PhB- and MCh-cytochromes P-450 were introduced in the wells E and F, respectively, both these cytochromes into well G, and rat liver microsomes induced by arochlor-1254, which induces the synthesis of both PhB- and MCh-forms of cytochrome P-450, into well H. Figure 1 shows clearly that polyspecific antibodies prepared artificially recognize homologous antigens, and give a picture of a double rocket in the case of a mixture of isolated preparations of cytochrome P-450 and rat liver microsomes induced by arochlor-1254.

Thus rocket immunoelectrophoresis, as applied to isolated forms of cytochrome P-450 and microsomal preparations, can be used to assess the monospecificity of antibodies on the basis of pattern of immunoprecipitation lines.

Incidentally, Ouchterlony's double immunodiffusion method is widely used for the qualitative study of induction of microsomal enzymes. However, this method is not sensitive enough when determining monospecificity of antibodies if the preparation of immunoglobulins obtained against a certain form of cytochrome P-450 contains antibodies against other antigens, and in the sample for testing, these antigens are present in a small quantity. The rocket immunoelectrophoresis technique possesses higher resolving power.

We consider that in most cases antibodies to a certain form of cytochrome P-450 can be regarded as monospecific if they give rise to a single rocket during analysis of a microsomal preparation known to contain the total number of forms of cytochrome P-450.

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EFFECT OF RUTHENIUM RED ON Ca^{++}-INDUCED β AND γ STATES OF COMUTON REGULATION OF RESPIRATION AND OXIDATIVE PHOSPHORYLATION IN RAT MITOCHONDRIA

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The writers showed previously that the tissues of rats [3, 5] and other vertebrates [1] contain a mechanism regulating energy metabolism in the tissue [4]. Its effectors are tissue-specific peptides known as comutons [6]. It has been shown that comuton regulation may exist in three states: in the α-state, expressed as tissue-specific stimulation of the respiration rate of mitochondria (MCh) in medium with succinate before addition of ADP (v_4), the β-state, when rotenone-insensitive uncoupling of oxidative phosphorylation of MCh is observed, and the γ-state, when this uncoupling becomes rotenone-stimulated [7]. In experiments *in vitro* the β- and γ-states of comuton regulation can be induced by brief preincubation of MCh from rat liver and kidney with Ca^{++} ions in the presence of 3 mM inorganic phosphate (P_i) [7]. To study the mechanism of the regulating action of Ca^{++} on the character of comuton control of oxidative

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phorphorylation, ruthenium red (RR), which interacts specifically with glyco- and mucoproteins of cell membranes, was used [13]. The dye inhibits active transport of Ca^{++} in MCh and other types of interaction of this cation with the outer surface of the inner membrane of MCh, but had no effect on Ca^{++} release from the mitochondrial matrix [13].

Previous experiments to study induction of the β- and γ-states of comuton regulation were conducted under conditions of massive Ca^{++} transport in MCh. An attempt to induce these states when the P_i concentration in the incubation medium of MCh was much lower than 1 mM, i.e., under conditions of limited Ca^{++} transport in MCh, was therefore interesting on its own account [12]. The results are described in this paper.

EXPERIMENTAL METHOD

Experiments were carried out on noninbred male albino rats weighing 200-230 g. The conditions for obtaining MCh from the liver and kidney, as well as for the comuton-containing liver cell soluble phase (CSP) were described previously [3, 7]. The effect of the liver CSP on respiration and oxidative phosphorylation of liver and kidney MCh was assessed polarographically [1]. The incubation medium of MCh, allowing for the CSP or isolation medium of that fraction, added in the ratio of 1:1, consisted of 210 mM sucrose, 40 mM KCl, 10 mM Tris-buffer (pH 6.8), and 5 mM succinate. To a polarographic cell filled with this medium $CaCl_2$ was added to induce a particular state of comuton regulation, after which liver or kidney MCh were added (2 mg protein/ml), and recording of respiration began. After 20 sec of preincubation of MCh with Ca^{++}, 3 mM EDTA with 3 mM H_3PO_4 was added. The value of v_4 was recorded for 40 sec, after which ADP was added and the respiration rate measured in state 3 (v_3), and also the rate v_4 on completion of state 3, until all the oxygen in the cell was used up. RR (5 nanomoles/mg protein of MCh) was added before addition of Ca^{++} to the medium, simultaneously with EDTA and P_i. Rotenone (0.5 μg/mg protein of MCh) was added only at the end of preincubation of MCh with Ca^{++}. Coupling of oxidative phosphorylation was judged from the quantity of oxygen used in the active metabolic state (ΔO_{act}), and the time of phosphorylation of the added ADP (t_p) also was calculated. Comuton activity in liver CSP was measured by recording the magnitude of tissue-specific changes in the recorded parameters of respiration and oxidative phosphorylation of liver MCh in the presence of this fraction, which was calculated by comparison of its effects on liver MCh with those on kidney MCh. The data given are the mean values of 3-5 experiments. Reagents of USSR origin were of the chemically pure and highly pure grades, the sucrose corresponded to State Standard 5883-54, the EDTA and Tris-buffer were obtained from Koch-Light (England), the RR from Fluka (Switzerland), and the ADP from Reanal (Hungary).

EXPERIMENTAL RESULTS

In a concentration of 5 ng/mg mitochondrial protein RR inhibits Ca^{++} transport in MCh, without affecting oxidative phosphorylation. In a dose of 10 ng/mg mitochondrial protein these effects are supplemented by inhibition of v_4' [13]. In the incubation medium used in the present experiments (with lower pH), RR also was found to inhibit Ca^{++} transport, but inhibition of v_4' was not found in either liver or kidney MCh. It was also shown that RR in concentrations of 5 to 40 ng/mg mitochondrial protein had no effect on tissue specificity or on the degree of stimulation of v_4' induced in liver MCh by homologous CSP (Fig. 1), in the α-state of comuton regulation. It will be clear from Figs. 2 and 3 that Ca^{++} ions induce β- and γ-states of comuton regulation and oxidative phosphorylation at a low P_i concentration also. The higher the K^+ concentration in the incubation medium of MCh, the more effect the inducing action of Ca^{++} was found to be. For instance, in the presence of 40 mM KCl the β-state was induced by 0.20 ± 0.12 mM Ca^{++} and the γ-state by 0.70 ± 0.15 mM. In the presence of 6 mM KCl, 1.8 mM Ca^{++} was now required to induce the β-state, and 2.2 ± 0.3 mM to induce the γ-state. The Ca^{++} concentrations mentioned had virtually no effect on coupling of oxidative phosphorylation of liver and kidney MCh in the absence of liver CSP. Preincubation of liver MCh with Ca^{++} in medium containing 40 mM KCl induced a tissue-specific increase in ΔO_{act} and t_p, insensitive to rotenone, and also inhibition of tissue-specific stimulation of v_4' — induction of the β-state of comuton regulation. When 6 mM KCl was used these effects were not present. If RR was added before Ca^{++}, instead of preincubation of MCh with this cation, the uncoupling effect of CSP was reduced by half, but both tissue-specific and tissue-nonspecific stimulation of v_4' were strongly stimulated. In the γ-state of comuton regulation rotenone stimulated not only tissue-specific uncoupling of oxidative phosphorylation of liver MCh, but also the tissue-specific component of v_4' (Fig. 3). Effects of RR here also were exhibited only when it was added before preincubation of MCh with Ca^{++}. The action of RR was similar to its effect on comuton regulation in the β-state, but inhibition of tissue-specific uncoupling of oxidative phosphorylation was complete.

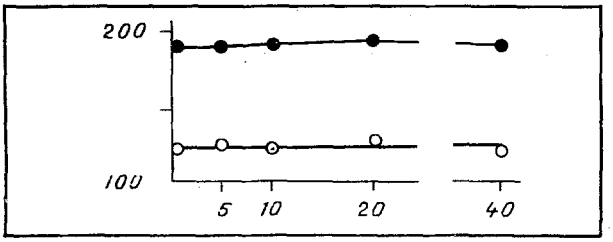

Fig. 1

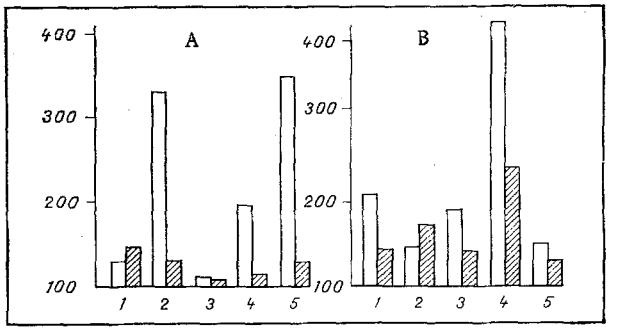

Fig. 2

Fig. 1. Effect of RR on tissue-specific stimulation of v_4' in liver MCh in the presence of homologous CSP, in the α-state of comuton regulation. Abscissa, RR concentration in incubation medium (in nmoles/mg mitochondrial protein); ordinate, v_4 for liver MCh (1) and kidney MCh (2) in presence of liver CSP (in percent of values of these rates in MCh from the above-mentioned tissues in the absence of CSP). Besides RR, EDTA (3 mM), H_3PO_4 (3 mM), and rotenone (0.5 μmole/mg mitochondrial protein) also were added to the incubation medium before the beginning of measurement of the rates.

Fig. 2. Effect of RR on tissue-specific changes in ΔO_{act} (A) and v_4' (B) observed in liver MCh as a result of induction of the β-state of comuton regulation by Ca^{++} ions. Ordinate, ΔO_{act} and v_4' in percent of values of these parameters in liver MCh (unshaded columns) and kidney MCh (shaded columns) in the absence of CSP. 1) Addition of of CAP without Ca^{++}; 2) preincubation with Ca^{++} ions; 3) preincubation with Ca^{++} ions in the presence of 6 mM KCl in medium; 4) RR added to medium before preincubation of MCh with Ca^{++} ions; 5) RR added to medium after preincubation of MCh with Ca^{++} ions.

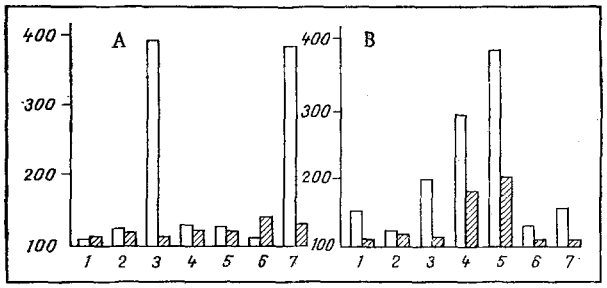

Fig. 3. Effect of RR on tissue-specific changes in ΔO_{act} (A) and v_4' (B) observed in liver MCh as a result of induction of the γ-state of comuton regulation by Ca^{++} ions. 1) addition of CSP without Ca^{++} in the presence of rotenone; 2-7) preincubation with Ca^{+} ions; 2,4,6) in the absence of rotenone; 3,4,7) addition of rotenone; 4, 5) RR added before preincubation of MCh with Ca^{++} ions; 6, 7) RR added after preincubation of MCh with Ca^{++} ions. Remainder of legend the same as to Fig. 2.

The β- and γ-states of comuton regulation can thus be successfully induced in liver MCh when Ca^{++} transport into the matrix of these organelles is limited. Under those conditions, however, the inducing action of Ca^{++} is 3-3.5 times less effective than during mass transport of Ca^{++} in the presence of 3 mM P_i [7]. It is well known that P_i stimulates active Ca^{++} transport in MCh [12]. It can therefore be tentatively suggested that active Ca^{++} transport in MCh makes the main contribution to induction of β- and γ-states in the present experiments. This explanation is supported by the fact that the inducing effect of Ca^{++} is inhibited by the addition of RR before preincubation of MCh with this cation. Incomplete inhibition of induction of the β-state under these conditions may be due to release of Ca^{++} from the mitochondrial matrix. In fact, release of Ca^{++} from MCh can be intensified by the addition of Ca^{++} in the presence of RR [8, 11], and this process, moreover, is not inhibited by RR [11]. Inhibition of oxidation of NADH in MCh is known to prevent Ca^{++} release from the organelles [10]. Meanwhile induction of the β-state of comuton regulation in liver MCh was inhibited by addition of rotenone before preincubation of MCh with Ca^{++} in the presence of 3 mM P_i [7]. This last

fact indicated that Ca^{++} participates in the induction of this state. Induction of the β- and γ-states of comuton regulation by Ca^{++} ions is effected through a K^+-dependent mechanism, with the participation of mitochondrial phospholipase A [7]. Activation of this phospholipase A has been shown to be essential also for Ca^{++} release from MCh, not inhibited by RR [11].

It can be concluded from the data described above that active Ca^{++} transport in MCh and also, perhaps, binding of Ca^{++} with sites of low affinity for this cation induce activation of phospholipase A, which is not complately abolished by RR [11]. As a result the K^+-permeability of the mitochondrial membrane is increased and comution regulation switches from the α-state into the β- and γ-states. The role postulated for K^+ in this process is in agreement with our data on induction of β- and γ-states by valinomycin [9]. Ca^{++} ions evidently affect comuton regulation through their action on the components of CSP also. This may explain the strengthening of tissue-specific stimulation of v_4 after preincubation of MCh and CSP with Ca^{++} in the presence of RR, which is not observed in the absence of RR, and also when RR is added after this preincubation. In the last two cases the Ca^{++} concentration in the medium evidently falls rapidly because of its transport into the mitochondrial matrix and interaction between Ca^{++} and CSP is shorter in duration. None of the states of comuton regulation was abolished by RR after their induction by Ca^{++} ions. This state of affairs suggests that glyco- and mucoproteins are not components of the receptor which interacts with the comuton molecule on the surface of the mitochondrial membrane.

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