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The use of neurally released agonist in the measurement of antagonism at α-adrenoceptors

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1 Graded contractile responses of the rat vas deferens were obtained to trains containing varying numbers of pulses delivered transmurally at 500 Hz, allowing construction of pulse-response curves.

2 In the presence of postjunctional adrenoceptor blocking drugs, these curves were displaced to the right in a parallel manner, allowing the estimation of pA_2 values for the drugs.

3 Activity of drugs at prejunctional adrenoceptors was estimated from the reduction of inhibition of the response to a second pulse delivered 3 s after trains containing different numbers of pulses.

4 It is suggested that the technique is more physiological than others used to determine the antagonistic activity of drugs at adrenoceptors.

Introduction

The most frequently quoted measurement of drug antagonism is the pA_x value introduced by Schild (1947). This is defined as the $\log_{10}$ of the reciprocal of the concentration of antagonist which reduces the effect of a multiple dose (x) of agonist to that of a single dose. The most common value used is the pA_2 value, where $x = 2$. Various assumptions are implicit in the use of this scale, e.g. that both agonist and antagonist drugs compete for the same receptor population and that the antagonism is competitively surmountable by increasing the concentration of agonist. All measurements of pA_2 may be subject to inaccuracies due to effects such as the metabolism or uptake of either the agonist or antagonist drugs (Warming, Shipley, Leedham, Hartley, Handberg & Pennefather, 1982). This is particularly true in the case of drugs competing for receptors in adrenergically innervated tissues where the processes of neuronal and extra-neuronal uptake may alter the local concentration of agonists, and where the variety of receptors (α_1, α_2, β, presynaptic and postsynaptic etc.) may contribute not only to the generation of an observable tissue response, but may also affect the output of transmitter, especially in electrically stimulated tissues.

Various attempts have been made to overcome these problems: use has been made of agonists which are resistant to neuronal uptake, such as methoxamine (Trendelenburg, Maxwell & Pluchino, 1970; Lattimer, Rhodes, Ward, Waterfall & White, 1982). Experiments are frequently performed in the presence of inhibitors of the uptake mechanisms (Doxey, Smith & Walker, 1977; Doggerell, 1981), and of agents which block the complicating 'unwanted' species of receptor, such as propranolol, prazosin, etc. Another approach is to use agonists which are relatively specific for certain receptor subtypes such as the α_1-specific amidephrine (Flavahan & McGrath, 1981) or the α_2-specific xylazine (Drew, 1977; Eltze, 1979).

This paper proposes that by appropriate stimulation of the intramural motor nerves in rat vasa deferentia, it is possible to obtain graded contractile responses which, it is suggested, are due to a graded output of transmitter either from individual varicosities, or because of the involvement of varying numbers of varicosities. It should also be possible to cause a displacement to the right of the stimulus-response curve in the presence of antagonists at the postjunctional receptors. This will then permit the calculation of a value analogous to the pA_2 value (Schild, 1947) for drugs which are antagonists at postjunctional adrenoceptors. This will be referred to as pA_{2s}; the suffix 's' refers to the fact that stimulus ratio is being measured instead of dose ratio.

It has been shown previously that application of high-frequency trains of pulses transmurally to either end of the bisected rat vas deferens results in a contractile response, the magnitude of which is directly related to the number of pulses in the train (French & Scott, 1983a). The frequency of pulses used (500 Hz) coupled with the short duration of the trains should make it most unlikely that mechanical summation of discrete contractile responses is re-

sponsible for the increased contractions in response to increased number of pulses.

Application of a single pulse within 2–10 s of such a train of pulses will result in a single twitch response, its size being dependent on the time interval between the train and the single pulse; short intervals result in small responses to the single pulse, while longer intervals result in a larger response. It has been suggested that these effects are related to (a), release of transmitter in amounts which are related to the number of pulses in the train, and (b), the inhibition of subsequent release of transmitter by an action of the released noradrenaline on the prejunctional α_2-receptors (French & Scott, 1983). This observation suggests that it should be possible to measure the effects of drugs which are antagonists at α_2-receptors by measuring the extent of their ability to antagonize the inhibition of the response to the testing, single pulse.

The epididymal portion of the rat vas deferens responds to single pulse stimulation of the intramural nerves with a biphasic twitch, the dominant phase, being due to the release of noradrenaline, which occurs approximately 650 ms after stimulation. The prostatic end, on the other hand, responds with a dominant peak at 250 ms its origin being non-adrenergic: non-cholinergic (NANC), (McGrath, 1978). This latter twitch response is virtually unaffected by concentrations of α_1-adrenoceptor blocking agents which markedly reduce the size of the response which is dominant in the epididymal end of the tissue. However, the size of this response can be affected by the actions of substances which are agonists at pre-junctional α_2-receptors, suggesting that the release of the transmitter responsible is subject to restraint in the same manner as is the release of noradrenaline (French & Scott, 1983).

Methods

Measurement of pA_2 values at postjunctional α_1-receptors

Epididymal ends of vasa deferentia taken from mature Sprague-Dawley rats were suspended between parallel platinum wire electrodes, 1 cm apart, in a tissue bath containing 100 ml of Krebs-Henseleit solution as previously described (French & Scott, 1981). Threshold twitches were obtained to single pulses of 0.3 ms duration, while maximum responses to single pulses were achieved when the pulse width was increased to 3 ms. Application of trains containing 1, 2, 4, 8, 16 or 32 pulses (0.3 ms) at 500 Hz resulted in twitch responses with magnitudes related to the logarithm of the number of pulses in the train in a sigmoid manner resembling the classical relationship between a stimulant drug and a tissue response (Figure 1). Pulse-response curves were thus determined in the absence of any drug, and after 30 min contact with three concentrations of the α-adrenoceptor antagonists prazosin (1, 3 and 10×10^{-9} M); phentolamine (1, 3 and 10×10^{-7} M); yohimbine (0.3, 1 and 3×10^{-6} M) and RS 21361 (2-(1-ethyl-2-imidazolylmethyl)-1, 4-benzodioxan), (1, 3 and 10×10^{-5} M). The pA_{2s} values were then calculated by firstly estimating the ratio of the number of pulses which produce identical responses in the presence and absence respectively of each antagonist. This was done either by interpolation from the pulse-response curves, or algebraically. This ratio (x) was estimated for three concentrations of each antagonist and is equivalent to the dose ratio obtained when the stimulating agency is an agonist drug rather than a discrete number of applied electrical pulses. A plot of log $(x-1)$ against log [antagonist] then gives a straight line with an intercept, when $x = 2$, and thus log $(x-1) = 0$, of $-pA_2$ (Arunlakshana & Schild, 1959). The calculations give rise to a practical impossibility in most instances, viz. that the calculated ratios imply the application of numbers of pulses which are not integers. However, if it is assumed that fractions of pulses can be applied, then the method is valid. The pA_{2s} values were thus calcu-

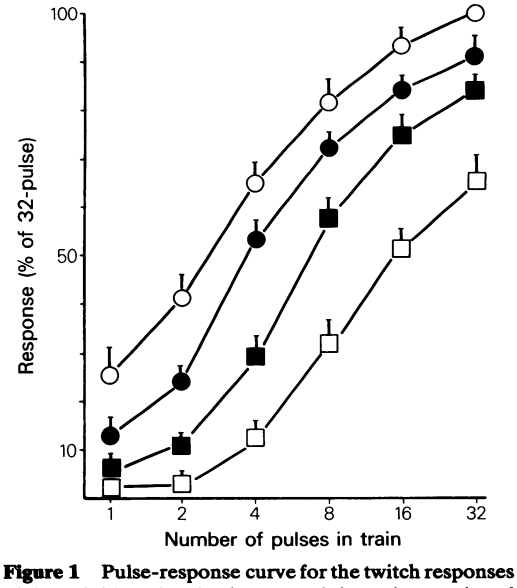

Figure 1 Pulse-response curve for the twitch responses of the epididymal ends of rat vasa deferentia to trains of high frequency stimulation in the absence of antagonist (control) (○), and in the presence of yohimbine 3×10^{-7} M (●), 1×10^{-6} M (■) and 3×10^{-6} M (□). Each point is the mean of at least 6 separate determinations and vertical bars show s.e.mean.

lated from the above intercept of the regression line for three points with the log [antagonist] axis.

Measurement of pA_{2s} values at prejunctional α_2-receptors

Prostatic ends of the rat vasa deferentia were used in this estimation, since the peak of the response is associated with the NANC transmitter and is thus virtually unaffected by any postjunctional α_1-receptor blocking activity of the drugs used (McGrath, 1978). However, the size of this response is modified by drugs acting on prejunctional α_2-receptors. Thus, the noradrenaline released during trains of pulses will also feed back and modify the release of the NANC transmitter (French & Scott, 1981), so causing a reduction in the size of the response to the following testing pulse. The assumption is made that the extent of the feedback inhibition and hence of the reduction in size of the response to the testing pulse will be directly related to the output of noradrenaline which in turn is related to the number of pulses in the conditioning train (see Introduction). It is antagonism of this inhibition which is measured by the following technique.

The tissues were set up under conditions identical to the epididymal ends, but the stimulation parameters were as follows: each train (of 1 to 32 pulses) was followed 3 s later by a single, testing pulse of 3 ms and 300 mA. These parameters were chosen from the results of preliminary experiments in which intervals between 2 s and 8 s were used between the train and the testing pulse. A pulse interval of 3 s was chosen for the following reasons: at intervals greater than 3 s, the effect of the transmitter (released during the train) begins to wane due to diffusion away from receptors and to the processes of neuronal and extraneuronal uptake. Intervals of 2 s or less are barely sufficient to allow the tissue to relax completely after the train response. The estimates of pA_{2s} obtained from these preliminary experiments, in which train-pulse intervals other than 3 s were used, did not differ significantly from those obtained by the final protocol as described. The testing pulse was 3 ms (i.e. ten times the width of the pulses in the train) in order to elicit a measurable response from the tissue, since the response to a testing pulse of 0.3 ms duration within 3 s of a preceding pulse (or train) is subject to considerable reduction due to the processes of negative feedback (French & Scott, 1981a; 1983b). The response to this testing pulse was inversely related to the size of the response in the preceding train, (see Figure 2) and the reduction in size was taken as an indicator of the extent of feedback inhibition due to the noradrenaline released in response to the conditioning train. The measured responses from this type of experiment can therefore be described as the *reduction* of the size of the contraction to the single testing pulse from that to the train containing one pulse. The experiment was then repeated after 30 min contact with each of three concentrations of antagonist drug, and the pA_{2s} calculated from the displacement to the right of the pulse-inhibition curves. The concentrations of antagonists used were: prazosin 0.3, 1 and 3×10^{-5} M; phentolamine 1, 3 and 10×10^{-8} M; yohimbine 0.3, 1 and 3×10^{-8} M; RS 21361 0.3, 1 and 3×10^{-7} M.

Results

Table 1 shows the pA_{2s} values for four adrenoceptor antagonists which have been shown to have various degrees of activity at prejunctional α_2 and postjunctional α_1-adrenoceptors. As expected, prazosin was the most active agent at the postjunctional receptors and the least active at the prejunctional receptors. At the other end of the scale, RS 21361 exhibited little postjunctional activity, but had marked activity against α_2-receptors, confirming previous reports of its selectivity in this respect (Michel & Whiting, 1981). The most potent α_2-receptor blocker was

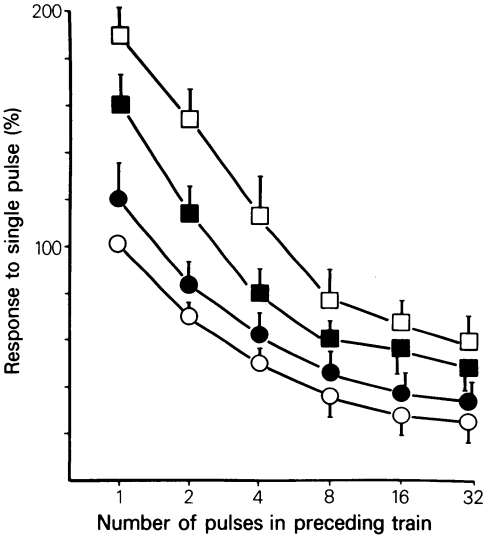

Figure 2 Responses of the prostatic ends of rat vasa deferentia to a single pulse (3 ms duration) delivered 3 s after a train containing the number of pulses shown on abscissa. The ordinate scale shows the size of the contractile responses expressed as a percentage of the response to a single pulse delivered after a train containing one pulse in the absence of antagonist (control) (○) and in the presence of phentolamine 1×10^{-8} M (●), 3×10^{-8} M (■) and 1×10^{-7} M (□). Each point is the mean of at least six separate determinations and vertical bars show s.e.mean.

Table 1 The pA_{2s} values at α_1- and α_2-adrenoceptors for four adrenoceptor antagonists

Antagonist	*pA_{2s} at α_1-adrenoceptors*	*pA_{2s} at α_2-adrenoceptors*	*α_2/α_1 selectivity ratio*
Prazosin	8.64±0.11	6.21±0.31	0.004
Phentolamine	6.98±0.19	7.60±0.19	4.2
Yohimbine	6.26±0.13	7.92±0.19	14
RS21361	4.93±0.20	7.01±0.32	120

Each value is the mean of at least 6 determinations on separate tissues. The α_2/α_1 selectivity ratio is the antilog of the difference of the pA_{2s} values for α_2- and α_1-receptors.

shown to be yohimbine, but its marked activity against α_1-receptors reduced its selectivity below that of RS 21361. Phentolamine, as expected, was a potent antagonist of both α_1- and α_2-receptors, but exhibited little selectivity.

In the estimates of the pA_2 values at α_1-receptors, the slopes of the regression lines are not significantly different from 1. The slopes of the regression lines for the α_2-receptor estimates of pA_2 are not significantly different from 1 for phentolamine and RS 21361. The slopes for prazosin and yohimbine are lower than expected, but no ready explanation is obvious (Figures 3 and 4).

Discussion

The variety of techniques used to measure pA_2 values for drugs with antagonistic actions at adrenoceptors has led to doubts about the accuracy of estimates obtained by different workers. These variations may be due to the different agonists used. Use of exogenous noradrenaline is complicated by the fact that it has actions at both α_1- and α_2-adrenoceptors, and also by the processes of neuronal and extraneuronal uptake (Warming *et al.*, 1982). However, in rat vas deferens the extraneuronal uptake processes may not be particularly marked (Hermann & Graefe, 1977).

Use has been made of inhibitors of the neuronal uptake processes such as cocaine or desipramine. However, both of these substances have other actions which can complicate the estimate of pA_2. For example, it is well known that desipramine blocks α_1-adrenoceptors (Bowman & Rand, 1980) while the local anaesthetic actions of cocaine could interfere with the processes of nerve conduction, and may also interfere more directly with transmitter release (Pennefather, Handberg, Shipley & Taylor, 1979).

Separation of α_1- and α_2-receptor effects has frequently involved the blocking of the unwanted receptor population with the use of, for example, propranolol to block β-receptors, or of prazosin to block α_1-receptors when looking at α_2-receptors. The problem here is that no drug has yet been described which is entirely specific for any one receptor type or subtype although so-called specific receptor agonists have been used; e.g. methoxamine has been used as a specific α_1-receptor agonist (Lattimer *et al.*, 1982) or xylazine as a specific α_2-receptor agonist (Docherty & McGrath, 1980). Agents which are less prone to neuronal uptake have also been used as agonists, e.g. amidephrine (Michel & Whiting, 1981). However, each technique involves the addition of complicating drug action or the use of non-physiological agonists.

The techniques presented in this study provide a means of examining the effectiveness of antagonists at α_1- and α_2-adrenoceptors with the least possible interference from other drug actions. The activity of antagonists at α_1-receptors should be unaffected by any antagonist action at prejunctional α_2-receptors since the processes of feedback inhibition of transmitter release are not operative when single pulse stimulation is used (or short trains of very high fre-

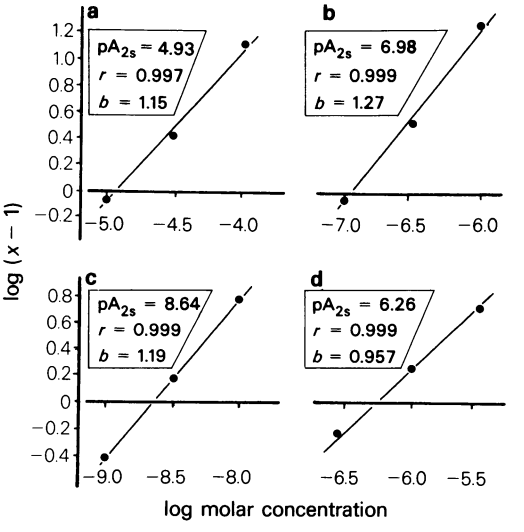

Figure 3 Schild plots for estimation of pA_{2s} values of antagonists RS 21361 (a), phentolamine (b), prazosin (c) and yohimbine (d), at postjunctional α_1-adrenoceptors. For explanation of $(x-1)$ value see Methods. The calculated pA_{2s} value for each antagonist is shown, along with the correlation coefficient (r) and the slope of the regression line (b).

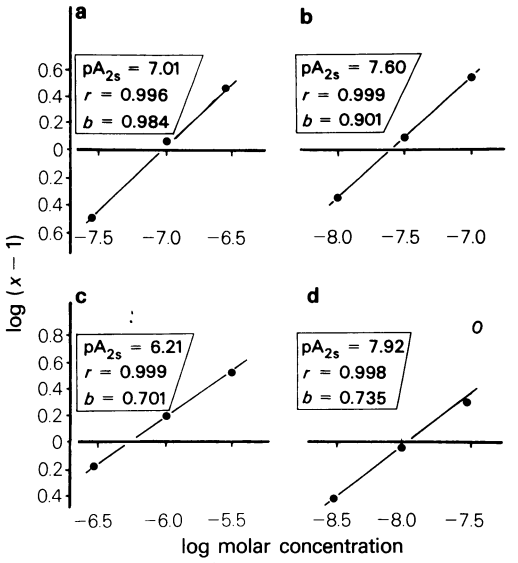

Figure 4 Schild plots for estimation of pA_{2s} values of antagonists RS 21361 (a), phentolamine (b), prazosin (c) and yohimbine (d), at prejunctional α_2-adrenoceptors. For explanation of $(x-1)$ value see Methods. The calculated pA_{2s} value for each antagonist is shown, along with the correlation coefficient (r) and the slope of the regression line (b).

quency stimulation as used in this study). Similarly, any α_1-receptor blocking activity of the compounds under investigation should not interfere with the responses of the prostatic end of the vas which is used for the pA_{2s} determinations at α_2-receptors, since it is the NANC response which is being measured, and this is unaffected by adrenoceptor blocking agents (McGrath, 1978).

The sigmoidal relationship which was observed between the number of pulses in each train and the magnitude of the evoked response supports the suggestion that the transmitter output is directly related to the number of pulses in each train. The frequency of stimulation may be in excess of the rate of physiological trains. However, this high rate of 500 Hz was chosen so that, even when 16–32 pulses were delivered in a train, the total duration of stimulation would be 32–64 ms. Also, this is the reason that the number of pulses in the trains was not increased beyond 32, even in the presence of concentrations of antagonists which caused significant shifts to the right of the pulse-response curve. If the number of pulses is increased even to 64, this means that the total duration of stimulation is 128 ms which becomes a significant portion of a twitch response, which, in the epididymal end of the vas deferens peaks at around 650 ms after a single pulse. The use of greater numbers of pulses, which, from Figure 1 it might be predicted would be required to match the 32 pulse response in the control situation, would start to approach the period when the onset of the feedback mechanisms might occur.

In guinea-pig atria the negative feedback processes are detected 1.5 s after stimulation of the adrenergic nerves (Story, McCulloch, Rand & Standford-Starr, 1981) and a similar time course has been suggested for the vas deferens (Rand, McCulloch & Story, 1982). Therefore, it is assumed that the output of noradrenaline in response to the early pulses in each train used in this study would not have time to feed back and inhibit the subsequent release to the later pulses in the train, up to a limit of 32 pulses. The refractory period of the small diameter sympathetic C fibres has been quoted as being of the order of 2 ms (Ruch, Patton, Woodbury & Towe, 1965). If the refractory period is in fact less than this, it would allow the generation of discrete action potentials to each pulse delivered at 500 Hz with the release of separate 'packets' of transmitter. Although 500 Hz is well outside the normal physiological frequency, previous experiments indicated that it is probable that the tissue does respond to each pulse delivered at this frequency.

Figure 5 shows the twitch response to 2 pulses (0.3 ms duration, 300–400 mA) delivered at pulse intervals between 1 ms and 512 ms. The response to 2 pulses 1 ms apart, which was shown to be not significantly different to the response to 1 pulse, is taken as the control value. When the interval is increased to 2 ms, there is a 'quantal jump' in twitch response, which then changes relatively little until the pulse interval reaches 64 ms. Further increases result in a gradual reduction in twitch responses. This is not related to feedback (i.e. the release of transmitter in response to the first pulse activating prejunctional

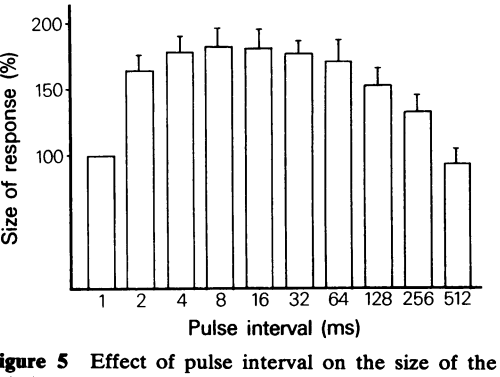

Figure 5 Effect of pulse interval on the size of the twitch response of the epididymal ends of rat vasa deferentia to pairs of stimuli (0.3 ms, 350 mA). Ordinate scale, size of twitch response expressed as a percentage of the response to two pulses 1 ms apart: abscissa scale, interval between pulses (in ms).

Table 2 Comparison of pA_{2s} and pA_2 values, for four adrenoceptor antagonists, obtained by different groups

	A (pA_{2s})			B (pA_2)			C (pA_2)		
Antagonist	α_1	$\alpha 2$	α_2/α_1	α_1	α_2	α_2/α_1	α_1	α_2	α_2/α_1
Prazosin	8.64	6.21	0.004	8.20	6.62	0.03	8.76	5.56	0.00063
Phentolamine	6.98	7.60	4.2	7.70	8.38	4.8	8.10	8.10	1
Yohimbine	6.26	7.92	45	6.40	8.18	60.3	6.25	7.72	29.5
RS 21361	4.93	7.01	120	—	—	—	4.00	6.79	>616

A This paper
B Doxey *et al.* (1977)
C Michel & Whiting (1981)

α-adrenoceptors and causing inhibition of release in response to the second of the pair of pulses) since α_2-receptor blockers did not restore the size of contraction: The parallel shift to the right of the pulse-response curve, in the presence of adrenoceptor blocking agents, tends to verify the assumptions about transmitter release. Comparison of the pA_{2s} values for these α_1 and α_2 antagonists obtained by this technique with the pA_2 values obtained by other workers using exogenous agonists shows considerable accord (Table 2). For example, the estimates of the pA_{2s} at α_1-adrenoceptors for prazosin and yohimbine are in reasonable agreement with the pA_2 values for these compounds and the pA_{2s} for RS 21361 is not grossly different from its pA_2 value. However, there is some variation in the results for phentolamine; there is a difference of more than one log unit between the pA_{2s} value and the pA_2 value quoted by Michel & Whiting (1981) and the estimate of the pA_{2s} value at α_2-adrenoceptors shows a similar difference from the pA_2 value estimated by Doxey *et al.* (1977). It is therefore all the more interesting that our estimates of the selectivity of phentolamine for α_2 and α_1-adrenoceptors are in complete agreement with those of Doxey *et al.*, (1977) and are not grossly different from those of Michel & Whiting (1981).

In conclusion, since the above method produces results which compare reasonably well with previously published results from other sources, it is suggested that the technique may be applicable to other tissues, where electrical stimulation of the motor nerves, either directly or indirectly, may be applicable. The technique may be more physiological than are those which utilize synthetic or exogenous agonists, since it involves the release of the natural transmitter from nerve terminals and the action of this transmitter substance on the appropriate physiological receptors.

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