Threshold responses in production of reactive oxygen metabolites in individual neutrophils detected by flow cytometry and microfluorimetry

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1. The fluorescent compound 2',7'-dichlorofluorescein was used as an indicator of intracellular H_2O_2 production by neutrophils in order to compare the response of the cell population with that observed with individual cells determined by flow cytometry and quantitative fluorescence microscopy. 2. 2',7'-Dichlorofluorescein diacetate was deacetylated by intracellular esterases to form reduced 2',7'-dichlorofluorescein. The polar non-fluorescent intermediate remained trapped within both intracellular granules and the cytoplasm. Reduced dichlorofluorescein was oxidized by H_2O_2 , a product of the oxidative burst, to yield the highly fluorescent product dichlorofluorescein. 3. A population of neutrophils stimulated by suboptimal concentrations of fMet-Leu-Phe (*N*-formylmethionyl-leucyl-phenylalanine) or phorbol ester (phorbol 12-myristate 13-acetate) resulted in an oxidation of 45–50% of the cellular dichlorofluorescein (non-fluorescent) to oxidized dichlorofluorescein within 30 min. Subcellular fractionation showed that, although dichlorofluorescein (non-fluorescent) occurred both in the cytoplasm and the granules, oxidation of dichlorofluorescein (non-fluorescent) occurred predominantly in the granules of stimulated neutrophils. 4. Flow cytometry showed that unstimulated cells consisted of a single population of cells with low cellular fluorescence. Activation of neutrophils (to produce reactive oxygen metabolites) resulted in the appearance of a second population of cells, with high fluorescence. The number of cells in this new population increased with time. fMet-Leu-Phe (0.1 μM) or phorbol ester (1 ng/ml) activated 45% of the cells within 8 min and 42% within 30 min respectively. 5. Analysis of individual cells by quantitative fluorescence microscopy demonstrated that, in the presence of a suboptimal concentration of stimulus, cells either failed to respond or were activated after different time delays, 4–120 s (39 ± 18.4 s) by fMet-Leu-Phe or 12–200 s (59 ± 17.4 s) by phorbol ester. Furthermore the oxidative bursts were of different magnitudes. 6. It is concluded that, in order for an individual cell to cross the activation threshold for the 'end response', a critical concentration of stimulus together with the necessary changes in intracellular signals are required.

INTRODUCTION

It is well established that changes in the intracellular concentrations of free Ca^{2+} , cyclic AMP, cyclic GMP and diacylglycerol play key roles in enabling primary electrical and chemical stimuli, acting on the cell membrane, to activate the end response of a cell (Campbell, 1983; Berridge & Irvine, 1984; Nishizuka, 1984). Much of the evidence for this in small cells has been based on biochemical measurements in populations of thousands of cells or more. A major problem in rationalizing the biochemical and evolutionary significance of intracellular messengers has been the difficulty in assessing the heterogeneity in the responses of individual cells within the population. There are three possible sources of this heterogeneity, which must be taken into account if the molecular basis of cell activation is to be fully understood (Campbell, 1983, 1987a,b). Firstly, a critical concentration of stimulus may be necessary to evoke an end response. Secondly, the time at which the end response is evoked may vary between individual cells and the concentration of stimulus. Thirdly, the magnitude of the eventual end-point

response may vary from cell to cell. Furthermore, it is necessary to establish whether secondary regulators, which may inhibit or enhance the population response, act by altering one or more of these parameters.

Neutrophils are the first cells to infiltrate a tissue following the acute inflammatory response. They can be activated to produce reactive oxygen metabolites (including O_2^- , H_2O_2 and OCI^-) by a range of natural occurring and experimental phagocytic or chemotactic stimuli (Babior *et al.*, 1973; Segal & Jones, 1979). The former can activate cells via a mechanism independent of an increase in intracellular free Ca^{2+} , the latter require an increase in intracellular free Ca^{2+} concentration (Hallett & Campbell, 1982, 1984; Campbell & Hallett, 1983; Pozzan *et al.*, 1983; Lew *et al.*, 1985). Adenosine acts as a secondary regulator, via cyclic AMP, but only inhibits those stimuli dependent on an increase in intracellular Ca^{2+} concentration (Roberts *et al.*, 1985a,b).

In the present paper we report the application of a fluorescent indicator (Bass *et al.*, 1983) for neutrophil oxidase activation, in order to establish that individual cells pass through a 'threshold' for activation at different times after the addition of the stimulus. The response of

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the whole population, monitored in a fluorimeter, was resolved into individual responses by flow cytometry, and the time course of response in individual cells was determined by quantitative fluorescence microscopy.

EXPERIMENTAL

Materials

Chemotactic peptide fMet-Leu-Phe, phorbol 12-myristate 13-acetate, 4-methylumbelliferyl 2-acetamide-2-deoxy- β -D-glucopyranoside, β -NADH, horseradish peroxidase and carboxylic-ester hydrolase were purchased from Sigma Chemical Co., Poole, Dorset, U.K. 2',7'-Dichlorofluorescein diacetate and 2',7'-dichlorofluorescein (fluorescent) were obtained from Eastman Kodak Co., Hemel Hempstead, Herts., U.K.

All other chemicals were AnalaR grade from BDH Chemicals, Poole, Dorset, U.K.

Isolation of neutrophils

Neutrophils were isolated by using a modification of the procedure described by Holt *et al.* (1984). Heparinized blood (20 ml) from normal volunteers was mixed with 5 ml of Dextran 110 (Dextraven 110; Fisons Pharmaceutical Division, Loughborough, Leics., U.K.) and left to stand for 1 h. The plasma was layered over 10 ml Ficoll-Paque (Pharmacia Fine Chemicals) and centrifuged at 500 *g* for 20 min to obtain a neutrophil pellet. The cells were resuspended in 2 ml of 0.9% NaCl. Contaminating erythrocytes were lysed in 6 ml of ice-cold double-distilled water for 60 s. Iso-osmotocity was restored with the addition of 2 ml of cold 3.5% (w/v) NaCl. The cells were centrifuged at 720 *g* for 5 min, and washed twice in Ca^{2+} -free Krebs Hepes buffer containing 120 mM-NaCl, 4.8 mM-KCl, 1.2 mM- KH_2PO_4 , 1.2 mM- MgSO_4 , 25 mM-Hepes and 0.1% bovine serum albumin, adjusted to pH 7.4 with NaOH. The cells were finally resuspended in Krebs Hepes buffer containing 1.3 mM- CaCl_2 . Whole blood (20 ml) yielded 25×10^6 – 30×10^6 cells, of which 98% were neutrophils, as seen by phase-contrast microscopy. Neutrophils were diluted to 1×10^6 – 2×10^6 cells/ml and kept on ice.

Preparation of reduced 2',7'-dichlorofluorescein

Reduced 2',7'-dichlorofluorescein was prepared fresh by treating 0.5 ml of 1 mM-dichlorofluorescein diacetate (dissolved in ethanol) with 2 ml of 0.01 M-NaOH at room temperature for 30 min. The resultant dichlorofluorescein (non-fluorescent) was diluted with 10 ml of phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4) and kept on ice. Conversion of dichlorofluorescein diacetate (R_f 0.97) into reduced dichlorofluorescein (R_f 0.82) was monitored chromatographically on Alufolien cellulose F_{254} plates, with a developing solvent of butan-1-ol/acetic acid/water (12:3:5, by vol.).

Fluorimetric assay for the indicators dichlorofluorescein diacetate and dichlorofluorescein (non-fluorescent)

Non-fluorescent compounds dichlorofluorescein diacetate and dichlorofluorescein were assayed after conversion into the stable fluorescent indicator dichlorofluorescein (excitation wavelength 500 nm, emission wavelength 530 nm). Briefly, 290 μ l samples containing dichlorofluorescein diacetate were deacetylated with 0.5 unit of carboxylic-ester hydrolase (non-specific esterase),

and the resultant dichlorofluorescein (non-fluorescent) was oxidized to dichlorofluorescein (fluorescent) with H_2O_2 (final concentration 1 mM) and 0.1 unit of horseradish peroxidase at 37 °C over 50 min. Fluorescence of the samples was measured, and equivalent concentrations of dichlorofluorescein (fluorescent) were determined from a standard curve.

Samples containing dichlorofluorescein (non-fluorescent) were assayed without carboxylic-ester hydrolase.

Optimal loading of neutrophils

Uptake of dichlorofluorescein diacetate by neutrophils was determined from total intracellular concentrations of dichlorofluorescein (non-fluorescent) and dichlorofluorescein (fluorescent). Neutrophils (16 ml , 1×10^6 cells/ml) in phosphate-buffered saline were incubated with 5 μ M-dichlorofluorescein diacetate at 37 °C for 30 min. Neutrophils (1×10^6 cells) at defined intervals were washed and intracellular concentrations of dichlorofluorescein (fluorescent) were determined. Cells were then lysed in 3.5% (v/v) Nonidet P40 and intracellular concentrations of dichlorofluorescein (non-fluorescent) were determined. The time taken for optimal loading of neutrophils was determined from maximal concentrations of intracellular dichlorofluorescein (non-fluorescent) (a potential substrate for reactive oxygen metabolites).

Subcellular fractionation of neutrophils

Neutrophils (5.0×10^7 cells/ml) in 0.25 M-sucrose were homogenized in a Polytron homogenizer. After separation of intact cells, the particulate and cytoplasmic fractions were separated by centrifugation at 13400 *g* for 15 min. After preincubation in Nonidet P40 (3.5%, v/v), the marker enzymes lactate dehydrogenase and myeloperoxidase together with fluorescent dichlorofluorescein were assayed. No further myeloperoxidase was sedimented from the 13400 *g* supernatant by centrifugation further at 100000 *g*. The content of dichlorofluorescein in the fractions is expressed as pmol/ 10^6 cells.

Flow cytometry

Fluorescence of dichlorofluorescein diacetate-treated neutrophils was analysed on a Becton Dickinson FACS III flow cytometer, fitted with a 50 μ m-diameter nozzle. Fluorescence emitted from cells containing dichlorofluorescein (fluorescent) was detected through a 520 LP filter by using an argon laser excitation wavelength at 488 nm. The fluorescence and scatter distribution was accumulated in a multichannel (127-channel) pulse-height analyser. A flow rate of 2000 cells/s was used to analyse 10000 cells.

Fluorescence measurements from single neutrophils

Human neutrophils loaded with dichlorofluorescein diacetate as described above were allowed to adhere to glass cover-slips by incubation at 37 °C for 10 min. After washing to remove non-adherent cells and extracellular dichlorofluorescein diacetate, the cover-slips were mounted on an adapted microscope slide with a clearance gap of 0.17 mm between the surface of the slide and the cells on the cover slip. The slide was mounted on a micro-foil stage heater (Lowndes & Hallett, 1986), which maintained the cells at 37 ± 0.1 °C during observation with an adapted Zeiss III fluorescent photomicroscope (Hallett, 1985). The fluorescence was stimulated by light passing

through an excitation filter BP450–490 (peak transmission 490 nm). The illuminated field with an oil objective $\times 100$ was restricted to one cell. The emitted light passing through an emission filter BP520–560 was projected on to the face of a photon-counting photomultiplier tube (EMI 9924B) coupled to the optical axis of the microscope. The power to the photomultiplier tube was maintained at 850V and the digital signal was processed by an integrator and rate meter ($t = 0.1$ s) to produce both integrated photon counts over a timed interval and a continuous trace of changes in photon emission rate with time.

Stimuli were applied to the cells during measurement of fluorescence by allowing a drop of Krebs Hepes buffer containing $0.1 \mu\text{M}$ -fMet-Leu-Phe or 1 ng of phorbol ester/ml to touch the clearance gap, capillary attraction bringing this stimuli into contact with the cells attached to the cover-slip. The interface between the incoming solution and the air gap produced a useful optical signal that timed the arrival of the stimulus of the cell under observation.

RESULTS

2',7'-Dichlorofluorescein as an indicator of production of reactive oxygen metabolites

In order to establish that 2',7'-dichlorofluorescein could be used to detect activation of neutrophils, it was first necessary (i) to quantify intracellular dichlorofluorescein (non-fluorescent) in neutrophils, (ii) to establish the intracellular distribution of dichlorofluorescein (non-fluorescent), and (iii) to demonstrate oxidation of intracellular dichlorofluorescein (non-fluorescent) to yield the highly fluorescent dichlorofluorescein in a population of cells stimulated by chemotactic peptide (fMet-Leu-Phe) and phorbol ester.

Neutrophils (1.0×10^7 cells in 1 ml) incubated with $5 \mu\text{M}$ -dichlorofluorescein diacetate resulted in an intracellular deacetylation of $9\text{--}10 \text{ pmol}$ of dichlorofluorescein diacetate to dichlorofluorescein (non-fluorescent). Subcellular fractionation of neutrophils showed that this deacetylation by intracellular esterases occurred in the granules and the cytoplasm, entrapping the polar intermediate dichlorofluorescein (non-fluorescent) in the granules (52%), in the cytoplasm (43%) and to a lesser extent in the nucleus (5%). Optimal accumulation of intracellular dichlorofluorescein (non-fluorescent) to dichlorofluorescein (non-fluorescent) in neutrophils was achieved within 12–15 min. Activation of these neutrophils by fMet-Leu-Phe or phorbol ester resulted in a metabolic burst producing H_2O_2 , which then oxidized dichlorofluorescein (fluorescent). Subcellular fractionation (Table 1) showed that in unstimulated cells more than 90% of the oxidized dichlorofluorescein was present in the granules with myeloperoxidase. Stimulation of the cells resulted in a 3-fold increase in total oxidized dichlorofluorescein, more than 70% of which was also sedimented with the myeloperoxidase-containing granules. Approx. 25–30% of the dichlorofluorescein and myeloperoxidase was found in the supernatant after subcellular fractionation of stimulated cells. Any myeloperoxidase and dichlorofluorescein secreted during cell stimulation was removed before homogenization. Since the production of dichlorofluorescein occurred only in the presence of peroxidase, under the conditions of

Table 1. Subcellular fractionation of neutrophils

Neutrophils (5×10^7 cells/ml) in Krebs Hepes buffer, pH 7.4, were incubated at 37°C for 30 min in the absence or in the presence of $1 \mu\text{M}$ chemotactic peptide fMet-Leu-Phe. The cells were fractionated as described in the Experimental section. The particulate (I) and cytoplasmic fractions (II) were assayed for marker enzymes lactate dehydrogenase and myeloperoxidase. Percentage distribution of dichlorofluorescein was determined, and also expressed as pmol/ 10^6 cells in parentheses.

	Distribution (%)			
	Control cells		Stimulated cells	
	I	II	I	II
Lactate dehydrogenase	5.0	95.0	4.5	95.5
Myeloperoxidase	> 99.0	< 1.0	75.0	25.0
Dichlorofluorescein	92.4 (0.29)	7.6 (0.03)	70.8 (0.7)	29.2 (0.3)

experiments reported here, release of oxygen metabolites from the NADPH oxidase located on the plasma membrane would not be detected. This method monitors intragranular H_2O_2 as an indicator of cell activation.

The response of a population of 'dichlorofluorescein diacetate-loaded' neutrophils was analysed fluorimetrically after activation by suboptimal concentrations of fMet-Leu-Phe ($0.1 \mu\text{M}$) or phorbol ester (1 ng/ml), which induced approx. 50% of maximal activation of the oxidase, determined either by O_2 uptake or luminol-dependent chemiluminescence. These concentrations of stimuli resulted in 45% (fMet-Leu-Phe) to 50% (phorbol ester) oxidation of intracellular dichlorofluorescein to fluorescent product. The fluorescence increase was detectable for both stimuli within 5 min (Fig 1). This increase reached a plateau within 10 min with fMet-Leu-Phe and 30 min with phorbol ester. Two questions arose. Firstly, was the difference in time course between fMet-Leu-Phe and phorbol ester explicable by individual cells switching 'on' at different times? Secondly, was the end point of approx. 50% oxidation to dichlorofluorescein the result of total oxidation of dichlorofluorescein (non-fluorescent) in only half of the cells?

Spectrum of individual neutrophil activation detected by flow cytometry

In the flow cytometer, the resting fluorescence of unstimulated neutrophils containing dichlorofluorescein (non-fluorescent) consisted of a single population of cells with low cellular dichlorofluorescein fluorescence. Activation of the oxidase in neutrophils by fMet-Leu-Phe or phorbol ester resulted in a second, distinct, population of cells with increased cellular dichlorofluorescein fluorescence (Fig. 2a). Addition of the stimulus resulted in a gradual rise in the number of cells with increased cellular dichlorofluorescein fluorescence. Suitable light-scatter fluorescence windows were therefore selected to differentiate cells with low, 'unstimulated' fluorescence (population A) and high, 'stimulated', fluorescence (population B). Activation of the oxidase was then quantified by measuring the number of individual cells appearing in

Fig. 1. Stimulation of dichlorofluorescein diacetate-loaded neutrophils by fMet-Leu-Phe or phorbol ester

Dichlorofluorescein diacetate-loaded neutrophils (2.5×10^7 cells) were washed and stimulated by (a) $0.1 \mu\text{M}$ -fMet-Leu-Phe or (b) 1 ng of phorbol ester/ml at 37°C . Samples (2×10^6 cells) were centrifuged and mean cellular fluorescence was determined (●). Residual intracellular dichlorofluorescein (non-fluorescent) (■) was determined and quoted as a percentage of total dichlorofluorescein diacetate loaded into neutrophils. Cellular fluorescence (○) and intracellular dichlorofluorescein (non-fluorescent) (□) of unstimulated cells were also monitored.

population B, expressed as a percentage of total cells analysed (Fig. 2b). fMet-Leu-Phe caused an activation of 45% of the total cells within 8 min, and phorbol ester-induced response was detectable after an initial lag of 4 min, and thereafter increased to 42% over the next 30 min. No further increase in population B was observed with either stimulus. In contrast, only 3% of all the cells were found in a population B in the absence of fMet-Leu-Phe or phorbol ester.

The data from flow cytometry showed that individual cells were activated at different times following addition of stimulus. Furthermore the results showed that partial oxidation of dichlorofluorescein (non-fluorescent) in the whole population resulted from activation of a fraction of the cells. In the flow cytometer, any particular neutrophil could only be observed for 0.5 ms as it passed through the laser beam. Therefore it was not possible to measure the lag time or the time course of the response in individual cells by this method. This was achieved by using a quantitative fluorescence microscope.

Production of reactive oxygen metabolites in individual cells

The fluorescence of dichlorofluorescein (fluorescent) was observed by fluorescence microscopy to be only in the granules of activated neutrophils (Fig. 3), despite the presence of dichlorofluorescein (non-fluorescent) in both the cytoplasm and granules. This observation was consistent with subcellular fractionation of activated neutrophils, which showed that cellular dichlorofluorescein fluorescence, due to the production of reactive oxygen metabolites, had increased by 0.41 pmol in the granules of 10^6 cells compared with unstimulated cells (Table 1).

The time course of the production of reactive oxygen metabolites in individual neutrophils was determined by using a quantitative fluorescence microscope. A total of 27 cells were investigated in different experiments. No significant changes in fluorescence were observed in unstimulated cells over the time period of these experiments. Typical fluorescence changes in seven representative cells stimulated by fMet-Leu-Phe or phorbol ester are shown in Fig. 4. These traces consisted of a lag, followed by an increase to maximum fluorescence, after which the fluorescence decays, probably because of photobleaching. Although the lag time with fMet-Leu-Phe varied widely from 4 to 120 s ($39 \pm 18.4 \text{ s}$, $n = 5$, coefficient of variation 94.5%) between individual cells, the maximum fluorescence intensity of responding cells was far less variable (coefficient of variation 18%). A concentration of fMet-Leu-Phe of $0.1 \mu\text{M}$ was unable to stimulate the production of reactive oxygen metabolites in 56% of the cells (e.g. cell 5). Production of reactive oxygen metabolites stimulated by a suboptimal concentration of phorbol ester (1 ng/ml) occurred after widely varying lags of 12 to 200 s ($59 \pm 17.4 \text{ s}$, $n = 12$, coefficient of variation 97%), whereas the magnitude of responses was less variable (coefficient of variation 13%). However, like fMet-Leu-Phe, this suboptimal concentration of phorbol ester was insufficient to activate the oxidative burst in 33% of the cells (e.g. cell 1). Non-responding cells were shown to be viable by Trypan Blue exclusion. These results support the conclusion from flow cytometry on a population of neutrophils, that approx. 42–45% of the cells were activated to produce reactive oxygen metabolites by a half-maximal concentration of fMet-Leu-Phe or phorbol ester (Figs. 1 and 2b). The data obtained by quantitative fluorescence microscopy also suggest that individual neutrophils stimulated by fMet-Leu-Phe and phorbol ester respond heterogeneously in terms of magnitude.

Fig. 2. Fluorescence distribution of dichlorofluorescein diacetate-loaded neutrophils by flow cytometry

Neutrophils (1×10^6 cells) containing dichlorofluorescein diacetate were stimulated and cellular fluorescence was analysed by flow cytometry. (a) Plot of cellular fluorescence against number of cells show (i) unstimulated cells, (ii) fMet-Leu-Phe-stimulated neutrophils or (iii) phorbol ester-stimulated neutrophils after 10 min. (b) Cells with high cellular fluorescence (population B) are quoted as a percentage of total cells analysed. Results represent a plot of percentage of neutrophils with high cellular fluorescence against time for (i) unstimulated cells (○), (ii) fMet-Leu-Phe-stimulated cells (■) or (iii) phorbol ester-stimulated cells (●).

Fig. 3. Fluorescence micrographs of a single neutrophils loaded with dichlorofluorescein diacetate

The Figure shows fluorescence of a cell stimulated by $0.1 \mu\text{M}$ -fMet-Leu-Phe at 37°C : (a) light field; (b) fluorescence field.

DISCUSSION

Most studies on the relationships between stimuli and cellular responses are performed on large populations of cells. Two implicit assumptions have been made when using data from such experiments as the basis for describing the molecular basis of cell activation: firstly, that the cells respond uniformly and to the same extent, and secondly, that they respond synchronously.

Examination of individual neutrophils exposed to a half-maximal concentration of stimulus (fMet-Leu-Phe or phorbol ester) by flow cytometry has shown that only some (42–45%) individual cells were activated (Fig. 2b), whereas a maximal concentration of stimulus appeared to activate more than 95% of the cells. Furthermore, the time course of the onset of the population response (Fig. 1) was explained by differences in the delay before each cell was switched 'on' (Fig. 2b). Suboptimal concentrations of stimuli failed to activate a significant proportion of the cells (Figs. 1 and 2b), and individual cells had different time delays before activation of the end response occurred. This was demonstrated both by flow cytometry and by direct measurement of the time course of responses from individual cells by the use of quantitative fluorescence microscopy (Fig. 4). Quantitative fluorescence microscopy also confirmed that some cells failed to respond at all. The 'all-or-none'

nature of neutrophil activation by phorbol ester has also been observed from measurements of NADPH auto-fluorescence (Hafeman *et al.*, 1982) and more recently confirmed by using dichlorofluorescein fluorescence measurements (Hirabayashi *et al.*, 1985), although one group (Bass *et al.*, 1983) has failed to observe this phenomenon. The reason for this apparent discrepancy is not clear. The time courses of dichlorofluorescein fluorescence in individual stimulated cells determined by quantitative fluorescence microscopy (Fig. 4) confirmed the 'all-or-none' activation of neutrophils. 'All-or-none' responses have also been demonstrated following complement-induced cell injury (Patel & Campbell, 1987). Such a phenomenon has important consequences for understanding the molecular basis of the action of primary stimuli and secondary regulators. Adenosine, acting through cyclic AMP, appears to modify the population response to chemotactic peptide (Roberts *et al.*, 1985b) by decreasing the number of cells crossing the activation threshold for the end response.

The key question now is: what is the molecular basis underlying the chemical thresholds for activation in these and other cells? There are three obvious possibilities, as follows. (i) The concerted action of a number of molecular reactions leads to a critical event that is necessary for activation to occur. This event could be fusion of a granule with the plasma membrane, or an internalized

Fig. 4. Typical fluorescence signals from single neutrophils loaded with 2',7'-dichlorofluorescein diacetate

Traces show responses of single neutrophils to suboptimal concentration of (a) phorbol ester (cells 1–4) and (b) fMet-Leu-Phe (cells 5–7).

vesicle from it, or the concerted action of covalently modified protein molecules. (ii) The gradient of O_2 varies within different cells, and the concentration of the primary substrate, O_2 determines both the time at which the enzyme complexes responsible for producing reactive oxygen metabolites switch 'on' and the magnitude of this response. We have previously shown that half-maximal stimulation of a population of neutrophils occurred at a concentration of approx. $3.7\text{--}9.6\ \mu\text{M}$. Although the most likely relationship between O_2 concentration and the production of reactive oxygen metabolites is a graded one, the possibility that the oxidase within the cell requires a critical O_2 concentration to be activated now needs to be investigated. This may be a mechanism particularly relevant *in vivo* (Edwards *et al.*, 1983). (iii) The concentration of intracellular regulator(s) must reach a critical level in the necessary region within the cell for activation to occur. The size, and thus location, of an intracellular Ca^{2+} cloud, or diacylglycerol, will be determined by three main factors: the receptor occupancy of the stimulus, the size and location of the inositol trisphosphate produced to release Ca^{2+} from its internal store, the internal buffering of Ca^{2+} , or the synthesis or degradation of other intracellular regulators that may prevent the critical concentration of Ca^{2+} to be reached or may prevent molecular events at its site of

activation. It is now also necessary to establish whether secondary regulators, such as adenosine, that alter the time to 'threshold' and the magnitude of the response once the 'threshold' has been passed, act through one or more of these mechanisms.

The chemi-symbiotic hypothesis (Campbell, 1983, 1987b) proposes that there are only a distinct number of states in which a cell can exist between the resting state, the fully activated state and cell death. It further proposes that a vital feature in the biochemical evolution of eukaryotic cells has been the development of an intracellular signalling system that enables the intracellular reactions and organelles responsible for these different states to be co-ordinated. The results reported here have established the validity of the touchstone of this hypothesis, namely the concept of chemical thresholds as the molecular basis for quantal events in cells. This hypothesis also provides a new framework for explaining the dichotomy between reversible and irreversible injury in diseases, particularly when cells are attacked by components of the immune system and membrane-pore-forming pathogens (Morgan *et al.*, 1986; Campbell, 1987b; Patel & Campbell, 1987). The question now is how the family of intracellular regulators, which include cations such as Ca^{2+} , H^+ , Na^+ and possibly Zn^{2+} , cyclic nucleotides, inositol phosphates and diacylglycerol, act either in a symbiotic or competitive manner to enable individual cells to transform rapidly from one state into another and not to remain in an intermediate one. These questions can only be answered experimentally by using single-cell analysis (Campbell *et al.*, 1985; Tsien & Poenie, 1986; Roberts *et al.*, 1987).

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