MEETING REPORT

Are molecular and electrophysiological data refining models of agonism?

The way pharmacologists think about agonism is rapidly evolving and theoretical models must now incorporate the stream of experimental information emerging from biochemical, electrophysiological and molecular biological studies of agonist action. The benefit of improved understanding will not be limited to pure science, but will extend to the practical matter of drug development. A recent international meeting† reported a wide range of approaches to this problem and reflected a growing consensus in areas where controversy has only recently raged.

Theoretical models

In the 1950s, the existence of spare receptors was demonstrated by Nickerson[1] and Stephenson[2] who showed that tissue response was not always linearly related to receptor occupancy; indeed large responses could be elicited when only a few receptors were occupied. Del Castillo and Katz[3] in 1957 suggested that the activation of the nicotinic cholinoceptor was a two-step process:

$$A + R \rightleftharpoons AR \rightleftharpoons AR^* \quad (1)$$

where A is agonist, R is receptor and only AR* is active. It now seems likely that a scheme of this general kind applies to all receptors that incorporate ion channels and have fast (~ 1 ms) onset of action, although elaborations are needed to take into account the phenomenon of desensitization and the existence of more than one binding site (e.g. two for nicotinic receptors) on the receptor macromolecule.

Progress in understanding the mechanisms of another group of receptors whose response times are 10–100 ms has been slower. However it is now well established that these receptors (considered 'intermediate-speed') all act by coupling to a G protein and the minimum mechanism required to explain their actions is:

$$A + R \rightleftharpoons AR + G \rightleftharpoons ARG^* \rightarrow \text{response} \quad (2)$$

The 1980s saw two important advances. The first was the formulation of an operational model of receptor action (Black and Leff[4]) which sought to describe the ability of drugs to activate receptors in terms of affinity and efficacy parameters, and which also took account of variations in the coupling of receptors to responses in different tissues. This scheme shared the concept implicit in the Stephenson formulation that affinity and efficacy under certain conditions were separable, and invokes a 'black box' of largely unknown events between agonist binding and tissue response. The second development came when David Colquhoun[9] pointed out that if either of the schemes shown above (1 and 2) held, then it would no longer be possible to regard 'affinity' (as measured by standard methods such as Furchgott's irreversible antagonist procedure) as an independent measure of the initial combination of the drug with its receptor, or to regard 'efficacy' as an independent measure of the ability of the drug–receptor complex to elicit a response. To some degree this ran counter to the Black and Leff approach (the operational model) and gave rise to controversy which has been covered in *TiPS*[5–8].

However at the meeting there was a pleasing degree of agreement. Colquhoun's arguments[9] have now been incorporated into newer models[10,11] of agonist action which explore the ternary-complex model (2) in fuller detail. Unlike the previous formulations of Black and Leff[4], these new models take into account the effect of coupling to the G protein on the overall affinity of the agonist.

How well do these new models reflect physiology? In practice, does G protein coupling significantly affect estimates of receptor affinity? Studies of this point have begun to be published[10,12] and new evidence was provided by Nigel Shankley (James Black Foundation, London). In keeping with other work[12], irreversible antagonists were used to reduce receptor number and the results were in agreement: in the tissues studied, the influence of G protein coupling appears not to be great. However, more detailed models being developed by Dennis Mackay (University of Leeds)[11] show that apparent affinity constants for agonists, as determined by classical irreversible antagonism procedures, are very complicated functions of the equilibrium and rate constants for the processes that underlie the activation of G-protein-linked signalling enzymes such as adenylyl cyclase. However, Mackay has also been able to show that, theoretically, conditions may occur under which at least some of these factors may not be important; clearly more experimental evidence is required to test whether these conditions are applicable. (For further discussion see also last month's *TiPS*[12].)

Further theoretical issues were clarified in spirited exchanges between David Colquhoun (University College London) and James Black (JBF, London), the progenitor of the operational model (sometimes referred to as the 'black box' model with no pun intended). There was general acceptance at the meeting that black box modelling is inappropriate for fast receptors because in principle all the parameters are now accessible to measurement. However, this is not so for the G-protein-coupled receptors. Although there was general agreement that the parameters generated by the black box might well be useful in drug development (e.g. by virtue of the empirical observation that they are helpful in suggesting new compounds, by extrapolation from existing ones) there was still disagreement about whether these parameters can be interpreted reliably as having any clearly defined physiological meaning (e.g. is 'affinity' a genuine equilibrium constant for the initial binding reaction?). At a heated moment, Colquhoun suggested that it might be better to call them X and Y rather than

† *Understanding and Using Agonists to Develop Drugs, Cambridge, UK, 9–10 January 1990.*

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affinity and efficacy; not surprisingly this was not acceptable to the other side.

The validity or otherwise of applying models based on the law of mass action to structures free to move only in two dimensions – and even then to a limited extent – was also discussed (D. Mackay; Nigel Birdsall, National Institute for Medical Research, London; Alan North, Vollum Institute, Portland). However, Colquhoun suggested that the same kinetics would apply provided that discrete states of the system behaved in a memory-less fashion.

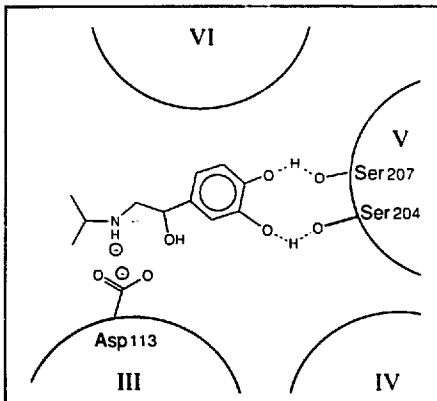

Fig. 1. Model of the ligand binding site of the β-adrenoceptor. Asp113 is one of the three conserved acidic residues postulated to be involved in ligand binding. Ser204 and Ser207 may stabilize hydroxyl groups in the catechol moiety by hydrogen bonding. (Reproduced from Ref. 20.)

Measuring response

Surprisingly little direct evidence has been generated specifically to investigate the validity of practical models for G-protein-linked receptors. However, support is now emerging from molecular biological sources that conformational changes can occur following agonist binding to receptor even in the absence of G protein. Birdsall reported that it has been possible to demonstrate agonist-specific phosphorylation of pure reconstituted muscarinic M_2 receptors[13] and α_2- and β_2-adrenoceptors[14] by the β-adrenoceptor kinase βARK. Since there was no phosphorylation in the absence of agonist and reactions were carried out in the absence of G proteins, these results imply that agonist-specific conformational changes can take place at the receptor level.

The meeting agreed that modelling is most accurate when response is measured as close to the agonist–receptor interaction as possible. A nice example of this was described by Mary Keen (University of Birmingham) who measured both binding of muscarinic agonists and the very early inhibition of cAMP formation in membrane preparations from rat corpus striatum and myocardium under the same experimental conditions. Interestingly her results could not be described by a simple form of the operational model[4] but were well explained by the general form of the model in which agonist binding is significantly affected by activation of the receptor.

A further example of measuring responses closely coupled to the receptor was provided by Steve Freedman (Merck Sharp & Dohme, Harlow) whose group are attempting to develop potent muscarinic agonists that also cross the blood–brain barrier. Their approach is to compare the ability of new ligands to displace the antagonist [^{3}H]*N*-methylscopolamine on the one hand and the agonist [^{3}H]oxotremorine-M on the other. The ratio of the derived affinity constants provided an index of efficacy which correlated closely with measurements of the ability of the same compounds to increase PI turnover in rat cerebral cortex. In this way they have been able to develop compounds of the appropriate profile, a matter of obvious clinical importance in relation to Alzheimer's disease. As has commonly been seen, they also showed that agonists can be distinguished from antagonists by their relatively flat displacement curves and the relative reduction of affinity produced by guanine nucleotides.

Molecular biology

Nigel Birdsall (NIMR, Mill Hill) discussed the new information becoming available through advances in molecular biology in relation to both fast and intermediate-speed (G-protein-linked) receptors. The muscle-type nicotinic receptor has been studied in the most detail and has been clearly defined at the molecular level as a heterologous pentameric protein of stoichiometry $\alpha_2\beta\gamma\delta$ or $\alpha_2\beta\epsilon\delta$. The α-subunit is assumed to contain the acetylcholine binding site. Each subunit has four transmembrane domains predicted from hydropathy plots.

The situation for the neuronal-type receptor is much less clear. To date seven distinct subunits have been cloned from rat and chick brains: α2, α3, α4, α5, β2, β3, β4. All α subunits from neuronal or muscle sources share 90% homology and are typified by the presence of a pair of cysteines at residues 192 and 193, lying close to the acetylcholine binding site. Neuronal β subunits do not share this feature. β2 and β4 can substitute for β subunits in muscle-type receptors expressed in oocytes – β3 appear to be non-functional in this regard. No other type of subunit analogous to the muscle type γ, δ or ϵ subunits has yet been found in brain. There is no real evidence for the arrangements of the subunits of these receptors that exist *in vivo*. Moreover Alan North pointed out that reconstitution studies, where appropriate mRNAs for subunits are injected into *Xenopus* oocytes, are not always decisive because the systems required for post-translational modification may not be the same as in mammalian cells.

Many of the G-protein-linked receptors have now been sequenced and their topographies, deduced from hydropathy plots, appear to conform to the model first proposed by the Findlay group for rhodopsin[15]. Birdsall reported that for the first time experimental support for this model has been published. Wang *et al.*[16] have used antibodies directed against various non-transmembrane domains of the β_2-adrenoceptor in permeabilized and non-permeabilized cells to demonstrate that intracellular and extracellular locations of loops between transmembrane domains had indeed been correctly predicted. A further remarkable development has been the systematic mutation/deletion studies

of the amino acids in the seven transmembrane segments by the Merck group. Combined with the mutation studies of the Lefkowitz and Venter groups, these have led to important insights into the location of binding and 'transduction' sites (see Fig. 1). Thus mutation of any of the three highly conserved acidic residues (usually aspartate) in TM3 affects binding to different, and sometimes profound, extents without abolishing function. Interestingly, one such mutation results in the conversion of an antagonist to an agonist[17]. This is an unusual event in pharmacology where conversion of agonist to antagonist is more easily achieved. Mutation of the conserved aspartate residue in TM2 results in a receptor that is essentially inactive.

Mutation or deletion of either of the pair of cysteines located in the extracellular loop between TM2 and TM3 perturbs both binding and G protein coupling. In addition, two intracellular sections of the region between TM5 and TM6, and a site of palmitoylation on the carboxy terminal, have all been shown to be important for G protein coupling.

Birdsall also reported further evidence from his group that two ligand binding sites exist on the M_2 muscarinic receptor which can be identified by differences in responsiveness to pH. This is particularly important pharmacologically because it becomes possible to distinguish between cardioselective and non-cardioselective ligands on this basis.

Partial agonists

The maximum response to a partial agonist depends on the extent of receptor reserve and therefore is likely to vary from tissue to tissue. The extent and consequences of this variation formed a major theme of the meeting.

Alan North demonstrated that variation in response could also be demonstrated at the single-cell level. In his study on the effects of the partial agonist clonidine and the full agonist noradrenaline on single submucosal plexus neurons, clonidine was shown to be inactive in some cells while acting as a full agonist in others – opposite ends of a spectrum, and remarkable to see at the single-cell level. These changes were not associated with any comparable variation in the response to noradrenaline. An additional twist to the story is that in low Mg^{2+} solutions, clonidine was reversibly converted from an agonist to an antagonist in all the cells he studied.

James Woods (University of Michigan Medical School) also demonstrated variability in response to partial agonists using nalbuphine, a partial agonist acting at μ-opioid receptors. As is generally seen with all opioid receptor agonists, potency appeared to be dependent on stimulus intensity (e.g. the temperature applied in the tail-withdrawal test) [see also M. J. Millan (1990) *TiPS* 11, 70–76]. At high temperature nalbuphine is less effective as an analgesic agent although it can still occupy receptors; efficacy is also lost in situations where receptor reserve may be diminished (e.g. by tolerance/downregulation).

Variability in receptor reserve between tissues is now being exploited by drug developers. If the differences are great enough tissue selectivity could be achieved by using partial agonists with suitable efficacy. Heather Giles (Wellcome, Beckenham) reported studies of the action of a new partial agonist acting at DP receptors, the receptor for the prostaglandin PGD_2. The aim of the study had originally been to test whether receptors inhibiting platelet aggregation (human) and those mediating vasodilation (rabbit jugular vein) were pharmacologically different. Use of the operational model[4] and also of selective antagonists showed this not to be the case. However, they found a higher receptor reserve in platelets and attempted to exploit this to determine whether inhibition of platelet aggregation could be achieved in humans in the absence of vascular effect. Although, in the event, this did not prove practicable, presumably because of the species-specific differences in receptor reserve, the pharmacological elegance of this approach was widely appreciated.

Clinical relevance

Partial and full agonists have an established place in drug therapy (e.g. salbutamol, morphine) although most currently used drugs are in fact antagonists. Drug development using agonists is however now becoming more sophisticated as evidenced by several examples reported at the meeting.

• A long-acting β_2-adrenoceptor agonist salmeterol has been developed by the Glaxo group (Malcolm Johnson, Glaxo, Ware) which is related to salbutamol but differs in possessing a long (mainly) hydrophobic chain. This

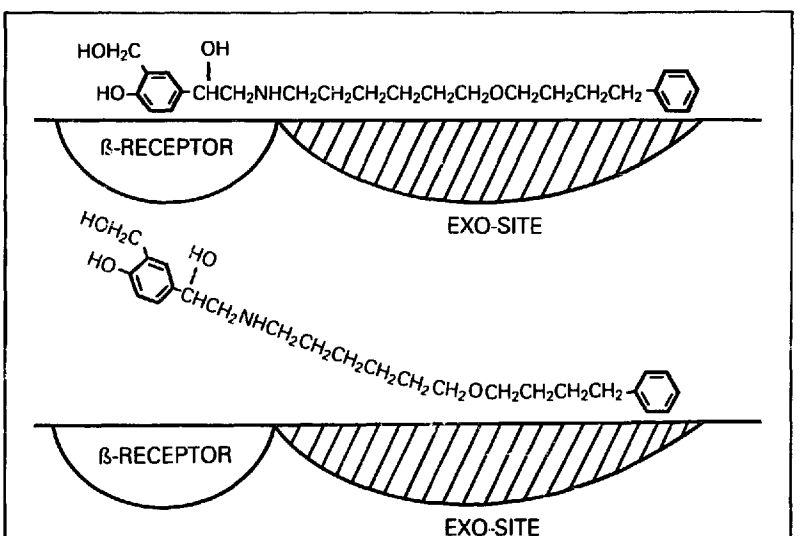

Fig. 2. Model of the proposed mechanism of salmeterol showing binding of the hydrophobic chain to the 'exo-site' which keeps the β-adrenoceptor binding end of the molecule in the vicinity of the receptor. (Figure courtesy of M. Johnson.)

feature is considered to underlie its prolonged duration of action (12–14 h)[18]. It is hypothesized that the chain becomes attached to an 'exo-site' in the vicinity of the β-adrenoceptor from where the rest of the molecule can interact freely with the receptor (Fig. 2). In keeping with this unusual proposed mechanism, the long duration of action is maintained in isolated tissue even when free drug has been washed away. A β-adrenoceptor antagonist at high enough concentration can reverse the response, but remarkably the response re-establishes after washout of the antagonist, in line with the idea that the drug is still anchored near the receptor. Clinical evaluation is under way.

• The development by ICI of a β_1-adrenoceptor partial agonist, xamoterol, for the treatment of heart failure was based on the supposition that a partial agonist of appropriate efficacy could 'buffer' the response to excess sympathetic activity while maintaining a low basal drive (Mike Snow, ICI Pharmaceuticals, Macclesfield). Thus inotropic support at rest and during light exercise would be provided while heart rate would be reduced during severe exercise[19]. Results of interaction studies where isoprenaline and xamoterol were administered to animals showed the characteristic pattern of responses to be expected during co-administration of a partial and a full agonist; human volunteer studies have also endorsed a partial agonist profile for xamoterol. Early clinical trials are showing some potential benefit in mild-to-moderate heart failure.

• The phenomenon of partial agonism can also be seen with substances acting on accessory sites of receptors (Leslie Iversen, Merck Sharp & Dohme, Harlow). The MSD group have tested a series of compounds that bind to the benzodiazepine site on the $GABA_A$ receptor using a procedure that measures the affinity of test compounds in displacing flumazenil (Ro151788) from sites in brain membranes in the presence and absence of added GABA. The measured 'GABA shift' has been found to be predictive of efficacy. Using this method the partial agonist FG8205, an oxadiazolylimidazobenzodiazepine, was identified. This compound possesses anxiolytic and anticonvulsant activity but lacks the sedative and muscle relaxant properties of other benzodiazepines. Moreover, animal models suggest that FG8205 has a much lower tolerance and dependence liability than diazepam. This favourable profile could be accounted for by FG8205 acting as a partial agonist at the GABA receptor accessory site and could be explained by a greater receptor reserve for the targeted as compared with the non-targeted effects. However, Iversen agreed that interpretation is complicated by the recently recognized possibility that GABA receptor subtypes may exist (see above). [FG8205 was withdrawn from development because of safety problems.]

The MSD group have also identified agonists, partial agonists and antagonists interacting with the glycine accessory site on the NMDA receptor which has a permissive effect on glutamate action.

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The meeting made clear that familiar homilies relating to the logic of interdisciplinary communication are not mere clichés; diverse approaches are – sometimes inadvertently – now starting to answer questions about agonism first posed by pharmacologists many years ago. A further and particularly interesting take-home message from the meeting was the extraordinary variation in the extent to which formal models are being used in practical drug development – from scarcely at all (Iversen) to fundamentally (Black, Giles).

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