Heparin and Heparan Sulfate Disaccharides Bind to the Exchanger Inhibitor Peptide Region of Na\(^+/\)Ca\(^{2+}\) Exchanger and Reduce the Cytosolic Calcium of Smooth Muscle Cell Lines

REQUIREMENT OF C4-C5 UNSATURATION AND 1→4 GLYCOSIDIC LINKAGE FOR ACTIVITY

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Heparin and heparan sulfate fragments, obtained by bacterial heparinase and heparitinases, bearing an unsaturation at C4-C5 of the uronic acid moiety, are able to produce up to 80% reduction of the cytosolic calcium of smooth muscle cell lines. Unsaturated disaccharides from chondroitin sulfate, dermatan sulfate, and hyaluronic acid are inactive, indicating that, besides the unsaturation of the uronic acid, a vicinal 1→4 glycosidic linkage is needed. An inverse correlation between the molecular weight and activity is observed. Thus, the ED\(_{50}\) of the N-acetylated disaccharide derived from heparin sulfate (450 Da) is 88 \(\mu\)M compared with 250 \(\mu\)M of the unsaturated disaccharide (650 Da) derived from heparin. Except for enoxaparin (which contains an unsaturation at the non-reducing end and 1→4 glycosidic linkage), other low molecular weight heparins and native heparin are practicably inactive in reducing the cytosolic calcium levels. Thapsigargin (sarcoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor), vanadate (cytoplasmic membrane Ca\(^{2+}\)-ATPase inhibitor), and nifedipine and verapamil (Ca\(^{2+}\) channel antagonists) do not interfere with the effect of the unsaturated disaccharide upon the decrease of the intracellular calcium. A significant decrease of the activity of the unsaturated disaccharide is observed by reducing extracellular sodium, suggesting that the fragments might act upon the Na\(^+/\)Ca\(^{2+}\) exchanger promoting the extrusion of Ca\(^{2+}\). This was further substantiated by binding experiments and circular dichroism analysis with the exchanger inhibitor peptide.

Heparin is a highly sulfated glycosaminoglycan, which has been widely used in medicine as an anticoagulant and anti-thrombotic drug. It was previously shown that the bleeding effect of heparin was not related to its anticoagulant activity, because its enzymatic fragments such as sulfated tetra- and disaccharides, which are devoid of anticoagulant action, were equally effective in disrupting the normal hemostatic mechanism. For instance, the basic disaccharide unit of heparin (AU,2S(1→4GlcNS,6S)) is at least 10 times more potent than heparin itself in inhibiting the normal bleeding arrest after topical application. This and other results led to the hypothesis that heparin and its fragments could interfere with the muscular tonus of blood vessels through receptors present in the smooth muscle cell surface (1, 2).

Changes in cytosolic calcium concentrations play a central role in determining the force of contraction of vascular smooth muscle cells (3), and the low cytosolic calcium concentration reflects the balance between influx and efflux in the cytoplasm. Plasma membrane plays an important role in allowing Ca\(^{2+}\) influx from the extracellular medium through various types of Ca\(^{2+}\) channels, including L-type channels activation (4) and removing it to the extracellular space, by activation of the Ca\(^{2+}\)-ATPase and Na\(^+/\)Ca\(^{2+}\) antiport exchangers (5, 6). Two intracellular organelles are also involved in decreasing Ca\(^{2+}\) concentrations: the sarcoplasmic reticulum by stimulation of Ca\(^{2+}\)-ATPases and mitochondria (7). Ca\(^{2+}\) can be released from the sarcoplasmic reticulum through ryanodine receptors, which are activated during the process of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (8) and inositol 1,4,5-trisphosphate receptors (8–10).

These combined results led us to study the effect of heparin and other glycosaminoglycans and their respective unsaturated fragments (prepared by the action of lyases) upon cytosolic Ca\(^{2+}\) levels of smooth muscle cell lines derived from rabbit aorta. Of particular significance was the finding that only unsaturated heparin-derived fragments were capable of decreasing the cytosolic Ca\(^{2+}\) levels in smooth muscle cell lines by increasing the rate of Ca\(^{2+}\) extrusion.

**EXPERIMENTAL PROCEDURES**

Glycosaminoglycans—Heparins from porcine intestine of 12 and 8 kDa were gifts from Prof. P. Bianchini (Opocrin, Italy) and Dr. Valentina Baigorria (KinMaster, Brazil), respectively. Chondroitin 4- and 6-sulfates, hyaluronic acid, and dermatan sulfate were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Cleoxine (enoxaparin) and fragment (dalteparin) were from Rhodia and Pharmacia & UpJohn (São Paulo, Brazil), respectively.

Glycosaminoglycan Enzymatic Fragments—Enzymatic fragments from glycosaminoglycans were obtained from heparin, heparan sulfate, chondroitin sulfate, and hyaluronic acid by degradation with Flavobacterium heparinum heparinase, heparitinase I, heparitinase II, and chondroitinase AC, respectively, as previously described (2, 11). The following fragments were prepared: heparin and heparan sulfate frag-
Intracellular Calcium and Unsaturated Heparin Fragments

Established vascular smooth muscle cells from rabbit aorta were used (12, 13). All protocols were approved by the Animal Care Research Committee. A culture of primary smooth muscle cells was obtained from rabbit thoracic aorta according to procedures already described (14–16). The cells were dispersed by incubating the tissue with a solution containing 1 mg/ml collagenase Type 1, 12 units/ml elastase, 100 units/ml penicillin, and 100 µg/ml streptomycin. The medium was changed every 3 days. The cells reached confluency after 10 days and were then subcultured. The identity and homogeneity of the cells were confirmed with a solution containing 1 mg/ml collagenase Type 1, 12 units/ml elastase, 100 units/ml penicillin, and 100 µg/ml streptomycin. The medium was changed every 3 days. The cells reached confluence after ~10 days and were then subcultured. The identity and homogeneity of the cells were indicated by positive fluorescence with antibodies against myosin and actin (16).

The viability of the cells was determined by the trypan blue exclusion method and was found to be greater than 90%.

Measurement of Intracellular Ca2⁺—Calcium was measured after incubation of the cells with the fluorescence indicator Fura-2/AM in the form of acetoxymethyl ester (AM). Smooth muscle cells at a concentration of 10⁶ cells/ml were suspended in 2.5 ml of Tyrode’s solution (137 mM NaCl, 2.68 KCl, 1.36 mM CaCl₂, 0.49 mM MgCl₂, 6.14 mM Na₂HPO₄, 0.36 mM NaH₂PO₄, and 5.5 mM d-glucose) containing 0.2% bovine serum albumin and left to stand in a CO₂ incubator at 37 °C for 30 min. The suspension was then centrifuged at 100 × g for 4 min, the supernatant was aspirated, and the resulting pellet was suspended in 2.5 ml of bovine serum albumin-free Tyrode and transferred to the quartz cuvette of a SPEX fluorometer (AR CM System) for autofluorescence determination. Measurements were made at 340- and 380-nm excitation wavelengths, with an emission at 505 nm. The autofluorescence ratio was less than 10% and therefore was not subtracted from the fluorescence measurements before calculation of the fluorescence ratio.

The cells were then incubated with 0.01% pluronic 127 detergent and 2 µM Fura-2/AM, and the cuvette was transferred to a PerkinElmer Life Sciences spectrofluorometer (LS 5B, Buckinghamshire, UK) to determine the fluorescence spectrum of the indicator in the excitation range of 300 to 400 nm, with emission at 520 nm. In the esterified form, maximum fluorescence was observed at 390 nm. As the indicator Fura-2/AM was transformed to the acid form, the fluorescence peak shifted to 350 nm within an average period of 2 h, thus indicating the maximum amount of indicator incorporation into the cell suspension. At the time the cell suspension was washed with 15 ml of Tyrode and centrifuged at 100 × g for 4 min. The supernatant was discarded, and the pellet was suspended in 2.5 ml of Tyrode and transferred to a SPEX fluorometer programmed for excitation at two wavelengths (340 and 380 nm) with emission at 505 nm, under constant stirring at 37 °C. The first reading was taken before the addition of each of the five doses to the same suspension prepared with the structures of known proteins according to the analysis of Sreerama et al. (17). The results were expressed by cumulative doses of drug as a function of time and by percentage of Ca²⁺ extrusion as calculated by the ratio between the percentage of Ca²⁺ extrusion and basal Ca²⁺.

The Emax levels of the compounds were calculated as the dose that promotes 50% of Ca²⁺ extrusion.

**Peptide Synthesis and Purification**—The XIP peptide corresponding to residues 238–251 of the cytoplasmic loop of the human Na⁺/Ca²⁺ exchanger (NCX) have been synthesized by the solid phase method using t-butyloxycarbonyl chemistry. Optimized coupling conditions were introduced according to the resin solvation theory (18). The XIP peptide, RRLFYKYVKYRKAGKQRG-CONH₂, was purified by high pressure liquid chromatography. The molecular mass (2621.13 Da) and the purity of synthesized peptide were checked by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (ToFSpec-E, Micromass) and/or peptide sequencing using a protein sequencer (PPSQ-23, Shimadzu, Tokyo, Japan).

**Heparin Fragments Binding to XIP**—Heparin fragments binding to XIP was measured at 37 °C in 10 mM Tris-HCl buffer, pH 7.4, except where different conditions are stated. The fluorescence of tyrosine residues of the XIP solutions was monitored by measuring the fluorescence at λem = 304 nm (20-nl slit) after excitation at λex = 278 nm (10-nl slit) in a Hitachi F-2000 fluorometer. The 1-cm path-length cuvette containing 2 ml of the buffered peptide solution was placed in a thermostatically controlled cell compartment under constant magnetic stirring. The spectrophotometer was prewarmed for 5 min prior to the addition of small aliquots of a highly concentrated heparin solution with minimal dilution (less than 5%), and the instantaneous increase in fluorescence signal was read. The dependence of fluorescence change, i.e., ΔF = (Fobs − F0), where F0 is the initial peptide solution fluorescence value and Fobs is the observed fluorescence value after each addition of heparin fragments, was analyzed by non-linear least-squares data fitting by a binding equation (Equation 1) using Grafit software.

\[
\Delta F = \frac{P \times \left(1 + \frac{nH}{K_D}\right)}{2P} \times \left(1 + \frac{nH}{K_D}\right)^2 - 4 \times \frac{P \times nH}{2P} \quad (\text{Eq. 1})
\]

where P is the total peptide concentration, H represents the added heparin fragments concentration, n is the stoichiometry, KD is the dissociation constant, and ∆Fmax is the maximum fluorescence change.

**Circular Dichroism Experiments**—Circular dichroism measurements in far ultraviolet regions (190–280 nm) of XIP-heparin fragments interactions were conducted in a JASCO J-810 spectropolarimeter scanning at a rate of 50 nm/min at 37 °C. Cells of 0.1 cm for the far UV were used. The experiments were done in 10 µM Tris, pH 7.4, containing 25 µM EDTA. The observed dichroicity was normalized to units of degrees cm².dmol⁻¹. All dichroic spectra were corrected by subtraction of the background for the spectrum obtained with buffer alone or buffer containing heparin fragments. The CD spectra for the XIP was analyzed for the relative amount in percentage of the secondary structural elements by a program based on comparison to the spectra obtained for the structures of known proteins according to the analysis of Sreevaram and Wood (19).

**RESULTS**

**Reduction of Cytosolic Calcium Levels by Heparin Trisulfated Disaccharide**—Fig. 1 shows the effect of heparin trisulfated disaccharide upon cytosolic calcium levels of the primary (Fig. 1A) and established (Fig. 1B) smooth muscle cell line from rabbit aorta. The tracing was obtained by addition of five doses to the same suspension prepared with the structures of known proteins according to the analysis of Sreevaram and Wood (19).

The effect of the trisulfated disaccharide is dependent on the intracellular calcium concentration. Thus, a progressive decrease of calcium levels in the cytosol leads to a decrease in the effect of the disaccharide upon the calcium levels (Fig. 2A). It
was also observed that higher doses of the trisulfated disaccharide are needed when the concentration of extracellular calcium is progressively decreased (Fig. 2B).

ATP promotes a transient influx of calcium in smooth muscle cells as shown in Fig. 3A. An experiment was then designed to observe the effect of the trisulfated disaccharide upon the effect of ATP in the cells. Fig. 3B shows that ATP potentiates the effect of the trisulfated disaccharide upon the decrease of intracellular calcium.

Effect of Different Glycosaminoglycan Fragments on Calcium Levels of Smooth Muscle Cell Line—Among the different disaccharides prepared from heparin, heparan sulfate, hyaluronic acid, and chondroitin sulfate, only heparin- and heparan sulfate-derived fragments led to a significant decrease of intracellular Ca$^{2+}$ in the established smooth muscle cell line (Fig. 4). The mono-, di-, and non-sulfated disaccharides were much more effective than the trisulfated disaccharide in producing the phenomena. Thus 350 $\mu$M of these disaccharides produces 80% decrease of the cytosolic calcium in the smooth muscle cell line. The chondroitin sulfate and hyaluronic acid disaccharides, namely $\Delta U(1 \rightarrow 3)\text{GalNAc}, 6S$, $\Delta U(1 \rightarrow 3)\text{GalNAc}, 4S$, and $\Delta U(1 \rightarrow 3)\text{GlcNAc}$, were weak or inactive in modulating cytosolic calcium.

The ED$_{50}$ levels of these disaccharides as well as the tetra- and hexasaccharides derived from heparin upon the Ca$^{2+}$ intracellular concentration are shown in Table I. It seems that the glycosidic linkage plays an important role on the decrease of calcium, because only disaccharides bearing 1→4 linkages are active. This is reinforced by comparing the activity of the N-acetylated disaccharide derived from heparan sulfate with the one derived from hyaluronic acid. The only difference between the disaccharides is the glycosidic linkage. The one derived from hyaluronic acid has an ED$_{50}$ of more than 5000 $\mu$M, whereas the one derived from heparan sulfate has an ED$_{50}$ of 88 $\mu$M.

The effects of unmodified heparin of two different molecular
weights and of two low molecular weight (LMW) heparins upon the intracellular Ca\(^{2+}\) concentration of smooth muscle cell line. 

Among the two LMW-heparins tested, only enoxaparin produced a significant decrease in the intracellular calcium of the smooth muscle cell line. This LMW-heparin is prepared by an elimination reaction from heparin producing a C4-C5 unsaturation at the non-reducing end of the molecules. This unsaturation was also present in the fragments produced by the different lyases (chondroitinases and heparinases).

An inverse correlation between the molecular weight of the fragments containing 1\(\rightarrow\)4 linkages and the activity is also apparent (Fig. 6). Thus, the heparin hexasaccharide has about 10% of the activity (ED\(_{50}\) 880 \(\mu\)M) when compared with the heparan sulfate N-acetylated disaccharide (ED\(_{50}\) 88 \(\mu\)M).

**Effect of Drugs on the Activity of the Trisulfated Disaccharide in the Reduction of Cytosolic Calcium Levels**—Several experiments were performed with 1 to 10 \(\mu\)M thapsigargin (sarco-plasmic reticulum Ca\(^{2+}\)-ATPase inhibitor), 1–100 \(\mu\)M vanadate (cytoplasmic membrane Ca\(^{2+}\)-ATPase inhibitor), 0.01–10 \(\mu\)M nifedipine, and 0.1–10 \(\mu\)M verapamil (Ca\(^{2+}\) channel antagonists) (18). None of the drugs at different doses interfered with the effect of the trisulfated disaccharide upon the decrease of fragments containing 1\(\rightarrow\)4 linkages and the activity is also apparent (Fig. 6). Thus, the heparin hexasaccharide has about 10% of the activity (ED\(_{50}\) 880 \(\mu\)M) when compared with the heparan sulfate N-acetylated disaccharide (ED\(_{50}\) 88 \(\mu\)M).
Intracellular Calcium and Unsaturated Heparin Fragments

**TABLE II**
The influence of heparin fragments upon XIP secondary structure

| Compound                | α-Helix (%) | β-Sheet (%) | Remaining (%) |
|-------------------------|-------------|-------------|---------------|
| Control 25 μM XIP       | 39.6        | 60.4        |               |
| XIP + 50 μM disaccharide| 51.4        | 48.6        |               |
| XIP + 50 μM tetrasaccharide | 54.0     | 46.0        |               |
| XIP + 50 μM hexasaccharide | 4.80     | 95.2        |               |
| XIP + 50 μM LMW         | 10.2        | 79.8        | 19.4          |
| XIP + 50 μM heparin     | 12.9        | 70.8        | 16.3          |

**TABLE III**
Dissociation constant (K_D) and stoichiometry (n) of XIP-heparin fragment interactions

| Heparin fragments | K_D (μM) | n   |
|------------------|----------|-----|
| Disaccharide     | 24 ± 2   | 0.81|
| Tetrasaccharide  | 13 ± 1   | 0.88|
| Hexasaccharide   | 7.4 ± 0.8| 0.94|
| LMW              | 2.7 ± 0.3| 1.00|
| Heparin          | 1.2 ± 0.1| 1.00|

**Fig. 9. Effect of heparin fragments upon XIP circular dichroism spectra.** Circular dichroism spectra of XIP were determined in 10 mM Tris-HCl, pH 7.4, at 37°C. The observed ellipticity was normalized to units of degrees.cm^2.dmol^-1. All dichroic spectra were corrected by subtraction of the background for the spectrum obtained with buffer alone or buffer containing heparin fragments. 25 μM XIP in absence (○) or in presence of 50 μM heparin fragments: disaccharide (▲), tetrasaccharide (△), hexasaccharide (○), heparin LMW (□), and heparin (■).

The intracellular calcium levels in primary or established smooth cell lines, despite the fact that thapsigargin and vanadate have, by themselves, effects upon the basal calcium levels.

The activity of the trisulfated disaccharide are significant decreased after reduction of 50% of extracellular Na^+ (Fig. 7). This suggests that the disaccharide might act upon Na^+ /Ca^2+ exchanger promoting the extrusion of Ca^2+. To substantiate the proposal we have studied the binding of the fragments with the exchanger inhibitor peptide (XIP).

**Heparin Binding to XIP—**Fig. 8 presents the variation of 2.0 mM XIP fluorescence versus heparin disaccharide concentration according to Equation 1. This is a representative curve of all fluorescent titrations performed in this work. The kinetic analysis shows that heparin and its fragments bind XIP by a saturable bimolecular reaction. The obtained dissociation constant (K_D) values as well as the stoichiometry for the interaction of the XIP peptide with heparin fragments are presented in Table II. The shorter heparin fragments, the disaccharide ΔU,2S-GlcNS,6S, binds XIP with a dissociation constant of 24 ± 2 μM, but as the molecular size of the heparin fragments increased, the K_D values decreased. The values of K_D for tetra-, hexasaccharide, LMW, and heparin were 13 ± 1, 7.4 ± 0.8, 2.7 ± 0.3, and 1.2 ± 0.1 μM, respectively, indicating that the affinity of the heparin fragments for XIP is dependent on their sizes and on the number of negative charges distributed along the sequences. All heparin fragments containing the minimum disaccharide unit (IdoU,2S-GlcNS,6S) have stoichiometry values around 1, despite their K_D values.

**Fig. 10. Minimum-energy structure of N-acetylated disaccharides from heparan sulfate and hyaluronic acid.** A, N-acetylated disaccharide from heparan sulfate; B, N-acetylated disaccharide from hyaluronic acid; the full linkage indicates the double bond. For details see Ref. 25.

**Circular Dichroism Spectra XIP—**The far UV-visible spectra of XIP are shown in Fig. 9, and the fractional percentages of secondary structures content calculated according to the Sreerama and Woody analysis (19) are presented in Table III. The CD spectra of XIP indicate the existence of a multiple conformation equilibrium, showing 60.4% of random structure in equilibrium with 39.6% of β-sheet structure in aqueous solution, and these values were not affected by changes in concentration within the 20–200 μM range. However, upon addition of heparin fragments, it was found that XIP exhibited a transition to β-sheet conformation. These data suggest that heparin and heparin fragments induce a significant amount of β-sheet on XIP. The shorter heparin fragment, disaccharide ΔU,2S-GlcNS,6S, was able to induce about 12% of β-sheet structure on XIP, but, as the size of the heparin fragments increased, the amount of β-sheet structure also increased; heparin was able to induce 31% of β-sheet structure on XIP, indicating that the amount of β-sheet structure induced by heparin fragments on XIP is dependent on their molecular sizes. Moreover, heparin fragments showing molecular sizes bigger than a hexasaccharide structure were also capable of inducing a small percent of α-helix structure on XIP (Table III).

**DISCUSSION**

Intracellular calcium is tightly regulated and critically important for vascular smooth muscle contraction. It is required for many cellular processes, and changes in concentration play an important role for activation of contractile systems (19). The contractile state of vascular smooth muscle cells is dependent on availability of Ca^2+ for activation of their contractile systems. During the muscle contraction, there is a rapid elevation of intracellular calcium. Maintenance of calcium concentration above basal levels is known to be associated with phasic contraction and continued tone of vascular smooth muscle (20). After the contraction period, the intracellular calcium returns to basal levels, which is about 100 nM. This process is possible because of many mechanisms that play a role in regulating Ca^2+ modulation. These include initial release of Ca^2+ from the sarcoplasmic reticulum, Ca^2+ influx via the sarcolemma, and Ca^2+ pumping both into the sarcoplasmic reticulum by the
sarcoplasmic reticulum Ca\(^{2+}\)-ATPase or out of the smooth muscle cells by the sarcolemmal Ca\(^{2+}\)-ATPase (21, 22). In addition, Ca\(^{2+}\) may be transferred from cytoplasm to extracellular medium, through Ca\(^{2+}\)-ATPases or Na\(^+\)/Ca\(^{2+}\)-exchangers. In this last case, the Na\(^+\)/Ca\(^{2+}\)-exchanger may play a significant role in the Ca\(^{2+}\) extrusion, across the plasma membrane, from vascular smooth muscle cells (21).

Elucidation of the Ca\(^{2+}\) mobilization mechanisms in smooth muscles is important not only for the understanding of the regulatory mechanisms of smooth muscle contraction but also for the better understanding of the mode of action of the drugs that modulate smooth muscle activities.

We now show that heparin fragments bearing a C4-C5 unsaturation at the non-reducing end of the molecules promote a decrease of the intracellular calcium. This activity is stereospecific, because only heparin and heparan sulfate unsaturated disaccharides bearing (1→4) glycosidic linkages are effective. The fragments derived from chondroitin sulfate and hyaluronic acid, which have (1→3) glycosidic linkages, have no activity in the intracellular Ca\(^{2+}\) concentration. This is clearly shown by comparing the activities of the two N-acetylated disaccharides from heparan sulfate and hyaluronic acid whose only difference is the glycosidic linkage. Previous findings on the conformation of these disaccharides represent a possible explanation for this.

Fig. 10 shows the minimum-energy structure of these two disaccharides that have been previously reported (23). Considering the plan formed by the hexosamine moieties, the uronic acid ring of the heparan sulfate disaccharide is above the plane, whereas the uronic acid of the disaccharide derived from hyaluronic acid is below. It was also shown, by \(^1\)H NMR spectroscopy, that the uronic residues proton coupling constants of the heparan and hyaluronic acid disaccharide differ substantially, \(J_{\text{H},\text{H}}\) of 5.95 and 4.8, respectively (23).

The magnitude of the decrease of Ca\(^{2+}\) (IC\(_{50}\)) promoted by the trisulfated disaccharide is inversely proportional to the concentration of intracellular Ca\(^{2+}\). This also occurs when the intracellular Ca\(^{2+}\) is transitorily increased by the increase of its extracellular concentration. These combined results suggest that the trisulfated disaccharide blocks the Ca\(^{2+}\) influx and leads to the Ca\(^{2+}\) extrusion from the cells.

This activity produced by the trisulfated disaccharide is not due either to the activation of Ca\(^{2+}\)-ATPase from plasma membrane or from sarcoplasmic reticulum, because vanadate, which inhibits the Ca\(^{2+}\) pumps (24, 25), and thapsigargin, which inhibits the microsomal Ca\(^{2+}\)-ATPases (26–31), do not block the extrusion of Ca\(^{2+}\) by the disaccharide. On the other hand, reduction of extracellular Na\(^+\) significantly decreases the disaccharide activity, suggesting an effect upon the Na\(^+\)/Ca\(^{2+}\) exchanger described by Bolton (21).

The effect of the trisulfated disaccharide in reducing the intracellular Ca\(^{2+}\) may have some therapeutically use in pathologies in which the decrease of this compound might be useful, such as atrial fibrillation (32, 33) and unstable angina (34, 35), and other pathologies (36, 37). In this context it is worth mentioning the effect of the unsaturated LMW-heparin (enoxaparin), which is effective in reducing intracellular calcium concentration as presently shown, and in unstable angina when compared with native heparin (34).

The Na\(^+/\)Ca\(^{2+}\) exchanger (NCX) undergoes an inactivation process in which exchange activity decays to a steady state following activation by intracellular increase of Na\(^+\) concentration. This process, called intracellular sodium-dependent inactivation, also designated regulation \(I_2\) (38), is mediated by endogenous exchange inhibitor peptide (XIP) region, amino acids 219–238, located at the N-terminal portion of the large intracellular loop (residues 218–764) of protein (39, 40). Increasing intracellular Ca\(^{2+}\) activates transport activity, whereas removal of cytoplasmic Ca\(^{2+}\) drives the NCX into an inactive state, also termed regulation \(I_2\) (41). Binding of Ca\(^{2+}\) at the amino acid residues 371–508 of the intracellular loop (42), the high affinity Ca\(^{2+}\) regulatory site, removes the sodium-dependent inactivation process and stimulates transport activity, showing that Ca\(^{2+}\) also involves the XIP region in its regulation (40). It has been proposed by Saba et al. (43) that, in the absence of Ca\(^{2+}\), the endogenous XIP domain binds to the large cytoplasmatic loop inducing the auto inhibited conformation of the exchanger, and the exogenous addition of XIP peptide has no effect upon tertiary structure of the protein. Ca\(^{2+}\)-binding to its regulatory site leads to the dissociation of the endogenous XIP domain from its binding site and induces an active protein conformation characterized by the folding of secondary structure into a more compact configuration. Also, in the presence of Ca\(^{2+}\), the exogenous addition of XIP promotes an opposite effect to that observed upon Ca\(^{2+}\) addition, i.e. at this stage, XIP addition gives an unfolded conformation inhibiting Na\(^+\)/Ca\(^{2+}\) exchange activity (43).

Zhaoping et al. (44) reported that the anionic phosphatidylinositol 4,5-bisphosphate interacted with the cationic XIP region, and this interaction abolish the sodium-dependent inactivation process of the Na\(^+\)/Ca\(^{2+}\) exchanger. Our results also show that the anionic heparin fragments and heparan sulfate disaccharides are able to interact with XIP inducing a secondary \(\beta\)-sheet structure in this peptide. It is important to note that, in vascular smooth muscle cells, when cytosolic Ca\(^{2+}\) concentration is low, <150 nM, the effect observed for heparin disaccharide upon Ca\(^{2+}\) extrusion is very depressed, i.e. the observed IC\(_{50}\) value is very high. On the contrary, when cytosolic Ca\(^{2+}\) concentration is high, heparin disaccharide has a powerful effect in the activation of cytosolic Ca\(^{2+}\) extrusion (Fig. 2). In a similar manner to XIP described above, at low cytosolic Ca\(^{2+}\) concentration, heparin disaccharide has low effect probably because the XIP domain is not accessible in the autoinhibited state. Thus, as Ca\(^{2+}\) concentration increases in the cytoplasm of vascular smooth muscle cells, above 300 nM, Ca\(^{2+}\) binding dislodges the endogenous XIP domain from its binding site favoring the interaction of the XIP domain with heparin disaccharide. The interaction of XIP with heparin fragments inhibits the autoinhibited state promoting the binding of XIP to the intracellular loop. Moreover, our results suggest that the XIP domain is accessible to extracellular heparin fragments, despite the location of this region at the amino-terminal region of the large intracellular loop where it contacts the plasma membrane.

To promote the increase of the rate of cytosolic Ca\(^{2+}\) extrusion, we observed that heparin disaccharide and heparan sulfate disaccharides were more potent drugs than were the bigger heparin fragments, i.e. tetra-, hexasaccharide, LMW, and heparin, because disaccharides probably have better diffusion at the internal XIP domain than that of the other fragments (Fig. 4).

Although we have shown that heparin fragments can interact with the XIP domain and increase the rate of cytosolic Ca\(^{2+}\) extrusion, there is no direct evidence that this interaction governs the biological activity of the Na\(^+\)/Ca\(^{2+}\) exchanger. However, the present results suggest that heparin fragments can be a novel class of drugs capable of activating the NCX activity, thereby providing a basis for development of clinically useful drugs for treatment of hypertension, cardiac ischemia/reperfusion, digitalis intoxication, and arrhythmia (36, 37).

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