not occur with fasting. These considerations emphasize the importance of the trapping mechanism in the maintenance of muscle creatine concentrations. Further work is needed to determine whether creatine is trapped primarily because it is phosphorylated, bound to a muscle constituent, or compartmentalized or because the process that moves creatine across muscle membranes serves only for entry.

1 Acknowledgment. This work was supported in part by grant AM 16623 from the National Institutes of Health. To whom reprint requests should be addressed.

2 Fitch, C.D., in: Pathogenesis of human muscular dystrophies, p.328. Ed. L.P. Rowland (Excerpta Med. Int. Cong. Ser.404). Excerpta Medica, Amsterdam 1977.
3 Walker, J.B., Adv. Enzymol. *50* (1979) 177.
4 Ennor, A.H., and Morrison, J.F., Physiol. Rev. *38* (1958) 631.
5 Fitch, C.D., Lucy, D.D., Bornhofen, J.H., and Dalrymple, G.V., Neurology *18* (1968) 32.
6 Fitch, C.D., and Shields, R.P., J. biol. Chem. *241* (1966) 3611.
7 Fitch, C.D., and Chevli, R., Metabolism *29* (1980) 686.
8 Fitch, C.D., Shields, R.P., Payne, W.F., and Dacus, J.M., J. biol. Chem. *243* (1968) 2024.
9 Fitch, C.D., Jellinek, M., and Mueller, E.J., J. biol. Chem. *249* (1974) 1060.
10 Chevli, R., and Fitch, C.D., Biochem. Med. *21* (1979) 162.
11 Shields, R.P., Whitehair, C.K., Carrow, R.E., Huesner, W.W., and Van Huss, W.D., Lab. Invest. *33* (1975) 151.
12 Mahanna, D.A., Fitch, C.D., and Fischer, V.W., Exp. Neurol. *68* (1980) 114.
13 Petrofsky, J.S., and Fitch, C.D., Pflügers Arch. *384* (1980) 123.
14 Laskowski, M.B., Chevli, R., and Fitch, C.D., Metabolism *30* (1981) 1080.
15 Fitch, C.D., and Rahmanian, M., Proc. Soc. exp. Biol. Med. *131* (1969) 236.
16 Berlet, H.H., Analyt. Biochem. *60* (1974) 347.
17 Fitch, C.D., Robbins, L.J., Jellinek, M., and Nelson, J.S., Experientia *29* (1973) 956.
18 Griffiths, W.J., J. clin, Path. *21* (1968) 412.
19 Bruno, G.A., and Christian, J.E., Analyt. Chem. *33* (1961) 1216.
20 Chanutin, A., and Beard, H.H., J. biol. Chem. *78* (1928) 167.
21 Chanutin, A., and Silvette, H., J. biol. Chem. *80* (1928) 589.

0014-4754/83/121360-03$1.50+0.20/0

Inhibition of erythrocyte ATPase activity by aclacinomycin and reverse effects of ascorbate on ATPase activity

T. Kitao and K. Hattori

Department of Medicine, Kanazawa University School of Medicine, Kanazawa 920 (Japan), January 14, 1983

Summary. We studied the effect of aclacinomycin on human erythrocyte membrane enzymes. Aclacinomycin inhibited ATPase, including Na-K-dependent ATPase, ouabain insensitive ATPase and Ca-ATPase. However acetylcholinesterase was not inhibited by aclacinomycin. The ATPase activities were not inhibited by aclacinomycin if ascorbate was added to the incubation mixture. However other reducing agents, α-tocopherol, superoxide dismutase and catalase had no effect on ATPase activity. Ascorbate may protect membrane proteins and lipids from peroxidate damage.

In 1975, a new anthracycline antibiotic, aclacinomycin, was isolated from a culture filtrate of *Streptomyces galilaeus*. The compound has shown wide antitumor activity against ascitic and solid forms of various malignancies in animal tumor systems. Objective responses to aclacinomycin were observed in patients with lymphoma, acute leukemia, chronic leukemia, breast cancer, ovarian cancer, lung cancer, gastric cancer and bladder carcinoma[1]. The cardiotoxicity of aclacinomycin was estimated as approximately $^1/_{15}$ that of doxorubicin.

Henderson demonstrated that anthracycline antibiotics generated oxygen radicals in erythrocytes[2]. Doxorubicin has the potential of inhibiting erythrocyte ATPase activity, including both Na^+-K^+-ATPase and ouabain insensitive ATPase, at concentrations not inhibitory to other enzymes. The net Na^+ content increased and K^+ content decreased after incubation of erythrocytes with doxorubicin. Such effects of doxorubicin on membrane ATPase and cation permeability may explain, in part, the cardiotoxicity observed in its clinical use and also the potential hemolyzing effect on erythrocytes[3].

The purpose of this study was to obtain further knowledge of the effects of aclacinomycin on erythrocyte membrane enzymes and the effects of radical scavengers on the enzyme activities.

Materials and methods. Commercially available aclacinomycin from Yamanouchi Pharma Co. was dissolved in isotonic NaCl and added to erythocyte suspensions in varying concentrations.

Adenosine triphosphate (ATP-Na), ouabain, acetylcholine iodide, 5,5′-dithio(bis)nitrobenzoic acid, etyleneglycolbis(2-aminoethylether)tetracetate (EGTA), ascorbate, α-tocopherol, coenzyme Q, riboflavin, catalase and superoxide dismutase were of the highest purity commercially available.

Heparinized blood was washed 3 times with isotonic NaCl and the buffy coat was removed after each concentration.

Erythrocyte ghosts were prepared by the method of Dodge et al.[4]; the protein concentration was measured by biuret method; ATPase activity was measured by the method of Nakao et al.[5]. All tubes contained Tri-HCl 40 mM pH 7.7, NaCl 140 mM, KCl 14 mM, $MgCl_2$ 5 mM, ATP-Na 3 mM and erythrocyte ghost 200 µg at a final volume of 1 ml. The reaction was started at 37 °C by the addition of ATP-Na. At the end of the incubation period (30 min) 2.0 ml of trichloracetic acid was added to each tube and the mixture was shaken vigorously. All the tubes were centrifuged at 2000×g for 15 min and the concentration of inorganic phosphorus in an aliquot of the supernatant solutions was measured by the method of Fiske and SubbaRow[6]. The activity of Na^+-K^+-dependent ATPase was determined by the difference between total activity and that in the presence of ouabain. Ca^{++}-ATPase activity was estimated by following procedures. All tubes contained Tris-maleate buffer 50 mM pH 7.0, $MgCl_2$ 5 mM, $CaCl_2$ 0.47 mM, EGTA 0.5 mM, KCl 100 mM, ATP 40 mM and erythrocyte ghost 200 µg at a final volume of 1 ml. The reaction was started by the addition of ATP. At the end of the incubation period (4 min) 1.0 ml of trichloroacetic acid (10%) was added and centrifuged. The concentration of inorganic phosphorus in the supernatant solutions was measured by the method of Martin and Doty[7].

Acetylcholinesterase activity was measured by the method of Beutler et al.[8]. The assay mixture contained 0.1 M Tris-HCl, pH 8.0, EDTA 0.5 mM, 5,5′-dithio(bis)nitrobenzoic acid 0.025 mM prepared in 1% sodium citrate and 10 µl of hemolysate (dilute to 1:200 in H_2O) and 0.5 mM acetylcholine iodide at a total volume 0.1 ml. The reaction was started by the addition of acetylcholine iodide and followed at 412 nm in a recording spectrophotometer.

Results. Aclacinomycin inhibited erythrocyte ATPase (fig. 1). Na^+-K^+-ATPase, ouabain-insensitive ATPase and Ca^{++}-ATPase were inhibited after 30 min incubation at aclacinomycin concentration of 0.1 mg/ml or greater. Acetylcholinesterase was not inhibited after 30 min incubation with aclacinomycin (fig. 2).

The ATPase activities were not inhibited by aclacinomycin if ascorbate was added in a concentration of 50 µM to the incubation mixture. But other radical scavengers such as α-tocopherol, coenzyme Q, riboflavin, catalase and superoxide dismutase had no effect on ATPase activity (figs 3 and 4).

Discussion. Aclacinomycin is an important agent in cancer chemotherapy. Biochemical evidence indicates its antineoplastic property is due to strong intercalative binding to nucleic acids with consequent inhibition of DNA replication and/or RNA synthesis. Anthracyclines (aclacinomycin, doxorubicin and daunorubicin) contain quinone structures that potentially may be reduced in vivo and then auto-oxidized to produce hydrogen peroxide and possibly other reactive oxygen metabolites. Incubation of doxorubicin and daunorubicin with microsomal fractions or with purified cytochrome p450 reductase and NADPH, results in the generation of superoxide and hydrogen peroxide and produces lipid peroxidation[9]. In this system anthracycline undergoes cyclic oxidation-reduction through a free radical semiquinone intermediate with the transfer of electrons from NADPH to molecular oxygen.

In non-enzymatic system, anthracyclines change to semiquinones with the transfer of one electron from ascorbate. Free semiquinone then auto-oxidized to produce hydrogen peroxide and other reactive oxygen metabolites.

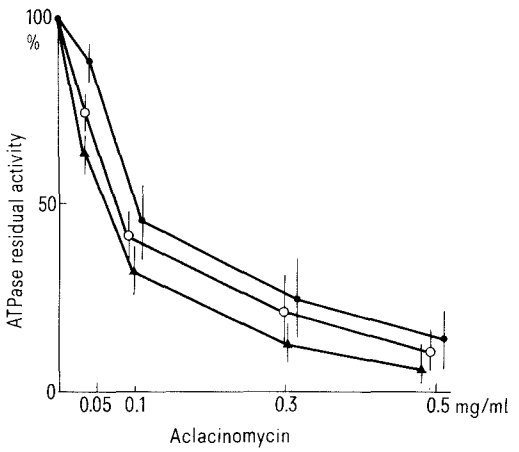

Figure 1. Inhibition of ATPase activity by aclacinomycin. Erythrocytes were suspended in PBS (phosphate buffer-NaCl, 300 mosmol, pH 7.4) at a hematocrit of 30% with various concentrations of aclacinomycin. 30 min after the incubation at 37 °C the erythrocytes were washed 5 times, then the ATPase activities were measured. ●—●, Na-K-ATPase; ▲—▲, Ca-ATPase; ○—○, ouabain insensitive ATPase. Bars indicate SE. n = 6.

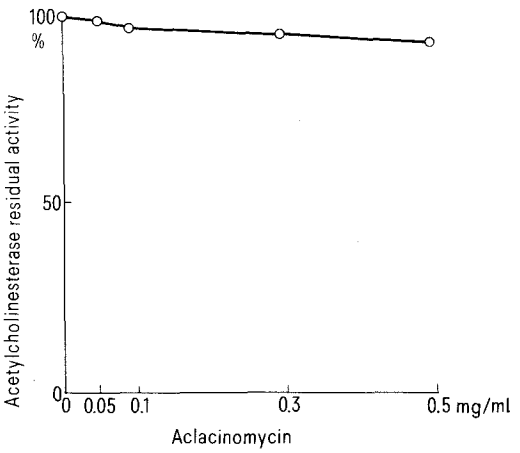

Figure 2. Effect of aclacinomycin on the erythrocyte acetylcholinesterase activity.

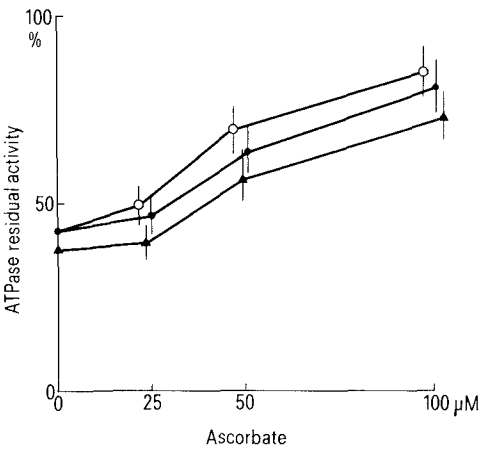

Figure 3. Effect of ascorbate on the ATPase activity inhibited by aclacinomycin. Various concentrations of ascorbate were incubated with the erythrocytes and aclacinomycin (0.1 mg/ml) at 37 °C for 30 min. ●—●, Na-K-ATPase; ▲—▲, Ca ATPase; ○—○, ouabain insensitive ATPase. Bars indicate SE. n = 6.

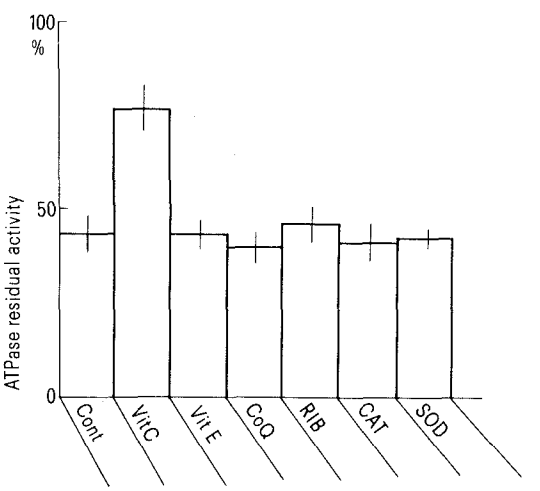

Figure 4. Effects of radical scavengers on the ATPase activities inhibited by aclacinomycin. Cont, no radical scavenger; Vit C, ascorbate (50 µM); Vit E, α-tocopherol (1 mg/ml); Co Q, coenzyme Q (0.1 mg/ml); RIB, riboflavin (0.1 mg/ml); CAT, catalase (1 mg/ml); SOD, superoxide dismutase (30 units/ml). Bars indicate SE. n = 5.

Anthracycline do not inhibit the enzymes of the reduction system (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione reductase, glutathione peroxidase, catalase, superoxide dismutase) of the erythrocytes[3]. Peroxidative damage to erythrocyte membrane proteins and lipids may have a role in the inhibition of ATPase. Indeed, ATPase is known to be lipid dependent. However, another lipid dependent membrane enzyme, acetylcholinesterase is not inhibited by aclacinomycin. Ascorbate is essential to a variety of biological oxidation systems. The most prominent chemical property is its ready oxidation to dehydroascorbic acid. Massive dose of ascorbate may prevent the membrane protein and lipid from peroxidative damage, however the definite generation of reactive oxygen species in intact erythrocytes has not been demonstrated. The erythrocyte model we have described might be useful in the detection and screening of additional compounds capable of producing such radicals. Although our in vitro data show that the effects of aclacinomycin on erythrocytes are dependent on concentration and time of incubation, the clinical use of aclacinomycin would not be expected to affect erythrocytes except perhaps transiently after i.v. administration. However, the possibility that administration at high dosage schedules may cause sufficient oxidative injury to erythrocytes to result in hemolysis should be considered. Pretreatment with massive ascorbate before administration of aclacinomycin may produce semiquinone effectively and reduce lipid peroxidation in the erythrocyte. Ascorbate may ameliorate reverse effects of aclacinomycin without impairing tumor response.

1 Ogawa, M., Inagaki, J., Horikoshi, N., Inoue, K., Chinen, T., Ueoka, H., and Nagura, E., Cancer Treatment Rep. *63* (1979) 931.
2 Henderson, C.A., Metz, E.N., Balcerzak, S.P., and Sagone A.L., Jr, Blood *52* (1978) 878.
3 Shinohara, K., and Tanaka, K.R., Hemoglobin *4* (1980) 735.
4 Dodge, J.T., Mitchell, C., and Hanahan, D.J., Archs Biochem. Biophys. *100* (1963) 119.
5 Nakao, T., Nagano, K., Adachi, K., and Nakao, M., Biochem. biophys. Res. Commun. *13* (1963) 444.
6 Methods in Enzymology, vol.3, p.843. Academic Press, New York 1957.
7 Martin, J.B., and Doty, D.M., Analyt. Chem. *21* (1949) 965.
8 Beutler, E., Blume, K.G., Kaplan, J.C., Lohr. G.W., Ramot, B., and Valentine, W.N., Br. J. Haemat. *35* (1977) 331.
9 Goodman, J., and Hochstein, P., Biochem. biophys. Res. Commun. *77* (1977) 797.

0014-4754/83/121362-03$1.50+0.20/0

Stimulation of cyclo-oxygenase by lidocaine, a pro-lipoperoxidant

C. Deby, G. Deby-Dupont, P. Hans, J. Pincemail and J. Bourdon-Neuray

Laboratoire de Biochimie Appliquée, Faculté de Médecine, Université de Liège, Blvd. de la Constitution, 32, B-4020 Liège (Belgium), February 3, 1983

Summary. Lidocaine, which was recently demonstrated to be a good pro-lipoperoxidant, was tested on in vitro PGs biosynthesis, and on arachidonate-induced arterial hypotension, in the rabbit. In the in vitro experiments, lidocaine alone was a poor stimulant of cyclo-oxygenase, but it enhanced significantly the cyclo-oxygenase activation of uric acid. In the rabbit, lidocaine lowered the i.v. arachidonate dose necessary to obtain a significant drop of blood pressure.

In 2 preliminary communications, we demonstrated that lidocaine has a pro-lipoperoxidant effect in that it accelerates the arachidonic acid autoxidation and the decomposition of arachidonic lipoperoxides into malonaldehyde[1,2].

It is well known that lipoperoxides are necessary to trigger and to maintain the cyclo-oxygenase activity[3]. Therefore, it would be of interest to know the effect of lidocaine on that enzyme activity in 'in vitro' and 'in vivo' conditions. To answer those questions, we have performed 2 kinds of experiment. First, we have studied the influence of lidocaine on prostaglandin biosynthesis by microsomes of bull seminal vesicles. In order to avoid the auto-inactivation of cyclo-oxygenase by excited oxygen species during the production of peroxides, uric acid, a potent free radical scavenger[5], was added to the reaction medium. In a second kind of experiment, we observed the arachidonate-induced hypotension in rabbits treated or not treated with lidocaine.

Material and methods. In vitro PGs biosynthesis. Microsomes of bull seminal vesicles were prepared following a procedure published elsewhere[6]. 14-C arachidonate was incubated in the presence of glutathione (10^{-4} M), in a phosphate buffer (6.10^{-2} M) at pH 7.8. After extraction with diethylether, the prostanoids were separated on TLC plates, and the labeled spots were visualized by autoradiography and automatically eluted by Eluchrom devices. Radioactivity counting was performed using a Nuclear Chicago beta scintillation counter. Uric acid was carefully dissolved in a phosphate buffer (pH 7.8), heated in a water bath at 40 °C and then added to the medium at a final concentration of 10^{-3} M. Lidocaine (Xylocaine®) was also used, at the same final concentration.

Arachidonate-induced hypotension in a rabbit model. This model has been described elsewhere[6,7]. It consists of rabbits anesthetized with nembutal (40 mg/kg) and heparinized i.v. (3 mg/kg) 1 h before the assays. They were intubated and artificially ventilated by a Servo-Ventilator Siemens Elema 900 B respirator, with an N_2/O_2 mixture. A 22% FiO_2 was maintained, monitored by a polarographic gauge. Arterial blood pressure, venous pressure and electrocardiogram were continuously registered by a Sanborn 7700 device.

In heparinized rabbits, after 1 h, the AA_{50} (see fig.2) falls from 400–500 µg/kg to ± 150 µg/kg[6,7], and the animals can be used for in vivo arachidonate conversion studies.

To compare the effects of different agents on the AA_{50}, we use a sensitization coefficient. This is the number by which the previous AA_{50} must be divided to obtain the new AA_{50}, observed after the administration of the agent. The larger the sensitization coefficient, the more the agent stimulates the in vivo arachidonic acid conversion.