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INHIBITION OF Na^+ AND K^+-STIMULATED ATPase OF RABBIT HEART SARCOLEMMA AFTER ADMINISTRATION OF HEPARIN

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SUMMARY - Intravenous heparin administration caused a marked inhibition of Mg^{++}-dependent (Na^++K^+)-stimulated ATPase activity of sarcolemmal (SL) membranes prepared from rabbit heart, whereas basal Mg^{++}-ATPase was not affected. The inhibition depended on K^+ concentration and was reversed only in the presence of albumin. Plasma free fatty acid (FFA) concentrations were raised in all animals, after heparin administration. The results obtained support the concept that FFA or other lipids originating in the plasma by the action of lipolytic enzymes released by heparin are involved in the mechanism of inhibition.

INTRODUCTION - Heparin intravenously injected releases into the circulating blood, several lipid hydrolysing enzymes as at least two different lipoprotein lipases LPL (1) and phospholipase A_1 (2,3). The action of these enzymes on blood lipids leads to increase concentrations of plasma FFA (1) and lysophospholipids (4,5). Both of these products have inhibitory effect on the (Na^++K^+)-ATPase activity in vitro (6-9). It is known that this enzyme is involved in active ion transport across the cell membrane, a phenomenon, which is of paramount importance for the performance of the heart, which also utilizes fatty acids as the major source of energy. For these reasons and since heparin is widely used in therapeutics of cardiovascular diseases, it was of interest to examine if this enzyme activity is affected in the SL of the heart following administration of the drug to experimental animals.

ABBREVIATIONS - SL: Sarcolemma, FFA: Free fatty acids, SCF: Soluble cytoplasmic fraction, glc: Gas liquid chromatography, tlc: Thin layer chromatography, cpm: Counts per min.

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MATERIALS AND METHODS - Animals: Male New Zealand rabbits of 2-2.5 kg body weight were given intravenously sodium heparin (166 units/mg, Leo Pharmaceutical Products, Ballerup, Denmark) in NaCl 0.9% solution and killed after 40 min by intravenous injection of 1 ml thiopental sodium 10% solution, followed by an air bubble. Samples of blood for FFA determination were taken prior to heparin administration and before killing the animals. Control animals were given only NaCl 0.9% solution.

Preparation of heart SL: Hearts were excised immediately after death and immersed in ice cold Tris-sucrose buffer (0.25 M sucrose, 0.5 mM EGTA and 20 mM Tris-HCl pH 7.4). Ventricles were freed of visible fat, cut into small pieces, washed free of blood, weighed, placed in 4 volumes of Tris-sucrose buffer and homogenized for 20 sec (2 times x 10 sec) in a Virtis Model 45 Omnimixer, cooled at -5°. All operations were performed at low temperature (0°-4°C). The following steps for the isolation of the heart SL are essentially those described by Dietze and Hepp (10) based on the work of Stam et al. (11). The first homogenate was given 4 strokes in a motor driven Potter Elvehjem homogenizer with plastic pestle and centrifuged at 2300 g for 15 min. The pellet was resuspended and washed 6 times with decreasing time and g-forces down to 470 g x 3 min. Filtration of the homogenate took place before the third centrifugation. The supernatants of the first three centrifugations were pooled and used for isolation of mitochondria and microsomes (10). The final pellet was suspended in 5 vols (based on the initial weight of tissue) of 0.9% NaCl. To this suspension an equal volume of a solution containing 6.6 mM NaI, 50 mM Cysteine, 5 mM $MgCl_2$, 3 mM ATP and 5 mM EDTA (dipotassium salt) (adjusted to pH 7.4 with Tris, was slowly added under stirring. The suspension was stirred for 60 min, diluted with cold water to a NaI concentration of 0.8 M and centrifuged for 20 min at 4300 g. The pellet was washed twice with Tris-sucrose buffer, taken up in the same buffer (about 1 ml per 2 mg of protein), and stored in several aliquots at -40°. The SL was usually isolated from about 8 g of ventricles from two or three control and experimental animals.

Enzyme assays: The Mg^{++}-dependent $(Na^{+}+K^{+})$-activated ATPase activity was measured as described by Post and Sen (12) except the ATP and Mg^{++} were 3 mM. Glucose-6-phosphatase was assayed as described by Harper (13). Inorganic phosphate was determined by the method of Fiske and Subbarow (14). Protein was determined by the method of Lowry et al. (15). Free fatty acids of the plasma were determined by the method of Laurell and Tibbling (16). To obtain the soluble cytoplasmic fraction (SCF) a small aliquot of the tissue homogenate was filtered immediately after homogenation through Millipore filter (0.45 µm). Isolation of FFA from SL and SCF was performed as follows. Total lipids (17) were treated with diazomethane and methylesters of the FFA separated by tlc and analysed by glc (18). An internal standard of heptadecanoic acid was used for quantitation. Counting of radioactivity was performed as described previously (19).

RESULTS AND DISCUSSION - The SL fraction prepared by the procedure described in the Methods was examined by phase contrast microscopy and showed the presence of empty cell segments almost completely free of visible striations. This fraction was also characterized by a high ratio of $\frac{(Na^{+}+K^{+})\text{ATPase}}{Mg^{++}\text{-ATPase}} = 1.4$ (6) as compared to the microsomal and mitochondrial fractions that had a ratio of less than 0.1. A low microsomal contamination was deduced by the low

TABLE I. EFFECT OF ADMINISTRATION OF HEPARIN ON $(Na^+ + K^+)$-STIMULATED ATPase OF RABBIT HEART.

Exp.	$Mg^{++}+Na^+ +K^+$ (a)		Mg^{++} (b)		ΔPi (a-b)	
	Control	Heparin	Control	Heparin	Control	Heparin
1	7.9	5.3	3.1	3.2	4.8	2.1
2	8.8	5.3	4.5	3.2	4.3	2.1
3	9.1	5.6	4.5	3.7	4.6	1.9
4	8.8	6.5	3.0	4.0	5.8	2.5
5	9.0	7.1	3.5	3.9	5.5	3.2
6	11.1	7.8	4.2	3.7	6.9	4.1
7	10.2	5.6	4.6	4.0	5.6	1.6
Mean value ± SE			3.9±0.26	3.7±0.13	5.3±0.33	2.5±0.32
						p<0.005
8	18.7	14.9	10.4	11.1	8.3	3.3

Heparin was given intravenously 30 mg/kg body weight for exp. 1 to 7 and 4 mg/kg for exp. 8. SL membranes were isolated from the hearts 30-40 min after the drug administration and assayed for ATPase activity in triplicate samples as described in Methods. Time of incubation was 20 min and SL membrane protein 20-60 µg per ml. The same quantities of protein from control and heparin SL were used for the enzyme assay.

glucose-6-phosphatase activity of the SL fraction (0.2 µmoles Pi/mg protein/hr) as compared to the microsomes (1.2 µmoles Pi/mg protein/hour). The $(Na^+ + K^+)$-stimulated ATPase was completely inhibited in the presence of 0.1 mM ouabain. The effect of heparin on the Mg^{++}-dependent $(Na^+ + K^+)$-stimulated ATPase activity of the heart SL is shown in Table I. It can be seen that 30 or 4 mg per Kg body weight of heparin injected intravenously results to inhibition of the $(Na^+ + K^+)$-activated enzyme to about 50%. In contrast, the basal Mg^{++}-dependent ATPase was not affected, suggesting the specificity of the inhibition. Heparin had no direct in vitro effect on the $(Na^+ + K^+)$-activated

ATPase because 1) when (N-sulfonate-^{35}S) heparin was injected to the animals together with non-radioactive drug, it was not detectable in the sarcolemma fraction and 2) when the enzyme reaction mixture was preincubated with heparin in concentrations from 7 to 130 µg per ml the (Na^++K^+)-ATPase activity was not affected.

Free fatty acids inhibit the (Na^++K^+)-ATPase in vitro (6-8). This finding has been confirmed in these experiments (myristate 0.05 mM and 0.1 mM inhibited the enzyme activity of the SL of the control animals by 33% and 55% respectively). Increased levels of FFA were found in the plasma of the experimental animals following heparin administration mean 0.57 ± 0.063 mEq/l as compared to mean 0.33 ± 0.021 mEq/l; $p<0.005$ before the drug administration for 10 animals. Contrary, the FFA concentration of the SCF of the heart was remarkably constant and did not differ in the two groups of animals (0.12 ± 0.012 µEq/mg protein without heparin and 0.13 ± 0.022 µEq/mg protein with heparin, n=5). These results strongly suggest that if the inhibitory effect of heparin derives from fatty acids they should originate in the plasma. Evidence suggesting the participation of FFA in the mechanism of the inhibition are presented in Table II. Albumin, which binds fatty acids with high affinity, can restore the in vivo inhibited (Na^++K^+)-ATPase activity. Also the same table shows that the percent inhibition of the enzyme in the heparin group increased inversely with K^+ concentration. This finding is in accordance with the competitive effect of K^+ on the inhibition of (Na^++K^+)-ATPase which is reported to exist for the in vitro inhibition by fatty acids (7,8).

It is known that in certain tissues uptake of fatty acids at superficial sites of the cell membrane occurs at low temperatures (20). In this connection ^{14}C labelled fatty acids were used in order to test the possibility that FFA of the SCF during homogenation of the heart are taken up by the SL. The results shown in Table III clearly demonstrate that in each of the two experiments the SL fractions of the control and heparin groups have absorbed at 4^o the same amount of radioactive fatty acids. It is understood

TABLE II. EFFECT OF K^+ AND ALBUMIN ON THE $(Na^+ + K^+)$-ATPase OF THE HEART SL OF CONTROL AND HEPARIN INJECTED RABBITS

Additions	Specific activity of $(Na^+ + K^+)$-ATPase (ΔPi)			
	Experiment I		Experiment II	
	Control	Heparin	Control	Heparin
K^+ 20 mM			8.3	3.8(54)
K^+ 10 mM			6.5	2.6(60)
K^+ 2 mM			5.2	1.6(69)
No Albumin	4.5	1.1		
Albumin 1.5 mg	5.1	3.3		

Assay of Na^+ and K^+ stimulated ATPase activity was performed as described in Methods except that K^+ or albumin were added in the incubation medium as shown above. Numbers in parentheses are percentage of inhibition.

that these fatty acids prior to absorption to the SL were diluted with the endogenous FFA of the SCF, on this basis the calculated amount of FFA taken up by the SL was negligible (<5 nmoles/100 µg protein) and could not interfere with the $(Na^+ + K^+)$-ATPase activity. Furthermore, as shown in the same Table most of the FFA taken up at 4^o were washed out from the SL at 37^o without any change in the degree of inhibition, which is completely reversed only in the presence of albumin. The finding strongly suggests that the FFA or other inhibitors of the $(Na^+ + K^+)$-ATPase are bound in vivo at specific sites of the SL where from they can be removed only with the aid of albumin.

The rate of uptake of FFA by the heart under normal cardiac work depends to a large extent on the FFA:albumin-molar ratio and up to a certain ratio the FFA uptake by the cells increases with FFA concentration of the plasma (21). Therefore, the inhibition of the $(Na^+ + K^+)$-ATPase activity in the SL of the hearts of the heparin-treated animals, where increased concentration of plasma FFA was detected, can be explained on the basis of an increased flux of FFA through the cell membrane. Other possible inhibitors related to the

TABLE III. INHIBITION OF THE (Na^++K^+)-STIMULATED ATPase ACTIVITY OF THE SL AND FATTY ACID UPTAKE AT 4° AND RELEASE AT 37°

Additions or Conditions	Experiment I		Experiment II	
	Control	Heparin	Control	Heparin
	(Na^++K^+)-ATPase (ΔPi)			
	μmoles Pi/mg protein/h			
SL(1)	4.5	1.1	8.5	3.0
SL(2) washed at 37°	3.8	1.2	5.2	2.5
Same as above plus albumin 1.5 mg	4.2	4.0	5.0	4.8
	FFA uptake at 4° and release at 37°			
	μmoles/100 μg protein)			
SL(1)	2.6(860)	2.8(780)	3.1(800)	2.3(600)
SL(2)	0.5(160)	0.7(200)	0.7(200)	0.6(150)

Numbers in parentheses are cpm/100 μg protein.

SL(1) membranes were isolated as described in Methods except that a mixture of [U-^{14}C]stearic acid (sp.act. 102 mCi/mmol) and [1-^{14}C]oleic acid (sp.act. 58 mCi/mmol) 5 μCi each (total 7.3x10^6 cpm) was added in the first homogenate dissolved from 50 λ alcoholic solutions to the Tris-sucrose buffer. FFA of the SL were isolated and counted for radioactivity, FFA of the SCF were isolated, counted for radioactivity and estimated quantitatively. Assuming that the added FFA are diluted with the FFA of the SCF the amounts of FFA (nmol/100 μg protein) taken up by the SL were calculated as follows: $\frac{\text{cpm/100 μg protein of the SL}}{\text{cpm/nmol FFA of the SCF}}$.

SL(2) is SL(1) washed at 37° as follows: membrane material was incubated in Tris-sucrose buffer for 5 min at 37° (600 μg protein in 8 ml) centrifuged and the sediment used for the enzyme assay. The amount of protein present in the incubation medium was 40 μg for Exp. I and 20 μg for Exp.II.

action of heparin are the lysophospholipids, which are found increased in the blood shortly after heparin injection (4). These lipids are known as inhibitors of the (Na^++K^+)-ATPase in vitro (6,9) and can be bound to albumin, which restored the inhibited enzyme activity (Table II), experiments are now in pro-

gress to investigate this possibility.

The physiological consequences of the present findings are not yet known, but voluminous data are accumulated that indicate the close relationship between the inhibitory effect on $(Na^+ + K^+)$-ATPase and the in vivo inotropic effect of cardiac glycosides (22), however the manner in which the inhibition of this enzyme induces the inotropic effect is not at all clear. To our knowledge it has never been reported that heparin has a positive inotropic effect though as shown here its administration caused a marked inhibition of the $(Na^+ + K^+)$-ATPase. It seems therefore reasonable to believe that the mechanism by which heparin administration to the animal results in inhibition of $(Na^+ + K^+)$-ATPase of heart SL is not related to the development of the positive inotropic effect. In other words the present findings provide strong evidence for the dissociation of inhibition of $(Na^+ + K^+)$-ATPase from the positive inotropic effect of digitalis which is gaining much support since the past few years (23,24). In this connection useful information may derive by further investigating the mechanism involved in the inhibition of $(Na^+ + K^+)$-ATPase described in this report. In conclusion the present experimental work provides for the first time evidence for the inhibition of the $(Na^+ + K^+)$-stimulated ATPase following heparin administration a drug, which is widely used in therapeutics.

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REFERENCES

1. Olivecrona, T., Hernell, O., and Egelrud, T., (1975) Heparin, Structure, Function and Clinical Implications, pp. 269-279, Bradshaw, R. and Wessler, S. Eds., Plenum Press, New York and London.
2. Zieve, F., and Zieve, L., (1972) Biochem.Biophys.Res.Comm., 47, 1480-1485.
3. Waite, M., and Sisson, P., (1973) J. Biol. Chem., 248, 7201-7206.
4. Besterman, E., and Gillet, M., (1973) Atherosclerosis, 17, 503-509.
5. Brown, W., Shaw, W., Baginsky, M., Boberg, J., and Augustin, J., (1975) Lipoprotein Metabolism, p. 2 Grêten H., Heidelberg, Springer-Verlag.
6. Skou, J., (1964) Progr.Biophys.Mol.Biol. 14, 133-166.
7. Ahmed, K., and Thomas, B., (1971) J.Biol. Chem., 246, 103-109.
8. Lamers, J., and Hülsmann, W., (1977) J. Mol. Cell Cardiology, 9, 343-346.
9. Godman, S.S., and Albers, R.W., (1973) J. Biol. Chem., 248, 867-874.

10. Dietze, G., and Hepp, K., (1971) Biochem. Biophys. Res. Comm., 44, 1041-1049.
11. Stam, Jr., A.C., Weglicki, Jr. W., Feldman, D., Shelburne, J., and Sonnenblick, E., (1970) J. Mol. Cell. Cardiol. 1, 117-130.
12. Post, R.L., and Sen, A.K., (1967) Methods in Enzymology, 10, pp. 762-768, Estabrook, R., and Pullman, M. Eds., Academic Press, New York and London.
13. Harper, A.E., (1963) Methods of Enzymatic Analysis, pp. 788-792, Verlag Chemie, New York.
14. Fiske, C.H., and Subbarow, Y., (1957) Methods in Enzymology, 3, pp. 843-844, Colowick S.P., and Kaplan, N.O., Eds., Academic Press, New York.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., (1951) J. Biol. Chem. 193, 265-275.
16. Laurell, S., and Tibbling, G., (1967) Clin. Chim. Acta, 16, 57-62.
17. Folch, J., Lees, M., and Sloane-Stanley, G.H., (1957) J. Biol. Chem., 226, 497-509.
18. Karli, J.N., and Levis, G.M., (1974) Lipids, 9, 819-824.
19. Levis, G.M., Efstratiadis, A.A., Mantzos, J.D., and Miras, C.J., (1975) Rad. Res., 61, 342-349.
20. Mishkin, S., Yalovsky, M., and Kessler, J., (1972) J. Lipid Res. 13, 155-168.
21. Neely, J.R., and Morgan, H.E., (1974) Ann.Rev. Phys., 36, 413-459, Camroe, J.H. ed., Annual Reviews Inc., Palo Alto, California.
22. Askari, A., (1974) Properties and functions of Na^+, K^+ activated-Adenosine triphosphatase, 242, pp. 1-741, Ann. N.Y. Acad. Sc., New York.
23. Peters, T., Raven, R.H., and Wassermann, O., (1974) Eur.J.Pharm. 26, 166-174.
24. Okita, G.T., Richardson, F., and Roth-Schechter, B.F., (1973) J. Pharmacol. Exp.Ther., 185, 1-11.