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The determination of receptor constants for histamine H_2-agonists in the guinea-pig isolated right atrium using an irreversible H_2-antagonist

T.J. Rising & A. Steward

Dept. of Drug Discovery, Hoechst Pharmaceutical Research Laboratories, Walton Manor, Walton, Milton Keynes MK7 7AJ

1 From measurements of chronotropy in the guinea-pig isolated right atrium, a compound (E1309) was found which behaved as an irreversible antagonist at the histamine H_2 receptor.

2 E1309 was used to block irreversibly a proportion of the H_2 receptors and the dissociation constants, relative efficacies and receptor reserves of four H_2-agonists were determined.

3 The calculated dissociation constants were similar to the K_i values reported from H_2-radioligand binding studies but different from the observed EC_{50} values.

4 The order of potency for the four H_2-agonists was impromidine >> histamine > dimaprit > 4-methylhistamine.

5 The order of relative efficacy was 4-methylhistamine > dimaprit > histamine > impromidine, the natural agonist not being the most efficacious. This atypical finding is discussed in relation to other receptor classes.

Introduction

The earlier work of Stephenson (1956) and Nickerson (1956) led Furchgott & Bursztyn (1967) to establish a procedure for the calculation of various receptor constants for full agonists including dissociation constants, relative efficacies and receptor reserves. This procedure required the availability of an irreversible receptor blocking agent, and has subsequently been applied to α-adrenoceptors (Besse & Furchgott, 1976), muscarinic receptors (Ringdahl, 1984) and β-adrenoceptor agonists (Broadley & Nicholson, 1978).

During routine *in vitro* screening of potential histamine H_2-antagonists, a compound, namely 3-(2-diethylaminoethylamino)-5-[2-[(2-guanidino-thiazol-4-yl)methylthio]-ethylamino]-4-methyl-1, 2, 4, 6-thiatriazine-1, 1-dioxide (E1309) was found, which behaved atypically in the various assay procedures. Subsequent work indicated that E1309 possessed the properties of an irreversible H_2-antagonist and thus could be employed in studies to determine H_2-receptor agonist constants.

This paper describes studies to demonstrate the irreversible nature of E1309 binding to the histamine H_2-receptor in the guinea-pig right atrium and its use in the determination of receptor constants of four H_2-agonists, histamine, dimaprit, 4-methylhistamine and impromidine. The results are discussed in relation to similar constants derived for other receptor sub-types. A brief summary of this work has recently appeared (Bottomley *et al.*, 1985).

Methods

Chronotropic measurements in the guinea-pig isolated right atrium

Chronotropic effects in the guinea-pig isolated right atrium were measured essentially by the method of Reinhardt *et al.* (1974). Basically, guinea-pigs of either sex (300–500 g) were killed by cervical dislocation and the whole heart excised and placed in Krebs-Henseleit solution. The right atrium was dissected free and suspended under a tension of 0.8–1.0 g in an organ bath containing Krebs-Henseleit solution aerated with 5% CO_2 in O_2. The signal from the spontaneously beating right atrium was used to trigger an electronic ratemeter which was connected to a calibrated chart recorder. All preparations were allowed to stabilize at a bath temperature of 32 ± 1.0°C for 1–1.5 h with frequent washing. Responses to an agonist were recorded as cumulative dose-response curves. The tissue fluid was changed and the atrium allowed to

stabilize before construction of a subsequent dose-response curve following preincubation with an antagonist.

Establishment of irreversible antagonism

In wash-out experiments to establish irreversible antagonism, the atria were set up as described above. Histamine, at a level which gave a response approximately 70% of the maximum, was added. After washing the tissue, E1309, ranitidine or both antagonists were added and incubated for the times shown in Figure 3. At the end of this period the tissue was washed three times over 15 min and rechallenged with the same fixed dose of histamine at 18 min. This washing and challenge procedure was repeated with the histamine being given at 36, 54 and 72 min. In the study with E1309 alone, noradrenaline at a level which gave a near maximum response, was added at the start and end of the experiment to check the viability of the tissue.

Determination of agonist constants

Dissociation constants (K_D), relative efficacies and receptor reserves were calculated using equations derived by Furchgott & Bursztyn (1967) from Stephenson (1956). Dose-response curves were prepared and the following equation applied:

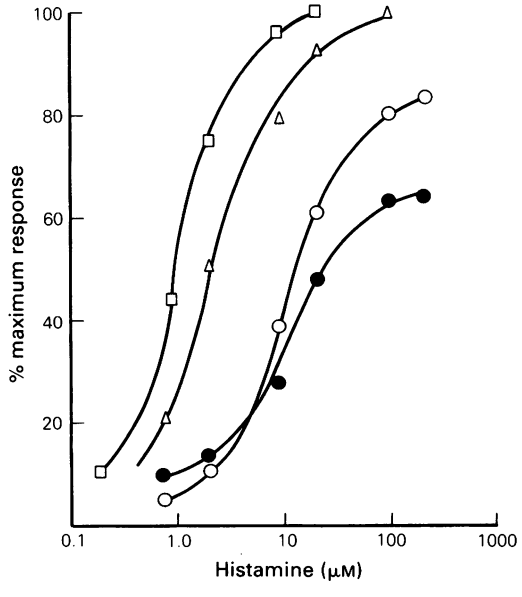

Figure 1 Preliminary dose-response curves to histamine alone (□) and in the presence of E1309 at concentrations of 0.133 μM (△), 0.267 μM (○) and 0.533 μM (●).

$$\frac{1}{[A]} = \frac{q-1}{K_D} + \left(\frac{1}{q}\right)\frac{1}{[A']}$$

where q is the fraction of receptors remaining unoccupied by the irreversible H_2-antagonist E1309 and [A] and [A'] are the concentrations of agonist in the absence and presence of E1309 respectively which give the same response. The reciprocal values were plotted as 1/[A] against 1/[A'] and from the resultant straight line the values of q and K_D were calculated. Using the value of K_D, the fraction of receptors occupied ([RA]/[R_t]) can be determined since

$$\frac{[RA]}{[R_t]} = \frac{[A]}{K_D + [A]}$$

For a number of different agonist concentrations a plot of log [RA]/[R_t] against the response [E_A] gives a graph from which the receptor reserve can be estimated. Also from this relationship the relative efficacy (E) of two agonists (a and b) is

$$\frac{E_b}{E_a} = \frac{[RA_a]/[R_t]}{[RA_b]/[R_t]}$$

where the fraction of receptors occupied is calculated for the maximum chronotropic response.

Results

Histamine dose-response curve

Concentrations of 0.1 μM to 0.1 mM were used to establish a dose-response curve to histamine for chronotropic activity in the guinea-pig isolated right atrium. Higher concentrations of histamine (and the other H_2-agonists) produced a diminution in the maximum response. However, there was no significant difference ($P > 0.05$) between the maximum responses for all four agonists. The addition of E1309 to the tissue resulted in a shift in the dose-response curve to the right with apparent non-surmountable inhibition at the higher concentrations (Figure 1). The Schild slope, calculated from a plot of log (dose ratio − 1) against log antagonist concentration (Schild, 1949), was 2.5 and the apparent pA_2 value for E1309 was 7.04. Under the same experimental conditions the derived pA_2 values for the competitive antagonists cimetidine and ranitidine were 6.32 and 6.92 respectively, with slopes not significantly different from unity.

Establishment of irreversible antagonism

The nature of the E1309-induced antagonism was further investigated by following the effect of different pre-challenge incubation times on the degree of inhibi-

tion. As can be seen from Figure 2, increasing the incubation time of E1309 from 20 to 40 min produced a dramatic change in the chronotropic profile with a marked depression of the maximum response. Since the longer incubation time of 60 min appeared to cause no additional change in the inhibitory pattern, 40 min was chosen for subsequent studies of this nature.

To demonstrate that E1309 was binding in an irreversible manner to the H_2-receptor, wash-out and receptor protection experiments were carried out (Figure 3). With a concentration of E1309 that gave sub-maximal inhibition of histamine-induced positive chronotropy, the atrial response following the fourth histamine challenge was no different from that of the first, even though the tissue had been extensively washed (Figure 3a). The similarity in the noradrenaline responses was indicative of no significant loss of tissue viability during the course of the study. The sustained receptor blocking action of E1309 can be compared with the effect of the competitive H_2-antagonist, ranitidine, under the same experimental conditions (Figure 3b), where the response returns to normal following one series of washings. To confirm that the E1309-induced irreversible antagonism was a direct result of binding to the H_2-receptor, a receptor protection experiment was undertaken using ranitidine. As shown in Figure 3c, pre-incubation of the atrium with ranitidine, prior to E1309 and subsequent histamine challenge, resulted in no significant dimunition of the maximum response.

Determination of receptor constants

With a level of E1309 (0.27 μM) that gave approximately a 30% inhibition of the maximum chronotropic response, the agonists concentrations giving equal responses in the presence [A'] and absence [A] of E1309 were determined. This procedure was adopted for all four agonists, a typical example for histamine being shown in Figure 4. From a reciprocal plot of these agonists concentrations, the values of q (fraction of receptors unoccupied in the presence of antagonist) and K_D were derived (Figure 4, inset). Further calculations based on the relationships described in the Methods section gave a measure of the receptor reserve and relative efficacies of the agonists (Table 1). Also shown in Table 1 for comparison are the corresponding values of EC_{50} and K_i (derived from specific H_2-receptor binding).

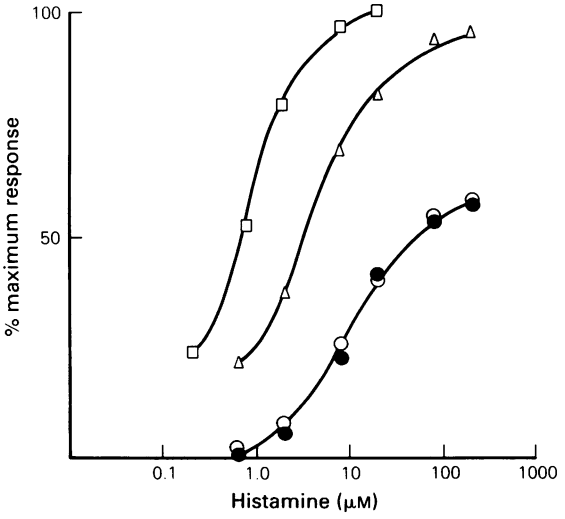

Figure 2 Dose-response curves to histamine in the presence of 2.67×10^{-7}M E1309 at different equilibrium times. A preliminary dose-response curve to histamine alone (□) was constructed before examining the effect of E1309 at 20 min (△), 40 min (○) and 60 min(●) incubation times.

Discussion

The concepts of spare receptors and relative efficacy are now generally accepted (Kenakin, 1984; Ruffolo,

Table 1 Histamine H_2-receptor constants in the guinea-pig right atrium

Constant	*Agonist*			
	Histamine	*4-Methylhistamine*	*Dimaprit*	*Impromidine*
EC_{50}(μM)	0.67 ± 0.33 (10)	1.76 ± 0.21 (9)	1.12 ± 0.25 (10)	0.0012 ± 0.0002 (8)
K_D (μM)	15.2 ± 5.1 (8)	171 ± 86 (7)	77.1 ± 26 (5)	0.0184 ± 0.0058 (8)
K_i (μM)	43	270	44	0.063
Receptor reserve (%) at maximum stimulation	16	68	56	19
Relative efficacy	1	3.46	2.49	0.57

The presented data are mean values ± s.e.mean for the number of experiments shown in parentheses. The K_i values are those reported by Gajtkowski *et al.* (1983) in guinea-pig cerebral cortex using an H_2-specific [^{3}H]-tiotidine radioligand binding assay. The relative efficacies quoted are related to histamine which has been given an arbitrary value of 1.

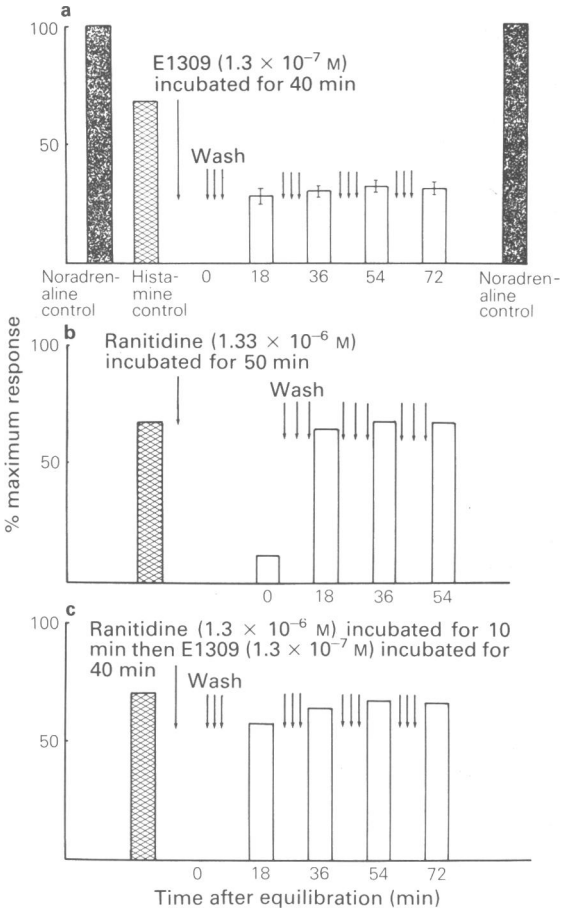

Figure 3 Experiments to determine the reversibility of antagonism by E1309 and ranitidine. Values represent means (± s.e. for (a) for the number of determinations indicated. (a) E1309 alone, noradrenaline was added at the beginning and at the end to demonstrate the viability of the tissue ($n = 4$); (b) ranitidine alone ($n = 2$); (c) ranitidine and E1309, ranitidine being added 10 min prior to E1309 ($n = 6$).

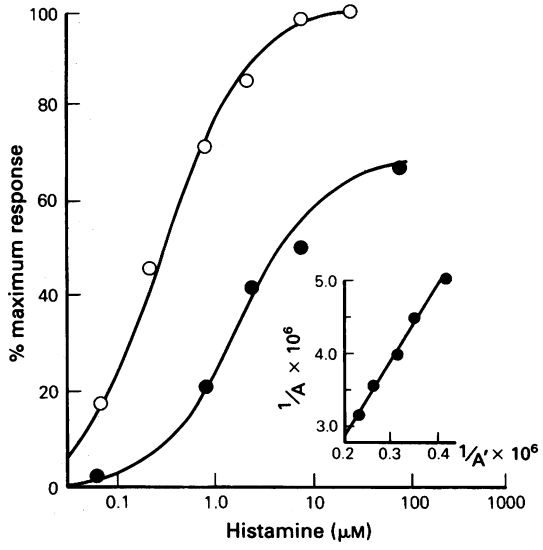

Figure 4 Determination of K_D, the dissociation constant for histamine in the guinea-pig right atrium. Cumulative dose-response curves are illustrated in the presence (●) and absence (○) of E1309. Pairs of concentrations (A and A') having equal effects are then plotted as reciprocals 1/A versus 1/A' (as shown in the inset) which obey a straight line equation (calculated by linear regression analysis) from which K_D is derived.

1982), and have been investigated in relation to a number of different receptor types. However, to date no results have been described for the histamine H_2-receptor, presumably due to the lack of a specific compound that would satisfy all the criteria for being an irreversible H_2-antagonist. Care must be taken to avoid compounds with a general non-specific depressant effect as recently reported for the β-adrenoceptor antagonist Ro 03-7894 on atrial preparations (Baker & Posner, 1983; Kostew *et al.*,1984). Using the guinea-pig isolated right atrium, a tissue highly suited for the investigation of histamine H_2-specific effects (Black *et al.*,1972), we have shown that the compound E1309 meets the necessary criteria.

Firstly, by inhibiting the chronotropic response induced by H_2-agonists in a dose-dependent manner, E1309 can be classed as a histamine H_2-antagonist. Specificity for the H_2-receptor is indicated from the observation that the atrium still remained fully responsive to the β-agonist noradrenaline although the histamine-induced chronotropic effect was markedly reduced. Moreover the inhibition at higher doses was non-surmountable. Secondly, since increasing the incubation time produced greater inhibition, this would suggest a slow on-rate or a slow off-rate or both. Thirdly, the wash-out experiments demonstrated that once bound to the receptor, E1309 apparently remained bound for at least 112 min after its initial introduction to the organ bath. This period was substantially longer than that employed in later experiments for the determination of agonist receptor constants. Lastly, the receptor protection study with the competitive H_2-antagonist ranitidine (Brittain & Daly, 1981) confirmed that the E1309 effects were related to the H_2-receptor. Although other explanations could be advanced to account for the observed E1309 activity, such as metabolism by cardiac tissue or internalization and resultant non H_2-effects, these are

extremely unlikely. Ideally, absolute confirmation of H_2-receptor occupancy could be obtained from radioligand binding studies. However, although from such studies with [^{3}H]-tiotidine in the cerebral cortex E1309 had definite H_2-properties, the binding method is not applicable to cardiac preparations (Gajtkowski, Norris & Rising, personal communication). Thus on the available data, E1309 can be classified as an irreversible H_2-antagonist. The chemical mechanism for this phenomenon has not been investigated, but would probably be related to the terminal nitrogen on the thiatriazine ring. This nitrogen is very basic and will undergo protonation, thus leading to very strong co-valent bond formation on nucleophilic sites.

In experiments based on those of Furchgott & Bursztyn (1967), and in which E1309 was used, the H_2-agonists, with the exception of impromidine, had low affinities for the H_2-receptor. There was a good correlation between the derived dissociation constants and the K_i values determined from binding studies in the guinea-pig cerebral cortex. Although this correlation is based on only limited data, it provides additional evidence confirming the validity of the method. The relative activities of two of the agonists compared to histamine were similar to those reported by others. We found 4-methylhistamine and dimaprit to have 38% and 60% the activity of histamine respectively, compared with corresponding values of 43% (Black *et al.*, 1972) and 71% (Flynn *et al.*, 1979). Although the rank orders of potency and affinity (EC_{50} and K_D values) were the same, being impromidine > histamine > dimaprit > 4-methylhistamine, the potency values were markedly lower. The disparity between EC_{50} and K_D values reinforces the danger of assuming that EC_{50} measurements are an accurate reflection of the dissociation constant.

However, in contrast to the order of potency, the relative efficacy was 4-methylhistamine > dimaprit > histamine > impromidine. These differences in efficacy were reflected in a low receptor reserve for histamine and impromidine at maximum chronotropic activity, demonstrating that they are close to becoming classified as partial agonists in the tissue. Impromidine has been reported to be a partial agonist in a number of tissues including rat uterus (Durant *et al.*, 1978), rabbit atrium (Tenner, 1981) and the isolated whole stomach of the rat (Parsons & Sykes, 1980), suggesting a lower receptor reserve than that for histamine. In the guinea-pig right atrium, we and others (Durant *et al.*,1978) have shown impromidine to be a full agonist, the receptor reserves for the two agonists being similar. It is difficult to explain these findings, although Al-Gadi & Hill (1985) have recently suggested differences in agonist selectivity of the H_2-receptor in different tissues. Also in tissues where impromidine acts as a partial agonist, K_D should equal the EC_{50}. Hence in the isolated stomach preparation and the rabbit atrium the K_D values would be 0.3 μM and 11.7 nM respectively, lying either side of the value of 18.4 nM obtained by us.

The relatively low capacity of histamine to elicit a response compared to dimaprit and 4-methylhistamine, would appear to be atypical for a natural agonist. For example, acetylcholine in the circular smooth muscle of rabbit stomach fundus was the most efficacious muscarinic receptor agonist (Furchgott & Bursztyn, 1967) as was noradrenaline acting on α-receptors in the isolated rabbit aorta (Besse & Furchgott, 1976).

Compounds with high relative receptor affinities need not have high relative intrinsic efficacies. Such is the case with isoprenaline, which had a higher affinity than both terbutaline and orciprenaline on β-adrenoceptors mediating positive chronotropic responses in the guinea-pig isolated atria but a lower relative efficacy than the other β-agonists (Broadley & Nicholson, 1978). This situation was not found when inotropic effects were measured in the atrial preparation. Differences in relative efficacies relating to different effects (e.g. chronotropy and inotropy) in the same tissue could have therapeutic implications. Recently, Baumann *et al.* (1982) proposed the use of H_2-agonists in the non-ischaemic surviving myocardium damaged by excessive exposure to catecholamines. The stimulation of uninvolved H_2-receptors by H_2-agonists like impromidine to produce contractile responses was thought to be beneficial in this disease state. It is envisaged that a large receptor reserve for inotropic activity relative to chronotropic effects (and indeed parietal cell H_2-receptors mediating gastric acid secretion) would be required. The relatively low receptor reserve for impromidine found in the present study, albeit in guinea-pig and not man, would appear to be compatible with part of this requirement.

The work from our laboratory has led to the first reported determinations of receptor constants for histamine H_2-agonists. This has been achieved by comparison of dose-response curves before and after irreversible blockade of a portion of the total H_2-receptor pool by a compound (E1309) specific for the H_2-receptor. This study in the guinea-pig isolated right atrium preparation could be extended to other H_2-receptor containing tissues (e.g. rat uterus) where relative affinities and efficacies might be different.

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