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STIMULATION OF INTRACELLULAR FREE CALCIUM INCREASES BY PLATELET-ACTIVATING FACTOR IN HT29 COLON CARCINOMA CELLS

SPECTROFLUORIMETRIC AND PRELIMINARY SPATIO-TEMPORAL ANALYSIS USING CONFOCAL LASER SCANNING FLUORESCENCE IMAGING MICROSCOPY

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Abstract—We examined the ability of platelet-activating factor (PAF) and its lyso derivative (lyso-PAF) to elicit increases in intracellular free calcium concentration ($[Ca^{2+}]_i$) in HT29 human colon carcinoma cells. Using spectrofluorimetric analysis with indo-1 as the $[Ca^{2+}]_i$ reporter molecule, we found that 1–10 μM concentrations of both lipids stimulated substantial, reversible, monophasic $[Ca^{2+}]_i$ elevations. Evidence was obtained that the two lipids may act via specific receptors to release Ca^{2+} from internal stores. Homologous desensitization was observed in both cases and PAF and lyso-PAF were also able to desensitize cells reciprocally (heterologous desensitization). The potent PAF receptor antagonist WEB 2086 {3-[4-(chlorophenyl)-9-methyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo-[4,3-*a*][1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanone} successfully blocked PAF-induced $[Ca^{2+}]_i$ elevations, but did not affect rises in response to lyso-PAF, suggesting that lyso-PAF may act through a different cellular receptor or mechanism. Higher concentrations (>10 μM) of PAF resulted in non-reversible $[Ca^{2+}]_i$ elevations which were caused by Ca^{2+} influx following membrane lysis. However, the WEB 2086 insensitivity of these effects and the resultant cellular toxicity clearly showed that such events were mechanistically distinct from the reversible $[Ca^{2+}]_i$ elevations apparently operating via WEB 2086-sensitive receptors. Preliminary spatio-temporal observations, using confocal microscopy and fluo-3 as the $[Ca^{2+}]_i$ reporter molecule, suggested that PAF can also induce $[Ca^{2+}]_i$ elevations in the absence of cell lysis in monolayer HT29 cells. Visual impressions were obtained of cellular and subcellular heterogeneity and of $[Ca^{2+}]_i$ oscillations in responding cells. However, these need to be interpreted with caution because of the intrinsic limitations of the methodology, particularly using non-ratiometric dyes. The significance of a receptor-mediated, reversible elevation of $[Ca^{2+}]_i$ by sub-toxic concentrations of PAF in HT29 colon cancer cells remains to be elucidated, but it is tempting to speculate that PAF might function as a locally acting signalling mediator in these and possibly other tumour cells.

Key words: platelet-activating factor; cancer cells; intracellular calcium

PAF** is a potent, locally acting, alkyl ether phospholipid signal mediator [1]. It is involved in platelet and neutrophil aggregation, hypotension, bronchoconstriction, and reproduction [2, 3]. The signal transduction cascade in these cells is initiated by the binding of PAF to its membrane receptor [4, 5], resulting in G-protein-mediated activation of a PLC and concurrent inhibition of adenylate cyclase [1, 6]. Increased PLC activity converts phosphatidylinositol-4,5-bisphosphate to the second messengers IP_3 and DAG. IP_3 proceeds to release Ca^{2+} from endoplasmic reticulum stores, while DAG and Ca^{2+} are jointly responsible for activating PKC. Another byproduct of PAF-stimulated PLC activity is free arachidonic acid, which is readily oxygenated via the cellular lipoxygenase and cyclooxygenase pathways to bioactivate lipids such as prostaglandins, thromboxanes, leucotrienes and HETEs [1]. The

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** Abbreviations: AM, acetoxymethyl; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; $Ca^{2+}{}_i$, intracellular free Ca^{2+}; DAG, diacylglycerol; HETEs, hydroxyeicosatetraenoic acids; IP_3, inositol-1,4,5-trisphosphate; LPA, lysophosphatidic acid; lyso-PAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PAF, 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine, platelet-activating factor; PKC, protein kinase C; PLC, phospholipase C; WEB 2086, 3-[4-(chlorophenyl)-9-methyl-6*H*-thieno[3,2-*f*][1,2,4]-triazolo-[4,3-*a*][1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanone.

interactions of these diverse signalling pathways are not yet clear, but one of the main elements in the cascade appears to be the activation of a cytoplasmic PKC, which in turn phosphorylates strategic effector proteins in the cell.

A possible role for PAF in the control of tumour cell growth does not appear to have been investigated. Although there are practical difficulties associated with the use of PAF in ligand–receptor binding studies [7, 8], evidence for a PAF receptor has been reported for the murine macrophage cell line P338D$_1$ [8] and HL-60 human promyelocytic leukaemia cells [9]. Synthetic alkyl ether lipids, quite closely related to PAF, are under investigation as antitumour agents [10, 11]. We have previously shown that PAF itself exhibits a direct cytotoxic action on tumour cells [12]. However, the IC_{50} value for PAF was around 40–50 μM in serum-containing medium; these concentrations are much higher than the nanomolar to micromolar amounts required for many other pharmacological actions of PAF. The metabolically stable antitumour ether lipids were about 10–20-fold more potent as antiproliferative agents [12].

The potent PAF receptor antagonist WEB 2086 does not modulate ether lipid cytotoxicity in a number of cell lines [13, 14] and *in vitro* antitumour activity does not correlate with PAF agonist or antagonist activity [15]. Some PAF antagonists can protect against the direct cytotoxic effects induced by antitumour ether lipids *in vitro*, but it is now thought that the active agents protect tumour cells by inhibiting drug uptake via endocytosis [16–18]. These results clearly suggest that the mechanism of action of the antitumour ether lipids does not involve PAF receptors. Nevertheless, antitumour ether lipids can elicit an increase in $[Ca^{2+}]_i$ in some tumour cells at non-lytic concentrations [19–21]. In addition, antitumour ether lipids can block various aspects of cell signalling by growth factors in cancer cells, including, paradoxically, inhibition of agonist-stimulated Ca^{2+} release [21]. However, the precise contribution of these effects to the antitumour action of ether lipids remains to be established.

As part of a series of experiments to distinguish the Ca^{2+}-mobilizing effects from the anticancer action of PAF and the antitumour ether lipids, we noted that PAF was able to induce a significant elevation in $[Ca^{2+}]_i$ in human HT29 colon carcinoma cells. The aim of the work described in this paper was to investigate the kinetics of this interesting $[Ca^{2+}]_i$ response to PAF and also to lyso-PAF in suspensions of HT29 colon carcinoma cells, using the Ca^{2+}-sensitive ratiometric fluorophore indo-1 as the intracellular reporter molecule. The inhibitory effect of the potent and selective PAF receptor antagonist WEB 2086 on the $[Ca^{2+}]_i$ elevations induced by PAF and lyso-PAF was also determined. In addition, given that the effects of PAF and lyso-PAF occur at relatively high concentrations, we characterized the quantitative dose–response relationships for acute membrane lysis in HT29 cells using ^{51}Cr release and for cytotoxicity using the MTT tetrazolium dye-reduction assay. Finally, we carried out a preliminary study of the spatio-temporal characteristics of the $[Ca^{2+}]_i$ elevation in attached monolayers, as visualized by confocal imaging fluorescence microscopy with fluo-3 as the intracellular reporter molecule.

MATERIALS AND METHODS

Cell culture. The human colon carcinoma cell line HT29 was cultured routinely in Eagle's minimum essential medium (Gibco, Paisley, U.K.) with 10% foetal calf serum (Seralab, Crawley Down, U.K.), glutamine and antibiotics (penicillin and streptomycin at 100 IU/mL and 100 μg/mL, respectively). Stock cultures were maintained in 75-cm^2 tissue culture flasks (Falcon) at 37° in a humidified atmosphere of 92% air and 8% CO_2. Cells were mycoplasma free and used in log-phase throughout.

Cytotoxicity testing. The antiproliferative potency of PAF was assessed using the MTT dye-reduction assay as described previously [14]. HT29 cells were seeded into 96-well flat-bottomed microtitre plates at 2×10^3 cells/well in a total vol. of 200 μL. Prior to drug addition, cells were incubated for 4–24 hr to allow the cells to attach to the plastic of the flat-bottomed wells. PAF was then added to the wells in a vol. of 20 μL to give the required final concentration. Cells were exposed to drug continuously for 4 days under normal cell culture conditions. This allowed a roughly 12–15-fold increase in control cell number. At the end of this incubation, 20 μL of MTT solution was added to each well and after 2–3 hr the enzyme reaction was terminated by gentle aspiration of the medium from the wells. The formazan crystals were then dissolved in 200 μL of DMSO, aided by gentle agitation on a plate shaker for 10 min. Absorbances were read on a Titertek Multiscan MCC MKII ELISA plate reader using a test wavelength of 540 nm and a reference of 690 nm. The results from four to eight replicate wells per treatment dose were averaged, expressed as a percentage of the untreated vehicle control and plotted against drug concentration. The IC_{50} values, corresponding to the concentrations at which absorbance values were reduced to 50%, were then interpolated from the concentration–response curve.

Membrane damage assay. The ^{51}Cr-radiolabel release assay is based on the ability of membrane-intact cells to retain radiolabelled chromate in the cytosol [14]. Briefly, 2–5 $\times 10^6$ cells were harvested, pelleted and resuspended in 100–200 μL of sodium [^{51}Cr]chromate in PBS (total activity = 100 μCi). The cells were incubated in the presence of [^{51}Cr]-chromate for 1 hr, then washed and incubated in full medium for a further hour to avoid analysis during the initial period of high spontaneous release. Following this, the cells were washed twice, counted and seeded into 96-well round-bottomed plastic microtitre plates at a density of 10^4 cells/well. The test agents were added immediately thereafter. After 3–4 hr of incubation, the plates were centrifuged and 100 μL aliquots of supernatant were drawn from each well for analysis on a LKB 1282 Compugamma CS gamma counter. The activity in cpm was calculated for each well from duplicate 1-min counts. The results from four replicate wells per treatment dose were then averaged and the extent of [^{51}Cr]-chromate release was calculated as a fraction of [^{51}Cr]chromate incorporated according to the

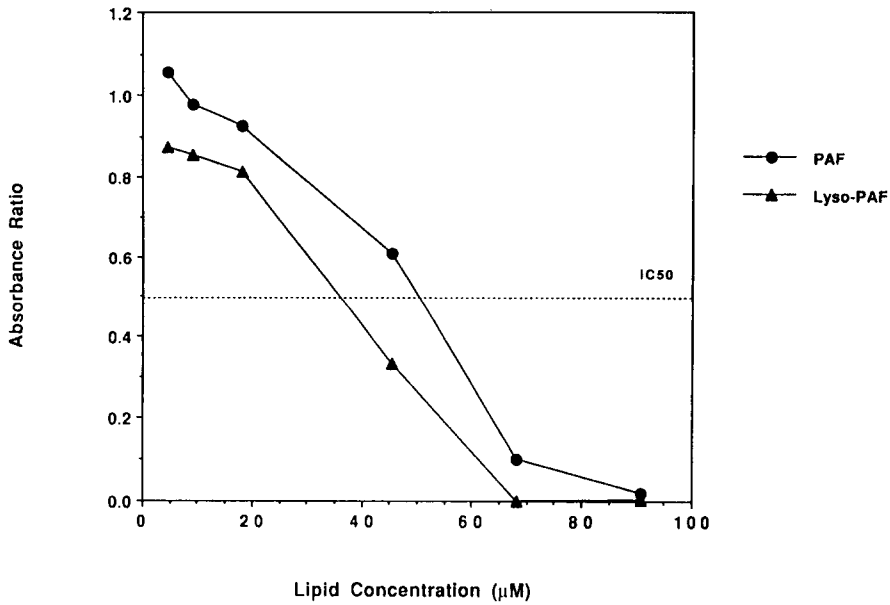

Fig. 1. MTT cytotoxicity dose–response profiles for PAF and lyso-PAF in HT29 cells. HT29 cells were exposed to the lipids as detailed in Materials and Methods. The IC_{50} concentrations (dotted line) are those at which MTT dye absorbance was reduced to 50% of control values. Error bars have been omitted as SDs of the eight replicate wells per treatment dose were routinely below 15% of the mean. The data illustrated are representative of at least four independent experiments. Means and SDs for replicate experiments are given in the text.

formula: $(EXR - SR)/(TU - SR)$, where *EXR* is the observed experimental release, *SR* is the spontaneous release, and *TU* represents total uptake of the ^{51}Cr-label, all in cpm [22]. The concentration causing 50% release of the isotope (R_{50}) was then interpolated from the resulting concentration–response curve.

Spectrofluorimetry. Cellular Ca^{2+}_i responses were measured using the indo-1 fluorophore [23] (Molecular Probes Inc., Eugene, OR, U.S.A.). Cells in full medium were loaded with 2 μM of the membrane-permeable AM ester of indo-1 for 40 min, and then washed and resuspended in full medium at a density between 2 and 5×10^6 cells/mL. After loading, the cells were kept in a gassing incubator. For each assay, an aliquot of cell suspension was withdrawn, washed and resuspended in 3 mL of assay buffer (145 mM NaCl, 5 mM KCl, 2.8 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 60 μM $MgSO_4$, 5.6 mM glucose and 15 mM HEPES, pH 7.2) at a density of 10^6 cells/mL, as described previously [19]. Cells were allowed to acclimatize to the buffer for about 5 min at 37° before being transferred to a quartz cuvette for analysis. Fluorescence measurements were performed on a Perkin Elmer LS50 spectrofluorimeter fitted with a thermostatted stirring cell holder. Cell suspensions were agitated during fluorescence acquisition by a rotating glass microflea. Data were collected using the LS50's Intracellular Biochemistry software in its alternating (medium-speed) ratio mode. Indo-1 was excited at 355 nm and fluorescence responses were monitored alternately at 410 and 480 nm.

Laser scanning confocal imaging microscopy. HT29 cells were seeded onto ethanol-washed and sterilized 10-mm circular coverslips and grown on that support for 2 days. The coverslip was then mounted face-down in a medium-filled perfusion chamber. The cells were visualized on the Bio-Rad MRC-500 confocal laser scanning microscope in transmitted light mode after preloading for 25 min with 1–2.5 μM of the Ca^{2+}-sensitive fluorophore fluo-3 (fluo-3-AM, Molecular Probes) in HEPES-buffered Hanks' medium (137 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.2 mM $MgSO_4$, 20 mM HEPES, 5 mM glucose, pH 7.2). Suitable preparations loaded rapidly and uniformly with no marked localization of the fluorescent dye within cells. This was ascertained visually by examining cellular fluorescence levels at maximal gain settings. After loading, the cells were washed twice in Hanks' buffer. Fluo-3 was excited at 488 nm as described previously [24] and Ca^{2+}-dependent fluorescence collected at intervals of 5 sec. An approximate calibration can be performed by saturating dye fluorescence using 10 μM ionomycin (F_{max}), then quenching fluorescence with 10mM $MnCl_2$ (F_{min}) before finally lysing the cells with 50 μM digitonin. These values are used to calculate approximate Ca^{2+}_i concentrations, as detailed by Minta *et al.* [24].

RESULTS

Cytotoxicity and membrane damage

PAF and its natural metabolite lyso-PAF did exhibit cytotoxicity towards HT29 colon carcinoma

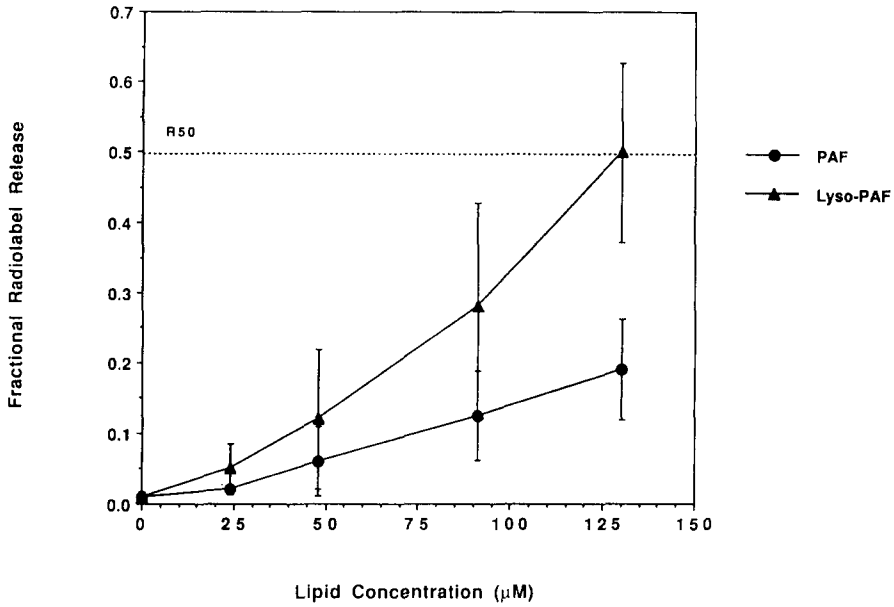

Fig. 2. [^{51}Cr]Chromate radiolabel release dose–response profiles for PAF and lyso-PAF in HT29 cells. Fractional radiolabel release was calculated as described in Materials and Methods and R_{50} concentrations were interpolated. Error bars indicate the standard error of three independent replicate experiments.

cells in serum-containing medium, as determined by the 4-day MTT viability assay. The IC_{50} values (±SD) for PAF and lyso-PAF were 54 ± 9 and 45 ± 13 μM, respectively (N = 6). A typical dose–response curve for both lipids is illustrated in Fig. 1. Reducing the nominal exposure time from 4 days to 20 hr and 2 hr resulted in a progressive decrease in relative potency. Under these conditions, the IC_{50} values (±SD) for PAF were 72 ± 15 and 107 ± 18 μM, respectively (N = 3).

These latter values are in excellent agreement with those found for the acute membrane-damaging potency, as determined by [^{51}Cr]chromate release, also in serum-containing medium. PAF and lyso-PAF both resulted in half-maximal radiolabel release (R_{50}) at concentrations of around 100–110 μM. The typical dose–response relationship for direct membrane damage is illustrated in Fig. 2.

$[Ca^{2+}]_i$ *measurements by spectrofluorimetry*

The Ca^{2+}-mobilizing effects of PAF were initially noted in cell suspensions using spectrofluorimetric analysis with the indo-1 dye. Figure 3 shows a typical $[Ca^{2+}]_i$ elevation induced in HT29 human colon carcinoma cells by 3 μM PAF in a standard serum-free assay buffer. After PAF addition, there followed a lag period of 25–35 sec before a $[Ca^{2+}]_i$ response was seen. PAF stimulated a monophasic rise in $[Ca^{2+}]_i$ which returns back to basal levels within 100–150 sec. Pre-incubating the cells in Ca^{2+}-free, EDTA-containing buffer had no effect on the onset or magnitude of the resulting $Ca^{2+}{}_i$ response to PAF (not shown). This observation is consistent with Ca^{2+} release primarily from internal stores.

Figure 4 (Trace A) shows that repeat dosing with PAF failed to elicit further rises in $[Ca^{2+}]_i$, until cumulative concentrations approached 10–12 μM (see later). The failure of PAF to induce sizeable $[Ca^{2+}]_i$ elevations after a previous PAF response points to a mechanism of homologous receptor desensitization in HT29 colon carcinoma cells.

The above data suggest that the $[Ca^{2+}]_i$ elevation seen in response to PAF may be mediated by specific PAF receptors in HT29 tumour cells. This hypothesis is further supported by the observation illustrated in Fig. 4 (Trace B). Pre-incubating HT29 cells with 20 μM of the potent and specific PAF receptor antagonist WEB 2086 successfully blocked the $[Ca^{2+}]_i$ response elicited by PAF.

Generally, lipid concentrations below 1 μM failed to induce reproducible $[Ca^{2+}]_i$ elevations, while 1.5–5 μM gave strong responses (Fig. 3). Higher concentrations up to 10 μM did not produce significant further increases in $[Ca^{2+}]_i$ and doses in excess of 10 μM, whether administered singly or cumulatively, routinely caused an immediate and sustained increase in dye fluorescence (Fig. 4). In contrast to the transient rise observed with lower doses, the sustained increase in fluorescence represents an artefact caused by the effects of PAF on plasma membrane integrity. PAF destabilizes the plasma membrane, leading to permeabilization and the release of fluorophore into the Ca^{2+}-containing assay medium. This was shown by analysing the assay buffer after pelleting the cells to determine the amount of dye which had leaked into the medium. A further control involved determination of the amount of fluorescence quenching, following addition of excess EDTA or EGTA [20]. Both of these controls confirmed that substantial cell lysis was occurring at concentrations >10 μM. It should be noted that WEB 2086 failed to protect cells

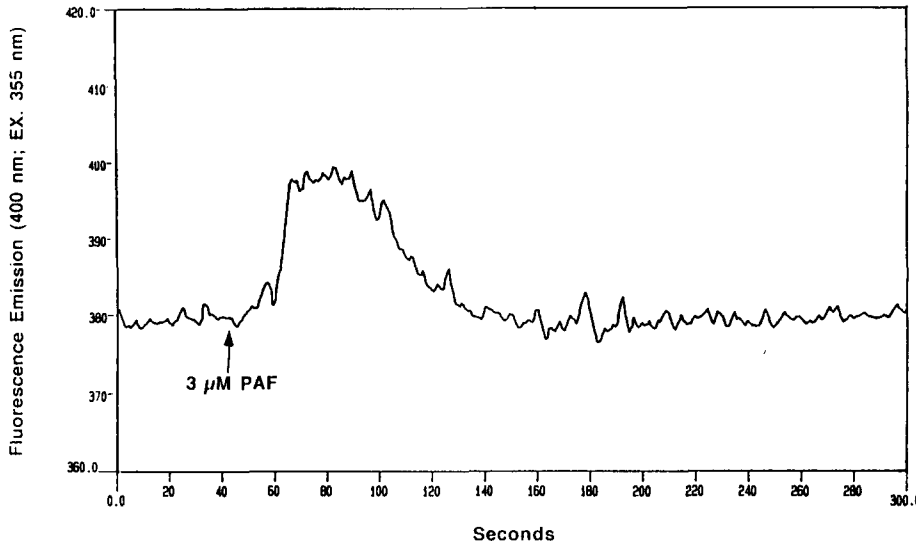

Fig. 3. Elevation in intracellular calcium levels induced by 3 μM PAF. Fluorescence changes were monitored spectrofluorimetrically using HT29 cells preloaded with the indo-1 fluorophore. The experimental conditions are detailed in Materials and Methods.

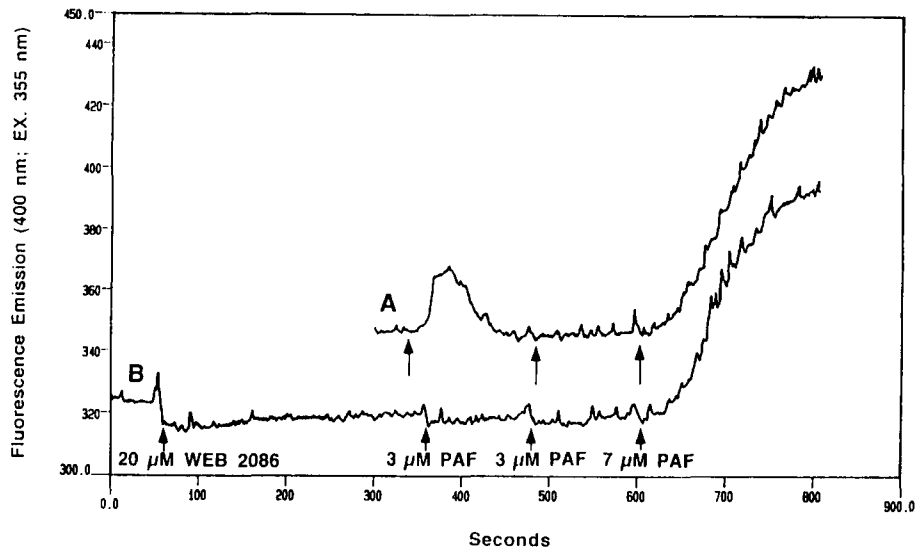

Fig. 4. Effect of repeat dosing of PAF on intracellular calcium levels in the presence and absence of WEB 2086. Trace A shows that an initial challenge with 3 μM PAF induced a significant response, while repeat doses of 3 and 7 μM PAF had no calcium-mobilizing effect. The elevation in dye fluorescence after addition of 7 μM PAF was due to membrane disruption (see text). Trace B illustrates that pre-incubation with 20 μM WEB 2086 can abolish PAF agonist activity in HT29 cells, but does not affect the lytic potency after a cumulative dose of 13 μM PAF. Fluorescence changes were monitored spectrofluorimetrically using HT29 cells preloaded with the indo-1 fluorophore (see Materials and Methods).

against the lytic effects of PAF at high concentrations (Fig. 4, Trace B), indicating that membrane toxicity did not involve a PAF receptor. This was confirmed directly using both ^{51}Cr release and MTT dye reduction as endpoints—in neither case did WEB 2086 have any effect on PAF cytotoxicity (Table 1).

In addition to PAF, lyso-PAF also elicited $[Ca^{2+}]_i$ elevations in HT29 cells. These responses were of a similar magnitude to those elicited by PAF, but the kinetics of the rise in $[Ca^{2+}]_i$ differed somewhat between the two lipids (Fig. 5). In contrast to the responses elicited by PAF, which routinely

Table 1. The effect of 49 μM WEB 2086 on cytotoxicity and membrane damage induced by PAF and lyso-PAF in HT29 cells

	Cytotoxicity (IC_{50})		Membrane damage (R_{50})	
	−WEB 2086	+WEB 2086	−WEB 2086	+WEB 2086
PAF	45.6 ± 2.4	43.2 ± 4.8	121 ± 14	114 ± 14
Lyso-PAF	41.4 ± 1.7	45.5 ± 2.2	110 ± 11	140 ± 10

IC_{50} and R_{50} values are given in μM as the means ± SE.
None of the comparative data reached statistical significance ($P > 0.05$; unpaired *t*-test).
The data are representative of between three and 17 independent experiments.
Cytotoxicity was determined by MTT assay (see Materials and Methods) and within each individual experiment, four or eight replicate wells per concentration were analysed. Cells were exposed to WEB 2086 and drug continuously for 4 days. Membrane damage was measured by ^{51}Cr-radiolabel release and, within each experiment, four replicate wells per concentration were analysed after drug exposures of 3–4 hr (see Materials and Methods).

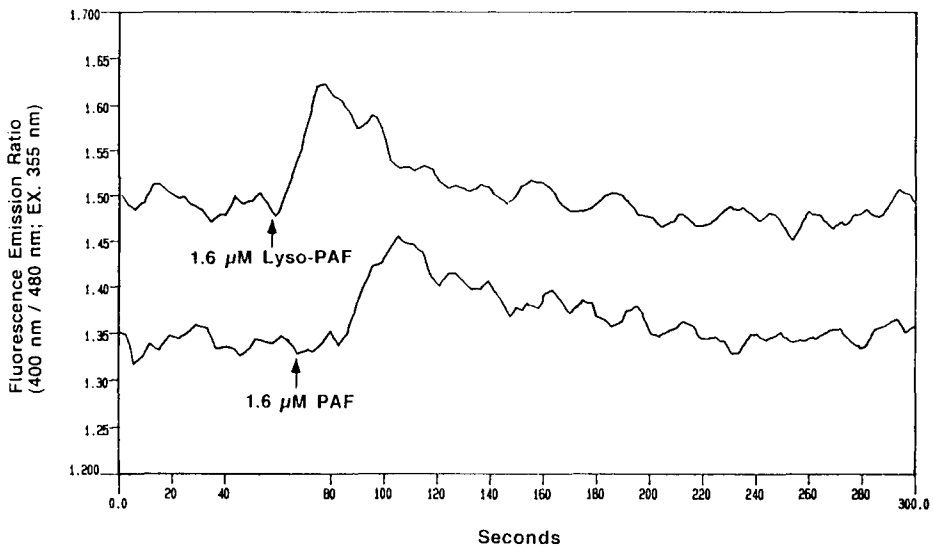

Fig. 5. Comparison of the intracellular calcium responses induced by 1.6 μM PAF and lyso-PAF. HT29 cells preloaded with indo-1 (see Materials and Methods) were challenged where indicated (arrows). The fluorescence emission ratio was plotted to facilitate comparison between the two different batches of cells used.

entailed a lag time of 25–35 sec, the lyso-PAF-induced $[Ca^{2+}]_i$ elevations were immediate. We also noted slight differences in the kinetics of the elevation and decline of $Ca^{2+}{}_i$ levels. Lyso-PAF generally elicited a rapid, sharp increase in $[Ca^{2+}]_i$, followed by a speedy initial decline, while $[Ca^{2+}]_i$ rises stimulated by PAF rose to their peak more slowly and decayed more gently back to basal levels (Figs 3 and 5). In general, the responses elicited by lyso-PAF were more similar to those noted for the structurally related antitumour ether lipids [20].

Homologous desensitization was seen with lyso-PAF as well as PAF. Interestingly, the two lipids were also found to antagonize each other's $[Ca^{2+}]_i$ elevations reciprocally (not shown). This demonstrates that, with respect to PAF and lyso-PAF, a mechanism of heterologous desensitization is operating in HT29 colon carcinoma cells. However, the two lipids do not appear to act via the same receptor, since PAF responses could be abolished by WEB 2086 (Fig. 4, Trace B), while lyso-PAF responses remained unaffected by the potent PAF receptor antagonist (not shown).

$[Ca^{2+}]_i$ *measurements by confocal microscopy*

We went on to conduct a preliminary study of the PAF-induced $Ca^{2+}{}_i$ responses in HT29 tumour cells by confocal microscopy. Firstly, we wished to visualize the effects in attached monolayers rather than in cell suspensions. Secondly, we wanted to examine any possible heterogeneity of the response between cells and its spatio-temporal characteristics within individual cells. Because of the optical

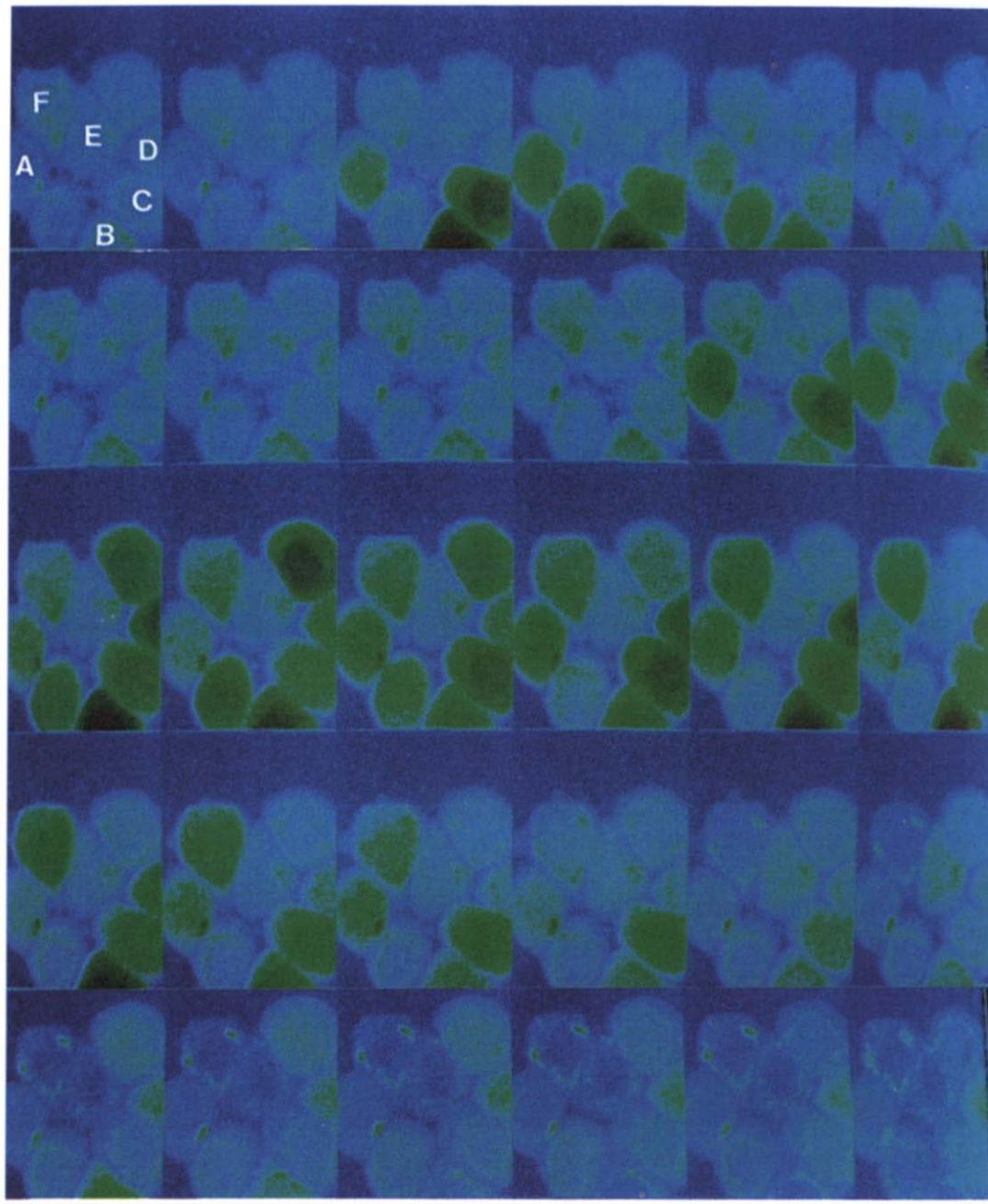

Fig. 6. Sequential screen shots of attached HT29 monolayer cells preloaded with fluo-3 and exposed to 100 μM PAF. Each frame represents the average of four scans within a 5-sec interval (see Materials and Methods). Note that the first frame shown was taken 55 sec after exposure to PAF and that localized elevated fluorescence levels appear to be the precursors to the first strong response seen in frames 3–5.

constraints of the instrument, we used the single wavelength Ca^{2+} fluorophore fluo-3. Cells were washed in serum-free Hanks' buffer which was exchanged rapidly with Hank's buffer containing 50–100 μM PAF. The effective concentration, however, will be less, due to the dilution inherent in the medium-replacement procedure. Lower added agonist concentrations were without effect. No evidence of cellular damage was seen in monolayer cells during these experiments.

A rise in $[Ca^{2+}]_i$ could be readily demonstrated in attached HT29 cells with this technique (Fig. 6). The first $Ca^{2+}{}_i$ responses were usually seen 70–100 sec after challenge. Figure 6 provides a visual impression

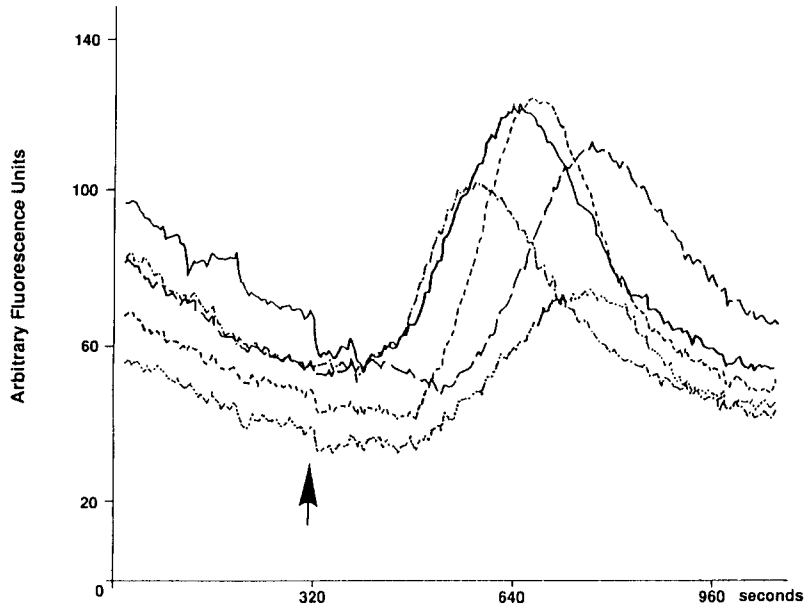

Fig. 7. Computer analysis of confocal microscope fluorescence intensity values for five different HT29 cells (denoted by different broken lines) within the viewing frame. PAF (100 μM) was added where indicated (arrow). Clearly evident are the different lag times and the heterogeneous magnitude of the elevations in intracellular calcium levels.

of the cellular heterogeneity in the $Ca^{2+}{}_i$ responses. Some cells showed a response comparatively rapidly after addition of the agonist, while others appeared to respond more slowly. A third small subset of cells failed to respond at all. Interestingly, a number of responding cells produced three or four distinct $[Ca^{2+}]_i$ oscillations. For example, the cell marked "A" in Fig. 6 showed an initial rise after 65 sec, reaching maximal fluorescence at 70 sec and declining back to near basal by 80 sec. This was followed by three further fluorescence maxima 105, 130 and 155 sec after drug addition. Cell "B" responded similarly with fluorescence maxima at 65, 115, 140 and 175 sec. In general, about 90% of cells responded to the PAF stimulus. The remaining 10%, such as cell "C", were either insensitive to PAF stimulation, non-viable, or possibly failed to load sufficient of the fluo-3 fluorophore to visualize the increase in intracellular Ca^{2+}.

The subcellular localization of $[Ca^{2+}]_i$ changes also appeared heterogeneous. Figure 6 shows that areas of high fluorescence emission (orange) were located in and around the nuclear region (see especially frames 3, 4, 13 and 14). Since there was no evidence of preferential dye localization to the nucleus, as judged by visual examination of the cells at maximal gain settings, these results suggest that Ca^{2+} concentrations in the nuclear and perinuclear regions appear to be particularly elevated. Interestingly, it seems that the $[Ca^{2+}]_i$ elevations originate from one "focal point" and sweep across the cell in a wave (e.g. cells "A" and "F"). These observations are consistent with release of Ca^{2+} from internal stores. As seen in the spectrofluorimetric analyses, repeat challenge with PAF failed to elicit further Ca^{2+} responses (not shown). However, pre-incubation of cells with WEB 2086 failed to block Ca^{2+} responses in attached cells.

Figure 7 illustrates quantitative changes in $[Ca^{2+}]_i$ in selected cells over time. Basal cell $[Ca^{2+}]_i$ levels were about 120 nM, while maximal $[Ca^{2+}]_i$ levels approached 500 nM. However, these values are likely to be in excess of true $Ca^{2+}{}_i$ concentrations, because of photobleaching [24].

DISCUSSION

We show here for the first time that PAF and its natural metabolite lyso-PAF can both stimulate human colon carcinoma cells (HT29) to elicit a rise in intracellular free Ca^{2+}. This effect was seen in cell suspensions as well as with attached cells. The $[Ca^{2+}]_i$ elevations did require relatively high concentrations of PAF (1–10 μM) compared to those needed for other pharmacological and pathophysiological effects of this lipid mediator. Nevertheless, clear evidence was obtained in cell suspensions for a specific receptor-mediated mechanism, distinct from the membrane-lytic effects seen at even higher concentrations (>10 μM)

The results obtained with spectrofluorimetry of cell suspensions, using indo-1 as the $[Ca^{2+}]_i$ probe, represent the average response of the sampled cell population. No $[Ca^{2+}]_i$ changes were detectable in HT29 tumour cell suspensions at lipid concentrations below 1 μM. At high concentrations (>10 μM), both PAF and lyso-PAF induced an immediate rise in intracellular $[Ca^{2+}]_i$ which was sustained for longer than 5–10 min. However, as we have pointed out previously in studies with alkyl lysophospholipids

[20], this sustained rise was an artefact of cell permeabilization. The detergent properties of PAF and related alkyl ether lipids induce direct membrane damage at high concentrations and this effect was about 20-fold more pronounced under serum-free conditions [20]. Thus, only the single, reversible, monophasic rise seen below membrane-toxic levels represents a genuine elevation in $[Ca^{2+}]_i$.

Similar quantitative changes were seen with both PAF and lyso-PAF, although some differences in kinetics were noted, suggestive of possible differences in mechanism (see below). Although the $[Ca^{2+}]_i$ elevations in response to PAF were of a similar magnitude to those elicited by lyso-PAF, the response to PAF was always preceded by a lag time of 25–35 sec. With lyso-PAF, the cellular response was swift and no lag time was evident.

The unusually narrow dose–response range clearly raises concerns about the nature of the phenomenon and the extent to which it can truly be distinguished mechanistically from changes resulting from non-specific membrane perturbations which cause lysis at higher doses (see also Refs 20, 25, 26). For this reason, we carried out further experiments, in particular to probe for the involvement of a receptor-mediated mechanism. Interestingly, the $Ca^{2+}{}_i$ responses elicited by PAF could be blocked successfully by pre-incubation of the cells with the potent, structurally unrelated PAF receptor antagonist WEB 2086. However, the $[Ca^{2+}]_i$ rise stimulated by lyso-PAF remained unaffected by WEB 2086. These results suggest that PAF acts through a conventional WEB-sensitive PAF receptor in HT29 colon carcinoma cells, whereas lyso-PAF does not.

We also found that an initial challenge with PAF successfully desensitized responsive cells to further doses of the lipid mediator. Similar results were seen with lyso-PAF. These findings point to a mechanism of homologous desensitization, operating either at the level of the proposed receptor or a point further downstream in the putative signal transduction pathway. Interestingly, heterologous desensitization was also evident, since PAF and lyso-PAF were capable of inhibiting each other's Ca^{2+} agonist activity. This may be occurring at a common downstream point in the signal cascade or through temporary depletion of internal Ca^{2+} stores. Future experiments could be designed to determine whether depletion of internal Ca^{2+} stores by other agonists has any effect on the responses to PAF and lyso-PAF and also whether WEB 2086 affects heterologous desensitization.

In considering the demonstration of WEB 2086 antagonism of PAF-induced elevation of $[Ca^{2+}]_i$ as evidence of a receptor-mediated mechanism within the narrow dose range of 1–10 μM, it is important to stress that WEB 2086 had no effect whatsoever on the acute membrane lytic effect measured by ^{51}Cr release over the PAF concentration range 25–130 μM, nor on the acute cytotoxicity assayed by MTT tetrazolium dye reduction over the PAF concentration range 5–90 μM. Furthermore, WEB 2086 did not antagonize the secondary apparent $[Ca^{2+}]_i$ rise which was immediate, sustained and non-reversible, and is a consequence of the membrane-lytic effects of PAF, which begin to be apparent in the spectrofluorimetric assay above 10–12 μM. On the basis of these collective observations, we can conclude with some confidence that a WEB 2086-sensitive biochemical mechanism is involved in the reversible rise in $[Ca^{2+}]_i$ in the concentration range 1–10 μM. This phenomenon appears to be independent of the membrane-lytic effects occurring at higher doses. In view of the evidence for agonist desensitization, it seems reasonable to suggest the involvement of a WEB 2086-sensitive PAF receptor. The PAF concentrations required would, however, indicate a low affinity or low abundance receptor in HT29 colon cancer cells.

The measured increases in response to PAF or lyso-PAF appear to be almost exclusively due to Ca^{2+} mobilization from intracellular stores such as the endoplasmic reticulum. This was demonstrated by spectrofluorimetry experiments in Ca^{2+}-free, EDTA-containing buffer, in which no effect on the onset or magnitude of the resulting $[Ca^{2+}]_i$ responses was noted. Confirmation was also provided by confocal microscopy where $[Ca^{2+}]_i$ increases could be seen to originate from defined intracellular locations.

Confocal microscope analysis, using fluo-3 as the reporter fluorophore, has the advantage of providing data on the temporal and spatial resolution of $[Ca^{2+}]_i$ responses at a single cell level. Although very much preliminary in nature and subject to a number of potential problems associated with this methodology, particularly the possible heterogeneity of cellular dye loading and intracellular dye localization, our experiments have provided a visual impression that PAF was able to induce a $[Ca^{2+}]_i$ rise in relatively unperturbed HT29 cells, growing attached in a monolayer. This initial analysis clearly suggested a heterogenous $[Ca^{2+}]_i$ signal, with some cells responding strongly with several distinct oscillations in $[Ca^{2+}]_i$ levels, while others appeared unresponsive to PAF challenge. There was also some variation in the onset time of the $[Ca^{2+}]_i$ rise. In line with previous spectrofluorimetry experiments, rechallenge failed to stimulate further responses. However, high lipid concentrations were required in this system and pre-incubation with WEB 2086 failed to block the $Ca^{2+}{}_i$ elevations. It is not clear whether this reflects differences due to cell attachment or is related to technical disparities. Further studies are needed in this area. The use of ratiometric Ca^{2+}-sensitive probes would also be an advantage to compensate for any heterogeneity of dye loading.

The biological and pharmacological significance of the rise in $[Ca^{2+}]_i$ induced by PAF in HT29 human colon carcinoma cells remains unclear at this stage. A sustained rise in $[Ca^{2+}]_i$ has been implicated in the mechanism of cell death induced by various drugs and toxins [27, 28]. Thus, the prolonged, non-reversible, WEB 2086-insensitive elevations in apparent $[Ca^{2+}]_i$ arising from Ca^{2+} influx might contribute to the cytotoxic effect of PAF at concentrations >10 μM, but cytotoxicity could equally well arise from other consequences of acute membrane perturbations at these very high dose levels. Since WEB 2086 failed to antagonize

membrane toxicity and cell killing, we can conclude that the more subtle, reversible WEB-sensitive Ca^{2+} mobilization mechanism is not involved in the cytotoxicity of high-dose PAF or lyso-PAF. However, we can be less certain about the possible biological consequences of the WEB 2086-sensitive "receptor"-mediated $[Ca^{2+}]_i$ elevation.

Previous studies have reported a rise in $[Ca^{2+}]_i$ in Rat-1 fibroblasts and HF cells treated with 20–30 μM LPA [29]. Moreover, substantial mitogenic stimulation was also noted for similar concentrations of this very simple, natural phospholipid [29]. Recently, a putative LPA-specific receptor has been purified [30]. It is an interesting but speculative possibility that PAF or closely related lipids may also have mitogenic potential, since we could clearly show that PAF and lyso-PAF can elicit sizeable $[Ca^{2+}]_i$ responses at concentrations which were not cytotoxic and lower than those reported for LPA. Although our observations are currently restricted to the HT29 colon carcinoma cell line, our results raise the intriguing possibility that PAF may function as a signalling molecule in colon carcinomas (and possibly other tumours) and its potential mitogenic effects are currently under investigation.

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