Biochemistry

 Volume 23, Number 25 December 4, 1984

Accelerated Publications

Assignment of Acyl Chain Resonances from Membranes of Mammalian Cells by Two-Dimensional NMR Methods†

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ABSTRACT: Two-dimensional nuclear magnetic resonance (NMR) methods have been successfully used to assign resonances in the ^{1}H NMR spectrum of intact viable rat mammary adenocarcinoma cells. Two-dimensional scalar-correlated spectroscopy identifies connectivities for resonances of the lipid acyl chains in the plasma membrane of these cells. We expect that two-dimensional scalar-correlated methods may be of general use for providing unequivocal assignments in the complex and often poorly resolved ^{1}H NMR spectra of cells.

Nuclear magnetic resonance spectroscopy (NMR)[1] has developed into a powerful tool for study of the biochemistry of intact cells and tissue samples. While most studies have utilized ^{13}C or ^{31}P NMR [see Roberts & Jardetzky (1981) and Kuchel (1981) for reviews], ^{1}H NMR is being applied increasingly (Brindle et al., 1979; Ogino et al., 1980; Agris & Campbell, 1982; Behar et al., 1983). ^{1}H NMR is particularly attractive because of the much higher sensitivity of this nucleus relative to ^{13}C or ^{31}P. A major problem encountered in studies of cell or tissue samples is the resolution and assignment of proton resonances to particular cellular constituents. Assignments are frequently made on the basis of comparable chemical shifts in the spectra of the cells and of the isolated constituents. This is a hazardous procedure at best. Chemical shifts may be displaced for molecules within the cell or tissue as a result of interactions between cellular constituents (Daniels et al., 1976, 1978). Differences in magnetic susceptibility inside and outside the cell can also lead to perturbation of chemical shifts (Fabry & San George, 1983). In some instances spin–echo pulse sequences have been used very effectively to assign resonances on the basis of multiplet structure (Brown et al., 1977; Agris & Campbell, 1982). Such methods are still limited since they fail to establish complete spin system connectivities. What is clearly needed is a direct method to make spin system assignments based on spin–spin (scalar) coupling within the spin system. Two-dimensional ^{1}H NMR spectroscopy has been used with great success to investigate scalar connectivities and assign spectra of proteins (Wagner et al., 1981; Arseniev et al., 1982). We demonstrate here for the first time that two-dimensional scalar-correlated spectroscopy (COSY) can also be used to make unequivocal assignments in the spectra of intact cells.

It has previously been reported that the plasma membrane lipids of intact viable human lymphocytes and rat mammary adenocarcinoma cell lines give rise to narrow ^{1}H NMR resonances (Mountford et al., 1982, 1984). These narrow lines have been called quasi-Lorentzian to characterize the superposition of Lorentzian lines of different widths and are thought to arise from lipids assembled in small (60–200-Å) domains (Mountford et al., 1984). Using COSY methods, we are now able to identify and assign resonances in the spectra of these cells arising from the lipid acyl chains.

Materials and Methods

The cultured rat mammary adenocarcinoma cell line, J clone, was supplied by Dr. I. Ramshaw, Australian National University (Ramshaw et al., 1982). Cells were grown at 37 °C in RPMI-1640 medium supplemented with 10% fetal calf serum. Cells were maintained and studied in the log phase of growth. The doubling time was 24–36 h.

Cell samples were prepared for NMR experiments as previously described (Mountford et al., 1982, 1984). Cell samples were kept at 37 °C in the NMR tube for 1 h prior to commencement of experiments to allow establishment of a stable

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[1] Abbreviations: NMR, nuclear magnetic resonance; COSY, two-dimensional scalar-correlated spectroscopy; rf, radio frequency; 2D, two dimensional.

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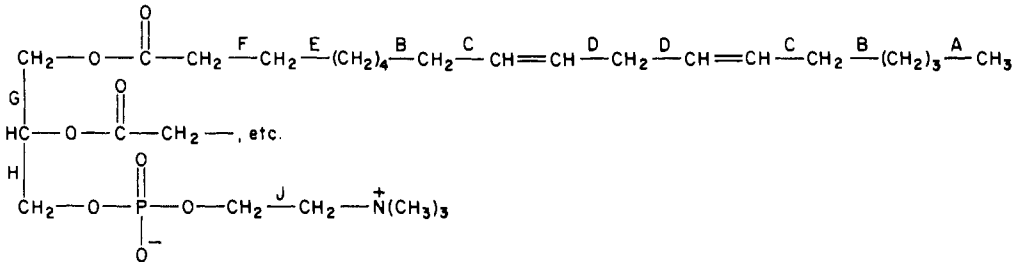

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state following increase in cell–cell contact. Cell viability was measured before and after each experiment. Only those data obtained from cells with at least 90% viability were used. The longest time for which this could be achieved was 3–4 h.

^{1}H NMR spectra were recorded on a Bruker WM-400 spectrometer equipped with an Aspect 2000 computer. Spectra of egg lecithin (Sigma) in $CDCl_3$ (20 mg/mL) were measured at 25 °C. The residual $CHCl_3$ resonance at 7.26 ppm was used as an internal chemical shift reference. Cell spectra were recorded at 37 °C and were referenced to aqueous sodium 3-(trimethylsilyl)propanesulfonate as an external standard.

COSY spectra of intact cells were recorded with a pulse sequence modified according to the principles of Levitt & Ernst (1983) to compensate for rf inhomogeneity. The complete sequence is

$$[(\pi/2)_\phi(\pi/2)_{\phi+90°}]-t_1-[(\pi/2)_\psi(\pi/2)_{\psi-90°}(\pi/2)_\psi(\pi/2)_{\psi+90°}]-t_2$$

where phase angles ϕ and ψ are cycled as in a conventional COSY experiment (Bax et al., 1981). We have found that the compensated COSY pulse sequence gives greatly improved results with inhomogeneous suspensions of cells compared to the conventional uncompensated pulse sequence. The duration of 2D experiments is limited by cell viability. Acquisition of 32 free induction decays for each of 200 increments in t_1 resulted in a total acquisition time of approximately 3 h. The resulting 200 × 2048 data point matrix was zero filled and Fourier transformed to 1024 × 1024 data points for each two-dimensional spectrum. A Lorentz–Gaussian window function (Ferridge & Lindon, 1978) was used in the t_2 domain with sine-bell weighting in the t_1 domain. The choice of window function in the t_2 domain was dictated by the requirement that the resolution enhancement procedure used did not substantially degrade the signal to noise ratio.

For COSY spectra of lecithin, 80 free induction decays were acquired for each of 256 increments in t_1. The 256 × 2048 data point matrix was Fourier transformed to a 1024 × 1024 data point 2D spectrum. A sine-bell squared weighting function was used in both the t_1 and t_2 domains.

Results and Discussion

The COSY spectrum of egg lecithin in $CDCl_3$ is shown in Figure 1. The off-diagonal cross peaks (labeled A–H and J) indicate spin–spin coupling between protons on adjacent carbon atoms. For example, cross peak A indicates spin–spin coupling between the terminal methyl proton resonance at 0.89 ppm and the methylene proton resonance at 1.25 ppm and establishes a connectivity between these resonances. All expected cross peaks appear in the spectrum. Connectivities for the acyl chain and head group are indicated in the figure. The connectivities corresponding to each cross peak are summarized in structure **1**.

The COSY spectrum confirms previous assignments of the ^{1}H NMR spectrum of lecithin (Finer et al., 1972). The spectrum immediately reveals the magnetic inequivalence of

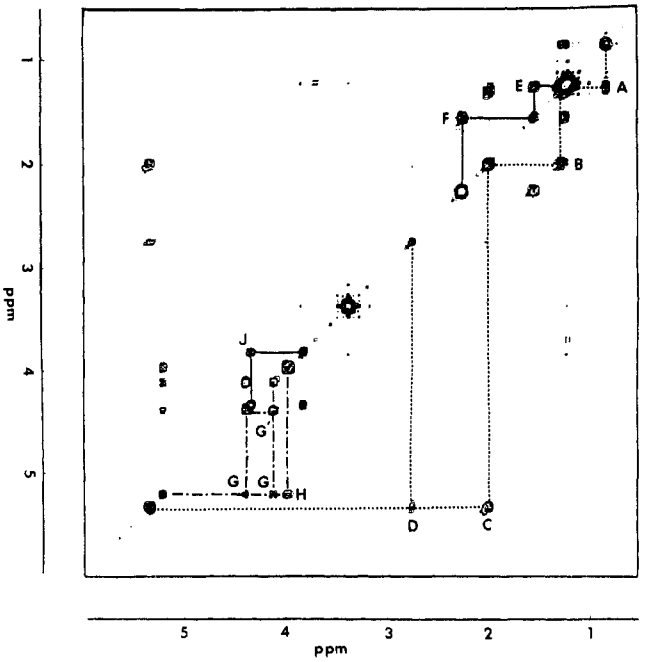

FIGURE 1: Symmetrized COSY spectrum of egg lecithin in $CDCl_3$ solution (20 mg/mL). Lipid acyl chain glyceride backbone and choline head group connectivities are indicated. The assignments of the cross peaks are given in the text.

the CH_2OCO methylene protons of the glyceride backbone (resonances at 4.12 and 4.39 ppm). This is evident from the two correlations labeled G in Figure 1 together with the strong cross peak G′ arising from the geminal coupling within this methylene group. Only one strong cross peak (J) is observed for protons of the choline head group. The $^+N(CH_3)_3$ resonance is a singlet and thus appears only as a strong diagonal peak (at 3.37 ppm).

The COSY spectrum of the rat mammary adenocarcinoma cell line, J clone, is shown in Figure 2. In addition to the prominent diagonal a number of cross peaks are observed. Although some of the cross peaks are weaker than those shown in the lecithin spectrum, all those shown in Figure 2 are significantly above the background noise. Many cross peaks arise from lipid acyl chain protons, which give rise to the same connectivity pattern shown for lecithin in Figure 1. These are identified by using the same labeling scheme as in Figure 1. These results confirm that the unknown lipids contained in the membrane domains in J-clone cells contain unsaturated acyl chains. The observation of cross peak D indicates that, in at least some of these, there is an adjacent pair of double bonds. It is significant that none of the cross peaks expected for glyceride backbone protons (G, G′, and H in Figure 1) are observed in the spectrum of the intact cells. This may reflect low abundance of glyceride lipids in the mobile membrane domains or reduced molecular mobility in the region of the glyceride backbone. Further experiments are necessary to distinguish between these possibilities.

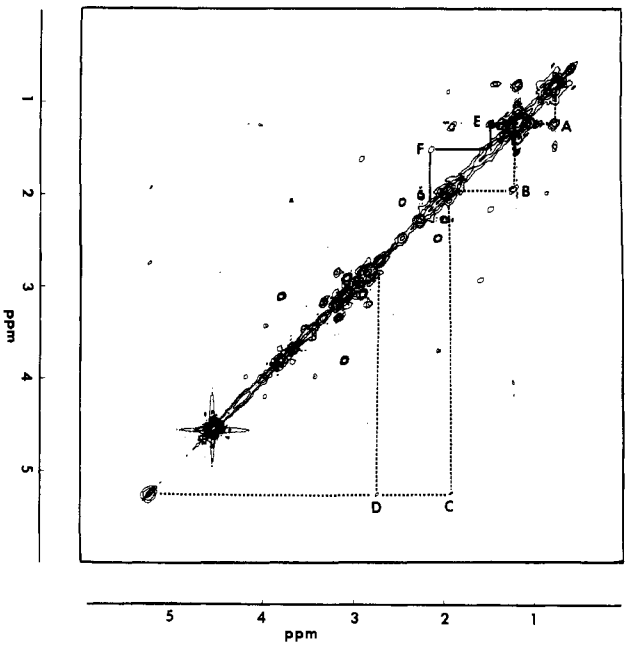

FIGURE 2: Symmetrized COSY spectrum of a suspension of J-clone cells (1×10^8 cells) in phosphate-buffered saline in D_2O. Spectra were obtained at 37 °C with the sample spinning and suppression of the residual HOD peak by gated irradiation. Lipid acyl chain connectivities are indicated, and cross peaks are designated according to the same scheme as Figure 1. Sine–bell and Gaussian (LB = −16, GB = 0.22) window functions were applied in the t_1 and t_2 domains, respectively.

The COSY spectrum of Figure 2 provides the first unequivocal assignments of lipid acyl chain resonances in the spectrum of intact cells. Additional cross peaks are also observed. These conceivably arise from various lipid head groups or from intracellular constituents. Definitive assignment must await the results of detailed biochemical and NMR analysis. Differences have been observed in this region of the spectra of different cell lines (unpublished observations).

The present experiments represent the first reported application of two-dimensional NMR methods to studies of cells. The observation that COSY spectra can be acquired for intact and viable cells is of considerable importance. Such spectra can readily provide unequivocal resonance assignments, a prerequisite for any detailed investigation of cell metabolism or biochemistry by ^1H NMR spectroscopy. While we have demonstrated the technique for a particular cell line (J clone) in the present paper, we expect that it will be of much more general applicability. Indeed, we have used COSY methods to study a wide range of cultured human and animal tumor cell lines. The results will be reported in detail elsewhere. The principal limitations that must be taken into account in applying two-dimensional methods concern cell viability and T_2 relaxation. Clearly the cells must remain in a metabolic steady state for the period of the COSY acquisition. In addition, T_2 relaxation must be sufficiently slow that the resonances of interest retain their multiplet structure (albeit unresolved in the normal one-dimensional spectrum). Any cell systems or excised tissue samples that meet these requirements should be amenable to analysis by two-dimensional scalar-correlated NMR spectroscopy.

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