Table 1. Measurements of the new Solnhofen specimen of *Archaeopteryx lithographica*.

Skeletal element	Length (mm)
Tail, restored	240*
Coracoid	24.5
Humerus	83.0
Ulna	72*
Radius	69*
Digit I	45.9
Digit II	65.4
Digit III	43.6
Hand length	105*
Ilium	38*
Ischium	24.5
Pubis	59.3
Femur	70*
Tibia	89.5
Fibula	82.4
Foot length	95*

*Approximate measurements.

of *Archaeopteryx* are forgeries, one is eager to see whether the Solnhofen specimen shows traces of feathers. Under low-angle illumination there are distinct parallel impressions originating from the lower arm of the left wing skeleton. These features (Fig. 2) are interpreted as imprints of feather shafts. Furthermore, impressions subparallel to the left fingers may mark the outline of the wing. No traces of tail feathers, as in the London, Berlin, and Eichstätt specimens, are visible, however. Because a question of a forgery in the new specimen does not arise, the impressions of the wing feathers are direct evidence that *Archaeopteryx* had feathers (*9*).

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10. I wish to thank G. Viohl of the Jura-Museum in Eichstätt, who recognized the new *Archaeopteryx* specimen first and who drew my attention to it. I also thank the former owner and collector, F. Müller, the former mayor of Solnhofen, who offered it to me for scientific study as well as Mayor O. Güllich of Solnhofen who agreed to lend it to me and allowed further preparations. Preparation was carried out by E. Schmieja, chief preparator at the State Collections in Munich. I am especially indebted to J. H. Ostrom, for valuable discussions and for reviewing an early draft of the manuscript.

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Neuroregulatory and Neuropathological Actions of the Ether-Phospholipid Platelet-Activating Factor

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Platelet-activating factor (PAF) is a naturally occurring phospholipid that serves as a critical mediator in diverse biological and pathophysiological processes. In this study of the interactions of PAF with neuronal cells, it was found that PAF increased the intracellular levels of free calcium ions in cells of the clones NG108-15 and PC12. The increase was dependent on extracellular calcium and was inhibited by the antagonistic PAF analog CV-3988 and by the calcium-influx blockers prenylamine and diltiazem. A functional consequence of this interaction was revealed by measuring a PAF-elicited, Ca^{2+}-dependent secretion of adenosine triphosphate from PC12 cells. Exposure of NG108-15 cells for 3 to 4 days to low concentrations of PAF induced neuronal differentiation; higher concentrations were neurotoxic. Thus, by influencing Ca^{2+} fluxes, PAF may play a physiological role in neuronal development and a pathophysiological role in the degeneration that occurs when neurons are exposed to circulatory factors as a result of trauma, stroke, or spinal cord injury.

PLATELET-ACTIVATING FACTOR (PAF, PAF-acether) is a naturally occurring alkyl-ether phospholipid: 1-*O*-alkyl, 2-acetyl-*sn*-glyceryl-3 phosphorylcholine (*1*). In addition to being a potent activator of platelets, PAF serves as an extracellular mediator in the process of inflammation, has antihypertensive activity, induces smooth muscle contraction, increases vascular permeability, and has been implicated in several pathological processes involving various tissues and cell types (*2*). Brain tissue contains relatively high levels of enzymes for the synthesis and metabolism of alkyl-ether phospholipids (*3*), and it has been suggested that PAF may play a role in the regulation of neuronal function (*4*). We have now examined directly the interactions of PAF with neuronal cells of the hybrid cholinergic clone NG108-15 and the adrenergic clone PC12 (*5*). By means of the bioluminescent calcium probe aequorin, it was found that in these cells PAF induces an increase in intracellular free Ca^{2+}. This increase is associated with stimulated uptake of Ca^{2+} from the extracellular environment and increased vesicular release.

Neural cells of the hybrid clone NG108-15 were grown in culture in a chemically defined medium (*6*). Within 24 to 48 hours after the addition of 50 n*M* PAF to the medium, the growth of the cells was arrested followed by morphological differentiation with neurite extension (Fig. 1A). This differentiating effect of PAF was concentration and time dependent (*7*): maximal effects were observed at 2.5 μ*M* PAF after 3 to 4 days of exposure. Addition of higher concentrations of PAF (3.5 to 10 μ*M*) produced cytotoxic effects. With increasing exposure time to these PAF concentrations the cells gradually rounded up and then lost viability (Fig. 1B). Thus, low concentrations of PAF may play a role in neuronal development, whereas higher PAF concentrations cause neurodegeneration.

Elevated concentrations of intracellular free Ca^{2+} are known to induce morphological changes in neurons, whereas a pathophysiological increase in intracellular calcium can be neurotoxic. To examine whether the dual effects (neurite extension and neurotoxicity) of increasing PAF concentrations on NG108-15 cells are mediated by this mechanism, we used cells loaded with aequorin. The addition of PAF resulted in a marked increase in the level of intracellular Ca^{2+} as measured by aequorin (Fig. 2A, left side); from a basal level of <1 μ*M* it increased to 7 μ*M* Ca^{2+} after stimulation with 4.5 μ*M* PAF. When 3 m*M* EDTA or 1.8 m*M* EGTA were added to the medium, the aequorin signal induced by PAF was completely blocked (Fig. 2A, right side). These results suggest that the PAF-induced increase in intraneuronal Ca^{2+} involves uptake of extracellular Ca^{2+}, a suggestion supported by the effects of inhibitors of Ca^{2+} influx, discussed below.

We also tested the effects of PAF on an adrenergic cell line, PC12 cells. The results were similar to those obtained with the cholinergic NG108-15 cells (Fig. 2, B and C). Although the cells showed responsiveness to nanomolar concentrations of PAF (see Fig. 2B, inset), the aequorin signal could be best quantitated when we used micromolar PAF concentrations. A hyper-

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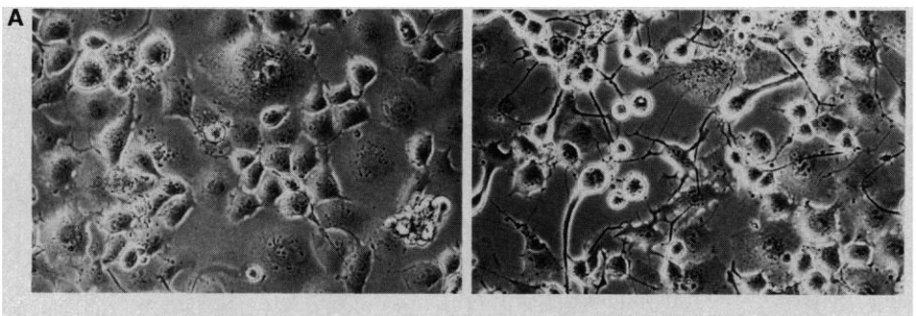
bolic curve was produced in response to PAF with saturation achieved at ~9 μ*M* PAF (Fig. 2B). The PAF-induced aequorin signal in PC12 cells could be completely blocked by EDTA and EGTA and by the structural PAF analog CV-3988 (Fig. 2C, right side). The antagonist CV-3988 also inhibited the PAF-induced aequorin signal of NG108-15 cells in a dose-dependent manner.

Elevation of intracellular free Ca^{2+} can cause vesicular release from neurons. We tested this functional aspect of the interaction of PAF with neural cells by measuring the release of adenosine triphosphate (ATP) from PC12 cells. In these cells ATP is stored with catecholamines in synaptic vesicles and coreleased with the neurotransmitter upon stimulation (*5*). The exposure of PC12 cells to PAF resulted in a release of ATP (Fig. 3) that approached a maximum level within 3 to 4 min after the addition of PAF, namely, after the peak increase in intracellular Ca^{2+}. In the presence of CV-3988, the PAF-induced release reaction was completely inhibited (Fig. 3). The addition of increasing concentrations of CV-3988 resulted in a dose-dependent inhibition of PAF-induced ATP release from PC12 cells; 50% inhibition was produced by 30 μ*M* CV-3988 and complete inhibition by 107 μ*M* CV-3988, when 20 μ*M* PAF was used to induce ATP release. Addition of EGTA (1.8 m*M*) to the extracellular medium also inhibited the PAF-induced release of ATP from PC12 cells, which indicates that the observed secretion is Ca^{2+} dependent.

Other agents were tested for their possible effects on the PAF-induced responses of neural cells. In contrast to the antagonist CV-3988, we found that the triazolobenzodiazepines, triazolam, alprazolam (*4*), and brotizolam, had only minimal effects on the PAF-induced response of aequorin-loaded NG108-15 or PC12 cells. In contrast, calcium-influx blockers inhibited the effects of PAF; prenylamine inhibited the PAF-induced aequorin response of NG108-15 cells in a dose-dependent manner [5 μ*M* prenylamine produced 50% inhibition (IC_{50}) of



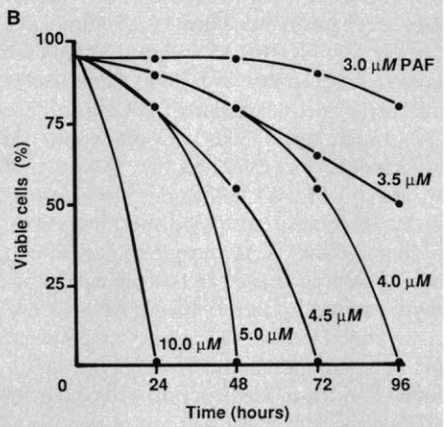


Fig. 1. Effects of PAF on the morphology and viability of NG108-15 neural cells. (**A**) Neuronal differentiation. NG108-15 cells were grown in 24-well cluster plates in a chemically defined, serum-free medium as described (*6*). PAF (Calbiochem) was added 24 hours after plating. The medium ± PAF was then changed daily. Cells were photographed 3 days after the addition of 50 n*M* PAF. The rounded cell bodies and the extended neurites characteristic of the differentiated state of these cells are clearly seen after PAF treatment (right side) compared to the flat appearance of control cells (left side). (**B**) Neurotoxicity of PAF. NG108-15 cells were grown as described above. Each of the PAF concentrations indicated was added to 16 individual wells, 4 of which were harvested every day for measuring cell count and determination of viability by trypan-blue exclusion. Results shown are the average of four wells for each point from an experiment that was repeated three times. Viability of control cells maintained without PAF was also determined in four wells every day and averaged 80 to 95%. Results from the three experiments agreed to within 10% of the shown means.

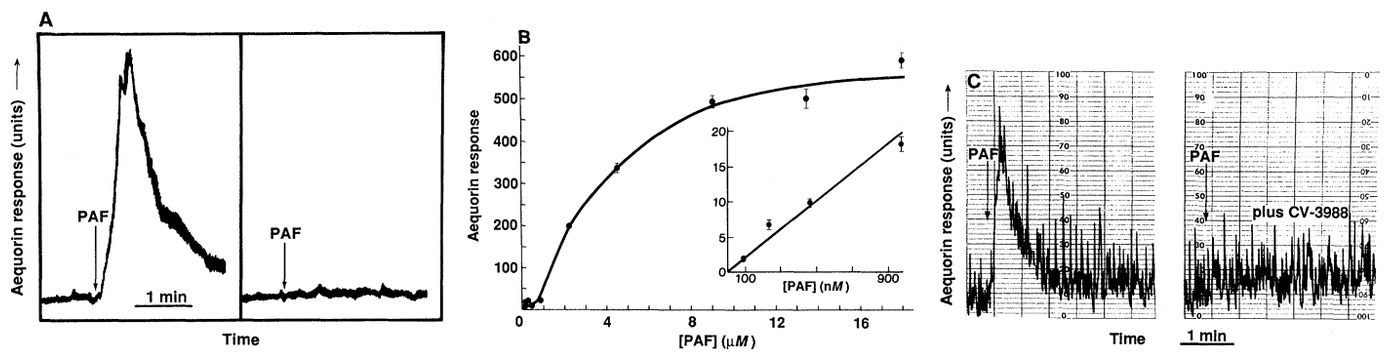


Fig. 2. PAF induces an increase in intracellular free Ca^{2+} in neural cells. NG108-15 cells were grown in T-150 culture flasks as detailed by Ehrlich *et al.* (*16*). PC12 cells were cultured in RPMI 1640 medium containing 10% heat-inactivated horse serum and 5% fetal calf serum. Cells at 80 to 95% confluency were harvested, washed, and resuspended in a Hepes buffer (*p*H 7.4) containing EGTA (5 m*M*), sodium phosphate (10 m*M*), NaCl (150 m*M*), and 1% glucose. The cells were washed and resuspended in the same buffer, then loaded with aequorin according to the dimethyl sulfoxide (DMSO) method of Yamaguchi *et al.* (*17*) with a slight modification or by the method of Ware *et al.* (*18*). Similar results were obtained with both procedures. For measuring aequorin signals, aequorin-treated cells were resuspended at a final concentration of 10^6 cells per milliliter in a buffer containing 1 m*M* $MgCl_2$ and 1 m*M* $CaCl_2$ (without EGTA). Aliquots (1.1 ml) were placed in cuvettes and examined in a PICA instrument (Chrono-Log) set at 400 rpm and 37°C. Agents tested for inducing changes in intracellular free-Ca^{2+} were added by injection through a rubber septum, and aequorin luminescence was plotted by a chart recorder. (**A**) Aequorin signal recorded from NG108-15 cells preloaded by the procedure of Ware *et al.* (*18*), in response to 4.5 μ*M* PAF (left). In the right side, 3 m*M* EDTA (final concentration) was added to the medium 1 min before the PAF. Addition of EGTA (1.8 m*M*) instead of EDTA gave the same results as those shown in the right side. (**B**) Dose-response curve of peak aequorin signals induced by PAF in PC12 cells preloaded by the DMSO procedure (*17*). Error bars are the mean ± SEM for three separate experiments performed in triplicate. (**C**) Recording of aequorin-signals from PC12 cells stimulated by PAF (2.2 μ*M* final concentration) in the absence (left) and presence (right) of 350 μ*M* of the PAF antagonist CV-3988 (*19*), added 1 min before the PAF. Cells were loaded by the DMSO method. Experiments with NG108-15 cells gave similar results (see also Table 1).

Table 1. Comparison of A23187 and PAF-induced aequorin responses in NG108-15 cells: differences in inhibition by CV-3988.

Addition	Concentration (μM)	Aequorin signal* (units)
A23187 alone	0.84	84 ± 9.7
A23187 + CV3988	179	86 ± 4.1
PAF alone	4.4	150 ± 16
PAF + CV-3988	179	0

*Means ± SEM, $n = 5$.

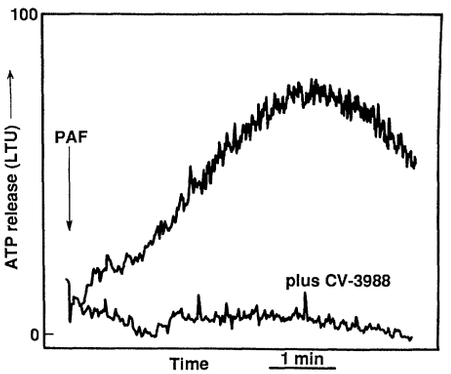


Fig. 3. PAF-induced release of ATP from PC12 cells. The cells were harvested and washed in phosphate-buffered saline (Gibco) supplemented with 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1% glucose. Aliquots (0.45 ml) of cell suspension (10^6 cells per milliliter) in glass cuvettes were placed in a Lumi-Aggregometer (Chrono-Log) set at 37°C and 400 rpm. A luciferin/luciferase reagent (25 μl of Chrono-Lume reagent) was added, followed by PAF (20 μM), in the presence and absence of CV-3988 (51 μM). Addition of EGTA (1.8 mM) to the buffer had effects similar to those shown here with CV-3988. ATP was measured in luminescence units (LTU). An ATP-standard curve prepared under these assay conditions provided a value of 22 ± 2 nM for the peak concentration of ATP released by PC12 cells in response to PAF.

the maximal extent of the aequorin signal induced by PAF]. Diltiazem also inhibited the aequorin signal induced by PAF, but with a much higher IC_{50} value (140 μM). Nitrendipine had minimal or no effects. Interestingly this order of potency is inversely related to the effectiveness of these agents in blocking voltage-dependent calcium channels, suggesting that a different mechanism of Ca^{2+} influx is activated by PAF, possibly a receptor-operated Ca^{2+} channel.

To show that the inhibition by the antagonist CV-3988 is specific to PAF responses, we used the calcium ionophore A23187, which induced an aequorin signal when added to NG108-15 cells (Table 1). However, CV-3988 at 179 μM, a concentration that completely blocked the aequorin signal generated by PAF, did not inhibit the aequorin response produced by A23187.

The properties of the PAF-induced increase in intracellular Ca^{2+} in neural cells are consistent with the reports that, in platelets, PAF increases cytoplasmic Ca^{2+} by inducing Ca^{2+} influx (*8*). In neurons, this effect of PAF on Ca^{2+} levels could lead to activation of phospholipase A_2 and release of free polyunsaturated fatty acids (*9*). The stimulation of aequorin signals in the neural cells studied here was most pronounced when PAF in the micromolar range was used, although small responses were observed in the nanomolar range. Since the sensitivity of aequorin to Ca^{2+} is most pronounced in the micromolar range (*10*), the use of fluorescent probes with sensitivity to Ca^{2+} in the range of 100 to 600 nM should enable the determinations of the effect of very low PAF concentrations on neuronal Ca^{2+} fluxes. The mechanism by which PAF stimulates free Ca^{2+} in NG108-15 and PC12 cells may involve interaction with low affinity sites that serve in the uptake of PAF into cells (*11*). Such an uptake process would be expected to be inhibited by a structural analog of PAF (CV-3988) but not necessarily by antagonists that bind specifically only to high-affinity PAF receptors, as shown here. The importance of the regulatory activity exerted by intracellular PAF was discussed by Henson (*12*), who emphasized the potential membrane effects of intracellular PAF. Such mode of action will be particularly important in the nervous system, since Bussolino *et al.* (*13*) have demonstrated that retinal cells synthesize PAF in response to stimulation by neurotransmitters but do not secrete it to the extracellular environment. The influx of Ca^{2+} plays a significant role in neuronal function, and PAF acting through this mechanism may serve as an important neuroregulator. It has been reported (*14*) that PAF stimulates secretion of hypothalamic corticotropin-releasing hormone. This PAF-induced secretion suggests that also in the central nervous system (CNS) PAF may act by increasing intraneuronal free Ca^{2+}. This possibility can be tested directly by studying primary CNS neurons differentiated in culture (*15*), as shown here with neural cell lines. Such studies could also reveal the presence of high-affinity PAF receptors in CNS neurons that may be antagonized by triazolobenzodiazepines (*4*).

The neurotoxicity (Fig. 1B) exerted by PAF concentrations that produce an exaggerated increase in intracellular free Ca^{2+} levels (Fig. 2) may have important clinical implications. PAF at concentrations originating from cells in the circulation may cause a pathophysiological increase in Ca^{2+}-influx, resulting in irreversible neurodegeneration. Inhibition of this process by specific drugs could provide a novel therapeutic approach for the prevention or treatment of damage caused to neurons in trauma, stroke, and spinal cord injury.

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