Original Article

The effect of cyclic nucleotide analog drugs on the mediators release from basophils

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Abstract

Background: The cyclic nucleotides, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), are intracellular second messengers that play an important role in modulating inflammatory cells involved in allergic diseases. In general, cAMP suppresses the activity of immune and inflammatory cells. We aim to evaluate the roles of cAMP and cGMP in regulating basophil activity.

Materials and Methods: Basophil-enriched preparations were incubated with analogs and then challenged with anti-IgE or IL-3 (4 or 24 hours). Supernatants were assayed for histamine, IL-4, and IL-13 release. The effects of Sp-8-CPT-cAMPS and Sp-8-CPT-cGMPS on IL-3-dependent mediator release from basophils were determined. The cells were pre-incubated with an analog and then incubated with IL-3 for 24 hours.

Results: Sp-8-CPT-cAMPS was an effective ($P < 0.05$) inhibitor of IL-4, IL-13, and histamine release from basophils. However, paradoxically, Sp-8-CPT-cGMPS enhanced histamine release and IL-13 generation, but by contrast, had little effect on IL-4 generation. Sp-8-CPT-cGMPS inhibited cytokine generation, but enhanced the release of histamine release to a modest extent.

Conclusion: This study shows that the cAMP/protein kinase A (PKA) pathway may be inhibitory to the IgE- and non-IgE-dependent release of mediators from basophils.

Key Words: Allergy, basophils, cytokine, drug

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INTRODUCTION

The cyclic nucleotides cAMP and cGMP are intracellular second messengers that play an important role in modulating inflammatory cells involved in allergic diseases.[1-5] Alteration in the levels of intracellular cAMP and cGMP can cause a wide range of functional effects in cells, from changes in the rates of ion entry to gene transcription.[6-11] cAMP suppresses the activity of immune and inflammatory cells such as basophils. Our laboratory has shown that agents that elevate and sustain increases in cAMP levels, inhibit IgE-triggered histamine release from basophils.[12] Adenylate cyclase, which is activated by G-proteins, generates increased levels of cAMP in intact cells or tissues.[13] By contrast, guanylate cyclase is activated by nitric oxide and guanylate cyclase — linked receptors that mediate the

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actions of certain peptides such as natriuretic peptides, which cause cGMP accumulation in cells.[14-16]

As cAMP and cGMP are considered to be impermeable to the cell membrane, due to the polar nature of the phosphate group, structural modifications are used to make the compounds more cell-permeant. Analogs with hydrophobic substitutions (e.g., Bu_2-cAMP, 8-Br-cAMP) and phosphorothioate modification (Sp-8-CPT-cAMPS) are generated. These analogs are more lipophilic, hence, more membrane permeable and show reduced susceptibility to hydrolysis by phosphodiesterases (PDEs).[17] In the present study, we have investigated and compared the effects of several analogs of cAMP and cGMP on histamine and cytokine release from basophils stimulated by IgE-dependent or IgE-independent mechanisms. The novelty of this study is to try to evaluate the role of the cAMP/PKA pathway, if any, in modulating human basophil activity in allergic diseases.

MATERIALS AND METHODS

Basophil isolation

Basophil-enriched preparations were isolated from whole fresh blood of healthy individuals (50-100 ml of venous blood was anti-coagulated with 5-10 ml of 0.1 M Ethylenediaminetetraacetic acid (EDTA)). Briefly, whole venous blood was layered over a two-step discontinuous Percoll gradient consisting of 15 ml of 62% Percoll overlaid with 15 ml of 53% Percoll and a basophil-rich layer (10-15% purity) was harvested. The cells were then washed once in 1 x PIPES (piperazine-N,N′-bis(2-ethanesulfonic acid), twice in Phosphate Buffered Saline (PBS) — EDTA and counted with Alcian Blue. The enriched-basophils were used in experiments investigating the release of histamine, IL-4, and IL-13.

Mediator release

The release of histamine, IL-4, and IL-13 was assessed from the basophil-enriched preparations activated with either anti-human IgE or IL-3. The effects of a Zaprinast inhibitor on the generation of these mediators were also determined. Mediator-release experiments were performed in the Roswell Park Memorial Institute RPMI buffer supplemented with bovine serum albumin (BSA), gentamicin, and calcium chloride. Typically, the basophils (80,000-300,000 basophils per sample) were incubated (30 minutes) with an inhibitor or buffer, before challenge with a stimulus. Cells incubated in buffer alone served as measures of spontaneous mediator release, and all values cited for stimulus-induced mediator generation were corrected by subtracting this spontaneous mediator release. In experiments monitoring IL-4 generation, the basophils were activated for four hours with an optimal releasing concentration of anti-IgE (1/100,000), and in monitoring IL-13 generation, the basophils were activated for 24 hours. These conditions for optimal production of IL-4 and IL-13 generation have been reported by others[18,19] and have been confirmed by us in a series of preliminary experiments. After activation, the cells were centrifuged and the supernatants analyzed for mediator release. The histamine content was analyzed using a modification of the automated fluorometric technique. Also the supernatant was assayed for IL-4 and IL-13 content by the enzyme-linked immunosorbent assay (ELISA). The limit of sensitivity was 0.2 and 0.5 pg/ml for the IL-4 and IL-13 assays, respectively. The Optical Density (OD) of the samples was measured at 450 nM using a Dynatech plate reader.

Materials

The following were purchased from the sources indicated; 8-bromo-cAMP, 8-bromo-cGMP, Dimethyl sulfoxide (DMSO), goat anti-human IgE, PIPES (free acid), Percoll, BSA, zaprinast (Sigma, Poole, U.K.);gentamicin, and RPMI 1640 (Gibco BRL, Dundee, U.K.); IL-3 (Peprotech, Rocky Hill, NJ, U.S.A.); Sp-8- CPT-cAMPS and Sp-8-CPT-cGMPS (Biolog Life Science Institute, Bremen, Germany); and ELISA kits for human IL-4 and IL-13 (Mast Diagnostics, Amsterdam, Netherlands).

Data analysis

Data were expressed as means ± S.E.M. EC50 values were determined using GraphPad Prism software (version 3). In order to establish whether drug treatments caused statistically significant effects, either paired t-tests or ANOVA, followed by the Dunnett test, were performed.

RESULTS

Effects of cAMP and cGMP analogs on histamine release from basophils

To investigate the role of cAMP and cGMP in human basophils, a number of structurally distinct analogs of cAMP and cGMP were investigated for the inhibitory effects on histamine release induced by anti-IgE.

The effects of Bu_2-cAMP and Bu_2-cGMP on IgE-mediated histamine release from basophils were assessed. The cells were pre-treated for 30 minutes in the presence of increasing concentrations ($3 \times 10^{-5} - 3 \times 10^{-3}$ M) of both nucleotide analogs. Then the cells were triggered with an optimal releasing concentration of anti-IgE (1/3000) for a further 45 minutes for the release of histamine. The data show [Figure 1] that Bu_2-cAMP inhibits histamine release in a dose-dependent manner with maximal inhibition of 80 ± 7% at 3 mM and an EC_{50} of about

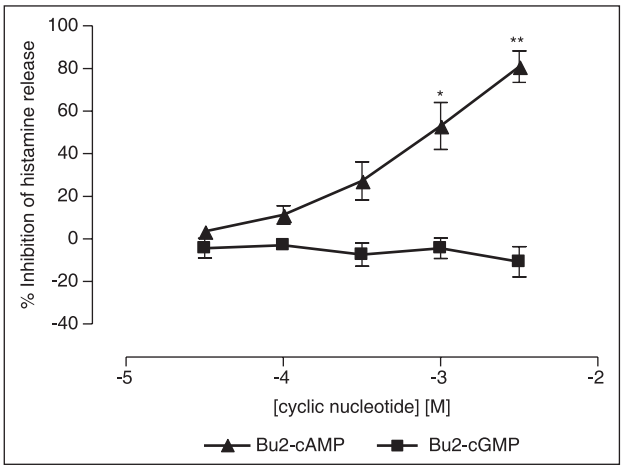

Figure 1: Effects of Bu_2-cAMP (▲) and Bu_2-cGMP (■) on histamine release from human basophils. Results are the percent inhibition of the control histamine release, which was 48 ± 4%. Values are means ± S.E.M, $n = 4$. Asterisks denote statistically significant levels of inhibition; $^{**}P < 0.01$, $^{*}P < 0.05$

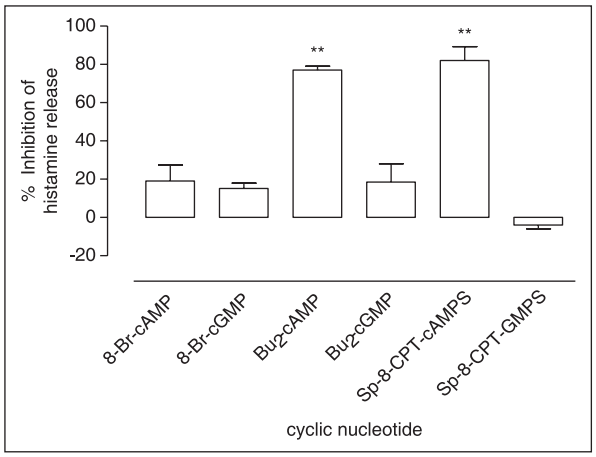

Figure 2: Effect of different cyclic nucleotide analogs on histamine release from basophils. Results are expressed as the percent inhibition of control histamine release, which was 34 ± 7%. Values are means ± S.E.M., $n = 4$. Asterisks denote statistically significant levels of inhibition; $^{**}P < 0.01$

1 mM. By contrast, Bu_2-cGMP was ineffective in inhibiting histamine release.

The effects of Bu_2-cAMP, Bu_2-cGMP, 8-Br-cAMP, 8-Br-cGMP, Sp-8-CPT-cAMPS, and Sp-8-CPT-cGMPS (1 mM) on IgE-mediated histamine release from basophils were investigated [Figure 2]. Both Bu_2-cAMP and Sp-8-CPT-cAMPS were very effective ($P < 0.01$) inhibitors of histamine release, whereas, 8-Br-cAMP was relatively ineffective. None of the cGMP analogs, Bu_2-cGMP, 8-Br-cGMP or Sp-8-CPT-cGMPS, were effective inhibitors of histamine release.

Effects of cAMP and cGMP analogs on cytokine generation from basophils

We examined the effects of Sp-8-CPT-cAMPS and Sp-8-CPT-cGMPS (1 mM) on the generation of IL-4 and IL-13, as well as histamine, from basophils activated with anti-IgE. Basophil-enriched preparations were incubated for 30 minutes with analogs and then challenged with anti-IgE for 24 hours [Figure 3]. The data showed that Sp-8-CPT-cAMPS was an effective ($P < 0.05$) inhibitor of IL-4, IL-13, and histamine release from basophils. However, paradoxically, Sp-8-CPT-cGMPS enhanced histamine release and IL-13 generation, but by contrast, had little effect on IL-4 generation.

Subsequently, the effects of Sp-8-CPT-cAMPS and Sp-8-CPT-cGMPS on IL-3-dependent mediator release from basophils were determined. The cells were pre-incubated for 30 minutes with an analog (1 mM) and then incubated with IL-3 (100 ng/ml) for 24 hours. Sp-8-CPT-cAMPS was effective (at least $P < 0.05$) at inhibiting histamine release, IL-4, and IL-13 generation from basophils. Sp-8-CPT-cGMPS inhibited cytokine generation, but enhanced the release of histamine release to a modest extent [Figure 4].

Effect of zaprinast on mediator release from basophils

To investigate the role of cGMP further, the effect of a zaprinast was assessed. The basophils were pretreated for 15 minutes with zaprinast (10 μM) and then challenged with anti-IgE for a further four hours (IL-4, histamine) or 24 hours (IL-13, histamine) [Table 1]. The results showed that zaprinast had little effect ($P > 0.05$) on histamine release or cytokine generation. The effects of zaprinast on histamine and cytokine generation induced by IL-3 (100 ng/ml) were examined [Table 2]. The basophils were incubated for 15 minutes with zaprinast (10 μM) and then further incubated with IL-3 for 24 hours. Zaprinast had no significant effect ($P > 0.05$) on mediator release from basophils.

Previous studies have shown that sodium nitroprusside elevates cGMP and activates protein kinase G (PKG) through activation of guanylate cyclase.[20-23] The cells were incubated with increasing concentrations of this compound (10^{-7}-10^{-3} M) for 30 minutes, before challenge with anti-IgE (data not shown). Sodium nitroprusside had no effect on histamine release except at the highest concentration used.

DISCUSSION

A number of analogs of cAMP and cGMP were studied for effects on the IgE-mediated release of histamine.[24] Bu_2-cAMP was an effective inhibitor of histamine release, but 8-Br-cAMP was ineffective. However, 8-Br-cAMP, although recognized as a superior probe

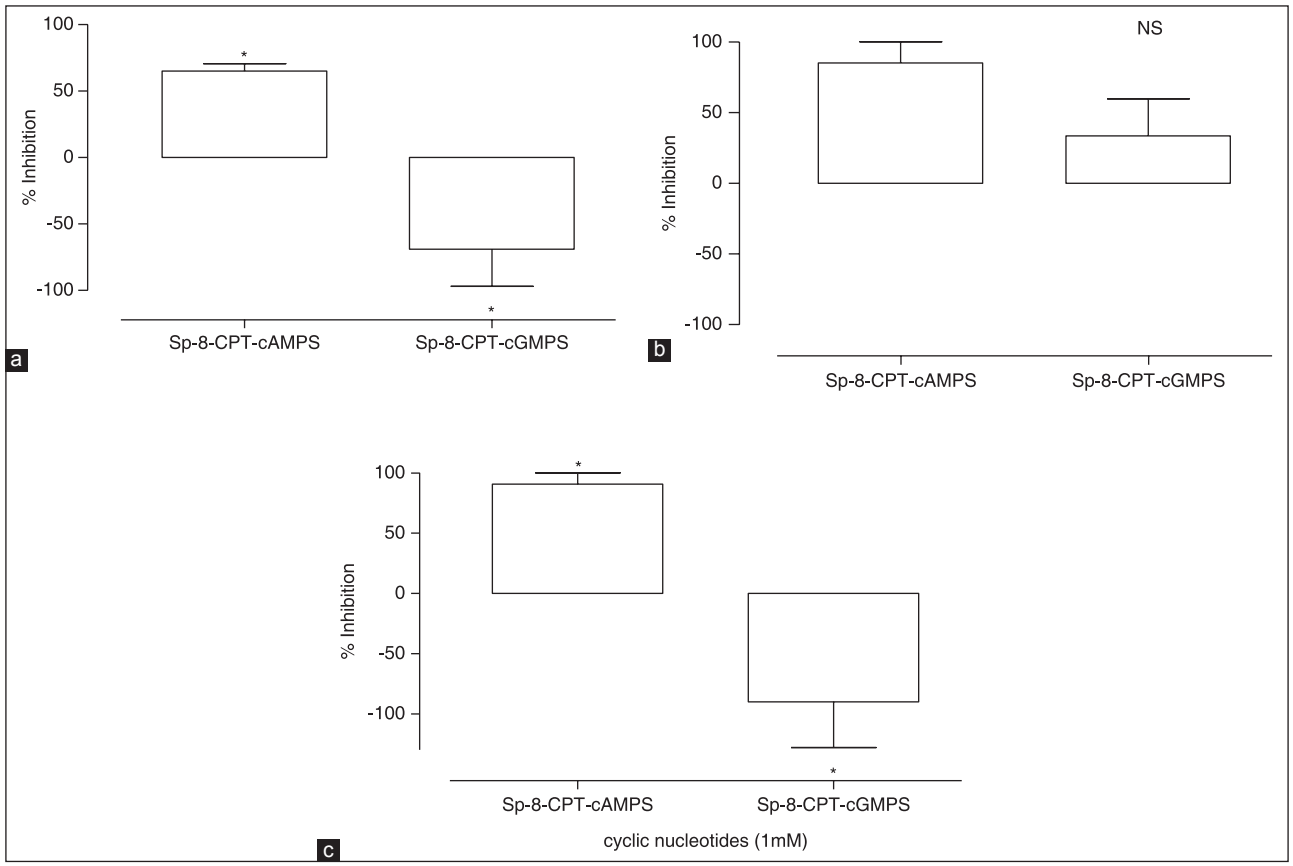

Figure 3: Effect of Sp-8-CPT-cAMPS and Sp-8-CPT-cGMPS analogs on IgE-dependent histamine (a), IL-4 (b), and IL-13 (c) release from human basophils. Results are expressed as the percent inhibition of control releases, which were 18 ± 3% histamine release, 8.5 ± 1 pg IL-4 per 10^6 basophils, and 39 ± 17 pg IL-13 per 10^6 basophils, respectively. Values are means ± S.E.M., n = 4-6. Asterisks denote statistically significant ($P < 0.05$) changes. Compared to control values; $^*P < 0.05$. NS, not significant

to Bu_2-cAMP in terms of specificity and resistance to hydrolysis, was found to be less permeable to the cell membrane than Bu_2-cAMP.[17] The inhibitory effects seen with Bu_2-cAMP, however, might not be due to the activation of PKA by Bu_2-cAMP, as it is known that Bu_2-cAMP could be converted intracellularly to butyrate and it was possible that butyrate was responsible for the inhibitory effects.[25-28] However, an alternative, highly lipophilic and nonhydrolyzable analog, Sp-8-CPT-cAMPS,[17,29] was a very effective inhibitor of stimulated histamine release from basophils. In contrast to these cAMP analogs, none of the cGMP analogs studied had any effect on the release of histamine induced by anti-IgE, arguing against a role for cGMP in the regulation of histamine release.

In further experiments to analyze the role of cAMP and cGMP analogs on cytokine generation, experiments with Sp-8-CPT-cAMPS, indicated that this analog was an effective inhibitor of cytokine generation from basophils activated by IL-3 or anti-IgE. This suggested that targeting the cAMP/PKA pathway was effective in attenuating the generation of cytokines and histamine release from basophils. However, in contrast to Sp-8-CPT-cAMP, Sp-8-CPT-cGMP enhanced IgE-mediated histamine release and IL-13 generation, but had no significant effect on IL-4 generation. On the other hand, when IL-3 was used as the stimulus, cytokine generation was effectively inhibited by Sp-8-CPT-cGMPS, whereas, no inhibition of histamine release from basophils was observed. These conflicting findings make it difficult to determine the role of cGMP in basophils. It is possible that Sp-8-CPT-cGMPS interferes with different pathways when different stimuli are used to activate basophils to modulate histamine and cytokine generation. Sp-8-CPT-cGMPS is considered as a superior nucleotide analog in terms of PDE resistance and lipophilicity, but it is not selective for PKG activation.[30,31] This suggests that the varied effects seen with Sp-8-CPT-cGMP in basophils are not necessarily mediated exclusively through the cGMP/PKG pathway. In addition, we found that zaprinast had no significant effect on mediator release induced by anti-IgE or IL-3. These findings with zaprinast do not support a role for cGMP in the regulation of basophil activity.

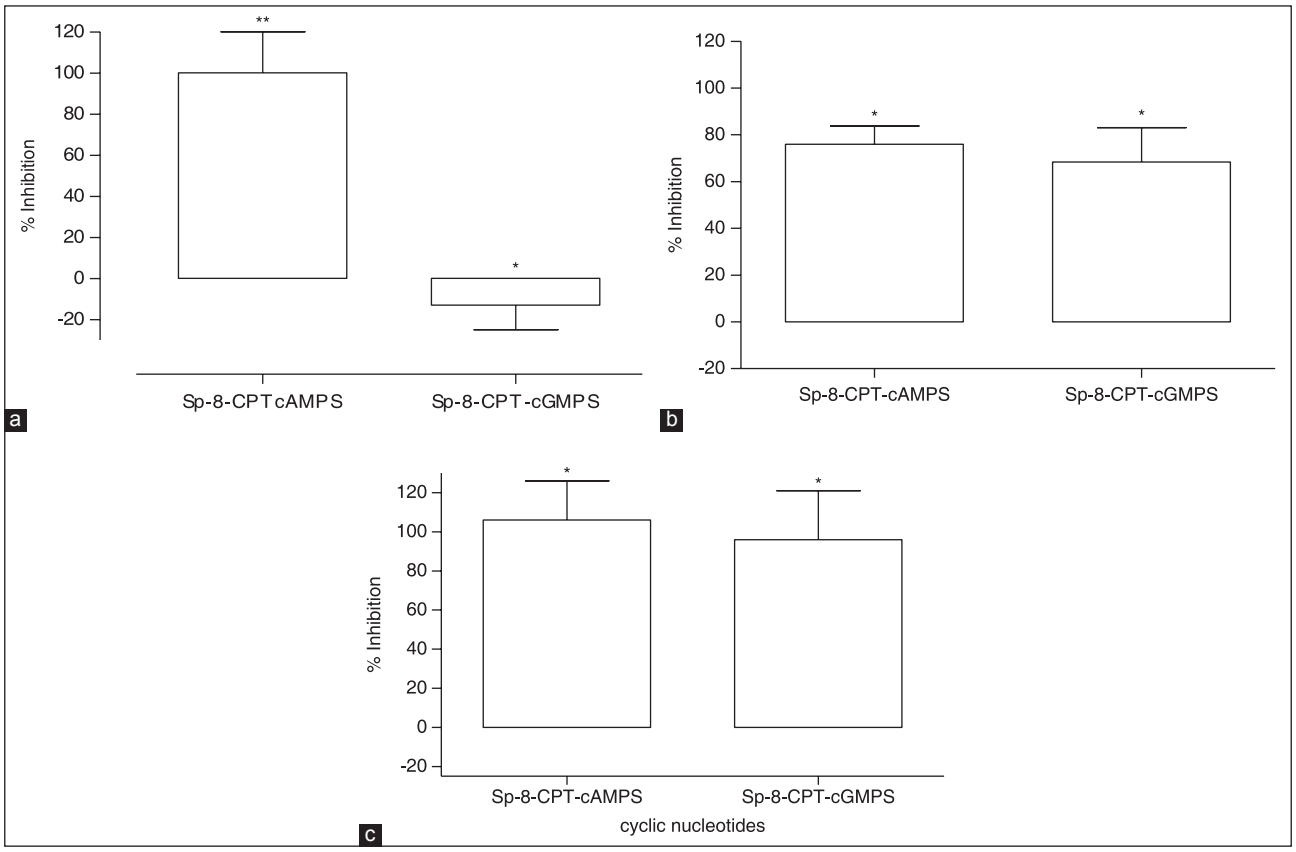

Figure 4: Effect of Sp-8-CPT-cAMPS and Sp-8-CPT-cGMPS analogs on IL-3-dependent histamine (a), IL-4 (b), and IL-13 (c) release from human basophils. Results are expressed as the percent inhibition of control releases, which were 15 ± 5% histamine release, 15 ± 7 pg IL-4 per 10^6 basophils, and 300 ± 84 pg IL-13 per 10^6 basophils. Values are means ± S.E.M., n = 4-6. Asterisks denote statistically significant ($P < 0.05$) changes. Compared to control; $^{*}P < 0.05$, $^{**}P < 0.01$

Table 1: Effect of the cGMP-specific PDE (PDE5) inhibitor, zaprinast, on the generation of histamine (a: 4h, b: 24h incubation), IL-13, and IL-4 from basophils activated with anti-IgE

	Percent Inhibition			
Inhibitor	**Histamine[a]**	**IL-4**	**Histamine[b]**	**IL-13**
Zaprinast	23±3	21±10	23±8	26±7

*All values are means ± S.E.M

Table 2: Effect of the cGMP-specific PDE (PDE5) inhibitor, zaprinast, on the generation of histamine, IL-13 and IL-4 from basophils activated with IL-3

	Percent Inhibition		
Inhibitor	**Histamine**	**IL-4**	**IL-13**
Zaprinast	14±7	34±11	10±4

*All values are means ± S.E.M

Sodium nitroprusside is known to activate gluanylate cyclase and this leads to elevations in cGMP levels.[32,33] In agreement with other data,[34-36] it has been found that sodium nitroprusside has no effect on histamine release except at a high concentration. Although these data suggest an inhibitory role for cGMP in basophils, a more complete assessment of the effects of nitroprusside on cytokine generation induced by anti-IgE or IL-3 would provide a clearer indication of the role of cGMP in basophils.

We conclude that the cAMP/PKA pathway is inhibitory to the IgE and non-IgE-dependent release of mediators from basophils. By contrast, the role, if any, of the cGMP / PKG pathway in modulating basophil activity is uncertain.

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