Mini-Review

Pharmacological versus binding analysis of receptor systems: How do they interplay? Myometrial cell receptors for oxytocin as a paradigm

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Summary. Binding studies in various biological systems frequently indicate the presence of several binding sites for a biologically active ligand. They differ in their affinity for the ligand in question, binding capacity, and Hill coefficient, which suggests differences in the mechanisms of the binding site-ligand interactions. Identification of the 'true' receptors (sites initiating a cellular response) appears to be difficult. Three clusters of binding sites for oxytocin were found on rat myometrial cells. The oxytocin receptor seems to be linked to the medium-affinity site; the cooperation between the high- and medium-affinity sites in eliciting the uterotonic response seems likely, but lacks experimental proof. Dose-response analysis in partially irreversibly inhibited uterus preparations, the method of equipotent doses (Furchgott-Bursztyn method), and structure-activity analysis of oxytocin-like peptides acting as competitive inhibitors of oxytocin, turned out to be suitable for pharmacological analysis of this receptor system.
Key words. Uterus; myometrial cells; oxytocin receptor; myometrium; dose-response analysis; structure-activity relationships; oxytocin; structure-activity relationships; binding isotherm.

Introduction

In the studies of the interactions of drugs, hormones and neurotransmitters with their respective receptors, a binding experiment is the most frequently chosen experimental method [1]. Few doubts can be raised about its usefulness for these aims, providing that the experimental conditions are properly designed and that the receptor is the only membrane binding-site present in the tissue studied. The latter condition might be fulfilled in some exceptional cases, but the majority of nonpurified receptor preparations, such as intact cells, isolated cell membranes or organelles, display several binding-site populations, differing both in their density per unit area (binding capacity) and in the dissociation constants of the ligand-binding site complex (K_d). They reflect binding moieties of various kinds, including those that bind ligand very weakly and are commonly designated as 'nonspecific'. Under these circumstances, the receptors cannot be identified by the use of binding studies alone. Only very few of the enthusiastic users, however, give more profound consideration to the limits of the conclusiveness of binding data in this regard. These limits are, as a matter of fact, rather narrow, and it appears to be inevitable that the question will arise of which tools, other than binding experiments, can be used for unambiguous identification and characterization of a receptor. Might pharmacological concepts bring us closer to this goal?

Receptors are understood as molecular units on which a cellular transformation called 'biological effect' is initiated. Hence, one would expect that a suitable pharmacological experiment, complementary to the binding study, might give the final word. The term 'suitable', however, seems to be the crux of the matter and the recent literature offers many examples that wishful thinking is more common here than a pharmacological analysis of the data.

Thus, receptor features are frequently assigned to the binding site with the highest affinity for the ligand. To do so without further strict proof can by no means be approved; no facts and no reasons, thermodynamic or regulatory, speak in favour of such an assumption. 'Low affinity' receptors have indeed been described in several regulatory systems and the concept that they exist may be justified from various viewpoints.

Furthermore, it has become a widespread practice to assign as receptors those binding sites for which the K_d is numerically similar to the ligand concentration that elicits one half of the maximal attainable biological response in the corresponding in vitro preparation (this concentration is referred to as EC_{50} or ED_{50}, frequently transformed as $pD_2 = -\log EC_{50}$). The following well-known experiment described by Nickerson many years ago [2] shows that this notion is vastly oversimplified. When a fraction of receptors was irreversibly inhibited, the half-maximal dose increased, although one would expect the affinity of the receptors to remain unchanged. The correspondence between EC_{50} and K_d obviously ceases to exist. In addition, the maximal response began to decrease only when a very considerable fraction of receptor, 99 percent or more, was inactivated; the concept of a 'receptor reserve' or 'spare receptors' therefore developed.

Another frequently heard argument is that high affinity receptors account better for 'physiological' concentrations of the substance in question. This line of reasoning is not only highly questionable, it obviously does not

hold: in most cases, such a judgment is based on in vitro experiments and the actual in vivo tissue or plasma agonist concentrations are several orders of magnitude lower than the K_d of the high affinity site(s).

We have recently been confronted with these questions in our experiments with the oxytocin receptor on rat and sheep myometrial cells [3]. Results of binding experiments in the rat and other species, carried out over a twelve-year period in various laboratories, have shown some divergency in several points. One can, however, infer the existence of three distinct binding site populations. Their dissociation constants, found by affinity spectrum analysis [3], are of the order of 5×10^{-9}, 3×10^{-7} and 10^{-5} mol/l; binding capacities are in the ratio of 1:20:180. We have addressed ourselves to the questions of how these data can be analysed pharmacologically, and what the safest way to identify the receptors is. Ultimately, our analysis rested on three approaches for which the necessary data were available: dose-response analysis, partial irreversible inhibition of oxytocin receptors in the rat uterus, and structural analogy in the receptor binding of oxytocin and its antagonists. These tools, in our opinion, may possess a more general character and will therefore be briefly reviewed in the following.

Dose-response analysis

The EC_{50} of oxytocin for the phasic contraction of rat uterus in vitro corresponds numerically to the high affinity K_d [4–9]. Having followed the argumentation mentioned above, several authors assigned this site as the oxytocin receptor. However, dose-response curves in rat uteri incubated during a constant time period with varying concentrations of two irreversible – or 'pseudoirreversible' – inhibitors [10, 11], showed a typical 'Nickerson shift', as I would like to call this profile in the present communication (fig. 1). This behaviour indicates a substantial receptor reserve. Providing that Nickerson's assumption does hold, and that the K_d of the still active receptors is not changed by a partial irreversible inhibition of the total population, the K_d of the receptors may attain any value between, roughly, 10^{-9} and 10^{-7} mol/l (cf. curves in fig. 1).

The simplified model drawn schematically in figure 2 may provide at least a partial explanation of this behaviour. It assumes that the kinetics of signal transduction consist of a number of steps with independent kinetic and steady state characteristics; three steps (receptor, mediator and effector) are considered. Furthermore, it is postulated that the response at each step depends solely on the level of response in the preceding step, and that a rectangular hyperbola is a sufficient approximation of this dependence within a rather broad interval of conditions. By simple mathematical operations, each step (m, e) can be expressed as a function of drug concentration; such relationships are frequently measurable. Under the conditions given above, these new relationships

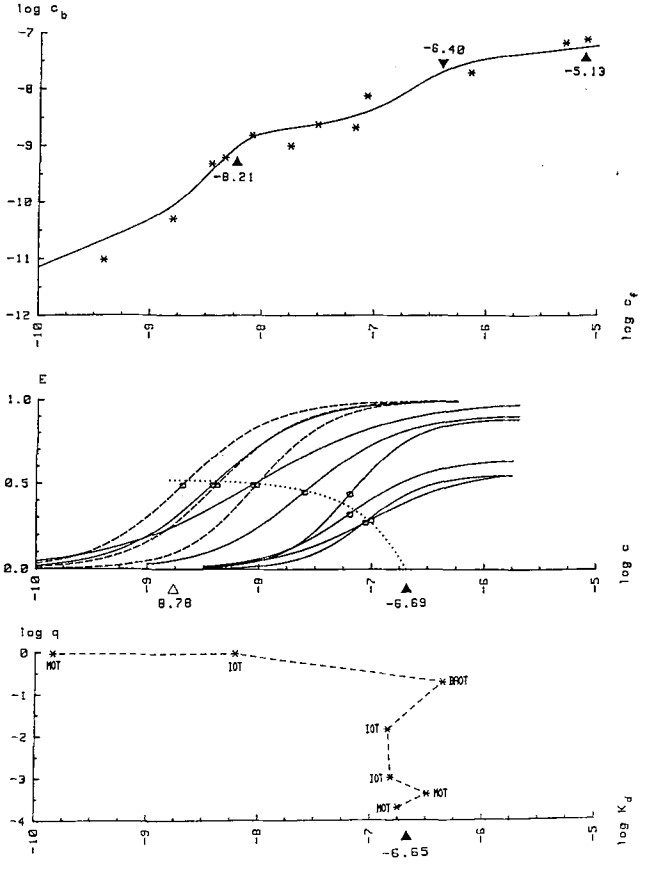

Figure 1. Relationship between binding of oxytocin to rat myometrial cells and the response of rat uterus in vitro to oxytocin. Upper panel: binding isotherm presented as a fit to the Hill equation containing three terms (for details cf. [2]). Detected pK_d are indicated by black triangles. Middle panel: dose-response curves for oxytocin at various levels of irreversible inhibition [15] (broken curves: noninhibited controls). Open triangle: pD_2 value for noninhibited uteri (arithmetic mean of pD_2's). Closed triangle: extrapolated value for fully inhibited receptors (see text). Lower panel: relationship between K_d and fraction of inhibited receptor (q) found by modified Furchgott-Bursztyn analysis [18]; curves of the middle panels were used. Symbols: c_f, c_b are concentrations of the free and bound ligand, respectively, E is an isometric response of rat uterus to oxytocin in a concentration c. MOT, IOT, BAOT are abbreviations for inhibitors used for partial inhibition (cf. [13]).

are again rectangular hyperbolas, with parameters determined by those of the isolated steps [12, 13]. This is obviously a very general model with regard to the form of the relationship between any two consecutive steps. (The recently published model by Leff and Harper [14], which postulates the existence of a ternary ligand-receptor complex and yields similar dose-response profiles, is apparently a specific case of it). It can be recursively extended to any number of steps, with similar results. It explains the 'Nickerson shift' of half-maximal concentrations and also shows that in general the equality between the dissociation constant (represented here by K_r) and the half-maximal dose (K_E) occurs only when the signal transduction chain is extremely inefficient (the product of K_m, K_e must be very large), or when the total number of receptors (ϱ in the model given in fig. 1) approaches zero. The

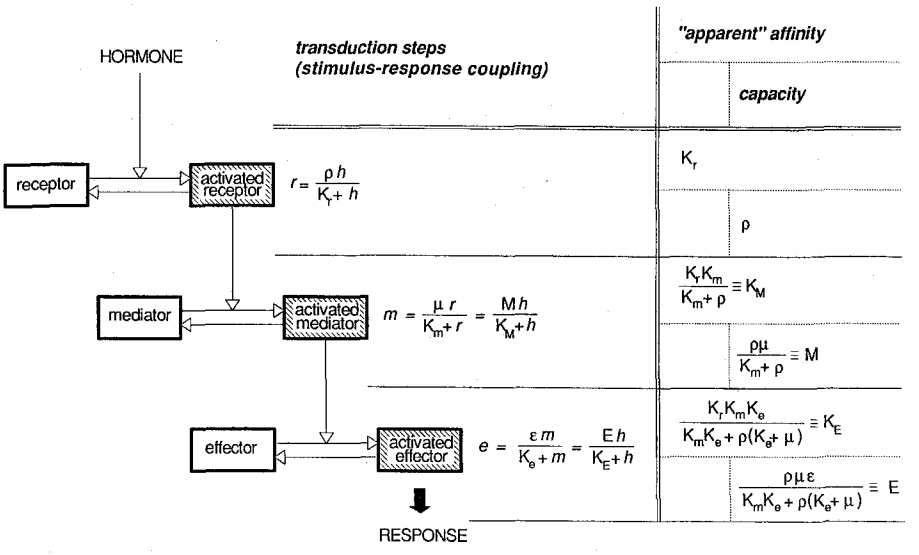

Figure 2. Transduction of a hormone (drug) stimulus from receptor to the responding unit (effector). Equilibria between 'resting' and 'activated' (hatched) forms of individual responding units (receptor, mediator, effector, etc.), expressed by rectangular hyperbolic relationships, are assumed. Formulas on the right-hand side of the hatched boxed express levels of activated forms (r, m, e) as a function of the preceding step, and as a function of the hormone concentration (h). Note that the latter function is also a rectangular hyperbola; parameters standing for affinity and capacity are indicated in the right-hand column. Response is proportional to the level of activated effectors (e).

latter condition is fulfilled as a limiting case in the stepwise irreversible inhibition experiment when the maximal attainable response (E) reaches zero. When dose-response relationships in several partially inhibited states are available, one obtains the EC_{50} limit by extrapolation for maximal response E approaching zero (cf. fig. 1, middle panel).

For oxytocin on myometrial cells, this estimate of 'receptor-linked' K_d (as seen in fig. 1, middle panel) is at 3×10^{-7} mol/l, which is very close to the medium affinity binding site, whereas the high affinity binding site approaches the EC_{50}-value of the noninhibited system. However, the dose-response analysis suggests that the high affinity site for oxytocin ($\sim 10^{-9}$ mol/l) may in some way also be involved in the response induction [15]. Our case is no exception with regard to this behaviour: it has been observed in a number of other instances where precise binding and dose-response data are available [13,16,17].

Furchgott-Bursztyn method

Further analysis of the inhibition experiment described by Nickerson led to a method which enabled one to estimate K_d from a dose-response relationship [18]. It rests on the assumption that an irreversible inhibitor removes a part of the receptors (q being the fraction of receptors remaining, referred to the noninhibited state; $0 \leq q \leq 1$), without causing any further changes in tissue properties, and that the response is a function (f) of the fraction of activated receptors. Independently of the nature of this function, comparison of several pairs of equipotent doses in h_0 (noninhibited system) and in h_i (inhibited system) at various response levels (e_{eq}) yields values of q, K_r and an empirical parameter ν [19] used for computational reasons (for better fit of the data). The procedure (our own modification [13]) is outlined in figure 3. Results for our system obtained by evaluation of the dose-response curves in figure 1 are demonstrated, in the form of a q, K_d-diagram, in the lower panel of figure 1, and show that in a

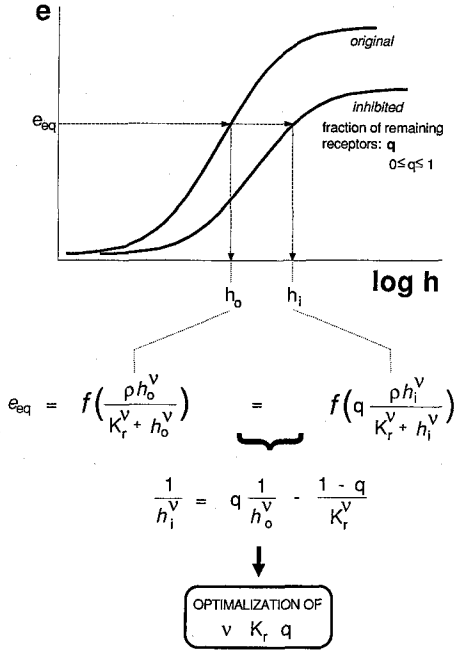

Figure 3. Furchgott-Bursztyn method [18] of stepwise irreversible inhibition. e_{eq} is a response elicited by equipotent doses (concentrations) of the hormone in the noninhibited (h_0) and irreversibly inhibited (h_i) preparation. ν is a power ('Hill') coefficient [19], f symbolizes a function describing the relation between the response e and the drug concentration h; other symbols cf. fig. 1. Optimalization of the parameters is attained by use of suitable numeric procedures (cf. [13]).

very broad interval of q obtained at various levels of inhibition (q from up to 0.98 to about 0.02), the dissociation constant is again around 2×10^{-7} mol/l. For low degrees of inhibition ($q > 0.98$), however, dissociation constants of the order of 10^{-9} mol/l or even lower were obtained. The reason for this may rest either with the decreased accuracy of the method applied close to its limit conditions, which is rather unlikely, or with the existence of two classes of simultaneously-existing receptors (or perhaps of two states of a single receptor), both participating in the initiation of the same response.

As a note of caution, we wish to remark that these results indicate certain discrepancies with the current receptor model assuming a simple equilibrium between just three reaction components, the ligand, the receptor, and their intermediary complex. The question arises as to whether any model *so* designed can sufficiently approximate the 'real' responding system, and if so, to what extent.

So far, the scope of applicability of the Furchgott-Bursztyn method has not been examined on a broader base; it depends undoubtedly upon the form of both the binding isotherm and the function f. The method is applicable, for instance, to the stepwise model in figure 2, and to any of its forms extended for further steps. On the other hand, the assumption that the binding isotherm is in the form of a Hill function [19] (cf. fig. 1) requires a modified evaluation routine.

Structural analogy in competitive inhibitors: Free-Wilson analysis

Small modifications of the primary structure of an agonist frequently result in a loss of agonistic activity, while the binding to the receptor is affected little, if at all. These substances act as competitive inhibitors (in this context, the term to be preferred to the almost synonymous 'antagonist'). Dissociation constants of competitive inhibitors, in contrast to agonists, can be assessed by pharmacological methodology in an in vitro experiment, assuming, however, that their receptor binding does not influence the stimulus-response coupling of the system, and that their concentrations in the vicinity of the receptors, the so-called 'receptor compartment' [20], and in the medium do not differ. The last condition may not fit in cases of uneven inhibitor partitioning between medium and receptor compartments [11].

The structural similarity opens a way toward estimation of K_d of *any* peptide in which the essential structural features of the inhibitors are preserved, i.e. also for 'analogical' agonists. The 'essential features' encompass, in the first place, the backbone conformation which ought not to be modified by the substitution, and independent effects of substituents in individual positions on physicochemical behaviour of the peptide.

Around 160 inhibitors of oxytocin on the rat uterus, obtained by substitution in positions 1, 2, 3, 4, 6, 7 and 8, have been investigated so far. Their pA_2 values, negative logarithms of K_d [11,21–23], have been recently listed [24]. They include very potent inhibitors, such as those substituted with pentamethylene propionic acid in position 1, *D*-amino acids in position 2, threonine, valine and asparagine in position 4 and ornithine in position 8. A direct comparison of the K_d's with oxytocin is hardly justified, but the pA_2 sample allows a closer look at the significance of individual peptide chain positions for binding to the receptor.

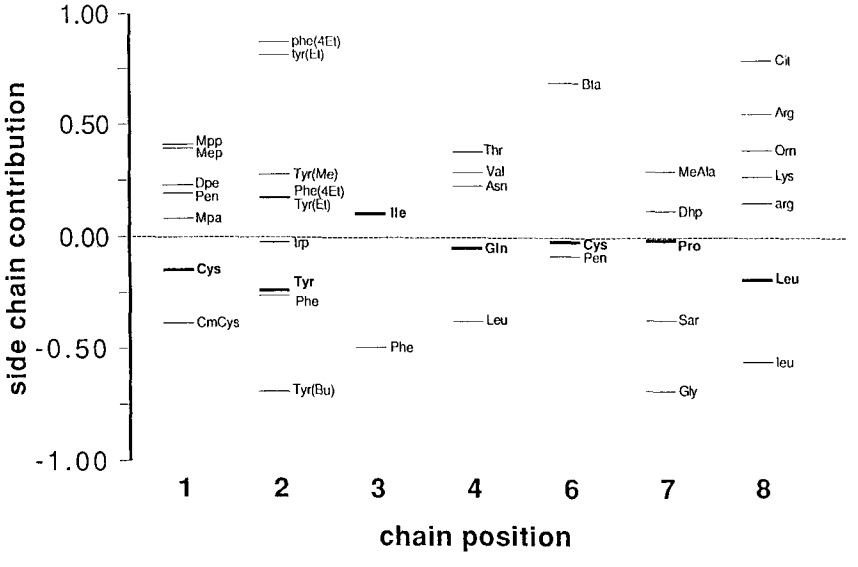

Figure 4. Free-Wilson analysis of oxytocin analogues acting as competitive inhibitors of oxytocin on rat uterus: level diagram. Bars indicate levels of side chain contributions to the pA_2 values of inhibitors, as related to an overall mean of all substances (broken line). Thick bars represent native amino acids of oxytocin. No analogues with modified positions 5 and 9 were available; these positions were taken as a constant backbone sequence and do not appear on the level diagram. Abbreviations of amino acid side chains and description of the method are given in ref. [24] (lower case first letter in the three-letter code for amino acids denotes *D*-enantiomer).

One simple way of looking at this problem follows from the idea that the effect upon K_d of a substituent at a certain chain position is *independent* of that of substituents in other positions. The effect will then be reflected in the pA_2 value in a stochastic manner, since effects of other positions are not considered. If the effects of substituents at individual positions (j) are not only independent, as formulated above, but also constant, then a biological descriptor A (pA_2, in our case) can be formulated as a sum of contributions (α_j) by these substituents ('additivity rule'):

$$A = \varphi + \sum_j \alpha_j.$$

The addition symbol stands for a sum over all positions j and φ is a constant backbone contribution. Computation of α's for a group of substances with a constant backbone and varying substituent groups in several positions does not usually pose any appreciable problems; a simple method to these aims was proposed by Free and Wilson[25]. The validity of the additivity rule for inhibitors of oxytocin was recently verified and 'fragmental contributions' to pA_2 for several substituents have been computed[24]. They are shown in the 'level diagram' on figure 4. When summing up the fragmental contributions for substituents in oxytocin (bold bars in the level diagram), an estimate of K_d for this or several other interesting peptides can be obtained[15,24]. For oxytocin, this amounts to 1.7×10^{-7} mol/l and verifies again the value found by other approaches.

A short comment should be added to these results. A comparison between substituent groups is, for obvious reasons, applicable if the substituent exercises a 'local' effect only, without causing a complex conformational change of the peptide backbone. Is this the case in the oxytocin series? So far, we lack a definitive answer since only very few analogues have been subjected to conformational studies[26–28]. Correlation of pA_2 with substituent constants describing 'local' effects like bulkiness, hydrophobicity, electronic properties etc.[29] suggests an absence of global conformational changes[30]. This chapter, however, is far from being closed.

Are new receptor models necessary?

Relationships between binding and pharmacological data have been under investigation for more than ten years, and some interesting facts have been revealed. Firstly, in cases in which several binding sites were assessed by binding studies, the K_d of the low affinity site correlates with the pA_2 of competitive inhibitors, and the K_d of the high affinity site with EC_{50}[31], the concentration eliciting a half-maximal response. Secondly, in these instances, K_d values estimated pharmacologically by means of irreversible inhibitors or by other analogous methods (analysis of pA_2 values has been mentioned above) correspond numerically by those of low affinity sites. And thirdly, if binding studies indicate a single K_d value and this value happens to correspond with the K_d obtained pharmacologically, then the binding is usually of low, or very low, affinity (for instance, in an extensive study of β-adrenergic substances by Furchgott[32], K_d's are in the range of 10^{-8} to 10^{-4} mol/l for both agonists and inhibitors). This must indeed awake a suspicion that another, high-affinity binding site exists but was not identified by binding studies.

At this point, a question arises as to the nature of the low affinity sites occasionally found in binding studies (cf. $K_d > 10^{-5}$ mol/l for oxytocin, as mentioned above). They do not seem to possess any obvious pharmacological correlates, and frequently their saturability cannot be proven. They may well reflect an accumulation of the ligand in the surface layer of the membrane aided by electrostatic forces[33] and/or by hydrophobic interactions[11]. These processes may change the ligand concentration in the receptor compartment[20] considerably and yield 'apparent' K_d's which are several orders of magnitude away from the actual ones. The relevance of such factors to biological systems was demonstrated in the case of dynorphin binding to the opioid κ-receptors[34].

It seems, therefore, plausible that several receptors, or states of a receptor, may be involved in eliciting a particular biological response. Such structures of receptor systems might perhaps be associated with a particular feature of the ligand-receptor interaction, like the 'induced fit' in the case of conformationally flexible ligands. Also, models postulating a 'receptor switch', a discrete change of binding affinities after attachment of the ligand to the receptor, ought to be mentioned in this context. Except in a few cases, proofs of the existence of this effect were usually insufficient in certain critical points, owing to the incompleteness of the binding study (e.g. because it was carried out using restricted ligand concentration intervals or evaluated by unsuitable methods) or to the absence of comparative pharmacological data. Besides appropriate binding studies, enabling the identification of *all* relevant binding sites in the system, the problem of verification resides largely in the methods of pharmacological investigation. No general protocol can be designed to these ends, and the methods mentioned above frequently fail in specific cases. Dose-response analysis, for example, would represent a very general approach, but the lack of powerful and sufficiently specific irreversible inhibitors is a prohibitive condition for many groups of substances. Also, the concept of structural analogy may fail in most cases, be it for a lack of comparative material, failure of the Free-Wilson analysis (or similar computational procedures), or simply a poor similarity of substance properties ('weak analogy'). On the other hand, other approaches such as response analysis on a cellular level, analysis of intermediate steps (second messengers) or comparison of drug behaviour in specific biological systems (e.g. 'normal' animals and animals with receptor failure) are conceivable in many instances.

It is likely that these combined efforts will become decisive for formulation of future receptor models, which might better match all the experimental evidence than the contemporary schemes.

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Research Articles

The use of significance limits in graphical data representations

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Summary. Significance limits are proposed as an alternative to the use of standard deviation, standard error, or confidence or tolerance limits when experimental data are presented in a graphical form. This measurement of uncertainty allows graphical t-tests to be used both for the estimation of data variance and for an approximate statistical comparison between two or more data sets.
Key words. Uncertainty limits; error bars; graphical t-test; significance limits.

Graphical representations are used in all areas of experimental science as an efficient tool for comparing two or more data sets. Over the past years, the new approach of exploratory data analysis (EDA)[1] has elevated data visualization to the status of an accepted statistical technique. Also, because of the increasing availability of computers, graphical representations have become more feasible. Since the values of the means or medians do not by themselves give enough information for the comparison of data sets, it is essential that they be accompanied by an appropriate measurement of uncertainty. EDA proposes the characterization of data sets graphically by medians, hinges, quartiles, and depth. This allows the representation of many features of a set, such as amount of spread, symmetry, and the presence of outliers. However, this representation, and also all classical uncertainty ranges (standard error, confidence limits, standard deviations, and tolerance limits), do not permit statistical differences between data sets to be inferred from the graphical representations alone.

Figure 1 shows the individual values, the means, and a variety of representations of uncertainties in increasing