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Development of the Gastrointestinal Mucosal Barrier V. Comparative Effect of Calcium Binding on Microvillus Membrane Structure in Newborn and Adult Rats

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Summary

Electron spin resonance (ESR) and the spin label method, with 5-doxyl stearic acid as a probe, were used to investigate the structure of microvillus membrane (MVM) from small intestine of adult and newborn rats. It was shown that the spin label in MVM of newborn was maintained in a more disordered environment than the spin label in adult animals. Calcium ion was used as an external stimulus to study the structural response and organization of these two membrane preparations. Ca^{++} enhanced the order of 5-doxyl stearic acid labeled MVM from mature and immature rats in a concentration-dependent saturable process, but Ca^{++} exerted a greater ordering effect on MVM from immature than MVM from the mature rat. Ca^{++} binding to MVM was also a concentration-dependent, saturable process. MVM from immature rat bound significantly more Ca^{++} in $CaCl_2$ concentration ranges from 12.5 μm to 4mM. Scatchard analysis of the binding data showed two classes of binding sites with a high affinity constant of 3.1×10^4 M^{-1} and a low affinity constant of 9.1×10^3 M^{-1}, with corresponding maximum binding capacities for each class site of 129.8 nmole of calcium/mg protein and 252.7 nmole calcium/mg of protein in newborn and 13-day-old MVM. Only one high affinity constant of $2.6 \times 10^4 M^{-1}$ with a corresponding maximum binding capacity of 106.4 nmole/mg of protein was observed in adult MVM. Proteolytic hydrolysis of the membranes by trypsin produced an increase in Ca^{++} binding in adult MVM and a decrease in Ca^{++} binding in newborn MVM. Neuraminidase and phospholipase C reduced the amount of bound Ca^{++} in both adult and newborn MVM. These results indicate a more disordered structure of newborn MVM and a differential effect of Ca^{++} on MVM during development.

Abbreviations

ESR, electron spin resonance
G, Gauss units
MVM, microvillus membrane

The microvillus surface (glycocalyx) of the small intestine represents a highly differentiated and metabolically active compartment that contributes to the final stages of digestion and active transport of nutrients, as well as providing an important barrier to the penetration of toxic and antigenic macromolecules and pathogens present in the luminal environment (10). In previous studies, we had begun to characterize the microvillus surface of the small intestine from developing animals and to compare its barrier function with that of the adult animals. We have thus far separated differences in the glycoprotein composition of isolated MVM and binding characteristics of lectins and enterotoxins to MVM from adult and newborn intestinal mucosal preparations (1, 2). In more recent comparative studies, we determined actual structural differences in MVM preparations from small intestine of adult and newborn animals and their differential structural response to the binding of enterotoxin using a membrane probe, 5-doxyl stearic acid (22). Because calcium ion has been used successfully to study plasma membrane characteristics from liver and kidney as well as mitochondrial membranes and endoplasmic reticulum (3, 14, 16, 21), we began in this study to examine the comparative Ca^{++} ion binding characteristics of MVM and the effect of Ca^{++} ion binding on the structure of MVM from intestinal preparations of newborn, 13-day-old, and adult rats.

MATERIALS AND METHODS

Membrane preparation. Microvillus membranes were prepared from newborn, 13-day-old, and 6-month-old female Sprague Dawley rats (Charles River, Wilmington, MA) by a modification of the method of Schmitz et al. (25), as previously reported (22). The final membrane preparation was maintained in 10 mM Hepes buffer, pH 7.4 for studies. Purity of membrane preparations was assessed by determining the specific activity of marker enzymes sucrase (adult) and lactase (newborn and 13-day-old) (18). The final ratio of specific activities of sucrase from the purified membrane preparation compared to the initial homogenates ranged from 13–20. The enrichment factor of lactase activity in a MVM preparation from immature animals ranged from 20–30. No DNA was detected in either of the final membrane preparations (7). Membrane protein concentration was determined by the method of Lowry *et al.* (15).

Measurement of membrane structure. The spin label method was used to monitor the effect of calcium ion on MVM isolated from newborn and adult rats. The spin label probe, 5-doxyl stearic acid (Sylva Co., Palo Alto, CA) at a concentration of 6.5 mM, for labeling membranes was stored at −20°C in absolute methanol. Ten to twenty microliters of spin label stock solution were dried under nitrogen gas and mixed with 500 μ of MVM preparations in 10 mM Hepes buffer, the mixture was vortexed for 1 min to ensure dispersion of the spin label into MVM. To study the effect of Ca^{++} on MVM structure, 100 μl of $CaCl_2$ solution in concentrations from 0.1 mM to 50 mM was mixed with equal volume of spin labeled MVM. The mixture was then incubated for 30 min at room temperature before an ESR spectrum was obtained. ESR spectra were recorded on a Varian E-9 spectrophotometer equipped with a variable temperature accessory. All spectra were taken at 24°C.

The flexibility of the spin label in MVM was quantitated by first measuring the hyperfine splitting parameters $2T'_{\parallel}$ (parallel) and $2T'_{\perp}$ (perpendicular) expressed in G units. These measurements correspond to the separation of the outer and inner

spectral extrema, respectively, in ESR spectrum of the labeled MVM (Fig. 1), and reflect the rotational diffusion of the spin label about the molecular axis in the membrane. Both $2T'_{\parallel}$ and $2T'_{\perp}$ were used to calculate the order parameter S′ according to the method of Hubbel and McConnell (11).

$$S' = \frac{T'_{\parallel} - T'_{\perp}}{T_{zz} - T_{xx}} \cdot \frac{a}{a'} \quad (1)$$

The order parameter S′ is an index for the flexibility of the spin label in the membrane environment or more accurately, S′ measures the angular deviation of the probe from its average orientation in membrane, an amplitude of its anisotropic motion. The value of S′ ranges from 0–1; 0 represents a completely disordered structure and 1 represents a completely ordered structure. Both S′ and $2T'_{\parallel}$ are highly sensitive to the changes in motional environment of the spin label, and have been shown to be inversely related to the flexibility of the label in membrane; and, therefore, directly related to the order in the membrane. In some cases, where a highly immobilized probe was observed, $2T'_{\parallel}$ was a good substitute for S′ for indexing flexibility in membrane.

Ca^{++} binding. MVM from newborn, 13-day-old, and adult at 1 mg/ml of membrane protein in 10 mM Hepes buffer pH 7.4 was mixed with equal volume of $CaCl_2$ solutions in the concentration ranges from 25 μM to 8 mM with trace amount of [$^{45}CaCl_2$] (NEZ-013 New England Nuclear, Boston, MA). The mixture was incubated at room temperature for 30 min. Aliquots (50–100 μl) of the mixture were centrifuged at 30 psi for 15 min at 178,000 × *g* in a Beckman airfuge to separate the unbound $CaCl_2$ from MVM. The radioactivity in 25 μl of supernatant and 25 μl of incubation mixture were measured in a scintillation counter. (Delta 300 Scintillation system, Searle Analytic Inc. Des Plaines, IL). The amount of [$^{45}Ca^{++}$] bound was calculated.

The nature of Ca^{++} binding sites in the MVM was further investigated by employing specific enzymes to modify the membrane surface. One hundred micrograms of trypsin (T-0134, Sigma, St. Louis, MO); 10 μg of neuraminidase (N-2133, Sigma St. Louis, MO), 50 μg of phospholipase C (P-0264, Sigma, St. Louis, MO) or 100 μl of buffer (control) were mixed with 0.3 mg MVM from adult and newborn preparations in 1 ml of 25 mM tris buffer, pH 7.3. The mixture was incubated at 37°C for 1 h and centrifuged at 36,000 × *g* for 30 min to separate membrane from enzyme. The precipitate was resuspended in 0.3 ml of 10 mM Hepes buffer at pH 7.4. The treated MVM was then mixed with 0.3 ml of $CaCl_2$ at 50 μM containing trace amount of [$^{45}Ca^{++}$]. Again the mixture was incubated at room temperature for 30 min and the unbound Ca^{++} was separated from MVM by ultracentrifugation in an airfuge. The amount of [$^{45}Ca^{++}$] bound was calculated as previously described.

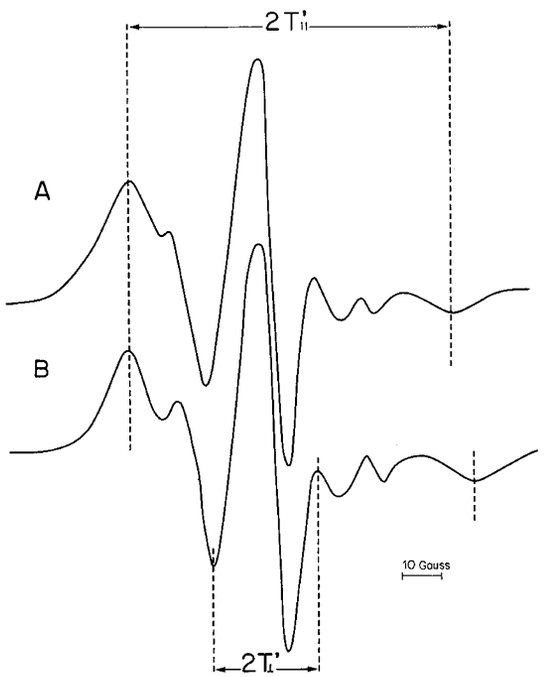

Fig. 1. A comparison of the typical electron span resonance spectrum of 5-doxyl stearic acid label newborn (*A*) and adult (*B*) microvillus membrane.

RESULTS

Microvillus membrane structure in newborn and adult animals. A comparison of representative ESR spectra of 5-doxyl stearic acid labeled MVM from adult and newborn rats at 24°C are shown in Figure 1. These spectra indicate that the spin label undergoes a rapid, anisotropic motion about its long molecular axis in a fairly restricted environment (8, 11), *i.e.*, flexing motions of the probe are relatively restricted. In Figure 1, it is clear that $2T'_{\parallel}$ in MVM from adult preparation is greater than $2T'_{\parallel}$ in MVM from newborn preparation. Using the expression in equation (1), order parameters were found to be 0.746 ± 0.010 for adult MVM and 0.677 ± 0.002 for newborn MVM (with six membrane preparations). Both $2T'_{\parallel}$ and S′ have been noted to be inversely related to the flexibility of the probe in membrane and therefore, related to the order of the membrane. At the same temperature, the spin probe of MVM from newborn rats reported a smaller order parameter than that of the spin probe in MVM from adult rats, suggesting a less ordered environment in MVM from newborn.

Effect of calcium on MVM structure. The effect of calcium on the MVM structure was tested by adding $CaCl_2$ to 5-doxyl stearic acid labeled microvillus membrane at 24°C. Millimolar $CaCl_2$ decreased the membrane flexibility of MVM from newborn and adult, as indicated by a positive increase in $2T'_{\parallel}$ (Fig. 2). The

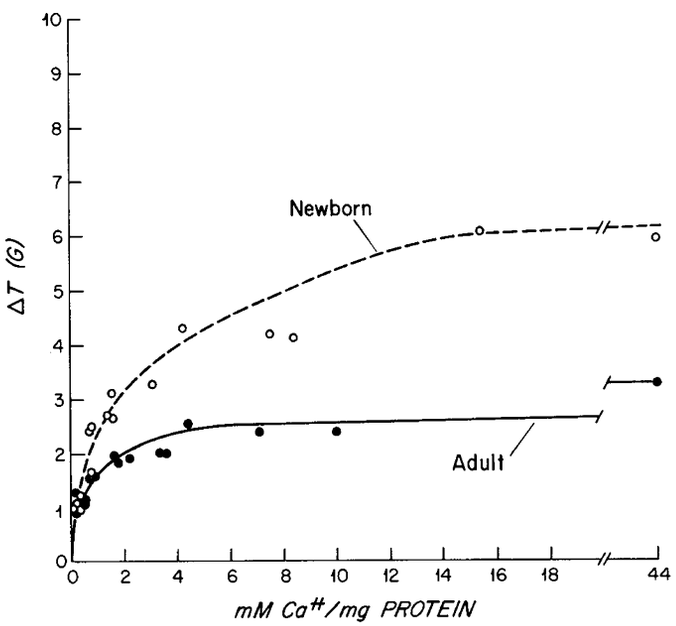

Fig. 2. Effect of increasing $CaCl_2$ concentration on 5-doxyl stearic acid labeled microvillus membranes (MVM) from adult (●) and newborn (○). The experimental procedures are described in "Materials and Methods." $\Delta T = (2T'_{\parallel})_{Ca^{++}} - (2T'_{\parallel})_{MVM}$. Where $(2T'_{\parallel})_{Ca^{++}}$ is the hyperfine splitting constant of 5-doxyl stearic acid labeled MVM with $CaCl_2$ at that concentration; $(2T'_{\parallel})_{MVM}$ is the hyperfine splitting constant of 5-doxyl stearic acid labeled MVM in 10 mM hepes, pH 7.4 without $CaCl_2$. Membrane protein concentrations were in the range from 0.8–4.5 mg/ml. Spin label concentrations ranged from 1–5 × 10^{-5} M. The mean $2T'_{\parallel}$ for adult MVM is 57.85 ± 0.39 G (n = 6) and 53.00 ± 0.13 G (n = 6) for newborn MVM.

calcium effect on these membranes appeared to be a concentration-dependent saturable process. There were major differences observed between the immature and mature MVM in their reactions to calcium ions. Ca^{++} had a greater effect in ordering the MVM from immature rats than that from adult rats. The ordering effect reached a maximum at 5 mM $CaCl_2$/mg of protein in adult MVM whereas no saturation was observed in MVM from newborn up to 15 mM $CaCl_2$/mg of protein.

The nature of the Ca^{++}-induced ordering in 5-doxyl stearic acid labeled MVM was characterized in additional experiments. The ordering effect of $CaCl_2$ remained unchanged in the presence of 150 mM NaCl, indicating that the action of Ca^{++} was not due to the increase in ionic strength.

[$^{45}Ca^{++}$] binding to MVM. Next, the binding characteristic of Ca^{++} to MVM was examined by ultracentrifugation. Ca^{++} binding reached a steady state by 15 min at all concentrations studied. It was a concentration-dependent process (Fig. 3) reaching a plateau at about 200 μM in adult MVM, and at about 4 mM in 13-day-old and newborn MVM. Scatchard analysis of a typical binding study at 10 mM Hepes pH 7.4 after correction for nonspecific binding (4, 24) is shown in Figure 4. Two classes of binding sites with a high affinity constant of 3.1×10^4 M^{-1} and a low affinity constant of 9.1×10^3 M^{-1} with the corresponding maximum binding capacities for each class site being 129.8 nmole of calcium/mg protein and 252.7 nmole calcium/mg protein in newborn and 13-day-old MVM. In contrast, only one high affinity constant of 2.6×10^4 M^{-1} with a corresponding maximum binding capacity of 106.4 nmole/mg of protein was observed in adult MVM. There was excellent agreement among three different microvillus membrane preparations with standard errors for Bmax and Ka values of less than 10% of the mean values.

The Ca^{++} binding to MVM was also carried out at high (0.5 mM) and low (0.1 mM) $CaCl_2$ concentrations, with increasing amounts of MVM to further characterize the nature of the binding (Fig. 5). At low $CaCl_2$ concentration, the amount of bound Ca^{++} increased with greater MVM concentration and yet, no saturation effect was observed up to 2.0 mg/ml of MVM in both adult and newborn preparations; however, there was a considerably larger amount of Ca^{++} bound to MVM from newborn than from adult animals in the entire range of MVM concentrations. Again at 0.5 mM $CaCl_2$, more Ca^{++} was bound to newborn MVM than MVM from adults, and both MVM preparations eventually reached saturation.

[$^{45}Ca^{++}$] binding to modified MVM. To determine which components in the microvillus membrane were involved in the Ca^{++} binding, MVM were modified with various enzymes and binding experiments repeated. Proteolytic hydrolysis of the membranes by trypsin produced a 8% increase in the amount of Ca^{++} bound in adult MVM and 17% decrease in newborn MVM at 25 μM $CaCl_2$ (Fig. 6). These data suggested a differential effect of trypsin on these membrane preparations. Since the carboxyl group of neuraminic acid residues are possible binding sites for Ca^{++} (27), the effect of their removal by neuraminidase was studied. The results are shown in Figure 6. Treatment of neuraminidase decreased Ca^{++} binding in both adult and newborn MVM. These results suggest that neuraminic acid may play an important role in Ca^{++} binding to MVM. Phospholipase C removes the entire polar group of phospholipids and would be expected to decrease the number of binding sites available to Ca^{++} (21). The results from phospholipase C experiments (Fig. 6) show a dramatic decrease in Ca^{++} binding in both MVM.

DISCUSSION

The microvillus surface of the small intestine represents a highly differentiated and metabolically active compartment which contributes to the end states of digestion and active transport of nutrients, as well as providing an important barrier to the penetration of toxic substances present in the external

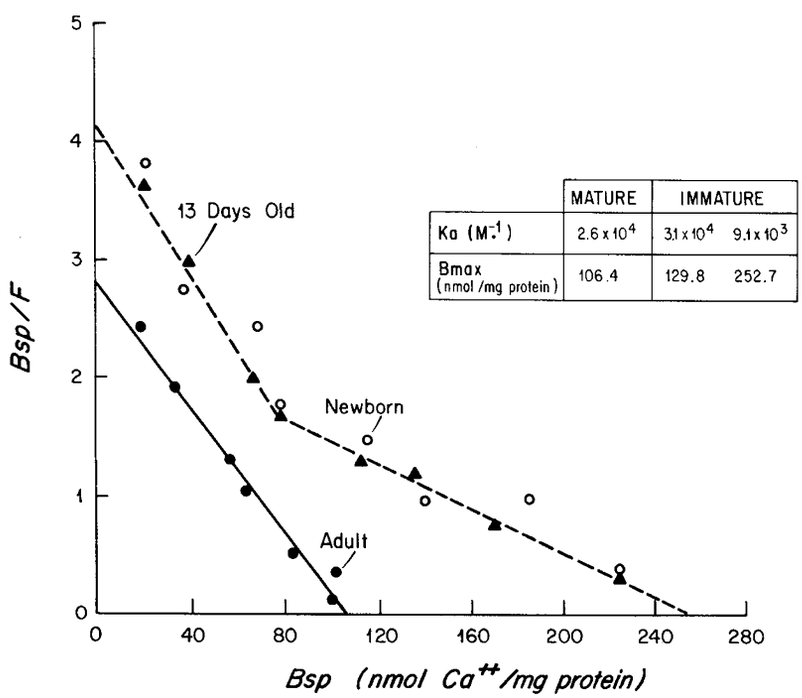

Fig. 3. Dependence of calcium chloride concentration on calcium binding to microvillus membranes, (MVM) from adult (●) to 13-day-old (▲), and newborn (○) rat small intestine. The experiments were performed as outlined in "Materials and Methods." The incubation mixture contained 0.5 mg/ml MVM in 10 mM Hepes, pH 7.4 and varying amount of unlabeled $CaCl_2$ in a final concentration ranging from 12.5 μM to 4 mM and a trace amount of [$^{45}CaCl_2$]. Results were obtained from three different membrane preparations with duplicate or triplicate determinations.

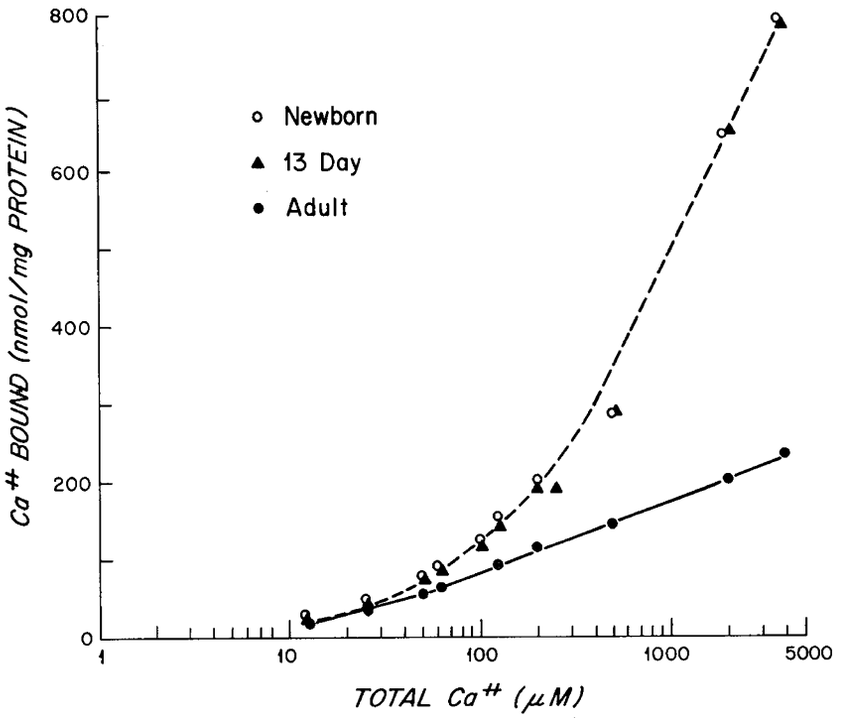

Fig. 4. The Scatchard analysis of Ca^{++} binding to microvillus membrane (MVM) from adult (●) 13-day-old (▲) and newborn (O) rat small intestine. Binding data are taken from Figure 3. The affinity constant (Ka) and the maximum binding capacity (Bmax) were determined from the slope and extrapolated x intercept of each line.

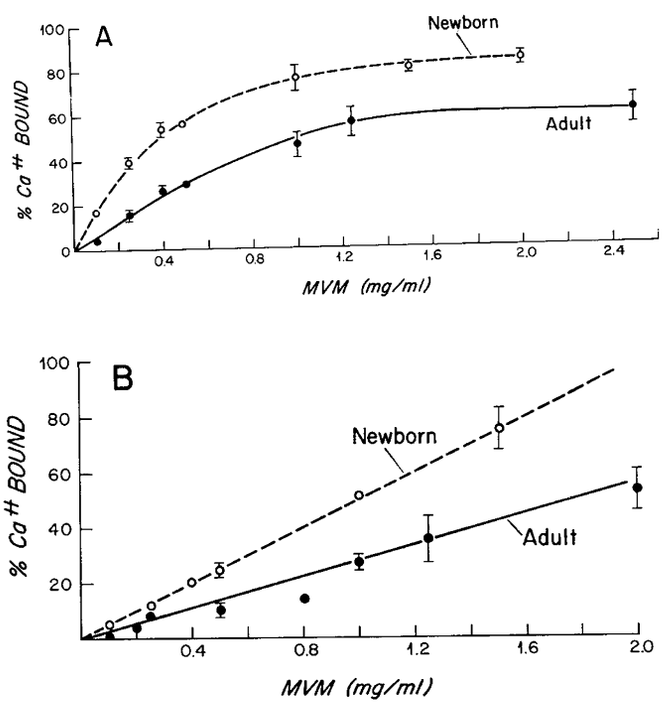

Fig. 5. Dependence of membrane concentration on calcium binding to microvillus membrane (MVM) from adult (●) and newborn (O) rat small intestine (*A*) at 0.1 mM $CaCl_2$ and (*B*) at 0.5 mM $CaCl_2$. Experiments were carried out as follows: microvillus membrane concentration ranging from 0.1–5 mg/ml in 10 mM Hepes, pH 7.4 were mixed with 0.2 or 1.0 mM $CaCl_2$ and a trace amount of [$^{45}CaCl_2$]. The mixture of $CaCl_2$ and microvillus membrane was incubated at room temperature for 30 min and bound [$^{45}Ca^{++}$] was separated from unbound [$^{45}Ca^{++}$] by ultracentrifugation as described in "Materials and Methods." The % of Ca^{++} bound was calculated. These results are the means of four different membrane preparations with duplicate samples. Error bars are standard errors. In the cases where the standard error is smaller than the symbol; the error bar could not be seen.

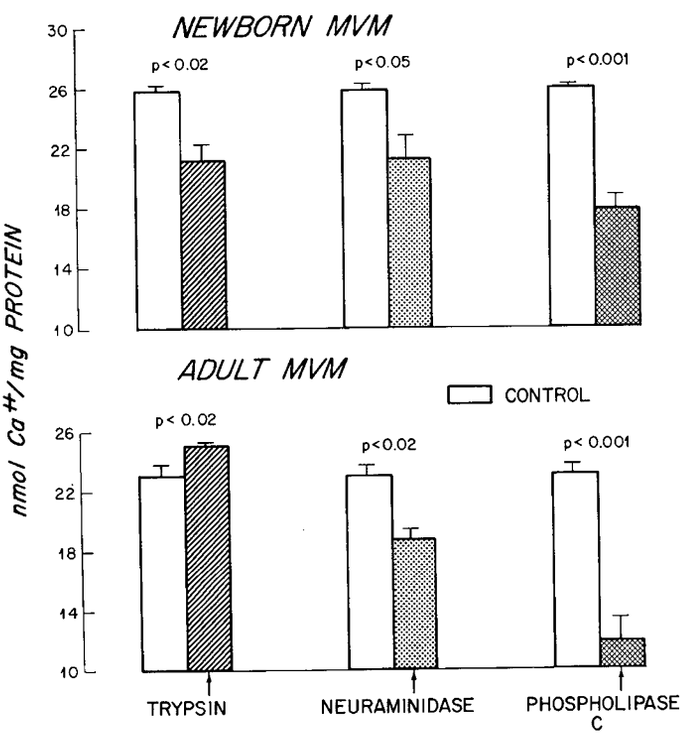

Fig. 6. Effect of trypsin, neuraminidase, and phospholipase C on Ca^{++} binding to microvillus membrane from adult and newborn rat small intestine. The experiments were carried out as described in "Materials and Methods." These results are the means of three different membrane preparations with duplicate samples. Student's *t* test is used to establish the significance between the control and the enzyme-treated membrane preparations.

environment. We have previously reported that macromolecules are transported intracellularly by enterocytes as metabolically-active or antigenic molecules via a pinocytotic process involving absorption to and invagination of the microvillus surface (5, 31, 32). In a separate study, we have shown that bovine serum albumin was transported in larger quantities across the small intestine of newborn and young rabbits compared with adults after oral ingestion of the protein (29, 30). More recently, studies from this laboratory have shown that microvillus membranes from immature animals are more disorganized than the MVM from mature animals (22) and the glycoprotein composition in these two membrane preparations are quite different (1, 2). These results strongly suggest that the differences in the mucosal barrier function in developing animals may be related to differences in MVM composition and structure. Schlatz and Marinetti (27) reported that structural integrity of rat liver plasma membrane was essential for optimal Ca^{++} binding. Palmer and Posey (21) have shown that phospholipase C-treated renal microsomal membrane bound less Ca^{++} than untreated membranes. These studies demonstrated the potentials of Ca^{++} ion in probing membrane characteristics. The present study was designed to examine the effect of Ca^{++} binding on preparation of newborn and adult MVM in order to further characterize structural differences in these two groups of animals.

Using 5-doxyl stearic acid as a standard spin label probe for isolated microvillus membranes, we were able to reproduce spectra similar to that previously reported for 5-doxyl stearic acid labeled model membranes and other biologic membranes (6, 8, 11) (Fig. 1). In comparing MVM from adult and newborn animals, both $2T'_{\parallel}$ and S' were significantly higher than corresponding values of newborn MVM. These results strongly suggested that the spin probe in newborn MVM was maintained in a much more disordered environment than the probe in adult MVM. Similar results had been observed in MVM from adult and newborn rabbit using ESR technique (22) and fluorescence spectroscopy (26). A more disordered membrane from immature animals renders it easier for the newly synthesized protein and lipid molecules to be inserted into the fluid portion of the bilayer and after lateral diffusion take their position in the membrane (17). At the same time, a more disordered membrane is probably more vulnerable to external stimuli such as Ca^{++}, consequently, a greater ordering effect by Ca^{++} on immature MVM.

Ca^{++} enhanced the order of 5-doxyl stearic acid labeled microvillus membrane from the small intestine of adult and newborn rats in a concentration-dependent, saturable manner, probably by binding to specific membrane sites. It has been previously reported that calcium ion caused ordering in model membranes as well as biologic membranes such as rat liver plasma membrane, adipocyte ghosts and lymphocyte ghosts (13, 14, 23). This ordering effect has been associated with the regulatory role Ca^{++} ion played in membrane transport (Glucose, Na^+) and membrane enzyme activities (12, 13, 19). The differential ordering effect by Ca^{++} on spin labeled adult and newborn MVM observed here suggested structural differences between mature and immature membranes. These differences in MVM may in turn alter the effect of Ca^{++} on physiologic functions in these mature and developing animals.

The nature of these differences can in part be explained in terms of differential Ca^{++} binding to these membranes. With an additional binding site and a greater binding capacity for Ca^{++} (Fig. 4), MVM from immature animals binds more calcium ions than MVM from mature animals in $CaCl_2$ concentration ranges from 12.5–4 mM (Fig. 2). This in turn causes a greater ordering effect observed in spin labeled immature MVM.

Because it has been shown that protein, neuraminic acid, and phospholipids played important roles in calcium binding to plasma membranes (20, 21, 27), the nature of Ca^{++} binding sites was further investigated in this study by using the proteolytic enzymes, neuraminidase and phospholipase C. Trypsin-treated adult MVM binds 8% more Ca^{++} than the untreated ones, suggesting the net gain in Ca^{++} binding components or an increase in its binding capacity after the proteolytic enzyme digestion whereas in a similarly treated newborn MVM, a 17% decrease in Ca^{++} binding was observed indicating a loss of binding components or a decrease in its binding capacity. These results seem to indicate a differential modification of the membrane structure in MVM from adult and newborn animals after trypsin treatment. The greater amount of Ca^{++} binding to the unmodified membrane and the net decrease in Ca^{++} binding to trypsin-treated membrane imply that the Ca^{++} binding components—proteins, phospholipids, and neuraminic acids—are more superficially located on the surface of the immature MVM than that in mature MVM, and therefore more vulnerable to Ca^{++} ion and enzymatic modification. These explanations are consistent with findings reported from our laboratory and others that there is a higher lipid to protein ratio in newborn MVM compared to adult MVM (1, 23, 28). These results suggest a fundamental difference in organization in mature and immature MVM.

Neuraminidase removes the carboxyl groups of neuraminic acid on the surface of membrane. Microvillus membranes from both preparations treated with neuraminidase showed a decrease in Ca^{++} binding to MVM. The effect of a polar group in membrane phospholipids on Ca^{++} binding was examined by using phospholipase C. Phospholipase C-treated MVM from both preparations also showed a significant decrease in Ca^{++} binding, suggesting an important role the polar group in phospholipids played in Ca^{++} binding.

We reported here a differential ordering effect of Ca^{++} ion on mature and immature MVM from rat small intestine. This effect is probably the result of the presence of an additional binding site and a greater maximum binding capacity of newborn MVM. In addition to polar phospholipids and neuraminic acid, calcium also probably binds to protein components on the surface of MVM from newborn. Results from this study suggest that MVM from immature rats was structurally more disordered and more vulnerable to an external stimulus like Ca^{++} ions. These fundamental differences in MVM organization could account for many physiologic phenomenon such as increased attachment and penetration of macromolecules (29) and increased uptake of calcium ion noted during the perinatal period (9).

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The Diagnosis and Staging of Hypocortisolism in Progressing Autoimmune Adrenalitis

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Summary

The course of development of hypocortisolism was studied in 20 patients with autoimmune polyendocrinopathy-candidosis-ectodermal dystrophy (APECED) for 1.3–9.3 years during which time the patients underwent at least three 2-h ACTH tests (2hAT). A slow progression of the disease was evident and could be staged. The earliest indicators of incipient failure were subnormality of the 2-h cortisol level alone or with subnormality of the 2-h increment. The increment was then abolished. A normal basal level was maintained longer. Longer forms of the ACTH tests produced normal responses even after the early stages of failure. A constantly elevated ACTH concentration and low cortisol/ACTH ratio in plasma were likewise signs of advanced hypocortisolism. Current criteria of primary hypocortisolism are thus indicators of the late stages of failure only. The presence of circulating adrenocortical antibodies is predictive of hypocortisolism. Some patients had normal 2hAT responses, but antibodies and subnormal cortisol/ACTH ratios. This may represent a state of compensatory activation of the hypothalamic-pituitary-adrenocortical axis.

Abbreviations

APECED, autoimmune polyendocrinopathy-candidosis-ectodermal dystrophy
4dAT, 4-day ACTH test
2hAT, 2-h ACTH test
17-OGS, 17 oxogenic steroids
RIA, radioimmunoassay

Autoimmune adrenalitis is a major cause of Addison's disease. In patients with polyendocrine deficiency diseases and their siblings, frequent testing of cortisol reserve is essential for early detection of failure of cortisol secretory capacity. Long lasting ACTH tests are impractical, because they entail hospitalization. Furthermore, such tests may not be sensitive enough to detect incipient failure. To our knowledge, no systematic study has appeared on the progression of hypocortisolism in adrenalitis. Neither have criteria been established for early stages of primary hypocortisolism. Such criteria would be important for the safety of individuals at risk. We have used an ambulatory 2-h ACTH test (2hAT) (13) for both detection and follow-up of progression of hypocortisolism in our large series of patients with APECED (18, 23). The 2hAT was clearly more sensitive than the 4dAT for the detection of incipient failure and even more sensitive than determinations of plasma ACTH. Our experience with these patients calls for a revision of the current diagnostic criteria of Addison's disease.