FORMATION OF COMPLEXES OF SUPEROXIDE ANION-RADICALS WITH Ca^{++}

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Since a leading role in the initiation of lipid peroxidation (LPO) in biological membrane is played by superoxide ion-radicals ($O_2^{\cdot-}$) [2, 6], it is important to elucidate the mechanisms controlling the content of these products of single-electron reduction of oxygen in the cell. The writers showed previously that Ca^{++} ions (in concentrations over 10^{-5} M) can inhibit LPO in membranes of cardiomyocyte microsomes and mitochondria induced both by enzymic and by non-enzymic systems generating $O_2^{\cdot-}$ [1]. This effect is absent in systems of LPO induction in which $O_2^{\cdot-}$ does not participate in the initiation of the process (organic peroxides — cumyl hydroperoxide + Fe^{++}).

The aim of this investigation was to study interaction of Ca^{++} with $O_2^{\cdot-}$. For this purpose the ability of Ca^{++} to inhibit reduction of tetranitrotetrazolium blue (TNTB) into formazan by the $O_2^{\cdot-}$ formed with one of two systems, xanthine—xanthine oxidase (X—XO) or phenazine methosulfate (PMS)—NADH, was studied.

EXPERIMENTAL METHOD

For the investigation 0.08 M carbonate buffer, pH 10.1, at 25°C was used. Reduction of TNTB was recorded as formazan formation at 560 nm ($\varepsilon = 3 \times 10^4$ M^{-1}/cm) [3] on a Shimadzu MPS-SOL spectrophotometer (Japan). The xanthine concentration was determined from its optical density at 250 nm. The X was obtained from Merck (West Germany), the XO from Olaine Pharmaceutical Chemical Factory, USSR), PMS from Gee Lawson (England), NADH from Reanal (Hungary), catalase and EGTA from Serva (West Germany), and TNTB from Chemapol (Czechoslovakia).

EXPERIMENTAL RESULTS

Kinetic curves of TNTB reduction in the control and after addition of different concentrations of Ca^{++} are shown in Fig. 1. With an increase in the Ca^{++} concentration the rate of reduction of TNTB fell (Table 1). This effect was not connected with inhibition of the enzyme itself by Ca^{++} ions, for even in a concentration of 10^{-4} M, Ca^{++} ions do not affect the conversion of X into hypoxanthine (as judged by the decrease in X), recorded as the change in optical density at 250 nm. Addition of the complexone Ca^{++}-EGTA (5×10^{-4} M) to incubation medium containing Ca^{++} (10^{-4} M) weakened the inhibitory action of Ca^{++} on reduction of TNTB by 2.5 times.

Ca^{++} ions in a PMS—NADH system had a similar inhibitory action on reduction of TNTB by $O_2^{\cdot-}$. Since one of the reaction products formed in X—XO and PMS—NADH systems is H_2O_2, the action of catalase (20 units/ml) on reduction of TNTB was investigated in the presence of Ca^{++}. It was found that catalase does not affect formazan formation. Hence it is clear that Ca^{++} ions interact with $O_2^{\cdot-}$ but not with H_2O_2.

When the velocity constant of interaction between Ca^{++} and $O_2^{\cdot-}$ was calculated the possibility of utilization of $O_2^{\cdot-}$ in the following reactions was considered:

$$HO_2^{\cdot} + O_2^{\cdot-} + H_2O \xrightarrow{k_1} H_2O_2 + O_2 + OH^-,$$

$$O^{\cdot-} + \text{TNTB} \xrightarrow{k_2} \text{formazan}, \quad \bar{O}_2^{\cdot-} + Ca^{2+} \xrightarrow{k_3} \text{product}.$$

The velocity of the reaction of direct dismutation of $2O_2^{\cdot-}$ can be disregarded. With a constant $O_2^{\cdot-}$ concentration $\left(\frac{d[O_2^{\cdot-}]}{dt} = 0\right)$ we have:

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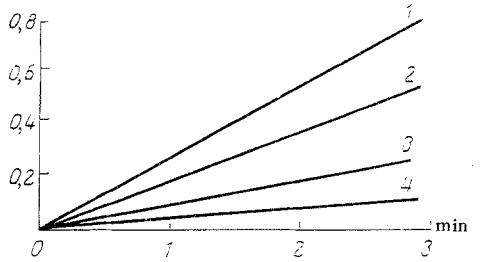

Fig. 1. Kinetic curves of reduction of TNTB into formazan by $O_2^{\cdot-}$ generated in X–XO system in the presence of different Ca^{++} concentrations. 1) Control; 2-4) Ca^{++} concentration 5×10^{-5}, 10^{-4}, and 5×10^{-4}, respectively. X 10^{-4} M, XO 0.058 unit/ml. Abscissa, time (in min); ordinate, optical density.

TABLE 1. Reduction of TNTB in PMS–NADH and X–XO Systems in the Presence of Ca^{++} (M × m)

Conditions	Velocity of reduction of TNTB, 10^{-5} $M^{-1} \cdot sec^{-1}$
PMS ($5 \cdot 10^{-5}$ M) + NADH (10^{-4} M) + TNTB ($5 \cdot 10^{-5}$ M)	
Control	7,0±0,8
Addition of:	
Ca^{2+} ($5 \cdot 10^{-5}$ M)	5,6±0,6
Ca^{2+} ($2 \cdot 10^{-4}$ M)	1,9±0,2
Mg^{2+} (10^{-4} M)	6,8±0,7
Zn^{2+} (10^{-4} M)	7,2±0,7
X (10^{-4} M + XO (0.058 unit/ml) + TNTB ($5 \cdot 10^{-5}$ M)	
Control	2,4±0,3
Addition of:	
Ca^{2+} (10^{-5} M)	2,3±0,3
Ca^{2+} ($5 \cdot 10^{-5}$ M)	1,3±0,2
Ca^{2+} ($6{,}6 \cdot 10^{-5}$ M)	1,2±0,2
Ca^{2+} (10^{-4} M)	0,8±0,1
Ca^{2+} ($5 \cdot 10^{-4}$ M)	0,7±0,1

$$V_{O_2^{\cdot-}} = k_1 \frac{[H^+]}{K_a} [O_2^{\cdot-}] + k_2 [TNTB] [O_2^{\cdot-}] + k_3 [O_2^{\cdot-}] [Ca^{2+}] \quad (1)$$

where $[O_2^{\cdot-}]$ denotes the rate of generation of $O_2^{\cdot-}$.

Since under the chosen conditions of pH 10.4, $k_1 \frac{[H^+]}{K_a} = 4 \times 10^2$ $M^{-1} \cdot sec^{-1}$ [5] and $K_2 = 6 \times 10^4$ $M^{-1} \cdot sec^{-1}$ [4] and since $[TNTB] \gg O_2^{\cdot-}$, the first term of equation (1) can be disregarded and we obtain:

$$[O_2^{\cdot-}] = \frac{V}{k_1 [TNTB] + k_3 [Ca^{2+}]}. \quad (2)$$

After transformation, in the presence of an excess of X and TNTB in the system we obtain:

$$k_3 = \frac{k_2 [TNTB]}{Ca^{2+}} \left(\frac{1}{\rho} - 1 \right), \quad (3)$$

where ρ is the ratio between the velocity of reduction of TNTB in the presence and absence of Ca^{++}, respectively.

Values of the constants of interaction of Ca^{++} with $O_2^{\cdot}$ in X–XO and PMS–NADH systems, calculated by equation (3), have values of $5.5 \pm 0.7 \times 10^4$ and $3.0 \pm 0.4 \times 10^4$ $M^{-1} \cdot sec^{-1}$, respectively, i.e., they agree sufficiently closely. Considering that the value obtained for $K_{Ca^{++}}$ was close to the value of K_{TNTB}, it can be concluded that interaction of Ca with O_2 is not catalytic but stoichiometric in character.

This effect of Ca^{++} is specific, for other bivalent cations (Mg^{++} and Zn^{++}) have no such action on $O_2^{\cdot}$ (Table 1).

Considering that the intracellular Ca^{++} concentrations vary between 10^{-7} and 10^{-5} M, and the extracellular Ca^{++} concentrations are between 10^{-4} and 10^{-3} M, and also considering values for the constant of interaction between Ca^{++} and $O_2^{\cdot}$ obtained in the present experiments, it can be concluded that Ca^{++} ions may regulate the concentration of $O_2^{\cdot}$ – a product of single-electron reduction of oxygen *in vivo*.

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PROTECTION OF SARCOPLASMIC RETICULAR MEMBRANES AGAINST DAMAGE BY FREE FATTY ACIDS BY VITAMIN E

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Free fatty acids (FFA) are present in small quantities in membranes of the sarcoplasmic reticulum (SR) of heart and skeletal muscles [9, 11]. The FFA content rises during the development of certain pathological states (ischemia, stress) up to a level where it disturbs the transport function of SR membranes [10, 11]. It is therefore important to seek ways of protecting the Ca^{++}-pump of SR membranes against damage by FFA. The latter increased the passive permeability of SR membranes for Ca^{++} and essentially reduced the resistance of Ca^{++}-dependent ATPase to thermal denaturation [5]. It was shown previously that α-tocopherol reduces the passive permeability of membranes for Ca^{++}, in agreement with the widely held view that vitamin E plays a stabilizing role in biological membranes [3, 8].

The object of this investigation was to study the protective action of α-tocopherol against temperature inactivation of the Ca^{++} pump of SR membranes in the presence of arachidonic acid (AA).

EXPERIMENTAL METHOD

Fragments of SR from rat skeletal muscles were isolated by differential centrifugation from a homogenate of hind limb muscles [12]. A highly purified fraction of SR membranes from rabbit muscles was isolated from white muscles of the hind limbs by the method described in [7]. ATPase activity and efficiency of Ca^{++} transport by SR membranes were determined by pH-

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