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Agonist and antagonist effects of histamine H_3 receptor ligands on 5-HT_3 receptor-mediated ion currents in NG108-15 cells

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Abstract

The ability of histamine H_3 receptor ligands to interact with 5-HT_3 receptors in NG108-15 cells was studied using the whole cell patch clamp recording technique. Imetit, a histamine H_3 receptor agonist, generated inward currents and exhibited weak partial agonist activity at the 5-HT_3 receptor ($EC_{50} = 11.8$ μM). Imetit-induced currents were slow to desensitize and at a high concentration reduced in size. The histamine H_3 receptor antagonists iodophenpropit and thioperamide did not generate inward currents but were able to inhibit 5-hydroxytryptamine (5-HT) responses with an IC_{50} of 1.57 ± 0.3 μM and 13.7 ± 3.5 μM, respectively. Thioperamide is probably a non-competitive antagonist which may have more than one binding site on the receptor.

Keywords: 5-HT_3 receptor; NG108-15 cell; Histamine H_3 receptor ligand; Thioperamide; Imetit; Iodophenpropit

1. Introduction

A large number of histamine H_3 receptor ligands have been developed to enable pharmacologists to probe the role of the histamine H_3 receptor. The potency and selectivity of these ligands have been investigated using radioligand binding and in vitro techniques. These studies have generally shown selectivity for histamine H_3 receptors over H_1 and H_2 subtypes, but less is known about the affinities for other non-histamine receptors. Recently, binding studies by Leurs et al. (1995) have established that the histamine H_3 agonist imetit and the histamine H_3 receptor antagonists thioperamide and iodophenpropit can displace the selective 5-HT_3 receptor ligand [^{3}H] GR65630 from rat cortex membranes. In addition, these workers have shown that iodophenpropit and thioperamide are 5-HT_3 receptor antagonists in the guinea pig ileum, whilst imetit is a potent 5-HT_3 receptor agonist in this preparation. Some of these compounds may therefore have the capacity to interact with ligand-gated ion channels and these aspects of their pharmacology have not been investigated using electrophysiological techniques. In the present study, the patch clamp technique was used to study the influence of histamine H_3 receptor ligands on macroscopic 5-HT_3 receptor-mediated currents in NG108-15 cells. A rapid drug application system was used to study whole cell currents and this system allowed the measurement of kinetic events such as desensitisation.

2. Materials and methods

2.1. Culture of neuroblastoma × glioma hybrid cells

Mouse neuroblastoma × rat glioma hybrid cells, NG108-15, were grown in Dulbecco's modified Eagle's medium (DMEM Glutamax) supplemented with 10% foetal calf serum, 2% hypoxanthine, aminopterine and thymidine (HAT) supplement and 50 μg/ml gentamicin. The undifferentiated cells were plated at a density of approximately 5×10^4 cells per 35 mm diameter culture dish, at least 24 h before use in electrophysiological experiments.

2.2. Whole cell patch-clamp recording

Agonist-activated macroscopic currents were recorded from whole cells by use of standard patch clamp techniques (Hamill et al., 1981) using a PC501A patch clamp amplifier (Warners Instruments). Patch electrodes were fabricated from borosilicate glass tubing and, when filled with the pipette solution detailed below, had a resistance of between 2–5 MΩ. NG108-15 cells were viewed through

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an inverted microscope and continuously superfused with an extracellular solution containing (in mM): 136 NaCl, 2.6 $CaCl_2$, 2.4 KCl, 1.2 $MgCl_2$, 15 HEPES, 10 glucose, (titrated with 3 M NaOH to pH 7.4). Perfusion of the experimental chamber was at a rate of approximately 1 ml min^{-1}, the volume of fluid within the chamber being held constant by continuous aspiration and the temperature of the perfusion fluid was maintained at 23°C ± 2°C. Patch electrodes were filled with a solution containing (in mM): 110 KCl, 3.0 $MgCl_2$, 40 HEPES, 3 EGTA, (titrated with 3 M KOH to pH 7.4) (Docherty et al., 1991).

2.3. Agonist application

Agonist induced currents were recorded from NG108-15 cells voltage clamped at a membrane potential of −80 mV. The cell recorded from was continuously superfused with extracellular solution via one of the two square capillaries (width 800 μm) glued together and mounted on a step motor-operated SF-77 perfusion fast-step system (Warner Instruments). Currents were evoked by stepping from one capillary with extracellular solution (± ligand) to the other capillary with agonist (± other ligands) containing extracellular solution. Application of agonist was for 5 s and the applications were repeated every 90 s to avoid desensitization.

The speed of the solution exchange was tested with open pipette experiments. An open pipette voltage clamped at 0 mV was exposed to test solutions of different ionic composition from the control solution. The change in clamp current occurred in less than 20 ms, measuring the rise time as the time interval between 10% and 90% of the total response (Bufler et al., 1996b; Dudel et al., 1992).

2.4. Data analysis

Agonist responses from each cell were normalised to the 10 μM 5-HT response. Concentration–response curves for agonists were fitted iteratively to the following equation using Fig P software (Biosoft Cambridge):

$$I = I_{max} / \left(1 + \left(\left([A]/EC_{50}\right)^{-n_H}\right)\right),$$

where I_{max} is the maximum normalised current response (in the absence of antagonists), $[A]$ the concentration of agonist, EC_{50} the concentration of agonist to produce a 50% maximum response, and n_H is the Hill coefficient.

Concentration–inhibition curves were fitted to the following equation:

$$I = 1 - \left(I_{max} / \left(1 + \left(\left([I]/IC_{50}\right)^{-n_H}\right)\right)\right),$$

where $[I]$ is the concentration of antagonist, IC_{50} is the concentration of antagonist producing 50% inhibition.

Values reported are mean ± S.E.M. Mean values were compared using paired two tailed *t*-tests as appropriate; $P < 0.05$ was considered significant.

2.5. Drugs

Drugs used were: D-tubocurarine chloride (Sigma–Aldrich); tropisetron hydrochloride (Sigma–Aldrich); 5-hydroxytryptamine creatine sulphate (Sigma–Aldrich); imetit dihydrobromide (*S*-[2-(4-imidazolyl)ethyl]isothiourea) (Tocris Cookson, UK); thioperamide maleate (*N*-cyclohexyl-4-(imidazol-4-yl)-1-piperidinecarbothioamide) (Tocris Cookson, UK); iodophenpropit dihydrobromide (*N*-[2-(4-iodophenyl)ethyl]-*S*-[3-(4(5)-imidazolyl)propyl]isothiourea) (Tocris Cookson, UK).

3. Results

In undifferentiated NG108-15 cells held at −80 mV, superfusion with 5-HT triggered inward currents which were completely blocked by 10 nM D-tubocurarine, consistent with the activation of 5-HT_3 ligand-gated channels (data not shown). Typically 5-HT-induced currents had a rapid onset followed by a slower biphasic decay due to receptor desensitisation. During the first 10 min of recording there was an increase in amplitude and slowing of the rate of decay of the 5-HT responses. These changes are probably due to changes in the cytoplasmic milieu that occur during whole-cell recording which lead to a decreased rate of desensitization (Yakel et al., 1991). All experiments were therefore undertaken after this initial period of stabilisation.

3.1. Imetit as a 5-HT_3 receptor agonist

5-HT-induced currents showed saturation with increasing concentration and displayed an EC_{50} of 3.0 ± 0.1 μM and a Hill coefficient of 3.1 ± 0.5 (Fig. 1A,B). Imetit appeared to be a much weaker agonist than 5-HT at the $5HT_3$ receptor, requiring larger concentrations and producing a smaller maximal response. High concentrations of imetit (> 100 μM) produced smaller than maximum responses (Fig. 1B). These higher concentrations were not used in the determination of the EC_{50}, the Hill coefficient (n_H), or maximal response for imetit (Table 1). Bell-shaped concentration response relationships such as this are indicative of 'agonist block'. Imetit therefore appears to be a weak partial agonist at the 5-HT_3 receptor. In addition, the time course of imetit responses was different from that induced by 5-HT itself where currents rose rapidly to a peak and then desensitized despite its continued presence (Fig. 1A).

Imetit-induced currents, however, showed a slow increase during the course of its application, were of small size and showed no tendency to desensitise (Fig. 1A). Most 5-HT_3 receptor agonists are potent desensitisers of the 5-HT_3 receptor producing desensitisation within sec-

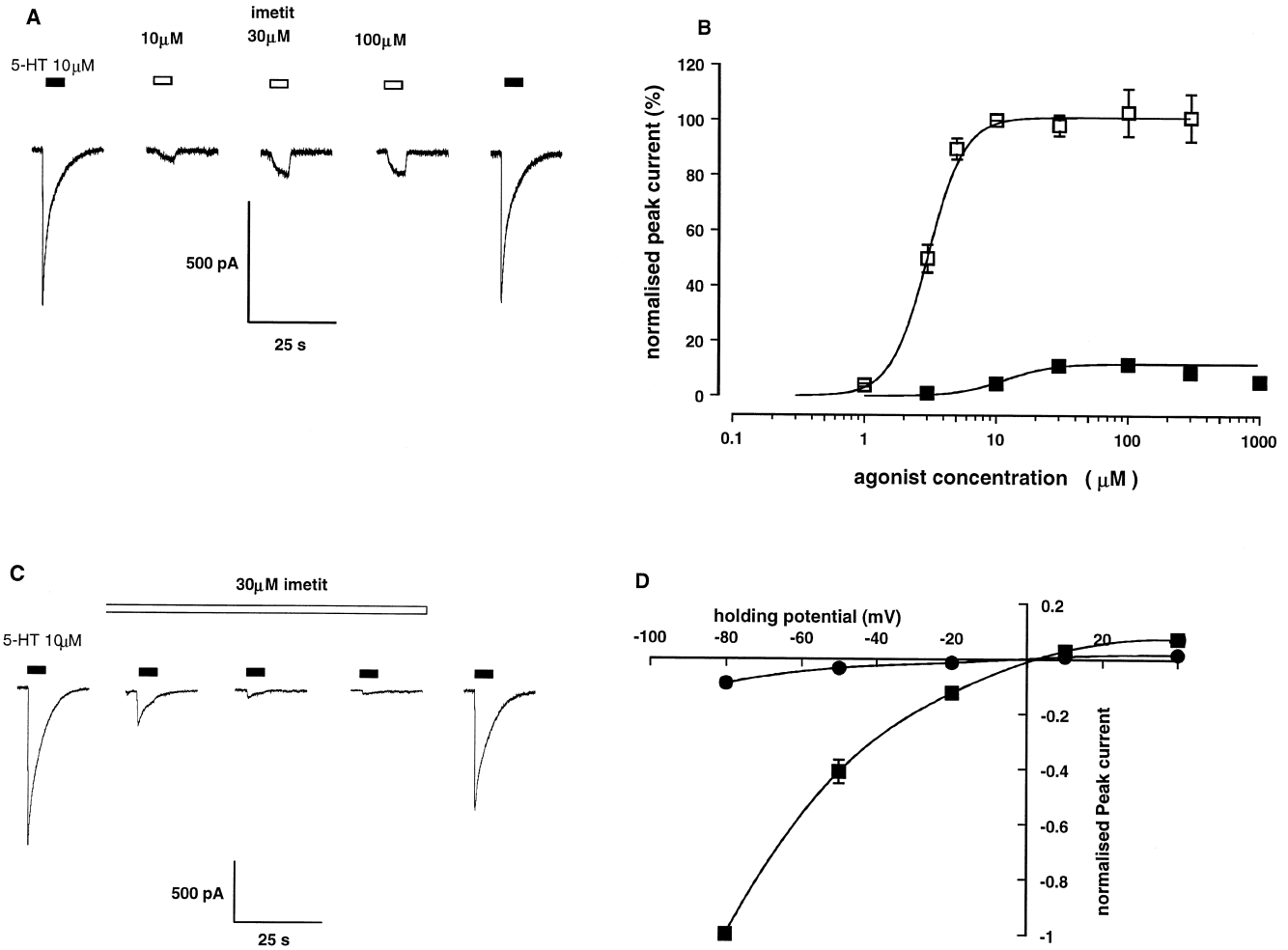


Fig. 1. Concentration–response data for imetit-evoked currents recorded from NG108-15 cells held at −80 mV. (A) Typical current responses to increasing concentrations of imetit in a single NG108-15 cell. Solid horizontal bars indicate the period of 5-HT application. Open horizontal bars indicate the period of imetit application. (B) Concentration–response curve for 5-HT- and imetit-evoked currents. (□) Represents responses to 5-HT alone, and (■) represents responses to imetit alone. Peak inward currents were normalized to currents evoked by 10 μM 5-HT. Each point represents the mean (±S.E.M.) of at least four cells. (C) The effect of continuous application of 30 μM imetit on the response to 10 μM 5-HT in a single NG108-15 cell. Solid horizontal bars indicate the period of 5-HT application. Open horizontal bars indicate imetit application. Cells were pretreated with imetit for 70 s, to allow desensitisation to develop, before the first simultaneous application of 5-HT (10 μM). (D) The current–voltage relationship for 5-HT (10 μM, ■) and imetit-(30 μM, ●) induced current in voltage clamped cells ($n = 3$). The curve was obtained by measuring the ligand-induced current at different membrane potentials (from −80 to +40 mV). Peak inward currents were normalized to currents evoked by 10 μM 5-HT.

onds. To investigate this apparent lack of desensitisation more prolonged applications (5 min) of 10 or 30 μM imetit were employed. These applications produced a small response that completely desensensitised within 50 s (data not shown). During the subsequent desensitised period, responses to brief applications of 10 μM 5-HT were also inhibited (Fig. 1C), hence confirming the phenomenon of cross desensitisation between 5-HT_3 receptor agonists already described in this cell line (Bartrup and Newberry, 1996).

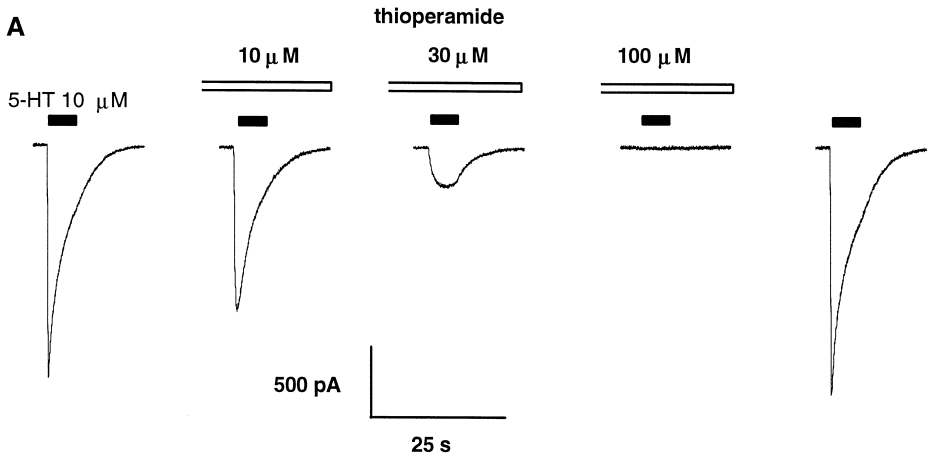
Table 1
Summary of EC_{50}, IC_{50}, and Hill coefficients for histamine H_3 receptor ligands with the 5-HT_3 receptor in NG108-15 cells

Ligand	EC_{50} (μM)	E_{max} (%)	IC_{50} (μM)	n_H	n
5-HT	3.0±0.1	100	–	3.1±0.5	5
Imetit	11.7±1.9	11.9±0.9	–	2.3±0.9	7
Thioperamide	–	–	15.3±1.6	1.7±0.3	9
Iodophenpropit	–	–	1.6±0.3	1.2±0.2	5

Values represent mean±S.E.M. of parameters estimated from n cells.
E_{max} values are normalised to the fitted E_{max} value of 5-HT.
Holding potential −80 mV.

Fig. 1D shows the current–voltage relationship for 5-HT (10 μM) and imetit (30 μM) induced current in voltage clamped NG108-15 cells. The curves were obtained by measuring peak agonist-induced currents at a series of different test membrane potentials. Both I/V plots display a degree of inward rectification and reverse at approximately 0 mV.

At a holding potential of −80 mV, D-tubocurarine (10 nM) strongly inhibited the imetit and 5-HT response by 82% ± 9.1% ($n = 6$) and 88% ± 1.3% ($n = 6$) respec-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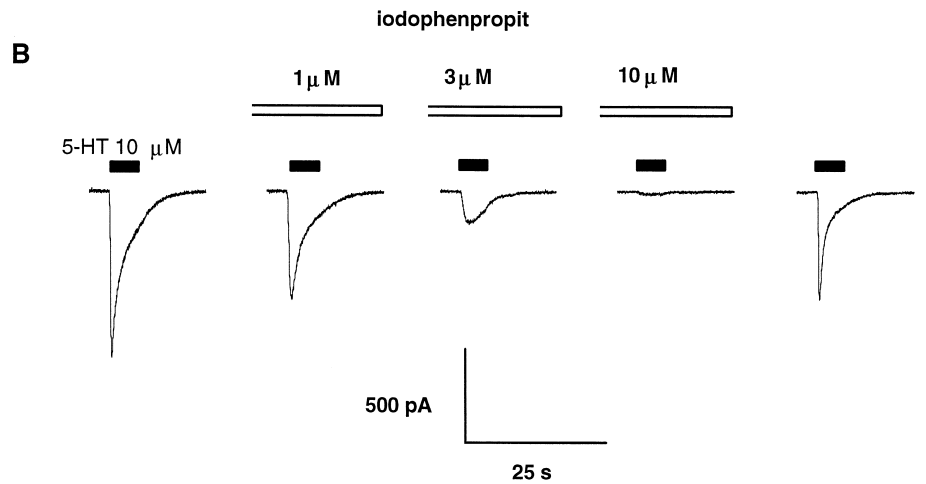
B
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1μM
3μM
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5-HT 10 μM
500 pA
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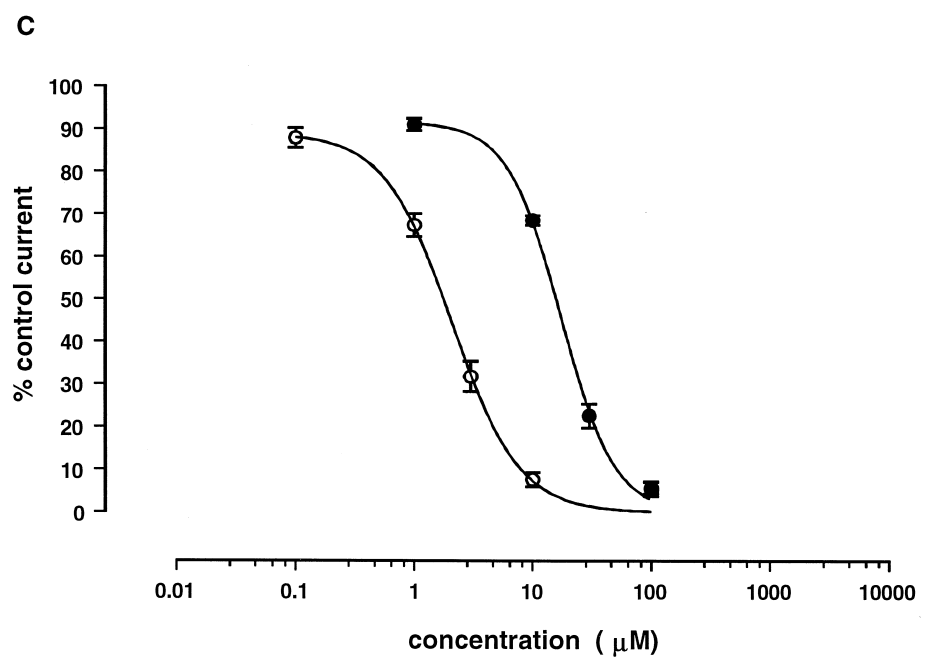
C
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tively, which is consistent with both agonists activating the murine 5-HT_3 receptor. In addition, the selective 5-HT_3 receptor antagonist, tropisetron (10 nM), completely inhibited both 5-HT and imetit responses ($n = 4$).

3.2. Effect of histamine H_3 receptor antagonists on 5-HT induced currents

Both iodophenpropit and thioperamide failed to generate inward current at concentrations up to 100 μM and showed no agonist activity on NG108-15 cells.

Thioperamide and iodophenpropit inhibited the 5-HT (10 μM) response in a reversible concentration-dependent manner (Fig. 2A,B,C) with iodophenpropit being approximately tenfold more potent than thioperamide. Table 1 summarises the potencies, efficacies and Hill coefficients for activation and inhibition of 5-HT_3 receptor-mediated currents in NG108-15 cells. Thioperamide resulted in a steep inhibition with a Hill coefficient of greater than one. Because of this its competitive nature was further investigated and it was found that increasing concentrations of thioperamide moved the log concentration response relationship to the right whilst at the same time depressing the maximum response (Fig. 3A). This indicates a non-competitive antagonism of 5-HT responses. Many non-competitive antagonists at ligand-gated ion channels show a voltage dependent blocking of the open channel. However, in this study inhibition of 5-HT responses did not appear to show such dependence (Fig. 3B).

3.3. Effect of histamine H_3 receptor antagonists on the desensitisation of the 5-HT current

Many non-competitive inhibitors of 5-HT_3 receptors in neuronal cell lines have been shown to increase the rate of desensitisation (Fan, 1994a,b). Consequently, the effect of thioperamide and iodophenpropit on this process was investigated. After an initial period of stabilisation upon achieving whole cell recording conditions, stable 5-HT responses could be recorded and the desensitisation of the inward current could be described by a bi-exponential decay process (Fig. 4). The time course of desensitisation was quantified by curve fitting the decaying phases of responses to a sum of two exponentials. The kinetic parameters obtained from the double exponential fits were τ_f (the fast time constant)and τ_s (the slow time constant). Following application of thioperamide or iodophenpropit the decaying phase of the response could only be fitted to

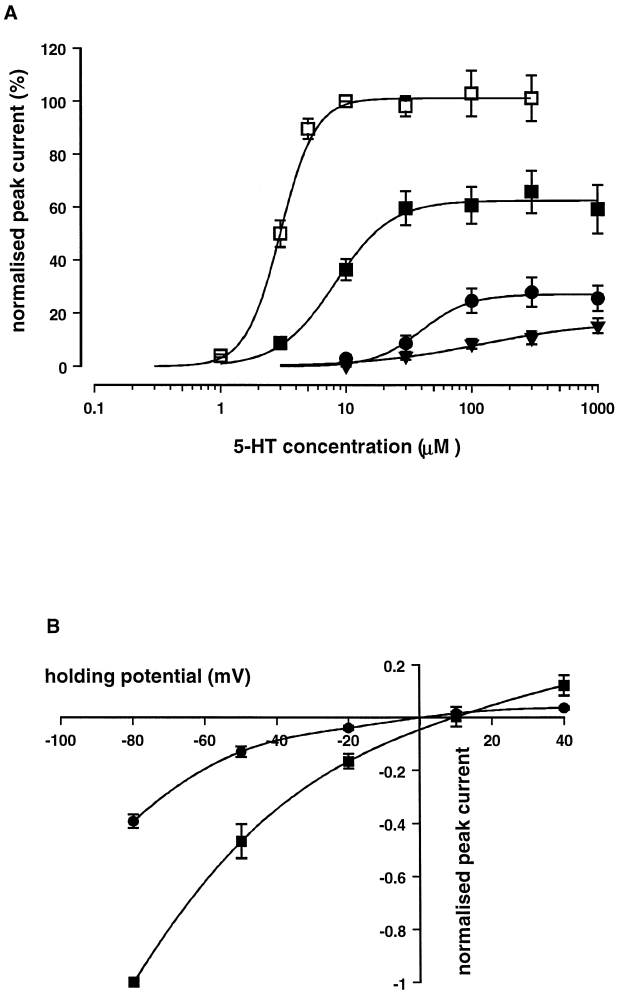

Fig. 3. Inhibition of 5-HT response by thioperamide. (A) The concentration response relationship for 5-HT in the absence (□) or presence of 10 μM thioperamide (■), 30 μM thioperamide (●) and 100 μM thioperamide (▼). Each point represents the mean ± S.E.M. of responses obtained from at least 4 cells. (B) Plots of 5-HT (10 μM)-induced current amplitude vs. holding membrane potential in the absence (■) and presence (●) of 10 μM thioperamide ($n = 4$). In both A and B peak inward currents were normalized to currents evoked by 10 μM 5-HT.

Fig. 2. The inhibition of 5-HT-evoked currents by thioperamide and iodophenpropit. (A) Effect of thioperamide on 5-HT-evoked inward currents in a single NG108-15 cells held at −80 mV. Solid horizontal bars indicate the period of 5-HT application. Open horizontal bars indicate the period and concentration of thioperamide application. Cells were pretreated with thioperamide for 70 s before the simultaneous application with 5-HT (10 μM). (B) Effect of iodophenpropit on 5-HT evoked inward currents in a single NG108-15 cells held at −80 mV. Solid horizontal bars indicate the period of 5-HT application. Open horizontal bars indicate the period and concentration of iodophenpropit application. Cells were pretreated with iodophenpropit for 70 s before the simultaneous application with 5-HT (10 μM). (C) Concentration dependence of block of 5-HT-induced current by iodophenpropit (●) and thioperamide (○). Each point is the mean (± S.E.M.) of at least four cells.

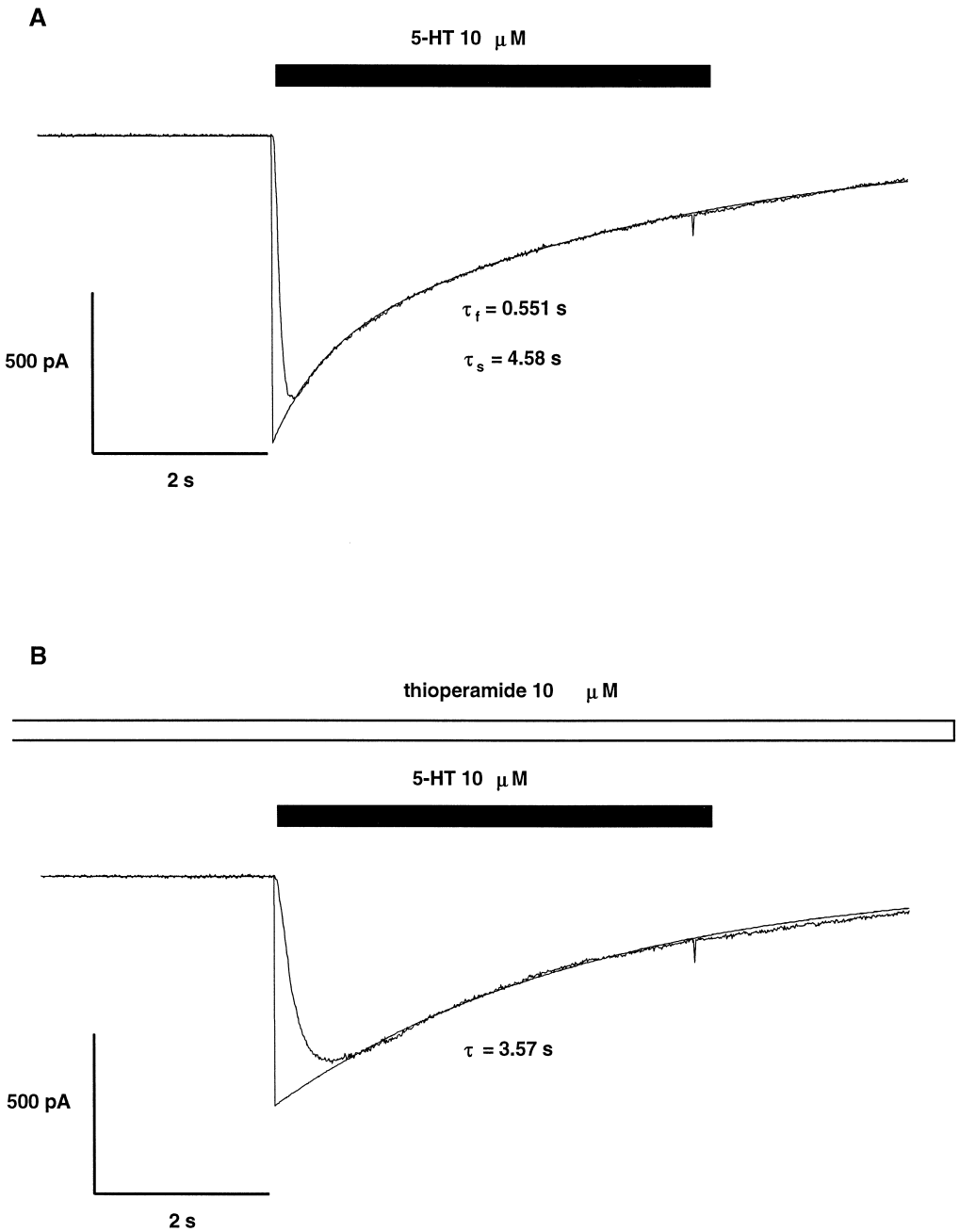

Fig. 4. The effects of thioperamide on the desensitisation of 5-HT responses. (A) The time course of a 5-HT (10 μM)-induced current in the absence of thioperamide. The desensitisation phase can be fitted to the sum of two exponential decays. (B) The time course of a 5-HT (10 μM)-induced current in the same cell but in the presence of thioperamide (10 μM). The desensitisation phase can be fitted to one exponential decay.

Table 2
The kinetic parameters of desensitisation before and following the addition of thioperamide or iodophenpropit

Treatment	τ_f (s)	τ_s (s)	Rise time (s)	n
Control	0.57 ± 0.04	6.21 ± 0.75	0.10 ± 0.01	6
Thioperamide 10 μM	–	3.75 ± 0.37[a]	0.34 ± 0.01[a]	6
Control	0.55 ± 0.03	4.41 ± 0.45	0.14 ± 0.03	5
Iodophenpropit 1 μM	–	1.55 ± 0.25[a]	0.29 ± 0.04[a]	5

Holding potential −80 mV.
Rise time is equal to the time to transverse between 10% and 90% of peak current,
[a] $P < 0.05$ compared with paired controls.

a single slow exponential (τ_s) and the rise time of the response was considerably slowed (Table 2; Fig. 4).

4. Discussion

5-HT_3 receptors, like other ligand gated ion channels, are thought to have more than one agonist binding site per channel. This results in a steep agonist concentration–response curve and a Hill coefficient greater than one. A pronounced feature of the 5-HT_3 receptor is its rapid

desensitisation in response to 5-HT, the presence of which can lead to an underestimation of the Hill coefficient for agonist responses. In the present investigation, 5-HT was applied using a fast application system that allowed solution exchange within 20 ms to minimise the effects of desensitisation. Analysis of the concentration response relationship for 5-HT yielded a Hill coefficient of 3.1, indicative of at least 2 and possibly 3 agonist binding sites on the 5-HT_3 receptor. The imetit concentration–response curve was also steep, yielding a Hill coefficient of 2.3 close to that for 5-HT. Leurs et al. (1995) have described imetit as a spasmogen and 5-HT_3 receptor agonist in the isolated guinea pig ileum ($pD_2 = 4.72$) only 7-fold less potent than the selective 5-HT_3 receptor agonist 2-methyl 5-HT ($pD_2 = 5.54$). However the data presented here show that, in voltage clamped NG108-15 cells, imetit exhibits weak partial agonist activity ($EC_{50} = 11.8$ μM). In the guinea pig ileum agonist binding to the 5-HT_3 receptor is distinct from the recorded contractile response, consequently few receptors may need to be occupied in order to initiate a maximal contraction. The presence of these spare receptors may mean that a weak partial agonist can produce a maximal response. In voltage clamped cells this phenomenon cannot occur as all agonist-induced channel opening can be directly recorded as an increase in clamp current, consequently there can be no spare receptors and partial agonists produce reduced maximal responses.

The currents evoked by imetit in voltage clamped NG108-15 cells were markedly different from the characteristic currents induced by 5-HT. In particular, they were slow in onset and showed little desensitisation over the usual 5 s application period. Consequently, it could be argued that imetit is not an agonist at 5-HT_3 receptors in this cell line. However, despite the slow time course of the imetit response other data suggests that it is a partial agonist. First, the imetit and 5-HT responses showed similar voltage dependence which is also consistent with both compounds being agonists at the same receptor. Second, both 5-HT and imetit responses were blocked by low concentrations of D-tubocurarine and tropisetron which is indicative of an action at murine 5-HT_3 receptors. Lastly, prolonged application of 30 μM imetit (greater than 50 s) was able to desensitize 5-HT_3 receptors and completely inhibit the response to 5-HT. The cross-desensitization observed between imetit and 5-HT in these experiments is consistent with both ligands acting at the same receptor (Neijt et al., 1988; Bartrup and Newberry, 1996). An alternative explanation for the observed inhibition of 5-HT responses by prolonged application of imetit is partial agonism which, in the presence of high concentrations of 5-HT, may behave more as an antagonist than an agonist. Whatever the mechanism of inhibition, the possibility that imetit acts on distinct receptors can also be excluded, since imetit completely suppressed the 5-HT response.

Partial agonism has been thoroughly studied at ligand-gated ion channels and a number of explanations for this phenomenon have been proposed. del Castillo and Katz (1957a,b) suggested that partial agonism results from a poor ability of bound agonist to induce channel opening. Van Hooft and Vijverberg (1996) have proposed that conformational selection may account for partial agonist activity at the 5-HT_3 receptor. They suggest that partial agonists act on a resting receptor conformation that is less prevalent than that available for full agonists. However, these theories cannot explain the bell-shaped concentration response relationship observed with imetit. Studies at the single channel level with nicotinic ligands suggest that many 'partial agonists' are in fact full agonists with intrinsic channel blocking activity. These compounds produce bell-shaped log concentration–response curves where responses to high concentrations of agonist are lower than the observed maximum response (Marshall et al., 1990, 1991). In the experiments reported here imetit, but not 5-HT, consistently produced small responses at high concentrations and this may be indicative of ion channel blockade. Single channel analysis should help resolve the nature of imetit's action.

This study confirmed the work of Leurs et al. (1995) by showing that micromolar concentrations of thioperamide and iodophenpropit can block 5-HT_3 receptors. Several lines of evidence suggest that the antagonism of the 5-HT_3 receptor by thioperamide is non competitive. First, the steep inhibition curve for thioperamide with a Hill coefficient greater than one is not compatible with a simple competitive inhibition at a single site. Second, the concentration response curves for 5-HT in the presence of increasing concentrations of thioperamide showed a depression of the maximal response as well as a shift to the right indicative of a non-competitive mechanism. Third, both thioperamide and iodophenpropit increased the rate of receptor desensitisation and this phenomenon is often associated with non-competitive mechanisms.

Such mechanisms include allosteric modulation, open channel block and closed channel block (Allen et al., 1998). Of these, open channel block is unlikely because thioperamide (i) slowed the rise time of the response, (ii) depressed the peak amplitude, (iii) showed little voltage-dependent inhibition and (iv) showed no obvious tail currents due to channel unblock on antagonist removal (Bufler et al., 1996a,b; Dudel et al., 1992; Fan, 1994a,b; Prior et al., 1995). This leaves allosteric modulation or closed channel block which are difficult to distinguish. Finally, the steep inhibition curves for thioperamide with a Hill coefficient greater than one suggest multiple binding sites on the 5-HT_3 receptor for thioperamide. Consequently, thioperamide could block 5-HT responses by a mixture of mechanisms.

The ability of these H_3 ligands to affect 5-HT_3 receptors could compromise their use as selective probes for H_3 receptor function. For example, Coruzzi et al. (1995) have demonstrated that imetit can decrease basal arterial pressure but that these effects are inhibited by atropine and

ondansetron and are probably due to imetit stimulating 5-HT_3 receptors and the von Bezold–Jarisch reflex. Leurs and others (1996) have also demonstrated that thioperamide can inhibit this reflex by inhibiting 5-HT_3 receptors in vivo. This action should therefore be considered in future when using these compounds in vivo especially when the true concentration of the drug at the site of action is unknown.

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