chemical structure of EAA (sodium salt).

the release induced by compound 48/80. No detailed studies have been carried out yet on the mode of inhibitory action of EAA on histamine release from mast cells.

In the present study, which aimed at elucidating the mechanism of action of EAA, the effect of EAA on the substance P-induced histamine release (3, 4) from rat peritoneal mast cells and the influence of extracellular alkaline-earth metal ions on this effect were examined. The mode of action of EAA was compared with that of DSCG (4).

MATERIALS AND METHODS

Animals. Male 10-14 week-old Sprague-Dawley rats weighing 300-460 g (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were used.

Chemicals and drugs. Substance P triacetate was obtained from the Protein Research Foundation (Minoh, Japan); bovine serum albumin (fraction V) from Armour Pharmaceutical Co. (Kankakee, IL) and DSCG from Fujisawa Pharmaceutical Co. (Osaka, Japan). Sodium salt of EAA was a gift from Eisai Co. (Tokyo).

Histamine release from rat peritoneal mast cells. Unless otherwise specified, phosphate-buffered saline (PBS) of the following composition was used : NaCl, 154 mM ; KCl, 2.7 mM ; $CaCl_2$, 0.9 mM ; Na_2HPO_4-KH_2PO_4 buffer, final pH 7.1, 6.7 mM ; glucose 5.6 mM ; bovine serum albumin, 0.05 %. Suspensions of rat peritoneal cells containing 5-8 % mast cells were prepared as described previously (3). The cells were obtained by peritoneal lavage with 20 ml of ice-cold PBS not containing bovine serum albumin. Usually the cells collected from 3 rats were pooled and used for one experiment. The cells were washed once with a large volume of ice-cold PBS.

Amounts of the cell suspension such that each tube contained 0.7-1.8 $\times$ 10^5 mast cells were put into glass centrifuge tubes. After centrifuging at 350 $\times$ *g* for 5 min at 4 ℃, the precipitated cells were resuspended in fresh PBS. Before the addition of EAA or DSCG, the suspended cells were preincubated for 5 min at 37 ℃. Unless otherwise specified, substance P was added to the cell suspension 10 sec after drug addition, and the incubation was continued for 5 min at the same temperature. The final reaction volume was 1.0 ml. Substance P, EAA and DSCG were dissolved in PBS to 10 times their final concentrations, and 0.1 ml of each was added to the cell suspension. After incubation, each tube was immediately transferred to an ice bath and centrifuged at 650 $\times$ *g* for 10 min at 0 ℃. Both the supernatant and the precipitate were assayed for histamine content. Siliconized glassware was used throughout. Histamine release was determined in duplicate samples.

In experiments to test the effect of alkaline-earth metal ions, Ca^{2+}-free PBS (PBS without added Ca^{2+}) was used for collecting and subsequently suspending the peritoneal cells. $CaCl_2$, $MgCl_2$, $SrCl_2$ or $BaCl_2$ was added to the cell suspension usually at the start of the preincubation as described by Nishibori and Saeki (4). When the development of tachyphylaxis to EAA and DSCG in Ca^{2+}-free PBS was studied, $CaCl_2$ was added 10 sec before the addition of substance P.

Determination of histamine. The histamine content of each sample was determined fluorometrically by the method of Shore *et al.* (5), omitting the extraction procedures with organic solvents, as described by Loeffler *et al.* (6). After fluorophore formation, 2 M citric acid instead of 3N HCl was used as the acidifying agent according to Anton and Sayre (7). Substance P, EAA and DSCG did not interfere with the histamine assay to any significant extent.

The percentage of histamine release was calculated using the following equation: histamine release (%) = (histamine content of the supernatant) × 100/[(histamine content of the supernatant) + (histamine content of the precipitate)]. Percent inhibition of histamine release was calculated as follows: % inhibition = [(% release from non-treated cells) − (% release from treated cells)] × 100/(% release from non-treated cells).

RESULTS

Effect of Ca^{2+} on the inhibition by EAA of the substance P-induced histamine release. Consistent with previous results (3, 4), substance P (10^{-5} M) induced a release of histamine from rat peritoneal mast cells both in the presence and absence of extracellular Ca^{2+} (Fig. 2).

When the usual PBS was used throughout the experimental procedure, EAA produced a marked and significant inhibition of the substance P-induced histamine release at concentrations of 10^{-6}-10^{-3} M. The histamine release in the presence of different concentrations of EAA formed a reversed bell-shaped curve which showed the lowest value at an EAA concentration of 10^{-5} M. EAA (10^{-6}-10^{-4} M) was effective in inhibiting the histamine release, even when the peritoneal cells were at first collected and suspended in Ca^{2+}-free PBS and Ca^{2+} (0.9 mM)

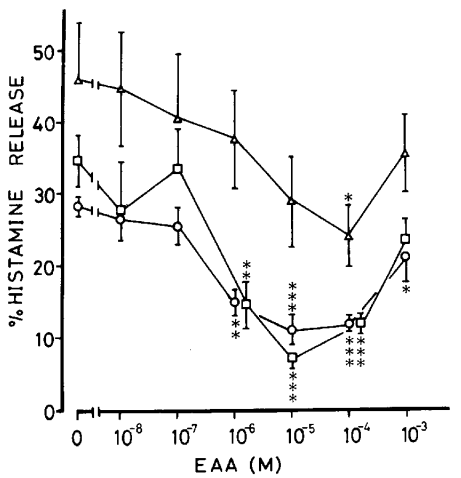

Fig. 2. Effect of Ca^{2+} on the inhibition by EAA of the substance P-induced histamine release from rat peritoneal mast cells. After the preincubation in the presence (0.9 mM) or absence of Ca^{2+}, EAA and substance P (10^{-5} M) were added to the cell suspension at an interval of 10 sec. O, Ca^{2+} present in the medium throughout the experimental procedure; △, Ca^{2+} absent throughout; □, Ca^{2+} added to the medium at the beginning of the preincubation. The results are the means ± S.E.M. of 4 experiments on different pools of cells corrected for spontaneous release. Spontaneous histamine release ranged from 2.6 to 8.6 %. EAA at concentrations tested had no significant effect on spontaneous release. Significantly different from the corresponding control value in the absence of EAA as determined by Student's unpaired *t*-test: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

was added to the medium at the start of the preincubation.

On the contrary, the inhibitory activity of EAA in Ca^{2+}-free medium was far weaker than that in Ca^{2+}-containing medium. When Ca^{2+}-free PBS was used throughout, EAA was ineffective at concentrations up to 10^{-5} M. The release was significantly inhibited at 10^{-4} M in the absence of Ca^{2+}, but the activity at this concentration was far less than that observed in the presence of Ca^{2+}.

Effect of alkaline-earth metal ions on the inhibition by EAA of the substance P-induced histamine release. The substance P-induced histamine release was greater in the presence of 0.9 mM Mg^{2+} or 0.9 mM Sr^{2+}, but less in the presence of 0.9 mM Ca^{2+} or 0.9 mM Ba^{2+}, than in the medium lacking alkaline-earth metal ions (Table 1).

EAA (10^{-5} M) had a moderate effect on the substance P-induced histamine release in the medium not containing alkaline-earth metal ions, but the inhibitory activity was far higher in the presence of 0.9 mM concentrations of these metal ions.

Effect of Ca^{2+} on the development of tachyphylaxis to EAA andDSCG. Incubation of the rat peritoneal cells with EAA (10^{-5} M) or DSCG (10^{-5} M) for 5 min or more prior to the addition of substance P resulted in a marked decrease in the inhibitory effect of these compounds on the histamine release, irrespective of the presence or absence of Ca^{2+} (Fig. 3).

Development of cross-tachyphylaxis between EAA and DSCG. When the peritoneal

TABLE 1. EFFECT OF ALKALINE-EARTH METAL IONS ON THE INHIBITION BY EAA OF THE SUBSTANCE P-INDUCED HISTAMINE RELEASE FROM RAT PERITONEAL MAST CELLS.

Drugs	% Histamine release in the presence (0.9 mM) of different divalent metal ions[a]				
	None	Mg^{2+}	Ca^{2+}	Sr^{2+}	Ba^{2+}
Substance P (10^{-5} M)	24.6 ± 1.6	33.6 ± 2.7[b]	19.1 ± 1.2[b]	32.7 ± 2.2[b]	18.2 ± 3.2
Substance P (10^{-5} M) + EAA (10^{-5} M)	15.4 ± 1.0[c] (37.5 ± 3.5[d])	14.7 ± 1.9[c] (56.4 ± 3.6[d])	2.7 ± 1.4[c] (86.9 ± 5.8[d])	9.2 ± 1.6[c] (72.5 ± 3.7[d])	2.8 ± 2.2[c] (89.1 ± 8.4[d])

The cells were collected and suspended in Ca^{2+}-free PBS. At the beginning of preincubation, alkaline-earth metal ions were added to the cell suspension. EAA and substance P were added at 10 sec intervals. The results are the means ± S.E.M. of 4 experiments on different pools of cells. The figures in parentheses represent % inhibition of histamine release.

a The spontaneous release (%) in the medium containing no added metal ion was 12.9 ± 0.9, and the release in the presence of Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} was 8.5 ± 1.3, 7.7 ± 1.3, 6.6 ± 0.6 and 6.8 ± 1.3, respectively. The spontaneous release in the presence of alkaline-earth metal ions was significantly lower than that in the absence of divalent metal ions as determined by Student's unpaired *t*-test ($p < 0.05$). EAA had no significant effect on spontaneous release. The results in the table have been corrected for spontaneous release.

b $p < 0.05$ compared with the corresponding value in the absence of alkaline-earth metal ion (unpaired *t*-test).

c $p < 0.05$ compared with the control value in the absence of EAA (unpaired *t*-test).

d $p < 0.05$ compared with the control value (paired *t*-test).

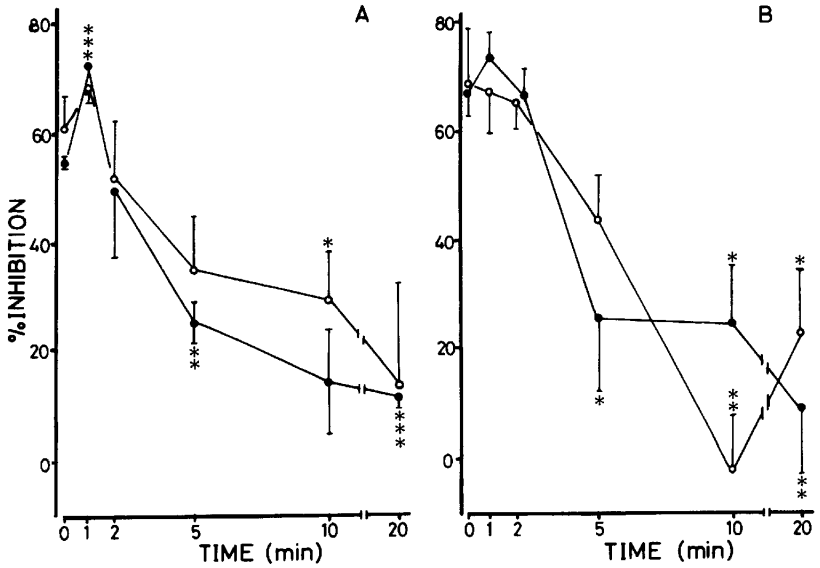

Fig. 3. Effect of Ca^{2+} on the development of tachyphylaxis to EAA (A) and DSCG (B). After preincubation in Ca^{2+}-free PBS, the peritoneal cells were incubated with EAA (10^{-5} M) or DSCG (10^{-5} M) for periods indicated on the abscissa in the presence (0.9 mM) (●) or absence (○) of Ca^{2+}. When the cells were treated with each drug in Ca^{2+}-free medium, Ca^{2+} (0.9 mM) and substance P (10^{-5} M) were added to the cell suspension 10 sec before and at the end of the periods, respectively. The results are the means ± S.E.M. of 4 experiments on different pools of cells. Significantly different from the corresponding control value obtained by adding substance P 10 sec after the addition of each drug as determined by Student's unpaired *t*-test: $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.001$.

TABLE 2. DEVELOPMENT OF CROSS-TACHYPHYLAXIS BETWEEN EAA AND DSCG ON THE SUBSTANCE P-INDUCED HISTAMINE RELEASE FROM RAT PERITONEAL MAST CELLS

Exp.	Treatment		% Histamine release
	First addition	Second addition	
1	None	None	39.2 ± 0.8
2	None	EAA	11.7 ± 0.9[a]
3	None	DSCG	19.2 ± 2.4[a]
4	EAA	None	36.5 ± 1.9
5	DSCG	None	41.4 ± 1.1
6	EAA	DSCG	41.1 ± 1.5
7	DSCG	EAA	36.7 ± 2.8

After preincubation, the cells were incubated at 37° C for 20 min in the absence or presence (10^{-5} M) of one of the two drugs (first treatment), after which no drug or the other of the two drugs (10^{-5} M) was added. The incubation was continued for a further 1 min (second treatment) before the addition of substance P (10^{-5} M). The results are the means ± S.E.M. of 4 experiments on different pools of cells and have been corrected for spontaneous release. *a* Significantly different from the value in Exp. 1 : $p<0.001$.

cells were first incubated for 20 min (a period long enough for the full development of tachyphylaxis) with one of the two drugs (10^{-5} M), the other drug (10^{-5} M) added at the end of this incubation period was completely ineffective in inhibiting the histamine release induced by substance P added 1 min later. The striking ineffectiveness of the second drug was observed irrespective of the order of addition of EAA and DSCG (Table 2).

DISCUSSION

DSCG effectively inhibits the release of histamine from rat peritoneal mast cells, as induced by interaction of a specific antigen with cell-bound IgE antibody (8-10) as well as by a variety of chemical substances such as dextran (10), compound 48/80 (3, 8, 11, 12), substance P (3) and neurotensin (3). Possible mechanisms of the action of DSCG include: blocking of the influx of Ca^{2+} into mast cells (11, 13), inhibition of phosphodiesterase (14, 15) and regulation of phosphorylation of mast cell protein (16). The influence of alkaline-earth metal ions on the DSCG inhibition of the substance P-induced histamine release has been studied (4). The results of the present experiments showed some similarities as well as dissimilarities between the modes of action of EAA and DSCG.

DSCG (10^{-5} M) has no effect on the substance P-induced histamine release in the absence of alkaline-earth metal ions, and only Ca^{2+} is effective in restoring the DSCG activity lost in the absence of alkaline-earth metal ions (4). It has been observed that an intense histamine release reaction is generally more resistant to the inhibitory action of drugs than a weak reaction (8, 17). In the present study, the control substance P-induced histamine release was more marked in the medium lacking alkaline-earth metal ions than in the presence of Ca^{2+}. These facts should be taken into consideration when comparing the intensity of the effect of EAA in different media. However, the effect of EAA was more marked in the presence of Mg^{2+} or Sr^{2+} than in the absence of alkaline-earth metal ions, although the control substance P-induced histamine release was more intense in the presence of these metal ions. Therefore, it seems reasonable to conclude that extracellular alkaline-earth metal ions are required for the optimal action of EAA. In the present study, no clear selectivity was observed among alkaline-earth metal ions for enhancing the EAA activity. The lack of a selective requirement for extracellular Ca^{2+} seems to distinguish the mode of action of EAA from that of DSCG (4).

Mazurek *et al.* (18) reported that DSCG covalently conjugated to fluorescent polyacrylamide-polyglutaraldehyde beads bound to rat peritoneal mast cells only in the presence of extracellular Ca^{2+}, and that these DSCG-bead conjugates inhibited anaphylactic histamine release in the presence of extracellular Ca^{2+}.

Nishibori and Saeki (4) proposed, from their own data as well as that of Mazurek *et al.* (18), that Ca^{2+} may be selectively required for the binding of DSCG to the specific binding sites on the mast cell surface or for some steps in the

DSCG action. The results of the present experiments indicate that there is no strict and specific Ca^{2+} requirement for the EAA binding to the binding sites on mast cells. The optimal binding of EAA may occur in the presence of not only Ca^{2+}, but also other alkaline-earth metal ions tested, although further studies are necessary to draw any definite conclusions.

It has been shown that marked tachyphylaxis develops to DSCG in the inhibition of histamine release from rat mast cells (19). The development of tachyphylaxis to EAA suggests a similarity between the actions of DSCG and EAA. However, the presence of cross-tachyphylaxis between these drugs seems to be much superior evidence that they possess some common sites of action. The mechanism involved in the development of tachyphylaxis has not been clarified. In contrast to the Ca^{2+} dependence of the DSCG inhibition of histamine release (4), we found that tachyphylaxis developed irrespective of the presence or absence of extracellular Ca^{2+}. This is consistent with the results reported by Sung *et al.* (19, 20). Ca^{2+} had no influence on the development of tachyphylaxis to EAA, either. Mazurek *et al.* (18) clearly showed that DSCG does not bind to the mast cell surface in the absence of extracellular Ca^{2+}. Sung *et al.* (20) observed that the inhibitory effect of DSCG was maintained by keeping mast cells at 2-4 ℃ after treatment with DSCG at 37 ℃. Based on such observations, they proposed that development of tachyphylaxis may be due to a temperature-dependent modification of the receptor sites which occurs after DSCG binds to these sites. However, in the absence of extracellular Ca^{2+}, this proposal seems to contradict the results reported by Mazurek *et al.* (18). Consequently, the mechanism of tachyphylaxis to EAA as well as DSCG remains enigmatic.

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