Uncoupling of Muscle and Blood Platelets Ca\textsuperscript{2+} Transport ATPases by Heparin

REGULATION BY K\textsuperscript{+*}

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Heparin (1-10 µg/ml) inhibits the Ca\textsuperscript{2+} transport ATPases found in the sarcoplasmic reticulum of skeletal muscle, the plasma membrane of red cells, and the dense tubular system of blood platelets, but not the (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase or the mitochondrial F\textsubscript{1}-ATPase. In the reversal of the Ca\textsuperscript{2+} pump, heparin uncouples the synthesis of ATP from Ca\textsuperscript{2+} efflux and inhibits the phosphorylation of the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase by P\textsubscript{i}, but has no effect the phosphorylation by ATP.

The effect of heparin on the muscle Ca\textsuperscript{2+}-ATPase is abolished by KCl and NaCl (100 mM) and to a lesser extent by LiCl. These monovalent cations are not effective as antagonists of heparin on the platelet Ca\textsuperscript{2+}-ATPase.

The effects of heparin are antagonized by the polyamines spermine, spermidine, and putrescine. Unlike KCl, polyamines are equally effective in counteracting the effects of heparin in both muscle and platelet Ca\textsuperscript{2+}-ATPases. In addition to heparin, the Ca\textsuperscript{2+}-ATPases of muscle and blood platelets are also inhibited by dextran sulfate and fucose-branched chondroitin sulfate.

The inhibition promoted by these glycosaminoglycans is antagonized by monovalent cations and polyamines in the same manner as heparin. Heparan sulfate, chondroitin sulfate, and hyaluronic acid have no effect on the Ca\textsuperscript{2+}-ATPases studied.

Heparin is a sulfated glycosaminoglycan found in the granules of mast cells. These cells are found alongside blood vessels, and tissues that are well vascularized are also rich in heparin. They include liver, lung, skin, and the peritoneal wall (1-3). For a long time, heparin has been widely used as an anticoagulant (4, 5). Clinical observation reveals that 5-10% of patients treated with heparin develop thrombocytopenia (6, 7).

Recent observations suggest that heparin is involved in different physiological events. This includes modulation of the effects of growth factors on target cells (8-10) and modulation of Ca\textsuperscript{2+} release promoted by inositol triphosphate. The effect of heparin on Ca\textsuperscript{2+} release seems to vary among the different tissues studied. In brain, liver, and adrenal cortex, heparin blocks the binding of inositol triphosphate to its receptor, and thus inhibits the Ca\textsuperscript{2+} release from internal stores (8-15).

In muscle cells, however, the role of inositol triphosphate and heparin in the mechanism of Ca\textsuperscript{2+} release is controversial. While some authors have presented evidence that inositol triphosphate promotes the release of Ca\textsuperscript{2+} that triggers muscle contraction (16-19), other authors found that heparin has no effect and inositol triphosphate is not involved in the release of Ca\textsuperscript{2+} (20-23). Recently Rojas and Jaimovich (24) observed that in frog permeabilized skeletal muscle, heparin does not inhibit Ca\textsuperscript{2+} release; on the contrary, it enhances the release of Ca\textsuperscript{2+} promoted by inositol triphosphate. In this muscle preparation heparin promotes the release of a small amount of Ca\textsuperscript{2+} even in the absence of inositol triphosphate (24). This effect of heparin is different from that described for liver and brain. Ritov et al. (25) observed that heparin induces the release of Ca\textsuperscript{2+} from the terminal cisternae but has no effect on the longitudinal vesicles of skeletal muscle sarcoplasmic reticulum. In these experiments, 100 mM KCl was included in the assay medium (25).

In this report it is shown that heparin inhibits the Ca\textsuperscript{2+} pump found in different membrane preparations. These include vesicles derived from the sarcoplasmic reticulum of skeletal muscle (SR), vesicles derived from the dense tubular system of human blood platelets (PV), and the plasma membranes of red cells.

MATERIALS AND METHODS

Vesicles derived primarily from the longitudinal tubules of the SRV were isolated from rabbit skeletal muscle as described by Eletr and Inesi (26) and stored in liquid nitrogen. As previously shown (27), electrophoretic analysis of the preparation reveals practically no ryanodine-sensitive Ca\textsuperscript{2+} channels, which are found in junctional vesicles. This vesicle preparation is not sensitive to ryanodine or caffeine, nor does it exhibit the phenomenon of activation of Ca\textsuperscript{2+} efflux by external Ca\textsuperscript{2+} (i.e. Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-release).

Vesicles derived from the dense tubular system of PV were prepared as described by Le Peuch et al. (28). Plasma enriched with platelets was obtained from a blood bank. The PV were resuspended in 20 mM Hepes buffer, pH 7.5, containing 94 mM KCl, 5 mM MgCl\textsubscript{2}, 50 µM CaCl\textsubscript{2}, 0.25 mM sodium azide, 0.1 mM phenylmethylsulfonyl fluoride, and 20% glycerol (v/v) and stored in liquid nitrogen until use. Each sample was used only once. The Ca\textsuperscript{2+} uptake and Ca\textsuperscript{2+}-ATPase activity of the preparation did not vary after 2 months of storage in liquid nitrogen.

Vesicles derived from red cell plasma membranes (29), the (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase (30), and the soluble F\textsubscript{i} from bovine heart mitochondria (31) were prepared as previously described.

Ca\textsuperscript{2+} uptake and efflux were measured at 35 °C by a filtration method using 40 Ca in a medium containing 50 mM MOPS-Tris, pH 7.0, 10 mM MgCl\textsubscript{2}, 20 mM P\textsubscript{i}, 0.3 mM CaCl\textsubscript{2}, 2 mM ATP, and 0.06 mg of vesicle protein/ml. After 50 min at 40,000 x g for 20 min, the supernatant was discarded, and the walls of the tubes were blotted to minimize the volume of residual loading me-

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1 The abbreviations used are: SRV, sarcoplasmic reticulum vesicles; PV, platelet vesicles; Mops, 4-morpholino propane sulfonic acid.
RESULTS

Inhibition of Ca\(^{2+}\) Transport ATPases by Heparin.—SRV are able to accumulate a larger amount of Ca\(^{2+}\) and at a faster rate than the PV (Fig. 1). In both preparations the Ca\(^{2+}\) uptake is inhibited by heparin. Addition of heparin after the vesicles are filled promotes the release of Ca\(^{2+}\) (Fig. 1). The effect of heparin is more pronounced in PV (Fig. 2) than in SRV (Fig. 3). In eight preparations tested the concentration needed for half-maximal inhibition of Ca\(^{2+}\) uptake varied between 0.3 and 1 \(\mu\)g/ml, whereas for the SRV half-maximal inhibition was observed with 2–5 \(\mu\)g/ml heparin. The concentration of heparin needed to prevent blood coagulation is 10 \(\mu\)g/ml (4–7). In the presence of low heparin concentrations, both the PV and the SRV seem to become leaky: the decrease in Ca\(^{2+}\) uptake is not accompanied by a decrease in ATPase activity (33). At higher concentrations both the Ca\(^{2+}\) uptake and the ATPase activity are inhibited by heparin (Figs. 2 and 3). Inhibition of the ATPase activity by heparin is observed with both intact SRV (Fig. 3) and SRV solubilized with the detergent Triton X-100 (Fig. 4). The calmodulin-activated Ca\(^{2+}\) transport ATPase found in the plasma membrane of red cells is also inhibited by heparin, whereas the (Na\(^{+}\) + K\(^{+}\))-ATPase of plasma membranes and the mitochondrial F\(_{0}\)-ATPase are not (Fig. 4).

Effects of Cations—In SRV, the effects of heparin on both Ca\(^{2+}\) uptake and ATPase activity are antagonized by KCl, NaCl, and Mg\(^{2+}\), to a much lesser extent by LiCl (Figs. 3 and 5). In PV, however, KCl and NaCl abolish the effect of heparin on the ATPase activity but have only a modest effect on the inhibition of Ca\(^{2+}\) uptake (Figs. 2 and 5). Thus, in the presence of physiological concentrations of KCl and NaCl, an inhibition of Ca\(^{2+}\) uptake by heparin is observed in PV but not in SRV preparations. LiCl does not antagonize the inhibition of either Ca\(^{2+}\) uptake (Fig. 5) or ATPase activity (data not shown) measured with PV.

The inhibition by heparin of the Ca\(^{2+}\)-ATPase from red cells is also antagonized by KCl (Fig. 4). Notice in Fig. 4 that the cation concentrations used to test the effect of heparin on the (Na\(^{+}\) + K\(^{+}\))-ATPase were very low, and would be insufficient to antagonize the effect of heparin on the divalent cation ATPases. Free Mg\(^{2+}\), up to a concentration of 20 mM, did not antagonize the effects of heparin on either SRV or PV (data not shown). Higher concentrations of Mg\(^{2+}\) were not used since above 20 mM, this cation inhibits Ca\(^{2+}\) transport in both membrane preparations.

Ruthenium red and the polyamines spermine and spermidine, but not putrescine, are also able to antagonize the effects of heparin on Ca\(^{2+}\) uptake and Ca\(^{2+}\)-dependent ATPase activity measured in SRV (Fig. 6) and PV (data not shown). In the absence of heparin, these drugs had no effect on the Ca\(^{2+}\) transport and, unlike K\(^{+}\) and Na\(^{+}\), they were equally effective in antagonizing the effects of heparin in both muscle SRV and PV.

Reversal of the Ca\(^{2+}\) Pump.—The catalytic cycle of the Ca\(^{2+}\)-ATPase is reversed when a Ca\(^{2+}\) gradient is formed across the SRV membrane (36–40). A slow Ca\(^{2+}\) efflux is observed when SRV previously loaded with Ca\(^{2+}\) are incubated in a medium containing EGTA, P\(_{i}\), and Mg\(^{2+}\). This efflux is referred to as passive efflux. The addition of ADP enhances the rate of Ca\(^{2+}\) efflux, which is now referred to as total Ca\(^{2+}\) efflux. The difference between total efflux and passive efflux is coupled with the
Different Sensitivities of Ca\textsuperscript{2+}-ATPases to Heparin and K\textsuperscript{+}

**Fig. 4. Effects of heparin on different transport ATPases.** The assay medium composition was 50 mM Mops-Tris buffer, pH 7.0, 3 mM MgCl\textsubscript{2}, 1 mM ATP, and (A) 0.1 mM CaCl\textsubscript{2}, 0.1 µg/ml calmodulin, and 0.2 mg/ml red cells plasma membrane; (B) same plus 100 mM KCl; (C) 0.1 mM CaCl\textsubscript{2}, 0.1 mg/ml SRV, and 0.1 mg/ml Triton X-100; (D) same plus 100 mM KCl, 2 mM KCl, 10 mM NaCl, and 0.04 mg/ml (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase; (E) 0.02 mg/ml soluble F\textsubscript{1}-ATPase. In the figure, 100% activity represents for: the red cell plasma membrane, 2.8 (without KCl) and 3.2 (with KCl; (with 100 mM KCl) pmol of Pi/mg/5 min; for the (Na\textsuperscript{+}/K\textsuperscript{+})-ATPase, 13.6 µmol of P/mg/5 min; and for the soluble F\textsubscript{1}-ATPase, 2.30 umol of Pi/mg/5 min.

**Fig. 5. Antagonism of the heparin effect by K\textsuperscript{+}, Na\textsuperscript{+}, and Li\textsuperscript{+}.** The assay medium composition and experimental conditions were as described in the legend to Fig. 1 using either SRV (A) or PV (B). The incubation times were 5 min for SRV and 40 min for PV. Open symbols, control (without heparin) and closed symbols, with 10 µg/ml heparin. ○, KCl; △, NaCl; and □, LiCl.

**Fig. 6. Antagonism of the heparin effect by polyamines.** The assay medium composition and experimental conditions were as described in the legend to Fig. 1 using SRV. Open symbols, control (without heparin) and closed symbols, with 10 µg/ml heparin. A, Ca\textsuperscript{2+} uptake; B, Ca\textsuperscript{2+}-dependent ATPase activity; ○, ruthenium red; △, spermine; □, spermidine; and ▽, △, putrescine.

**Fig. 7. Reversal of the Ca\textsuperscript{2+} pump uncoupling by heparin.** A, passive (○) and total (●) Ca\textsuperscript{2+} efflux. SRV previously loaded with 3.2 µmol of Ca\textsuperscript{2+}ing of SV protein were diluted to a final concentration of 0.04 mg of protein/ml in a medium containing 60 mM Mops-Tris buffer, pH 7.0, 2 mM EGTA, 4 mM P\textsubscript{i}, and 1 mM MgCl\textsubscript{2} without ADP (○) or with 0.2 mM ADP (●). B, active Ca\textsuperscript{2+} efflux, calculated by subtracting the values of passive efflux from the values of total Ca\textsuperscript{2+} efflux measured in A. C, synthesis of ATP. The incubation time at 35 °C was 5 min.

**Table I**

<table>
<thead>
<tr>
<th>Additions</th>
<th>ATP synthesis</th>
<th>ATP\textsubscript{2+}P exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Heparin, 10 µg/ml</td>
</tr>
<tr>
<td>SRV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>188 ± 32 (4)</td>
<td>39 ± 9 (4)</td>
</tr>
<tr>
<td>100 mM KCl</td>
<td>176 ± 35 (4)</td>
<td>151 ± 21 (4)</td>
</tr>
<tr>
<td>100 mM LiCl</td>
<td>183 ± 33 (3)</td>
<td>52 ± 13 (3)</td>
</tr>
<tr>
<td>PV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>25 ± 3 (3)</td>
<td>6 ± 1 (3)</td>
</tr>
<tr>
<td>100 mM KCl</td>
<td>26 ± 4 (3)</td>
<td>22 ± 3 (3)</td>
</tr>
<tr>
<td>100 mM LiCl</td>
<td>24 ± 2 (3)</td>
<td>9 ± 3 (3)</td>
</tr>
</tbody>
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synthesis of ATP from ADP and P\textsubscript{i} and is referred to as active Ca\textsuperscript{2+} efflux (36–40). Heparin uncouples the synthesis of ATP from the Ca\textsuperscript{2+} efflux in both SRV and PV (Fig. 7 and Table I). The passive Ca\textsuperscript{2+} efflux is enhanced by heparin up to a rate similar to that measured with ADP. Thus, as the heparin concentration in the medium is raised, the total Ca\textsuperscript{2+} efflux remains constant but the synthesis of ATP is progressively abolished.

As shown above for Ca\textsuperscript{2+} uptake, KCl antagonizes the effect of heparin on the synthesis of ATP; LiCl has little or no effect (Table I). The synthesis of ATP is also restored by spermine and spermidine in both SRV and PV (Fig. 8 and PV (data not shown)).

The reversal of the Ca\textsuperscript{2+} pump can also be studied in steady-state conditions by measuring the ATP\textsubscript{2+}P exchange reaction (38–41). During Ca\textsuperscript{2+} uptake, a Ca\textsuperscript{2+} gradient is formed across the vesicle membrane and after the initial seconds of incubation, a steady exchange between 32P\textsubscript{v} and the γ-phosphate of ATP is observed. The ATP\textsubscript{2+}P exchange reaction indicates that the ATP\textsubscript{2+}P pump operates simultaneously forward (ATP hydrolysis) and backwards (ATP synthesis from ADP and 32P\textsubscript{v}). Heparin inhibits the ATP\textsubscript{2+}P exchange reaction catalyzed by the Ca\textsuperscript{2+}-ATPases of both SRV and PV (Table I). Monovalent cations counteract the effect of heparin in both preparations in the same manner as that observed for Ca\textsuperscript{2+} uptake and net synthesis of ATP measured during net Ca\textsuperscript{2+} efflux.

Phosphorylation of the Ca\textsuperscript{2+}-ATPase—These experiments...
were performed only with the Ca$^{2+}$-ATPase of SRV. During the catalytic cycle this enzyme can be phosphorylated by either ATP or Pi. In both reactions, an acylphosphoryl residue is formed at the catalytic site of the enzyme (38-43). The hydrolysis of ATP is initiated by phosphorylation of the enzyme by ATP while the synthesis of ATP observed during the reversal of the pump is initiated by phosphorylation of the enzyme by Pi. Heparin inhibits only the phosphorylation by Pi (Fig. 9A) and has no effect on the level of phosphoenzyme formed from Pi, thus decreasing the equilibrium level of phosphoenzyme formed from Pi, thus decreasing the equilibrium level of phosphoenzyme formed from Pi.

In the presence of 100 mM KCl, the inhibition by heparin was decreased. In 11 different experiments, the addition of 20 \mu g/ml heparin in the absence of K$^+$ promoted a 43 ± 5% decrease in the phosphoenzyme level formed by Pi, whereas in the presence of 100 mM KCl, the inhibition promoted by 20 \mu g of heparin was 23 ± 4%. The same results were obtained when the enzyme was phosphorylated by 4 mM $^{32}$P$_i$ at pH 6.0 in a medium without dimethyl sulfoxide (other conditions as in Fig. 9A).

**Polyasaccharide Specificity**—The Ca$^{2+}$-ATPases of both SRV and PV are inhibited by dextran sulfate (average molecular weight 8,000 and 500,000) and by a fucose-branched chondroitin sulfate recently purified from sea cucumber (44). The concentrations needed for half-maximal inhibition by these sulfated polysaccharides are practically the same as for heparin (Fig. 10). Dermatan sulfate also inhibits the two Ca$^{2+}$-ATPases, but the concentration needed for half-maximal inhibition is more than 100-fold higher than for heparin. Heparan sulfate, chondroitin sulfate (Fig. 10), and hyaluronic acid (not shown) have practically no effect. The inhibitory activity of the different polysaccharides seems to be related to the content of sulfate of the molecule because: (a) the sulfate contents of heparin, dextran sulfate, and the fucose-branched chondroitin sulfate are higher than those of the other polysaccharides tested and (b) the inhibitory activity of the fucose-branched chondroitin sulfate isolated from sea cucumber is greatly decreased after desulfation (Fig. 11). Similarly, in two experiments the Ca$^{2+}$ transport of SRV and PV was not inhibited by heparin de-N-sulfated (100 \mu g/ml). This was measured using the experimental conditions of Fig. 1 (data not shown).

The inhibition of Ca$^{2+}$ transport by the fucose-branched chondroitin sulfate (data not shown) and by dextran sulfate (Fig. 12) is antagonized by monovalent cations, ruthenium red, and polyamines in the same manner as heparin.

**DISCUSSION**

The inhibition of the different Ca$^{2+}$ transport ATPases by the glycosaminoglycans seems to be related to the sulfate content of the molecule (Figs. 10 and 11) but the antagonism of this effect by monovalent cations (Fig. 5) does not appear to be due to neutralization of the negative charges of the sulfate residues. Lithium is the most electropositive ion among the alkali ions tested. Potassium strongly antagonizes the effect of the different glycosaminoglycan sulfates in SRV but has only a modest effect on PV. Thus, if the effect of the alkali ions were to neutralize the negative charges of heparin, then Li$^+$ should be as effective as K$^+$ or Na$^+$ and the effects of heparin on SRV and PV should be antagonized equally by K$^+$.

In previous reports (45-49) it has been shown that the Ca$^{2+}$-ATPase of SRV can operate either as a pump (coupled) or as a Ca$^{2+}$ channel (uncoupled). Phenothiazines (47, 48) and local anesthetics (49) uncouple the pump and greatly increase the
leakage of Ca$^{2+}$ through the ATPase. Similar to heparin, these drugs inhibit phosphorylation of the SRV ATPase by Pi and the leakage of Ca$^{2+}$ is antagonized by K', ruthenium red, and LiCl (26).

**Ritov et al.** (25) reported that heparin had no effect on SRV vesicles and Ghosh et al. (14) observed that heparin does not inhibit the Ca$^{2+}$ transport measured in microsomal vesicles isolated from smooth muscle. This apparent discrepancy with our results is related to the use of KCl. In their experiments, Ritov et al. (25) included 100 mM KCl in the assay medium and Ghosh et al. (14) tested the effect of heparin in the presence of 140 mM KCl. As shown in Figs. 3 and 5, heparin has no effect on SRV when KCl is included in the assay medium. However, as was also found for vesicles derived from the terminal cisternae of the sarcoplasmic reticulum (25), KCl fails to impair the effect of heparin in PV, suggesting that the Ca$^{2+}$ transport ATPase from blood platelets bears some resemblance to that derived from muscle terminal cisternae. Perhaps, the sensitivity to K' represents a property that might discriminate among cells or cell compartments that can and cannot respond to heparin and other drugs that impair (or) uncouple the Ca$^{2+}$ pump.

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**REFERENCES**


**FIG. 12. Effects of polyamines and monovalent cations on the inhibitory activity of dextran sulfate. The assay medium and conditions were as described in the legend to Fig. 10, except that SRV to a final concentration of 0.05 mg/ml was used. A, Ca$^{2+}$ uptake; B, Ca$^{2+}$-dependent ATPase activity. Control without dextran (○); with 4 µmol/l dextran sulfate (□); with 0.4 µmol/l (△), spermine (□), spermidine (●), putrescine (×), KA (□), NaCl (●), or LiCl (○).**