Pergamon

Biochemical Pharmacology, Vol. 48, No. 9, pp. 1721–1728, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)00348-3

SELECTIVE POTENTIATION OF HISTAMINE H_1-RECEPTOR STIMULATED CALCIUM RESPONSES BY 1,4-DITHIOTHREITOL IN DDT_1MF-2 CELLS

JOHN M. DICKENSON* and STEPHEN J. HILL

Department of Physiology and Pharmacology, Medical School, Queen's Medical Centre, Nottingham NG7 2UH, U.K.

(*Received* 22 *December* 1993; *accepted* 26 *July* 1994)

Abstract—The effect of 1,4-dithiothreitol (DTT) on agonist-stimulated increases in intracellular free calcium concentration ($[Ca^{2+}]_i$) has been investigated in the smooth muscle cell line, DDT_1MF-2, derived from hamster vas deferens. Pretreatment with DTT (1 mM) produced a large leftward parallel shift in concentration–response curve for histamine H_1-receptor mediated increases in $[Ca^{2+}]_i$. The EC_{50} values for H_1-receptor stimulated increases in $[Ca^{2+}]_i$ in the absence and presence of DTT were 11.3 ± 1.5 μM (N = 6) and 0.52 ± 0.15 μM (N = 6), respectively. DTT had no significant effect on the maximum Ca^{2+} response elicited by histamine (100 μM). In the presence of DTT the partial H_1-receptor agonist 2-pyridylethylamine (100 μM) increased $[Ca^{2+}]_i$ from 112 ± 14 nM to 237 ± 24 nM (N = 10). In control cells 2-pyridylethylamine (100 μM) did not elicit a Ca^{2+} response. DTT had no significant effect on the maximum Ca^{2+} response elicited by 1 mM 2-pyridylethylamine. The enhancement of histamine H_1-receptor Ca^{2+} responses by DTT was reversed by the sulphydryl oxidizing agent dithiobis-(2-nitrobenzoic acid). DTT had no significant effect on adenosine A_1-, bradykinin and ATP-receptor stimulated increases in $[Ca^{2+}]_i$. [^{3}H]mepyramine binding experiments confirmed that DTT increased agonist affinity. DTT produced a small, but significant, leftward shift in concentration–response curve for histamine displacement of [^{3}H]mepyramine binding. These data suggest that DTT potentiates H_1-receptor mediated Ca^{2+} responses by increasing agonist affinity.

Key words: DDT_1MF-2 cells; histamine H_1-receptor; calcium mobilization; dithiothreitol; smooth muscle cells

Biochemical studies using the disulphide bond reducing agent DTT† have revealed the importance of disulphide bonds in histamine H_1-receptor structure and function [1–9]. For example, DTT pretreatment potentiates histamine H_1-receptor mediated contractile responses in rabbit aorta [1], colon [4] and guinea-pig ileal smooth muscle [4, 7, 8]. Interestingly, the contractile responses elicited by other agents such acetylcholine, 5-hydroxytryptamine and KCl were not potentiated by DTT [1, 7].

Histamine H_1-receptors are coupled to phospholipase C through a regulatory G_q protein, which upon activation hydrolyses the plasma-membrane phospholipid, phosphatidylinositol 4,5-biphosphate ($PtdInsP_2$), producing two second messengers inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol. In smooth muscle, $InsP_3$ stimulates the release or mobilization of sequestered Ca^{2+} from intracellular stores, producing a rise in $[Ca^{2+}]_i$ [10]. $InsP_3$-stimulated release of Ca^{2+} is associated with the initial phase of smooth muscle contraction in non-excitable cells. The potentiation of histamine H_1-receptor mediated contractions (in rabbit aorta and guinea-pig ileal smooth muscle) by DTT may be due to increased $InsP_3$ production. Indeed DTT potentiates H_1-receptor stimulated accumulation of [^{3}H]inositol phosphates in guinea-pig and rat cerebral cortex [5, 9] and guinea-pig cerebellum [5]. Interestingly the DTT had no effect on the accumulation of [^{3}H]inositol phosphates elicited by the muscarinic agonist carbachol in guinea-pig cerebral cortex [5]. However, to date there are no reports regarding the effects of DTT on histamine H_1-receptor stimulated inositol phospholipid hydrolysis or Ca^{2+} responses in cultured smooth muscle cells.

Studies on other G-protein coupled receptors have revealed the importance of disulphide bonds in receptor function. For example the role of cysteine (Cys) residues and disulphide bonds in muscarinic M_1-receptor function have recently been investigated using site-directed mutagenesis [11]. This study revealed that the disulphide bond between Cys^{98} and Cys^{178} is critical for maintaining the integrity of the ligand binding site. Biochemical studies of the adenosine A_2-receptor have demonstrated that antagonist binding can be inhibited following treatment with DTT [12].

The smooth muscle cell line, DDT_1MF-2 derived from hamster vas deferens expresses functional histamine H_1-receptors coupled to inositol phospholipid hydrolysis and calcium mobilization through a pertussis toxin insensitive G-protein [13–15]. The histamine H_1-receptor mediated Ca^{2+} signalling is

* Corresponding author. Tel. 0602 709468.

† Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; DTT, 1,4-dithiothreitol; FCS, foetal calf serum; CPA, N^6-cyclopentyladenosine; 2-PEA, 2-pyridylethylamine.

comprised of two components: (i) release of Ca^{2+} from intracellular stores (mediated through inositol 1,4,5-trisphosphate) and (ii) Ca^{2+} entry (influx) across the plasma membrane which requires the continued presence of histamine on the receptor [14]. DDT_1MF-2 cells also express adenosine A_1-, bradykinin and ATP-receptors coupled to inositol phospholipid hydrolysis and Ca^{2+} mobilization [15–17]. In this paper we have investigated the effect of DTT on histamine H_1-receptor Ca^{2+} signalling in DDT_1MF-2 cells.

MATERIALS AND METHODS

Chemicals

[pyridyl-5-^{3}H]mepyramine (21 Ci/mmol) was obtained from Amersham International (Amersham, U.K.). Fura-2/AM and ionomycin were from Calbiochem (Nottingham, U.K.). Histamine, bradykinin, adenosine-5′-triphosphate, CPA, DTT, promethazine, 5,5′-dithiobis-(2-nitrobenzoic acid) were obtained from the Sigma Chemical Co. (Poole, U.K.). The gift of 2-PEA (Smithkline Beecham, Welwyn Garden City, U.K.) is gratefully acknowledged. Dulbecco's modified Eagle's medium and FCS were from Northumbria Biologicals (U.K.). All other chemicals were of analytical grade.

Cell culture

The hamster vas deferens smooth muscle cell line (DDT_1MF-2) was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, U.K.). DDT_1MF-2 cells were cultured at 37° in a humidified air/CO_2 (90:10) atmosphere in 75 cm^2 flasks (Costar). The growth medium was Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine and 10% (v/v) FCS. Cells were passaged twice a week (1/8 split ratio) by vigorous shaking of the flask, placed into 75 cm^2 flasks and fed with fresh growth medium every 48 hr. Cells for $[Ca^{2+}]_i$ determinations were grown on 24 × 10 mm glass coverslips in 90 mm petri dishes. Cells for binding experiments were grown in 175 cm^2 flasks. All experiments were performed on confluent monolayers (passages 4–21, numbers assigned after receiving the cell line).

Measurement of intracellular free calcium

Intracellular free calcium was measured by loading confluent cell monolayers with the calcium-sensitive fluorescent dye fura-2. Individual coverslips were placed in 35 mm petri dishes with 1 mL of physiological buffer (145 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM $MgSO_4$, 10 mM HEPES, 2 mM $CaCl_2$, pH 7.45) containing 10% FCS (v/v), 3 μM fura-2/AM and incubated for 30 min at 37°. After this "loading" period the fura-2 containing buffer was replaced with fresh buffer, that was free of fura-2 and FCS but contained 0.1% bovine serum albumin, and left at 37° for a further 15 min. Loaded coverslips were then mounted in a specially designed holder which enabled the coverslip to be positioned across the diagonal of a polymethacrylate cuvette. Each cuvette contained 2.9 mL of physiological buffer (drugs were added to the cuvettes in 100 μL aliquots) and fluorescent measurements were made at 37° using a Perkin–Elmer LS 50 spectrometer. The excitation wavelengths were 340 and 380 nm, with emission at 500 nm. The slit-widths were set at 10 nm for both the excitation and emission wavelengths and the time taken to switch between 340 and 380 nm was 0.8 sec. Intracellular Ca^{2+} was calculated every 1.9 sec from the ratio (R) of 340 nm/380 nm fluorescent values using the equation of Grynkiewicz *et al.* [18]:

$$[Ca^{2+}]_i = \frac{(R-R_{min})}{(R_{max}-R)} \times (S_{380,min}/S_{380,max}) \times K_d$$

where K_d is the affinity of fura-2 for Ca^{2+} (224 nM at 37°) and $S_{380,min}/S_{380,max}$ is the ratio (β value) of the fluorescent values obtained at 380 nm in the absence and presence of saturating $[Ca^{2+}]_i$. The maximum and minimum R values (R_{max} and R_{min}) were determined on separate coverslips under saturating $[Ca^{2+}]_i$ (achieved by increasing the extracellular $[Ca^{2+}]$ to 20 mM followed by 10 μM ionomycin, pH 7.4) and calcium-free (achieved using 8.3 mM EGTA immediately followed by 25 μL of 1.0 M NaOH to compensate for the decrease in pH, in the presence of 10 μM ionomycin) conditions, respectively. Corrections for autofluorescence were made by measuring the fluorescence produced by coverslips that had not been loaded with fura-2. Where Ca^{2+}-free conditions were required, experiments were performed in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA.

[^{3}H]*mepyramine binding experiments*

(i) Membrane preparation. Cells from eight confluent 175 cm^2 flasks (sufficient for 96 tubes) were detached using a Ca^{2+}/Mg^{2+}-free phosphate-buffered saline solution (137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2SO_4, 6.5 mM Na_2HPO_4, 0.005% EDTA, pH 7.4) at 37° for 5 min. After centrifugation (150 g for 5 min) membranes were prepared by re-suspending the cells in 20 mL of ice-cold Tris buffer (50 mM, pH 7.7), followed by homogenization using a glass–teflon homogenizer (approx. 10 strokes) and centrifugation at 20,000 g for 15 min. The resulting pellet was re-suspended in 5 mL of Tris buffer and kept on ice until required. Freshly prepared membranes were incubated at 37° for 15 min in the presence or absence of DTT (1 mM).

(ii) Histamine displacement of [^{3}H]*mepyramine binding.* Displacement binding assays were performed in 50 mM Tris buffer, pH 7.4, containing [^{3}H]mepyramine (between 2.5 and 3.5 nM) in the absence and presence of 1 mM DTT. DDT_1MF-2 cell membranes (50 μL; 0.4–0.7 mg protein) were incubated in the presence of increasing concentrations of histamine (10^{-3}–10^{-10}) in a total volume of 1 mL (three replicates were performed for each competing ligand concentration, spread throughout the experiment). The incubation was for 30 min at 37° and was stopped by rapid filtration and washing with ice-cold Tris buffer (three times, approx. volume 10 mL) over Whatman GF/B filters (pre-soaked for 1 hr in 0.3% polyethylenimine to reduce non-specific binding) using a Brandel MR24 cell harvester. Filters were transferred to scintillation vial inserts and 5 mL of Emulsifier-Safe scintillator (Packard) added. The

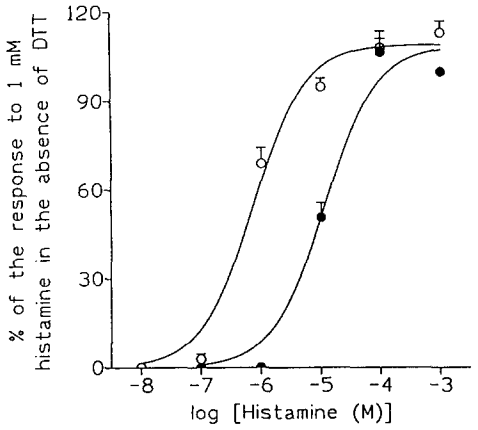

Fig. 1. Concentration–response curves for histamine H_1-receptor stimulated increases in $[Ca^{2+}]_i$ in the presence (open circles) and absence (closed circles) of 1 mM DTT. The data are expressed as a percentage of the maximum response to 1 mM histamine in the absence of DTT (expressed as an increase in F_{340}/F_{380} ratio minus the basal fluorescence ratio). Curves were fitted by use of a logistic equation as described in Materials and Methods (Hill coefficients were constrained to 1.0 in both curves). Data are the means ± SEM of six experiments.

filters were left at room temperature for at least 2 hr before liquid-scintillation spectrometry.

(iii) Saturation binding experiments. Saturation binding experiments were performed under similar conditions with increasing concentrations of [^{3}H]-mepyramine (0.25–8 nM; in 50 mM Tris buffer) in the absence and presence of 1 mM DTT. DDT_1MF-2 cell membranes (50 μL aliquots; 0.4–0.7 mg protein) were incubated in the presence (non-specific binding) or absence (total binding) of promethazine (2 μM) at 37° for 30 min in a total volume of 1 mL (three replicates were performed for each [^{3}H]-mepyramine concentration, spread throughout the experiment). In all experiments non-specific binding was determined using promethazine (2 μM; six replicates per 24 tubes spread throughout the experiment). Non-specific binding was between 60 and 75% of the total [^{3}H]mepyramine bound at circa 3 nM [^{3}H]mepyramine. The high levels of non-specific binding are due to the presence of a secondary binding site for [^{3}H]mepyramine in DDT_1MF-2 cells [19].

Protein determinations were made using the method of Bradford [20], following digestion in NaOH (0.5 M), using bovine serum albumin as the standard.

Data analysis

Rises in $[Ca^{2+}]_i$ were evaluated by importing the fluorescence data into the spreadsheet AsEasyAs (TRIUS Inc). Basal $[Ca^{2+}]_i$ levels were determined by calculating the mean of the 10 data points (measured every 1.9 sec) prior to drug addition, whereas the maximum Ca^{2+} signal was deemed to be the largest Ca^{2+} response obtained immediately after drug addition. Due to the fluctuations in basal $[Ca^{2+}]_i$, the mean of the 10 data points measured before and immediately after drug addition were also calculated to determine whether the measured response was significantly different from basal levels (Mann Whitney U-test).

Agonist and [^{3}H]mepyramine displacement concentration–response curves were fitted to a logistic equation using the non-linear regression programme GraphPAD (ISI). GraphPAD was also used to perform non-linear regression analysis for fitting data from saturation experiments. The expression used to derive B_{max} and K_d values was:

$$[^3H]\text{mepyramine bound} = B_{max} \times L/(K_d + L)$$

where L is the concentration of the radioligand. The function fits the data to a rectangular hyperbola. Data are shown as means ± SEM. Statistical analysis was performed using the Student's unpaired t-test. A P value <0.05 was considered as statistically significant. N in the text refers to the number of separate experiments.

RESULTS

Effects of DTT on agonist-stimulated increases in $[Ca^{2+}]_i$

Pretreatment with DTT (1 mM; 15 min) produced a large parallel shift in concentration–response curve

Table 1. The effect of dithiothreitol pretreatment on agonist stimulated increases in $[Ca^{2+}]_i$ in the presence of extracellular calcium

Agonist	+DTT (1 mM) Basal	+DTT (1 mM) Stimulated	(N)	−DTT Basal	−DTT Stimulated	(N)
Histamine	98 ± 12	368 ± 17	6	112 ± 9	352 ± 10	6
Bradykinin	105 ± 23	423 ± 9	4	123 ± 13	417 ± 16	4
ATP	87 ± 9	452 ± 11	5	99 ± 20	460 ± 18	5
CPA	103 ± 13	268 ± 12	3	78 ± 19	255 ± 17	3

Cells were preincubated with DTT (1 mM) for 15 min before stimulation with histamine (100 μM), bradykinin (100 nM), ATP (100 μM) and CPA (300 nM). Control responses were obtained by stimulating cells that had not been preincubated with DTT. Values represent the means ± SEM of basal and stimulated increases in $[Ca^{2+}]_i$ (nM) obtained in (N) experiments.

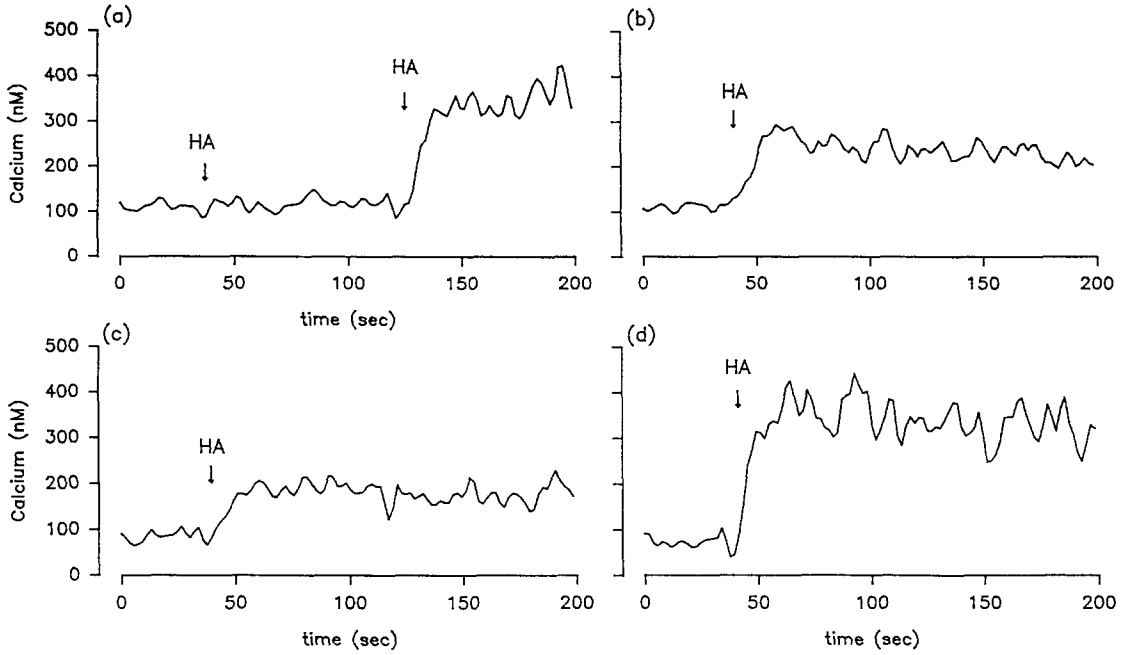

Fig. 2. The effect of DTT on histamine H_1-receptor stimulated increases in $[Ca^{2+}]_i$ in the presence of extracellular calcium. Profiles (a) and (c) represent control responses to 1 and 10 μM histamine, respectively. In profiles (b) and (d) cells were preincubated with DTT (1 mM) for 15 min before stimulating with 1 and 10 μM histamine, respectively. The addition of histamine (100 μM) at the end of experiment (a) was to show cell viability. The relevant concentrations of histamine (HA) were added where indicated. Similar results were obtained in five other experiments.

for histamine H_1-receptor mediated increases in $[Ca^{2+}]_i$ in DDT$_1$MF-2 cells (Fig. 1). The EC_{50} values for H_1-receptor stimulated increases in $[Ca^{2+}]_i$ in the absence and presence of DTT were 11.3 ± 1.5 μM (N = 6) and 0.52 ± 0.15 μM (N = 6), respectively. DTT had no significant effect on the maximum Ca^{2+} response elicited by histamine (Fig. 1 and Table 1). Figure 2 shows the effect of DTT on histamine H_1-receptor stimulated increases in $[Ca^{2+}]_i$ measured in the presence of extracellular Ca^{2+} (2 mM). No increase in $[Ca^{2+}]_i$ was observed with 1 μM histamine (N = 6; Fig. 2a). In contrast 1 μM histamine increased $[Ca^{2+}]_i$ from 110 ± 23 nM (N = 6) to 250 ± 17 nM (N = 6) in cells pretreated with 1 mM DTT. Similarly, DTT pretreatment potentiated the response to 10 μM histamine (Fig. 2d). In these experiments $[Ca^{2+}]_i$ increased from 97 ± 11 nM to 210 ± 23 nM (N = 6) in control cells and from 110 ± 24 nM to 405 ± 17 nM (N = 6) in DTT treated cells.

To assess the effect of DTT on the two components of the H_1-receptor Ca^{2+} response, i.e. intracellular Ca^{2+} release and Ca^{2+} influx, increases in $[Ca^{2+}]_i$ were measured using the Ca^{2+}-free/Ca^{2+}-reintroduction protocol. Pretreatment with DTT potentiated 1 μM histamine stimulated intracellular Ca^{2+} release ($[Ca^{2+}]_i$ increased from 113 ± 14 nM to 232 ± 34 nM (N = 4)) and Ca^{2+} influx ($[Ca^{2+}]_i$ increased from 179 ± 23 nM to 370 ± 45 nM (N = 4) see Fig. 3). In control experiments 1 μM histamine did not stimulate Ca^{2+} release or Ca^{2+} influx (N = 4; Fig. 3a).

DTT also potentiated Ca^{2+} responses produced by the lower efficacy H_1-receptor agonist 2-PEA [5]. In control experiments 100 μM 2-PEA did not stimulate increases in $[Ca^{2+}]_i$. However, in cells pretreated with 1 mM DTT 2-PEA (100 μM) increased $[Ca^{2+}]_i$ from 112 ± 14 nM to 237 ± 24 nM (N = 10). No increase in $[Ca^{2+}]_i$ was observed with 10 μM 2-PEA in the absence or presence of DTT. DTT had no significant effect on the maximum Ca^{2+} response elicited by 1 mM 2-PEA. In these experiments 2-PEA increased $[Ca^{2+}]_i$ from 117 ± 12 nM to 285 ± 23 nM (N = 4) in control cells and from 109 ± 11 nM to 273 ± 17 nM (N = 4) in DTT treated cells. In the same batches of cells 100 μM histamine increased $[Ca^{2+}]_i$ from 98 ± 9 nM to 376 ± 13 nM (N = 4).

The enhancement of histamine H_1-receptor Ca^{2+} responses by DTT was reversed by the sulphydryl oxidising agent dithiobis-(2-nitrobenzoic acid) (DTNB). Cells initially treated for 15 min with 1 mM DTT and then for a further 15 min with 1 mM DTNB (in the absence of DTT) did not respond to 1 μM histamine (Fig. 4c). The relevant control experiments obtained on the same experimental day are shown in Fig. 4a and b. DTNB alone had no significant effect on the H_1-receptor Ca^{2+} response. $[Ca^{2+}]_i$ increased from 88 ± 23 nM to 380 ± 17 nM (N = 3) in control experiments and from 97 ± 12 nM to 397 ± 20 nM (N = 3) in DTNB (1 mM) treated cells.

DTT had no significant effect on adenosine A_1-, bradykinin and ATP-receptor stimulated increases in $[Ca^{2+}]_i$. The effect of DTT on the maximum Ca^{2+}

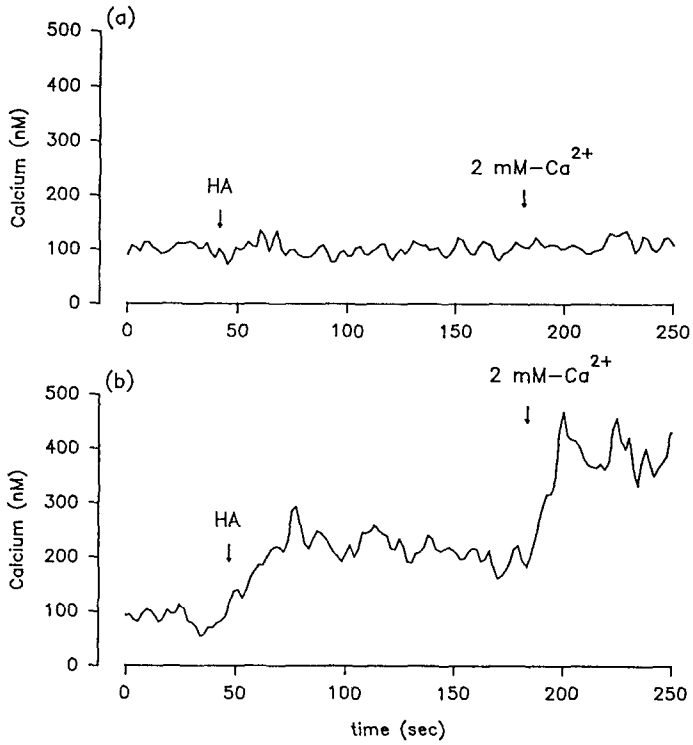

Fig. 3. The effect of DTT on histamine H_1-receptor stimulated increases in $[Ca^{2+}]_i$ in the absence of extracellular calcium. (a) Cells were initially stimulated with 1 μM histamine in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA (to measure intracellular Ca^{2+} release) after which Ca^{2+} (2 mM) was re-applied (to measure Ca^{2+} influx). (b) The same protocol as in (a), but cells were preincubated for 15 min with 1 mM DTT. Histamine (HA; 1 μM) and $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in two other experiments.

responses elicited by CPA (100 nM), bradykinin (100 nM) and ATP (100 μM) are summarized in Table 1. The EC_{50} values obtained for CPA, bradykinin and ATP in the absence and presence of DTT are given in Table 2.

Effects of DTT on [^{3}H]*mepyramine binding*

[^{3}H]mepyramine binding experiments were performed to confirm if DTT increased agonist affinity. DTT (30 min incubation; 1 mM) had no significant effect on the dissociation constant (K_d) for mepyramine determined from saturation binding experiments. The K_d values for mepyramine were 3.5 ± 0.6 nM (N = 5) and 3.2 ± 0.4 (N = 5), in the absence and presence of DTT, respectively. Similarly DTT had no significant effect on the B_{max} determined in the presence [33 ± 5 (N = 5) fmol/mg protein] and absence [36 ± 5 (N = 5) fmol/mg protein] of DTT.

Incubation of DDT_1MF-2 cell membranes with DTT (1 mM; 30 min) produced a small, but significant, leftward shift in concentration–response curve for histamine displacement of [^{3}H]mepyramine binding (Fig. 5). The IC_{50} values for histamine displacing [^{3}H]mepyramine were 2.5 ± 0.35 μM (N = 4) and 0.72 ± 0.17 (N = 4), in the absence and presence of DTT, respectively (Fig. 5; $P < 0.05$).

DISCUSSION

The present study set out to investigate the effects of the disulphide bond reducing agent DTT on histamine H_1-receptor mediated Ca^{2+} signalling in the hamster vas deferens smooth muscle cell line DDT_1MF-2. The results presented show that both components of the histamine H_1-receptor-mediated increase in $[Ca^{2+}]_i$, (i.e. intracellular Ca^{2+} release and Ca^{2+} influx) are potentiated by DTT. The mechanism(s) involved in the Ca^{2+}-influx component of the H_1-receptor response in DDT_1MF-2 cells remain to be established (for possible mechanisms see Ref. 14). These data confirm previous studies which have shown DTT potentiating H_1-receptor mediated contractions [1, 4, 7, 8] and [^{3}H]inositol phosphate accumulation [5, 9]. To our knowledge this is the first report showing the effect of DTT on H_1-receptor Ca^{2+} responses.

In marked contrast, however, DTT had no significant effect on the Ca^{2+} responses elicited by CPA (adenosine A_1-receptor agonist), bradykinin and ATP. The selective action of DTT on the histamine H_1-receptor suggests that the enhancement of H_1-receptor activity in DDT_1MF-2 cells is due to the reduction of disulphide bonds within the H_1-receptor and not within proteins (e.g. G-proteins, phospholipase C) shared by the other Ca^{2+} mobilizing

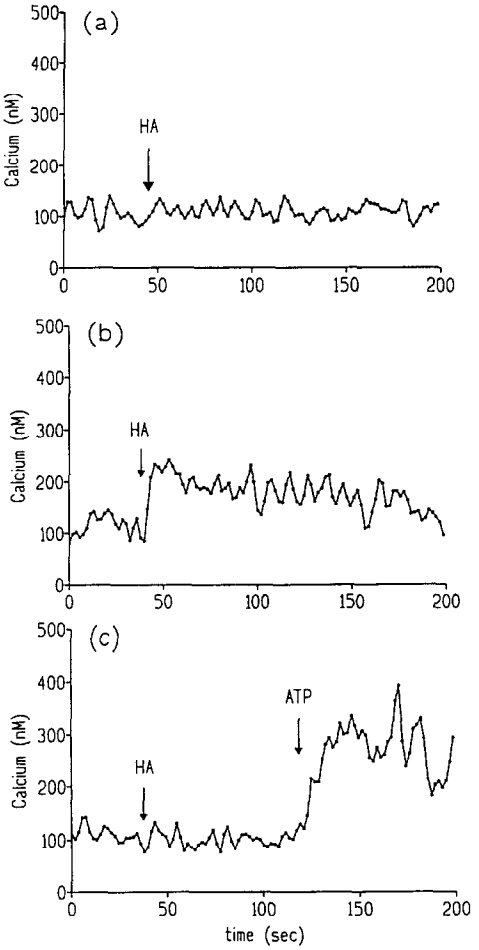

Fig. 4. Reversal of the effects of DTT by the sulphydryl oxidizing agent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). Calcium measurements were performed in the presence of extracellular Ca^{2+} (2 mM). (a) Response to 1 μM histamine in the absence of DTT. In these experiments cells were left at 37° for 30 min (after loading) in physiological buffer before stimulating with 1 μM histamine. (b) Cells preincubated for 15 min with 1 mM DTT. The coverslip was then washed and placed in physiological buffer for 15 min before stimulating with 1 μM histamine. (c) Cells were initially preincubated for 15 min with 1 mM DTT. The coverslip was then washed and incubated for a further 15 min with 1 mM DTNB before stimulating with 1 μM histamine. ATP (100 μM) was applied at the end of experiment to show cell viability. Histamine (HA; 1 μM) and ATP (100 μM) were added where indicated. Similar results were obtained in three other experiments.

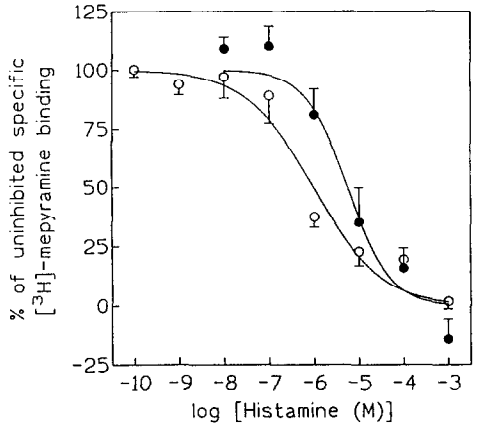

Fig. 5. The inhibition of [^{3}H]mepyramine binding by histamine in the presence (open circles) and absence (closed circles) of 1,4-dithiothreitol. Values represent the combined mean of triplicate determinations made in each of four experiments. Non-specific binding was defined using 2 μM promethazine. Curves were fitted to a logistic equation using the non-linear regression programme GraphPAD. Hill coefficients were 0.92 ± 0.17 (N = 4) and 0.57 ± 0.06 (N = 4) in the absence and presence of DTT, respectively.

receptors, i.e. bradykinin and ATP (assuming these receptors couple to the same G-protein and/or phospholipase C as the H_1-receptor). Alternatively, the reduction of disulphide bonds within a G-protein and/or phospholipase C specific to the H_1-receptor would also account for the selective action of DTT. A similar selective enhancement of H_1-receptor activity was observed in rabbit aorta [1] and guinea-pig ileal smooth muscle [7]. It is quite likely that DTT will reduce disulphide bond(s) within the other G-protein coupled receptors studied (i.e. adenosine A_1, bradykinin and ATP), but this study would suggest that these are not vital for agonist-induced calcium signalling.

Treatment of DDT$_1$MF-2 cell membranes with DTT produced a small but significant reduction in the IC_{50} value obtained for histamine displacement of [^{3}H]mepyramine binding (2.5 μM compared to 0.7 μM in treated cells). It is noticeable that the binding curve for histamine displacement of [^{3}H]-mepyramine lies to the left of the functional (Ca^{2+}) curve in DTT treated and control cells. This is difficult to explain but may be due to the different buffers used for the functional and binding studies.

Table 2. The effect of DTT on the EC_{50} values for histamine, CPA (adenosine A_1-receptor agonist), bradykinin and ATP stimulated increases in $[Ca^{2+}]_i$

Agonist	−DTT EC_{50}	(N)	+DTT (1 mM) EC_{50}	(N)
Histamine	11.0 ± 1.5 μM	6	0.50 ± 0.15* μM	6
CPA	7.3 ± 0.7 nM	3	11 ± 2 nM	3
Bradykinin	25 ± 5 nM	4	41 ± 9 nM	4
ATP	10.0 ± 0.5 μM	5	9.0 ± 0.5 μM	5

* Statistically significant ($P < 0.05$) from control response.

Several studies have shown that Na^{2+} ions selectively decrease the affinity of the H_1-receptor for histamine [21, 22]. Therefore in Na^{2+}-free Tris buffer the majority of receptors may adopt a higher affinity conformation. The lowering of the Hill coefficient in the presence of DTT would seem to suggest the presence of two binding sites (low and high affinity). Analysis of binding curves for histamine displacement of [^{3}H]mepyramine in guinea-pig cerebellum also revealed the presence of two binding sites in DTT treated membranes [6]. In contrast DTT had no significant effect on the dissociation constant (K_d) for mepyramine determined from saturation binding experiments. These binding data suggest that DTT increases the affinity of the H_1-receptor for histamine. Leftward shifts in the concentration–response curve for histamine displacement of [^{3}H]mepyramine binding have also been reported in guinea-pig ileal smooth muscle [8], rat cerebral cortex [9] and guinea-pig cerebellum [6].

In DDT_1MF-2 cells the H_1-receptor agonist 2-PEA produces a maximal inositol phosphate response which is 67% of the maximum response to histamine (100 μM histamine = 100%) [15]. In this study we investigated whether this partial H_1-agonist could elicit a Ca^{2+} response and if so how it could be modified by DTT. Cells stimulated with 1 mM 2-PEA elicited a Ca^{2+} response which was about 70% of the maximum response to 100 μM histamine. Interestingly, DTT had no significant effect on the Ca^{2+} response produced by 1 mM 2-PEA. However, DTT did potentiate Ca^{2+} responses elicited by 100 μM 2-PEA (a concentration which on its own did not produce a Ca^{2+} response). Furthermore no increase in $[Ca^{2+}]_i$ was observed with 10 μM 2-PEA in the absence or presence of DTT. 2-PEA is a low efficacy agonist in other systems [5] and in view of the 70% Ca^{2+} responses (compared to histamine) obtained in DDT_1MF-2 cells one might have expected efficacy to increase in the presence of DTT. These data suggest that DTT does not increase the efficacy of 2-PEA. However, the large 20-fold functional shift in the presence of DTT (compared to the 4-fold shift observed with the binding experiments) would suggest a possible increase in the efficacy of histamine. This might be achieved by an increase in the proportion of G-protein coupled receptors (which have higher affinity for histamine) and this would be consistent with the change in Hill coefficient of the histamine binding data obtained in DTT treated cell membranes. A similar observation has been made in guinea-pig cerebral cortex [6].

The majority of G-protein coupled receptors sequenced to date contain a critical disulphide bond between two conserved cysteine residues in the second and third extracellular domains [23]. However, the role of this conserved disulphide bond appears to vary between different classes of receptor. For example site-directed mutagenesis studies of the rat M_1-receptor have shown that disrupting the disulphide bond between Cys^{98} and Cys^{178} prevents carbachol-stimulated inositol phospholipid hydrolysis and antagonist binding [11]. In contrast in the β_2-adrenoceptor conversion of either Cys^{106} in the second extracellular domain or Cys^{184} in third extracellular domain to valine (to break the disulphide bond) causes a shift in the agonist-binding properties [24]. The mutant receptors display one high and one low affinity binding site for isoprenaline, whereas the wild-type receptor displays a single class of binding site with intermediate affinity [23]. The recent cloning of the bovine [25], rat [26] and guinea-pig [27] histamine H_1-receptor has also revealed a disulphide bond between cysteine residues located in the second and third extracellular domains. The role of this disulphide bond in agonist binding to the histamine H_1-receptor should become clear with the application of site-directed mutagenesis.

In summary, the present study has shown that the disulphide bond reducing agent DTT potentiates histamine H_1-receptor mediated Ca^{2+} responses (intracellular Ca^{2+} release and Ca^{2+} influx) in DDT_1MF-2. [^{3}H]mepyramine binding studies suggest that this effect is associated with an increase in affinity of the H_1-receptor for histamine. The precise mechanism(s) underlying the potentiation of H_1-receptor responses by DTT remain to be established. One possibility is that DTT may increase the coupling between the H_1-receptor and G-protein leading to an increase in agonist affinity. Site-directed mutagenesis studies should enable the role of disulphide bonds in agonist binding to the H_1-receptor to be determined.

Acknowledgement—The authors thank the Wellcome Trust for financial support.

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