Br. J. Pharmac. (1985), **85**, 65–73

Histamine is an antagonist of the acetylcholine receptor at the frog endplate

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1 The effects of histamine on the acetylcholine (ACh) receptor-channel complex were examined by means of voltage-clamp at the frog endplate. ACh was ionophoretically applied to the endplate. Histamine was added to the perfusate.

2 Histamine (100 nM – 1 mM) reversibly depressed the peak amplitude of the ACh-induced inward current in a dose-dependent manner.

3 The double reciprocal plot of the dose-response relationship between the peak ACh current and the amount of ACh applied suggested that histamine (100 μM) depressed the ACh-induced current in a competitive manner.

4 Histamine prevented the specific ACh binding site within the receptor-channel complex from binding erabutoxin, a sea-snake venom, which binds irreversibly to the specific ACh binding site.

5 Histamine had no detectable effects on the equilibrium potential of the endplate current but shortened the half-decay time of the endplate current in a voltage-dependent manner.

6 It was therefore concluded that histamine blocks not only the specific ACh binding site but also interacts with the ACh-channel site.

7 The present experiments strongly suggest that histamine can act as an antagonist to modulate nicotinic cholinergic transmission.

Introduction

It has been suggested that histamine, an endogenous amine and a putative neurotransmitter at aminergic synapses in the brain, may have physiological functions as a 'neuromodulator' at other (such as cholinergic) types of synapses (Yamada *et al.*, 1982). Activation of presynaptic histamine receptors (both H_1 and H_2 types) can either increase or decrease the release of acetylcholine (ACh) from preganglionic nerve terminals in bullfrog sympathetic ganglia (Yamada *et al.*, 1982) and motor nerve terminals at frog neuromuscular junctions (Scuka, 1973). On the other hand, histamine depresses the sensitivity of frog endplate receptors to ACh by some unknown mechanisms (Scuka, 1973).

The aim of the present study was to clarify the mechanisms which underlie the inhibitory postsynaptic actions of histamine at the frog endplate. Two main approaches were employed. ACh-receptor interactions were analysed by Lineweaver-Burk plot in the absence or presence of histamine. Next, the effect of histamine on the ACh current was compared to that of erabutoxin (Tamiya & Arai, 1966; Sato & Tamiya, 1971).

The results obtained demonstrated that histamine may act as an antagonist of ACh at the binding site of the nicotinic receptor-ionic channel complex. A preliminary account of this work has been published previously (Ohta *et al.*, 1984).

Methods

The sciatic nerve-sartorius muscle preparation of the frog (*Rana nigromaculata*) was used for the experiments. Muscles mounted on a Sylgard based lucite chamber were continuously perfused with Ringer solution of the following composition (mM): NaCl 112, KCl 2.0, $CaCl_2$ 1.8, $NaHCO_3$ 2.0 (pH was 7.2–7.3, adjusted using HCl). The pH of the histamine-containing Ringer solution was also adjusted to between 7.2 and 7.3 with NaOH. Glass microelectrodes filled with 3 M KCl (d.c. resistance; 10–20 MΩ) were used for intracellular recordings. The criteria for the acceptable positioning of the electrode at the endplate were a rapid ($<$1.0 ms) rise time of the endplate potential (e.p.p.) and the presence of min-

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iature e.p.ps. For voltage clamping, the two-microelectrode method was used, as has been described by Takeuchi & Takeuchi (1959) and Kuba *et al.* (1976). A current-passing electrode filled with 3 M KCl (d.c. resistance; $< 5\ M\Omega$) was inserted within 50 μm away from the point of impalement of the potential-recording electrode. Muscle contraction was prevented by pretreatment of the preparation with glycerol (400 mM)-containing Ringer solution for 60 min (Fujino *et al.*, 1961; Gage & Eisenberg, 1967). The quantal content of e.p.ps was estimated by means of the variance method in low Ca^{2+} (0.9 mM) and high Mg^{2+} (5.0–7.0 mM) Ringer solution (Del Castillo & Katz, 1954). Acetylcholine (ACh) was ionophoretical-

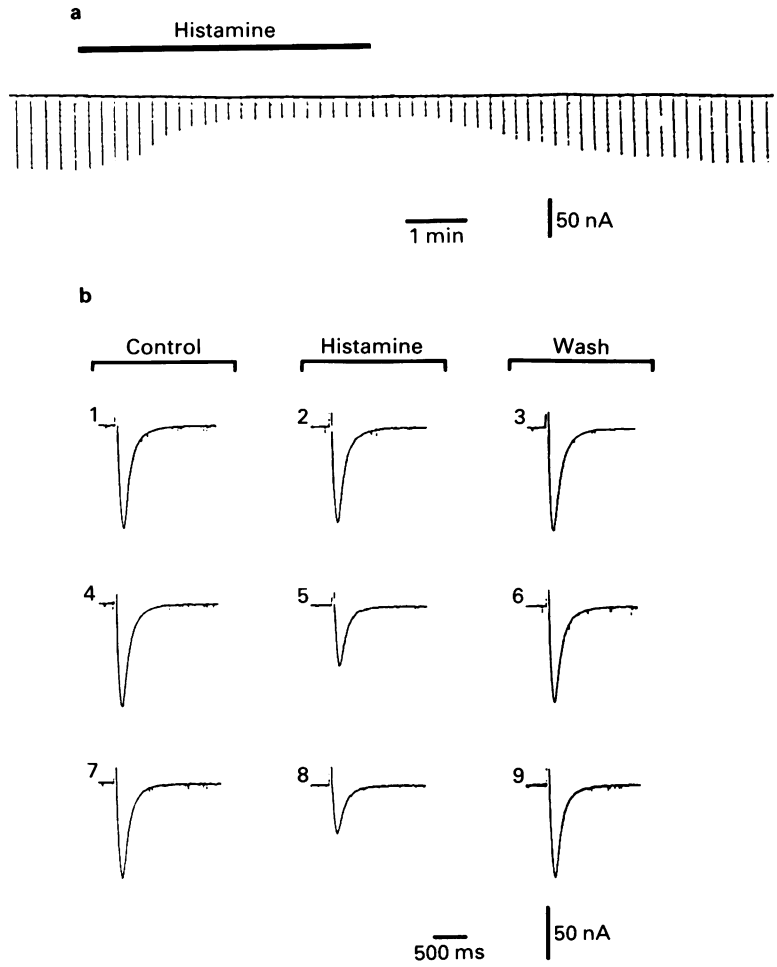

Figure 1 Depression of the acetylcholine (ACh)-induced current by histamine. (a) The entire time course of the effects of histamine. ACh was applied from an ACh-containing pipette (ejection current 30 nA for 40 ms). Downward deflections from the baseline denote ACh-induced inward currents. Histamine (100 μM) was added to the perfusate for the period indicated by the bar above the trace (about 5 min). The depressed ACh-induced current completely returned to the control level 6 min after washout of histamine. The straight baseline implied that histamine had no effect on the resting membrane potential. Holding potential was − 90 mV. (b) Concentration-dependent depression of the ACh-induced current by histamine. Left column (traces 1, 4 and 7): control. Middle column (traces 2, 5 and 8) represent the depressed ACh-induced current in the presence of 3 μM, 30 μM or 300 μM histamine. Right column (traces 3, 6 and 9): wash. Small downward (inward) deflections on each trace represent miniature endplate currents. Note that the amplitude of the miniature endplate current decreased in the presence of histamine, becoming virtually immeasurable in trace 8. Ejection current for ACh ionophoresis was 30 nA for 30 ms; holding potential − 90 mV. All traces in (b) were displayed on a chart recorder through a digitizing oscilloscope.

ly applied to the endplate from an ACh (2 M)-containing pipette (d.c. resistance; > 100 MΩ). Ejection current (30–60 nA for 2–50 ms) was passed through the pipette at a pulse frequency of 0.1 Hz. A small (up to 5 nA) anodal current was routinely applied to reduce diffusional leakage from ionophoresis pipettes.

Drugs used were: acetylcholine chloride (Wako Pure Chemical), histamine dihydrochloride (Wako Pure Chemical), (+)-tubocurarine chloride (Tokyo Kasei), pyrilamine as maleate salt (Sigma) and cimetidine (Sigma). Erabutoxin-b was purified by Dr N. Tamiya from sea-snake (*Laticuda semifasciate*) and kindly supplied to our laboratory. The structure of the toxin has been determined previously (Tamiya & Arai, 1966; Sato & Tamiya, 1971). All the experiments were carried out at room temperature (18–22°C).

Results

General observations

Histamine was added to the perfusate at concentrations between 100 nM and 1 mM. We confirmed the original observations of Scuka (1973) that histamine depresses cholinergic transmission at both presynaptic and postsynaptic levels. Quantal analysis by means of the variance method revealed that histamine (100 μM) depressed the quantal content and the mean amplitude of the endplate potential by about 15% and 30% (30% and 55% in the case of 300 μM histamine) ($n = 4$ and 5, respectively). We have not included a detailed analysis of the inhibition of the evoked release of acetylcholine (ACh) by histamine in the present paper. Histamine did not cause any detectable shifts in the holding-current at − 90 mV (Figure 1).

The acetylcholine current

Histamine reversibly depressed the peak amplitude of the ACh-induced inward current (Figure 1). The depression of the ACh-induced current could be observed soon after the perfusate was changed from Ringer solution to a solution that contained histamine and reached the maximum level within 2 or 3 min (Figure 1). The maximum depression remained almost unchanged throughout the period (up to 30 min) during which the preparation was perfused with histamine-containing Ringer solution. Longer perfusion than 30 min was not tested. The peak amplitude of the ACh-induced current returned to the respective control value within 15 min after washout of histamine (Figure 1).

The extent of depression of the ACh current depended on the concentration of histamine in the bath (Figures 1 and 2). The peak amplitude of the ACh-induced current was depressed by about 45% in the

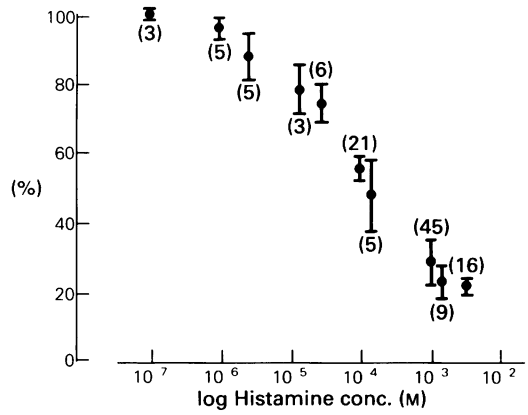

Figure 2 Summary of the concentration-dependent depression of the acetylcholine (ACh)-induced current by histamine. Ordinate scale; normalized amplitude of the ACh-induced current under the influence of histamine. The peak amplitude of the control ACh-induced current was taken as 100%. Each point represents the mean, vertical lines show s.e.mean, of the observations from the number of endplates indicated in parentheses.

presence of histamine (100 μM) at 21 different endplates (Figure 2) and the minimum effective concentration of histamine was about 1 μM (Figures 1 and 2). Also, the ACh-induced current was depressed by histamine (100 μM) even in Ringer solution which contained both pyrilamine (5 μM) and cimetidine (50 μM) implying that neither H_1-nor H_2-receptors mediate the depression of the ACh-induced current. The time course of the ACh-induced current remained unchanged in the presence of histamine (Figure 1; see also the latter section).

This effect of histamine was independent of the frequency of ACh ionophoresis (Figure 3); the interval between periods of ACh ionophoresis was changed from 10 s to 20, 30, 40, 50 or 60 s while histamine was present in the bath and an almost identical depression of the ACh current by a given concentration of histamine could be observed in each case ($n = 6$). Figure 3b demonstrates a particular example of this type where the interval of ACh ionophoresis was such that ACh was applied only twice; just after the addition of histamine and just before the washout of histamine. The ACh current was depressed by 50%; an identical effect to that in Figure 3a where ACh was applied every 10 s throughout the experiment. This indicated that the histamine effect on the ACh current was not use-dependent.

The dose-response curve of the acetylcholine current

The dose-response relationship between the peak ACh-induced current and the amount of ACh applied

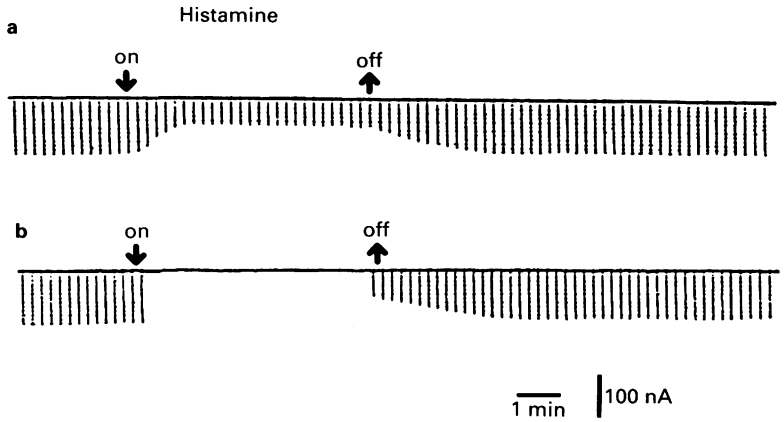

Figure 3 Use-independent depression of the acetylcholine (ACh)-induced current by histamine. Histamine (100 μM) was added to the bath between downward and upward arrows. (a) Control, ACh was applied at 0.1 Hz throughout the experiment. (b) Interval of ACh ionophoresis was changed such that ACh was applied only twice during bath-application of histamine (just after addition of histamine and just before washout). An identical depression (50% depression) was observed in both (a) and (b). Holding potential was − 88 mV throughout the experiments.

was examined in either the absence or the presence of histamine. The dose-response curve of the ACh current was obtained by plotting the peak ACh-induced current against the logarithm of the ejection charge used for the ionophoresis (Dreyer *et al.*, 1978). One of the representative examples from six experiments of this type is illustrated in Figure 4. The control dose-response curve seemed to be shifted in a parallel fashion to the right in the presence of histamine (Figure 4a). It was necessary to construct the double reciprocal plot of the dose-response curve (Lineweaver-Burk plot) to estimate the maximum response to ACh and to see whether histamine affected it. The Lineweaver-Burk plot was fitted by a straight line, assuming that the Hill coefficient is equal 2.5, and the point where the line crossed the ordinate gave an estimated measure of the maximum response (Figure 4b). The estimated maximum response was only

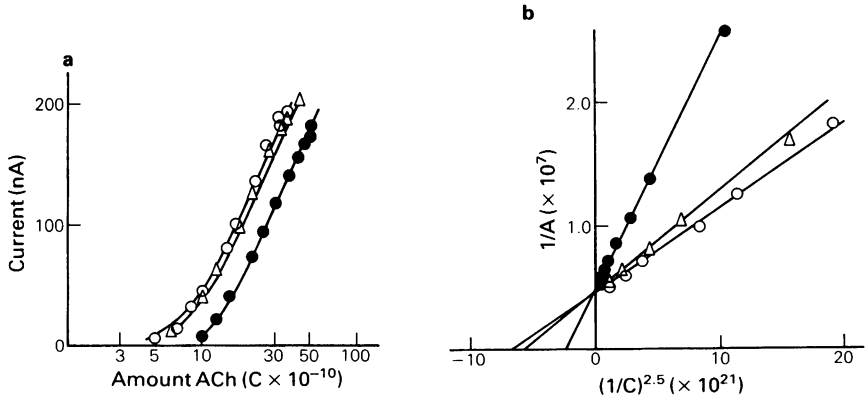

Figure 4 Dose-response curves of the acetylcholine (ACh)-induced current in the presence and absence of histamine. (a) The semi-log dose-response relationship between the amount of ACh applied and the peak amplitude of the ACh-induced current. Ordinate scale: the amplitude of the ACh-induced current. Abscissa scale: the amount of ACh applied, expressed as an electrical charge for ionophoresis in coulombs. (○) Control; (●) during bath-application of histamine (100 μM); (△) after washout of histamine (b) Lineweaver-Burk plot (double reciprocal plot) constructed from the data in (a) by assuming the Hill coefficient is equal to 2.5. The estimated maximum response was only slightly reduced in the presence of histamine; this was suggestive of a competitive antagonism. The symbols represent the same conditions as in (a).

slightly reduced in the presence of histamine (Figure 4b). Figure 4b shows that histamine increased an apparent dissociation constant. These findings were suggestive of a competitive antagonism, although quantitative measurement was not possible since the ACh-receptor interaction is not a first-order reaction.

Protection against erabutoxin

From the above results we hypothesized that histamine binds to the specific ACh binding site within the receptor-channel complex in a similar way to curare or α-bungarotoxin (Lee, 1972). It has been shown that erabutoxin-b (Tamiya & Arai, 1966; Sato & Tamiya, 1971) also binds to the specific binding site of ACh at the frog endplate and this action is almost irreversible (Kato *et al.*, 1978). The toxin effect is inhibited in the presence of curare indicating that both the toxin and curare compete at the same binding site (Kato *et al.*, 1978). If this irreversible blocking action of the toxin is also inhibited by the presence of histamine in the bath, then our hypothesis would be supported since the toxin would not be able to bind to specific binding sites which were already occupied by histamine (cf. Koketsu *et al.*, 1982). Figure 5 shows that the specific ACh binding site is protected by extremely high concentrations of histamine which sustains our hypothesis. In this particular example, histamine (5 mM) depressed the ACh-induced potential by 80% (Figure 5b–2; the same condition as in Figures 5a–2 and 3) and when the toxin (1 μM) was added to the bathing solution the residual ACh-induced potential was abolished (Figure 5b–3). The ACh-induced potential gradually returned to the control amplitude after washout of both histamine and the toxin, as would be expected from our hypothesis (Figure 5b-4). The predicted full recovery was to 80% of the control amplitude (80% of trace 1 in Figure 5a and b) but the observed recovery was only 55% (Figure 5b–4) and it was found that the recovery depended on the duration of the toxin's presence. In fact, no recovery was obtained when the toxin was perfused for more than 20 min. Quantitatively com-

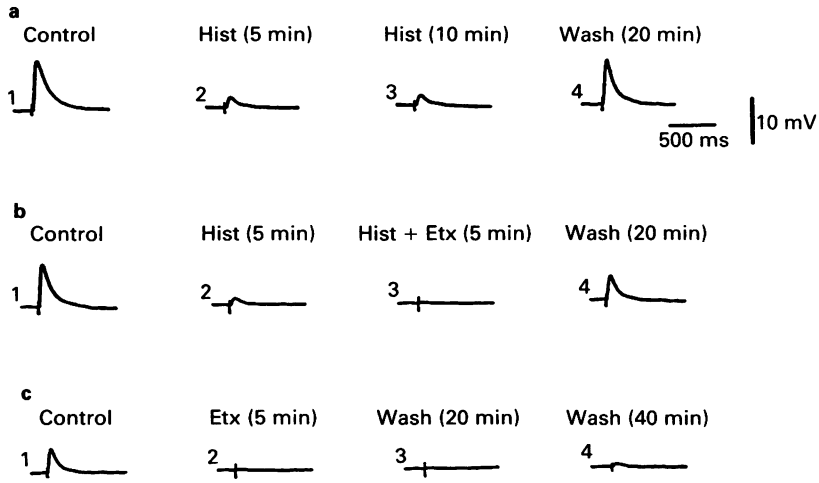

Figure 5 Traces showing the protective effect of histamine (Hist) against erabutoxin-b binding to the acetylcholine (ACh) binding site. Results were obtained from a single endplate. ACh was applied at 0.05 Hz (ionophoretic ejection current; 30 nA for 5 ms). (a) Effects of histamine (5 mM) alone on the ACh-induced potential. Trace 1, control; traces 2 and 3 were obtained 5 and 10 min, respectively, after the addition of histamine to the bath. Note that the amplitude of the ACh-induced potential was depressed by 80%. Trace 4 shows that the ACh-induced potential completely returned to the control amplitude 20 min after washout. (b) Effects of erabutoxin-b (Etx; 1 μM) on the ACh-induced potential in the presence of histamine (5 mM). Trace 1, control (same as a-1 and a-4); trace 2 shows effects of histamine alone (same as a-2). After trace 2 had been observed, erabutoxin was added to the histamine-containing Ringer solution and the residual ACh-induced potential (20% of the control) was abolished within 1 min. Trace 3 was observed 5 min after the addition of the toxin, then the toxin alone was washed out. The complete abolition of the ACh-induced potential persisted during the next 5 min in the Ringer solution which contained only histamine (data not shown). Trace 4 was obtained 20 min after washout of histamine (25 min after washout the of toxin) and shows that the ACh-induced potential recovered to 55% of its respective control amplitude. (c) Shows the irreversible action of erabutoxin (1 μM) alone. Trace 1; same condition as b-4 but after another 20 min. Trace 2; erabutoxin (5 min) and then the toxin was washed out. Traces 3 and 4 were observed 20 and 40 min after washout of the toxin. Only 10% (7% of its original control such as a-1 or b-1) recovery was observed after 40 min of washout. See Results for further details.

parable results were obtained from 8 other experiments. The toxin can associate with the specific binding site only when histamine dissociates from the site. In other words, if there are sufficient histamine molecules around the specific ACh binding site histamine can re-associate with the site before it is occupied by the toxin. This may result in a substantial proportion of the specific ACh binding site remaining unbound by the toxin (but bound by histamine) throughout the period during which the preparation was perfused with the Ringer solution containing both histamine and the toxin. Some of the specific ACh binding sites should thus be 'empty' after washout of both histamine and the toxin. The extent of the protection also depended on the concentrations of both histamine and the toxin. This was inferred by the following findings: decreasing the concentration of the toxin from 1 μM to 100 nM led to a greater recovery of the ACh potential ($n = 3$) and decreasing the histamine concentration from 5 mM to 300 μM caused the opposite to occur ($n = 2$).

The equilibrium potential of the acetylcholine current

The following two sections deal with the possibility that histamine may affect the channel site within the

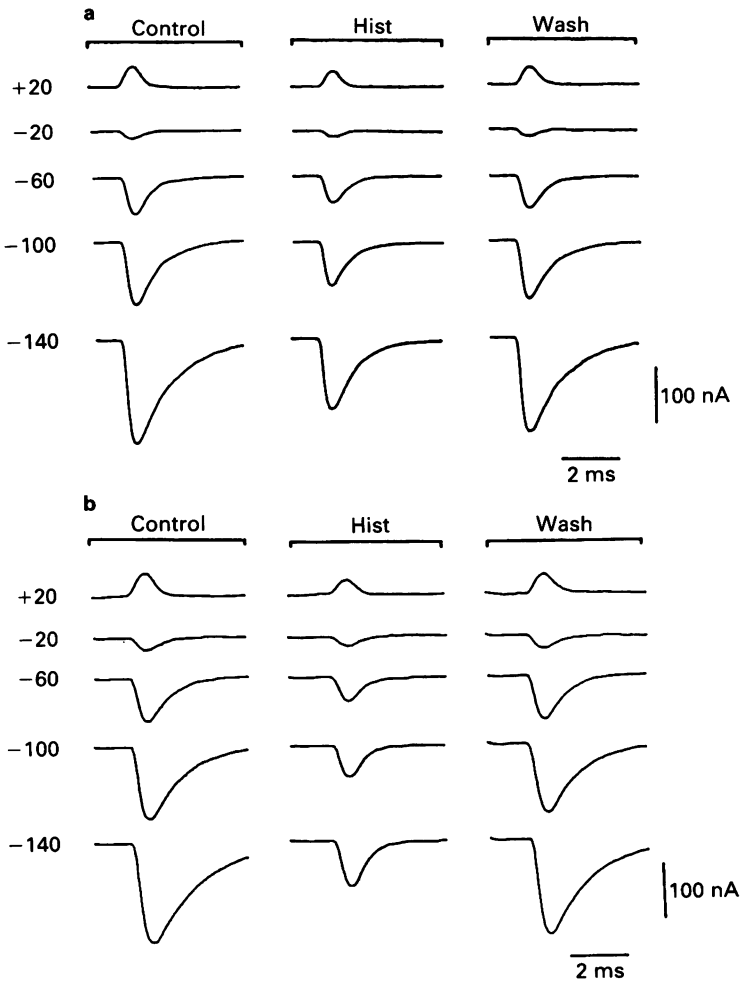

Figure 6 The reversal potential and the time course of the endplate current (e.p.c.) in the presence of histamine (Hist). The e.p.c. was observed at various induced potentials (see figure beside each trace in mV) before the addition of histamine, in the presence of histamine (control) (a) 100 μM and (b) 300 μM and after washout of histamine. Note that the e.p.c. was markedly shortened at − 100 mV and − 140 mV in (b). All the recordings were displayed on a chart recorder through a digitizing oscilloscope.

receptor-channel complex in addition to the receptor site. For this purpose, the endplate current (e.p.c.) was observed in the absence or presence of various concentrations of histamine (10 μM–1 mM). Muscles were pretreated with glycerol in order to prevent the muscle from contracting (see Methods). Curare (up to 2 μM) was present in the perfusate throughout the experiments. The peak amplitude of e.p.c. was a linear function of the membrane potential at between − 140 and + 40 mV (Figures 6 and 7). The reversal potential of the e.p.c. was − 11.7 ± 1.4 mV (mean + s.e.mean, $n = 18$). Both this linearity and the reversal potential remained almost unchanged in the presence of histamine (100 μM) although histamine depressed e.p.c. in an expected manner at each potential level ($n = 3$) (Figures 6a and 7a). This indicated that the depression of e.p.c. was not due to the changes in the reversal potential of the e.p.c. However, it was noted, in the presence of histamine at concentrations greater than 300 μM, that the relationship between e.p.c. amplitude and membrane potential became non-linear between − 60 mV and − 140 mV; the percentage depression at − 140 mV was greater than that at − 60 mV ($n = 6$) (Figures 6b and 7b). The reversal potential of the e.p.c. itself remained almost unchanged even in the presence of 1 mM histamine ($n = 3$).

The time course of the falling phase of the endplate current

The half-decay time of the e.p.c. (h.d.t.) was 0.84 ± 0.07 ms at − 100 mV (mean ± s.e.mean,

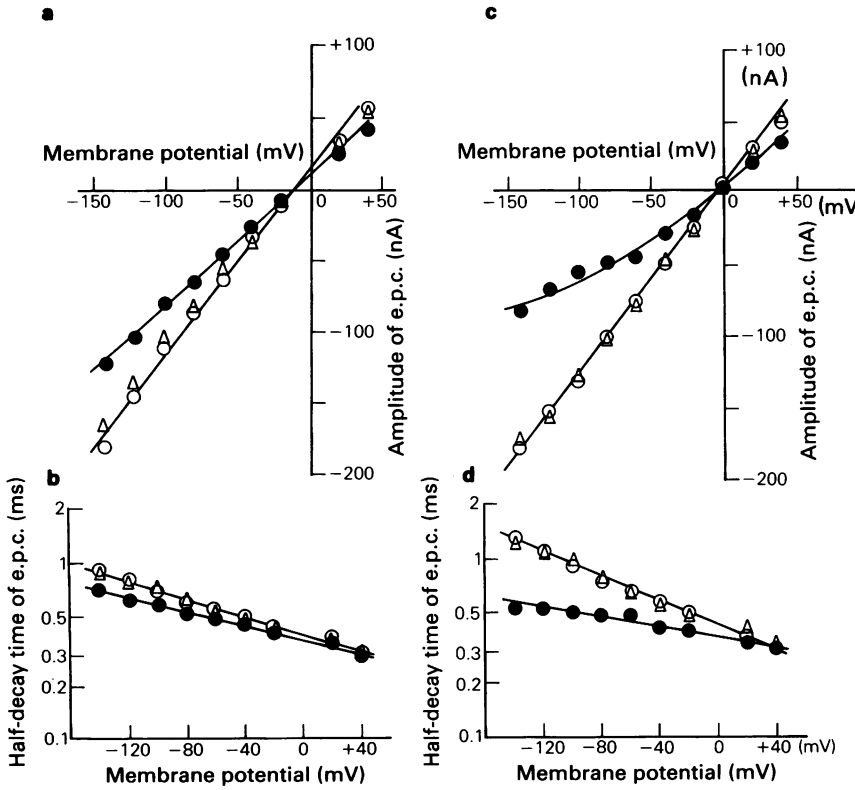

Figure 7 The half-decay time of the endplate current (e.p.c.) and its voltage-dependence in the presence of histamine. (a) and (c) The relationship between the e.p.c. amplitude and the membrane potential (measured from data in Figure 6a and b, respectively). Ordinates: amplitude of the e.p.c. in nA (inward negative). Open circles, closed circles and triangles represent control, histamine and wash, respectively. The amplitude of the e.p.c. was a linear function of the membrane potential in control and in 100 μM histamine but was non-linear in the presence of 300 μM histamine. The reversal potential of the e.p.c. remained unchanged in both cases. (b) and (d) The half-decay time of the e.p.c. (h.d.t.) and its voltage-dependence (measured from data in Figure 6a and b, respectively), Ordinates: half-decay time in ms (log scale). Symbols in (b) and (d) represent the same as those in (a) and (c). Note that the voltage-dependence of the h.d.t. was markedly reduced in the presence of 300 μM histamine.

$n = 11$). The h.d.t. can be expressed by ln(h.d.t. v)) = ln(h.d.t.(0)) + V/B, where h.d.t(v), h.d.t.(0) and B denote h.d.t. at a given membrane potential of V mV, h.d.t at 0 mV and the coefficient of the voltage-dependence, respectively. B was -143 ± 15 mV (mean ± s.e.mean, $n = 7$) (Figure 7); this value is consistent with previously published data (Magleby & Stevens, 1972; Gage & McBurney, 1975; Kuba *et al.*, 1976). Histamine (> 25 μM) shortened the h.d.t. in a voltage-dependent manner; the shortening of the h.d.t. was marked when the membrane was hyperpolarized (Figure 7). The h.d.t. and B, in the presence of histamine (100 μM), were 0.58 ± 0.11 ms (at -100 mV) and -244 ± 23 mV, respectively (mean ± s.e.mean, $n = 5$) (Figure 7). These were significantly smaller and larger, respectively, than those obtained in control conditions ($P < 0.05$; paired t test). Therefore, it was suggested that histamine may have some additional effects on the ACh-channel itself.

Discussion

The results of the experiments described demonstrate the possibility that histamine may act as an antagonist of ACh. The three main findings of the study are stated below.

(1) The depression of the ACh-induced current by histamine is unlikely to be mediated by the activation of histamine receptor since neither H_1- nor H_2-receptor antagonists could inhibit the depression of the ACh current. Rather, it was strongly suggested that, at relatively low concentrations (< 50 μM), histamine can directly couple with the specific ACh binding site with only minor effects on the channel itself. (2) Since histamine can, to some extent, protect ACh-receptors from irreversible block by erabutoxin we infer that histamine is binding to, or in the region of, the ACh-receptor site. (3) Finally, it is well established that curare ((+)-tubocurarine) blocks not only the receptor site but the channel site when it is used at relatively higher concentrations (usually more than 5 μM in many experimental conditions in various tissues; cf. Katz & Miledi, 1978). In this respect, the finding that histamine affects the ACh-channel itself might not be surprising, although the precise mechanism underlying the channel blocking action should be clarified by much more elegant experimental approaches such as single channel recording.

Hence, the modulation of cholinergic transmission at the frog neuromuscular junction by histamine is obviously inhibitory since histamine (100 μM, used in the present experiments) decreased not only the release of ACh from motor nerve terminals but also the sensitivity of the endplate receptor to ACh (see also results by Scuka, 1973; Ohta *et al.*, 1984). However, further experiments need to be performed to investigate the physiological significance of this effect of histamine.

We thank Professor N. Tamiya for his generous gift of erabutoxin. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. We are grateful to Dr T. Akasu for his critical comments.

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(*Received August 8, 1984.*
Revised January 2, 1985.
Accepted January 3, 1985.)