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BLEOMYCIN STIMULATES BOTH MEMBRANE (Na^+-K^+) ATPase AND ELECTROGENIC (Na^+-K^+) PUMP AND PARTIALLY REMOVES THE INHIBITION BY VANADIUM IONS

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SUMMARY: Bleomycin $2x10^{-6}$ and $6x10^{-6}$ $mol.l^{-1}$ increased the activity of specific (Na^+-K^+) ATPase of the rat brain microsomes. It also stimulated the electrogenic (Na^+-K^+) pump in intact skeletal muscle cells. The blocking effect of vanadyl (^{+4}V) on membrane (Na^+-K^+) ATPase was eliminated completely by the drug, but the action of vanadate (^{+5}V) was counteracted only partially. Electron paramagnetic resonance spectra revealed the formation of a ^{+4}V - bleomycin complex which is still able to activate the (Na^+-K^+) ATPase.

Bleomycin is extensively used in the treatment of cancer (1). It represents a group of glycopeptide antibiotics and its effect in vivo is thought to be in degradation of cellular DNA (2, 3). Bleomycin (BLM) might form complexes with transition metal ions and its metabolic activity may be increased by metal complexation (3-6). One of the transition metals, vanadium, can effectively block membrane (Na^+-K^+) ATPase (7-15). The aim of the present paper was to follow the effect of BLM on this membrane enzyme and on the electrogenic (Na^+-K^+) pump of muscle fibres, which is an electrophysiological correlate of the (Na^+-K^+) ATPase in intact cells.

METHODS

Bleomycin (Bleocina, metal-free) was kindly provided by Nippon Kayaku, Japan. Other chemicals were the purest commer-

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cially available reagents. Preparation of stock solutions of both forms of vanadium and electrophysiological measurements were described previously (14, 16). Brain subcellular fractions were prepared according to De Robertis et al. (17) from the cerebral cortex of white Wistar rats (180 g). The microsomal membrane particles were sedimented from the 12 000 x g supernatant by centrifugation for 60 min at 100 000 x g. The resulting sediments were resuspended in 0.32 mol.l^{-1} sucrose and stored at -25 °C.

The activity of (Na^+-K^+) ATPase was determined as inorganic phosphorus (P_i) production as described elsewhere (20). Brain microsomes[1](0.03-0.05 mg membrane protein per ml) were preincubated for 5 min at 37 °C in 1.25 ml of medium A (100 mmol.l^{-1} NaCl, 20 mmol.l^{-1} KCl, 5 mmol.l^{-1} $MgCl_2$, 100 mmol.l^{-1} TRIS-HCl, pH 7.4) or in a medium B without K^+ (120 mmol.l^{-1} NaCl, 5 mmol.l^{-1} $MgCl_2$, 100 mmol.l^{-1} TRIS-HCl, pH 7.4 and 2 x 10^{-4} mmol.l^{-1} ouabain, Sigma). The reaction was started by addition of ATP (Boehringer) (final conc. 2.5 mmol.l^{-1}), continued for 15 min at 37 °C and terminated by 0.3 ml of 10 % $HClO_4$. The precipitated protein was removed by centrifugation and P_i was determined according to Taussky and Shorr (21). The production of P_i in the medium B represented the activity of basal ouabain-insensitive Mg^{2+}- ATPase. (Na^+-K^+) ATPase was calculated as the difference between the total (medium A) and the basal Mg^{2+}-ATPase (medium B).

The measurements of paramagnetic ^{+4}V were performed at 77°K on a JEOL JES-PE-3X spectrometer at 100 KHz modulation frequency in a LC-01 aqueous solution cell with active volume of the sample 0.02 ml (22).

The electrogenic (Na^+-K^+) pump was estimated electrophysiologically (14, 16) on mouse diaphragm muscle fibres enriched with Na^+ by incubation for 6 hours in a K^+-free Liley (23) solution (16). Resting membrane potentials were recorded with an intracellular microelectrode (3 mol.l^{-1} KCl, 10-20 MΩ) from superficial muscle fibres before and after addition of 5x10^{-3} mol.l^{-1} K^+ into the muscle bath. The difference between these two values was considered as the expression of electrogenic (Na^+-K^+) pump activity (24, 25).

RESULTS AND DISCUSSION

In control experiments with oubain-insensitive Mg^{2+}-ATPase, bleomycin at 2x10^{-6} and 6x10^{-6} mol.l^{-1} had no effect on the basal activity (Tab.1,b,c). When added to microsomes in an ouabain-free, potassium containing medium, the activity of (Na^+-K^+) ATPase (total minus basal) was markedly potentiated by both concentrations (Tab.1,d-f). In the case of BLM 2x10^{-6} mol.l^{-1}, the activity was higher by 94 % and BLM 6x10^{-6} mol.l^{-1} led to a more than twofold increase of activity, by 127 %. This increase is much more pronounced than the known potentiation of (Na^+-K^+) ATPase by noradrenaline. Wu and Phillis (26) reported

TABLE 1. THE EFFECT OF BLEOMYCIN (BLM), NORADRENALINE (NOR) AND VANADIUM IONS ON THE BRAIN, MICROSOMAL (Na^+-K^+) ATPase

Assay	Activity (mean + S.E.M.)	%
a) Total ATPase (medium A)	46.0 ± 2.0 (41)	
b) Basal, Mg^{2+}-ATPase (medium B)	30.2 ± 2.0 (40)	
c) + BLM $6x10^{-6}$	31.1 ± 1.9 (5)	
d) (Na^+-K^+) ATPase	15.8 ± 1.1 (40)	100
e) + BLM $2x10^{-2}$	30.7 ± 3.1 (6)	194
f) + BLM $6x10^{-6}$	36.0 ± 2.4 (6)	227
g) + NOR $1x10^{-6}$	18.7 ± 2.6 (6)	118
h) + ^{+4}V $1x10^{-6}$, DTT $1x10^{-6}$	7.9 ± 1.2 (6)	50
i) + ^{+5}V $1x10^{-6}$	5.8 ± 1.3 (6)	36
j) + ^{+4}V $1x10^{-6}$, DTT, BLM $2x10^{-6}$	24.9 ± 0.9 (6)	157
k) + ^{+5}V $1x10^{-6}$, BLM $2x10^{-6}$	11.8 ± 0.9 (6)	75
l)§ + ^{+4}V $1x10^{-6}$, DTT, BLM $2x10^{-6}$	26.3 ± 3.0 (6)	166
m)§ + ^{+5}V $1x10^{-6}$, BLM $2x10^{-6}$	14.0 - 1.5 (6)	88

Concentrations correspond to $mol.l^{-1}$. Activity is expressed as $\mu mol\ P_i/h$ per mg protein. § l,m - Aqueous solutions of vanadium ions and BLM were mixed to final concentrations 1 $mmol.l^{-1}$ and 1.2 $mmol.l^{-1}$ respectively and allowed to stand overnight at laboratory temperature in the dark (18, 19). Aliquots of this mixture were then added to the microsomes suspended in ATPase reaction media A or B. After 5 min preincubation, the ATPase reaction was started by ATP and the ATPase activities were determined as described in Methods. In the presence of BLM, appropriate blanks were used to measure the nonenzymatic hydrolysis of ATP. Dithiothreitol (DTT) $1x10^{-6}$ $mol.l^{-1}$ used to prevent the ^{+4}V oxidation had no effect on specific (Na^+-K^+)$_4$ATPase activity itself (27). Ouabain in a concentration of $1x10^{-4}$ $mol.l^{-1}$ was used. Numerals in brackets - number of experiments. Right column - percentage changes (%).

that the specific activity was increased by about 35 % in brain microsomes in the presence of $2x10^{-6}$ $mol.l^{-1}$ noradrenaline and our own data show an increase of 18 % (Tab.1,g).

Potentiation was also observed in electrophysiological experiments with the electrogenic (Na^+-K^+) pump. It may be seen in Fig.1 (the representative records of two out of eight identical experiments) that the resting membrane potential (RMP) of

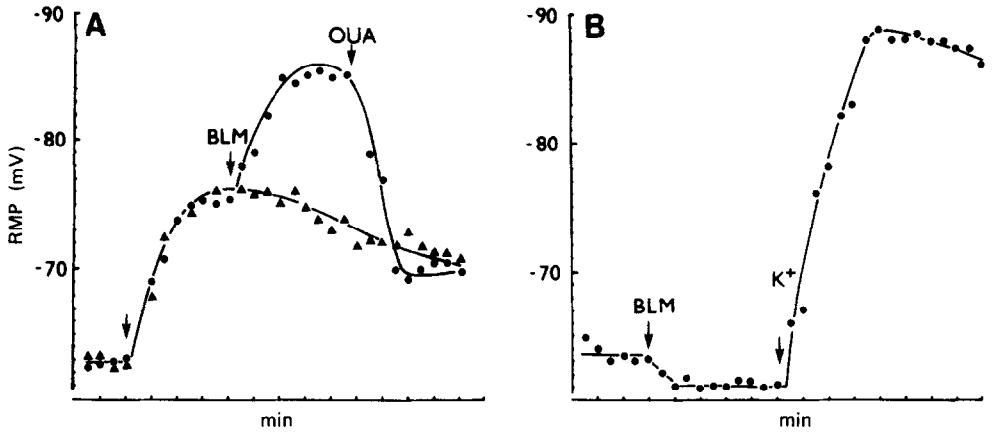

Fig. 1. The effect of $2x10^{-6}$ Bleomycin (BLM) on the electrogenic (Na^+-K^+) pump expressed as the increase of resting membrane potential (RMP) after adding 5 $mmol.l^{-1}$ K^+ to the perfusion solution. Each point represents the mean of five consequent measurements of RMP in different diaphragm fibres during one experiment. The standard intracellular recording technique was used. A: triangles - control experiment without BLM, full circles - BLM was added at the peak of the K^+ effect. Ouabain (OUA) $1x10^{-4}$ $mol.l^{-1}$ eliminated the effect of BLM completely. B: time course of RMP changes after adding of BLM and K^+. Temperature 22 °C, time on abscissae in minutes. For further description see text.

Na^+-enriched muscle fibres increased after adding 5 $mmol.l^{-1}$ K^+ from -63 mV (inside negative) to -75 mV and that this 12 mV hyperpolarization increased further during the course of the experiment by $2x10^{-6}$ $mol.l^{-1}$ BLM to -85 mV (Fig.1A). Similarly as in microsomal (Na^+-K^+) ATPase activity (Tab.1), the potentiating effect of BLM on electrogenesis was rapidly eliminated by $1x10^{-4}$ $mol.l^{-1}$ ouabain. Fig.1B shows the slight depolarizing effect of $2x10^{-6}$ $mol.l^{-1}$ BLM alone (by about 2 mV) and subsequent potentiation of the electrogenic pump when 5 $mmol.l^{-1}$ K^+ was added (cf. Fig.1A, triangles). This indicates that BLM potentiation occurs on the outer face of the membrane, probably at/or near the potassium binding site of the (Na^+-K^+) ATPase moiety.

According to our recent studies on brain microsomal (Na^+-K^+) ATPase (27), both redox forms of vanadium ions VO^{2+}

(^{+4}V) and VO_3^- (^{+5}V) inhibit the brain microsomal (Na^+-K^+) ATPase with similar affinities provided that ^{+4}V is protected by a reducing agent, e.g. dithiothreitol against oxidation in the air atmosphere. In the present experiments, the specific enzyme activity was decreased to 50 % and to 36 % by $1x10^{-6}$ $mol.l^{-1}$ ^{+4}V (plus $1x10^{-6}$ $mol.l^{-1}$ dithiothreitol) and by ^{+5}V, respectively (Tab.1,h,i). When BLM was added in slight excess to the microsomes (Tab.1,j,k) together with ^{+4}V, inhibition of the enzyme (50 %) was converted to activation which reached 157 % of the control values (cf. 194 % when BLM acted alone). Much less pronounced disinhibition (from 36 % to 75 %) was observed when BLM was used together with ^{+5}V. Similar results were obtained when BLM and vanadium were kept together in the dark for about 12 hours (Tab.1,l,m).

There are two possibilities how to explain the almost complete elimination of the inhibition by ^{+4}V. Either BLM and vanadium act in an opposite way as allosteric modulators on the ATPase moiety or more probably the effect of the metal is eliminated by the formation of an inactive complex with BLM. Although the former case cannot be excluded, the complexation capacity of both ^{+4}V (30, 31) and BLM (3-6, 32) favours the latter alternative. For this reason we tried to check whether the electron paramagnetic resonance spectra (EPR) of ^{+4}V plus BLM reveal some features of the complex formed in an aqueous solutions.

Fig.2 shows control experiments where no ^{+4}V signal could be observed when $VOSO_4$ was simply dissolved in water (A). In this case, all four valencies of the metal are attached to water molecules; after acidification with HCl (B) (pH 0.5) the changed relaxation time makes it possible to see the typical rigid matrix EPR spectrum of ^{+4}V in the frozen state (see e.g.

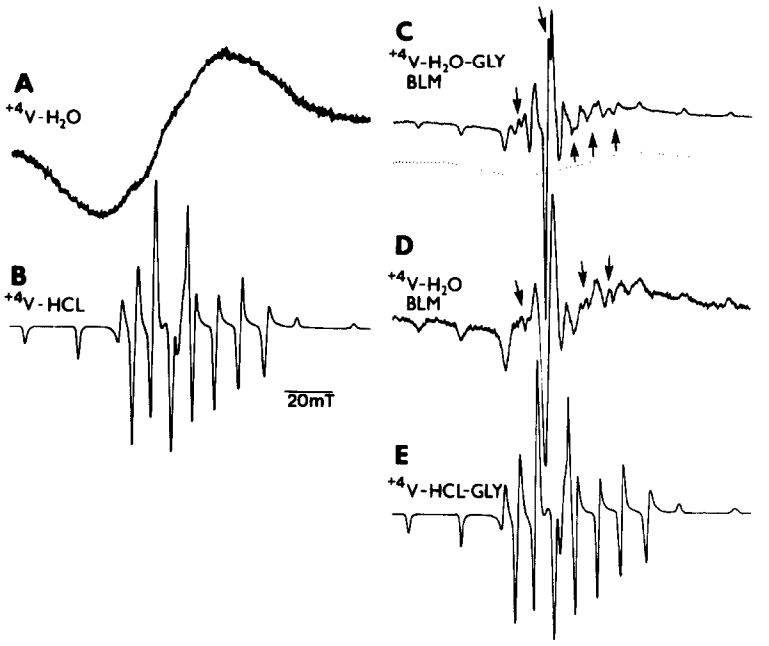

Fig. 2. EPR spectra of $1x10^{-3}$ $VOSO_4$($^{+4}$V) without and with $2x10^{-3}$ bleomycin (BLM) in H_2O and after acidification (HCl). Amplification in A is five times and in D two times higher than in B, C and E. Dotted line in C represents the basal envelope of the signal. Glycerine (Gly) to water ratio was 1:2 (32). Note the appearance of complex in the presence of glycerine in C (arrows) and without glycerine in D.

33, Fig.1). Figure 2 presents the spectrum obtained in the presence of BLM (C, D, plus or minus glycerine, which might increase the signal to noise ratio without changing the line positions, 32). On both records, the hyperfine components (arrows) are indicative of the formation of a single VO^{2+} -- BLM complex. Similar spectra were obtained in an ATPase medium pH = 7.4 (data not shown). The ESR data support the possibility that there exists a complex of both compounds, in which the $^{+4}$V is inactive as an inhibitor of (Na^+-K^+) ATPase. The ability of BLM to form (inactive?) complex with pentavalent vanadium cannot, however, be tested by the EPR method; because the blocking effect of $^{+5}$V was not eliminated to any appreciable extent by BLM, we are inclined to assume that there was no strong $^{+5}$V-BLM complex present in our experiments.

This is supported by the general view that ^{+5}V does not easily form complexes with biologically active substances (29, 33), whereas complexes of vanadyl are numerous (34).

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REFERENCES

1. Umezawa, H. (1976) Gann Monograph on Cancer Research 19, pp. 3-36, University of Tokyo Press, Tokyo.
2. Suzuki, H., Nagai, K., Yamaki, H., Tanabe, N., and Umezawa, H. (1969) J. Antibiotics 22, 446-448.
3. Sausville, E.A., Peisach, J., and Horwitz, S.B. (1978) Biochemistry 17, 2740-2746.
4. Freedman, J.H., Horwitz, S.B., and Peisach, J. (1982) Biochemistry 21, 2203-2210.
5. Margusan, N., Ehrenfeld, G.M., and Hecht, S.M. (1982) J. Biol. Chem. 257, 8600-8603.
6. Chang, C.H., Dallas, J.L., and Meares, C.F. (1983) Biochem. Biophys. Res. Commun. 110, 959-966.
7. Cantley, L.C., Resh, M.D., and Guidotti, G. (1978) Nature 272, 552-554.
8. Beaugé, L., and Glynn, I.M. (1978) Nature 272, 551-552.
9. Hudgins, P.M., and Bond, G.H. (1977) Biochem. Biophys. Res. Commun. 77, 1024-1029.
10. Karlish, S.J.D., Beaugé, L.A., and Glynn, I.M. (1979) Nature 282, 333-335.
11. Myers, T.D., Boerth, R.C., and Post, R.L. (1979) Biochim. Biophys. Acta 558, 99-107.
12. Wallick, E.T., Lane, L.K., and Schwartz, A. (1979) J. Biol. Chem. 254, 8107-8109.
13. Hansen, O. (1979) Biochim. Biophys. Acta 568, 265-269.
14. Vyskočil, F., Teisinger, J., and Dlouhá, H. (1981) Biochem. Biophys. Res. Commun. 100, 982-987.
15. Hansen O. (1983) Acta Pharmacol. Toxicol. 52, Suppl.I., 1-19.
16. Dlouhá, H., Teisinger, J., and Vyskočil, F. (1981) Physiol. Bohemoslov. 30, 1-10.
17. De Robertis, E., De Iraldi, A.P., De Lorez, A., and Salganicoff, A.L. (1962) J. Neurochem. 9, 23-34.
18. DeRiemer, L.H., Meares, C.F., Goodwin, D.A., and Diamanti, C.I. (1979) J. Med. Chem. 22, 1019-1023.
19. Dabrowiak, J.C., Greenaway, F.T., and Santillo, F.S. (1979) in Bleomycin: Chemical, biochemical and biological aspects (Hecht, S.M., ed.), pp. 137-155, Springer Verlag, New York.
20. Svoboda, P., and Mosinger, B. (1981) Biochem. Pharmacol. 30, 427-432.
21. Taussky, H.H., and Shorr, E. (1953) J. Biol. Chem. 202, 675-681.
22. Vyskočil, F., Pilař, J., Zemková, H., and Teisinger, J. (1982) Lancet, 1078-1079.
23. Liley, A.W. (1966) J. Physiol. 132, 650-666.
24. Kernan, R.P. (1962) Nature 193, 986-987.

25. Dlouhá, H., Teisinger, J., and Vyskočil, F. (1979) Pflügers Arch. 380, 101-104.
26. Wu, P.H., and Phillis, J.W. (1979) Int. J. Biochem. 10, 629-635.
27. Svoboda, P., Teisinger, J., Pilař, J., and Vyskočil, F. (1983) in press.
28. Selbin, J. (1966) Coord. Chem. Rev. 1, 293-314.
29. Ferguson, J.H., and Kustin, K. (1979) Inorg. Chem. 18, 3349-3357.
30. Sausville, E.A., Stein, R.W., Peisach, J., and Horwitz, S.B. (1978) Biochemistry 17, 2746-2754.
31. Mulks, C., Kirste, B., and van Willigen, H. (1982) J.Amer. Chem. Soc. 104, 5906-5911.
32. Albanese, N.F., and Chasteen, N.D. (1978) J. Phys. Chem. 82, 910-920.
33. Rubinson, K.A. (1981) Proc. R. Soc. Lond. B 212, 65-81.
34. Sillén, L.G., and Martell, E.A. (1971) Chem. Soc., Spec. Publ. No. 17 and 25.