Nuclear Magnetic Resonance Studies of Drug-Receptor Interactions

The Binding of Epinephrine to Isolated Mouse Liver Cells

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SUMMARY

Using a technique based on the relaxation phenomenon of nuclear magnetic resonance, the interaction of a drug and its receptor site has been directly observed in an intact cellular system. The NMR spectrum of epinephrine is altered by the presence of liver cells in a manner characteristic of a binding interaction. The spectral changes permit certain conclusions to be drawn about the nature of the binding. Dichloroisoproterenol, a *beta*-adrenergic blocking agent, competitively inhibits the changes in the epinephrine spectrum while its own spectrum exhibits evidence of binding.

Several years ago a technique was described, making use of the relaxation phenomenon of nuclear magnetic resonance spectroscopy, for the study of weak, reversible binding between small molecules and macromolecules or cell structures. This method was first demonstrated using the well-known penicillin–serum albumin system as an example of this type of binding (1, 2). Although it has subsequently been applied to a variety of interesting systems, including enzyme-substrate (3), enzyme-coenzyme (3, 4), and enzyme-inhibitor (5) interactions, other examples of drug–plasma protein binding, and haptene-antibody complexes (6), all previous attempts to achieve our ultimate objective of actually observing a drug-receptor interaction in an intact cellular system have met with failure. This lack of success was due mainly to the limitation in sensitivity of the available spectrometers and, to a certain extent, to the choice of experimental systems.

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This preliminary report describes the observation of the interaction between epinephrine and intact liver cells, a mixed *alpha*- and *beta*-type adrenergic interaction which leads via the adenyl cyclase system ultimately to release of glucose (7). This system seemed to be particularly well suited for such a study for many reasons: it is representative of an important class of pharmacological effects; it has been extensively investigated by indirect classical methods, including structure-activity and inhibition studies; drug binding is thought to occur on the cell membrane; and the dose-response curve for this effect seemed to extend to relatively high drug concentrations, approximately 10^{-5} M, thus perhaps indicating that the concentration of receptor sites would also be relatively great (8).

A single-cell suspension of mouse liver cells was prepared by a modification of the method of Rappaport and Howze (9). Male C3H mice, 3–4 months of age, were killed by cervical transection. The livers were immediately removed, chopped coarsely, and incubated for 2 hr with stirring at room temperature in a solution containing

0.05 M sucrose, 0.14 M NaCl, 0.005 M sodium phosphate buffer (pH 7.8), and 0.003 M sodium tetraphenylboron as the dissociating agent. Following incubation, some gross fragments of undissociated liver remained, but a substantial portion of the parenchymal cells existed as single cells dispersed in the medium and could be easily drawn through a fine-tipped pipette. Intact single cells were harvested from the suspension by centrifugation. The cells were prepared for use in the spectrometer by being repeatedly washed and allowed to stand overnight in the phosphate buffer made with D_2O. Cells prepared in this way have a normal morphology when viewed with the light microscope, and they are resistant to staining with trypan blue dye, a finding which has previously correlated well with viability. When packed by centrifugation, there are approximately 3×10^7 cells/ml.

NMR spectra were obtained using a 90-MHz Bruker HFX-3 spectrometer with an internal lock system. The cell suspensions were contained in spinning sample tubes 13 mm in diameter. Both external TMS[1] and internal DSS were used as the reference and lock signals. With the large sample tubes, easily measurable signals could be obtained from single hydrogen nuclei present in 0.001 M concentrations, provided that the line widths were less than 1 Hz. When necessary, signal to noise ratio enhancement was achieved using a Fabri-Tek 4096-channel time-averaging computer; 5-fold enhancement usually proved to be convenient.

The line widths due to inhomogeneity of the applied magnetic field over the large sample tubes could be maintained at less than 1 Hz. For samples containing the cell suspension, widths of less than 3–5 Hz have not been achieved. It is believed that this value is determined by microscopic field inhomogeneities within the sample, which are inherent in a structured system such as this. This conclusion is supported by the fact that the solvent signal and the peaks of other small molecules added to the suspension all showed line widths in excess of this value although their measured widths in the solvent alone were much less. Compounds chosen as controls, such as penicillin G, which were not expected to interact with the cells, produced spectra with this common line width, a finding most characteristic of such a nonspecific broadening mechanism. The time-averaging procedure further increased the observed widths to a minimum of approximately 6–8 Hz. For relatively broad lines this presents little difficulty; however, it does prevent accurate measurements of some of the narrow peaks to be described here, and thus represents one area for future improvement in technique.

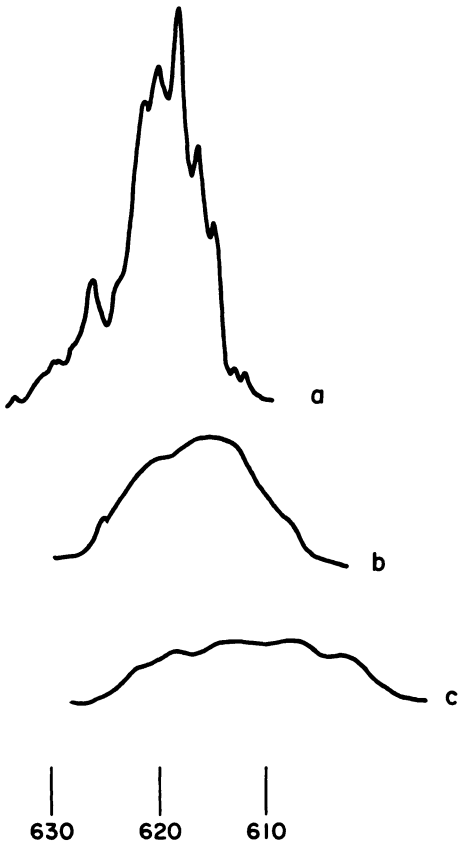

FIG. 1. *NMR signal arising from the phenyl protons of epinephrine*

Scale: chemical shift in Hertz relative to internal DSS. a. Epinephrine. b. Liver cells with 0.01 M epinephrine. c. Liver cells with 0.005 M epinephrine.

[1] The abbreviations used are: TMS, tetramethylsilane; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

TABLE 1
Chemical shifts and line widths of NMR signal from epinephrine

System	Phenyl		Methylene		Methyl	
	W^a	Δ	W	Δ	W	Δ
	Hz		*Hz*		*Hz*	
Epinephrine	8–10	618	11 (3)	282	<3	236
Liver cells						
+Epinephrine, 0.001 M	30	606		281	21	236
+Epinephrine, 0.005 M	22	612		282	17	236
+Epinephrine, 0.01 M	15	615		280	12	237
+Epinephrine, 0.02 M	12	617	15–18	282	9[b]	236
+Epinephrine, 0.05 M	12	618	13	282	7[b]	237
+Epinephrine, 0.01 M; dichloroisoproterenol, 0.02 M	12	618			9[b]	238

[a] W, width at half-maximum height; Δ, chemical shift relative to internal DSS.
[b] Measured values determined primarily by instrument resolution.

The general experimental design was similar to that previously described for NMR binding studies. Spectra of epinephrine alone, liver cells alone, and the two in various combinations were obtained. In this case, however, since the concentration of binding sites, proportional to the concentration of cells, was a limiting factor, the use of dilute cell suspensions quite predictably failed to show significant effects even at the lowest drug concentrations which could be observed.

The epinephrine spectrum is relatively easy to interpret; some of its important parameters are given in Table 1. The 3 ring protons give an incompletely resolved pattern, shown in Fig. 1a. Slight broadening removes the fine structure and produces a single peak 8–10 Hz wide at half-maximum height. The methylene protons produce a doublet with 7-Hz splitting and inherent line widths of approximately 3 Hz. Again slight broadening produces a single peak approximately 11 Hz in width. There is a single methyl peak of width less than 3 Hz. The CH group produces a triplet, which is obscured by the water peak in the samples containing cells and is thus not suitable for measurement. For epinephrine alone, there is no significant concentration effect on either the line widths or the chemical shifts over the range of interest.

The cells alone produce a broad elevation of the baseline in the aromatic proton region of the spectrum, but this does not interfere at all with measurements of the epinephrine phenyl peaks. However, a number of distinct peaks are present in the aliphatic region, which do in fact make the examination of the spectra of small molecules much more difficult. The estimated accuracy of the line widths is thus 1–2 Hz in the aromatic region and 2–3 Hz in the aliphatic region.

The results obtained from spectra of samples containing epinephrine and packed liver cells are given in Table 1. The epinephrine concentration ranged from 0.001 to 0.05 M. All the epinephrine peaks are broadened. Figure 1b and c shows the effect on the phenyl peak for two epinephrine concentrations. For the methylene peak the broadening is so great that accurate line widths cannot be determined for drug concentrations lower than 0.02 M. Only the phenyl peak shows a significant change in chemical shift. The finding that these changes are greatest for the lowest epinephrine concentrations, and that the observed widths and shifts approach those of free epinephrine as the concentration of the drug increases, is characteristic of the binding of a large excess of small molecules to a fixed number of receptor sites and is totally inconsistent with any nonspecific broadening mechanism.

The presence of dichloroisoproterenol, which is thought to be a competitive inhibitor of this action of epinephrine by virtue of stronger binding to the same receptor site, partially eliminates the effect of the cells on the epinephrine spectrum. The dichloroisoproterenol phenyl and methyl peaks fortunately do not overlap those of the epinephrine, although the methylene peaks do so and thus cannot be evaluated for the inhibition studies. Although a full concentration range was not studied with dichloroisoproterenol, it is interesting that even at the relatively high concentration used, 0.02 M, its phenyl peaks showed considerable broadening to 20–30 Hz, and the methyl peaks were almost completely obliterated; both findings are consistent with stronger binding.

Because of the difficulty in obtaining really accurate data in a system of this complexity, a detailed kinetic study and analysis, such as was carried out for the original penicillin-albumin work, hardly seems indicated. Nevertheless, the observed effects are so striking that several conclusions concerning the nature of the drug-receptor interaction seem justified.

The marked broadening of the phenyl and methylene peaks indicates that the molecule must be bound both at the ring itself and somewhere distal to the methylene group on the side chain. The obvious choice for the latter site is, of course, the nitrogen atom, most likely involved in hydrogen bonding. The large upfield shift of the phenyl peak could result only from proximity to the diamagnetic region of another unsaturated ring structure. If it were possible to estimate the amount of change in the chemical shifts of the separate components of this complex peak, even more might be learned of the geometry of the complex, but unfortunately this becomes impossible with this degree of broadening. The effect on the methyl signal is less striking and is compatible with this group's retaining considerable freedom of motion even when the complex is formed. Since this portion of the receptor site seems adequate to accept isopropyl groups, this is not a surprising result. Interestingly, the measurements of the dichloroisoproterenol methyl peaks, while incomplete, seem to indicate much more restriction of the motion of this bulkier group.

These conclusions concerning the nature of the drug-receptor complex are not inconsistent with those that have evolved from the classic structure-activity determinations (10). It should prove most profitable to expand these studies to include examples of the other types of adrenergic receptors, some of which probably will prove to be quite different.

Examples of investigations of drug-receptor interactions involving direct physical-chemical measurements of the complex in systems containing intact viable cells must be rare, if indeed any others do exist. The theoretical considerations responsible for this situation and the characteristics of NMR spectroscopy which make it uniquely suited for such studies have recently been reviewed (11). Unlike most common spectroscopic methods, NMR is carried out at a frequency for which most biological materials show little background absorption. Furthermore, the technique described above provides detailed information about the nature of the interaction, which can hardly be duplicated by any other method even in the simplest of experimental systems. The results presented here clearly indicate that drug-receptor interactions can be studied with the instrumentation now available. Certainly there exist innumerable examples in all fields of pharmacology which would now be suitable for such studies.

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REFERENCES

1. J. J. Fischer, The Mechanism of Binding of Penicillins to Serum Albumin Studied by NMR Spectroscopy. Ph.D. thesis, Harvard University, 1964.
2. J. J. Fischer and O. Jardetzky, *J. Amer. Chem. Soc.* **87**, 3237 (1965).
3. D. P. Hollis, *Biochemistry* **6**, 2080 (1967).
4. O. Jardetzky, N. G. Wade and J. J. Fischer, *Nature* **197**, 183 (1963).

5. J. T. Gerig, *J. Amer. Chem. Soc.* **90**, 2681 (1968).
6. A. S. V. Burgen, O. Jardetzky, J. C. Metcalfe and N. Wade-Jardetzky, *Proc. Nat. Acad. Sci. U. S. A.* **58**, 447 (1967).
7. E. W. Sutherland and G. A. Robison, *Pharmacol Rev.* **18**, 145 (1966).
8. F. Murad, Y.-M. Chi, T. W. Rall and E. W. Sutherland, *J. Biol. Chem.* **237**, 1233 (1962).
9. C. Rappaport and G. B. Howze, *Proc. Soc. Exp. Biol. Med.* **121**, 1010 (1966).
10. B. Belleau, *Ann. N. Y. Acad. Sci.* **139**, 580 (1967).
11. J. J. Fischer, *in* "Methods in Pharmacology" (A. Schwartz, ed.). Appleton-Century-Crofts, New York. In press.

The Effect of Ca^{++} Omission on the Secretion of Catecholamines and the Incorporation of Orthophosphate-^{32}P into Nucleotides and Phospholipids of Bovine Adrenal Medulla during Acetylcholine Stimulation

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SUMMARY

Bovine adrenal medullary slices were incubated at 30° in Locke's solution containing orthophosphate-^{32}P (50 μCi/ml) with and without Ca^{++}, and were stimulated with acetylcholine (10^{-5} M) in the presence of eserine (10^{-5} M).

The omission of Ca^{++}, as expected, abolished the increment in catecholamine secretion due to acetylcholine stimulation.

Acetylcholine stimulation increased the incorporation of ^{32}P into phospholipids, particularly into phosphatidic acid and into phosphatidylinositol, in both the presence and absence of Ca^{++}. Thus, a lack of correlation between catecholamine release and ^{32}P incorporation into phospholipids upon acetylcholine stimulation was observed.

It has been demonstrated that acetylcholine stimulation increases the incorporation of ^{32}P into the phospholipids of the adrenal medulla (1, 2), particularly phosphatidic acid and phosphatidylinositol. This effect of acetylcholine was due to an increase in the turnover of phosphorus in these two phosphatides, rather than to secondary changes in the specific activity of precursors (2). Since ^{32}P incorporation into phospholipids is also increased in other tissues where acetylcholine seems to be the naturally occurring transmitter (3–6), it was postulated that phospholipids may play an active role in the secretory process (1, 3, 6). The results published in a previous paper (2), showing that following acetylcholine stimulation the ^{32}P

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