that changes in membrane electrical potential difference or changes in the transport of specific ions into the receptor cells might serve a similar function in influencing the level of intracellular calcium. The present studies do not provide sufficient information to distinguish between these various alternative mechanisms. Nevertheless, our results indicate that the activity of the intracellular calcium system of the recepter cell serves a critical role in coupling the events occurring within the distal tubular lumen with the resultant alterations in glomerular vascular resistances.

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Neutrophil Activation Monitored by Flow Cytometry: Stimulation by Phorbol Diester Is an All-or-None Event

Abstract. *The population dynamics of single-cell stimulation was analyzed by monitoring autofluorescence by flow cytometry. Stimulation of the respiratory burst in human neutrophils by 12-O-tetradecanoyl phorbol-13-acetate (TPA) caused a decline in highly fluorescent cells (characteristic of resting neutrophils) and a corresponding increase in the number of weakly fluorescent cells (characteristic of activated neutrophils). Increasing concentrations of TPA caused increasing numbers of cells to shift from the highly fluorescent population to the weakly fluorescent population without the appearance of intermediate populations. Thus the neutrophil respiratory burst, a component of neutrophil cytotoxic response, is triggered in an all-or none fashion.*

Phagocytosis of foreign bodies by macrophages and neutrophils is associated with a respiratory burst of superoxide (O_2^-) production because of the activation of a membrane-bound pyridine nucleotide oxidase. Superoxide, or subsequently formed "active oxygen" species such as hydrogen peroxide, hydroxyl radicals, and possibly singlet oxygen, participate in the oxidative cytotoxic mechanisms of these cells. These oxidative mechanisms are used by phagocytic cells to kill foreign organisms [for review, see (*1–3*)]. The formation and release of O_2^- from activated immunologic cells also appears to play a role in the pathology of autoimmune and arthritic diseases (*4*). For these reasons, the mechanism and control of this response have been the subjects of intense investigation.

Triggering of the respiratory burst does not require phagocytosis since surfaces that are coated with immunoglobulin G (IgG) and that do not undergo phagocytosis trigger the response (*5*). The response is also triggered by certain soluble agents such as chemotactic peptides, anionic detergents, calcium ionophore A23187, concanavalin A, and various phorbol diesters. In general, increasing concentrations of stimulating substances cause an increased mean metabolic response of a cell population (*1–3*, *6*). It has not been known whether this is due to a dose-dependent increase in the metabolic response of all cells, or whether individual cells are quantized in completely activated or resting modes and vary in the concentration of agonist required for activation.

To test whether the metabolic response in neutrophils is quantized, we monitored cell autofluorescence by flow cytometry. In principle, any fluorescence change due to metabolic stimulation could be monitored. We chose excitation wavelengths in the region of 350 nm and monitored emission > 425 nm in order to observe the decreases in intracellular reduced pyridine nucleotides [NAD(P)H] that have been reported to occur after stimulation of the neutrophil respiratory burst (*7–9*). The fluorescence of reduced pyridine nucleotides is much greater than that of the oxidized species and fluorescence changes have previously been monitored in intact cells (*10*).

As shown in Fig. 1a, the fluorescence distribution of neutrophils is clearly bimodal (*11*). Most of the cells belong to the highly fluorescent population. The fraction of weakly fluorescent cells varied between neutrophil preparations; in some cases, it was less than 0.05, in others (see Fig. 1a) it was as great as 0.3. Both cell populations were viable as determined by analysis of propidium iodide exclusion (*12*).

Next we added progressively increasing amounts of the respiratory burst stimulator 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) to the cell suspension prior to incubation. As shown in Fig. 1, b to f, TPA caused a dose-dependent decrease in the population frequency of the highly fluorescent cells and a corresponding increase in the population frequency of the weakly fluorescent cells. This result is consistent with the expected decrease in the ratio of reduced to oxidized pyridine nucleotides in metabolically stimulated neutrophils (*7–9*). No decrease in fluorescence was observed when the cells were incubated with TPA at 0°C. The lower fluorescence intensity of the stimulated neutrophils was similar in magnitude to that of the initial variable subpopulation of neutrophils with low fluorescence intensity. The presence of weakly fluorescent cells in the initial unstimulated neutrophil preparation may be due to stimulation of a fraction of the neutrophils in vivo or during the cell purification procedure.

Analysis of these results yielded additional information about the mechanism of stimulation of individual cells. A downward shift in fluorescence intensity of the entire neutrophil population as the concentration of TPA is increased would indicate that higher TPA concentrations

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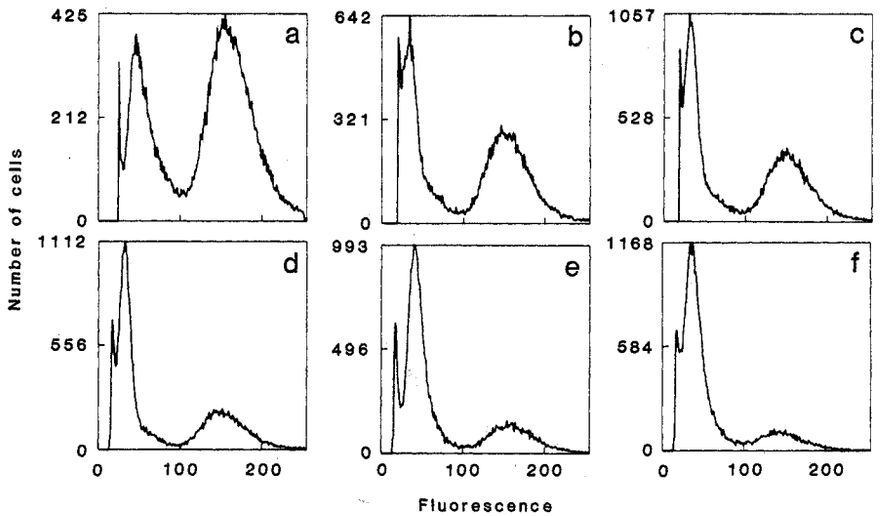

Fig. 1. Distribution of NAD(P)H fluorescence from human neutrophils incubated with various concentrations of TPA. The fluorescence scale is in arbitrary units. (a) No TPA, (b) 0.6 n*M*, (c) 1.5 n*M*, (d) 3.0 n*M*, (e) 4.5 n*M*, and (f) 6.0 n*M* TPA. The switching of the cells from the highly fluorescent population to the weakly fluorescent population with increasing concentrations of TPA is clearly visible.

stimulate greater metabolic activity within individual cells. However, our results showed that as the concentration of TPA was increased there was an increase in the fraction of neutrophils that displayed the fluorescence shift and a corresponding decrease in the highly fluorescent population, indicating that the neutrophil respiratory burst is an all-or-none event within individual cells (Fig. 1). Cells with fluorescence intensity intermediate between the two populations were insignificant in number. The fraction of the total neutrophil population that appears in the weakly fluorescent (activated) cell population as a function of TPA concentration is shown in Fig. 2. The response was near maximum in the presence of 6.0 n*M* TPA. We had previously found that this concentration was sufficient for nearly maximum stimulation of the neutrophil respiratory burst (CN^--insensitive oxygen consumption).

Because neutrophil stimulation can induce cell-cell adhesion (*13*), it was possible that stimulated neutrophils were aggregating and thus were not analyzed as single cells. Cell clumping and adhesion was minimized in our experiments by the addition of 10 m*M* EDTA. Hemacytometer counts of single cells before and after stimulation were similar, indicating that cells were not lost as a result of clumping or adhesion to test tube surfaces. This eliminated the possibility that the fluorescence distributions were not representative of the entire cell population. Thus neutrophil stimulation by TPA induces a large quantum decrease in the NAD(P)H fluorescence in individual cells. The dose-dependent stimulation of the respiratory burst in a large population of neutrophils is therefore due to triggering of the respiratory burst in a large fraction of the cells by high concentrations of TPA rather than to greater stimulation of individual cells.

Visible fluorescence emission from unstained intact cells with the use of ≃ 350-nm excitation energy is mainly due to reduced pyridine nucleotides (*10*). We found that addition of 50 m*M* 2-deoxyglucose to inhibit glucose-dependent pyridine nucleotide reduction decreased the fluorescence intensity of neutrophils incubated for 30 minutes at 37°C. In experiments similar to that shown in Fig. 1, we found that the fluorescence intensity of activated neutrophils was about 20 to 60 percent of unactivated cells. These measurements do not alone establish the magnitude of pyridine nucleotide changes in individual cells during stimulation. Quantitative analysis would require several additional factors to be taken into account including the background fluorescence of substances other than NAD(P)H and possible changes in fluorescence quantum yield during metabolic stimulation.

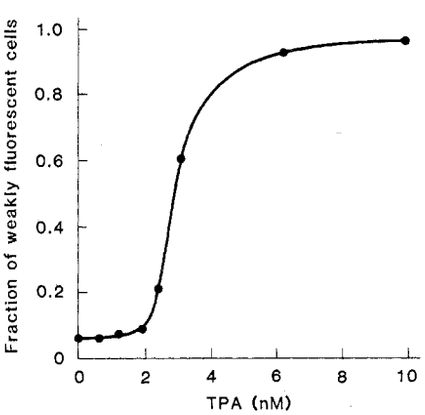

Fig. 2. Fraction of weakly fluorescent (activated) cells in the total neutrophil population as a function of TPA concentration. The plot was constructed by determining the area under the two neutrophil population peaks in an experiment similar to that shown in Fig. 1.

Phorbol diesters such as TPA are responsible for the tumor-promoting activity in croton oil (*14*) and stimulate a wide variety of cellular responses including the degranulation of mast cells at concentrations similar to those necessary for stimulation of the neutrophil respiratory burst (*15*). These responses appear to be mediated through high-affinity membrane receptors for phorbol diesters since the concentrations necessary for biological activity are similar to the receptor dissociation constants (*15, 16*). The present results demonstrate that individual neutrophils are triggered by TPA in an all-or-none, single quantum step rather than in a continuous fashion. This mode of metabolic stimulation rules out a stoichiometric triggering mechanism, such as alteration of enzymatic activity through direct binding of the agonist to the membrane-bound NAD(P)H oxidase that catalyzes O_2^- production. Our results suggest instead that the stimulation process involves a single event which is sufficient for maximum or near maximum metabolic stimulation. Such a single event might be the fusion of two intracellular membrane compartments. There is evidence that the membrane-bound NAD(P)H oxidase is a multicomponent system similar to the mitochondrial electron transport chain (*17*). Furthermore, it has been proposed that fusion of two such membrane compartments, each bearing only a fraction of the essential components, is responsible for stimulation of the respiratory burst (*18*). Alternatively, a highly cooperative process such as the opening of membrane ion channels may give rise to the observed all-or-none response. Electrical depolarization and loss of membrane-bound Ca^{2+} have been observed during stimulation of the neutrophil respiratory burst (*19, 20*).

Under physiological conditions, neutrophils bind foreign target organisms chiefly through interaction of plasma membrane receptors with target-bound antibody molecules (Fc fragment of IgG), or alternatively, with target-bound activated components of the complement cascade. It is the target-bound IgG that triggers phagocytosis, lysosomal degranulation, and stimulation of the respiratory burst (*21*). A logical but unanswered question is whether these physiological processes are triggered by target-

bound IgG in a quantum fashion similar to that induced by TPA.

The use of flow cytometry to analyze intracellular metabolic processes promises to yield additional comparative information about the mechanism of cell stimulation by a wide variety of agonists. The technique requires only that the agonist produce a discernible change in either the intrinsic cell fluorescence properties or in fluorogenic substrates added to the cell *(22, 23)*. Changes in intracellular *p*H during metabolic events can also be monitored *(24)*. This type of analysis should be useful for investigation of the mechanisms of immune cell stimulation and cell-cell communication and for studying the mechanism of hormone action at the single cell level.

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Corticotropin-Releasing Activity of α-Melanotropin

Abstract. *Synthetic α-melanotropin stimulated the release of immunoreactive adrenocorticotropin from primary cultures of rat anterior pituitary cells. The effect of the α-melanotropin was dose-dependent. Cells incubated with synthetic arginine-vasopressin and α-melanotropin simultaneously produced an amount of adrenocorticotropin that was greater than the sum of the amount that the cells produced in response to each peptide added separately. Other peptides structurally similar to α-melanotropin, such as, β-, γ_1-, γ_2-, and γ_3-melanotropin, were also tested for adrenocorticotropin-releasing activity. Only the γ_3-melanotropin demonstrated a statistically significant effect. A vasopressin preparation (Pitressin, Parke-Davis) purified from posterior pituitaries and previously shown to contain some α-melanotropin was much more potent in releasing adrenocorticotropin than the synthetic vasopressin.*

Corticotropin-releasing factor was the first hypothalamic hypophysiotropic hormone to be demonstrated *(1)*, but still remains to be identified. By using primary cultures of rat anterior pituitary cells and measuring the corticotropin (ACTH) released into the incubation medium by radioimmunoassay, we observed that Pitressin (Parke-Davis), a purified vasopressin from posterior pituitaries, was much more potent than the synthetic arginine-vasopressin (AVP) in stimulating ACTH release. It was previously reported that Pitressin preparations contain α-melanotropin (α-MSH) *(2)*. Here we report the effect of synthetic α-MSH on the release of ACTH by rat pituitary cells in vitro and the interaction of α-MSH with synthetic AVP.

Our method of primary culture of rat pituitary cells is a modification of the procedure of Vale *et al.* *(3)*. The modifications can be summarized as follows. The cells are digested by a mixture of Dispase (Boehringer Mannheim, Canada), collagenase, deoxyribonuclease, and chicken serum as described previously *(4)*. The cells are then attached to polylysine-coated plastic multiwell dishes in Hepes buffered Dulbecco's modified Eagle's medium supplemented with L-glutamine and 10 percent fetal bovine serum and containing gentamycin *(5)*. The cells are then incubated for 2 days at 37°C in an atmosphere of 5 percent CO_2 and 95 percent air. The cells are washed and incubated for 2 hours before they are used. Our cell preparations contain mostly cells of the anterior lobe of the pituitary. We have found previously that the remaining cells of the intermediate lobe are more fragile than those of the anterior lobe and cannot withstand the enzymatic treatment used here to separate the cells.

For the radioimmunoassay we used an antiserum that is specific to the NH_2 terminus of ACTH and shows only weak cross-reactivity with α-MSH (0.16 percent at 50 percent displacement of ^{125}I-labeled ACTH). No cross-reactions were observed with β-, γ_1-, γ_2-, or γ_3-MSH, or with AVP. We did not identify the peptides in the incubation medium but con-

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