STUDIES ON THE MECHANISMS OF THE INHIBITORY EFFECT OF N-5' ON HISTAMINE RELEASE FROM RAT PERITONEAL EXUDATE CELLS

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Abstract—To investigate the mechanisms for the inhibition of IgE-mediated histamine release from rat peritoneal exudate cells (PEC) by N-5', we studied the relation between the inhibitory effect of N-5' on histamine release and the intracellular levels of adenine nucleotides such as ATP and cAMP. Evident histamine release was induced by the addition of specific antigen to rat PEC sensitized with IgE antiserum in vitro, and the release showed a maximum 30 sec after the antigen challenge. In the same time course as the histamine release, the intracellular levels of ATP and cAMP decreased. N-5' significantly inhibited the histamine release and a decrease in ATP level as a result of the antigen-antibody reaction. A decrease in cAMP level showed a tendency to be suppressed by N-5'. Antigen-induced 14CO2 production for 6-14C-glucose in the sensitized PEC was 3 times that seen in the case without antigen. N-5' dramatically suppressed the acceleration in the production of 14CO2. Differing from the action of papaverine, the inhibitory effect of N-5' on the IgE-mediated histamine release from rat PEC was identical both in the presence or in the absence of glucose. N-5' scarcely affected the ATP level in the non-sensitized PEC in the glucose-free medium. On the other hand, N-5' inhibited the activity of Na+, K+-ATPase, one of the ATP-consuming enzymes, in a dose-dependent fashion. From these results, it is presumed that the suppression of ATP-utilization through the inhibition of Na+, K+-ATPase activity is involved in the inhibition of histamine release by N-5'. The relation between the inhibitory effect of N-5' on histamine release and both nucleotides was also discussed.

N-(3,4-dimethoxycinnamoyl)anthranilic acid (N-5'), a new anti-atopic agent, was first reported to have a significant suppressive effect on homologous passive cutaneous anaphylaxis (PCA) in rats with the oral application by Koda et al. (1). A clinical study for the atopic type of asthma has shown good results (2). Azuma et al. reported that the suppressive effect of N-5' on homologous PCA may be principally due to the inhibition of histamine release mediated by IgE antibody from mast cells (MC) (3). The mechanism for the inhibition of histamine release has been reported to be different from that of disodium cromoglycate (DSCG) and to differ from those of metabolic inhibitors such as papaverine and 2,4-dinitrophenol (4). However, the mechanism for the inhibition of allergic histamine release by N-5' is not yet clear.

It is well known that cyclic 3',5'-AMP (cAMP) plays an important role in IgE-mediated histamine release (5–7), and it is
also known that intracellular adenosine triphosphate (ATP) is important in the histamine secretory process (8–10). The present work was carried out to study the relation between these nucleotides and the inhibitory effect of N-5' on histamine release.

Materials and Methods

1) Preparation of the suspension of rat peritoneal exudate cells

Peritoneal exudate cells (PEC) were isolated in the same manner as described in the previous report (3). The PEC obtained was suspended in phosphate buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂ · 6H₂O, 5.6 mM glucose, 5 units/ml of heparin, and adjusted to pH 7.2 with 5% (v/v) of 0.1 M Sörensen phosphate buffer, and they were prepared in a concentration of 5 x 10⁴ MC/ml of PBS unless otherwise noted.

2) Sensitization of PEC and induction of histamine release

The PEC were sensitized in vitro by incubation with rat IgE antiserum against 2,4-dinitrophenyl-coupled ascaris extract (DNP-As) as described in the previous report (3). The amount of histamine released in the medium by the challenge with 100 μg/ml of DNP-As as an antigen at 37°C for various periods and the amount of residual intracellular histamine were determined by the method of Shore et al. (11).

3) Assay of cAMP in PEC

One milliliter of the sensitized or non-sensitized PEC suspension was incubated for various periods at 37°C. The PEC suspension was immersed into a dry ice-acetone bath until frozen, and this was followed by subsequent immersing into a boiling water bath for 5 min. The PEC were homogenized and centrifuged for 10 min at 1,100 x g. An aliquot of the supernatant was used for cAMP determination by the binding assay method of Gilman (12).

4) Assay of ATP in PEC

The sensitized or non-sensitized PEC suspension was treated in the same manner as described for the cAMP assay, and the amount of ATP was assayed using an ATP kit (Boehringer Co.).

5) Enzyme activity

Na⁺, K⁺-dependent adenosine triphosphatase (Na⁺, K⁺-ATPase) activity: Rat brain microsomes were treated with 2 M NaI; and Na⁺, K⁺-ATPase was separated and activated by the method of Nakao et al. (13). Inorganic phosphorus liberated during the enzyme reaction was determined by the method of Fiske and Subbarow (14), while protein content was determined by the Lowry method (15).

6) Antigen-induced ¹⁴C0₂ production in sensitized PEC

Sensitized PEC (comprising 10⁶ mast cells) suspended in 2 ml of PBS containing 0.1 μCi of 6-¹⁴C-glucose were incubated with DNP-As in a final concentration of 100 μg/ml in a Warburg flask at 37°C. The reaction was terminated after an established period by the addition of 0.3 ml of perchloric acid (70%). The ¹⁴CO₂ generated was incubated so it could adsorb in 0.4 ml of hyamine for 30 min according to the method described by Tamers et al. (16), and the radioactivity of the adsorbed ¹⁴CO₂ was determined by a liquid scintillation counter (Packard).

7) Drugs used

The drugs used were as follows: N-5' (Kissei Pharmaceutical Co.), DSCG (Fujisawa Pharmaceutical Co.), epinephrine hydrochloride (Sankyö Pharmaceutical Co.), theophylline (Nakarai Chemicals), papaverine hydrochloride (Dainippon Pharmaceutical Co.), ouabain (Merck), ATP-2Na (Sigma), cAMP (Wako Chemicals), NaI (Nakarai Chemicals), phosphatidylserine (Nakarai Chemicals), ³H-cAMP (RCC Amersham, 27 mCi/mmol) and D-6-¹⁴C-glucose (RCC Amersham, 60.4 mCi/mmol).
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N-5' was dissolved in a 1% NaHCO₃ aqueous solution and diluted to the required concentration with PBS. The other drugs used were dissolved in PBS, and phosphatidylserine was dissolved in ethanol. In the antigen-antibody reaction, drugs to be tested were applied 1 min prior to the antigen challenge, at which time inhibition of allergic histamine release by N-5' is most potent as described in the previous report (3).

Results

1) Influence on antigen-induced histamine release from PEC and intracellular cAMP and ATP levels: Histamine release was quite evident when 100 μg/ml of the antigen was added to the rat sensitized PEC. The release showed a maximum at 30 sec after the antigen challenge. Such a release was obviously inhibited by 100 μM N-5' and 100 μM DSCG (Fig. 1-A). Conversely, the cAMP level in the PEC decreased in virtually the same time course as the histamine release. A minimum value was exhibited at 30 sec after the antigen challenge. A decrease in cAMP level induced by the antigen showed a tendency to be suppressed by 100 μM N-5' and 100 μM DSCG (Fig. 1-B). The ATP level in PEC also decreased in the same time course as the histamine release and the decrease in cAMP level discussed above. N-5' in a dose of 100 μM significantly inhibited the decrease in ATP level induced by the antigen (Fig. 1-C).

2) Influence on antigen-induced production of ¹⁴CO₂ in sensitized PEC: When DNP-As was added to the sensitized PEC suspension containing 0.1 μCi of 6-¹⁴C-glucose, significant generation of ¹⁴CO₂ was seen 1 min after; and thereafter, ¹⁴CO₂ production increased in accordance with the incubation period. The amount of ¹⁴CO₂ at 20 min after the antigen challenge was approx. 3 times that seen in the case without antigen.

![Fig. 1. Effect of N-5' and DSCG on antigen-induced histamine release (A) from rat PEC and changes in the intracellular levels of cAMP (B) and ATP (C). PEC were sensitized with rat IgE antiserum against DNP-As in vitro. Each point represents the mean of 4 experiments and vertical bars indicate standard error. *: Statistical significance from the control at P<0.05 and P<0.01, respectively. O: Control. ●: 100 μM N-5'. △: 100 μM DSCG.](image)

![Fig. 2. Effect of N-5' on antigen-induced ¹⁴CO₂ production from 6-¹⁴C-glucose in PEC of rats. PEC were sensitized with rat IgE antiserum against DNP-As in vitro. Each point represents the mean of 3 to 5 experiments and vertical bars indicate standard error. *: Statistical significance from the control at P<0.05 and P<0.01, respectively. O: Control. ●: 100 μM N-5'. △: Spontaneous ¹⁴CO₂ production without antigen.](image)
without antigen. Application of 100 \( \mu M \)
N-5' clearly suppressed the increase in generation of \(^{14}CO_2\) induced by the antigen challenge at any observation time (Fig. 2).

3) Influence on allergic histamine release in the presence or absence of glucose: Histamine release from PEC mediated by IgE was clearly lower during the absence of glucose than during the presence of glucose in the medium used, as shown in Table 1. The inhibitory effect of N-5' and that of theophylline on the histamine release were identical, both in the presence or in the absence of glucose. In contrast, the inhibitory

| Drug (\( \mu M \)) | With glucose | Without glucose |
|-------------------|--------------|----------------|
|                   | Histamine release (%) | Inhibition (%) | Histamine release (%) | Inhibition (%) |
| Control           | 42.6\(\pm\)1.25 | 27.8\(\pm\)0.47 |
| Spontaneous       | 10.8\(\pm\)0.06 | 5.7\(\pm\)0.21 |
| N-5' (100)        | 25.5\(\pm\)1.36* | 53.8 | 14.5\(\pm\)0.98* | 60.2 |
| Papaverine (100)  | 36.9\(\pm\)0.60 | 17.9 | 9.0\(\pm\)0.23* | 85.1 |
| Theophylline (1000)| 21.4\(\pm\)1.36* | 66.7 | 15.4\(\pm\)1.21* | 56.1 |

Inhibitory activity of drugs was examined in the medium containing glucose (5.6 mM) or without glucose. The sensitized PEC was incubated at 37°C for 20 min after the antigen challenge. Each experiment represents the mean\(\pm\)S.E. of 3 observations. *: Statistical significance from the control at P<0.01.

| Drug                | \( \mu M \) | 1 min | 20 min |
|---------------------|-------------|-------|--------|
| Control             | 10          | 1.94\(\pm\)0.16 | 1.86\(\pm\)0.12 |
| N-5'                | 100         | 1.89\(\pm\)0.34 | 1.74\(\pm\)0.27 |
| Papaverine          | 1000        | 1.84\(\pm\)0.11 | 1.69\(\pm\)0.20 |

Non-sensitized PEC were incubated in the medium without glucose for 1 or 20 min. Each datum indicates \(\mu g/tube\) of ATP and represents the mean\(\pm\)S.E. of 5 observations. *: Statistical significance from the control at P<0.01.

| Drug                      | \( \mu M \) | ATP liberated (\(\mu m mole/mg\) protein/30 min) | Inhibition % |
|---------------------------|-------------|-------------------------------------------------|--------------|
| Control                   |             | 82.5\(\pm\)7.2 |                                |              |
| N-5'                      | 10          | 70.6\(\pm\)6.4 | 14.4 |                              |
|                           | 100         | 61.8\(\pm\)7.2* | 25.1 |                              |
|                           | 1000        | 38.0\(\pm\)6.4* | 54.0 |                              |
| Ouabain                   | 10          | 54.3\(\pm\)8.6* | 34.2 |                              |
| Phosphatidylserine        | 100         | 90.4\(\pm\)7.2 | -9.6 |                              |

Each experiment included 8 to 20 observations. *: Statistical significance from the control at P<0.05 and P<0.01, respectively.
4) Influence on ATP level in non-sensitized PEC: The effects of 10 to 1,000 nM N-5' and of 100 nM papaverine on the ATP level in non-sensitized PEC in glucose-free medium were studied. Although N-5' had virtually no effect on the ATP level after a 1- or 20-min incubation, papaverine reduced the ATP level in both cases (Table 2).

5) Influence on Na+, K+-ATPase activity: The activity of crude Na+, K+-ATPase prepared from rat brain microsomes was markedly inhibited by 10 nM ouabain, while it increased slightly with the addition of 100 nM phosphatidylserine. On the other hand, 10 to 1,000 nM N-5' exhibited a dose-dependent suppressive effect (Table 3).

Discussion

Histamine release induced by the specific antigen (DNP-As) from the rat PEC sensitized in vitro with IgE antibody reached a maximum 30 sec after challenge. In virtually the same time course, the levels of cAMP and ATP in the cells decreased. N-5' exhibited a pronounced inhibitory effect on the histamine release and a suppressive effect on the decrease in the intracellular levels of cAMP and ATP. It is well known that an energy requiring process is essential when histamine release is induced by the antigen challenge (8–10). In our study, we observed that the ATP level decreased in accordance with the time course of histamine release. Accordingly, it was presumed that ATP was consumed during histamine release. An increase in the production of $^{14}$CO$_2$ from 6-$^{14}$C-glucose by the antigen challenge in the Embden-Meyerhof cycle, a de novo synthetic system of ATP, is conceivable to support this presumption. The fact that the inhibition of the histamine release in the absence of glucose by papaverine, an uncoupler of oxidative phosphorylation (17), disappeared by the addition of glucose to the medium also supports this assumption. Inhibitors of glucose metabolism such as 2-deoxyglucose are able to inhibit the allergic histamine release from mast cells (18). N-5' suppressed the acceleration of $^{14}$CO$_2$ production from 6-$^{14}$C-glucose by the antigen challenge in the sensitized PEC. However, as N-5' also suppressed the decrease in the intracellular level of ATP, the suppression of glucose metabolism by N-5' seemed to be the result of the inhibition of ATP consumption rather than direct inhibition of glucose metabolism.

Since the inhibition of the allergic histamine release by N-5' was not affected in the presence of glucose in the medium and N-5' scarcely affected the ATP level in non-sensitized PEC in glucose-free medium, N-5' has a different mechanism from those of the metabolic inhibitors such as papaverine and 2-deoxyglucose concerning the inhibition of histamine release. On the other hand, N-5' exhibited a clear inhibitory effect in a dose-dependent fashion on the activity of Na+, K+-ATPase, one of the ATP-consuming enzymes. As mentioned previously, a suppressive effect of N-5' on the decrease in ATP level was exhibited during allergic histamine release. Although, because we used rat brain microsomes as the source of Na+, K+-ATPase, tissue specificity of the inhibition to this enzyme by N-5' must be examined using purified mast cells; at least a part of the action of N-5' in inhibiting the histamine release appears to be due to its suppression of ATP utilization through the inhibition of Na+, K+-ATPase.

It has been well established that cAMP plays an important role in the process of allergic histamine release (5–7). There have been reports concerning the suppression of histamine release during the inhibition of phosphodiesterase activity due to xanthine derivatives and during the activation of
adenylate cyclase activity due to adrenergic 
β-receptor stimulants (19, 20). Kaliner and 
Austen (18) reported that the decrease in 
cAMP level during histamine release is due 
to the decrease in intracellular level of ATP, 
the substrate of cAMP synthesis. In our 
study, we demonstrated that the cAMP 
level in the PEC decreased in close correlation 
with the time course of histamine release 
mediated by IgE. Koda et al. (21) reported 
that degranulation of mesentric mast cells 
during the antigen-antibody reaction was 
inhibited by isoproterenol and theophylline 
dose-dependently, and each inhibitory effect 
of both drugs was potentiated synergistically 
by the combination with the other drug. They 
also reported that as N-5' and DSCG did not 
potentiate the inhibitory effect of isoproterenol 
or theophylline on the degranulation, the 
inhibitory effect of N-5' and DSCG on 
degranulation of mast cells is not dependent 
on the elevation of cAMP level in mast cells. 
In our study, as the suppression of decrease 
in the intracellular level of cAMP during the 
inhibition of histamine release by N-5' was 
less than that shown in the case of the ATP 
level, the role of suppression of the decrease 
in intracellular level of cAMP in the inhibition 
of histamine release by N-5' does not seem 
to be so large. 

As indicated above, at least a part of the 
effect of N-5' in inhibiting the histamine 
release mediated by IgE is presumed to 
originate in the suppression of ATP-utilization 
due to the inhibition of Na+, K+-ATPase 
activity. However, because we used "mixed" 
PEC in this experiment, changes in adenine 
nucleotides such as ATP and cAMP level in 
PEC during the histamine release may not 
accurately reflect those in mast cells. So these 
must be further studied using purified mast 
cells with the inhibition to Na+, K+-ATPase. 

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