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Surface charge, fluidity, and calcium uptake by rat intestinal brush-border vesicles

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Abstract

Biological membrane outer surfaces are negatively charged and interact with positively charged calcium ion during calcium uptake. Positively charged polycations such as polyarginine bind to membranes with high affinity, displacing bound calcium from the membrane. We tested the effect of polyarginine on uptake of calcium by brush-border membrane vesicles and examined the responses in terms of membrane fluidity by electron paramagnetic resonance (EPR). Polyarginine inhibited the saturable component of calcium uptake by a mechanism combining inhibition characteristics of strontium (competitive) and magnesium (non-competitive). Unlike the inhibition of non-saturable calcium uptake by strontium and magnesium, polyarginine increased k_D, the rate constant for non-saturable calcium uptake, by a concentration dependent mechanism. These effects of polyarginine on calcium uptake were associated with decreased membrane fluidity at the uptake temperature. These findings are consistent with a role for surface negative charge in determining both saturable and non-saturable calcium uptake. Increased membrane fluidity is associated with decreased saturable and increased non-saturable calcium uptake. Although increased fluidity might be involved in the increased k_D for non-saturable uptake, the concentration-specific stimulating effect of polyarginine suggests a gating mechanism.

Keywords: Brush-border membrane vesicle; Calcium ion transport; Inhibition; Polycation; EPR; (Intestine)

1. Introduction

Uptake of the calcium ion by membrane vesicles from small intestinal enterocytes provides an experimental model to examine mechanisms involved in calcium absorption by the small intestine [1]. When measured under conditions approaching initial rate, calcium uptake by brush-border membrane vesicles, as related to medium calcium concentration, comprises saturable and non-saturable processes [2]. The saturable process is inhibited by cognate divalent cations in the uptake medium: strontium inhibition is competitive; magnesium inhibition is non-competitive [3]. When the vesicle technique is used to localize the cognate ion inside the vesicle, magnesium is without effect, but strontium increases V_{max} for saturable uptake of calcium. These responses to strontium are consistent with competition by strontium ions with calcium ions at negatively charged calcium uptake sites of the transporter on the outer and inner surfaces of the membrane, and suggests a mobile carrier mechanism for calcium uptake. The inhibition of saturable uptake by magnesium is consistent with binding to a negatively charged transporter site different from the Ca^{2+}/Sr^{2+} binding site on the outside of the membrane. V_{max} for brush-border membrane vesicle calcium uptake is also increased by vitamin D, but non-saturable uptake is unaffected [4].

Non-saturable uptake measured over a concentration range under initial rate conditions demonstrates a binding component: inhibition of calcium uptake to a similar degree by both strontium and magnesium ions in the uptake medium [3]. Localized to the inside of the vesicle, neither cognate ion altered non-saturable uptake. The remaining component of non-saturable uptake is uninhibited in the presence of strontium or magnesium in the uptake medium, or both inside the vesicle and in the uptake medium, and is assumed to be diffusion [3]. Although, under conditions approaching initial rate (i.e., 3–12 s uptake period), non-saturable calcium uptake increases almost linearly with

Abbreviations: EPR, electron paramagnetic resonance; V_{max}, maximal rate of saturable calcium uptake at infinite medium calcium concentration; K_T, medium calcium concentration at half V_{max}; k_D, rate constant for non-saturable calcium uptake.

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time [3], at long time points uptake saturates [2]. Prior studies of calcium binding by rat brush-border vesicles were performed in the time frame of minutes and show saturation [5]. Since uptake of calcium is resolved into saturable and non-saturable components only in the time-frame of seconds [3,6], the prior binding study [5] provides no information on the components of surface binding in relation to the dynamics of calcium uptake. However, knowledge of the concurrent binding that accompanies calcium uptake is critical to quantitating the several components of calcium uptake by brush-border membrane vesicles that are identifiable under initial rate conditions.

Interaction between ions in the uptake medium and membrane charge have been examined using polyvalent cations that bind tightly to the membrane surface. Polycation inhibition of sodium ion driven brush-border membrane D-glucose uptake is thought to result from competition with sodium ion for the negatively charged sodium binding site on the hexose transporter [7]. Because polycation binding affinity to liposome surface is sufficiently great to displace calcium [8], we examined effects of polyarginine on the saturable and non-saturable components of calcium uptake. Access of polyarginine is limited to the outer membrane surface because of molecular weight and charge.

Brush-border membrane fluidity has been extensively studied because of its possible regulatory role in absorptive processes. For example, brush-border membrane fluidity is thought to be regulatory for uptake of calcium, and membrane fluidity change may be involved in the calcium uptake response to vitamin D [9]. Fluidity of rat brush-border membrane vesicles measured by fluorescence polarization is altered by treatment with 1α,25-dihydroxycholecalciferol [10]. Therefore, we also examined the membrane fluidity response to polyarginine using electron paramagnetic resonance (EPR) to explore a possible relationship to calcium uptake responses.

2. Materials and methods

All animal experiments conformed to laboratory animal guidelines of proper care and use by Public Health Service, NIH, and by the University of Iowa Animal Welfare Committee. Young growing male rats obtained from Harlan Sprague-Dawley (Madison, WI) were housed in the animal care unit with a dark cycle 6:00 p.m. to 6:00 a.m. Rats were fed Purina (Ralston Purina, St. Louis, MO) rat chow (1.01% calcium, 0.74% phosphorus, 0.20% magnesium, and 3.3 IU vitamin D-3/g) ad libitum up to the morning of the experiment. An average of eight rats were used for each of the 14 vesicle preparations. After cervical dislocation, the segment comprising the proximal 30 cm of small intestine was excised. The excised segments were flushed with iced saline, everted, placed on a glass plate over ice, and the mucosa was scraped from underlying tissue into 5 mmol/l EDTA, 1 mmol/l Hepes, 1 mmol/l Tris and homogenized. Calcium chelation by EDTA minimizes action of calcium-activated lipases on the brush-border [11]. Brush-border membrane vesicles were prepared as previously described [2] according to the method of Forstner [12] and Hopfer [13]. Sucrase [14] and protein [15] were assayed in mucosal homogenate and brush-border membrane vesicle preparation. Purification was expressed as specific activity of sucrase (U/mg protein) in brush-border vesicles compared with homogenate.

Calcium uptake was measured at 3 s using ^{45}Ca tracer (0.04 mmol/l, specific activity 28.61 mCi/mg, New England Nuclear, Boston, MA) [3]. Tracer was either diluted or calcium added to give eight final concentrations in uptake tubes ranging from 0.01–1 mmol/l. The uptake medium consisted of 100 mmol/l KCl, 20 mmol/l Hepes-Tris with tracer ^{45}Ca and a series of calcium concentrations as above. The vesicle medium was the same as the uptake medium but contained no calcium or ^{45}Ca. Calcium uptake was measured under control conditions and from uptake medium containing polyarginine at each calcium concentration. Transport experiments were performed on the day of vesicle preparation. Poly(L-arginine) hydrochloride, M_r 41400, was purchased from Sigma (St. Louis, MO). The nitroxide stable radical, 12-doxylstearic acid, was purchased from Aldrich, Milwaukee, WI.

Calcium uptake was determined at 25°C by a Millipore filtration technique. To initiate uptake, 12.5 μl of vesicle suspension containing approx. 50 μg protein was mixed with 62.5 μl of uptake medium containing calcium and ^{45}Ca, with or without polyelectrolyte. Uptake was determined in triplicate at each of the 8 concentrations of calcium. Uptake was terminated after 3 s by addition of 1 ml ice-cold stop solution containing (in mmol/l) 100 KCl, 20 Hepes-Tris, 5 $MgCl_2$, 1 EGTA, pH 7.5, and the suspension was immediately filtered (Millipore filters, type HA, 0.45 μm, 25 mm diam, Millipore, Bedford, MA). The filter was washed with 12 ml of the same stop solution. Radioactivity remaining on the filter was counted by liquid scintillation after dissolving the filter in 2 ml of ethylene glycol monoethyl ether (Fisher, Fairlawn, NJ) and adding 8 ml aqueous counting scintillant (Amersham, Arlington Heights, IL).

2.1. Electron paramagnetic resonance (EPR)

12-Doxylstearic acid dissolved in ethanol (1 mg/ml; 2.6 nmol/μl) stored under nitrogen in the freezer was pipetted into 7 ml screw capped vials and the ethanol was evaporated under nitrogen. Brush-border vesicle aliquots suspended in 100 mmol/l KCl, 20 mmol/l Hepes-Tris, without or with 10^{-6} mol/l polyarginine, were pipetted into the vials. Based on pilot studies, optimal signal was obtained with a control vesicle mixture of 3 mg vesicle protein with 300 nmol 12-doxylstearic acid. Vesicles containing 10^{-6} mol/l polyarginine gave optimal signal with

2 mg vesicle protein with 200 nmol of 12-doxylstearic acid. After agitation by vortexing, vial contents were transferred by pipetting into 7 ml polycarbonate tubes and centrifuged at $35\,000 \times g$ for 15 min to pellet the vesicles. Vesicles were extracted with diluted Hanks buffer [16] to remove excess 12-doxylstearic acid. Hanks buffer concentrated 10-fold contained 0.90 g/l $Na_2HPO_4 \cdot 7H_2O$ instead of 0.06 g/l $Na_2HPO_4 \cdot 2H_2O$; 1 g/l $MgCl_2 \cdot 6H_2O$ was added to the original formula and phenol red was omitted (purchased from Gibco, Grand Island, NY). The concentrated buffer was diluted 10-fold with distilled water and pH was adjusted to 7.4 with solid $NaHCO_3$. After the supernate was decanted, pellets were rinsed twice with 1 ml Hanks buffer, either without or with 10^{-6} mol/l polyarginine, and resuspended in 4 ml of corresponding Hanks buffer. Pellet extraction was promoted by rapidly aspirating and discharging the suspension with a 1 ml tuberculin syringe and mixing of the suspension by vortexing. After transferring the suspension to clean polycarbonate tubes, the cycle of centrifugation, decanting of supernate, and washing with Hanks solution was repeated three times. All supernates were analyzed by EPR to verify extraction of excess 12-doxylstearic acid. After rinsing the final pellet twice with 1 ml Hanks solution, the pellet was drawn up into a capillary tube, the tip of the tube was sealed with heat-softened beeswax, the sample was shaken down to the tip, and then placed in the spectrometer and the temperature probe inserted.

EPR spectra were measured using a Bruker ESP300 EPR Spectrometer with ERVIII VT variable temperature unit (Bruker, Karlsruhe, Germany). Spectra were measured at 1°C increments from 15 to 45°C in the ascending mode. Six control and six 10^{-6} mol/l polyarginine measurements were performed from three different preparations of brush-border membrane vesicles. The EPR spectra were stored on the computer, plotted, and the order parameter S was calculated [17] and analyzed statistically [18] by one-way ANOVA.

2.2. Calculations

The EPR spectrum of a control sample at 25°C is shown in Fig. 2. When molecular motions of the spin probes in membranes are fast on the EPR time scale, the hyperfine splitting, $A_{\parallel} = A'_{\parallel}$ and $A'_{\perp}$ can be measured from the spectrum [17]. $A'_{\perp}$ is corrected to give $A_{\perp}$ as follows:

$$A_{\perp} = A'_{\perp} + 1.4\Big(1 - \big(A_{\parallel} - A'_{\perp}\big) \Big/ \big(A_{zz} - 0.5(A_{xx} + A_{yy})\big)\Big)$$

where A_{xx}, A_{yy} and A_{zz} are the principal hyperfine splittings (in gauss, G) of the spin probe. The hyperfine splittings for 12-doxylstearic acid are 6.3, 5.8, and 33.6 G for A_{xx}, A_{yy}, and A_{zz} respectively [17]. Since the hyperfine splitting also depends on the polarity of the nitroxide environment, a polarity normalization term, a'_o/a_o, has been introduced into the calculation of the order parameter, S:

$$S = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - 0.5(A_{xx} + A_{yy})} \times \frac{a'_o}{a_o}$$

where $a'_o = 1/3(A_{xx} + A_{yy} + A_{zz})$, and $a_o = 1/3(A_{\parallel} + 2A_{\perp})$.

2.3. Data analysis

Kinetic constants were calculated from total calcium uptake by the Systat software program [18] nonlinear estimation procedure, using the model statement

$$V_T = \big((V_{max} \times A)/(K_T + A)\big) + (k_D \times A)$$

where V_T is total uptake rate; V_{max} is maximal rate of saturable calcium uptake at infinite medium calcium concentration; K_T is calcium concentration at half maximal saturable calcium uptake rate; k_D is rate constant for non-saturable calcium uptake; and A is medium calcium concentration. The model statement was estimated by the Simplex algorithm direct search procedure. After kinetic constants were calculated for each control and polyelectrolyte experiment, mean ± S.E. was calculated. The same vesicle preparation was used for comparing control and polyelectrolyte experiments.

Experimental design forced an incomplete statistical block analysis. A control is present in each block. For each control, 1–3 concentrations of inhibitor were compared by SAS (Statistical Analysis Systems) using the general linear models procedure for analysis of variance [19], blocking by preparation and inhibitor concentrations. Differences were considered significant at $P < 0.05$ by Tukey's studentized range test.

3. Results

Data on the rats, mucosa, and brush-border preparations used in these experiments are shown in Table 1. Rats were growing, as shown by the 42 g increase in weight from day of receipt to experimental day, i.e., 6–7 g/day. Brush-border protein recovery was 0.46% of mucosal homogenate protein. Sucrase recovery in brush-border based on total homogenate sucrase was 16%. Enhancement of sucrase activity in brush-border above homogenate was 35-fold.

The effects of increasing polyarginine concentration on kinetic constants for saturable calcium uptake by brush-border membrane vesicles are shown in Table 2. As the polyarginine concentration was increased, V_{max} was significantly decreased at $5 \cdot 10^{-6}$ mol/l. Above $5 \cdot 10^{-6}$ mol/l, V_{max} decreased progressively, whereas K_T shows corresponding increases. In comparison with controls, V_{max} was decreased significantly ($P < 0.05$) at the three highest

Table 1
Data on animals studied, mucosa, homogenate and brush-border fractions [a]

Body wt, g	
initial	186 ± 6
final	228 ± 5
Mucosa per rat	
wet wt, g	1.46 ± 0.03
protein, mg	
homogenate (H)	189 ± 5
brush-border (BB)	0.86 ± 0.06
recovery, % (BB/H × 100)	0.46 ± 0.04
Sucrase specific activity, U/mg protein	
homogenate (H)	0.053 ± 0.002
brush-border (BB)	1.834 ± 0.058
enhancement (BB/H)	34.8 ± 1.3
Sucrase per rat, U	
homogenate (H)	10.03 ± 0.34
brush-border (BB)	1.58 ± 0.12
recovery, % (BB/H × 100)	16.1 ± 1.4

[a] Values are means ± S.E. A total of 114 rats was used for 14 brush-border membrane vesicle preparations.

polyarginine concentrations, and K_T was increased at the two highest polyarginine concentrations. Unlike the pattern of greater effects on V_{max} and K_T with increasing concentrations, polyarginine elevated ($P < 0.05$) k_D above control at intermediate polyarginine concentrations, with peak elevation at 10^{-6} mol/l. Polyarginine at 10^{-7} mol/l had no effect on k_D, and the 50% increase above control with 10^{-4} mol/l was not significant.

Total, saturable and non-saturable uptake of calcium under control conditions and in the presence of 10^{-6} mol/l polyarginine is shown in Fig. 1. At 10^{-6} mol/l, polyarginine increased non-saturable uptake by 350%, but had no effect on saturable uptake. In contrast, at 10^{-4} mol/l, polyarginine decreased V_{max} to half the control, but had no significant effect on non-saturable uptake (Fig. 1).

The EPR spectrum of 12-doxylstearic acid in brush-border membrane vesicles from the control specimen suspended in Hanks buffer measured at 25°C is shown in Fig. 2. The 12-doxylstearic acid would be oriented essentially parallel to the fatty acid chains in the brush-border phospholipids with the carboxyl group at the aqueous interface. A membrane order parameter, S, can be determined from spectral measurement of the observed $2A'_{\parallel}$ and $2A'_{\perp}$ (Fig. 2), which results from motional averaging. Our instrumentation permitted a 5-fold amplification of the spectra (Fig. 2 inset, 5 ×) allowing reliable measurement of S up to 45°C. In the most fluid state where motion of the spin probe is isotropic, $S = 0$; in the most ordered environment, $S = 1$. The value of S is calculated from the EPR spectrum separations between the observed outer and inner hyperfine extremas, $2A'_{\parallel}$ and $2A'_{\perp}$. The EPR spectrum is typical for lipid dispersions in which the probe is undergoing rapid but anisotropic motion. The observed outer hyperfine extrema, $A'_{\parallel}$, decreased as the temperature increased because of increased wobbling of the probe within the bilayer.

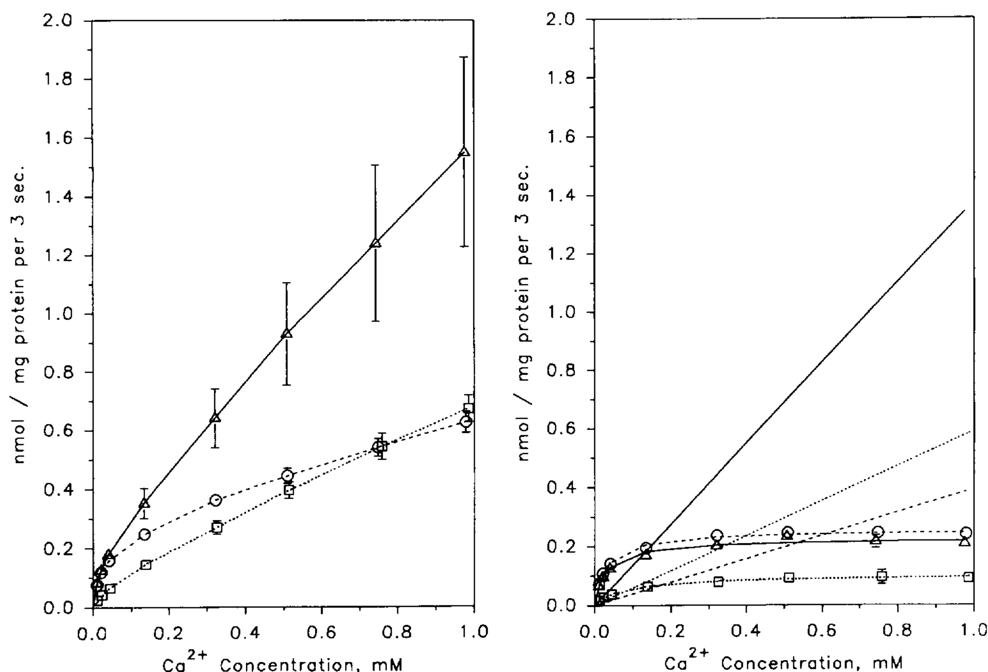

Fig. 1. Polyarginine and calcium uptake by brush-border membrane vesicles. Total (left panel) and saturable and non-saturable uptake (right panel) are shown under control conditions (O- - -O) and in the presence of 10^{-6} mol/l polyarginine (△——△) and 10^{-4} mol/l polyarginine (□ · · · · · □). (Left panel) In comparison with control, 10^{-6} mol/l polyarginine increased total calcium uptake, particularly at higher calcium concentrations. (Right panel) Although saturable uptake is not affected by 10^{-6} mol/l polyarginine, non-saturable uptake is increased by a factor of 3.5. Saturable uptake of calcium is decreased to less than half that of control by 10^{-4} mol/l polyarginine, but the small increase in non-saturable uptake is not significant.

Table 2
Kinetic constants for calcium uptake effects of polyarginine [a]

Experiment	N	V_{max} (pmol/mg protein per 3 s)	k_D (pmol/mg protein per 3 s at 1 mmol/l Ca^{2+})	K_T (mmol/l × 10^3)
Control	14	254 ± 14	392 ± 27	32 ± 3
Polyarginine (mol/l)				
$1 \cdot 10^{-7}$	5	203 ± 28	402 ± 37	23 ± 3
$1 \cdot 10^{-6}$	5	224 ± 12	1378 ± 339 [b]	30 ± 5
$5 \cdot 10^{-6}$	4	191 ± 19 [b]	1060 ± 133 [b]	48 ± 17
$1 \cdot 10^{-5}$	5	179 ± 22 [b]	923 ± 69 [b]	66 ± 5 [b]
$1 \cdot 10^{-4}$	6	103 ± 22 [b]	590 ± 34	68 ± 15 [b]

[a] Values are means ± S.E. Data from 14 vesicle preparations, each preparation consisted of one control and 1–3 experimental uptakes.
[b] Differs from control, $P < 0.05$.

Control and polyarginine-treated vesicles were measured over the temperature range of 15 to 45°C to compare temperature effects on membrane order (Figs. 3 and 4). For the control, apparent discontinuities in temperature dependence of S were observed at about 25–28°C, and possibly also in the range of 41°C (Fig. 3). Over the temperature range of 25–40°C, the rate of change of S with temperature was relatively constant. At temperatures below the discontinuity at 25–28°C, the rate of change in S decreased. At temperatures above the apparent discontinuity at 41°C, the slope of the curve also decreased when compared to the 25–40°C range.

In comparison with the 'S-shaped' curve of the control (Fig. 3), the plot for vesicles in the presence of polyarginine tended to approach linearity. Although discontinuities are suggested at 25°C and 41°C, they are much less evident than in the control. Thus, electrostatic binding of polyarginine to the membrane surface tended to suppress discontinuities at the membrane lipid bilayer level as measured using 12-doxylstearic acid.

The S plots of Figs. 3 and 4 are superimposed in Fig. 5. The two curves cross at about 28.5°C because the polyarginine-treated vesicles do not show the discontinuity of the control in the range of 25°C. Values of S are significantly greater for the control over the range 21–26°C and are greater for polyarginine-treated vesicles from 31 to 44°C. The lower S value of the polyarginine-treated vesicles at 25°C implies greater fluidity at the temperature of the calcium uptake studies. Since these effects on S occur in relationship to the apparent discontinuity in the range of 25°C, they may be related to thermotropic lipid phase transitions. Based on the S values, polyarginine alters

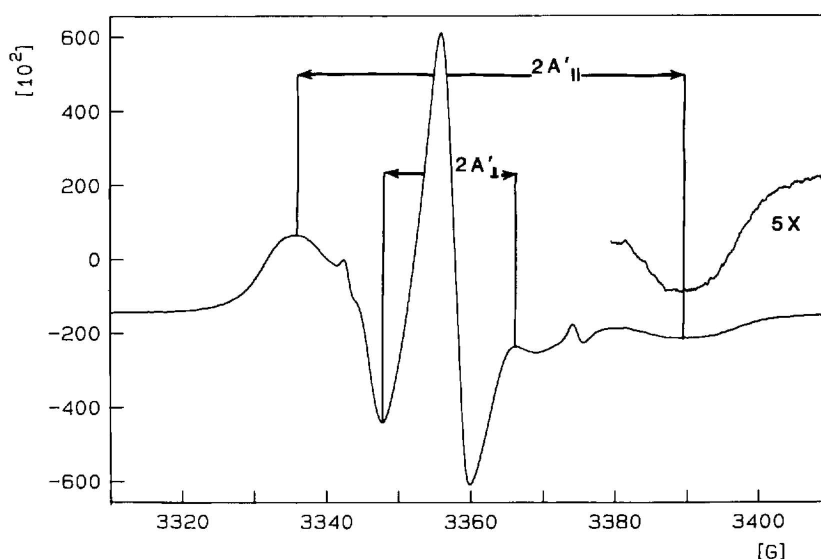

Fig. 2. Electron paramagnetic resonance spectrum of 12-doxylstearic acid in brush-border membrane vesicles suspended in Hanks buffer, control sample at 25°C. Measurements made for calculating S, i.e., $2A'_{\parallel}$ and $2A'_{\perp}$ are shown. Spectrum width is 100 G. The spectrum expanded 5-fold (5 ×) for precise measurement of $2A'_{\parallel}$ is shown in the inset.

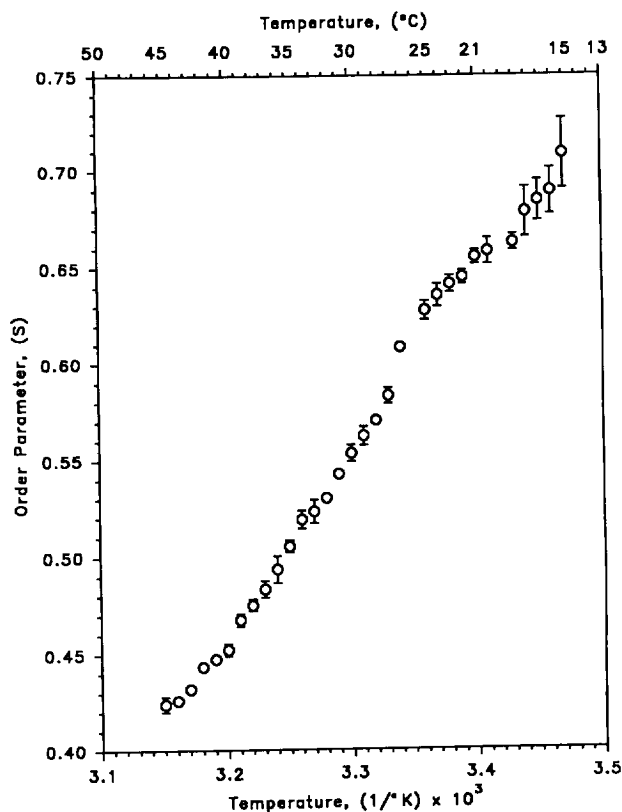

Fig. 3. Membrane order parameter (S, mean ± S.E.) in relation to temperature (°C; $(1/K)\times10^3$) for control brush-border membrane vesicles. Discontinuities are present in the region of 25°C and possibly also at 41°C.

membrane lipid bilayer fluidity by stabilizing the membrane from 31–44°C while simultaneously increasing fluidity from 21 to 26°C by eliminating the apparent phase transition.

4. Discussion

4.1. Calcium transport

Anionic binding sites on the brush-border are involved in saturable and non-saturable uptake of calcium [3]. Saturable calcium uptake involves negatively charged sites on a mobile carrier transporter. The calcium binding site on the transporter also accepts strontium. A second negatively charged site on the transporter binds magnesium. Both strontium and magnesium inhibit non-saturable calcium uptake similarly, apparently by binding non-specifically to anionic sites on the membrane surface. Polyvalent cations such as polyarginine would be expected to bind to anionic sites on the membrane surface but, unlike divalent cations, remain unabsorbed.

We found polyarginine in the uptake medium to exert opposite effects on saturable (inhibition) and non-saturable (acceleration) uptake of calcium. These findings contrasted with response to the cations we previously studied (strontium and magnesium), which were inhibitory for both uptake processes under the same conditions. In the case of saturable uptake, polyarginine response mimicked that of both strontium (increased K_T) and magnesium (V_{max} was not restored to control by increasing calcium concentration). Thus, the inhibition by polyarginine is neither competitive nor non-competitive, although it shows features of both. This suggests that polyarginine bound to the membrane combines with both the negative charge on the transporter that accepts calcium and the negative charge on the transporter that is regulated by magnesium, and thus both increases K_T and depresses V_{max}.

Polycations have the potential to disrupt membranes, thereby increasing membrane permeability. The increased k_D for non-saturable uptake of calcium is most readily explained by increased membrane permeability with resultant increase in diffusive uptake of calcium. Increased permeability is in accord with increased membrane fluidity demonstrated by EPR. The enhanced diffusive uptake of calcium prevents detection of possible effects of polyarginine binding to decrease the vesicle surface binding component of non-saturable calcium uptake previously demonstrated with strontium and magnesium in the uptake medium [3].

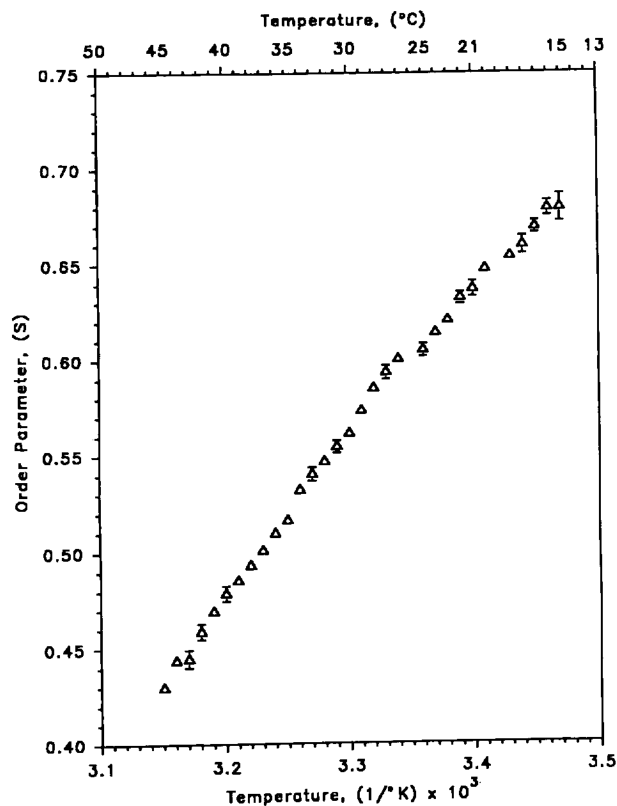

Fig. 4. Membrane order parameter (S, mean ± S.E.) in relation to temperature in the presence of polyarginine. Discontinuities are greatly decreased in comparison with control.

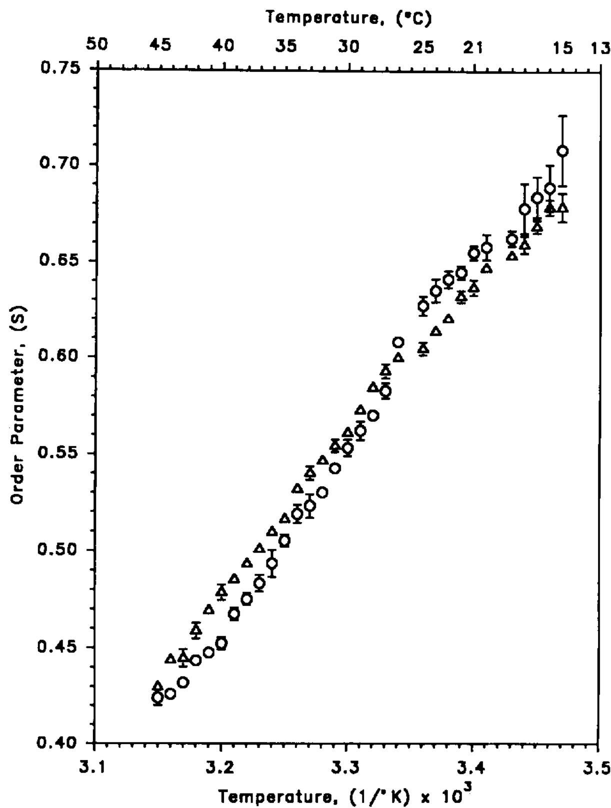

Fig. 5. Membrane order parameter (S) in relation to temperature, °C, in the absence (○) and presence (△) of 10^{-6} mol/l polyarginine. S is lower ($P < 0.05$; greater fluidity) in the presence of polyarginine at the uptake temperature used in these experiments (25°), but is greater ($P < 0.05$) at physiological temperatures (38–41°C). S is significantly lower in polyarginine-treated vesicles over the range 21–26°C and is greater over the range 31–44°C ($P < 0.05$).

In prior experiments, we showed the converse of the polyarginine effect on k_D, decreased ($P < 0.05$) non-saturable uptake of calcium in response to cholesterol enrichment of brush-border membrane vesicles [20]. Fluidity, expressed as r values from diphenylhexatriene fluorescence polarization measurements, was decreased over the entire temperature range of the plot, and approached statistical significance ($P = 0.09$). It is also possible that polyarginine acts on a specific component of non-saturable calcium uptake. The calcium channel antagonists diltiazem and verapamil increased and nicardipine decreased k_D for non-saturable calcium uptake by intestinal brush-border membrane vesicles [21]. k_D was also inhibited by trifluoperazine (Schedl et al., unpublished data). Effects of calcium channel blockers to increase renal brush-border membrane calcium uptake have recently been reviewed [22].

Prior experiments with polycations also showed effects on both saturable and non-saturable transport processes [7,23]. Polycations inhibited neutral amino acid uptake competitively, but inhibition of monosaccharide uptake was of 'mixed type' in experiments using everted rings. Glucose uptake by brush-border membrane vesicles was also inhibited. Similar to our experiments with saturable uptake of calcium, inhibition increased with increasing polycation concentration. It was hypothesized that the polycation inhibited transport by binding to the sodium binding anionic site of the cotransporter. Two substrates showing non-saturable transport, mannitol and 2-deoxy-D-glucose, showed increased in vitro [7] and in vivo [23] transport in the presence of polycation.

4.2. EPR: order parameter

The purpose of the EPR studies was to examine effects of the polyarginine surface binding on the brush-border membrane and to relate these findings to responses in calcium uptake mechanisms. The EPR data demonstrate effects of polyarginine on the membrane order parameter, S, of the brush-border. The response to polyarginine appears to be related to the discontinuity of the temperature dependence of S of the native membrane at 25°C. Below the discontinuity of the control at 25°C, where the membrane is more ordered, polyarginine increased fluidity. Above the discontinuity temperature of the control, in the physiologic temperature range (31–44°C), where the control membrane is more fluid, the polyarginine decreased fluidity.

Extensive studies by a variety of techniques including EPR have defined properties of rabbit small intestine brush-border membrane vesicles and extracted lipids [24,25]. EPR measurements utilized 12-doxylstearic acid among a number of probes [24]. In both the prior and present experiments spectra were typical for these probes in lipid dispersions in which the probe is undergoing rapid anisotropic motion. The values for S decreased with increasing temperature, and plots of S vs. $1/T$ showed apparent discontinuities. In experiments using 12-doxylstearic acid, our S values in rat were higher than in vesicles from rabbit. Values for the hyperfine splitting $2A'_{\parallel}$, an indication of anisotropy of motion, were also higher in rat. At 25°C, $2A'_{\parallel}$ values for rat were 53.8 ± 0.5 G for control and 51.9 ± 0.3 G for polyarginine vesicles, as compared with 45.8 G for the rabbit. At 40°C, corresponding values were 43.6 ± 0.2 G for control and 44.7 ± 0.2 G for polyarginine rat vesicles, as compared with 41.0 G for rabbit. Values for $2A'_{\perp}$ were similar for both rat and rabbit. The rate of change of $2A'_{\parallel}$ with temperature over the 25–40°C range was more rapid in rat, 10.2 G/15°C for control, than in rabbit, 4.8 G/15°C. Thus, fluidity of the more highly ordered rat membrane changed more rapidly with temperature. The plot of $2A'_{\parallel}$ vs. $1/T$ for the control showed the same two apparent discontinuities (25 and 41°C) more definitively than the plot of S values (Fig. 3) (data not presented). The plot of $2A'_{\parallel}$ vs. $1/T$ for the polyarginine treated vesicles was not as linear as the corresponding S plot (Fig. 4) and was suggestive of a discontinuity at 25°C but not at 41°C (data not presented). Another interesting difference between the present and

prior experiments in rabbit [24] is in the rate of change of S with temperature in relation to the discontinuity at 25°C. In the rat experiments, rate of change of S with temperature decreased below the discontinuity temperature, whereas with the rabbit it increased. The same pattern was even more clearly demonstrated by the plot of $2A'_{\parallel}$ (data not shown). This type of pattern has previously been observed in S plots of $2A'_{\parallel}$ data using 8-doxyl phosphatidylcholine with rabbit brush-border membrane vesicles [26].

The variation in the hyperfine splitting constant a_N can be used as a measure of the polarity of the spin label environment [27]. The isotropic a_N of 12-doxylstearic acid in the buffer was 15.90 ± 0.01 as compared with the anisotropic a_N $((A_{\parallel} + 2A_{\perp})/3)$ for the control 15.10 ± 0.09, and 14.80 ± 0.05 for polyarginine vesicles. The lower a_N values in the membrane are consistent with a non-polar, lipoidal medium.

The brush-border membrane vesicles used for EPR measurements in the prior studies [24,25] as well as in the current experiments should be representative of the native membrane lipids, since calcium chelation was used to prevent action of calcium-activated lipases [11]. The technique of vesicle preparation used in the present experiments differed from that used by prior workers [24]: differential centrifugation vs. magnesium precipitation. Thus, in addition to species differences, the method of vesicle preparation could influence the differences between rat and rabbit.

Based on the prior EPR spin probe studies [24] correlated with differential scanning calorimetry experiments [25], the discontinuity in S near 25–28°C (Fig. 3) in the present study probably results from thermotropic lipid phase transitions and/or phase separations. With the rabbit, this discontinuity was at 22°C [24]. Thus, the higher S values for rat are in the setting of a higher phase transition temperature. The 25°C temperature range is too far from mammalian body temperature to be physiologically significant except in the setting of hypothermia. A phase transition setting in the temperature range of 41°C (Fig. 3) would be of possible physiologic significance. Similar transitions in the temperature range of 23–39°C (peak temperature 31°C) have been demonstrated by differential scanning calorimetry of rat brush-border membrane vesicles [28]. This temperature is higher than that observed for rabbit [25]. Similarly, the discontinuity in the EPR order parameter S with 12-doxylstearic acid in rat is at a higher temperature than that observed in rabbit (25 vs. 22°C). The prior studies in rat [28] did not use chelation to minimize brush-border phospholipid hydrolysis by calcium-activated lipases.

Although not previously studied by EPR, rat brush-border membrane vesicle fluidity has been extensively studied by fluorescence polarization [10,28–31] and by differential scanning calorimetry [28]. Membrane fluidity showed temperature-dependent discontinuities and fluidity of brush-border membranes was lower than in plasma membranes such as basolateral membranes [30].

4.3. Polycations stabilize liposomes

Studies of bilayer stabilization of phospholipid liposomes by the polycation, polylysine, showed that binding strength was highly dependent on the degree of polymerization [8], requiring three or more lysine residues per molecule. The combined interaction of multiple lysines within the polymer promotes binding. The high degree of polyarginine polymerization in our experiments greatly increases binding of polyarginine. In the cardiolipin liposome experiments [8], calcium and polylysine compete for binding, but affinity of polylysine for cardiolipin negative charge was much greater than the calcium ion affinity. Binding of polylysine released calcium from the calcium-cardiolipin complex. This may be a model for interaction of calcium and other metal cations with polyvalent cations and brush-border surface charge.

In prior experiments fluidity of brush-border membrane vesicles has been altered by incorporating hydrophobic compounds such as cholesterol, amphipathic compounds such as phospholipids [20,31] or polar compounds such as benzyl alcohol [30] into the lipid bilayer. The present experiment describes a novel approach to altering membrane fluidity, while simultaneously virtually abolishing discontinuities in the S plot. Polyarginine probably produces its effects without entering the lipid bilayer, but by acting on charges on the membrane surface. Adhesion of biologically active charged macromolecules to specific sites on cell membranes has the potential to produce analogous effects on calcium transport and fluidity. However, actions of polycations may not be limited to the membrane surface, as demonstrated by their application to introduce exogenous DNA into cells [32].

In summary, we used binding of polyarginine to vesicle surface charge to determine the effect of charge neutralization on calcium uptake processes. Both saturable and non-saturable uptake processes were altered. Inhibition of saturable calcium uptake results from preventing binding of calcium to the calcium binding site of the mobile carrier transporter, as well as to the magnesium regulatory site. The relationship between the increase in non-saturable calcium uptake and membrane fluidity secondary to polyarginine binding requires further study.

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