Current Trends in Receptor Characterization

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Corpora non agunt nisi fixata (Ehrlich, 1909)

The first expression of receptor concept was given by Langley (1878). Langley (1905) observed that "the relationship between the receptive substance and the contractile substance is clearly very close and on the general lines of Ehrlich's immunity theory". These hypothetical specific chemical groupings of cell upon which drugs were assumed to act, Ehrlich (1913) gave the name 'receptors'. Since then receptors have been shown to be responsible for the effects of many hormones and neurotransmitters. It is now clear that cell surface receptors are involved in cellular transmembrane signalling. The development of Langley's idea into present rather detailed knowledge of receptorology has depended on several advancements in characterization and isolation, purification of receptors. An attempt is made to review the various techniques and methods available to characterize the receptors in this article.

For many years receptors could be studied only by physiological or pharmacological methods eg. whole animal behavioural tests, organ responses, electrophysiological methods. The use of isolated physiologically responding tissues to investigate neurotransmitter, drug and hormone receptors is quite old in comparison to the newer and more convenient radioligand binding studies that are becoming increasingly popular. Nowadays, many consider that these classical methods are obsolete. However, radioligand binding studies have not, and probably will not, replace the isolated physiologically responding tissues for the studying neurotransmitter receptors since radioligand techniques provide only a part of the information concerning drug and neurotransmitter action at the receptor level (Ruffolo, 1984).

Since Ariens' (1954) concepts of drug action, which have been modified and extended by Stephenson (1956) and Furchgott (1966), it has been accepted that the drugs will first combine to their receptors and those drugs which are agonists or partial agonists will stimulate the receptor subsequent to binding leading to effect. The extent with which the drug combine can be determined by the affinity constant of the drug-receptor complex. The property which is related to the ability of the drug to stimulate the receptor is referred to as intrinsic activity or efficacy. To know about the latter property, a biological response must be measured. The radioligand binding studies, which largely measure only affinity, cannot presently replace the more classical techniques and cannot provide a reliable index of the im-

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portant parameter of drug action, intrinsic activity or efficacy.

The basis of drug receptor characterization and classification is the unequivocal measurement and comparison of parameters that depend exclusively on drug-receptor interaction (Kenakin, 1984). The four commonly used pharmacological methods are (a) agonist rank order potency, (b) selective agonism, (c) comparison of agonist affinity and relative efficacy, and (d) quantification of competitive antagonist affinity.

Determination of antagonist affinity constant is much easier. Schild (1949) and Arunlakshana and Schild (1959) have proposed pA_x scale to study the competitive antagonism. pA_2 value of a competitive antagonist gives the affinity constant of the antagonist. The slope of Arunlakshana-Schild plot will also provide the information regarding the homogeneity of the receptor population. However, the pD_2 (– log ED 50) of agonist may not necessarily represent the affinity constant of the agonist. The quantification of the agonist-receptor interactions is complicated with the existence of varying degree of spare receptors from system to system and also within a system for different agonists (Ruffolo, 1982). Furchgott (1966) was largely credited for the development of the specific techniques associated with the determination of dissociation constant of agonists. These methods require the use of irreversible receptor antagonists. Further, on the basis of selective agonism receptors can be characterized. The circumstantial evidence of presence and absence of a particular receptor can be obtained by using highly selective drugs. However, one must be cautious in interpreting the data as none of the selective drugs available for the various subtypes of receptors are specific for that subtype of receptor.

Apart from measuring the tissue biological responses, one can directly estimate the secondary messengers in tissue extracts biochemically and determine the affinity of drug towards receptors. For example, activation of beta adrenoceptors leads to stimulation of adenylate cyclase system. So, estimation of cAMP levels reflect the extent of receptor activation.

Although the use of *in situ* preparations as the initial step in defining receptor subtypes has provided much of the early evidence in support of the existence of receptor subtypes, there are problems with the use of such preparations. For example the drugs can be biotransformed before they reach the biophase and may fail to interact with the receptors. There may also be differences in the tissue distribution of exogenously administered drugs. The factors affecting the pharmacological specificities of drugs *in vivo* or *in situ* are access (eg. blood brain barrier, metabolic transformation), removal (eg. uptake, enzymatic degradation, chemical degradation), and neuronal complexity (eg. feedback, receptor heterogeneity) (Molinoff *et al.*, 1981). In an ideal receptor characterization methodology, one must account for all these processes (Furchgott, 1972). The ideal approach is thus to combine studies of physiological responses with *in vitro* studies of radioligand binding.

Until recently, the binding of drug to its receptor has been inferred from the analysis of dose-response characteristics of specific whole tissue responses. This approach provided fairly accurate and self-consistent values for the affinities of antagonists for these receptors but, in most instances, only gave

estimates of potencies of agonists. For many receptor systems it is now possible to directly monitor the binding of drugs to their receptor binding sites on membrane. This article is not intended to be exhaustive and the interested reader is referred to additional presentations of this subject in Rodbard (1973), Boeynaems and Dumont (1975) Cuatrecasas and Hollenberg (1976), Weiland and Molinoff (1981) and Molinoff *et al.*, (1981).

A radio labelled drug, D, is allowed to bind to the receptor, R, and the radiolabelled drug bound to the receptor, B, is measured.

$$\underset{F}{D} + R \rightleftharpoons \underset{B}{DR}$$

F is the concentration of unbound radioligand. The binding process is described by the equation

$$\frac{B}{R_{total}} = \frac{K.[F]}{1+K[F]}$$

From an analysis of how B varies as F changes, it is possible to estimate both the affinity constant of D, and the number of receptor binding sites, R_{total}. The latter value is not accessible by analysis of dose-response curves.

Radioligand compounds are used in homogenates or in membrane fractions of the tissue that is presumed to contain the receptor to be studied. The advances in the procedures for fractionation, isolation and purification of these subcellular constituents and their high affinity for the labelled substance hold great promise for the identification of receptors. Of central importance in this identification of binding sites as receptors is an agreement in the values of drug receptor dissociation constants determined by both radioligand binding methods and standard methods. Furchgott (1978) cautions "the investigator who claims to be studying a specific type of receptor with the radioligand procedure must validate his claim by showing good correlation between values of the affinities of a series of antagonists and agonists for the specific sites to which the radioligand binds, and the values of affinities of the same series of agonists and antagonists for a specific receptor derived from the results of appropriate pharmacological testing." The dissociation constants of alpha adrenergic drugs determined by both methods were in good agreement (Furchgott, 1978).

The potential disadvantage of the direct radioligand binding technique is that the receptors in a crude tissue preparation might be labelled, whether of smooth muscle, vascular, neural or other region. The data, therefore, might represent the composite picture obtained by labelling different types of receptors, in proportion to their relative affinities for the ligand and their relative density (Wood *et al.*, 1979).

The graphical analysis of ligand-receptor binding studies is done in general by Scatchard plot (1949). Klotz (1982,1983) has called attention to some potential shortcomings of the Scatchard plot method. Further, Muson and Rodbard (1983) published a constructive critique on Scatchard and Klotz graphs.

Work with the radioligands as a tool to characterize the receptors is proceeding at a rapid pace for a variety of different receptors. Kebabian and Calne (1979), in their review of dopamine discussed the progress of this methodology in locating dopaminergic recep-

tors, while Hulme et al., (1978) have studied brain muscarinic receptors and Mohler and Okada (1977) benzodiazepines receptors.

For the study of the anatomical or histological distribution of receptors or the accumulation of drugs, the method autoradiography is often used. The most sensitive isotopic method is radioimmunoassay which is applicable to almost any molecule that can act as hapten. Fluoroscence probes can have multiple uses in receptor characterization. Photoaffinity labelling represents a special category of general affinity labelling developed out of desire to overcome the major limitations of the classical labelling techniques. In case of photoaffinity labelling the reagents undergo photoactivation which can be accomplished after reagent is bound, creates a highly reactive functional group capable of interacting with hydrophobic as well as polar residues. Guillory and Jeng (1983) concluded that photoaffinity labelling is likely to emerge as one of the major techniques for elucidating structure-function relationships within biological membranes.

Another approach in receptor characterization is kinetic binding methods. Most experiments based on this concept have been conducted in the area of neurotransmitter-receptor interaction because the receptor-effector interaction in such cases is directed and measurable by electrical methods. The discussion of the sophisticated techniques of fluctuation analysis, voltage jump relaxation, and concentration jump methods are beyond the scope of this article. Persons interested in these methods are referred to the reviews published by Colquhoun (1979,1981) and Stevens (1980).

However, a real insight of the molecular characteristics of the drug receptors will come only from the isolation, purification and full characterization of these complex proteolipids, including the mapping of their membrane environment. Even if isolation does succeed, the isolated 'receptor' may not necessarily be the same as the native functional entity in its membrane constraint because the function of amplification site may be lost. However, recently pure beta adrenergic receptors isolated from guinea pig lung and pure guanine nucleotide binding regulatory protein of adenylate cyclase isolated from human erythrocytes have been inserted into phospholipid vesicles resulting in the functional coupling of these two components (Cerione *et al.*, 1984).

The first step in receptor purification is solubilization of membrane bound receptors. In this technique the detergents are normally used. The lipophilic ends of the detergents will get attached to the lipophilic portion of the receptor protein. In aqueous medium the protein detergent complex becomes colloidal dispersion. The commonly used detergents are sodium dodecyl sulfonate (an anionic detergent), sodium deoxycholate (a natural anionic bile acid derivative), a large number of very gentle nonionic detergents (Tween, Triton etc.), and some specific glycoprotein solubilizers like lithium diiodosalycilate (Lindstorm, 1978). The solubilized receptor is purified by gel chromatography. By gel filtration and dialysis the detergent can be removed. Affinity chromatography is highly efficient separation method based on the function of the substance to be separated, i.e., receptor protein. Lerman and his coworkers in 1951 developed the technique, now referred to as 'affinity chromatography', when they covalently linked an antigen via an azo bond to a cellulose matrix for purification of

antibodies (Campbell, 1951). A specific ligand is attached to an insoluble, porous inert support by a long side chain. Thus a small molecule 'bait' attached by a fishing line to a fishing rod. Since the bait is highly specific, only a complimentary macromolecule will bind to it efficiently when the column chromatography system is set up.

Affinity labelling is a method for specifically and permanently marking the binding sites of drug receptors, neurotransmitter receptors and hormone receptors (Cuatrecasas and Anfinsen, 1971). A site specific ligand is modified by the incorporation of a functional group that is capable of covalently binding to a protein -OH, -NH, or -CH group. Binding specificity is provided by the ligand itself directing it to the receptor recognition site.

The immobilization or covalent bonding of drugs, hormones and neurotransmitters to soluble and insoluble supporting matrices is a technique that has found application to diverse endeavours ranging from elucidation of the sites and mechanisms of hormone action to drug receptor isolation. Immobilized drug and hormone preparations have been used to localize receptors specific parts of cells to isolate and purify soluble and membrane bound receptors, to understand structure activity relationships of drugs on specific receptors and understand the mechanism of receptor expression. Polymeric drugs and hormones covalently coupled to natural and synthetic polymers have numerous advantages over solid phase immobilized drugs and have received widespread attention from a number of areas. While the biological effects attributed to solid phase immobilizing drugs have not been substantiated, these agents have proven useful in localizing drugs to tissues and in affinity isolation of cells and receptors. The affinity isolation of cells and receptors is also of increasing importance as the re ceptor field moves away from binding phenomena towards a molecular resolution of receptor structure and function. An extensive review on applications of immobilized drugs was published (Venter, 1982a).

In the recent years, the monoclonal antibodies and autoantibodies are playing important role in the isolation and characterization of neurotransmitter receptors. Insulin receptor isolation and structural identification were made following the identification of autoantibodies to insulin receptors in patients with insulin resistance diabetes (Flier *et. al.*, 1975, 1976).

The hybridoma technology greatly changed the immunological approach to macromolecule characterization (Kohler and Milstein, 1975, Kohler *et al.*, 1976). The monoclonal antibodies obtained from hybridomas are single, homogenous, well-defined chemical species allowing for standardization of immunological reagents between single antigenic determinants on a variety of proteins. Recently, for the most of the receptor systems the monoclonal antibodies are available. Monoclonal antibodies do play important role in receptor purification. The development of monoclonal antibodies to receptor molecules has enabled the study of structural homology between receptor subtypes to progress to the level of single antigenic determinants. These agents will play important role in study of such phenomena as desensitization, synthesis and degradation of receptors. Although many of the potential applications of monoclonal antibodies in hormone and neurotansmitter receptor

research are yet to be realized, it is expected that monocional antibodies will eventually become primary reagents in receptor studies (Venter, 1982b).

Magnetic resonance—the absorption of electromagnetic radiation by an atom or electron spinning in an applied field—is a technique, which is increasingly used in macromolecule structure determination and drug-receptor interaction (Rama Rao, 1979). Peak broadening due to interaction and slight shift of various peaks are used in determination of receptor interaction pattern. Hydrogen bond formation and charge transfer complex formation which normally takes place in drug receptor interaction will shift resonance downfield or upfield respectively.

The various methods described in the previous few pages contributed a lot to the field of receptorology. Still newer and more sophisticated, sensitive and selective methods will follow in the next few years. Seeing the trend it is "clear that it will not be long before receptorology will be an essential and integral part of biological sciences, including pathlogy, diagnosis and therapeutics" (Ariens, 1983).

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