INTRODUCTION

Platelet activating factor (PAF) regulates a wide range of physiological and pathological processes in mammals (Braquet et al., 1987; Barnes et al., 1989; Saito and Hanahan, 1989; Hwang, 1990; Kato et al., 1994). At the cellular level, PAF modulates intracellular second messengers, such as cAMP, cGMP, Ca2+ and IP3, via a G protein-linked membrane receptor (Braquet et al., 1987; Snyder, 1989). Modulatory activities linked to intracellular receptors have also been suggested, based on differential effects of PAF antagonists, but they are less well characterized (Stewart et al., 1990; Hwang, 1990; Marcheselli and Bazan, 1994; Kato et al., 1994).

PAF has been found also in lower eukaryotes, such as Tetrahymena pyriformis (Lekka et al., 1990) and Dictyostelium discoideum (Bussolino et al., 1991).

Dictyostelium cells grow as free-living amoebae, by feeding on bacteria. Development is triggered by starvation, and results in the formation of multicellular aggregates which undergo a series of morphogenetic changes (Bonner, 1967; Loomis, 1982). A major regulatory role in aggregation is played by cAMP, which is secreted by the cells a few hours after starvation and acts as a chemoattractant.

In Dictyostelium, the intracellular concentration of PAF increases during development, and in response to cAMP (Bussolino et al., 1991). Spontaneous, periodic cellular oscillations, which occur during development to aggregation (Gerisch and Hess, 1974; Gerisch and Wick, 1975), are amplified by addition of exogenous PAF (Sordano et al., 1993). PAF showed a reduced response in the G protein β− strain LW14 and was unable to induce Ca2+ influx in the Gα2− strains HC85 and JM1. The latter expresses the cAMP receptors CAR1 constitutively, and exhibits cAMP-induced Ca2+ influx, albeit at a reduced level. In order to decide whether the inability of PAF to elicit a Ca2+ response in JM1 cells was due to the lack of differentiation and/or the lack of Gα2, we inhibited the IP3-dependent pathway with compound U73122 and found that Ca2+ entry was blocked, whereas a closely related inactive compound, U73343, did not alter the response. In agreement with this, NBD-Cl, an inhibitor of Ca2+ uptake into the IP3-sensitive store in Dictyostelium, also abolished PAF activity. The latter was not inhibited by the plasma membrane antagonists BN-52021 or WEB 2170. Therefore PAF seems to operate intracellularly via the IP3-signalling pathway at or upstream of the IP3-sensitive store.

SUMMARY

Stimulation of calcium influx by platelet activating factor in Dictyostelium

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MATERIALS AND METHODS

Chemicals

PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) and lyso-PAF (1-O-hexadecyl-sn-glycero-3-phosphocholine) were purchased from Bachem (Bubendorf, Switzerland), and cyclic AMP from Boehringer (Mannheim, FRG), 1-Palmitylthio-2-palmitoyl-amino-1,2-dideoxy-sn-glycero-3-phosphorylcholine (TPC) and compounds U73122 and U73343 were obtained from Biomol (Hamburg, FRG). NBD-Cl (7-chloro-4-nitrobenz-2-oxa-1,3-diazole) was obtained from Sigma (Munich, FRG). Geneticin (G418) was
purchased from Gibco-BRL (Paisley, Scotland). PAF antagonists BN-52021 and WEB 2170 were generous gifts from Dr P. Braquet (Institut Henry Beaufour, Paris) and D H. Heuer (Boehringer-Ingelheim), respectively.

**Cell cultures**

Cells of *Dictyostelium discoideum* AX2 strain, of the mutant HSB1, and of transformants LW14 (Lilly et al., 1993) and JM1 (Milne and Devreotes, 1993) were cultured axenically as previously described (Watts and Ashworth, 1970); mutant HCS5 was grown on *Klebsiella aerogenes* (Coukell et al., 1983; Menz et al., 1991). The antibiotic G418 was added during growth of LW14 and JM1 at a concentration of 20 |μ|g per ml.

The amoebae were harvested during exponential growth, washed twice with cold Sörensen phosphate buffer, pH 6.0, adjusted to 2×10^7 cells per ml in this buffer and shaken at 150 revs/min until used. Cultivation of cells and all experiments was at 23°C.

**Measurement of Ca^{2+} influx**

Ca^{2+} influx was measured in a suspension of 5×10^7 cells per ml in a total volume of 2 ml by using a calcium-ion-sensitive electrode (Möller, Zürich, Switzerland) as described by Bumann et al. (1984). Briefly, cells were washed and resuspended in 5 mM tricine buffer, pH 7.0, 5 mM KCl. The cell suspension was stirred and aerated with water-saturated oxygen. Electrode potentials were recorded with a pH meter 610 (Metrohm, Herisau, Switzerland), and calibration curves were run for each experiment. Cyclic AMP was dissolved in water and added, when required, in a volume of 2 |μ|l. In experiments with PAF or lyso-PAF, the lipid molecules were solubilized in 95% ethanol at concentrations high enough to allow for a thousand-fold dilution of the solvent upon addition to the cell suspension. TPC was dissolved in ethanol at 10 mM concentration whereas U73122 and U73343 were dissolved in DMSO at 5 mM concentration. BN-52021 and WEB 2170 were dissolved in DMSO (stock solution: 50 mM) and in water (stock solution: 5 mM), respectively. The final concentration of DMSO and ethanol did not exceed 0.1% and 0.4%, respectively.

**RESULTS**

**Specificity and concentration dependence of Ca^{2+} influx induced by PAF**

In our previous study (Sordano et al., 1993) we reported that PAF treatment resulted in a transient stimulation of Ca^{2+} influx, with kinetics similar to that evoked by cAMP. Fig. 1 demonstrates these effects of 1 |μ|M PAF, lyso-PAF or cAMP on Ca^{2+} influx in starving AX2 cells. The extent of Ca^{2+} influx in response to cAMP and PAF is subject to variation from experiment to experiment: sometimes cells responded better to cAMP than to PAF, as in the experiment shown in Fig. 1, but the opposite was also true. The dose-response curve revealed a steep increase of Ca^{2+} entry between 0.1 |μ|M and 1 |μ|M PAF; the half-maximal influx occurred at 0.3 |μ|M, with saturation above 1 |μ|M PAF (Fig. 2).

Lyso-PAF is an intermediate product of PAF metabolism, which is rapidly formed in *Dictyostelium* by deacetylation of PAF and then acylated to acyl-PAF (Brusati et al., unpublished data). At concentrations varying between 0.1 and 1 |μ|M, lyso-PAF did not affect Ca^{2+} influx in any way (Fig. 1) nor did it influence the cellular response to a subsequent PAF stimulus (not shown). In contrast, when PAF was added twice to the same cell suspension, with a delay of several minutes between additions, the cellular response to the second stimulus was strongly reduced or even absent (Fig. 3). The length of adaptation seemed to depend on the extent of Ca^{2+} influx elicited by PAF.

**Effect of PAF on Ca^{2+} influx is developmentally regulated**

When cells were treated with PAF at the beginning of starvation, no change in the extracellular concentration of Ca^{2+} was observed. This led us to test whether PAF stimulation of Ca^{2+} influx changed during development. As shown in Fig. 4, a clear effect of PAF is detected after 3 hours of development, reaches a maximum at 4 hours, and declines slowly thereafter. This initial pattern is similar to that observed for cAMP-induced Ca^{2+} influx, except that cAMP evokes a small transitory increase also at the beginning of starvation. In addition, maximal stimulation with cAMP is reached 2 hours later than with PAF (Fig. 4). It is noteworthy that the cellular sensitivity to PAF increases coincidentally with the onset of spontaneous, spike-shaped light-scattering oscillations (Fig. 4, arrows 1 and 2), whereas a sharp decline is observed at the transition from spike-shaped to sinusoidal oscillations (Fig. 4, arrow 3). Spike-shaped oscillations, but not sinusoidal ones, were previously found to be amplified by PAF (Sordano et al., 1993).

**Synergistic effects of PAF and cAMP on Ca^{2+} influx**

To test whether PAF and cAMP stimulate Ca^{2+} influx through independent or identical pathways, synergy experiments were performed, in which cells were treated first with a pulse of cAMP or PAF: 20 to 25 seconds later, as soon as the Ca^{2+} response reached its maximum, a second pulse of the other
compound was administered to the cells, while continuously monitoring the cellular response. As shown in Fig. 5, addition of 1 µM PAF followed by 1 µM cAMP induced a response which was larger than those evoked by PAF or cAMP alone. Usually, the extent of the change in Ca\(^{2+}\) concentration was about the sum of those evoked by PAF and cAMP added separately (Figs 5 and 6). Instead, in the reverse experiment the additional effect of PAF was usually smaller than that of PAF alone (Fig. 6), and sometimes even absent. It is interesting to note that two pulses of the same intensity of either cAMP or PAF, added sequentially under the same conditions, failed in both cases to evoke a larger influx (Figs 5 and 6).

When cAMP and PAF (1 µM each) were added simultaneously, the change in Ca\(^{2+}\) influx amounted to the sum of the effects of each compound administered separately. Note that the concentration of PAF used saturates the response (Fig. 2), whereas 1 µM cAMP elicits only 50% of maximum Ca\(^{2+}\) entry. At saturating concentrations of cAMP (10 µM), the two responses behaved additively (data not shown).

In conclusion, these experiments show: (1) that 1 µM cAMP and 1 µM PAF stimulate Ca\(^{2+}\) influx by acting on two apparently autonomous transduction pathways; (2) that the two pathways, however, are not totally independent, since cAMP interferes with PAF-induced Ca\(^{2+}\) influx; (3) that cells adapt to subsequent stimuli of PAF as is known for cAMP (Fig. 3).

**PAF-induced Ca\(^{2+}\) influx in cell lines with defective signal transduction**

Several mutant strains or transformants have been described, which are defective at various steps in the signal transduction pathways (for review see Devreotes, 1994). We have tested the effects of PAF on Ca\(^{2+}\) influx in mutants HSB1 and HC85 and in transformants LW14 and JM1. HSB1 is a synag mutant defective in adenylate cyclase activation by cAMP (Bozzaro et al., 1987); HC85 is a fgdA mutant in which the G\(\alpha\)2 subunit of the G protein is inactive (Coukell et al., 1983; Firtel et al., 1989); JM1 is a derivative of HC85 with constitutive expression of the cAMP receptor cAR1 (Milne and Devreotes, 1993). LW14, is a null mutant for the \(\beta\) subunit of the G protein, obtained by homologous recombination (Lilly et al., 1993).

As shown in Fig. 7, PAF, as well as cAMP, failed to stimulate Ca\(^{2+}\) entry in HC85 cells, whereas the response of HSB1 was normal with respect to PAF and slightly reduced with respect to cAMP, possibly reflecting incomplete differentiation of HSB1 to aggregation competence. The \(\beta^-\) mutant...
LW14 exhibited a strongly reduced Ca\(^{2+}\) influx for both PAF and cAMP, indicating that the $\beta$ subunit is required for differentiation and/or for Ca\(^{2+}\) influx. The latter explanation seems to be more likely, at least for cAMP, since the normal number of cAMP receptors are present in this strain (Lilly et al., 1993).

The $\text{G}_\alpha_2$ subunit could be directly involved in PAF-induced Ca\(^{2+}\) entry. However, it must be kept in mind that HC85, like all $\text{fgdA}$ mutants, is blocked at the beginning of development, and that the PAF response, as shown above, is developmentally regulated. It is, therefore, possible that HC85 cells do not express a developmentally regulated PAF effector that is necessary for induction of Ca\(^{2+}\) influx. Milne and Coukell (1991) reported that 50 nM cAMP pulses for 9 hours after the beginning of starvation led to a small but reproducible cAMP-induced Ca\(^{2+}\) influx, indicating that cAMP pulses induce limited differentiation of the $\text{fgdA}$ mutant. To test whether this limited differentiation is sufficient for a PAF response we applied their conditions to mutant HC85 cells and also found a small cAMP-induced Ca\(^{2+}\) influx of 20±6 pmol/10\(^7\) cells ($n=5$) at 1 $\mu$M cAMP (22±12 pmol/10\(^7\) cells at 10 $\mu$M cAMP; $n=6$) in two separate experiments. The PAF-induced Ca\(^{2+}\) influx, however, was still absent.

Milne and Devreotes (1993) have shown that constitutive overexpression of the, otherwise developmentally regulated, cAMP receptors cAR1 or cAR3 in a HC85-derived transformant was sufficient for restoring at least half of the cAMP-induced Ca\(^{2+}\) entry, based on receptor number. We have tested their transformant, JM1, under our conditions and we find that no PAF-induced Ca\(^{2+}\) influx occurred, in contrast to the response to cAMP, which amounted roughly to half of the influx obtained with AX2 cells (Fig. 7).

**Inhibition of Ca\(^{2+}\) influx by a PLC antagonist and NBD-Cl, an inhibitor of the IP\(_3\)-sensitive store**

Since PAF responses were absent in $\text{G}_\alpha_2$ negative mutant cells, we tested whether PLC or phospholipase A\(_2\) (PLA\(_2\)) activities are required for Ca\(^{2+}\) entry. Using a non-hydrolysable analog of lecithin, TPC, which exhibits an IC\(_{50}\) of 2 $\mu$M for cobra venom PLA\(_2\) activity (Yu et al., 1990), we found that 40 $\mu$M TPC did not affect the response to PAF (Table 1). By contrast,
compound U73122, an inhibitor of receptor-stimulated IP₃ formation in neutrophils (Smith et al., 1990) and the IP₃-sensitive Ca²⁺ store in pancreatic acinar cells (Willems et al., 1994), blocked PAF-induced Ca²⁺ entry completely, whereas a closely related inactive analog U73343 did not alter the response. Previously we have found that NBD-Cl, an inhibitor of V-type H⁺-ATPase, blocked cAMP-induced Ca²⁺ uptake into the IP₃-sensitive store (Flaadt et al., 1993); 50 µM NBD-Cl likewise abolished Ca²⁺ influx induced by PAF in two separate experiments (Table 1).

**DISCUSSION**

The only known direct effect of PAF on signal transduction in *Dictyostelium* is the stimulation of Ca²⁺ influx (Sordano et al., 1993). Therefore we used the Ca²⁺ response as a measure to investigate the mechanism of PAF action. The most important finding with respect to its role in *Dictyostelium* development was the restriction of PAF activity to the period of spike-shaped oscillations and its absence during sinusoidal oscillations (Fig. 4).

This result is in agreement with the previous finding that PAF enhanced cGMP and cAMP formation in the presence of exogenous or endogenous cAMP (Sordano et al., 1993). We interpret these data to mean that PAF is used in signal amplification in order to enhance cAMP relay. It is interesting in this respect that PAF increases postsynaptic neural transmission in hippocampal neurons (Kato et al., 1994). In *Dictyostelium*, PAF action seems to be required only a few times, since the

**Table 1. Effects of various inhibitors on PAF-induced Ca²⁺ influx**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Developmental time (hours)</th>
<th>Ca²⁺ influx of control cells (pmoles/10⁷ cells)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPC</td>
<td>4-6</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>(40 µM)</td>
<td>4-6</td>
<td>98</td>
<td>0</td>
</tr>
<tr>
<td>U73122</td>
<td>4-5.5</td>
<td>111</td>
<td>100</td>
</tr>
<tr>
<td>(5 µM)</td>
<td>5-6</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>U73343</td>
<td>4-5.5</td>
<td>174</td>
<td>0</td>
</tr>
<tr>
<td>(5 µM)</td>
<td>5-6</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>NBD-Cl</td>
<td>5</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>(50 µM)</td>
<td>6</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

1 µM pulses of PAF were applied to the cell suspension in the absence or presence of the compound indicated and the extent of Ca²⁺ changes was recorded with a Ca²⁺-sensitive electrode as described in Materials and Methods. Developmental time: time after washing from growth medium. The results of two independent experiments are shown.
cells adapted to the stimulus for a period of 30 minutes to 1 hour (Fig. 3). Augmentation may result in lowering the threshold or increasing the sensitivity for a certain step in signal transduction. This is indicated by the result shown in Fig. 6, where a somewhat greater response was obtained when PAF was given before cAMP, in comparison to the addition after cAMP.

At the concentration used cAMP and PAF acted synergistically, suggesting that at least initially two different transduction pathways are used, which are, however, not totally independent, since the prior response to cAMP reduced PAF-induced Ca\textsuperscript{2+} influx (Figs 5, 6). The fact that two different signal transduction pathways exist became apparent while testing strain JM1, which lacks the G protein subunit G\textsubscript{q2}, as does the fgdA strain HC85, but is constitutively expressing the cAMP receptor cAR1 at a high level (Milne and Devreotes, 1993). In this strain cAMP still induced a significant proportion of Ca\textsuperscript{2+} influx with respect to strain AX2 in agreement with the results of Milne and Devreotes (1993); however, the response to PAF was absent. Similarly, pulsing of HC85 cells failed to yield a response to PAF, though partially restoring the cAMP response. This result can be explained in two ways: either G\textsubscript{q2} is absolutely necessary for Ca\textsuperscript{2+} entry elicited by PAF and/or a PAF acceptor is required, which appears during differentiation to aggregate competence concomitantly with spike-shaped oscillations, and is absent in JM1 cells and also in pulsed HC85 cells. These cells do not differentiate due to the absence of G\textsubscript{q2}.

Do we have some evidence as to the location of the target of PAF action in signal transduction? PAF is synthesized in response to cAMP after about 2 to 3 minutes, but does not appear to be secreted (Bussolino et al., 1991). Therefore it seems unlikely that a cell surface receptor mediates PAF activity as occurs in many mammalian cells. PAF binding experiments to plasma membranes have failed so far, and application of the plasma membrane PAF-receptor antagonist WEB 2170, which is known to inhibit aggregation of human platelets and neutrophils (IC\textsubscript{50} values: 0.3 and 0.83 \mu M, respectively; Heuer et al., 1990), did not affect PAF induced Ca\textsuperscript{2+} influx up to 100 \mu M (data not shown). BN-52021, which binds to PAF receptors integrated in the plasma membrane (Marcheselli et al., 1990), did not reveal a clearcut inhibition of the PAF response at a concentration of 50 \mu M (not shown). Second messenger functions of PAF are known to occur in leukocytes and endothelial cells (Stewart et al., 1990) as well as intracellular binding sites in the rat cerebral cortex (Marcheselli et al., 1990); however, the mechanism of action is still unknown. Using a non-competitive inhibitor of receptor-mediated IP\textsubscript{3} and DAG formation, U73122, which is thought to interfere with PLC activation at the G protein level (Smith et al., 1990; Smallridge et al., 1992) and also inhibits specifically the IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} store (Willems et al., 1994), we found that PAF-stimulated Ca\textsuperscript{2+} entry was blocked, whereas an inhibitor of PLA\textsubscript{2} activity, TPC, was unable to interfere with the PAF response. Our results thus favour the hypothesis that PAF acts intracellularly, possibly at the G protein level, since both G\textsubscript{q2} and \beta subunits seems to be of importance for PAF-induced Ca\textsuperscript{2+} influx. On the other hand the inhibition of PAF-induced Ca\textsuperscript{2+} influx by U73122 and NBD-Cl indicates that the target of PAF resides at or upstream of the IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} store. In addition, our results indicate that two different signal transduction mechanisms exist for Ca\textsuperscript{2+} entry, one independent of differentiation and G\textsubscript{q2} and the other dependent on G\textsubscript{q2} and differentiation.

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