Dissociation of tumour-promoter-induced effects on prostaglandin release, polyamine synthesis and cell proliferation of 3T3 cells

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The phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate induces tumour promotion, inflammation, cell proliferation and prostaglandin release. Recent reports suggest that the prostaglandins released by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) initiate a cascade of events leading to polyamine synthesis and cell proliferation. In experiments designed to test this contention, it was found that addition of TPA (1 μ M to 1 nM) to confluent mouse 3T3 fibroblasts successively caused the release of prostaglandins E₂ and I₂, induction of the enzyme ornithine decarboxylase (EC 4.1.1.17), stimulation of [³H]thymidine incorporation into DNA, and cell proliferation. Pretreatment of the cells with the anti-inflammatory steroid dexamethasone (1 μ M) or the non-steroidal anti-inflammatory drug indomethacin (1 μ M) inhibited TPA-induced prostaglandin release. However, dexamethasone enhanced the other effects of TPA, whereas indomethacin was ineffective. Addition of prostaglandin E₂ to the cultures did not induce ornithine decarboxylase activity and cell proliferation. Pretreatment of the cells with 1,3-diaminopropane (1 mM) or α -methylornithine (5 mM), inhibitors of polyamine synthesis, decreased TPA-induced ornithine decarboxylase activity without affecting DNA synthesis. TPA stimulated [³H]thymidine incorporation into DNA, even when the ornithine decarboxylase activity was completely blocked. These data suggest that the proliferative effect of TPA on 3T3 cells is independent of prostaglandin release and polyamine synthesis.

Many macrocyclic diterpene esters possess tumour-promoting activity (Hecker & Schmidt, 1974). These agents do not themselves induce cancer, but markedly enhance the formation of skin tumours when repeatedly applied to mouse skin pretreated with low doses of chemical carcinogens (Van Duuren, 1969). The most potent tumour promoter of this group is 12-*O*-tetradecanoylphorbol 13-acetate. It is the active constituent of croton oil obtained from *Croton tiglium* seeds (Hecker, 1968). The tumour-promoting activity of TPA has been determined in a two-stage mouse skin carcinogenesis system developed by Berenblum (1975).

Phorbol esters also elicit a variety of biological effects in many different cell types *in vivo* and *in vitro*. These effects include enhanced cell growth (Sivak, 1977) and biochemical changes such as the induction of ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17), which is the rate-

limiting enzyme in polyamine formation (Yuspa *et al.*, 1976). TPA also causes inflammation (Hecker, 1971) and alterations in cellular morphology (Sivak, 1977; Brune *et al.*, 1978; Lowe *et al.*, 1978; Ohuchi & Levine, 1978) connected with prostaglandin release *in vitro* (Brune *et al.*, 1978; Ohuchi & Levine, 1978; Yamasaki *et al.*, 1979) and *in vivo* (Ashendel & Boutwell, 1979; Fürstenberger & Marks, 1980).

The importance of inflammation-related cell proliferation for tumour promotion has been studied extensively, but remains unclear (Belman & Troll, 1972; Scribner & Boutwell, 1972; Scribner & Slaga, 1973; Brune *et al.*, 1978; Slaga *et al.*, 1978; Weeks & Slaga, 1979). Recent studies have suggested that prostaglandins released by inflammagens may initiate cell proliferation, which will then allow for the expression of malignant cell transformation (Verma *et al.*, 1977, 1980; Fürstenberger & Marks, 1978, 1980; Ashendel & Boutwell, 1979).

In the present study we investigated if indeed TPA-induced prostaglandin release causes cell proliferation in cultures of Swiss mouse 3T3 fibro-

Abbreviation used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

blasts, and if ornithine decarboxylase induction is an obligatory event in TPA-stimulated DNA synthesis. The results show that TPA-induced prostaglandin release and ornithine decarboxylase induction can be dissociated by pharmacological means from incorporation of thymidine into DNA and cell proliferation. The biochemical messenger(s) leading to cell proliferation subsequent to TPA addition are not prostaglandins or polyamines and therefore remain(s) to be defined.

Materials and methods

Chemicals

TPA was kindly provided by Professor Dr. E. Hecker, German Cancer Research Centre, Heidelberg, Germany. Indomethacin was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Dexamethasone was used as the water-soluble sodium phosphate salt (Decadron; Merck, Sharp and Dohme). 1,3-Diaminopropane was from Merck-Schuchardt, München, Germany, α -methyl-ornithine was from Calbiochem, San Diego, CA, U.S.A. Pyridoxal 5'-phosphate was purchased from Boehringer Mannheim G.m.b.H., and dithiothreitol and 3,5-diaminobenzoic acid were from Serva, Heidelberg, Germany. Prostaglandin E_2 was from the Upjohn Company, Kalamazoo, MI, U.S.A. Hyamine hydroxide (Hyamine is the registered trade mark of Rohm & Haas) was obtained from New England Nuclear, Boston, MA, U.S.A. The labelled compounds [5,6,8,11,12,14,15(n)- 3H]-prostaglandin E_2 (sp. radioactivity 160 Ci/mmol), L-[1- ^{14}C]ornithine monohydrochloride (sp. radioactivity 59 Ci/mol) and [6- 3H]thymidine (sp. radioactivity 5 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Other chemicals were analytical or reagent grade.

Cell cultures

Swiss mouse 3T3 cells (non-transformed) were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) newborn calf serum and 1% (v/v) penicillin/streptomycin solution (10000 units/ml), all supplied by Gibco Bio-Cult, Glasgow, Scotland, U.K. Cells were trypsin-treated and seeded at a density of about 5×10^3 cells per cm^2 in 35 mm Falcon plastic culture dishes for determination of prostaglandin release and cell proliferation. For all other experiments 60 mm dishes were used. Cultures were incubated at 37°C in a humidified atmosphere containing 10% CO_2 . Experiments were performed 4–5 days after plating when the cells just reached confluency. The medium was changed before addition of TPA only in the prostaglandin release experiments. TPA and indomethacin were dissolved in dimethyl sulphoxide, and prostaglandin E_2 in 70% ethanol for use in cell cultures. The concentration of

dimethyl sulphoxide or ethanol in the culture medium never exceeded 0.5%.

Determination of prostaglandin release

Prostaglandin E_2 was measured directly in the culture fluid by a specific radioimmunoassay described before (Glatt *et al.*, 1974). Prostaglandin I_2 was measured by a specific radioimmunoassay of the stable hydration metabolite 6-oxoprostaglandin $F_{1\alpha}$ as described by Peskar *et al.* (1979).

Determination of ornithine decarboxylase activity

Ornithine decarboxylase activity was determined by measuring the release of $^{14}CO_2$ from L-[1- ^{14}C]ornithine according to the method of Russell & Snyder (1968). The cells were scraped off the 60 mm dishes at indicated times after treatment and subsequently disrupted by freezing and thawing in 0.3 ml of 50 mM-sodium phosphate/potassium phosphate buffer, pH 7.2, containing 5 mM-dithiothreitol and 0.2 mM-pyridoxal 5'-phosphate. Extracts were then centrifuged at 12000 g for 30 min and ornithine decarboxylase activity was measured in the supernatant fluids. The enzyme reaction was performed in 10 ml centrifuge tubes equipped with a rubber stopper supporting a centre well of polyethylene (Kontes Glass Co., Vineland, NJ, U.S.A.) containing 0.2 ml of 1 M-Hyamine hydroxide. Portions (0.1 ml) of the supernatant were incubated for 90 min at 37°C after addition of 10 μ l of L-[1- ^{14}C]ornithine solution (55 nmol, 0.25 μ Ci). The ornithine, dissolved in 0.05 M-HCl, was evaporated to dryness in a rotary evaporator before use in an aqueous solution to eliminate contamination by $^{14}CO_2$ present in the original commercial preparation. The enzyme reaction was terminated by addition of 0.5 ml of 40% trichloroacetic acid, and the incubation was continued for another 20 min to trap the released $^{14}CO_2$ in the Hyamine solution. The centre wells were then transferred into counting vials and radioactivity was determined by liquid-scintillation counting using Insta-Gel (Packard) as the scintillant. The protein content was determined in the supernatants, after destruction of dithiothreitol by chloramine-T (5 mg/ml), by the method of Lowry *et al.* (1951).

Determination of DNA synthesis

At indicated times after TPA addition, cells were treated with 4 μ Ci of [6- 3H]thymidine per 60 mm culture dish for 1 h. The cells were then washed three times with cold phosphate-buffered saline, scraped off the dishes and disrupted by freezing and thawing in 0.3 ml of phosphate-buffered saline. DNA was extracted and assayed fluorimetrically with 3,5-diaminobenzoic acid as described (Cattolico & Gibbs, 1975; Puzas & Goodman, 1978). [3H]Thymidine incorporation into DNA was determined by measuring radioactivity in a portion of the extract.

Determination of cell proliferation

Cells were trypsin-treated and portions of the cell suspension were counted in a Coulter counter.

Results

Effects of TPA on prostaglandin release, ornithine decarboxylase induction and cell proliferation

TPA at concentrations of $1\text{ }\mu\text{M}$ to 1 nM produced a dose-dependent release of prostaglandin E_2 and prostaglandin I_2 , which reached a maximum 2 h after TPA treatment (results not shown). The maximal amount (mean $\pm$ S.D.) of prostaglandin E_2 released by TPA was $12.92 \pm 0.97\text{ ng/ml}$ of culture fluid (control $2.69 \pm 0.55\text{ ng/ml}$), and the concentration of prostaglandin I_2 was $0.80 \pm 0.03\text{ ng/ml}$ (control $0.11 \pm 0.01\text{ ng/ml}$). TPA also markedly increased ornithine decarboxylase activity at the same concentrations at which prostaglandin release occurred (Fig. 1). The ornithine decarboxylase-inducing potency of TPA was most pronounced at day 4 after plating, at which stage the cells had just reached confluency (Fig. 2). Peak activity of ornithine decarboxylase was found 4 h after TPA treatment, i.e. shortly after the prostaglandin release had reached its maximum (Fig. 3). Incorporation of [^3H]thymidine into DNA was also stimulated by

Fig. 2. Effect of TPA on ornithine decarboxylase content of 3T3 cells at different times after plating

The number of cells was determined at different times after plating ($\square$). The ornithine decarboxylase activity was determined at the same times in dimethyl sulphoxide ($\blacksquare$)- or TPA ($\bullet$)-treated cultures. Exposure to dimethyl sulphoxide (solvent controls) or TPA ($0.1\text{ }\mu\text{M}$) was for 4 h. Results are means $\pm$ S.D. of four cultures in duplicate.

Fig. 1. Induction of ornithine decarboxylase by TPA and prostaglandin E_2

Confluent cells were treated with different concentrations of TPA or prostaglandin E_2 . At 4 h ornithine decarboxylase activity was determined. $\bullet$, TPA; $\circ$, prostaglandin E_2 (means $\pm$ S.D. of four experiments in duplicate).

$0.1\text{ }\mu\text{M}$ -TPA the maximum being at 18 h after TPA addition (Table 1). Also, addition of $0.1\text{ }\mu\text{M}$ -TPA increased the cell number per culture significantly within 2 days after treatment (Fig. 4), i.e. TPA consecutively stimulated prostaglandin release, ornithine decarboxylase activation and cell proliferation.

Effects of inhibitors of prostaglandin release on TPA-induced ornithine decarboxylase activity and cell proliferation

Pretreatment of cultures of 3T3 cells with dexamethasone ($1\text{ }\mu\text{M}$ to 1 nM) or indomethacin ($1\text{ }\mu\text{M}$ to 1 nM) caused a dose-dependent inhibition of TPA-induced release of prostaglandin E_2 and prostaglandin I_2 (results not shown). The TPA effect on ornithine decarboxylase and cell proliferation, however, was enhanced significantly by pretreatment of the cells with $1\text{ }\mu\text{M}$ -dexamethasone, whereas $1\text{ }\mu\text{M}$ -indomethacin was without measurable effects (Figs. 3 and 4a, Table 1), although both drugs almost completely blocked the prostaglandin release at these concentrations. Dexamethasone ($1\text{ }\mu\text{M}$) also increased the number of cells in culture when given alone (Fig. 4a). Addition of prostaglandin E_2 to unstimulated cells caused only a slight increase of ornithine decarboxylase activity (Fig. 1) and cell number (Fig. 4b) at high concentrations of about $10\text{ }\mu\text{M}$, i.e. it was not possible to mimic the effects of

Fig. 3. *Effect of dexamethasone and indomethacin on the TPA-induced content of ornithine decarboxylase*
 (a) Effect of dexamethasone. Confluent cells were exposed to 1 μ M-dexamethasone for 24 h. Then 0.1 μ M-TPA (final concentration) was added and the ornithine decarboxylase activity was subsequently determined. (b) Effect of indomethacin. Similar conditions as in (a), except that the cells were pretreated with indomethacin (1 μ M) for 1 h. ■, Control; ●, TPA (0.1 μ M); ▲, TPA (0.1 μ M) + dexamethasone (1 μ M); △, TPA (0.1 μ M) + indomethacin (1 μ M) (means $\pm$ s.d. for four experiments in duplicate). Significant differences ($P < 0.01$, by Student's t test) from TPA-treated groups were denoted by **.

Fig. 4. *Effect of TPA, prostaglandin E₂ and dexamethasone on cell proliferation*

At different times after the addition of drug, cells were harvested and counted in a Coulter counter. Results are means $\pm$ s.d. for five experiments. ** indicates a significant difference from the controls ($P < 0.01$); ++ indicates a significant difference from TPA-treated groups ($P < 0.01$) by Student's t test. (a) Effect of dexamethasone alone or in combination with TPA on cell growth. ■, Control; △, dexamethasone (1 μ M); ●, TPA (0.1 μ M); ▲, TPA (0.1 μ M) + dexamethasone (1 μ M). (b) Effect of TPA and prostaglandin E₂ on cell growth. ■, Control; ●, TPA (0.1 μ M); ○, prostaglandin E₂ (10 μ M).

TPA by prostaglandin E₂. No correlation was found between prostaglandin release, ornithine decarboxylase induction and cell proliferation when inhibitors of prostaglandin synthesis were applied.

Effects of inhibitors of polyamine synthesis on TPA-induced cell proliferation

Ornithine decarboxylase induction by 0.1 μ M-TPA was decreased by 80% after pretreatment of the cells

Table 1. Incorporation of [³H]thymidine into DNA

Confluent cells were exposed to 1 μM-indomethacin for 1 h or to 1 μM-dexamethasone for 24 h. Then TPA (final concentration 0.1 μM) was added and the incorporation of [³H]thymidine into DNA was determined at different times after TPA treatment (means ± s.d. for four experiments). Values that differ significantly from the controls by Student's *t* test are indicated (*, *P* < 0.05; †, *P* < 0.01).

		³ H Thymidine incorporation into DNA (d.p.m./μg of DNA) at indicated times after TPA addition					
Additions	Time (h)	9	15	18	21	24	30
No additions	...	1416 ± 96	1098 ± 274	1218 ± 138	1084 ± 146	1083 ± 221	1508 ± 72
TPA (0.1 μM) (controls)		1375 ± 37	12 336 ± 1322	13 166 ± 1448	8054 ± 813	4501 ± 320	1933 ± 69
TPA (0.1 μM) + indomethacin (1 μM)		—	—	12 655 ± 669	8451 ± 334	4312 ± 303	—
TPA (0.1 μM) + dexamethasone (1 μM)		—	14 315 ± 1626	15 265 ± 852*	12 208 ± 368†	6770 ± 309†	—

Fig. 5. Effect of 1,3-diaminopropane on TPA-induced ornithine decarboxylase activity and [³H]thymidine incorporation into DNA

Confluent cells were exposed to 1,3-diaminopropane (1–10 mM) for 2 h. Then 0.1 μM-TPA (final concentration) was added. Ornithine decarboxylase activity (a) (means ± s.d. for five experiments in duplicate) was determined 4 h and [³H]thymidine incorporation into DNA (b) (means ± s.d. for five experiments) 18 h after TPA treatment. ** indicates significant differences from the controls (*P* < 0.01); †† indicates significant differences from TPA-treated groups (*P* < 0.01) by Student's *t* test. A, Control; B, 0.1 μM-TPA; C, 0.1 μM-TPA + 1 mM-1,3-diaminopropane; D, 0.1 μM-TPA + 10 mM-1,3-diaminopropane.

with 1 mM-1,3-diaminopropane, which inhibits the synthesis of the enzyme. At this concentration, however, TPA-stimulated thymidine incorporation into DNA was not affected. Complete suppression

of ornithine decarboxylase activity by 10 mM-1,3-diaminopropane resulted in a decrease of TPA-stimulated thymidine incorporation by 60%. Nevertheless, TPA induced DNA synthesis in the total absence of measurable ornithine decarboxylase activity (Fig. 5). Similar results were obtained with a competitive inhibitor of ornithine decarboxylase activity, α-methylornithine (5 mM). This compound decreased TPA-induced ornithine decarboxylase activity by more than 60% when measured in extracts of pretreated cells without affecting TPA-stimulated DNA synthesis. No correlation was found between TPA-induced ornithine decarboxylase activity and thymidine incorporation into DNA when the synthesis or activity of ornithine decarboxylase was blocked by inhibitors.

Discussion

The studies described show that TPA triggers a cascade of events in 3T3 cells, leading from prostaglandin production to ornithine decarboxylase activation, followed by thymidine incorporation into DNA and cell proliferation. This chain of events does not appear to consist of causally connected links, in that we did not find evidence that the preceding step, e.g. prostaglandin production, initiates the following one, i.e. ornithine decarboxylase activation. We regard these findings as the main points emerging from our studies.

First, we have produced evidence that metabolic products of arachidonate, i.e. prostaglandins, are not required for the expression of cell proliferation in 3T3 cells as proposed for epidermal cells by Fürstenberger & Marks (1980). We have shown that TPA-induced ornithine decarboxylase activation, thymidine incorporation into DNA and cell proliferation proceed despite inhibition of prostaglandin synthesis. Moreover, prostaglandins, even in pharmacological concentrations (10 μM), could not mimic the effects of TPA in 3T3 cells. This is in

contrast with findings of other workers. Fürstenberger & Marks (1978) observed an inhibition of TPA-induced cell proliferation in mouse epidermis by non-steroidal inhibitors of prostaglandin synthesis at high doses (1–10 mM indomethacin). Hial *et al.* (1977) made similar observations with rat hepatoma cells or human fibroblasts in culture. At high concentrations inhibitors of prostaglandin synthesis have many pharmacological actions (Flower, 1974). It appears therefore possible that the effects observed by these authors may be unrelated to inhibition of prostaglandin synthesis. This interpretation is supported by the observation that indomethacin is a potent inhibitor of tumour growth (Hial *et al.*, 1976), an effect apparently unrelated to prostaglandin-synthesis inhibition (De Mello *et al.*, 1980). The apparent lack of importance of prostaglandins in TPA-stimulated cell proliferation is further supported by the observation that dexamethasone, despite inhibiting prostaglandin synthesis, enhances ornithine decarboxylase activation and cell proliferation after TPA administration. Glucocorticoids decrease the availability of arachidonate for further metabolism (Gryglewski *et al.*, 1975; Flower & Blackwell, 1979) and hence decrease the formation of eicosanoids other than prostaglandins, e.g. lipoxigenase products (Hamberg & Samuelsson, 1974; Nugteren, 1975). These data exclude the possibility that other eicosanoids not measured in the present study function as mediators in the TPA-stimulated proliferation of 3T3 cells. The observed small but definite enhancing effect of dexamethasone on DNA synthesis and cell proliferation is in agreement with other reports (Thrash & Cunningham, 1973; Clark & Duffy, 1976), and the attempts to explain this phenomenon are numerous. For example, one could argue that the increase in cell number by dexamethasone is due to better adhesion of the cells to the dish arising from increased production of collagen (Furcht *et al.*, 1979). But TPA enhances cell proliferation in the presence of inhibited collagen production (Delclos & Blumberg, 1979). Hence, both effects should antagonize each other. Also this interpretation leaves the increased DNA synthesis unexplained. All these observations are in contrast with the effects of glucocorticoids *in vivo*, where these compounds inhibit TPA-induced tumour promotion (Belman & Troll, 1972). Further studies designed to imitate the complex cell–cell interactions likely to occur *in vivo* are required to clarify this contradiction.

Like prostaglandins, cyclic nucleotides have often been proposed as important growth-regulating messengers (Sheppard & Bannai, 1974). However, they do not appear to play a major role in the expression of TPA-induced cell proliferation, since a single application of TPA has been found by others to be ineffective in changing the basal cyclic nucleotide

concentration (Mufson *et al.*, 1977a; Belman *et al.*, 1978). Moreover, increasing the concentrations of cyclic nucleotides by β -adrenergic drugs had no effect on TPA-induced ornithine decarboxylase activation (Mufson *et al.*, 1977b). From these results it is likely that cyclic nucleotides can be excluded as possible second messengers in TPA-induced cell proliferation.

On the other hand, polyamines released intracellularly by enhanced ornithine decarboxylase activity have been suggested to function as triggers of TPA-stimulated DNA synthesis *in vitro* (Yuspa *et al.*, 1976) and *in vivo* (O'Brien *et al.*, 1975). We have also tried to discover whether ornithine decarboxylase activation is a necessary step in TPA-induced proliferation of 3T3 cells. By using an inhibitor of ornithine decarboxylase synthesis (1,3-diaminopropane; Pösö & Jänne, 1976; Sunkara *et al.*, 1977) and a competitive inhibitor of ornithine decarboxylase activity (α -methylornithine; Abdel-Monem *et al.*, 1975) we found that (a) TPA-induced DNA synthesis proceeded in the absence of measurable ornithine decarboxylase activity (being blocked by 10 mM 1,3-diaminopropane). This concentration of 1,3-diaminopropane, however, decreased the TPA-induced thymidine incorporation into DNA by about 60%. This effect is likely to be unspecific since 1,3-diaminopropane at 1 mM decreased the TPA-induced ornithine decarboxylase activity by 80% without affecting the TPA-stimulated DNA synthesis at all. The lack of correlation between ornithine decarboxylase activity and DNA synthesis does not appear to be due to substitution of the polyamine requirement of the cells by the unphysiological diamine repressor, since similar results were obtained with (b) α -methylornithine. Again, TPA-induced thymidine incorporation proceeded uninhibited in the presence of 5 mM α -methylornithine, a concentration that is likely to suppress the ornithine decarboxylase activity in intact cells completely (Mamont *et al.*, 1976). These results clearly argue against an obligatory role of ornithine decarboxylase activation in TPA-induced cell proliferation as suggested by O'Brien *et al.* (1975). They are, however, in agreement with the observations of Clark & Duffy (1976), who found a poor correlation between increases in ornithine decarboxylase activity and DNA synthesis or proliferation of 3T3 cells stimulated by different growth factors.

In conclusion, we have shown that the tumour promoter TPA does not exert its cell-proliferative effect in 3T3 cells via prostaglandin production and ornithine decarboxylase activation, because thymidine incorporation after exposure of these cells proceeds almost uninhibited after total blockade of both biochemical events. The question remains, which messengers, if any, trigger cell proliferation subsequent to TPA administration. Two possi-

bilities are worth mentioning in this context. Recently, evidence was produced that the cytoskeleton might play a role in mediating cell proliferation induced by growth factors (Otto *et al.*, 1979). This suggestion appears attractive since TPA enters the cell interior almost instantaneously (Brune *et al.*, 1979) and changes the cell morphology of macrophages within minutes (Brune *et al.*, 1978) and of 3T3 cells within hours (Sivak, 1977). However, quantitative morphological studies correlating TPA-induced morphological changes with cell proliferation have yet to be produced. Also, TPA is known to initiate the release of the serine proteinase plasminogen activator. This enzyme appears to be involved in the malignant transformation of cells in culture subsequent to TPA exposure (Wigler & Weinstein, 1976). It may also lead to enhanced cell proliferation as shown for other proteinases (Noonan & Burger, 1973). This contention is probably irrelevant to the present study, since TPA-induced secretion of plasminogen activator takes about a day for full expression (Miskin *et al.*, 1978), which is longer than the time period between TPA addition and the maximum of thymidine incorporation. Nevertheless, the role of TPA-induced secretion of proteinases may contribute to cell proliferation at later times after TPA addition and hence requires further investigation.

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