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CATION BINDING TO ANIONIC BIOPOLYMERS OF VASCULAR CONNECTIVE TISSUE

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Résumé — Des variations de concentrations externes en protons et/ou en cations produisent des modifications des propriétés de liaison des macromolécules polyanioniques dans le tissu conjonctif vasculaire. Ainsi, la concentration extracellulaire des différentes espèces cationiques peut varier de façon rapide et importante dans les mailles du réseau conjonctif et au voisinage de la membrane des cellules vasculaires lisses. En particulier, l'adsorption potassique par acidification de la solution extracellulaire comme la liaison par coopération du potassium (consécutives aux changements conformationnels induits par les ions Mg^{2+} dans les polyanions) présentent une grande importance dans l'hyperpolarisation membranaire et la vasodilatation.

Abstract — The pH-dependent binding of *monovalent* cations to vascular connective tissue is dependent on the concentration and affinity constant of the ion species in question. The mode of interaction is competition. *Divalent* cation binding to vascular connective tissue and its proteoglycans is additionally dependent on conformational changes. For example, competition experiments in physiological Krebs solutions using multi-chain chondroitin sulphate and Mg^{2+} at pH 7.38 showed increasing $^{23}Na^+$ excess transverse relaxation rates during the initial phase [cf.5]. Above 4 mM Mg^{2+} simple exchange of bound Na^+ with Mg^{2+} was observed. The increased excess transverse relaxation rate seen upon Mg^{2+} additions was referred to an increase in the correlation time. In the presence of Mg^{2+} , cross-linking of chains, clustered together on the protein core, was promoted. Thus, physiological concentrations of Mg^{2+} ions can induce a specific change in the configuration of the anionic biopolymers, which enables K^+ ions to bind cooperatively ($n_H = 6.8$), while the adsorption of all the other cations is competitively inhibited. This means that with extracellular Mg^{2+} excess not only more Mg^{2+} ions are bound to vascular connective tissue but also more K^+ ions. $[K^+]_0$ would decrease near the cell membrane, hyperpolarization and vasodilatation would occur.

Since a tight electromechanical coupling exists in arterial smooth muscle, even small shifts of the membrane potential are sufficient to change the vascular lumen [12]. The extracellular H^+ , Na^+ , K^+ , Mg^{2+} and Ca^{2+} concentrations are important effectors for the adjustment of the membrane potential. The ion concentrations in the immediate neighbourhood of the cell membrane can be influenced by the microdynamic binding properties of the basal lamina and the surrounding connective tissue fibres, which are separated from the vascular smooth muscle cell membranes by tiny cleft spaces. These structures display a high binding capacity for small cations which has been ascribed to the presence of polyanionic proteoglycans [13]. These biopolymers owe their anionic character to many highly sulphated and carboxylated glycosaminoglycan (GAG) chains that are covalently attached to the protein core. Knowledge concerning the dynamics of cation-polyanion interactions could be of great importance for an understanding of the excitation-contraction coupling in smooth muscle cells [10]. Therefore, the ion binding properties of vascular connective tissue as well as of substances derived therefrom were studied in dependence on proton and cation concentration by tracer and NMR techniques. This paper is intended to deal essentially with K^+ ions and their binding, as this ion species plays the decisive part in passive potential genesis.

Fig.1 shows K^+ binding curves for normal and Ca^{2+} -free Krebs solutions as well as for a 4.7 mM KCl solution. In a normal Krebs solution, the K binding increases with increasing pH and attains a maximum at pH = 6.8. Surprisingly, it decreases in the basic pH range, which is a most remarkable fact for a pH-dependent cation binding curve [10,13]. Before an explanation for this behaviour is given, the significance of the K binding curve shall be discussed. Following transition from pH 7.3 to 6.8, 0.64 mmole K^+ /kg fibre wt. are bound to connective tissue structures, while 1.52 mmole K^+ /kg fibre wt. are released upon transition from pH 7.3 to 7.8. Application of a simple geometric model, which considers the narrow cleft spaces between cell membrane, basal lamina and connective tissue fibres, can lead to a reduction of the K^+ concentration in the cleft spaces to $\frac{2}{3}$ to $\frac{1}{2}$ in the first case, in the latter case to an increase to the double to triple of its normal value. Extracellular accumulation of potassium would lead to membrane depolarization of smooth muscle cells and sympathetic nerve fibres located at the adventitia-media boundary, and thus to contraction, depletion of potassium to hyperpolarization with vasodilatation.

The K binding characteristic in a Ca^{2+} -free Krebs solution explains the unusual course of the K binding curve under normal conditions [10,13]. Comparing both curves, one can immediately conclude that the decrease of K binding in the basic pH range for normal Krebs solution must be attributed to Ca^{2+} competition. The Ca^{2+} competition is least pronounced between pH 6 and 7. Therefore, the result of a Ca^{2+} -free Krebs solution is a

simple sigmoid K^+ titration curve. Furthermore, the regulating role of K^+ ions on the membrane potential is abolished by flattening its binding characteristic, since the K binding at $pH = 7.4$ has reached a steady state value.

Incubating vascular connective tissue in a 4.7 mM KCl solution, we found a pH-dependent K binding characteristic similar in principle to the K binding curve in Ca^{2+} -free Krebs solution. Also in this KCl solution, which contains KCl in physiological concentration but is free of Ca^{2+} and has a considerably diminished ionic strength in comparison to blood, a sigmoid K^+ titration curve was determined. Although there is no competition by other counterions, the binding curve lies distinctly below that in Ca^{2+} -free Krebs solution. Physico-chemically, there is the possibility of an 'occlusion' of unspecific and specific sites by their steric arrangement within a polyelectrolyte system [7]. Thus, the site-bearing proteoglycans embedded in a fine collagen mesh-work could be excluded from the fast cation exchange with increasing 'felting' by aggregation of collagenous molecules with each other and with the proteoglycans. Increasing macromolecular interactions with decreasing ionic strength of the incubation medium are well-known [2,6,8,9].

Fig. 1. K binding of isolated adventitial connective tissue from the canine carotid artery as a function of the pH of a normal ($\circ$) and a Ca^{2+} -free Krebs solution ($\square$) as well as of a 4.7 mM KCl solution (Δ).

Fig. 2. K ($\circ$) [$n=8$] and Mg binding ($\square$) [$n=9$] of isolated adventitial connective tissue of the canine carotid artery after a 3 hr incubation in pure, aqueous $MgCl_2$ solutions at $pH 7.0$.

In order to determine global affinity constants for *in vivo* vascular connective tissue, tracer efflux experiments were designed, which would supply us with the amplitudes and rate constants for calculating affinity constants. Isolated adventitial connective tissue was used as a polyelectrolyte, and $^{24}Na^+$, $^{42}K^+$ or $^{28}Mg^{2+}$ as counterions in three different concentrations [13]. Each series of flux experiments with one cation species resulted in equal and mutually uniform kinetic coefficients for the slower exchange fractions. The kinetic coefficients are an expression of the exchange deceleration by chemical binding and the affinity of binding sites. The homogeneity of the kinetic coefficients under the various experimental conditions shows that the negatively charged groups in the preparations after Na^+ , K^+ or Mg^{2+} accumulation can be subdivided into few classes of binding sites with uniform intrinsic affinity constants. The result of these flux experiments is summarized in Table 1. One notices that the $\log K_A$ values for $S_{i,1}$ and $S_{i,2}$, respectively, are nearly equal for both the ion species K^+ and Mg^{2+} , where $S_{i,2}$ binds more strongly than $S_{i,1}$. Also the total number of binding sites for K^+ and Mg^{2+} ions is nearly identical. This means that K^+ and Mg^{2+} ions with equal affinities strongly compete for the same molecular sites. In general, the $\log K_A$ values are between 1.8 and 3.7 indicating binding sites with high affinity and selectivity for cation binding. Computer models for chain association in proteoglycans [3] define binding sites formed by several ionized and hydroxyl groups, with a striking structural analogy to crown ethers [9]. These are strong and selective complexing agents whose affinity constant for K^+ is typically between $\log K_A$ 0.6 and 2.2 [1]. Investigating the mode of interaction of various counterions (Na^+ , K^+ , Mg^{2+} , Ca^{2+}) with polyanionic vascular connective tissue in dependence on the concentration of a single cation species, competition is found in all cases but one: a rise of the external Mg^{2+} concentration leads not only to an enhanced Mg^{2+} , but also to an increased K^+ binding. Consequently, the K

Table 1. Binding site $S_{i,j}$, affinity constant K_A and concentration of sites $[S_{i,j}]$ of arterial vascular connective tissue of the dog for the cation species Na^+ , K^+ and Mg^{2+} .

$S_{i,j}$	K_A (l/mole)	$\log K_A$	$[S_{i,j}]$ (mmole/kg fibre wt.)	$\Sigma [S_i]$ (meq/kg fibre wt.)
$S_{\text{Na},1}$	4,522	3.66	0.850	2.176
$S_{\text{Na},2}$	58	1.76	1.326	
$S_{\text{K},1}$	365	2.56	13.940	16.068
$S_{\text{K},2}$	1,009	3.00	2.128	
$S_{\text{Mg},1}$	559	2.75	6.407	15.672
$S_{\text{Mg},2}$	3,177	3.50	1.429	

binding was inspected more closely for a wide range of Mg^{2+} concentrations (Fig.2). In pure MgCl_2 solutions the K binding increases continuously in a low Mg^{2+} concentration range [13], then increases steeply between 15 and 20 mM, and finally decreases gradually after saturation. The initial rise of K^+ adsorption can be explained by a steady augmentation of K^+ binding sites with increasing ionic strength, and thus a more effective shielding by mobile cations of the high charge density of fixed anionic groups of GAG chains. This effect promotes an association of GAG chains whose share in the generation of specific sites of high affinity was suggested [3,6,9]. However, the steep increment of K^+ binding can be attributed only to an allosteric interaction due to further increasing Mg^{2+} concentrations, the following graded fall probably to a gradual configurational transition of the macromolecules. Fitting the measured values to the function

$$Y = \frac{a[S]^n \cdot C_{\max}}{(1 + a[S]^n)(1 + b[S]^m)} + c \ln[S] + d \quad (1)$$

results in the following parameters: $a = 2.7 \cdot 10^{-9} \text{ mM}^{-n}$; $b = 4.5 \cdot 10^{-3} \text{ mM}^{-m}$; $c = 0.19 \text{ mM}$; $d = 3.61 \text{ mM}$; $n = 6.77$; $m = 1.07$; $C_{\max} = 2.33 \text{ mmole/kg}$. Thus, the Hill coefficient of cooperative K binding is 6.8, the $[\text{Mg}^{2+}]_0$ concentration at half-maximal velocity $S'[0.5] = 18.5 \text{ mM}$ ($S''[0.5] = 153.7 \text{ mM}$). When the curves for K^+ and Mg^{2+} binding are compared, a distinct rise of Mg^{2+} binding is seen already at $[\text{Mg}^{2+}]_0 = 0.1 \text{ mM}$ as well as a saturation in the same $[\text{Mg}^{2+}]_0$ range, in which the K^+ binding increases drastically. This relation permits the conclusion that only saturation of all specific Mg^{2+} binding sites leads to the conformational transitions in the polyanions necessary for a cooperative K^+ binding. Finally, it shall be mentioned that the ionic strength of these pure MgCl_2 solutions is by no means physiologic. Preliminary studies in Krebs solutions of increasing Mg^{2+} concentration yielded also cooperative K binding for an $[\text{Mg}^{2+}]_0$ range between 0.3 and 0.8 mM.

Fig.3. The $^{23}\text{Na}^+$ excess longitudinal ($\square$) and transverse ($\circ$) relaxation rates, R_{1ex} and R_{2ex} , in dependence on the Mg concentration of a Krebs solution containing 0.1 M CSP at pH = 7.38. The initial fall of the longitudinal relaxation rate and the initial rise of the transverse relaxation rate in the range of physiological Mg concentrations is supplementarily illustrated in a separate inset. This behaviour is indicative of a conformational change of the CS-P macromolecules.

Cooperative binding is possible if a conformational change of the polyelectrolyte takes place via the allosteric effect of a ligand [11]. Monovalent cations are not suitable for such a change of proteoglycan structure, but rather cations like Mg^{2+} and Ca^{2+} with their two valences [5]. Stretched in between the side chains of these polyanions, they can rearrange the whole macromolecular structure. With the help of NMR techniques, the ability of Mg^{2+} ions in a low concentration range has been examined to cause such a change in configuration. In Fig.3, the ^{23}Na excess relaxation rates are plotted versus the Mg^{2+} concentration of a physiological Krebs solution at pH 7.38 in the presence of a physiological CS-P (multi-chain chondroitin sulphate-polypeptide complex) concentration. It is highly surprising that $R_{2\text{ex}}$ increases for small additions of MgCl_2 [cf.13], and decreases only with higher Mg^{2+} concentrations, that is Na^+ is expelled from its binding sites (normal competition). The remarkable initial increase of $R_{2\text{ex}}$ is drawn once again in a separate inset. The additional measurement of the longitudinal relaxation rate, $R_{1\text{ex}}$, which exhibits normal competition behaviour, gave further evidence for a conformational change of the macromolecules, which is indicated by the $R_{2\text{ex}}$ increase. With Mg^{2+} concentrations >150 mM both ^{23}Na excess relaxation rates rise steadily due to ion-ion and ion-solvent interactions.

The increased excess transverse relaxation rate seen upon Mg^{2+} additions is referred to an increase in the correlation time. Under certain prerequisites, the ratio of transverse and longitudinal relaxation time can be formulated as follows [4]

$$\frac{\Delta\left(\frac{1}{T_1}\right)}{\Delta\left(\frac{1}{T_2}\right)} = \frac{R_{1\text{ex}}}{R_{2\text{ex}}} = \frac{\frac{1.6}{1 + 4\omega^2\tau_c^2} + \frac{0.4}{1 + \omega^2\tau_c^2}}{0.6 + \frac{0.4}{1 + 4\omega^2\tau_c^2} + \frac{1}{1 + \omega^2\tau_c^2}} \quad (2)$$

From this equation the correlation time τ_c can be calculated, which is an approximate measure of the mobility of a molecule or an ion. A raised τ_c value means less mobility. In Fig.4 the τ_c value for Na^+ increases immediately in the range $0 < [\text{Mg}^{2+}]_0 < 5$ mM and then reaches a plateau value. The jump in correlation time cannot be explained by a thermic mobility change of the Na-CS-P complex, because CS-P should influence the correlation times only insignificantly due to its molecular size and the ensuing relative immobility. Therefore, the most probable explanation of the sudden change of τ_c remains the induction of intra- or intermole-

Fig.4. Correlation time for sodium ions bound to CS-P as a function of the Mg^{2+} concentration of a Krebs solution at pH = 7.38. The ratio $R_{1\text{ex}}/R_{2\text{ex}}$ was used in the calculation of τ_c .

cular cross-linking leading to conformational transitions of the polyelectrolyte, to molecular associations, or to both. This means that already physiological concentrations of Mg^{2+} ions can induce a specific change in configuration, which enables K^+ ions to bind cooperatively to connective tissue structures [13]. Therefore, with extracellular Mg^{2+} deficiency, not only less Mg^{2+} ions are bound to vascular connective tissue but also less K^+ ions, and additionally a K^+ release is initiated. $[\text{K}^+]_0$ would increase near the cell membrane of vascular smooth muscle cells and sympathetic nerve terminals, depolarization and vasoconstriction would occur [13].

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