^{14}N NMR of Lipid Bilayers: Effects of Ions and Anesthetics[†]

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ABSTRACT: The interaction of divalent and trivalent metal cations, ferricyanide, a lipophilic ion (tetraphenylborate), and a local anesthetic (tetracaine) with the phosphocholine head group of egg lecithin was investigated by using wide-line ^{14}N and ^{31}P NMR. Measurements of the ^{14}N quadrupolar splittings in the presence of a variety of perturbing agents demonstrated that the ^{14}N NMR technique can be used to directly monitor ion or anesthetic binding. The ^{14}N quadrupolar splitting ($\Delta\nu_Q$) is a measure of the order parameter of the C_β–N bond segment, and changes in $\Delta\nu_Q$ as large as 3.5 kHz were observed. Moreover, a comparison of the changes in the quadrupolar splittings induced by the binding of ions or anesthetics provided a sensitive method of discriminating between these perturbing agents in their ability to alter the orientational order of the C_β–N bond segment of the phosphocholine moiety. Without exception, addition of metal ions or anesthetics always resulted in a decrease of the ^{14}N $\Delta\nu_Q$. This reduction reflects a change in the average orientation or degree of motional averaging at the C_β–N bond segment position. In the case of metal ion binding, the strength of the interaction increased with the charge of the metal ion in the order $Ca^{2+} < Ln^{3+}$, in agreement with a previous ^2H NMR study [Akutsu, H., & Seelig, J. (1981) *Biochemistry 20*, 7366–7373]. However, distinct differences were also noted between ions of the same charge, and in the case of the tri-valent lanthanide ions, the ^{14}N $\Delta\nu_Q$ decreased in the sequence $La^{3+} > Pr^{3+} > Eu^{3+} > Lu^{3+}$, following the order of the lan-thanide contraction. The ^{14}N and ^{31}P line shapes in the presence of lanthanide ions showed that it is possible to clearly distinguish between the effects of paramagnetic (Pr^{3+}, Eu^{3+}, Dy^{3+}, Tm^{3+}) and diamagnetic (La^{3+}, Lu^{3+}) ions. Unusual, distinctive "asymmetric" ^{14}N NMR line shapes were observed in the presence of the paramagnetic lanthanides, apparently due to incomplete averaging of the magnetic dipolar interaction between the bound lanthanide ion and the nitrogen nucleus. Addition of the lipophilic anion tetraphenylborate led to a slight reduction in the ^{14}N $\Delta\nu_Q$, together with a dramatic *decrease* in the absolute value of the ^{31}P chemical shielding anisotropy ($\Delta\sigma$). By contrast, the reduction of the ^{14}N $\Delta\nu_Q$ due to the presence of tetracaine was accompanied by a sub-stantial *increase* in the ^{31}P $|\Delta\sigma|$. In general, the measurements of the ^{14}N $\Delta\nu_Q$ as well as the ^{14}N spin–lattice (T_1) relaxation times support the notion that there is a rapid exchange of ions from the bulk medium to the ligand binding sites and among the various binding sites, in agreement with ^2H NMR studies. The above results suggest that ^{14}N NMR can provide a useful complement to ^{31}P and ^2H NMR techniques in studies of the influence of various perturbing agents on the orientational order and dynamics of the phosphocholine head groups in membranes.

The topography of the cell membrane surface is determined by integral membrane proteins which span the lipid bilayer and by the lipid head groups of both monolayers. The physiological functions of many tissues depend, in part, on the interaction of compounds such as hormones or neurotrans-mitters with specific protein binding sites exposed at the membrane surface. Other functions depend on interactions which may be relatively nonspecific in nature, involving only the polar head groups of membrane lipids. In particular, the binding of metal ions such as Ca^{2+} or Mg^{2+} to the phospholipid constituents of biological membranes is most certainly the result of an interaction with the polar head groups of either charged or neutral phospholipids. A broad survey of mam-malian cell membranes indicates not only that phosphati-dylcholine (lecithin) is a major fraction of the total phos-pholipid content of these membranes but also that it is asym-metrically distributed between the two monolayers of the lipid bilayer, in such a way that the phosphocholine head groups are predominant in the outer monolayer (Rothman & Lenard, 1977). It is reasonable to anticipate that the interactions of metal cations with the phosphocholine head group, which is an electrically neutral zwitterion over the physiological pH range, would be somewhat weaker than those with negatively charged lipids such as phosphatidylserine. Nevertheless, it has been demonstrated by NMR (McLaughlin et al., 1978; Hauser et al., 1975, 1977; Hutton et al., 1977) and X-ray diffraction studies (Lis et al., 1981) of model membranes that lecithin does bind divalent cations such as Ca^{2+} or Mg^{2+}, and trivalent cations such as Pr^{3+} and Eu^{3+}, although the binding of the alkaline earth cations is significantly weaker than that of the lanthanides (Hauser et al., 1975).

The biological significance of metal ion binding to phos-phatidylcholine in vivo is not yet understood. It is possible that the modulation of many Ca^{2+}-sensitive functions of membranes is accomplished by ion-induced changes in the membrane surface potential. The electrostatic potential at the surface of the bilayer is very likely to be sensitive to the conformation of the polar head group (Zakrzewska & Pull-man, 1981) as well as to the presence of bound ions. If cation binding alters the head-group conformation, then there could be major effects on the electrostatic potential at the membrane surface. Much effort has therefore been directed toward determining what are the nature and the extent of the changes which accompany ion binding to the phosphocholine head group. In this regard, wide-line ^2H NMR was first used by Brown & Seelig (1977) to establish directly that the binding of lanthanide ions to 1,2-dipalmitoyl-*sn*-glycero-3-phospho-choline (DPPC)[1] causes a change in the conformation of the

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[1] Abbreviations: PC, phosphatidylcholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; Ph$_4$B$^-$ (ϕ_4B$^-$ in figures), tetraphenylborate; ESR, elec-tron spin resonance; EDTA, ethylenediaminetetraacetic acid.

head group. The sensitivity of the ^{2}H quadrupole splittings to ion binding was further exploited by Akutsu & Seelig (1981) to determine the concentration, temperature, and ion dependence of the lipid–metal ion interaction. In this paper, we report the results of a wide-line ^{14}N and ^{31}P NMR study of the effects of various lipophilic, diamagnetic, and paramagnetic ions on the phosphocholine head group of egg lecithin.

The primary advantage of natural-abundance probes such as ^{14}N or ^{31}P lies in the fact that no specific labeling is required, thus obviating the need for carrying out the synthesis of labeled molecules, as is required for ^{2}H NMR. At the same time, because the ^{14}N nucleus has nuclear spin $I = 1$, it has a number of physical properties in common with the ^{2}H nucleus which have important consequences for the use of wide-line ^{14}N NMR as probe of the lecithin head group in unsonicated lipid systems. First, the spin–lattice relaxation of the ^{14}N or ^{2}H nuclear spin is dominated by the quadrupolar interaction, and so the analysis of the relaxation rates is considerably simplified. Second, because the NMR line shapes of spin 1 nuclei are determined almost exclusively by the interaction between the quadrupole moment and the surrounding electric field gradient, the wide-line ^{14}N or ^{2}H NMR spectrum constitutes a powder pattern whose quadrupolar splitting is a direct measure of the orientational order parameter for the symmetry axis of the appropriate (axially symmetric) electric field gradient tensor. In the case of wide-line ^{2}H NMR, it has been demonstrated from studies of DPPC specifically labeled in the phosphocholine head group (Brown & Seelig, 1977; Akutsu & Seelig, 1981) that significant changes in the ^{2}H quadrupolar splittings can be induced by the interaction with ions. Here, we extend and complement these ^{2}H NMR studies by using ^{14}N NMR to monitor the response of the C_β–N bond segment to the binding of ions and anesthetics.

Materials and Methods

Egg lecithin was prepared by the method of Singleton et al. (1965), and its concentration was determined by using phosphorus analysis, assuming a molecular weight of 770. Unbuffered 1.00 M stock solutions of the lanthanide trichlorides were prepared from the hexahydrates (Alfa, Ventron); in some cases, their concentrations were checked by titration with a standardized solution of EDTA using xylenol orange as an indicator. A $CaCl_2$ stock solution was prepared from the tetrahydrate (ultrapure; Alfa, Ventron), and sodium tetraphenylborate ($Na^+Ph_4B^-$) was obtained from Aldrich (gold label). The pH of the stock solutions was checked and, if necessary, was adjusted to between 5 and 7 by using HCl or NaOH. Tetracaine hydrochloride was obtained from Sigma, and a 1.00 M stock solution was prepared in 0.10 M HOAc buffer, pH 6.6. Aliquots of the egg lecithin in $CHCl_3$ were evaporated to dryness, followed by exposure to high vacuum for at least 24 h; appropriate amounts of the stock solutions were then added together with sufficient doubly distilled H_2O such that the final water concentration was 50 wt %. The samples were then vortexed extensively, centrifuged repeatedly, and freeze–thawed several times to assure their homogeneity. For the NMR experiments, the lipid dispersions were transferred to 10-mm o.d. NMR tubes and sealed with silicone sealant under argon.

^{14}N NMR spectra were obtained at a frequency of 19.438 MHz by using a homebuilt spectrometer (Jeffrey, 1980) and a Bruker superconducting solenoid (6.3 T) as previously described (Siminovitch et al., 1980; Siminovitch & Jeffrey, 1981). ^{31}P NMR spectra were acquired on a Bruker WH-400 spectrometer at 161.98 MHz by using a single 90° pulse and
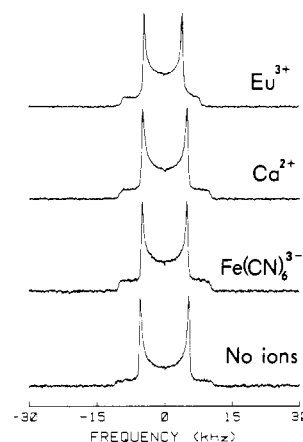

FIGURE 1: ^{14}N spectra of egg lecithin in the presence of the indicated ions at a lipid to ion mole ratio of 5.0 (lipid/ion = 5.0). To enhance the signal to noise ratio, typically 16K scans were signal averaged. The quadrupolar echo signal was digitized at a rate of once every 10 μs, corresponding to a spectral width of 100 kHz, and the spectra have a 200-Hz line broadening due to exponential multiplication of the echo signal prior to Fourier transformation. Quadrature phase detection was used, and the spectra were *not* folded about the resonance frequency of the spectrometer.
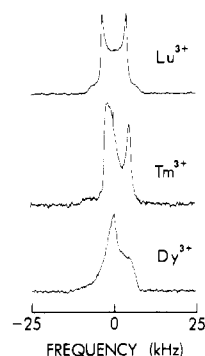

FIGURE 2: ^{14}N spectra of egg lecithin in the presence of the indicated lanthanide ions (lipid/Ln^{3+} = 1.6). Experimental conditions are identical with those described in the caption to Figure 1.

broad-band ^{1}H decoupling. A modified form of the quadrupole echo sequence (Davis et al., 1976; Siminovitch et al., 1980) was used to obtain the ^{14}N spectra. Typical 90° pulses were 8 μs in duration and were separated by a pulse spacing (τ) of 120 μs. The recycle time was 0.16 s. Quadrature phase detection was used throughout. All of the ^{14}N experiments were carried out at a temperature of 16 °C, while the ^{31}P experiments were performed at 20 °C. Both of these temperatures are well above the liquid-crystal to gel-phase transition, which in egg lecithin occurs near –10 °C.
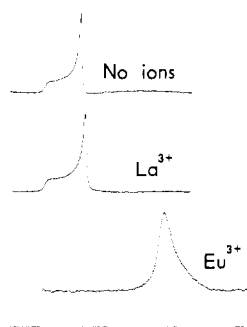
^{14}N T_1 relaxation times were measured by observing the recovery of the magnetization following the application of a single 180° pulse. The magnetization was sampled at various intervals following the inverting pulse using the quadrupolar echo; the Fourier transforms of the echo signals were then used to monitor the recovery of magnetization by measuring the spectral intensity corresponding to 90° orientations of the bilayer normal with respect to the main magnetic field.

Results

Metal Ions. Typical ^{14}N spectra of egg lecithin in the presence of various metal ions are shown in Figures 1 and 2. Figure 3 displays ^{31}P spectra of egg lecithin complexed with lanthanide ions. In the case of the La^{3+} and Eu^{3+} ions, similar ^{31}P spectra have been reported previously by Brown & Seelig (1977) for lanthanide ion binding to the head group of DPPC. Except for the unusual ^{31}P line shape which is observed in the

Table I: ^{14}N Quadrupolar Splittings ($\Delta\nu_Q$) as a Function of the Lipid/Ion Ratio and Values of the ^{31}P Chemical Shift Anisotropy ($|\Delta\sigma|$) at a Lipid/Ion Ratio of 1.6
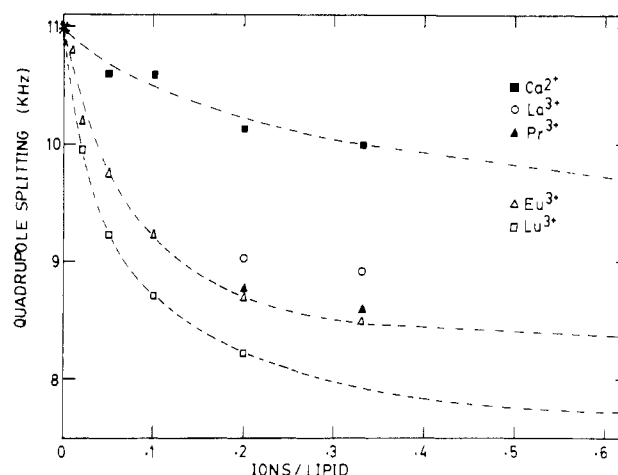
ion	$\Delta\nu_Q$ at a PC/ion ratio of							$|\Delta\sigma|$ (ppm) at a PC/ion ratio of 1.6
	1.6	3.0	5.0	10	20	50	100	
La^{3+}	8.65	8.92	9.03					55
Pr^{3+}	8.51	8.60	8.78					
Eu^{3+}	8.38	8.50	8.70	9.23	9.75	10.2	10.8	
Lu^{3+}	7.72		8.20	8.72	9.23	9.96		55
Fe(CN)$_6$$^{3-}$	9.14	9.84	10.2					
Ca^{2+}	9.73	10.0	10.1	10.6	10.6			55
Ph$_4$B$^-$	10.2	10.8	10.9					33
none	11.0							48

FIGURE 3: ^{31}P spectra of egg lecithin in the presence of the indicated lanthanide ions (lipid/Ln^{3+} = 1.6). To obtain these spectra, 125 free induction decays were signal averaged. The chemical shift scale has a total width of 300 ppm, corresponding to 25 ppm per division.

presence of the paramagnetic Eu^{3+} ion, the ^{31}P powder pattern line shapes in the presence of La^{3+} and Lu^{3+} (not shown) are both characteristic of a random dispersion of liquid-crystalline bilayers.

Regardless of the metal ion involved, only one ^{14}N quadrupolar splitting ($\Delta\nu_Q$) was observed for all of the dispersions in which ions were present at a lipid/ion ratio of 1.6. At lower lanthanide ion concentrations, two component spectra were sometimes observed. The presence of two components would appear to be indicative of a sample which is not properly mixed, since after further cycles of freeze–thawing, only one quadrupolar splitting was observed. Westman et al. (1982) have also reported that repeated cycles of freeze–thawing facilitate contact between metal cations in a water phase and lipid bilayers. The single quadrupolar splitting observed after freeze–thawing was identical in value with that of one of the two components initially detected. This suggests that one of the components, whose quadrupolar splitting is invariant and equal to that observed without ions, corresponds to head groups which are not interacting with ions. The other component, whose quadrupolar splitting varies as a function of ion concentration, corresponds to head groups which are interacting with ions. In the case of this latter component, the exchange between free and bound ions must be rapid on the NMR time scale. For this component alone, we have measured the quadrupolar splittings as a function of ion concentration, and these values are given in Table I, together with representative values of the absolute magnitude of the ^{31}P chemical shift anisotropy, $|\Delta\sigma|$, at a lipid/ion ratio of 1.6.

We observe that, without exception, addition of metal ions always results in a *decrease* of the ^{14}N $\Delta\nu_Q$. Relative to the changes observed in the ^{2}H $\Delta\nu_Q$ of the C_α position upon addition of metal ions to DPPC (Akutsu & Seelig, 1981), the magnitude of the decrease in the ^{14}N $\Delta\nu_Q$ is small. The largest decrease in the ^{14}N $\Delta\nu_Q$ ($\sim$30%) is observed when Lu^{3+} ions are added in a lipid/ion ratio of 1.6. The response of the ^{14}N $\Delta\nu_Q$ to ion binding should be contrasted with the changes in

FIGURE 4: Adsorption isotherms of egg lecithin bilayers (50 wt % H$_2$O) for the indicated metal cations at 16 °C. The ^{14}N quadrupolar splittings are plotted as a function of the ion/lipid ratio. (The dashed lines connecting the points do not have theoretical significance and are intended for clarity of presentation only.)

the ^{2}H $\Delta\nu_Q$ of the C_α and C_β positions of the DPPC head groups upon the addition of metal ions (Brown & Seelig, 1977; Akutsu & Seelig, 1981), where C_α denotes that segment closest to the phosphodiester moiety. The C_α position $\Delta\nu_Q$ always decreased when metal ions were added, whereas the β-position splitting always increased. The maximum change in the ^{2}H $\Delta\nu_Q$ is about twice as large for the C_α segment as for the C_β segment, while the maximum change in the ^{14}N $\Delta\nu_Q$ in egg lecithin is much the same as that reported for the ^{2}H $\Delta\nu_Q$ of the C_β segment of DPPC. In agreement with previous studies of ion binding to DPPC by Brown & Seelig (1977) and Akutsu & Seelig (1981), the ^{31}P results given in Table I show that there is a slight *increase* in the absolute magnitude of the chemical shift anisotropy upon the addition of diamagnetic ions such as Ca^{2+}, La^{3+}, or Lu^{3+}. Both of the lanthanide ions, La^{3+} and Lu^{3+}, also cause a small shift of the ^{31}P powder pattern to higher frequency.

If the size of the ^{14}N $\Delta\nu_Q$ is taken as a measure of the binding of the ions to the head group, then the quadrupolar splittings given in Table I and plotted in Figure 4 show that the strength of the lipid–ion interaction increases with the charge of the metal ion, such that Ca^{2+} < Ln^{3+}. However, the strength of the interaction is not determined exclusively by the ionic charge. For the trivalent lanthanide ions, the ^{14}N $\Delta\nu_Q$ decreases in the sequence La^{3+} > Pr^{3+} > Eu^{3+} > Lu^{3+}, following the order of the lanthanide contraction (Cotton & Wilkinson, 1980). Similar charge effects have been reported by Akutsu & Seelig (1981), only that their magnitude was somewhat greater. They found that the strength of the lipid–ion interaction increased with the charge of the metal ion in the order Na$^+$ < Ca^{2+} < La^{3+}, while for divalent ions, the

induced conformational change increased in the order Cd^{2+} < Mg^{2+} < Ca^{2+}. Since the ionic radii of calcium and the lanthanides are similar (Shannon & Prewitt, 1969; Martin & Richardson, 1979), lanthanide ions have often been used as "isomorphous" replacements for Ca^{2+} in a wide variety of ion-binding studies. It is important to note that both ^{2}H NMR and ^{14}N NMR studies show that at the same ion concentration, Ca^{2+} and the lanthanides do not have identical effects on the phosphocholine head group, and in this respect, the lanthanides are not completely suitable Ca^{2+} ion substitutes.

The ^{14}N and ^{31}P line shapes in the presence of lanthanide ions show that it is possible to clearly distinguish between the effects of paramagnetic (Pr^{3+}, Eu^{3+}, Dy^{3+}, Tm^{3+}) and diamagnetic (La^{3+}, Lu^{3+}) ions. The presence of the paramagnetic lanthanides gives rise to an unusual asymmetric ^{14}N NMR line shape. In contrast, completely symmetric quadrupolar powder patterns (except for minor misadjustments of phase or tuning) are observed in the presence of the diamagnetic La^{3+} or Lu^{3+} ion. The use of quadrature detection is essential for the acquisition of an asymmetric spectrum. The use of single phase detection with on-resonance irradiation (or the folding of a spectrum acquired in quadrature by zero filling the imaginary channel) will result in a symmetrized spectrum, and indeed Rothgeb & Oldfield (1981) have reported a ^{14}N spectrum of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) in the presence of Eu^{3+} ions which is symmetric for this reason. Using ^{2}H NMR, Akutsu & Seelig (1981) have also reported unusual asymmetric line shapes in the presence of Eu^{3+} ions, although the full asymmetric character was not revealed due to the fact that a single 90° pulse was used to acquire spectra. A more complete account of the significance and interpretation of ^{14}N and ^{2}H line shapes in the presence of paramagnetic lanthanides will be given in a separate publication (Siminovitch et al., 1984).[2]

While the paramagnetic lanthanide ions cause some of the largest perturbations of the head group, in terms of their effect both on the ^{14}N $\Delta\nu_Q$ and on the line shape, addition of the paramagnetic shift reagent $K_3Fe(CN)_6$ has relatively little effect. Table I shows that at equivalent molar concentrations, the ferricyanide ion results in a smaller reduction in the ^{14}N $\Delta\nu_Q$ than is the case for the lanthanides. Moreover, in the presence of the ferricyanide ion, we do not observe any asymmetry in the ^{14}N line shape, such as that observed when any of the paramagnetic lanthanides are present. Rothgeb & Oldfield (1981) have also observed that in comparison to the Eu^{3+} cation, the $Fe(CN)_6^{3-}$ anion has a relatively small effect on the ^{14}N $\Delta\nu_Q$ of DMPC. Brown & Seelig (1977) have shown that addition of $Fe(CN)_6^{3-}$ has little influence on the ^{31}P NMR line shape of unsonicated dispersions of DPPC, whereas binding of Eu^{3+} is accompanied by an apparent reversal in the sign of the residual chemical shift anisotropy, as well as a large chemical shift. $Fe(CN)_6^{3-}$ has been employed as a paramagnetic shift reagent in vesicle studies (Kostelnik & Castellano, 1972; Berden et al., 1975; Sim et al., 1975;

FIGURE 5: Comparison of the ^{14}N quadrupolar splittings vs. temperature for egg lecithin in the presence of Eu^{3+} ions (O) and La^{3+} ions (Δ) and without ions (□) (lipid/Ln^{3+} = 5.0). The estimated error is ±0.1 kHz.

FIGURE 6: Arrhenius plots of the ^{14}N spin–lattice (T_1) relaxation times for egg lecithin with and without the indicated lanthanide ions (lipid/Ln^{3+} = 5.0). The plotting symbols used correspond to those of Figure 5. The error in T_1 is estimated to be less than ±5%.

Albert & Yeagle, 1982), and a comparison of the effects of Eu^{3+} and $Fe(CN)_6^{3-}$ on the choline methyl resonance (Kostelnik & Castellano, 1972) indicates that the concentration of $Fe(CN)_6^{3-}$ ion must be at least 1 order of magnitude greater in order to produce the same shift in the resonance corresponding to outer monolayer lipids. Berden et al. (1975) and Albert & Yeagle (1982) have also found that relatively high concentrations of $Fe(CN)_6^{3-}$ ion are required to separate the inner and outer resonances of lecithin vesicles. All of the evidence from the high-resolution experiments and from the wide-line ^{31}P and ^{14}N experiments indicates that the influence of the $Fe(CN)_6^{3-}$ ion on the phosphocholine head group is the result of a relatively weak binding interaction with the positively charged trimethylammonium group.

In the case of the paramagnetic lanthanides Dy^{3+} and Tm^{3+}, the ^{14}N NMR line shapes are significantly perturbed by the large paramagnetic moments such that, without extensive simulations, it is not possible to assign accurate values of the ^{14}N $\Delta\nu_Q$ [cf. Siminovitch et al. (1984)]. However, with either Eu^{3+} or Pr^{3+}, the perturbation of the paramagnetic ion is small enough that it is possible to measure the ^{14}N $\Delta\nu_Q$ accurately. Although both of the lanthanides Eu^{3+} and Pr^{3+} result in characteristic asymmetric ^{14}N line shapes, the lanthanides La^{3+}, Pr^{3+}, and Eu^{3+} all have very similar effects on the orientational order and motional dynamics of the C_β–N bond

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[2] It may be noted that the asymmetric ^{14}N NMR spectra reported in this paper are inverted relative to some of those shown elsewhere (Siminovitch et al., 1984). Fourier transform NMR spectra are conventionally plotted with increasing frequency to the left, corresponding to decreasing magnetic field in a continuous-wave experiment. The asymmetric spectra depicted in Figures 1 and 2 are therefore inverted relative to convention; i.e., increasing frequency is to the right. In the presence of paramagnetic ions, the prominent asymmetric shoulder corresponds to the low-frequency side of the spectrum in all cases investigated. Normally this question does not arise in NMR studies of quadrupolar nuclei, since the spectra are symmetric about the central frequency to first order.
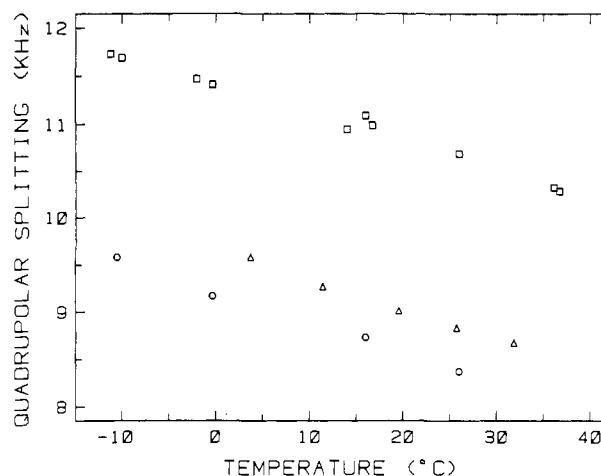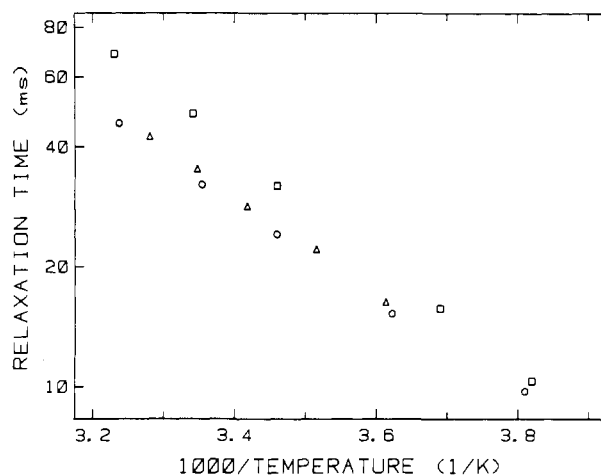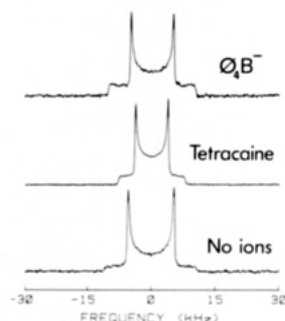
FIGURE 7: ^{14}N spectra in the presence of the Ph$_4$B$^-$ ion (lipid/Ph$_4$B$^-$ = 1.6) and tetracaine (lipid/tetracaine = 2.6). Experimental conditions are as in Figures 1 and 2.

FIGURE 8: ^{31}P spectra showing the effect of the Ph$_4$B$^-$ ion (lipid/Ph$_4$B$^-$ = 1.6) and tetracaine (lipid/tetracaine = 2.6). Experimental conditions are as in Figure 3. The chemical shift scale has a total width of 200 ppm, corresponding to 25 ppm per division.

segment. In Figure 5, the quadrupolar splittings of egg lecithin in the presence of the diamagnetic La^{3+} ion and the paramagnetic Eu^{3+} ion are compared as a function of temperature to those of egg lecithin alone. With or without ions, the same slope is observed, demonstrating that the temperature dependence of the ^{14}N quadrupolar splittings is not altered significantly by the presence of the lanthanide ions. This behavior is consistent with that observed by Akutsu & Seelig (1981), who found that the temperature dependence of the ^{2}H splittings of the C$_\alpha$ or the C$_\beta$ position is largely unaffected by the presence of metal cations and that almost the same slope is observed for each of the ions investigated.

Figure 6 shows that the T_1 relaxation times measured in the presence of the diamagnetic La^{3+} ion are indistinguishable from those measured with the Eu^{3+} ion, which demonstrates that the paramagnetic interaction has little effect on the relaxation of the ^{14}N nucleus. At least under these conditions, it would appear that the quadrupolar relaxation mechanism dominates the paramagnetic contribution. The T_1 relaxation times are slightly shorter in the presence of the lanthanide ions and become even shorter as the temperature increases. If the temperature dependence of the ^{14}N relaxation rates is interpreted in the standard fashion, by using an Arrhenius-type expression, then from the Arrhenius plots of ln T_1 vs. T^{-1} we find that for egg lecithin alone the activation energy is 27 kJ mol^{-1} (6.5 kcal mol^{-1}), and in the presence of Eu^{3+}, it is 23 kJ mol^{-1} (5.5 kcal mol^{-1}). These values appear slightly greater than those derived from ^{2}H T_1 studies for the C$_\alpha$ and C$_\gamma$ segment positions (Gally et al., 1975).

Tetraphenylborate and Tetracaine. ^{14}N and ^{31}P NMR spectra of egg lecithin in the presence of these two molecules are shown in Figures 7 and 8, respectively. Table I shows that even at the highest concentration of Ph$_4$B$^-$ ([PC]/[Ph$_4$B$^-$] = 1.6), binding of this ion results in the smallest decrease in $\Delta\nu_Q$ for all of the ions investigated. However, the ^{31}P spectrum of the same sample shows that binding of Ph$_4$B$^-$ results in a dramatic *reduction* in the absolute magnitude of the chemical shift anisotropy, from 48 ppm in egg lecithin alone to a value of 33 ppm with Ph$_4$B$^-$, in good agreement with previous work (Hubbell et al., 1980; unpublished results).

At a molar ratio of lipid to tetracaine of 2.6, a single quadrupolar powder pattern is observed, whose $\Delta\nu_Q$ of 7.91 kHz is (with the sole exception of the PC/Lu^{3+} $\Delta\nu_Q$) *smaller* than any value observed in the presence of metal ions. The absolute magnitude of the ^{31}P chemical shift anisotropy *increases* from a value of 48 ppm in egg lecithin alone to a value of 58 ppm with tetracaine, which is in reasonable agreement with other studies of the binding of local anesthetics such as tetracaine (Boulanger et al., 1981) and dibucaine (Browning & Akutsu, 1982) to lecithin model membranes. At a lower tetracaine concentration ([PC]/[tetracaine] = 5.2), we observe

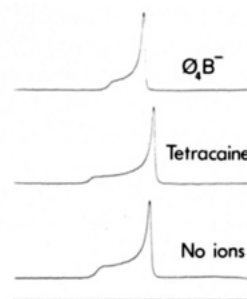a spectrum which can best be described as a superposition of two powder patterns, corresponding to two quadrupolar splittings. In a ^{2}H NMR study of dibucaine binding to DPPC, Browning & Akutsu (1982) also report two signals at low drug concentrations in the ^{2}H NMR spectra at the C$_\alpha$ and C$_\beta$ positions of the phosphocholine moiety.

Discussion

Phospholipid head groups exposed at the bilayer surface are the first interaction site for external perturbing agents such as ions or anesthetics. As a first step in determining the nature of their interaction with the lipid bilayer, it is therefore important to fully characterize the response of the polar head group to the binding of these molecules. Although high-resolution ^{1}H and ^{31}P NMR (Hauser et al., 1975, 1976, 1977; Hutton et al., 1977; Grasdalen et al., 1977; McLaughlin et al., 1978; Westman & Eriksson, 1979; Chrzeszczyk et al., 1981) experiments have provided valuable information on the stoichiometry and strength of metal ion binding to neutral phospholipids such as DPPC, most of these studies have relied upon the use of paramagnetic lanthanide ions to shift the head-group resonances of the complexed lipids (Bergelson et al., 1970; Bergelson, 1977; Barsukov, 1978; Lichtenberg et al., 1979; Hunt & Tipping, 1980). Moreover, the acquisition of the high-resolution NMR spectra necessary to detect the small shifts induced by the binding of these shift reagents has dictated the use of small unilamellar vesicles. The principal advantages of wide-line NMR studies of lipid–ion interactions using either the naturally abundant ^{14}N and ^{31}P probes or ^{2}H labels introduced at specific positions in the polar head group are that ion-induced perturbations may be monitored equally well in the presence of both diamagnetic *and* paramagnetic ions, over a wide range of ion concentrations, and without sonication of the lipid dispersion.

Without exception, we have found that the ^{14}N quadrupolar splitting $\Delta\nu_Q$ *decreases* in the presence of ions or anesthetics and that the magnitude of this change is somewhat smaller than the changes in the ^{2}H quadrupolar splittings at the C$_\alpha$ or the C$_\beta$ position of DPPC. In a ^{14}N NMR study of DMPC in the presence of various perturbing agents such as ions, drugs, antibiotics, and proteins, Rothgeb & Oldfield (1981) have also observed that the ^{14}N $\Delta\nu_Q$ always decreases. The ^{14}N quadrupolar splitting $\Delta\nu_Q$ can be written as

$$\Delta\nu_Q = [3e^2qQ/(4h)]S_{C_\beta-N} \qquad (1)$$

where the order parameter of the C$_\beta$–N bond segment is given by

$$S_{C_\beta-N} = \langle(3\cos^2\theta - 1)/2\rangle \qquad (2)$$

The quantity e^2qQ/h is the nuclear quadrupolar coupling

constant, which is determined by the nuclear electric quadrupole moment (eQ) and the principal value of the electric field gradient (eq). It has been assumed that the electric field gradient tensor is axially symmetric along the C_β–N bond axis, and therefore, the angle θ is between the C_β–N bond direction and the bilayer normal. The broken brackets indicate that an average must be taken over the molecular motions which cause changes in the angle θ. For the case of ^{2}H NMR, it is well established (Seelig, 1977) that the electric field gradient at the deuterium site is axially symmetric along the C–D bond direction and that the electric field gradient is determined almost exclusively by the electronic structure of the C–D bond; hence, e^2qQ is considered to be constant, and the observed changes in $\Delta\nu_Q$ are attributed to changes in the corresponding bond segmental order parameter S_{CD} as a function of ion binding (Akutsu & Seelig, 1981). There is a potential problem with the interpretation of the ^{14}N quadrupole splittings because it has not been shown experimentally that the observed changes in $\Delta\nu_Q$ arise solely from changes in $S_{C_\beta-N}$, such that e^2qQ remains constant. The C_{3v} symmetry of the trimethylammonium group suggests that the electric field gradient must be axially symmetric along the C_β–N bond direction (Cohen & Reif, 1957). Because the ^{14}N nucleus is at the center of this group, intermolecular contributions to the electric field gradient should be small in the liquid crystalline state; nonetheless, distortion of the nearly tetrahedral bonding of the nitrogen atom could potentially cause large changes in e^2qQ. The binding of metal ions is quite weak, however, and since the binding site is probably near the phosphate group, it is unlikely that changes in the electronic structure of the trimethylammonium moiety occur. Similar considerations hold for the binding of anesthetics and hydrophobic ions. Thus, it is probably a safe assumption to maintain that the observed changes in $\Delta\nu_Q$ are due to alterations in $S_{C_\beta-N}$, which arise from changes in either the *amplitude* of the molecular motions or the *average orientation* of the C_β–N bond.

It should be noted that although wide-line ^{2}H or ^{14}N NMR studies of lipid–ion interactions may be performed with either paramagnetic *or* diamagnetic ions, it is not true that the magnetic properties of the ions employed are immaterial. In this work, we have shown that binding of *paramagnetic* lanthanide ions gives rise to a unique, asymmetric ^{14}N NMR line shape. Contrary to the shift induced by paramagnetic ions in solution, which only arises if the magnetic moment of the lanthanide ion is anisotropic (Bleaney, 1972), a plausible explanation is that the asymmetric wide-line ^{14}N NMR spectra appear to result from incomplete averaging, in the anisotropic environment of unsonicated lipid bilayers, of the magnetic dipolar interaction between the bound lanthanide ion and the nitrogen nucleus (Siminovitch, 1982).

Ion-Induced Conformational Changes. By labeling both the C_α and C_β positions of the choline moiety, previous ^{2}H NMR studies of ion binding to the head group of DPPC (Brown & Seelig, 1977; Akutsu & Seelig, 1981) have provided evidence for a conformational change in the polar head group upon the binding of metal ions. The results of the ^{14}N NMR experiments on egg lecithin described here and on DMPC described elsewhere (Rothgeb & Oldfield, 1981) show only that ion binding to the phosphocholine head group results in a decrease in the $S_{C_\beta-N}$ order parameter. Because the value of $S_{C_\beta-N}$ will, in general, depend on both (i) the average orientation of the C_β–N bond segment with respect to the axis of rotational symmetry (the bilayer normal) *and* (ii) the amplitude of fluctuations around this average orientation, it is not possible to unequivocally attribute the observed decrease

in $S_{C_\beta-N}$ to a change in the average orientation of the C_β–N bond segment. Thus, the question of whether or not ion binding to the choline moiety results in a change in the average orientation of the C_β–N bond segment cannot be answered by ^{14}N NMR alone. However, given that the binding of metal ions to the DPPC head group affects the torsion angles of the phosphate segment and of both methylene groups (Akutsu & Seelig, 1981), it would not be surprising if these conformational changes are also accompanied by a change in the orientation of the C_β–N bond segment. Thus, although the binding of the metal cation takes place near the phosphate group, the effects seen at the nitrogen site may reflect a change in the orientation of the whole phosphocholine group, which encompasses the phosphate segment, both methylene groups, and the C_β–N bond segment.

Analysis of Adsorption Isotherms. Under the experimental conditions accessible to the use of paramagnetic shift reagents in high-resolution NMR studies of lipid–ion interactions, conformational changes in phospholipid head groups were difficult to detect because the concentration of paramagnetic ions was low (on the order of 50–100 phospholipid molecules per ion) (Hauser et al., 1977; Barsukov, 1978; Westman & Eriksson, 1979). The use of wide-line ^{14}N or ^{2}H NMR studies of unsonicated lipid–ion dispersions extends the accessible concentration range by almost 2 orders of magnitude (approaching a 1 to 1 phospholipid to ion mole ratio). Akutsu & Seelig (1981) have suggested that ion binding data such as shown in Figure 4 can be interpreted in terms of a two-state model in which the fraction of binding sites filled, θ, is defined by the relationship

$$\theta = (\nu_{obsd} - \nu_0)/(\nu_{max} - \nu_0) \tag{3}$$

Here ν_0 is the quadrupolar splitting in the absence of ions (11.0 kHz for egg lecithin at 16 °C), ν_{obsd} is the observed quadrupolar splitting at a given ion concentration, and ν_{max} denotes the limiting splitting when all of the binding sites are filled. To proceed further, it is necessary to assume a physical model for the interaction between the ions in the aqueous regions and the lipid bilayer surface. Consideration has been given to the simple mass action equation or Langmuir adsorption isotherm, the Bragg–Williams approximation (Hill, 1956) as used by Akutsu & Seelig (1981), and the Gouy–Chapman–Stern theory [see, e.g., McLaughlin (1977)]. At the present time, there is insufficient data to differentiate unequivocally among the various models. However, the data summarized in Figure 4 suggest that detailed analysis will be possible in the future when results at other concentrations are available.

Tetraphenylborate and Tetracaine. The Ph_4B^- ion is hydrophobic in nature, and its lipid solubility has been exploited to study the mechanisms involved in ion transport across lipid bilayers and to estimate transmembrane potentials. However, it has recently been recognized by several investigators (Levine et al., 1979; Hubbell et al., 1980; unpublished results) that relatively strong binding of this ion at the bilayer surface should be taken into consideration in its use for the assessment of transmembrane potentials. On the basis of ^{1}H and ^{13}C ring-current shifts (Levine et al., 1979; Hubbell et al., 1980; unpublished results), the binding site of Ph_4B^- at the membrane surface may be near the $N^+(CH_3)_3$ group, although an additional site deeper in the bilayer is not precluded. The transit of hydrophobic ions such as Ph_4B^- across the lipid bilayer has been modeled (Ketterer et al., 1971; Andersen et al., 1978) by constructing a potential energy profile of the ion within the bilayer. This profile contains potential energy minima near each of the membrane–solution interfaces, which may correspond to Ph_4B^- binding sites. An ESR study (Seidah

et al., 1976) of the perturbations caused by Ph_4B^- on a variety of spin probes labeled at different depths in the bilayer established that the interaction of Ph_4B^- occurs at the membrane surface. Levine et al. (1979) used the shifts induced by the ring currents of the aromatic phenyl groups of Ph_4B^- on the proton resonances of the DPPC head group to show that the bound Ph_4B^- ions lie at the membrane–water interface, in a manner which permits the electrostatic interaction between the anion and the $N^+(CH_3)_3$ group. The ^{13}C chemical shifts in the presence of Ph_4B^- measured by Hubbell et al. (1980) also suggest that the effects of binding Ph_4B^- are predominantly confined to the choline head group over the concentration range employed.

The preliminary results given in the preceding section showed an unexpectedly small change in the orientational order of the C_β–N bond segment caused by the binding of Ph_4B^-. It is difficult to reconcile this result with the other NMR evidence (vide supra), which suggests that the binding site of this ion may be closer to the $N^+(CH_3)_3$ moiety than it is to any other part of the polar head group, unless the binding of Ph_4B^- caused only a small change in the orientation of the phosphocholine group. In this respect, it is interesting that the largest spectral changes upon binding of Ph_4B^- appear to be observed in the ^{31}P chemical shielding anisotropy (Figure 8), in contrast to the results obtained upon binding of divalent or trivalent metal ions (Brown & Seelig, 1977, Akutsu & Seelig, 1981). Levine et al. (1979) have noted that the proton resonances of Ph_4B^- bound to the bilayer surface are not shifted in the presence of bound paramagnetic ions such as Pr^{3+}, which also suggests that the lanthanide binding site is different from the Ph_4B^- binding site. The above results are in agreement with previous unpublished ^{13}C and ^{31}P NMR studies by Hubbell et al.; however, further work is necessary before firm conclusions can be reached.

Tetracaine is a local anesthetic which is known to perturb the structure of the lipid bilayer in model membranes. The nature of the interaction of local anesthetics such as tetracaine, dibucaine, or procaine with biological membranes is still a matter under investigation (Lee, 1976; Richards, 1978). It is not clear at present whether the mechanism of local anesthetic action depends on an interaction with the lipid component alone or whether specific protein binding sites are involved. What is clear is that tetracaine does interact hydrophobically with model membranes composed of lipid alone (Fernandez & Cerbon, 1973) and that at least at physiological pH, the perturbing influence of the bound anesthetic molecule is felt in the head group and in the fatty acyl chain region of the surrounding lipid molecules.

The extent of the perturbation caused by tetracaine in the lipid bilayer is consistent with its amphiphilic nature. The tetracaine molecule consists of a hydrophobic portion connected to a polar tertiary amine group, whose charge state depends on the pH of the medium. At pH 5.5, the tertiary amine group is positively charged, and Boulanger et al. (1981) have developed a model for the interaction of tetracaine with DPPC bilayers which positions the hydrophobic portion of the anesthetic molecule within the bilayer but places the polar tertiary amine at the level of the phosphocholine head group. ^2H NMR studies of the interaction of tetracaine (Boulanger et al., 1981) and dibucaine (Browning & Akutsu, 1982) with specifically deuterated DPPC confirm that both of these local anesthetics interact with the polar head group. Between a pH of 5.5 and 7.0, the binding of tetracaine or dibucaine decreases the C_α position splitting, increases the C_β position splitting, and causes a slight increase in the C_γ splitting (Boulanger et

al., 1981; Browning & Akutsu, 1982). Browning & Akutsu (1982) have pointed out that metal cation binding (Brown & Seelig, 1977) and anesthetic binding induce essentially identical changes in the phosphocholine head group, although the magnitude of the effects caused by anesthetics is much larger. Using the natural-abundance ^{14}N probe, we observe similar effects demonstrating that tetracaine alters the conformation of the polar head group of egg lecithin.

The ^{14}N and ^{31}P NMR spectra of egg lecithin in the presence of tetracaine and tetraphenylborate (Figures 7 and 8, respectively) provide a striking illustration of the fact that the ^{14}N $\Delta\nu_Q$ and the ^{31}P $\Delta\sigma$ can change *independently* of each other. In so doing, they suggest that, whenever possible, *both* naturally abundant NMR probes should be utilized in future studies of anesthetic or lipophilic ion interactions with the phosphocholine head group.

Conclusions

The results of the present work demonstrate that the technique of ^{14}N NMR offers a number of significant advantages for the study of lipids and their interactions with external perturbing agents. First, the use of the natural-abundance (>99.6%) ^{14}N probe enables one to work not only with synthetic lipids but also with lipids of natural origin, such as the lecithins and the sphingomyelins. In principle, the technique could even be extended to the study of lipid–ion or lipid–drug interactions in intact biological membranes, and to studies of membranes containing proteins such as the visual pigment rhodopsin (Brown et al., 1982). Second, although the absolute changes induced in the ^{14}N $\Delta\nu_Q$ are relatively small in comparison to those induced in the ^2H $\Delta\nu_Q$ at the C_α position, for example, they nevertheless appear to provide a reliable method for comparing the effects of various perturbing agents on the phosphocholine head group. Thus, an initial ^{14}N NMR study may be extremely useful in determining the binding affinity of various ions of interest, in discriminating between perturbing agents in their ability to alter the orientational order of the C_β–N bond segment, or in pointing out phenomena worthy of further investigation by ^2H NMR. Follow-up ^2H NMR studies utilizing specifically labeled head groups may then be used to provide a complete profile of the conformational and dynamic changes which are induced by those perturbing agents of interest.

The observation of only one ^{14}N $\Delta\nu_Q$ and T_1 relaxation time in the presence of metal cations parallels the results of previous ^2H NMR studies (Brown & Seelig, 1977; Akutsu & Seelig, 1981) in which only one ^2H $\Delta\nu_Q$ or T_1 relaxation time was observed and supports the conclusion that there is a rapid exchange of ions from the bulk medium to the lipid binding sites and among the various binding sites. At least in the case of the Eu^{3+} and La^{3+} ions, the ^{14}N T_1 relaxation times are only slightly shorter in the presence of ions. Akutsu & Seelig (1981) also report that in the presence of La^{3+}, the ^2H T_1 relaxation times are shorter, and the activation energy is smaller. Thus, measurements of both ^2H and ^{14}N spin–lattice relaxation times in the presence of lanthanide ions would suggest that binding of these ions has only a small influence on the rapid segmental motions of the phosphocholine head group. Qualitatively, the above T_1 results can be understood as follows: in the presence of the bound lanthanide ions (Eu^{3+} or La^{3+}), the residual quadrupolar splitting $\Delta\nu_Q$ is smaller, and the fluctuating part of the quadrupolar interaction is correspondingly increased. Thus, the relaxation is more efficient, leading to a shorter relaxation time as observed experimentally. An alternative explanation, put forward recently (Brown, 1982, 1984a,b; Brown et al., 1983) to account for the ^2H T_1 relax-

ation rates in the hydrocarbon chain region of DPPC, is that relatively slow director fluctuations may provide a dominant relaxation mechanism in some instances. If this is the case, then for the head group the ^{14}N relaxation rate would vary as $S_{C_\beta-N}{}^2$, and the temperature dependence of T_1 could then be ascribed to the temperature dependence of $S_{C_\beta-N}$. However, a preliminary analysis suggests that this is not so (results not shown).

Finally, in the presence of the paramagnetic lanthanide ions, we have observed unusual, asymmetric ^{14}N quadrupolar powder patterns which appear to result from incomplete averaging of the dipolar interaction between the magnetic moment of the ^{14}N nucleus and the paramagnetic moment of the lanthanide ion. Because the interaction with the paramagnetic ion is dipolar in origin, the larger magnetogyric ratio of ^{2}H and, more importantly, the proximity of the C_α or the C_β position to the phosphodiester binding site of the lanthanide ion may result in a significant enhancement of the effects of paramagnetic ions in ^{2}H NMR spectra at these positions. These effects will be further discussed elsewhere (Siminovitch et al., 1984).

Acknowledgments

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Peptide Binding by Calmodulin and Its Proteolytic Fragments and by Troponin C[†]

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ABSTRACT: Calmodulin and troponin C exhibit calcium-dependent binding of 1 mol/mol of dynorphin$_{1-17}$. The dissociation constants of the complexes, determined in 0.20 N KCl–1.0 mM $CaCl_2$, pH 7.3, are 0.6 μM for calmodulin, 2.4 μM for rabbit fast skeletal muscle troponin C, and 9 μM for bovine heart troponin C. Experiments with deletion peptides of dynorphin$_{1-17}$ show that peptide chain length and especially charge affect the binding of the peptides by calmodulin. Dynorphin$_{1-17}$, but not mastoparan or melittin, inhibits adenosinetriphosphatase activity in a reconstituted rabbit skeletal muscle actomyosin assay. The inhibition is partially reversed by the addition of calmodulin or troponin C in the presence of calcium. Calmodulin also exhibits calcium-dependent binding of a synthetic peptide corresponding to positions 104–115 of rabbit fast skeletal muscle troponin I. Mastoparan is a tetradecapeptide from the vespid wasp having exceptional affinity for calmodulin, with K_d ~0.3 nM [Malencik, D. A., & Anderson, S. R. (1983) *Biochem. Biophys. Res. Commun. 114*, 50]. The addition of 1 mol/mol of mastoparan to the complex of calmodulin with dynorphin$_{1-17}$ results in complete dissociation of dynorphin. Similar titrations of the skeletal muscle troponin C–dynorphin$_{1-17}$ complex produce a gradual dissociation consistent with a dissociation constant of 0.2 μM for the troponin C–mastoparan complex. Fluorescence anisotropy measurements using the intrinsic tryptophan fluorescence of mastoparan X show strongly calcium-dependent binding by proteolytic fragments of calmodulin. Dissociation constants for the complexes with mastoparan X are ~0.9 nM for calmodulin, 0.9 μM for thrombic fragment 1–106, and ~0.15 μM for half-calmodulin fragment 72–148. Neither fragment 107–148 nor parvalbumin associates with mastoparan X in this concentration range. The fluorescence emission spectra of the tryptophan-containing peptides display varying shifts toward shorter wavelengths on the binding of either calmodulin or troponin C, indicating that the environment of the peptide binding site in these proteins is *either hydrophobic or rigid, hydrophilic*. Competition experiments show that melittin may have the highest affinity for calmodulin of any protein or peptide known to interact reversibly with it. The calculated relative affinities suggest that the dissociation constant for the melittin–calmodulin complex is smaller than 3 nM, the value originally estimated [Comte, M., Maulet, Y., & Cox, J. A. (1983) *Biochem. J. 209*, 269].

The binding of calcium stabilizes one or more specific conformations of the calmodulin molecule recognized by calmodulin-dependent enzymes such as cyclic nucleotide phosphodiesterase, adenylate cyclase, and myosin light chain kinase [cf. reviews by Means (1981) and Cheung (1980, 1982)]. Calmodulin also shows calcium-dependent binding of a number of small basic polypeptides. Weiss et al. (1980) first noted that adrenocorticotropin (ACTH)[1] and β-endorphin inhibit the purified cyclic nucleotide phosphodiesterase and demonstrated that these peptides compete with the enzyme for calmodulin. Malencik & Anderson (1982, 1983a) performed fluorescence binding measurements showing the interaction of calmodulin with ACTH, β-endorphin, substance P, glucagon, dynorphin$_{1-13}$, secretin, the gastric inhibitory peptide (GIP), and the vasoactive intestinal peptide (VIP) with dissociation constants ranging from several micromolar to 50 nM in the case of VIP. Several toxic peptides from Hymenoptera species proved surprisingly efficient in calmodulin binding. The complex of calmodulin with melittin, a 26-residue polypeptide from honey bee venom, has a reported dissociation constant

of 3 nM (Comte et al., 1983) while the complex with mastoparan, a tetradecapeptide from the vespid wasp (Hirai et al., 1979), has a dissociation constant of ~0.3 nM (Malencik & Anderson, 1983b). Coincidentally, Barnette et al. (1983) discovered that mastoparan is a potent inhibitor of calmodulin-stimulated phosphodiesterase activity. Giedroc et al. (1983a,b) have used the hydrophilic cross-linking reagent bis(sulfosuccinimidyl) suberate to couple β-endorphin and some deletion peptides from its C-terminal half to calmodulin.

The peptides which calmodulin binds well generally contain homologies consisting of a strongly basic tripeptide sequence, with at least two residues which are either Arg or Lys, three positions away from a pair of hydrophobic residues (Malencik

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[1] Abbreviations: Mops, 3-(N-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; cAMP, adenosine cyclic 3',5'-phosphate; CaM, calmodulin; TnC, troponin C; Dyn, dynorphin; ACTH, adrenocorticotropic hormone; VIP, vasoactive intestinal peptide; GIP, gastric inhibitory peptide; dansyl, 5-(dimethylamino)-1-naphthalenesulfonyl; ANS, 8-anilino-1-naphthalenesulfonate; NaDodSO$_4$, sodium dodecyl sulfate; K, dissociation constant; F, observed fluorescence; F_0, fluorescence of unbound ligand; F_∞, fluorescence of totally bound ligand; $\bar{A}$, average anisotropy; A_f, anisotropy of unbound ligand; A_b, anisotropy of bound ligand; [X$_0$], total concentration of ligand; [X], concentration of unbound ligand; [P$_0$], total concentration of protein (usually CaM or TnC); [P], concentration of unbound protein; f_b, fraction of fluorescent ligand bound; ϕ, fractional degree of saturation of calmodulin with peptide.