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Biphasic increase in intracellular calcium induced by platelet-activating factor in macrophages

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In single mouse macrophages stimulated by platelet-activating factor (PAF), the intracellular calcium concentration (Ca_i) monitored with fura-2 at room temperature presents a biphasic increase, including a transient and a more sustained component. After pulse administration of PAF, the first phase lasts for a few seconds and reaches a peak value of 0.5–1 μM Ca^{2+} at high PAF concentration. The amplitude of this peak is independent of extracellular Ca^{2+} concentration, suggesting that the initial Ca^{2+} transient is due to the release of Ca^{2+} from intracellular stores. The second phase of the response lasts for several minutes; its maximum amplitude is reached 1 2 min after the brief initial PAF stimulation. This phase, suppressed in zero external Ca^{2+} and increased in 10 mM Ca^{2+}, is probably due to influx of Ca^{2+} through the plasma membrane. This secondary Ca^{2+} increase is blocked by 10–50 μM lanthanum. At low PAF concentration, the initial Ca^{2+} transient is not followed by a second phase, showing that the initial rises of Ca^{2+} and of its activator (presumably inositol trisphosphate) are not sufficient to trigger the second phase of Ca^{2+} increase.

Ca^{2+}; Fura-2; Macrophage; Platelet-activating factor

1. INTRODUCTION

Platelet-activating factor (PAF), 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine, is a phospholipid secreted by various cell types including platelets, macrophages, mastocytes and endothelial cells (review [1]). Specific receptors for PAF have been described on platelets [2], polymorphonuclear lymphocytes [3] and some smooth muscles [4]. PAF exerts a variety of biological effects, such as platelet aggregation, contraction of smooth muscle, increased vascular permeability and release of histamine from mast cells (review [1]). When applied to macrophages, PAF stimulates the oxidative burst, release of arachidonic acid and some of its metabolites (prostaglandin E and thromboxane B_2), and cell spreading [5,6]. The mode of action of PAF is far from being understood. In platelets and neutrophils, PAF activates the phospholipase C-mediated hydrolysis of polyphosphoinositides [7]. In platelets and macrophages, PAF induces an increase in intracellular Ca^{2+} concentration (Ca_i); this increase results partly from the release of Ca^{2+} from intracellular stores, and partly from influx of Ca^{2+} from the extracellular solution [8]. The latter results were obtained with the quin-2 method [9]. The average signal obtained on a large number of cells was not suitable for a thorough analysis of the contributions of the two components to the PAF-induced Ca^{2+} rise.

Here, we have measured Ca_i with the fluorescent dye fura-2 [10] on single cells. With a double perfusion system, PAF could be applied to cells for various periods (as short as 2 s) and immediately washed, and the extracellular Ca^{2+} concentration could be changed in less than 200 ms. The resulting time resolution allowed us to separate clearly the two phases of the Ca^{2+} rise elicited by PAF, and to analyze quantitatively their kinetic characteristics and dependence upon PAF concentration.

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2. MATERIALS AND METHODS

Mouse peritoneal macrophages were collected and cultured as described [11]. Experiments were performed at room temperature 5–25 days after plating the cells.

2.1. *Solutions*

Bath solution contained (in mM) 5 KCl, 140 NaCl, 1 $CaCl_2$, 1 $MgCl_2$ (pH 7.2) fixed with 5 Hepes-Na. Some experiments were in high Ca^{2+} (4 or 9 mM Ca^{2+} added to the normal solution) or Ca^{2+}-free solutions (0.5 mM EGTA, 0 Ca^{2+}). PAF (Sigma) was dissolved in ethanol at 40 μg/ml and stored at −20°C. Diltiazem and (+)-tubocurarine were from Sigma, and QX 222 from Astra Pharmaceutical Products.

2.2. *Perfusion systems*

PAF was applied with a pipette (opening ~20 μm) rapidly brought close to the cell with a hydraulic micromanipulator, under red light. Solution changes taking place in less than 1 s could be achieved in this way. In addition, we used a fast perfusion system based on a U-tube [10], with which changes in bath solution could be effected in less than 200 ms. When a test solution was not flowing out of the U-tube, bath solution was continuously sucked through the same opening, contributing to the fast termination of PAF application by the leaking pipette. During prolonged PAF application, this aspiration avoided flooding of the rest of the dish with PAF and desensitization of other cells.

2.3. *Calcium measurements*

Measurements of intracellular calcium concentration on single cells with the Ca^{2+} indicator fura-2 were made as follows. Cells were incubated for 30 min at 37°C with 2 μM fura-2 acetoxy methyl ester plus 0.4 mg/ml Pluronic (Molecular Probes). Generally, this procedure resulted in a fura-2 loading which yielded homogeneous fluorescence within each cell. In a few preparations, brilliant intracellular granules were observed. These could be due to the endocytosis of fura-2, followed by its concentration in lysosomes [12] or to intracellular transport of the dye into cytoplasmic vacuoles [13]. Such cells had an apparent level of resting Ca^{2+} which was abnormally low and responded poorly to PAF stimulation. These preparations were discarded in the present study. Measurements of Ca_i were performed as in [14] (except that the photodiode was replaced by a Hamamatsu 647-01 photomultiplier).

3. RESULTS

3.1. *Relation between PAF concentration and characteristics of the Ca^{2+} response*

The resting level of Ca_i was usually around 100 nM except during the first 2–3 days after plating (200–400 nM). Application of 4 ng/ml PAF to these cells for a few seconds elicited, after some delay (see below), an increase in Ca_i composed of two phases, as shown in fig.1b: a transient increase (phase I) followed by a second period (phase II) where, for a few minutes, Ca_i remained higher

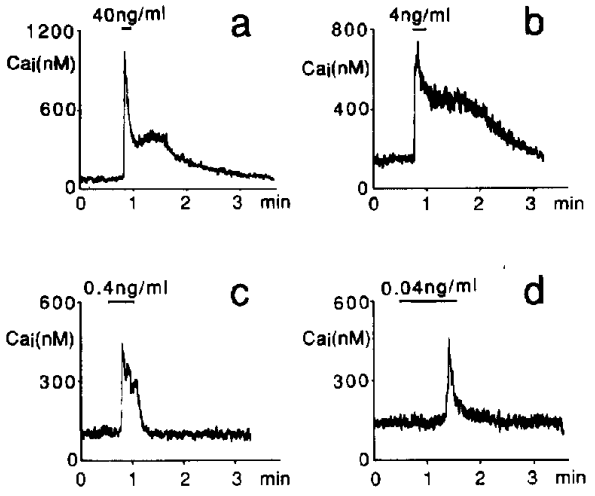

Fig.1. Typical changes in Ca_i induced by PAF applications of various concentrations. In this and subsequent figures the duration of PAF application is indicated by the bar above each record.

than the resting level. The changes in Ca_i induced by 40 ng/ml PAF (fig.1a) were similar to those elicited by 4 ng/ml PAF (fig.1b), except that the delay was reduced and barely measurable (<1 s).

PAF concentrations lower than 4 ng/ml induced Ca^{2+} changes (fig.1c,d) which differed from those induced by higher doses in several respects: (i) the initial delay was increased; (ii) the amplitude of the initial Ca^{2+} peak was slightly reduced; (iii) the magnitude of the second phase was reduced to a greater extent vs the first phase; (iv) with PAF at 0.04–0.004 ng/ml, only a fraction of the cells gave a response.

Fig.2a shows the curves relating the amplitude of the initial Ca^{2+} transient to PAF concentration. The upper curve was obtained by recording only those peaks which were different from zero, whereas the overall mean, including the failures to respond to PAF, is shown in the lower trace. In the upper curve, it appears that at a PAF concentration 1000-times smaller than that necessary for maximum response, when phase I is triggered, the response is still half of the maximum. The EC_{50} which can be derived from the lower curve is around 0.2 ng/ml, i.e. 0.4 nM PAF. The curve relating the duration of the initial delay to the reciprocal of the PAF concentration is shown in fig.2b. It shows an initial part which is linear. At very low PAF concentrations, the curve tends

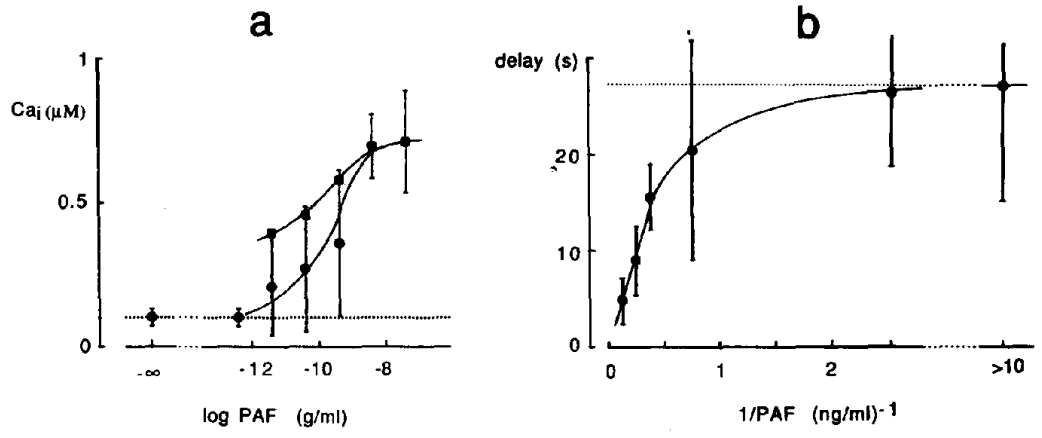

Fig.2. Effect of PAF concentration on the initial peak amplitude (a) and the preceding delay (b). (a) On the abscissa, a logarithmic scale was used because of the wide range of PAF concentrations explored (between 4×10^{-13} and 4×10^{-8} g/ml). Squares correspond to the average of the amplitude of the peaks of responding cells and circles (± SD) to the overall average, including the failures. The dotted line indicates the Ca_i level in the absence of PAF stimulation. (b) Average delays (± SD) preceding a response as a function of reciprocal PAF concentration. Dotted line: maximum average delay observed at low PAF concentration. All curves were drawn by eye. Averages ± SD determined on 5–9 experimental values in a and b.

towards a limit, which corresponds to the maximum delay that can precede a rise of Ca^{2+}: on average, 27 ± 12 s (mean ± SD, $n = 7$) for PAF concentrations ranging between 0.04 and 0.004 ng/ml.

3.2. *Effects of extracellular Ca^{2+} concentration*

We next examined the influence of extracellular Ca^{2+} (Ca_o) on the properties of the two phases of the Ca^{2+} rise elicited by 4 ng/ml PAF. The amplitude of the initial Ca^{2+} peak was scarcely affected by changes in Ca_o: PAF stimulation performed in a Ca^{2+}-free solution gave Ca^{2+} transients of normal amplitude. Increasing Ca_o from 1 to 10 mM did not modify significantly the amplitude of the initial peak.

In contrast, the amplitude of phase II was strongly dependent upon Ca_o being markedly enhanced in 10 mM Ca_o. The ratio of the amplitudes of phase II/phase I was 0.24 ± 0.36 (mean ± SD, $n = 8$) in 1 mM Ca_o and 0.91 ± 0.46 ($n = 6$) in 10 mM Ca_o. The two values are significantly different (*t*-test, $p < 0.01$).

Fig.3c shows the increase in Ca_i elicited by PAF in the absence of external Ca^{2+}. The cell was bathed in 1 mM Ca^{2+}, but PAF was applied for a few seconds in a Ca^{2+}-free solution, and another Ca^{2+}-free solution was applied by U-tube perfusion (see section 2) immediately after PAF stimulation. Under these conditions, only a transient rise in Ca^{2+} was obtained, and Ca_i returned to its resting level after 1 min. Upon readmission of 1 mM Ca^{2+} to the bath, Ca_i rose to several hundred nanomolar. For several minutes after PAF stimulation, the level of Ca_i could be varied by changing Ca_o. Similar changes of Ca_o before PAF stimulation had no effect on Ca_i (not shown).

These data suggest that phase I, which is sometimes well separated from phase II (figs 1a,3a), and sometimes less so (fig.1b), does not depend upon Ca_o and therefore probably results from a Ca^{2+} release from intracellular stores. On the other hand, the amplitude of phase II is increased in high Ca_o, and suppressed in Ca^{2+} free solutions, and thus probably arises from influx of Ca^{2+} through the plasma membrane.

These conclusions are confirmed by the experiment shown in fig.3d: this cell had been kept for more than 1 h in a Ca^{2+}-free solution. Nevertheless, a transient Ca^{2+} response could be evoked by PAF under these conditions (in the experiment of fig.3d, the amplitude of the Ca^{2+} transient is small, but not significantly different from that obtained on the same day with 1 mM Ca_o). Changes of Ca_o from 0 to 10 mM for brief periods induced an increase in Ca_i after PAF stimulation, but not

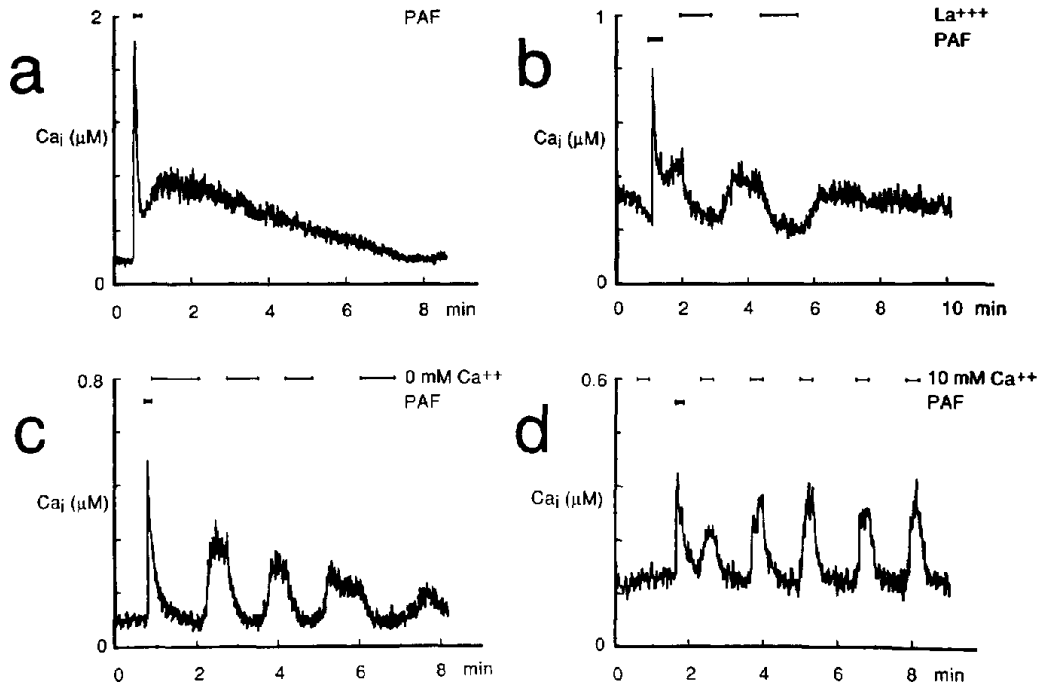

Fig.3. Ca_i variations induced in different cells by brief applications of PAF (4 ng/ml). Applications of PAF by the leaking pipette are indicated by thick bars and of various solutions by U-tube perfusion by thin bars. (a) Example of a response with a large initial peak and long duration (Ca_o = 5 mM). (b) Reduction of Ca_i during phase II induced by application of 50 μM lanthanum (Ca_o = 1 mM). (c) Reduction of Ca_i during phase II, induced by application of a Ca^{2+}-free solution (0.5 mM EGTA). Control Ca_o = 1 mM. PAF was diluted in the Ca^{2+}-free solution. (d) Ca^{2+} response to PAF in a cell bathed in Ca^{2+}-free solution (0.5 mM EGTA). Effect of brief applications of 10 mM Ca_i, before and after PAF stimulation.

before. Note that in this example, the maximum amplitude of phase II is reached several minutes after the brief stimulation by PAF.

The influx of Ca^{2+} giving rise to phase II is likely to be due to the opening of ionic channels. We have tried several substances which have been shown to block either Ca^{2+} channels or cationic channels. Three of them did not affect the second phase of the Ca^{2+} response to PAF, namely diltiazem (200 μM), (+)-tubocurarine (100 μM) and QX 222 (100 μM). When the trivalent lanthanum cation La^{3+} (10–50 μM) was applied during phase II, the level of Ca_i was rapidly reduced, as would be expected if La^{3+} efficiently blocked Ca^{2+} influx (fig.3b). This effect was immediately reversible upon washing La^{3+}.

3.3. *Kinetics of PAF response*

The initial rise of Ca_i during a response to PAF developed in less than 1 s. This cannot be shown with a good time resolution by measuring the absolute value of Ca_i, at a rate limited by that of the rotating filter (~5 Hz). For this experiment fura-2 was excited at a single wavelength (385 nm) and its fluorescence sampled at 100 Hz. It is reasonable to assume that the time courses of the PAF-induced fluorescence decrease and Ca_i increase are identical. Note that in this example (fig.4a), the sharp increase in Ca_i (indicated by the decrease in fluorescent signal) was preceded by a slower and smaller Ca^{2+} increase. This initial creep was not always so obvious. In this example, the change in fluorescence from 20 to 80% of the full amplitude took 200 ms, not much longer than the time required for the solution change. This rate of rise of Ca_i is similar to that observed after stimulation of muscarinic receptors in exocrine glands [15,16].

The rate of decay of phase I was better observed in Ca^{2+}-free solutions, in order to separate it from the second phase. In several instances (not shown),

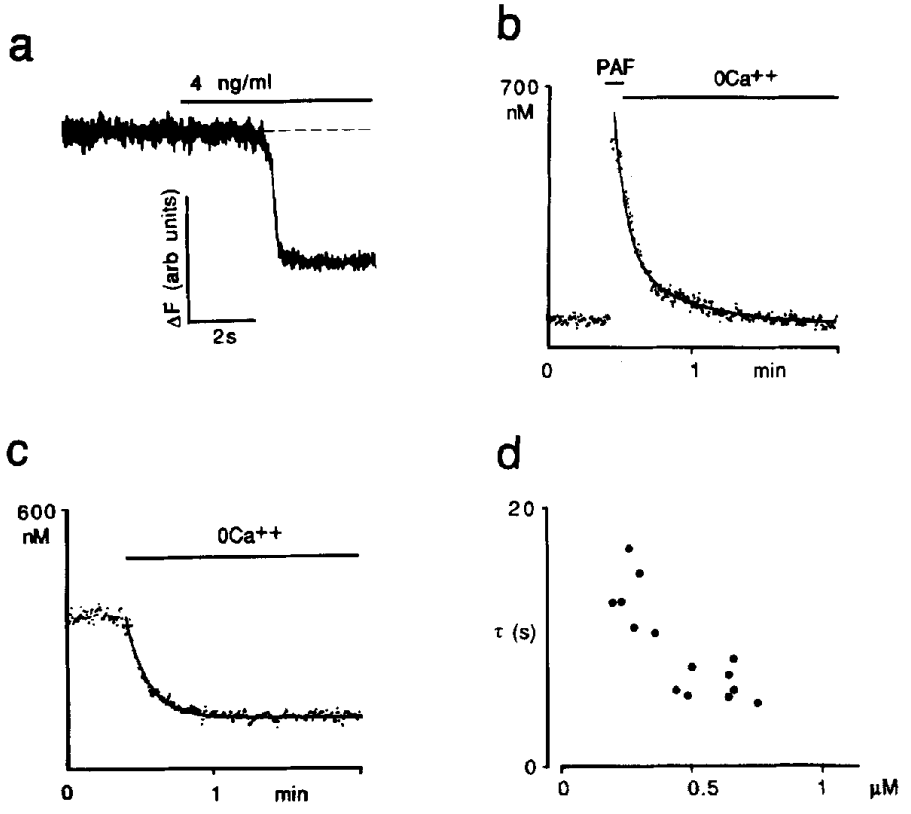

Fig.4. Kinetics of PAF-induced Ca_i changes. (a) Fluorescence measured after excitation at 385 nm. Fluorescence decrease corresponds to Ca_i increase induced by PAF (phase I). (b) The decay of the Ca_i peak induced by PAF in Ca^{2+}-free solution can be fitted by the sum of two exponentials (time constants 5.7 and 26 s). Here and in d, dots correspond to experimental points and the continuous line to the exponential fit. (c) Example of a Ca_i decrease induced by switching Ca_o from 1 to 0 Ca^{2+} during phase II, fitted by an exponential (time constant 10 s). (d) Time constant of decay of Ca_i in experiments of the type shown in (c), as a function of the plateau value of Ca_i before switching to 0 Ca^{2+}.

after a Ca^{2+} transient induced by PAF in a Ca^{2+}-free solution, Ca_i returned to a lower level than before PAF stimulation, suggesting that during phase I, activation of the Ca^{2+} pumps outlasts the Ca^{2+} transient. This decay was generally biphasic, and could be fitted by the sum of two exponentials (fig.4b). Our interpretation is that the initial fast decrease of Ca_i could be due to the activity of two pumps (plasmalemmal and of intracellular stores). After these stores have been filled, Ca^{2+} extrusion would depend only on the plasmalemmal Ca^{2+} pump, and Ca_i would decrease more slowly.

During phase II, an apparent rate of Ca^{2+} influx can be estimated by measuring the rate of Ca_i increase induced by switching the extracellular solution from 0 to 1 or 10 mM Ca^{2+}. The half rise times of these increases averaged 3.1 ± 2.2 s (mean ± SD, n = 16). This Ca^{2+} rise is thus much slower than during phase I.

When a Ca^{2+}-free solution was rapidly applied to the cell during phase II, Ca_i slowly returned to resting levels, generally with an exponential time course, as shown in fig.4c. The time constant of decay of Ca_i (τ) depended upon the Ca_i value attained during phase II, τ being smaller for higher plateau values of Ca_i. This is illustrated in fig.4d, which gives the relation between τ and the Ca_i value measured before the application of the Ca^{2+}-free solution. Assuming that in phase II intracellular stores are refilled, the decrease in Ca_i would be due to the activity of the plasmalemmal

pump, extruding Ca^{2+} after dissociation from Ca^{2+}-binding proteins. The accelerated return of Ca_i to basal levels when the initial Ca^{2+} value is high probably reflects the Ca^{2+} dependence of this pump.

4. DISCUSSION

In a variety of cells, the activation of some membrane receptors triggers an increase in Ca_i due partly to the release of Ca^{2+} from intracellular stores and partly to influx of Ca^{2+} through the plasma membrane. Such increases follow, for instance, the activation of muscarinic receptors in exocrine glands [17], substance P receptors in mast cells [18], purinergic receptors in endothelial cells [19], PAF receptors or ATP receptors in macrophages [8,20] or are triggered by the cross-linking of surface immunoglobulins in B cells [21].

Several conditions must be met in order to analyze such responses: (i) Since the response presents a fast component which is not synchronous in all the cells of a population, Ca^{2+} measurements should not be performed on a large number of cells, but on single cells. (ii) During prolonged stimulation, the second phase could be contaminated by a small but sustained release of Ca^{2+} from intracellular stores. Thus, the separation of the two phases should be drawn out by triggering the response via brief administration of the stimulating molecule. (iii) Fast changes of the extracellular solution are necessary for the kinetic analysis of Ca^{2+} changes during the two phases of the response. (iv) The amplitude of each phase should be sufficiently large.

Here, a quantitative analysis of the two phases of the Ca^{2+} rise elicited by PAF in macrophages could be performed because all these conditions were fulfilled.

4.1. *Dose-response curve: amplitude and delay*

The dose-response curve that we have established relates the amplitude of the peak of the first phase of the Ca^{2+} response to PAF concentration. The EC_{50} derived from such a functional dose-response curve (0.4 nM) is comparable to the K_d previously reported: 0.6 nM in neutrophils [22], 0.1 nM in polymorphonuclear leucocytes [3]. One peculiarity of this curve is that it spreads over several orders of magnitude of PAF concentration. A double-reciprocal plot of the data gives a relation which is less than linear. This apparent negative cooperativity could mean that the PAF-receptor stoichiometry is less than unity; one PAF molecule would then activate several receptors. Alternatively, several kinds of receptors could be involved in the PAF response.

However, there are two features of these responses which are not taken into account in this curve. The first is that, at low PAF concentration, the apparent low amplitude of the response is the average of a majority of complete failures to respond, and of a minority of responses of almost normal amplitude. At a PAF concentration where more than 60% of the cells failed to respond, the amplitude of the Ca^{2+} peak of the responding cells was still ~50% of the maximum. Such behaviour may reveal the existence of a threshold and of positive feed-back: if Ca_i reaches a certain level, then it increases very rapidly above this level [23].

A second feature of the Ca^{2+} response is the existence of a delay between PAF application and Ca^{2+} rise. A similar delay was observed after stimulation of various receptors in platelets [24] and of muscarinic receptors in exocrine glands; in these glands, with supra-maximal agonist concentration, a minimum delay of 70–700 ms was observed [15,16]. At high PAF concentrations, the delay preceding the response is linearly related to the reciprocal of the PAF concentration. A similar situation was described for the muscarinic response (to moderate and high doses of acetylcholine) in lacrimal glands [15]. The authors conclude that this delay should represent the time needed to build up the concentration of an intracellular component, X, directly involved in the response. We have observed that at very low PAF concentrations, when a response is obtained, the preceding delay is never longer than 30 s on the average. We suggest that this limiting delay could be the time at which the concentration of X passes through a maximum (after which it is degraded or diluted). Our data indicate that the time at which this maximum is reached should be independent of PAF concentration. At high PAF concentration, X reaches a threshold value well before this time. At lower PAF concentrations the most likely time where the threshold can be reached is at the X peak.

4.2. *Relative importance of the two phases of the Ca^{2+} response*

It has been claimed that after PAF stimulation the Ca_i increase due to Ca_i release from intracellular stores was much smaller than the Ca^{2+} influx [8]. In contrast, it appears in our data that the rate of Ca^{2+} entry in the cytoplasm is much larger when Ca^{2+} is released from intracellular stores than when it comes from the extracellular solution. As a result the Ca^{2+} concentration reached during phase I is greater than during phase II. This is true for pulse administrations of PAF as well as for prolonged PAF applications (not shown). There are two reasons why in previous estimates, the relative importance of Ca^{2+} release was underestimated. Firstly, in a cell population, PAF-induced Ca^{2+} transients are not necessarily synchronous in all the cells. As a consequence, the average peak is smaller than the individual peaks. Secondly, quin-2 measurements are generally carried out with a high intracellular concentration of dye (around 1 mM), which exerts a Ca^{2+}-buffering effect particularly marked on Ca^{2+} transients (see e.g. [18]). One can predict that biological processes with different Ca^{2+} requirements are regulated during phase I (large and brief Ca^{2+} rise) and during phase II (lower but prolonged increase in Ca_i).

4.3. *Control of the second phase of the PAF response*

In macrophages, Ca^{2+} influx during this phase is not due to the modulation of voltage-dependent Ca^{2+} channels which occurs in other cells (review [25]), since macrophages are devoid of such channels [11,26]. Rather, this influx is probably due to the opening of receptor-operated Ca^{2+}-channels. Such channels have been described in neutrophils [27], T lymphocytes [28], mast cells [18] and platelets [29]. They can be blocked by nickel or cadmium [28,29]. Since these channels could have a cationic selectivity, we have tried various blockers known to affect the cationic channel of the nicotinic receptor (QX222, (+)-tubocurarine; review [30]) or that of photoreceptors (diltiazem, see [31]). None of these blockers affected Ca^{2+} influx in macrophages. It would be of major importance to find a blocker of these channels more specific than the only one that was efficient here, lanthanum.

It has been suggested that these receptor-operated Ca^{2+} channels could be opened either by intracellular Ca^{2+} [27], or by IP_3 [18,32]. Our data do not support these hypotheses. The fact that at low PAF concentration, the initial Ca^{2+} (and presumably IP_3) peak is not followed by Ca^{2+} influx indicates that the initial increases in Ca^{2+} and IP_3 are not sufficient to activate receptor-operated Ca^{2+} channels. In addition, the peak of the second phase is reached between 1 min and several minutes after the Ca^{2+} (and presumably the IP_3) peaks have taken place. The final reason against the Ca^{2+} hypothesis being valid can be drawn from the observation that if a Ca^{2+}-free solution is applied to a cell during phase II for a duration sufficient to lower Ca_i to resting levels, upon readmission of external Ca^{2+}, Ca_i increases without delay. Therefore, the membrane channels responsible for phase II, once activated, may remain open even when Ca_i is low. Recent results obtained with various growth factors on 3T3 cells also argue in favour of a dissociation between hydrolysis of polyphosphoinositides and Ca^{2+} influx [33]. A similar conclusion can also be drawn from the observation that in salivary cells, a low dose of phorbol esters can block the Ca^{2+} release elicited by carbachol, without blocking the carbachol-induced Ca^{2+} influx [34].

Future work will be necessary in order to determine whether direct activation by G-proteins, or phosphorylation, or other mechanisms, are involved in the control of receptor-operated Ca^{2+} channels. The PAF-macrophage system, where a secondary Ca^{2+} rise of large amplitude clearly distinct from the initial Ca^{2+} transient can be observed, should prove useful in elucidating this problem.

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