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Ganglioside–Protein Interactions: Spin-Label Electron Spin Resonance Studies with (Na^+,K^+)-ATPase Membranes†

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ABSTRACT: Lipid–protein interactions in (Na^+,K^+)-ATPase-rich membranes from *Squalus acanthias* have been studied using spin-labeled derivatives of the mono- and disialogangliosides G_{M1}, G_{M2}, G_{M3}, and G_{D1b}, in conjunction with electron spin resonance (ESR) spectroscopy. Ganglioside–protein interactions are revealed by the presence of a second component in the ESR spectra of the membranes in addition to a component that corresponds closely to the ESR spectra obtained from dispersions of the extracted membrane lipids. This second component corresponds to spin-labeled gangliosides whose chain motion is significantly restricted relative to that of the fluid lipids in the membrane or the lipid extract. A small selectivity for the motionally restricted component associated with the protein is found in the order $G_{D1b} > G_{M1} \approx G_{M2} \approx G_{M3}$. Comparison with previous results from spin-labeled phospholipids in the same system [Esmann, M., Watts, A., & Marsh, D. (1985) *Biochemistry 24*, 1386–1393] shows that the spin-labeled monosialogangliosides G_{M1}, G_{M2}, and G_{M3} display little selectivity in the lipid–protein interaction relative to spin-labeled phosphatidylcholine. The spectral characteristics of both the fluid and motionally restricted spin-label components differ very significantly, however, between the gangliosides and the phospholipids. The outer hyperfine splitting of the motionally restricted component is smaller for the gangliosides than for the phospholipids, indicating a smaller degree of motional restriction on interaction of the ganglioside lipid chains with the protein. The effective order parameters of the fluid component are larger for the gangliosides than for the phospholipids, consistent with a location of the spin-labeled segment closer to the polar interface of the membrane in the case of the gangliosides.

The (Na^+,K^+)-ATPase[1] (EC 3.6.1.8) is a membranous active transport enzyme involved in maintaining ion levels and osmotic balance in the cell [for reviews see, e.g., Skou (1965) and Glynn (1985)]. Functionally, the enzyme acts as an electrogenic sodium pump, mediating the extrusion of three Na^+ ions and the uptake of two K^+ ions, coupled to the hydrolysis of one molecule of ATP. The catalytic transport cycle probably involves the formation of a phosphorylated enzyme intermediate and a conformational transition between the E_1 and E_2 forms of the enzyme, which display different affinities for Na^+ and K^+. It is possible that the lipid environment of the protein may have an influence on this conformational equilibrium.

The enzyme has a requirement for phospholipid for overall (Na^+,K^+)-ATPase activity. Bulk lipid from the membrane can be replaced by detergents such as $C_{12}E_8$, but removal of boundary lipid, i.e., the 60–70 molecules of phospholipid surrounding the protein at the hydrophobic interface between the protein and lipid, leads to inactivation (Esmann, 1984). The enzymatic activity can be, at least partially, restored by addition of phosphatidylcholine or phosphatidylethanolamine to a fully delipidated enzyme (Ottolenghi, 1979).

Shark rectal glands contain about 1.8 mg of ganglioside/g of dry weight, mostly as *N*-acetylneuraminylgalactosylglucosylceramide (Karlsson et al., 1974). The purified (Na^+,K^+)-ATPase preparation used in this study has a ganglioside content of approximately 1 mol % of the total lipid, and one molecule on average remains associated with the enzyme upon solubilization in $C_{12}E_8$ and removal of excess, bulk lipid by gel filtration (Esmann et al., 1980). The majority of the lipid in both the membrane and solubilized enzyme is phosphatidylcholine (70–80%) and phosphatidylethanolamine (about 20%).

Previous studies using electron spin resonance (ESR)[1] spectroscopy of spin-labeled phospholipids and fatty acid have revealed a selectivity for the protein in the following order: cardiolipin$^-$ > stearic acid$^-$ > phosphatidic acid^{2-} ≳ phosphatidylserine$^-$ > stearic acid^0 ≈ phosphatidylcholine ≈ phosphatidylglycerol$^-$ ≈ phosphatidylethanolamine ≳ phosphatidic acid$^-$ (Esmann et al., 1985; Esmann & Marsh, 1985). It was also found that ca. 65 nonselective lipids were motionally restricted by direct interaction with the protein, corresponding approximately to a single lipid shell surrounding the protein (Esmann et al., 1985).

In the present work we have extended this study to investigate the interaction with various spin-labeled mono- and

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[1] Abbreviations: (Na^+,K^+)-ATPase, sodium and potassium ion activated adenosinetriphosphatase (EC 3.6.1.8); EDTA, ethylenediaminetetraacetic acid; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; 14-GM1SL, II^{3}NeuAc-GgOse$_4$-*N*-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]sphingosine; 14-GM2SL, II^{3}NeuAc-GgOse$_3$-*N*-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]sphingosine; 14-GM3SL, II^{3}NeuAc-Lac-*N*-[14-(4,4-dimethyloxazolidine-*N*-oxyl)-stearoyl]sphingosine; 14-GD1bSL, II^3(NeuAc)$_2$-GgOse$_4$-*N*-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]sphingosine; 14-PGSL and -PCSL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoglycerol and -phosphocholine; $C_{12}E_8$, octaethylene glycol dodecyl monoether; ESR, electron spin resonance; His, histidine (buffer); SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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disialogangliosides. There have been several suggestions that gangliosides may interact specifically with various integral membrane enzymes, receptors, and transport systems (Nagai & Iwamori, 1980; Hakamori, 1981; Dacremont et al., 1984; Yamakawa & Nagai, 1978). The sodium pump seems a suitable system with which to look for specific ganglioside–protein interactions, since the interactions with other membrane lipids have been previously characterized in detail. These former studies also suggest that the use of spin-labeled gangliosides (Schwarzmann & Sandhoff, 1987) should provide a convenient means with which to undertake this investigation. ESR studies of the cellular incorporation of spin-labeled ganglioside analogues have also demonstrated that the spin-labeled analogues behave in a similar way to the authentic parent gangliosides (Schwarzmann et al., 1983, 1984).

Materials and Methods

Materials. (*A*) *Preparation of Enzyme.* (Na^+,K^+)-ATPase-rich membranes were prepared from the rectal gland of *Squalus acanthias* according to the method of Skou and Esmann (1979), but omitting the treatment with saponin. The (Na^+,K^+)-ATPase constituted typically 50–70% of the protein (determined as the content of α- and β-subunit judged from SDS gel electrophoresis), and the specific activity ranged accordingly from 1100 to 1500 μmol of ATP hydrolyzed (mg of protein)$^{-1}$ h^{-1}. Serum albumin was used as a standard for protein determination, with a concentration of 1 mg/mL giving an absorption of 0.67 at 280 nm. No further corrections were made.

The phospholipid content of the preparation was approximately 0.9 mg/mg of protein, and the cholesterol content was approximately 0.3 mg/mg of protein. Lipids were extracted from the (Na^+,K^+)-ATPase membranes with $CHCl_3/CH_3OH$ (2:1 v/v), according to the method of Folch et al. (1957). Dispersions of the extracted lipid were formed by adding a small volume of buffer (typically 10 μL) to ca. 1 mg of the dried lipid (vacuum desiccated). Approximately 1 mol % of spin-labeled lipid had been added to the $CHCl_3/CH_3OH$ (2:1 v/v) solution, prior to drying the lipid. The lipid suspension was vigorously vortexed and used for ESR measurements without further manipulations. Membrane suspensions were spin-labeled from a small volume of concentrated solution in ethanol, as previously described (Esmann et al., 1985).

(*B*) *Preparation of Spin-Labeled Gangliosides.* Stearic acid spin-labeled on the C-14 atom [14-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid] was prepared essentially according to the method of Hubbell and McConnell (1971). The *N*-succinimidyl ester of the spin-labeled stearic acid was prepared as described by Lapidot et al. (1967). Gangliosides were extracted and purified from brain and the corresponding lysogangliosides prepared as described by Schwarzmann and Sandhoff (1987). The spin-labeled gangliosides 14-GM1SL, 14-GM2SL, 14-GM3SL, and 14-GD1bSL were then prepared by selective N-acylation of the appropriate lysogangliosides using the methods described by Schwarzmann and Sandhoff (1987). The isolation and characterization of the spin-labeled products is described fully in this reference. The structures of the various spin-labeled gangliosides are given in Figure 1. The sphingosine moiety, which is in the D-erythro configuration, is only indicated schematically in Figure 1; the actual composition corresponds to that of the parent natural ganglioside (principally C_{18} and C_{20}-sphingosine, except for 14-GM3SL which contains only C_{18}-sphingosine).

ESR Measurements. For ESR measurements, spin-labeled membranes were suspended after washing in the appropriate buffer, usually 100 mM NaCl, 1 mM CDTA, and 20 mM

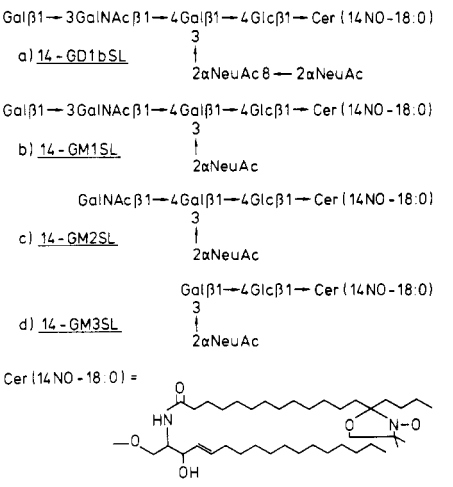

FIGURE 1: Structures of spin-labeled gangliosides: (a) 14-GD1bSL, spin-labeled G_{D1b}; (b) 14-GM1SL, spin-labeled G_{M1}; (c) 14-GM2SL, spin-labeled G_{M2}; (d) 14-GM3SL, spin-labeled G_{M3}.

histidine (pH 7.4 at 37 °C), and the membranes were pelleted in this buffer. The pellets were freed from excess buffer, and about 10 μL of buffer was then added to the pellet. By gentle resuspension the concentrated membrane suspension was transferred to a 1-mm i.d., 100-μL glass capillary. Lipid dispersions were transferred to the measuring capillaries directly, using a drawn-out Pasteur pipet.

ESR spectra were recorded on a Varian E-12 9-GHz spectrometer equipped with nitrogen gas flow temperature regulation. Samples were contained in the 1-mm diameter, 100-μL sealed glass capillaries, within standard 4-mm diameter quartz tubes containing light silicone oil for thermal stability. Spectral digitization and processing were performed by using a PDP 11/10 dedicated computer and a Digital Equipment Corp. LPS system with VT-11 display. Further details on the ESR spin-label techniques are given in Marsh (1982).

Apparent order parameters were calculated by using the expression

$$S_{app} = \frac{A_\parallel - A_\perp}{A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})} \frac{a'_0}{a_0} \quad (1)$$

where $2A_\parallel$ is equal to the outer, maximum hyperfine splitting ($2A_{max}$) and $A_\perp$ is obtained from the inner, minimum hyperfine splitting ($2A_{min}$) according to (Griffith and Jost, 1976)

$$A_\perp \text{ (G)} = A_{min} \text{ (G)} + 1.4\left[1 - \frac{A_\parallel - A_{min}}{A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})}\right] \quad (2)$$

The effective isotropic hyperfine splitting constant is given by

$$a_0 = \frac{1}{3}(A_\parallel + 2A_\perp) \quad (3)$$

and that corresponding to the single-crystal environment in which the principal values of the hyperfine tensor A_{xx}, A_{yy}, and A_{zz} were measured is given by $a'_0 = {}^1/_3(A_{xx} + A_{yy} + A_{zz})$. Line shape simulations which do not make the motional narrowing approximation have shown that the spectra of lipid spin-labels in fluid bilayers contain important contributions from slow molecular motions (Lange et al., 1985). Thus the effective order parameter calculated by using eq 1, which assumes fast motion, can only be considered as an apparent value, but is nonetheless useful for making intercomparisons between membranes and lipid dispersions or between spin-labels with different head groups. Details of the methods used

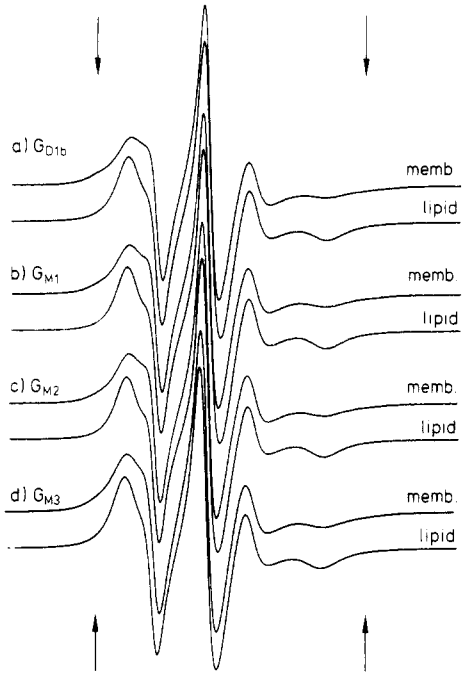

FIGURE 2: ESR spectra of C-14 nitroxide spin-labeled gangliosides in (Na^+,K^+)-ATPase membranes and extracted lipids from *S. acanthias* rectal gland. Buffer: 10 mM Tris, pH 7.5, T = 12 °C. The upper spectrum of each pair is from the membranes, and the lower spectrum in each pair is from the extracted membrane lipids. (a) Spin-labeled G_{D1b}, 14-GD1bSL; (b) spin-labeled G_{M1}, 14-GM1SL; (c) spin-labeled G_{M2}, 14-GM2SL; (d) spin-labeled G_{M3}, 14-GM3SL. Scan range = 100 G. The position of a second, motionally restricted component in the wings of the membrane spectra is indicated by the arrows.

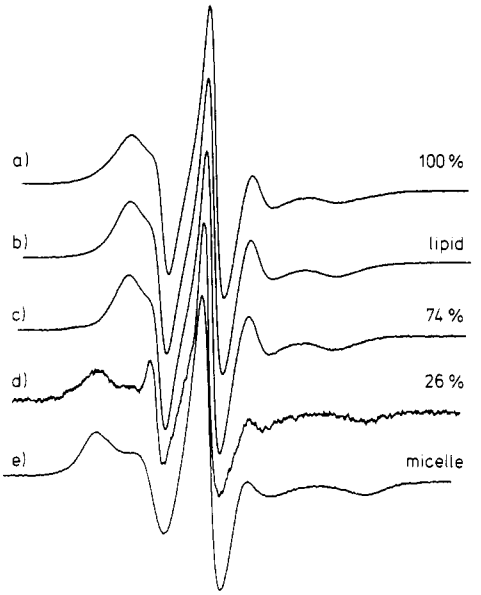

FIGURE 3: Spectral subtraction for the 14-GD1bSL spin-label in (Na^+,K^+)-ATPase membranes at 4 °C. Buffer: 30 mM His, 0.1 M NaCl, and 1 mM CDTA, pH 7.4. (a) Spectrum of 14-GD1bSL in membranes at 4 °C; (b) spectrum of 14-GM2SL in extracted membrane lipids at 4 °C; (c) fluid difference spectrum obtained by subtracting 26% of the double-integrated intensity from spectrum a by using spectrum e; (d) motionally restricted difference spectrum obtained by subtracting 74% of the double-integrated intensity from spectrum a by using the lipid spectrum b; (e) spectrum of 14-GM1SL in mixed ganglioside micelles at 12 °C, used for subtraction c. Scan range = 100 G.

for spectral subtraction and quantitation are given in Esmann et al. (1985).

Results

The ESR spectra of the four C-14 nitroxide ganglioside labels (cf. Figure 1) in (Na^+,K^+)-ATPase membranes, at 12 °C, are given in Figure 2. The ESR spectra of the ganglioside labels in aqueous dispersions of the extracted membrane lipids are also shown in Figure 2, for comparison. Each of the upper four spectra is composed of two components, corresponding to two motionally distinct lipid environments in the membranes. One component corresponds to the fluid lipid environment found in the extracted lipid (lower spectrum of each pair in Figure 2). The second component (indicated by the arrows in Figure 2) has a larger hyperfine splitting and is not seen in the spectra from the lipid alone. It represents a lipid environment with considerably more restricted mobility and is attributed to lipids interacting directly with the integral proteins in the membrane. Since solubilization of the membranes in the detergent $C_{12}E_8$ yields an insoluble precipitate, presumably of peripheral proteins, and a supernatant that contains practically pure (Na^+,K^+)-ATPase (Esmann et al., 1979), it can be concluded that the (Na^+,K^+)-ATPase is essentially the only integral protein in these membrane preparations. Thus the lipid–protein interactions investigated here are those with the (Na^+,K^+)-ATPase. From the spectra shown in Figure 2 it is clear that the amount of motionally restricted lipid component is rather similar for all four ganglioside spin-labels. The spectra of the four different ganglioside spin-labels in the extracted membrane lipids are virtually identical.

The method of analyzing the two-component membrane spectra by digital subtraction has been described previously (Esmann et al., 1985) but is illustrated here in Figure 3 because the motionally restricted ganglioside component differs significantly from that found for phospholipid labels in this and all other lipid–protein systems so far reported (cf. below). The motionally restricted component subtraction end point (Figure 3d) coincidentally corresponds to the spectrum obtained from 14-GM1SL in mixed ganglioside micelles at 12 °C (Figure 3e). Consistent values are obtained for the fraction of motionally restricted lipid from the two independent methods of subtraction (Figure 3c,d), validating the assumption of a two-component nature of the membrane spectra. It will be noted from this example that, even at temperatures for which the components are not well resolved, spectral subtraction is capable of separating the two components.

The results of the spectral subtraction indicate that there is only a small decrease in the fraction of motionally restricted ganglioside with increasing temperature: from approximately 0.26 to 0.22 for 14-GM1SL, 14-GM2SL, and 14-GM3SL. The temperature dependence is yet smaller for 14-GD1bSL, and the motionally restricted fraction is slightly higher than for the other three ganglioside labels: about 0.26–0.28, over the temperature range 0–37 °C.

The average values for the fraction of motionally restricted lipid obtained from the spectra of the ganglioside labels, over the temperature range 0–37 °C, are 0.26 ± 0.01 for 14-GD1bSL, 0.23 ± 0.02 for 14-GM1SL, 0.22 ± 0.03 for 14-GM2SL, and 0.21 ± 0.02 for 14-GM3SL. For comparison, values obtained previously (Esmann et al., 1985) for spin-labeled phospholipids are 0.23 ± 0.01 for 14-PCSL and 0.19 ± 0.03 for 14-PGSL. Of the four ganglioside spin-labels, only the disialoganglioside 14-GD1bSL can be considered to be different from the majority phospholipid phosphatidylcholine, with respect to selectivity of the motionally restricted component.

The spectra of the motionally restricted component and of the fluid component can be used for analysis of the dynamic

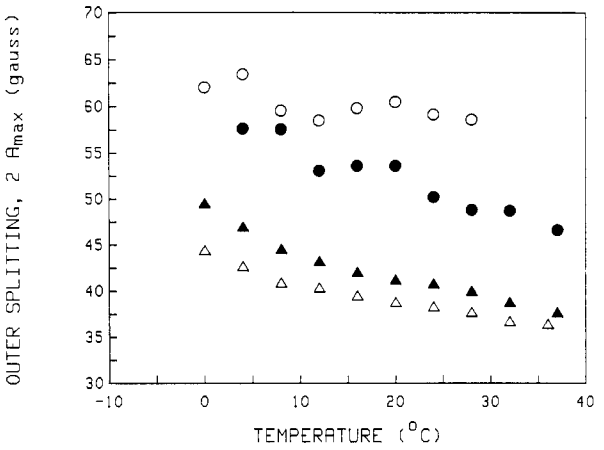

FIGURE 4: Temperature dependence of the maximum outer hyperfine splitting, A_{max}, for both the fluid and motionally restricted lipid components of the 14-GM1SL ganglioside spin-label, and of the 14-PGSL phosphatidylglycerol spin-label, in (Na^+,K^+)-ATPase membranes. (●) Motionally restricted component of 14-GM1SL; (○) motionally restricted component of 14-PGSL; (▲) fluid component of 14-GM1SL; (△) fluid component of 14-PGSL.

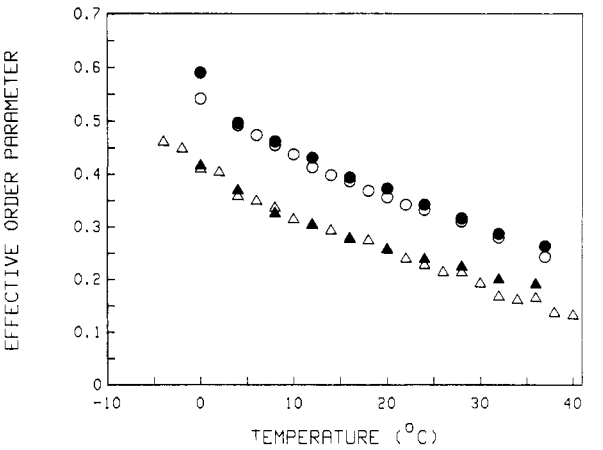

FIGURE 5: Effective order parameter for the fluid lipid component of the 14-GM2SL ganglioside spin-label, and of the 14-PGSL phosphatidylglycerol spin-label, in (Na^+,K^+)-ATPase membranes and in the extracted membrane lipids. (●) 14-GM2SL in membranes; (○) 14-GM2SL in extracted lipid dispersion; (▲) 14-PGSL in membranes; (△) 14-PGSL in extracted lipid dispersion.

properties of the lipid components, in terms of the maximum outer hyperfine splitting, $2A_{max}$ [cf. Freed (1976) and Marsh (1982)]. Figure 4 gives a comparison of the temperature dependence of the outer hyperfine splitting of both components for the 14-GM1SL ganglioside spin-label and the 14-PGSL phosphatidylglycerol spin-label. The data for the latter come from previous studies (Esmann et al., 1985) and were chosen as a representative negatively charged phospholipid that exhibits the same fraction of motionally restricted component as for the ganglioside. A large difference in the dynamic characteristics of both components is observed between the ganglioside spin-label and the phospholipid spin-label. The ganglioside label has a considerably smaller outer splitting of the motionally restricted component than the phospholipid label, indicating a significantly greater degree of rotational motion for the former. The decrease in splitting with temperature is much larger for the ganglioside label than for the phospholipid label, which is also consistent with greater rotational motional freedom. The difference between the fluid components of the ganglioside and phospholipid labels is seen to be opposite to that for the motionally restricted components. The outer splitting for the ganglioside label is consistently larger than that for the phospholipid label, indicating a decreased amplitude and/or rate of motion for the latter. Qualitatively consistent results, in terms of the differences in dynamic properties, are obtained for all the ganglioside and phospholipid labels studied. It is found that the difference spectra of all the ganglioside labels are very similar (data not shown, but cf. Figure 2), as are those for the phospholipid labels (Esmann et al., 1985).

The fluid component spectra of the ganglioside labels have also been analyzed in terms of the conventional apparent order parameter formalism. Figure 5 shows a comparison of the temperature dependence of the effective order parameter of the fluid component in the membrane spectra and in the extracted lipids, for the 14-GM2SL ganglioside spin-label, and also for the 14-PGSL phosphatidylglycerol spin-label. There is no significant difference between the effective order parameters in the membranes and in the extracted lipids, for both the ganglioside label and the phospholipid label. The effective order parameters are, however, consistently greater for the ganglioside label than for the phospholipid label, in both systems. No significant difference is observed between the fluid component in the membranes and the lipid extract for the other three ganglioside labels studied (data not shown), as found previously for the various phospholipid labels (Esmann et al., 1985).

Conjugate with the values for the effective order parameter are those for the effective isotropic hyperfine splitting, which is sensitive to environmental polarity. Values of the effective isotropic hyperfine splitting constant in (Na^+,K^+)-ATPase membranes for the four ganglioside labels are 14.15 ± 0.02 for 14-GD1bSL, 14.19 ± 0.07 for 14-GM1SL, 14.21 ± 0.18 for 14-GM2SL, and 14.22 ± 0.03 for 14-GM3SL, and this value is 14.23 ± 0.07 for 14-GM2SL in the extracted membrane lipids. The measured values remain essentially constant with temperature from 8 to 37 °C, indicating no significant artifacts from motional components in the slow regime. Within experimental accuracy the values for the ganglioside labels are identical in membranes and extracted lipids and are also identical with those previously obtained for C-14 position labeled phospholipids [cf. Table IV of Esmann et al. (1985)].

Discussion

The first interesting aspect of the present results regards the fraction of spin-labeled ganglioside whose motion is directly restricted by interaction with the protein [essentially the (Na^+,K^+)-ATPase; cf. Results]. For the spin-labeled monosialogangliosides G_{M1}, G_{M2}, and G_{M3} these values are very similar to those obtained previously for the spin-labeled analogue of phosphatidylcholine (Esmann et al., 1985), which constitutes the majority lipid in the membrane. Phosphatidylcholine was the spin-labeled lipid that showed least selectivity for the protein in this previous study. Thus it would appear that, in spite of their complex head group structure and negatively charged sialic acid residue, these gangliosides do not have any degree of specific association with the protein. The spin-labeled disialoganglioside G_{D1b} exhibits a limited selectivity for the protein relative to the monosialogangliosides and phosphatidylcholine. This effect is presumably due to the additional sialic acid residue, which alone differentiates G_{D1b} from G_{M1} (cf. Figure 1). However, the selectivity for G_{D1b} is, nonetheless, considerably smaller than that for the other negatively charged lipids studied previously (Esmann et al., 1985; Esmann & Marsh, 1985), with the exception of phosphatidylglycerol. It would thus appear that the bulk of the ganglioside head group prevents any preferential association with the protein and that there is little specific interaction with

the carbohydrate moieties of the head group which could overcome this simple steric effect. However, it should be noted that the effect of head group bulk is not so extreme as to cause a deselection of gangliosides relative to phosphatidylcholine. It is further possible that the large size of the head group leads to a greater separation of charge (also in the case of G_{D1b}), which attenuates the electrostatic interactions that were found previously to contribute to the specificity of interaction with other lipids (Brotherus et al., 1980; Esmann & Marsh, 1985).

Although it is difficult to generalize from this one example to the interaction of gangliosides with other ion transport proteins, it is perhaps significant that a very similar ganglioside selectivity pattern has been observed with the proteolipid protein from nerve myelin (L. I. Horvath, P. J. Brophy, D. Marsh, G. Schwarzmann, and K. Sandhoff, unpublished results). It is possible that this rather surprising lack of selectivity may arise from the interplay of various opposing contributions to the ganglioside protein interaction: head group size and hydration on the one hand and specific interactions and electrostatic attractions on the other. From the functional point of view, it is interesting to note that gangliosides were not found capable of increasing the (Na^+,K^+)-ATPase activity of brain microsomes, whereas the Mg^{2+}-dependent ATPase was greatly activated (Caputto et al., 1977). This result correlates well with the lack of selectivity for gangliosides reported here and suggests that more specific interactions possibly may be found with other membrane enzymes.

In contrast to the rather similar degree of association with the protein, the ganglioside spin-labels display rather different dynamic properties from those of the phospholipid labels. The motionally restricted ganglioside component exhibits far greater mobility than that of the phospholipid spin-labels. Whereas the spectra of the latter lie in the slow motion regime of nitroxide ESR spectroscopy, implying rotational correlation times in the region of 20–50 ns (Esmann et al., 1985), the motionally restricted spectra of the gangliosides lie in the intermediate motional regime, suggesting rotational correlation times in the region of 3–5 ns [cf. Polnaszek et al. (1981)]. This difference is clearly seen in the outer hyperfine splittings, and in the relative temperature dependences (Figure 4). A possible explanation for this increased mobility may be that the bulk of the ganglioside head group reduces the interaction between the chains and the protein, for purely steric reasons. A natural consequence of this would be that if the lipid–protein interface were enriched in gangliosides, the immediate environment of the protein would be considerably more fluid than in the phospholipid case, which might have functional consequences. However, it is possible that this increased mobility is not a general feature of ganglioside–protein interfaces, since in the case of the myelin proteolipid protein it is found that both phospholipid and ganglioside chains experience a similar degree of motional restriction at the lipid–protein interface (L. I. Horvath, P. J. Brophy, D. Marsh, G. Schwarzmann, and K. Sandhoff, unpublished results).

The fluid ganglioside component clearly displays higher effective order parameters than the corresponding phospholipid labels. This most probably arises from a difference in positioning of the chains in the gangliosphingolipids compared with the glycerophospholipids. A detailed comparison of the effective order parameters of three different chain-labeled positional isomers of the ganglioside spin-labeled analogues in dimyristoylphosphatidylcholine bilayers, with the order parameter profile established with a series of spin-labeled phosphatidylcholines, indicates that corresponding positions in the fatty acid chain of gangliosides are located 1–2 CH_2 groups closer to the polar–apolar interface of the bilayer than those in the *sn*-2 chain of the phosphatidylcholine (D. Epperlein, D. Marsh, G. Schwarzmann, and K. Sandhoff, unpublished results). The difference in relative positions of the nitroxides in the two sets of labels is not reflected in differences in the effective isotropic splitting factors because both sets of nitroxides are located in the plateau region of low polarity in the central region of the bilayer [cf. Marsh and Watts (1981)]. Thus the difference in apparent order parameters reflects the difference in sphingolipid backbone structure and hydrocarbon chain attachment from that in glycerophospholipids. This in turn may give rise to a different dynamic environment for those integral proteins, which unlike the (Na^+,K^+)-ATPase might be situated in an environment that is highly enriched in gangliosides. Proteins such as the acetylcholine receptor that are present in nerve endings, which are known to contain high concentrations of gangliosides, would seem to be attractive candidates for future study.

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REFERENCES

Brotherus, J. R., Jost, P. C., Griffith, O. H., Keana, J. F. W., & Hokin, L. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 272–276.

Caputto, R., Maccioni, A. H. R., & Caputto, H. L. (1977) *Biochem. Biophys. Res. Commun. 74*, 1046–1052.

Dacremont, G., de Baets, M., Kaufman, J. M., Elewaut, A., & Vermeulen, A. (1984) *Biochim. Biophys. Acta 770*, 142–147.

Esmann, M. (1984) *Biochim. Biophys. Acta 787*, 81–89.

Esmann, M., & Marsh, D. (1985) *Biochemistry 24*, 3572–3578.

Esmann, M., Skou, J. C., & Christiansen, C. (1979) *Biochim. Biophys. Acta 567*, 410–420.

Esmann, M., Christiansen, C., Karlsson, K.-A., Hansson, G. C., & Skou, J. C. (1980) *Biochim. Biophys. Acta 603*, 1–12.

Esmann, M., Watts, A., & Marsh, D. (1985) *Biochemistry 24*, 1386–1393.

Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497–509.

Freed, J. H. (1976) in *Spin Labeling. Theory and Applications* (Berliner, L. J., Ed.) Vol. I, pp 53–132, Academic, New York.

Glynn, I. M. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. N., Ed.) 2nd ed., Vol. 3, pp 35–114, Plenum Press, New York.

Griffith, O. H., & Jost, P. C. (1976) in *Spin Labeling. Theory and Applications* (Berliner, L. J., Ed.) Vol. I, pp 453–521, Academic, New York.

Hakamori, S. (1981) *Annu. Rev. Biochem. 50*, 733–764.

Hubbell, W. L., & McConnell, H. M. (1971) *J. Am. Chem. Soc. 93*, 314–326.

Karlsson, K.-A., Samuelsson, B. E., & Steen, G. O. (1974) *Biochim. Biophys. Acta 337*, 356–376.

Lange, A., Marsh, D., Wassmer, K.-H., Meier, P., & Kothe, G. (1985) *Biochemistry 24*, 4383–4392.

Lapidot, Y., Rappaport, S., & Wolman, Y. (1967) *J. Lipid Res. 8*, 142–145.

Marsh, D. (1982) in *Techniques in Lipid and Membrane Biochemistry* (Hesketh, T. R., & Metcalfe, J. C., Eds.) Vol. B4/II, pp B426/1–B426/44, Elsevier, Limerick, Ireland.

Marsh, D., & Watts, A. (1981) in *Liposomes: From Physical Structure to Therapeutic Applications* (Knight, C. G., Ed.)

pp 139–188, Elsevier/North-Holland, Amsterdam, New York, and Oxford.
Nagai, Y., & Iwamori, M. (1980) *Mol. Cell. Biochem. 29*, 81–90.
Ottolenghi, P. (1979) *Eur. J. Biochem. 99*, 113–131.
Polnaszek, C. F., Marsh, D., & Smith, I. C. P. (1981) *J. Magn. Reson. 43*, 54–64.
Schwarzmann, G., & Sandhoff, K. (1987) *Methods Enzymol. 138*, 319–341.
Schwarzmann, G., Hoffmann-Bleihauer, P., Schubert, J., Sandhoff, K., & Marsh, D. (1983) *Biochemistry 22*, 5041–5048.
Schwarzmann, G., Sonderfeld, S., Conzelmann, E., Marsh, D., & Sandhoff, K. (1984) *Inst. Natl. Sante Rech. Med.*, [*Colloq.*] *126*, 195–210.
Skou, J. C. (1965) *Physiol. Rev. 3*, 596–617.
Skou, J. C., & Esmann, M. (1979) *Biochim. Biophys. Acta 567*, 436–444.
Yamakawa, T., & Nagai, Y. (1978) *Trends Biochem. Sci.* (*Pers. Ed.*) *3*, 128–131.

Inhibition of Sodium–Calcium Exchange in Cardiac Sarcolemmal Membrane Vesicles. 1. Mechanism of Inhibition by Amiloride Analogues

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ABSTRACT: The mechanism by which terminal guanidino nitrogen substituted analogues of amiloride inhibit Na–Ca exchange in purified cardiac sarcolemmal membrane vesicles has been investigated. These inhibitors block both Na_i-dependent Ca^{2+} uptake and Na_o-dependent Ca^{2+} efflux. Inhibition of Na–Ca exchange monitored in K^+ is noncompetitive vs Ca^{2+} but competitive vs Na^+. Substitution of sucrose for K^+ results in mixed kinetics of inhibition vs Ca^{2+}, suggesting a complex interaction between inhibitor and carrier under this condition. Amiloride derivatives also block two other modes of carrier action: Na–Na exchange is inhibited in a competitive fashion with Na^+ and kinetics of Ca–Ca exchange inhibition are mixed vs Ca^{2+} in either sucrose or K^+. However, Ca–Ca exchange inhibition can be alleviated by increasing K^+ concentration. Dixon analyses of Na–Ca exchange block with mixtures of inhibitors suggest that these agents are interacting at more than one site. In addition, Hill plots of inhibition are biphasic with Hill coefficients of 1 and 2 at low and high inhibitor concentrations, respectively. These results indicate that amiloride derivatives are mechanism-based inhibitors that interact at two classes of substrate-binding sites on the carrier; at low concentration they bind preferentially to a site that is exclusive for Na^+, while at higher concentration they also interact at a site that is common for Na^+, Ca^{2+}, and K^+.

Na–Ca exchange has been postulated to play a key role in Ca^{2+} homeostasis of electrically excitable cells [for reviews see Baker and DiPolo (1984), Reeves (1985), and Kaczorowski (1985)]. This process transports Ca^{2+} across the plasma membrane coupled to Na^+ movement in the opposite direction and is controlled by transmembrane electrical and Na^+ concentration gradients. By use of cardiac sarcolemmal membrane vesicles, the Na–Ca exchange reaction has been demonstrated to be electrogenic (Reeves & Sutko, 1980), to possess a stoichiometry of $3Na^+:Ca^{2+}$ (Reeves & Hale, 1984), and to be bidirectional (Philipson & Nishimoto, 1982). Moreover, the kinetics of transport have been extensively characterized in this preparation (Kadoma et al., 1982; Reeves & Sutko, 1983; Philipson, 1985). The V_{max} of Na–Ca exchange in vesicles is very high, and operation of the transporter is kinetically symmetrical in most respects. The carrier possesses multiple classes of ion-binding sites, and Na^+ appears to interact in a highly cooperative manner. In addition to Na–Ca exchange, this system will also function in nonproductive Ca–Ca (Slaughter et al., 1983) and Na–Na (Reeves, 1985) exchange modes, studies of which have provided insight into the mechanism of carrier action.

A model based on flux measurements has been proposed to describe the operation of the cardiac transporter (Reeves et al., 1984; Reeves, 1985). In this scheme, two classes of ion-binding sites exist: a common site at which either one or two Na^+ or a single Ca^{2+} binds (A-site) and a distinct site (B-site) at which the third transported Na^+ binds. Occupation of the A-site by two Na^+ and the B-site by a single Na^+ promotes transport of Ca^{2+} bound to an A-site on the opposite face of the carrier. Binding of Ca^{2+} at the A-site allows broader substrate specificity at the B-site, where it has been postulated that alkali metal ions which stimulate Ca–Ca exchange interact. This mechanism is similar in many respects to the one proposed for Na–Ca exchange action in squid axon (Blaustein, 1977).

To test putative models of Na–Ca exchange and elucidate the physiological role of this transport reaction, specific mechanism-based inhibitors will be required. To date, few inhibitors of Na–Ca exchange have been identified [for a review see Kaczorowski et al. (1988)]. Recently, inhibition

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