ABEL® CELL ACTIVATION RESPIRATORY BURST ASSAYS

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Cell Activation Test Kits with Pholasin® are easy-to-use, rapid, ultra-sensitive and can even be used with capillary blood).They are chemiluminescent tests that measure real-time production of free radicals and degranulation. They can be used to monitor changes in the respiratory burst during different exercise regimes.

They can be used to:

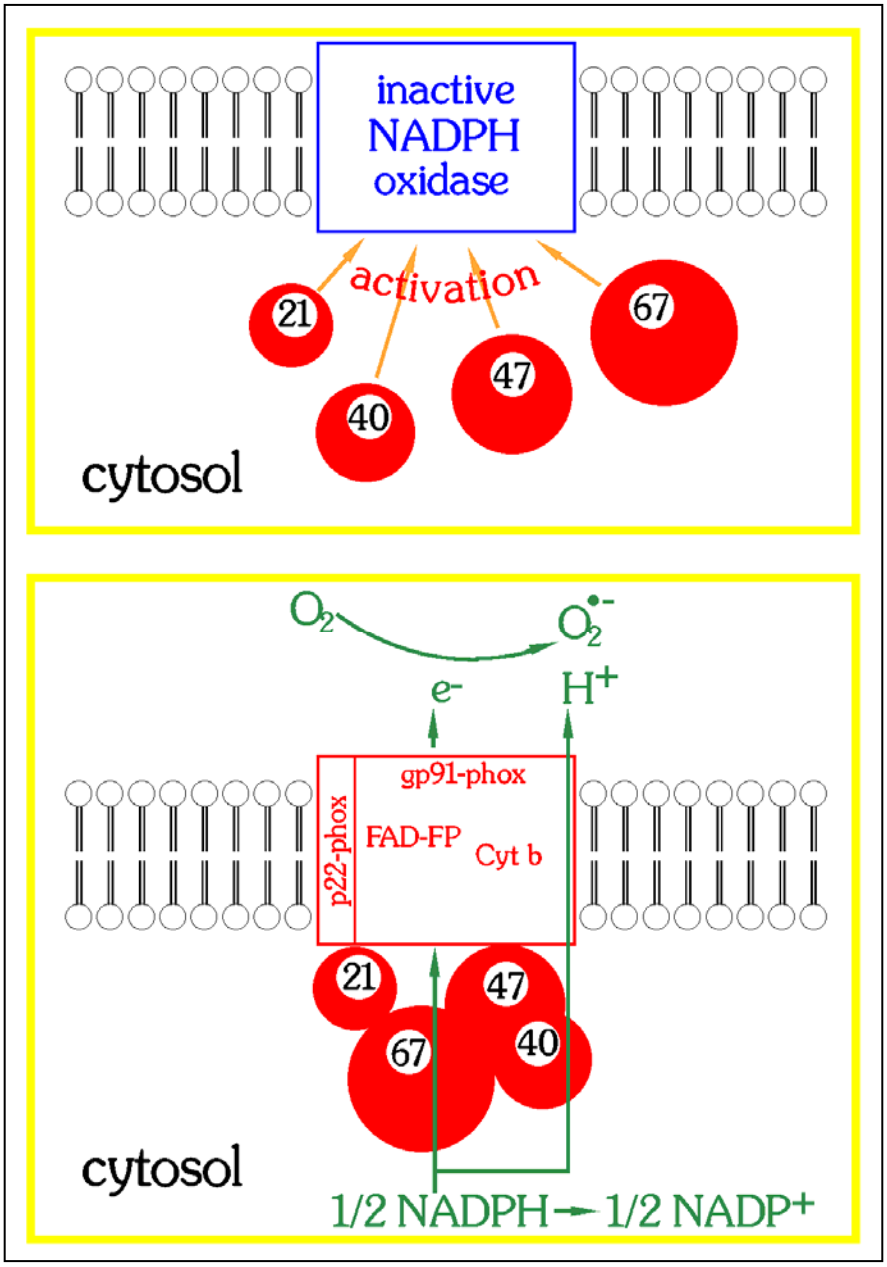
- measure the respiratory burst in leucocytes
- study abnormalities in NADPH oxidase such as occur in different types of chronic granulomatous disease (CGD) and various polymorphisms
- distinguish between receptor activation and intracellular activation of NADPH oxidase
- monitor changes in the activation of the NADPH oxidase in response to complement activation, infection, inflammation and medical intervention, thus having applications in:
    - biocompatibility monitoring
    - drug evaluation and testing
    - QC of vaccines
    - surgery
    - disease management
    - assessing neutrophil activity following chemotherapy, transplantation etc.
- monitor degranulation of myeloperoxidase
- ultra-sensitive detection of free radicals and oxidants produced by a range of cell types including brain cells.

The NADPH oxidase when activated forms a complex of proteins that assembles at the inner surface of the plasma membrane. There a constant stream of superoxide is released to the outside of the cells via a continual transfer of electrons (from NADPH) through the membrane to oxygen. The term respiratory burst was used to describe this event because of the very large amounts of oxygen (10-20 times above normal) that are consumed during the oxidation of glucose to NADPH, which ironically is not via mitochondrial respiration. The membrane component of the NADPH oxidase also occurs on secondary granule membranes which fuse with other granules as well as the plasma membrane during deganulation. Degranulation frequently follows the activation of the oxidase depending upon the nature of the stimulus. And because the overwhelming amount of free radicals, oxidants and enzymes are released to the outside of the cells, methods that only measure intracellular hydrogen peroxide from the dismutation of superoxide quantify only a small part of the respiratory burst.

SOME DETAILS OF THE FUNDAMENTAL MECHANISMS INVOLVED

Activation of the NADPH Oxidase

figure 1



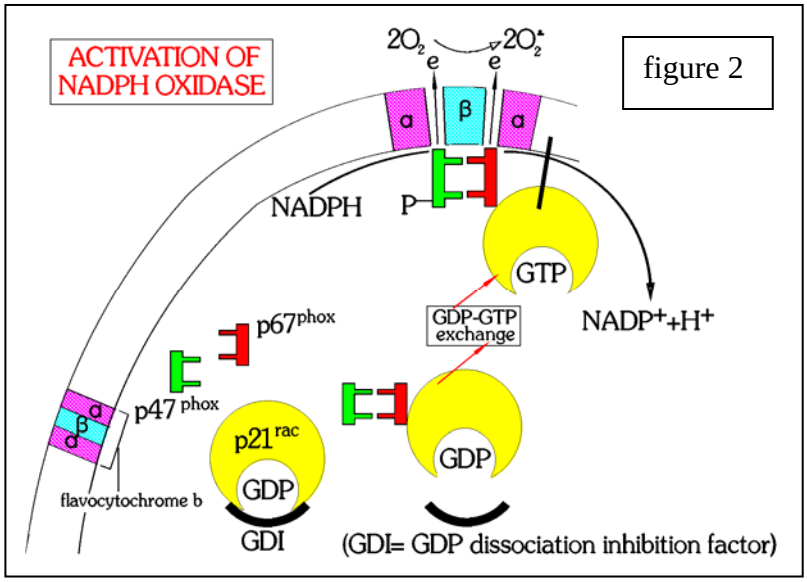
The NADPH oxidase (figure 1) consists of a membrane-bound flavoprotein and cytochrome B together with four other proteins in the cytosol[1] . The NADPH oxidase is inactive until stimulated. On activation all the components come together at the cell membrane with the result that the transfer of an electron through the membrane and the direct reduction of oxygen to superoxide is effected.

The fully active NADPH oxidase is a membrane-bound complex consisting of short electron transport chain[i] (flavocytochrome b_{245}) and four cytosolic proteins: $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and $p21^{rac}$; where the superscript "phox" represents phagocyte oxidase. The induction of electron transport is most likely related to translocation of cytosolic proteins to the membrane as that event coincides with oxidase activity[4]. The

$p21^{rac}$ is a GTP-binding protein-that occurs in the cytosol as a complex with Rho-GDI, a GDP-dissociation inhibition factor. The function of the oxidase is to accept an electron from NADPH and transfer it to the inside of the cell membrane whence it is passed to oxygen on the outside wall. This one electron reduction of oxygen leads to the direct production of superoxide at the surface of the cell. The dissociation of Rho-GDI from $p21^{rac}$ is the most probable switch that initiates the activation of the oxidase[ii]. Upon activation, $p21^{rac}$ dissociates from Rho-GDI and translocates to the cell membrane[iii] where an activation complex involving $p21^{rac}$, $p47^{phox}$ and $p67^{phox}$ docks with flavocytochrome b_{245} inducing a conformational change conducive to electron transport. NADPH, the substrate and the source of the electrons for reduction of oxygen, is produced from the oxidation of glucose via the hexose monophosphate shunt. This so-called respiratory burst of phagocytes has been recognised for some time as being distinct from mitochondrial respiration[iv].

In the plasma membrane and in the membrane of secondary granules of neutrophils is the membrane bound component of the NADPH oxidase. This component is a flavocytochrome that is in fact a heterodimer. The smaller subunit is approximately 22kDa and is referred to as $p22^{phox}$, while the larger ß subunit is approximately 91 kDa ($gp91\text{-}^{phox}$) but is heavily glycosylated. In unstimulated neutrophils, the oxidase is inactive and no other polypeptide is associated with the membrane. On activation, three further polypeptides are recruited from the cytosol to the membrane. These are: 67 kDa ($p67^{phox}$), 47 kDa ($p47^{phox}$) and 40kDa ($p40\text{-}^{phox}$). It is speculated that these cytosolic components aid the correct conformation of the flavocytochrome and facilitate substrate binding and electron flow through the complex.

The redox activity of the complex (figure 2) is associated with the $gp91\text{-}^{phox}$ subunit. Electrons derived from intracellular NADPH are passed through the membrane complex to extracellular oxygen which is reduced to superoxide.



Activation of the complex also involves two G proteins (see figure 2). These act as molecular switches, alternating between binding GDP in the inactive state and GDP in the active state. The G protein Rap1A copurifies with the flavocytochrome while the

G protein p21rac2 translocates to the membrane like the other cytosolic polypeptides. In the cytosol p21rac2 normally is associated with the guanine nucleotide exchange inhibitor rhoGDI. On activation GDS (a GDP dissociated stimulator protein) catalyses the exchange of GDP/GTP.

Phagocytosis

figure 3

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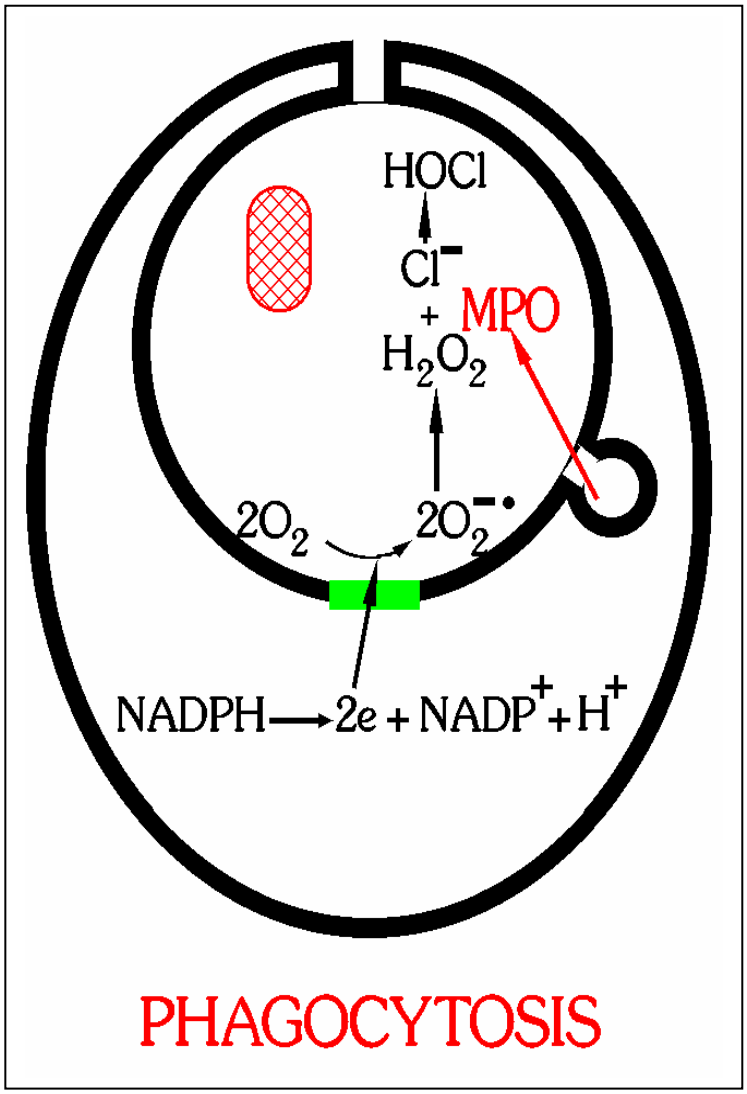


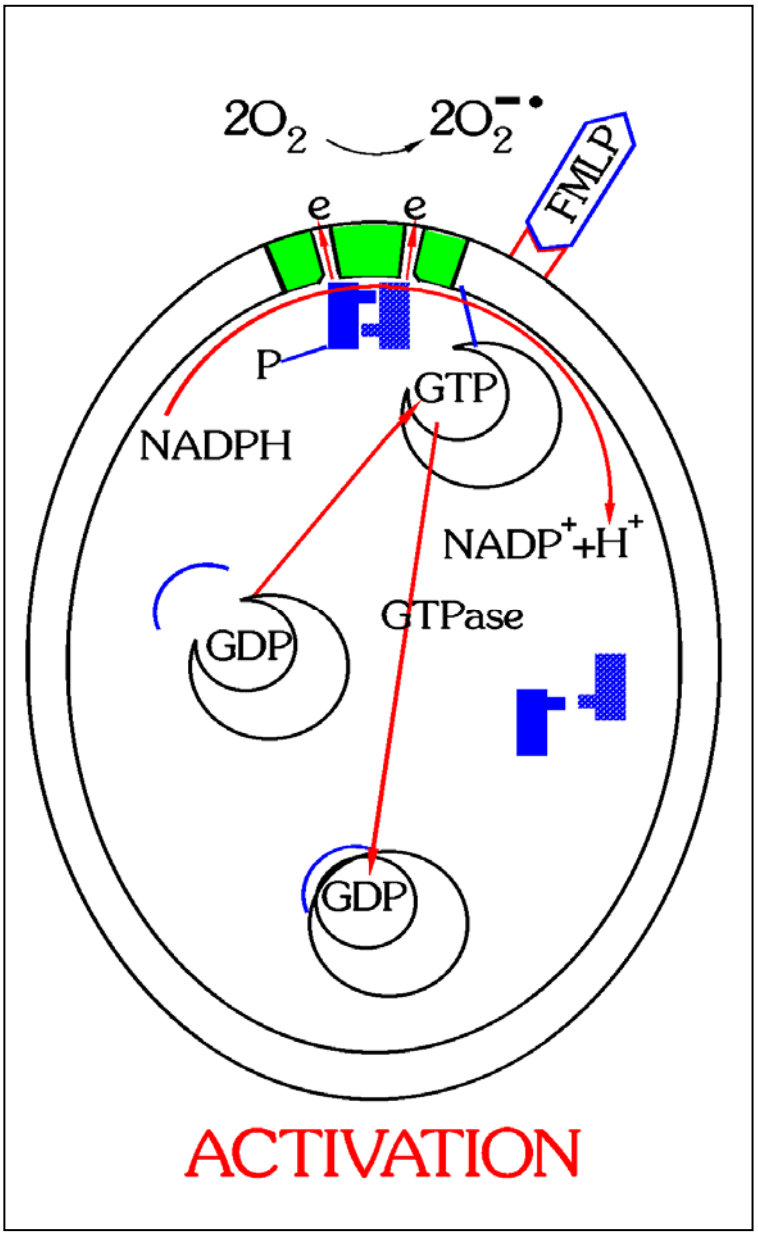
Phagocytic cells, such as neutrophils, eosinophils, monocytes/macrophages recognise the presence of a foreign body such as a bacterium or perhaps a particle derived from a medical device. The response of the phagocytic cells is initially via a receptor which leads to the invagination of the cell membrane to form a vacuole (phagosome), in which the foreign particle is trapped (see figure 3). The NADPH oxidase on the plasma membrane is activated to produce superoxide which is released into the vacuole. Accompanying the formation of the phagosome is degranulation, the fusing of the membrane of a granule within the cell with another membrane, usually the plasma membrane. During phagocytosis the primary granules fuse with the membrane of the vacuole and then release into the vacuole the enzyme

myeloperoxidase, MPO. MPO produces hypochlorous acid from hydrogen peroxide (H_2O_2)[1] and chlorine.

In phagocytosis, during which foreign bodies such bacteria, spent red blood cells or dead tissue are trapped and usually destroyed (figure 3) oxygen consumption can increase 10-20 fold. The consumption of oxygen in the "respiratory burst" leads to the production of NADPH from glucose via the hexose monophosphate shunt. This oxygen consumption **does not** occur in the mitochondria and is not affected by poisons such as cyanide.

Receptor-Mediated Activation

figure 4



Phagocytosis is initiated by receptors at the cell surface which detect chemotactic substances emanating from the foreign body or dead and dying cells. The complete

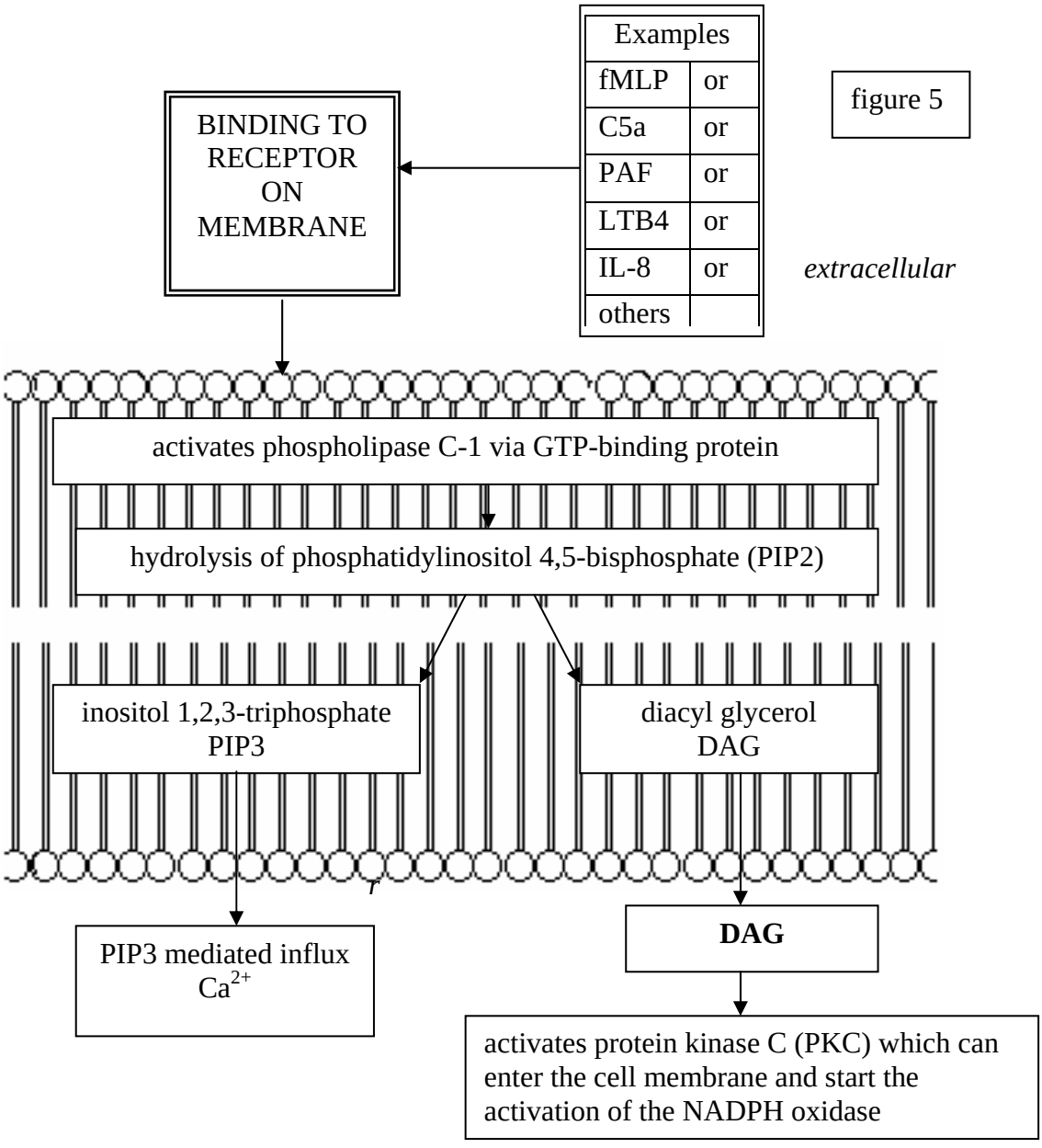
[1] hydrogen peroxide is formed from the dismutation of superoxide usually catalysed by superoxide dismutase.

process of phagocytosis can take a number of hours. However, certain soluble substances can activate the NADPH oxidase at the cell membrane without formation of a phagocytic vacuole (see figure 4). These include:

- activated complement fragment C5a,
- the chemotactic peptide derived from the cell wall of a bacterium formyl-leucyl-methionyl-phenylalanine (fMLP),
- bioactive lipids such as platelet activating factor (PAF) and leukotriene B4 (LTB4),
- and several neutrophil activation proteins including IL-8 and granulocyte macrophage colony stimulating factor (GM-CSF).

The time from presentation of stimulus to response (lag time) is usually 2-5 seconds with the maximum rate of production of superoxide attained after about 60 seconds.

The activation of the oxidase by receptor-mediated stimuli



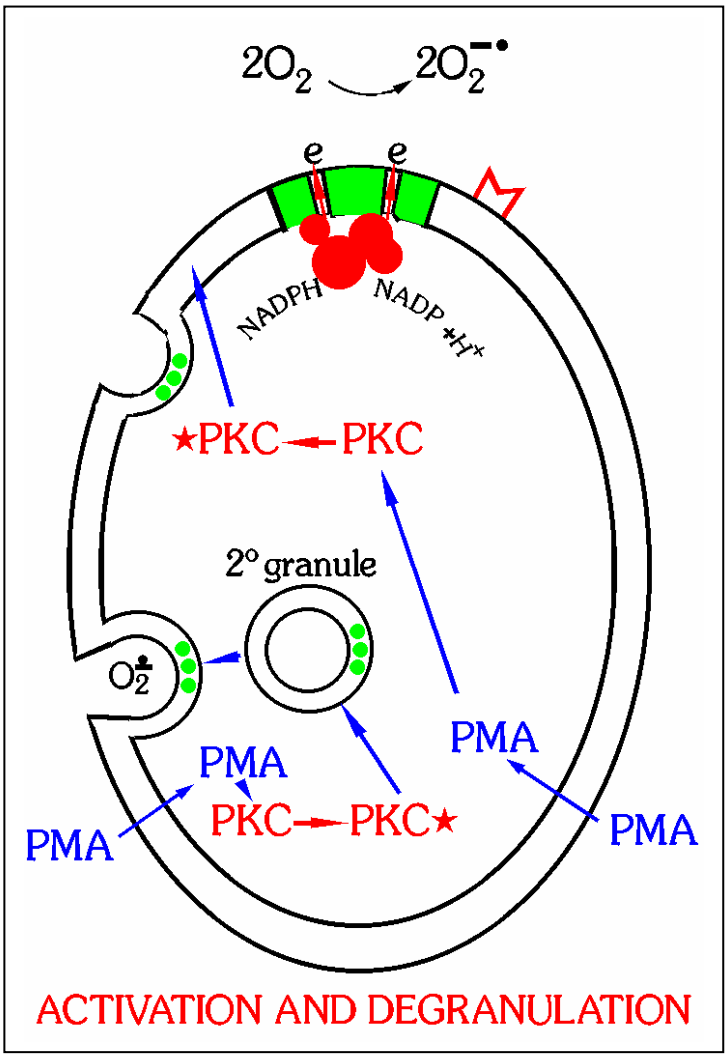
The stimulant must remain in contact with the receptor for activation to continue; removing the stimulant deactivates the oxidase. Phosphorylation and dephosphorylation also appear to be key in the activation and deactivation of the oxidase[v]. Tyrosine kinase inhibitors, such as erbstatin, inhibit receptor mediated activation such as that by fMLP. They do not inhibit activation by phorbol-12-myristate-13-acetate (PMA) which activates protein kinase C directly (see later for details).

Stimulation of the NADPH oxidase via receptors is calcium dependent and takes 2-5 seconds. The maximum rate of production of superoxide is attained after about 60 seconds and usually lasts for less than 5 minutes. Inhibitors of PKC have no effect on receptor activation[vi] even though activation of PKC is mediated by diacyl glycerol (see figure 6). The fungal metabolite 17-hydroxywortmannin, an inhibitor of P13 kinase, inhibits the receptor mediated activation[vii].but not the receptor-independent activation.

Receptor independent activation of the NADPH oxidase is not calcium dependent and occurs by direct activation of PKC (see figures 6 and 7). Long-chain unsaturated fatty acids and other PKC agonists, such as PMA, migrate into the cell, acting as a diacyl glycerol (DAG) analogue and activating PKC. Activated PKC then migrates into the cell membrane and presumably the membrane of secondary granules, which also contain the membrane components of the NADPH oxidase system, the flavocytochrome b (see figure 2).

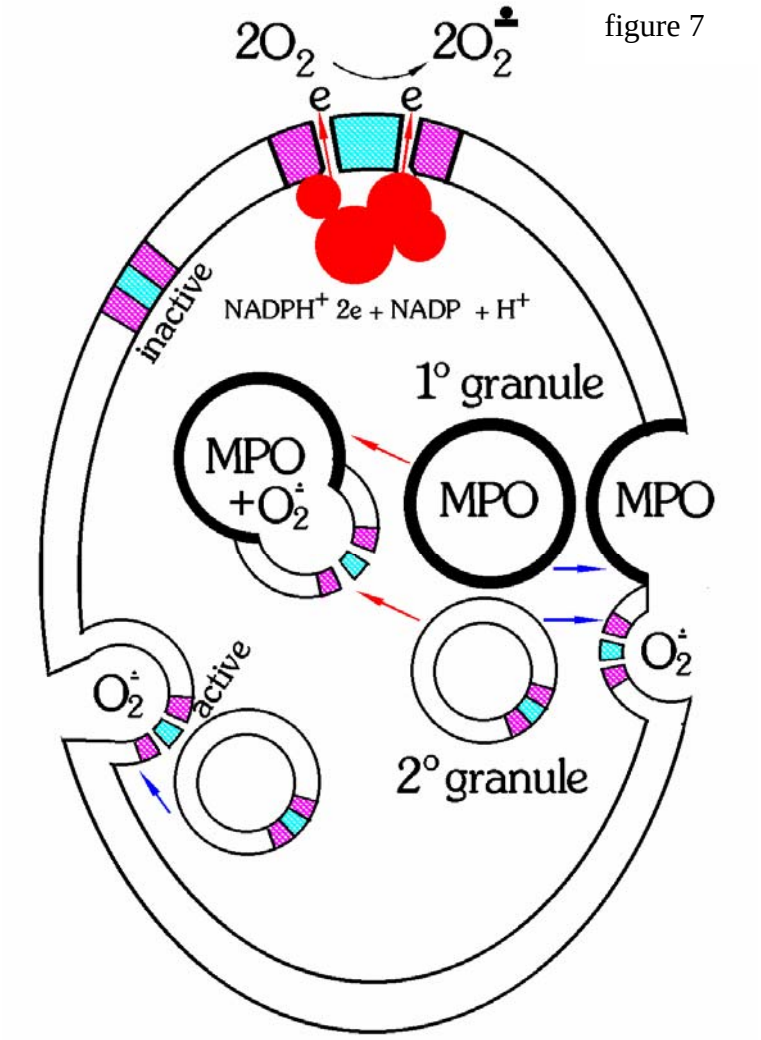
Receptor-Independent Activation

figure 6



The activation of the NADPH oxidase is often linked to degranulation, a phenomenon in which membrane bound intracellular granules containing a range of enzymes fuse with the plasma membrane of the cell and release their enzyme contents to the surrounding tissue. Degranulation of primary as well as secondary granules can be detected by Pholasin®[viii,ix] which reacts with myeloperoxidase (MPO) in a reaction which is very

figure 7



much enhanced by hydrogen peroxide. However, when degranulation occurs the MPO released from the primary granules can bind to adhesion molecules (CD11b/CD18) which are expressed on the membrane of the secondary granule, which also contains the NADPH oxidase. MPO is therefore bound at the membrane where hydrogen peroxide (derived from the dismutation of superoxide) can participate in luminescent reactions with Pholasin®.

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