# A NEW WAY FOR INDUCING A RESPIRATORY BURST IN GUINEA PIG NEUTROPHILS

## Change in the $Na^+$,$K^+$ concentration of the medium

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## 1. Introduction

During phagocytosis and when stimulated with appropriate membrane-perturbing agents, leucocytes undergo a dramatic stimulation of $O_2$ consumption and of $O_2^-$ and $H_2O_2$ production [1–4]. The key event of this respiratory burst appears to be the activation of a plasma membrane-bound NAD(P)H oxidase that is dormant in the resting state [3–10].

During the last decade information has accumulated on a series of molecular events that take place at the level of the plasma membrane following the interaction with the stimulating agents. These include, among others, changes in transmembrane potential [10–16], modification of $Ca^{2+}$ fluxes and availability [17,19], changes in $Na^+$,$K^+$ fluxes (18,19), increase in protein phosphorylation [20]. However, the precise nature and the key sequence of events triggering the respiratory response remains to be clarified.

We have observed a new phenomenon that might be relevant for the understanding of the molecular events at the level of the plasma membrane responsible for the activation of the NADPH oxidase. It involves an induction of a respiratory burst observed in guinea pig granulocytes by appropriate changes in the ionic composition of the suspending medium.

## 2. Materials and methods

### 2.1. *Collection of cells*

Guinea pig polymorphonuclear leukocytes (PMN) were prepared from casein-induced peritoneal exudate as in [6]. Cells, freed from erythrocytes by hypotonic lysis, were washed 3-fold and suspended ($1–4 \times 10^8$) in appropriately buffered solution. The temperature of all these manipulations was carefully maintained at 0–4°C. The viability of the cell was determined by trypan blue-exclusion test.

### 2.2. *Suspending buffered solutions*

The solutions employed, buffered at pH 7.4 and containing 5 mM glucose, were the following:

(1) Krebs-Ringer phosphate (KRP): [$Na^+$] 152 mM; [$K^+$] 5 mM; [$Cl^-$] 130 mM; [$PO_4^{3-}$] 16.6 mM; [$Mg^{2+}$] 1.22 mM.

(2) High $K^+$,$Na^+$-free KRP(KRP-K): [$K^+$] 157 mM; [$Cl^-$] 130 mM; [$PO_4^{3-}$] 16.6 mM; [$Mg^{2+}$] 1.22 mM.

(3) High $Na^+$,$K^+$-free KRP (KRP-Na): [$Na^+$] 157 mM; [$Cl^-$] 130 mM; [$PO_4^{3-}$] 16.6 mM; [$Mg^{2+}$] 1.22 mM.

(4) KRP-containing different concentrations of $Na^+$ and $K^+$.

(5) $Na^+$-free KRP containing different concentrations of $K^+$ and choline (KRP-ch).

### 2.3. *Metabolic studies*

The $O_2$ consumption of the cell was measured with a Clark oxygen electrode [6], $O_2^-$ production as SOD-sensitive cytochrome *c* reduction [21], $H_2O_2$ as homovanillic acid oxidation [22], NADPH oxidase as $O_2$ consumption or $O_2^-$ production [23].

The procedure employed for investigating the effect of the suspending solutions on the respiratory metabolism of PMN was: 1 ml of the solution selected for the experiments (i.e., KRP-K) was pre-warmed for 5 min at 37°C in the oxygen electrode chamber or in the cuvette for the measurement of $O_2^-$ and of $H_2O_2$,

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and after that time the recording was initiated. After a couple of minutes, 10–20 μl PMN, suspended in the same medium and till then kept at 0–4°C, were added and the recording of $O_2$ consumption or of $O_2^-$ or $H_2O_2$ production was continued for the time required.

2.4. *Membrane potential*

The procedure used was mostly that devised in [24] as applied in [13,25,26]. The probe used was 3,3′-di-propyl-thiocarbocyanine (di-S-$C_3$(5)) at 2 μM in 2 ml of the buffered solutions indicated above. The changes of fluorescence intensity of di-S-$C_3$(5) after the addition of 4 × $10^6$ PMN, suspended in the same buffered solution, were recorded at 37°C with a spectrophotometer (Ciampolini, Italy) with excitation and emission wavelength of 622 nm and of 660 nm, respectively. This measurement was performed in presence of 500 IU of catalase and of 25 μg superoxide dismutase (SOD) in order to avoid the quenching of the dye by the intermediate of oxygen reduction [26].

2.5. *Materials*

Phorbol myristate acetate (PMA), NADPH, cytochrome *c* type VI, catalase and superoxide dismutase were purchased from Sigma (USA); choline from Merck (FRG); the fluorescent dye di-S-$C_3$(5) was a generous gift from Dr Alan Waggoner and trifluorpyrazine from Smith, Kline and French Labs Ltd (Herts, England).

## 3. Results

Fig.1 reports the effect of buffered solutions of different ionic composition on $O_2$ consumption and on $O_2^-$ production by guinea pig PMN. It can be seen that when PMN, pre-suspended in cold KRP-K, are added to the same medium at 37°C, they undergo a very marked respiratory burst. This phenomenon does not occur when PMN are pre-suspended in KRP or in KRP-Na and added to the same media.

The stimulation of the respiratory activity induced

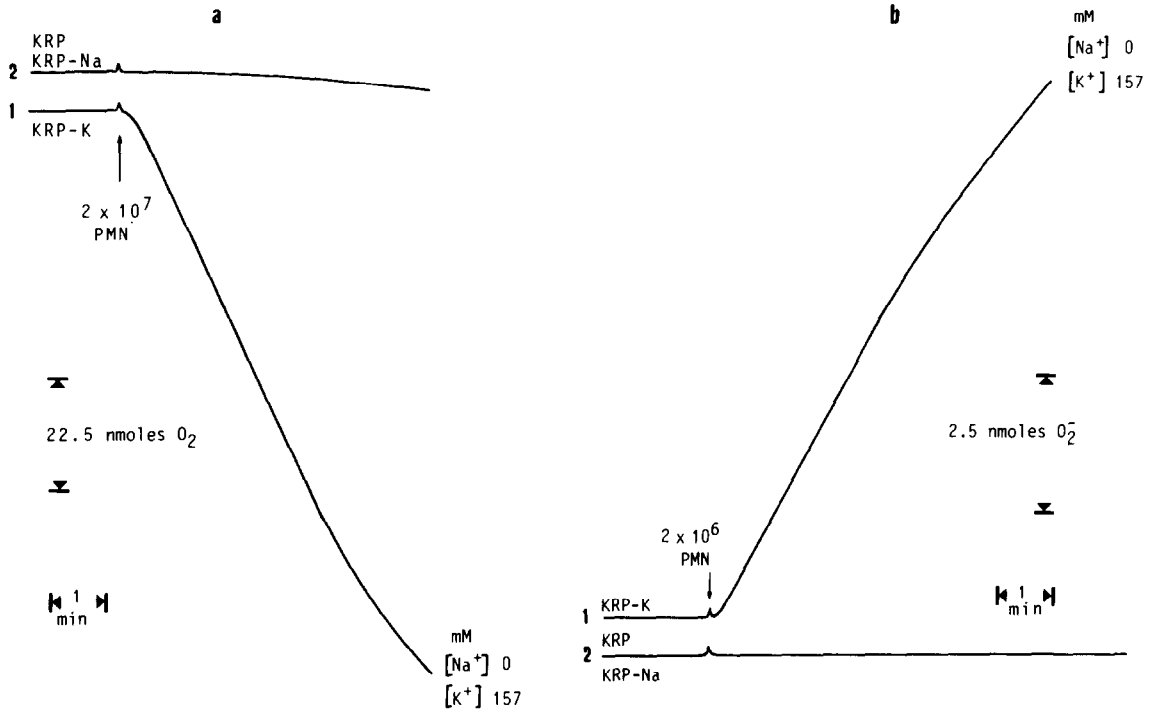


Fig.1. Recording of polarographic assay of $O_2$ consumption (a) and of spectrophotometric measurement of $O_2^-$ production (b) by PMN added to different buffered solutions: (1) experiments in KRP-K; (2) experiments in KRP and in KRP-Na. For details see section 2.

by high-$K^+$,$Na^+$-free medium (ion-dependent bursts) has the following characteristics:

(i) It is, as those caused by phagocytosis or by membrane perturbing agents, insensitive to cyanide, rotenone, antimycin A and azide;

(ii) The time lag, that is the time of activation, is very short. In fact the stimulation of $O_2$ consumption, of $O_2^-$ (fig.1) and of $H_2O_2$ production is measurable almost immediately after the addition of PMN. The maximal rates remain linear for 2–7 min depending on the batch of cell. When the burst is ceased the cells remain viable as shown by the exclusion of trypan blue and by the response to PMA.

(iii) It is associated with the stimulation of the membrane bound NADPH oxidase activity (not shown).

In order to understand the mechanism by which this particular ionic composition of the medium triggers the activation of the NADPH oxidase and hence of the respiratory metabolism in guinea-pig PMN we have searched for a possible relationship with a modification of membrane potential. It is widely known that in the cells, including guinea-pig granulocytes, the membrane potential depends largely on the $K^+$-gradient and that a high $[K^+]$ in the suspending medium causes a depolarisation [12–14,16,25]. In PMN a change of membrane potential correlates and preceeds the onset of the respiratory burst and of other functional responses [10–16].

Fig.2 shows the results of the modifications of membrane potential of PMN suspended in different buffered solutions monitored as changes in fluorescence intensity of di-S-$C_3$(5) after the addition of the cells. The method is based on the principle that the distribution of the fluorescent dye, a lipophilic ion, between the external medium and the cell is a function of the membrane potential, the more negative inside (i.e., more polarized) the more dye enters the cell. Since the fluorescence of di-S-$C_3$(5) inside the cell is quenched, the fluorescence that is monitored is that of the dye remaining outside the cell [26]. Therefore the more polarized is the cell, the less fluorescence is monitored. Fig.2 shows that the fluorescence intensity of the suspension of PMN in KRP-K at the end of equilibration (baseline fluorescence) reaches a value higher than that of PMN suspended in KRP and KRP-Na. This finding indicates that the suspension of PMN in KRP-K induces a depolarization as previously shown [12–14,16].

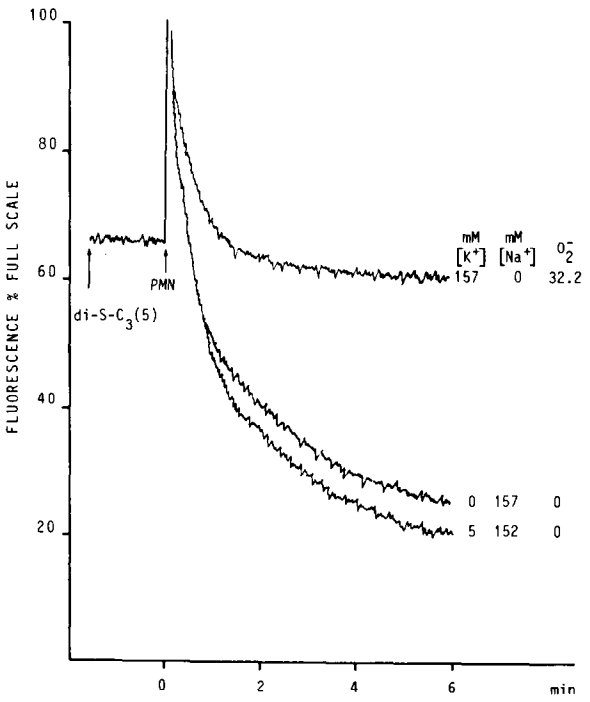


Fig.2. Membrane potential, measured as baseline fluorescence of di-S-$C_3$(5), of PMN in KRP, KRP-K, KRP-Na. For details see section 2. A lower fluorescence indicates a higher polarization. $O_2^-$ indicates nmol superoxide produced . $min^{-1}$ . 2 × $10^7$ $PMN^{-1}$ of the same batch in the same conditions of incubations; see section 2.

Since in this condition PMN have a respiratory burst it seems, at first sight, that a direct correlation exists between depolarization and the induction of the burst. This conclusion seems to be in agreement with the emerging view that the membrane depolarization of leucocytes is involved in the mechanism triggering the activation of the respiratory burst [12–16]. However, it cannot be excluded that in our experimental conditions the modification of the ionic composition of the interior of the cell and of particular sites of the membrane rather than the change of the membrane potential per se is the final signal for the activation of the oxidase.

We have tried to answer this question by comparing the respiratory activity and the state of membrane potential in PMN suspended in high-$K^+$,$Na^+$-free buffered solution (KRP-K) and in buffered solutions where the concentration of $K^+$ was progressively diminished and substituted with increasing $[Na^+]$.

Fig.3 shows that by substituting $Na^+$ for $K^+$ the

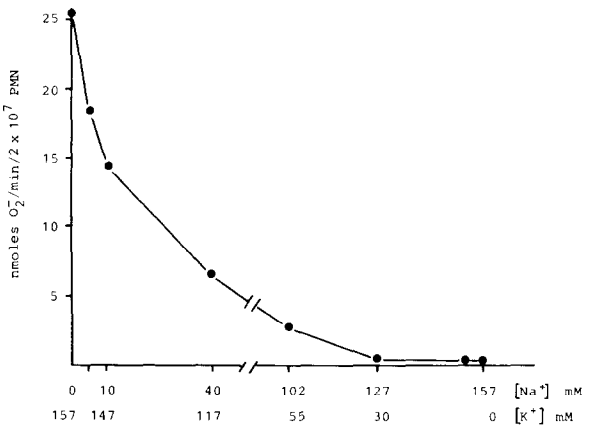


Fig.3. Respiratory burst ($O_2^-$ production . $min^{-1}$ . 2 × $10^7$ $cells^{-1}$) of PMN as function of different [$K^+$] and [$Na^+$]. For details see section 2.

respiratory burst progressively decreases. The decrease is already detectable at 5 mM $Na^+$ and at >100 mM $Na^+$ the respiratory burst is practically absent. By comparing the intensity of the burst with the state of depolarization of PMN in these different buffered solutions (fig.4), it can be seen that when suspended in KRP-K, in KRP with 152 mM $K^+$ and 5 mM $Na^+$, in KRP with 147 mM $K^+$ and 10 mM $Na^+$, in KRP with 117 mM $K^+$ and 40 mM $Na^+$, they present the same degree of depolarization and very different respiratory activity. This finding that the same states of depolarization do not agree with the same intensity of the respiratory burst suggests that other modifications rather than (or in addition to) the membrane depolarization are involved in the mechanism triggering this particular type of respiratory activation. By examining the effect of different [$Na^+$] on the burst (fig.3) the hypothesis can be advanced that the main cause is the absence of $Na^+$.

In an attempt to verify the validity of this hypothesis we have suspended the leukocytes in $Na^+$-free KRP-K, where $K^+$ was progressively substituted with choline (KRP-Ch). The results reported in fig.5 show a non-correlation between the occurrence of the burst and the depolarization. In fact in KRP-Ch containing 30 mM $K^+$, 127 mM choline and 5 mM $K^+$, 147 mM choline the cells present a respiratory burst without depolarization. The data of fig.5 show also that the length of the respiratory burst (that is the length of the linear rate of $O_2^-$ production) of non-depolarized PMN is very short. This finding indicates the existence of a direct correlation between the [$K^+$] and the degree of depolarization on one hand and the mecha-

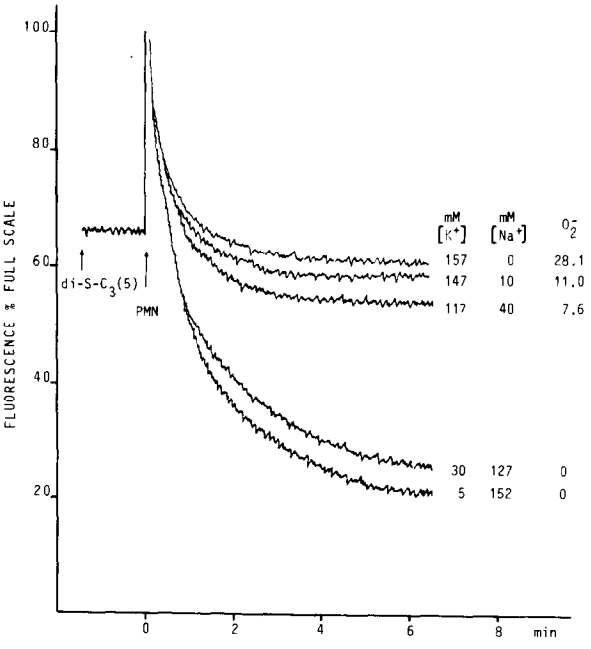


Fig.4. Respiratory burst ($O_2^-$ production . $min^{-1}$ . 2 × $10^7$ $cells^{-1}$) and membrane potential of PMN suspended in buffered solutions containing different [$K^+$] and [$Na^+$]. For details see section 2.

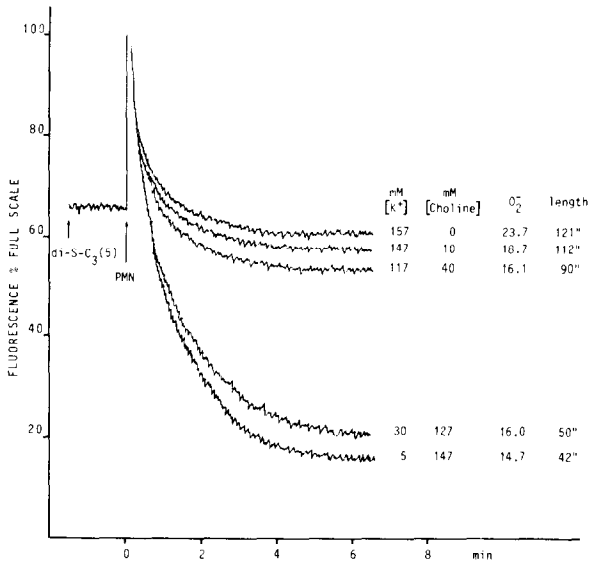


Fig.5. Respiratory burst (as $O_2^-$ production . $min^{-1}$ . 2 × $10^7$ $cells^{-1}$) and membrane potential of PMN suspended in $Na^+$-free KRP-K containing different [$K^+$] and [choline]. For details see section 2.

nism controlling the length of the activation state on the other.

On the basis of the results presented so far, it seems appropriate to advance the hypothesis that the absence of $Na^+$ (or the $[Na^+]$) is involved in the mechanisms that trigger the activation of this type of burst, while the $[K^+]$ and the consequent state of depolarization are relevant for the control of the length of the activation state and, it is likely, of the degree of activation.

Further investigations should clarify the sequence of molecular events at the level of the plasma membrane induced by the change of ion compositions and finally responsible of the transformation of the oxidase (or of the oxidase system) from inactive to active state. We have found that $\mu$M levels of trifluoperazine, added both at beginning of the burst and when the rate of the respiration has reached the maximum value, inhibits this ion-dependent burst without changing the state of depolarization (not shown). This finding may be in agreement with a role of $Ca^{2+}$ and of some calmodulin-like proteins on the activation of the oxidase as in [27].

The last point that should be discussed is the relationship between this ion-dependent burst of leukocytes and those induced by phagocytosis and by a variety of membrane-perturbing agents. With our present knowledge, we cannot say whether or not the hypothesis advanced before regarding the relations between the changes of membrane potential and of ionic compositions on one hand and the mechanism controlling the triggering and the state of activation on the other, can be applied also to the other types of burst. Future investigations should clarify whether the changes of ionic composition, that cause the burst shown here, mimick those induced (at least in a particular site on the plasma membrane) by all the other stimulants or if they represent one of the possible changes or conditions that cause a respiratory response in leukocytes.

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**References**

[1] Karnovsky, M. L. (1962) Physiol. Rev. 42, 143–168.
[2] Klebanoff, S. J. and Clark, R. A. (1978) The Neutrophil: Function and Clinical Disorders, Elsevier/North-Holland, Amsterdam, New York.
[3] Rossi, F., Romeo, D. and Patriarca, P. (1972) J. Reticuloendothel. Soc. 12, 127–149.
[4] Babior, B. M. (1978) New Engl. J. Med. 298, 659–668.
[5] Rossi, F. and Zatti, M. (1964) Br. J. Exp. Pathol. 45, 548–558.
[6] Patriarca, P., Cramer, R., Moncalvo, S., Rossi, F. and Romeo, D. (1971) Arch. Biochem. Biophys. 145, 255–262.
[7] Cross, A. R., Jones, O. T. G., Harper, A. M. and Segal, A. W. (1981) Biochem. J. 194, 599–606.
[8] Kakinuma, K. and Kaneda, M. (1980) FEBS Lett. 111, 90–94.
[9] Cohen, H. J., Chovaniec, M. E. and Davies, W. A. (1980) Blood 55, 355–363.
[10] Gallin, E. K. and Gallin, J. I. (1977) J. Cell Biol. 75, 277–289.
[11] Korchak, H. M. and Weissmann, G. (1978) Proc. Natl. Acad. Sci. USA 75, 3818–3822.
[12] Seligman, B. E. and Gallin, J. I. (1980) J. Clin. Invest. 66, 493–503.
[13] Within, J. C., Chapman, C. E., Simons, E. R., Chovaniec, M. E. and Cohen, H. J. (1980) J. Biol. Chem. 255, 1874–1878.
[14] Jones, G. S., Wilson, M. E., Vandyke, K. and Castranova, V. (1980) Fed. Proc. FASEB 39, 2613 (abst.).
[15] Castranova, V., Jones, G. S., Phillips, R. M., Peden, D. and Vandyke, K. (1981) Biochim. Biophys. Acta 645, 49–53.
[16] Utsumi, K., Sugiyama, K., Miyahara, M., Naito, M., Auai, M. and Inoue, M. (1977) Cell. Struct. Func. 2, 203–209.
[17] Hoffstein, S. T. (1979) J. Immunol. 123, 1395–1402.
[18] Dunham, P. B., Goldstein, I. M. and Weissmann, G. (1974) J. Cell Biol. 63, 215–226.
[19] Naccache, P. H., Showell, H. J., Becker, E. L. and Sha'afi, R. I. (1977) J. Cell Biol. 73, 428–444.
[20] Schneider, C., Zanetti, M. and Romeo, D. (1981) FEBS Lett. 127, 4–8.
[21] Babior, B. M., Kipnes, R. S. and Curnutte, J. T. (1973) J. Clin. Invest. 52, 741–744.
[22] Rossi, F., Zabucchi, G., Dri, P., Bellavite, P. and Berton, G. (1980) in: Macrophages and Lymphocytes, pp. 53–74, Plenum, New York.
[23] Bellavite, P., Berton, G., Dri, P. and Soranzo, M. R. (1981) J. Reticuloendothel. Soc. 29, 47–60.
[24] Sims, P. J., Waggoner, A. S., Chao-Huei Wang, and Hoffman, J. F. (1974) Biochemistry 13, 3315–3330.
[25] Jones, G. S., Vandike, K. and Castranova, V. (1980) J. Cell. Physiol. 104, 425–431.
[26] Seligman, B. and Gallin, J. I. (1981) Movement, Metabolism and Microbicidal Activity of Leukocytes (Rossi, F. and Patriarca, P. eds) Plenum, New York, in press.
[27] Takeshige, K. and Minakami, S. (1981) Biochem. Biophys. Res. Commun. 99, 484–490.