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HISTAMINE-STIMULATED INCREASES IN INTRACELLULAR CALCIUM IN THE SMOOTH MUSCLE CELL LINE, DDT$_1$MF-2

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Abstract—Suspensions of undifferentiated cultured vas deferens smooth muscle cells (DDT$_1$MF-2) were loaded with the calcium-sensitive fluorescent dye fura-2. Exposure to histamine elicited a rapid and maintained increase in intracellular free calcium ($[Ca^{2+}]_i$) with an EC_{50} of $1.3 \pm 0.7 \times 10^{-5}$ M. The initial rise is a consequence of calcium release from intracellular stores, whereas the maintained or plateau phase, which is dependent upon the presence of extracellular calcium, is associated with calcium influx. Experiments in nominally Ca^{2+}-free buffer attenuated the initial rise in $[Ca^{2+}]_i$ (i.e. peak height) and virtually abolished the plateau phase. Re-addition of 2 mM Ca^{2+} (during experiments performed in nominally Ca^{2+}-free buffer) resulted in a return of the plateau phase. Pretreatment with the H_1-antagonist mepyramine (100 nM; $K_d = 1.0 \pm 0.4$ nM, N = 3) completely blocks the response to histamine, whereas tiotidine (2 μM; H_2-antagonist) had no effect. In conclusion, the present data would suggest that functional H_1-receptors found in hamster vas deferens smooth muscle cells are typical of the "classical" H_1-receptor in both its control of intracellular Ca_{2+} and sensitivity to antagonism by mepyramine.

A notable feature of binding studies with radioactive H_1-receptor antagonists in mammalian brain membranes is the species differences in antagonist binding properties [1]. For example, in rat brain the antagonist K_d values for some, but not all, H_1-antagonists are an order of magnitude greater than those obtained in guinea-pig brain membranes [2] or those determined from functional H_1-responses in guinea-pig cerebral cortical slices and guinea-pig ileum [3, 4]. In most respects, however, the characteristics of ^{3}H-antagonist binding in rat brain are those expected of an H_1-receptor and K_d values obtained from functional studies in rat cerebral cortical slices agree reasonably well with the binding data [5, 6]. These observations suggest that the structural identity or membrane environment of the histamine H_1-receptor may differ between different tissues and species [1].

In the smooth muscle cell line DDT$_1$MF-2, derived from a hamster vas deferens tumour [7], the K_d values for H_1-antagonists determined from [^{3}H]-mepyramine binding are at least two orders of magnitude greater than those obtained in guinea-pig tissues [8, 9]. Interestingly, this low-affinity [^{3}H]-mepyramine binding site on DDT$_1$MF-2 cells has been purified to homogeneity, with a molecular weight of 38–40 kDa [10]. Preliminary studies of function responses in this cell line indicate that antagonism by H_1-antagonists can be demonstrated over a similar low affinity range [8, 9], raising the possibility that an H_1-receptor subtype may be present.

In mammalian cells, H_1-receptors are generally coupled to phospholipase C, via a regulatory G-protein, which upon activation hydrolyses the plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (for reviews see Refs 11 and 12]). Diacylglycerol can then activate protein kinase C while IP_3 triggers the release of calcium from intracellular stores resulting in a rapid rise in cytosolic calcium concentration [11, 12]. Cytosolic free Ca^{2+} can be measured in living cells using the calcium-sensitive fluorescent dyes fura-2 and quin-2 or the bioluminescence protein aequorin [13].

Histamine H_1-receptor induced rises in cytosolic $[Ca^{2+}]$ have been recorded using fluorescent dyes in a variety of tissue and cell types. For example, in airway and vascular smooth muscle [14–18], human fibroblasts [19], 1321N1 astrocytoma cells [20], N1E-115 neuroblastoma cells [21, 22], BC_3H1 smooth muscle cells [23–25] and human umbilical vein endothelial cells [26–32]. The H_1-receptor stimulation in human airway smooth muscle, rat vascular smooth muscle and endothelial cells produces a sustained rise in $[Ca^{2+}]_i$ that is biphasic in nature. The first component results from the release of calcium from intracellular stores, whereas, the sustained or plateau phase is predominantly calcium influx. In contrast, H_1-receptor stimulation in human fibroblasts and established cell lines of CNS origin (1321N1 and N1E-115) results in a transient rise in $[Ca^{2+}]_i$, with a considerably reduced or absent plateau phase [19, 21, 22].

The intracellular calcium response to H_1-receptor stimulation has previously been recorded in the DDT$_1$MF-2 smooth muscle cell line by Mitsuhashi and Payan [8, 9]. These authors reported a rapid and concentration-dependent increase in $[Ca^{2+}]_i$ in

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response to histamine which was sensitive to antagonism by high concentrations of the H_1-receptor antagonist chlorpheniramine (100 nM to 1 μM) [8, 9]. The aim of this study was to quantitatively evaluate the susceptibility of this response to the selective H_1-antagonist mepyramine and to investigate the possibility that the suggested low-affinity H_1-receptors in DDT_1MF-2 cells, may regulate intracellular Ca^{2+} increases differently from the "classical" high-affinity H_1-receptors found in other cell lines.

MATERIALS AND METHODS

Materials. 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.). Fura-2/AM and ionomycin were from Calbiochem. Histamine and mepyramine were obtained from Sigma. Dulbecco's modified Eagle's medium (DMEM) and foetal calf serum (FCS) were from Northumbria Biologicals (U.K.) and trypsin–EDTA (10× solution) from Gibco. All other chemicals were of analytical grade.

Cell culture. 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 once a week by treatment with trypsin (0.05%)–EDTA (0.02%) solution (1/20 split ratio) and fed with fresh growth medium every 48 hr. All experiments were performed on confluent cells (passages 4–11, numbers assigned after receiving the cell line).

Measurement of intracellular free calcium. Intracellular free calcium was measured by loading cells with the calcium-sensitive fluorescent dye fura-2. Confluent monolayers were removed from culture flasks 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) containing 0.005% EDTA, pH 7.4, at 37° for 5 min. After centrifugation, cells were resuspended in 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 20 min at 37°. After this "loading" period cells were washed, centrifuged, resuspended in fresh buffer that was free of fura-2 but contained 0.1% bovine serum albumin (BSA) and kept at 37° until required. Before each experimental run a 2 mL aliquot was taken, washed and resuspended in fresh buffer. Fluorescent measurements were made at 37° in quartz cuvettes (drugs were added to the cuvettes in 10-μL aliquots) containing a stirrer bar using a Perkin–Elmer LS 50 spectrometer. The excitation wavelengths were 340 and 380 nm, with emission at 500 nm. The time taken to switch between 340 and 380 nm excitation was 0.8 sec. Intracellular Ca^{2+} was calculated every 1.6 sec from the ratio (R) of 340 nm/380 nm fluorescent values using the equation of Grynkiewicz *et al.* [33]:

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

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 for each separate experiment under saturating $[Ca^{2+}]_i$ (using 20 μM ionomycin, pH 7.45) followed by calcium-free (achieved using 6.25 mM EGTA immediately followed by 25 μL of 1.0 M NaOH to compensate for the decrease in pH, in the presence of 20 μM ionomycin) conditions, respectively. Corrections for autofluorescence were made by the addition of ionomycin (20 μM) followed by 5 mM $MnSO_4$. In our system R_{max} was 35.4 ± 4.7 (N = 6), R_{min} was 1.5 ± 0.1 (N = 6) and $S_{380,min}/S_{380,max}$ was 7.2 ± 0.9 (N = 6). The R_{min} is comparatively high compared to values normally cited in the literature which are typically <1.0. This apparently "high" R_{min} value may be a consequence of sequestration of fura-2 into cytoplasmic organelles. Where Ca^{2+}-free conditions were required, fura-2 loaded cells were washed, centrifuged and resuspended in fresh buffer that was nominally Ca^{2+}-free.

Data analysis. Agonist and antagonist concentration–response curves were fitted to a logistic equation using the non-linear regression program GraphPAD (ISI).

Apparent antagonist equilibrium dissociation constants (K_d) were estimated from the relationship:

$$K_d = IC_{50}/([A]/EC_{50} - 1)$$

where IC_{50} is the concentration of antagonist (mepyramine) required to inhibit by 50% the maximal response elicited by 10^{-4} M histamine ($[A]$) and EC_{50} is the concentration of agonist (histamine) required to produce half-maximum stimulation. This concentration of histamine produces the same response as that obtained with 100 μM histamine in the presence of the IC_{50} concentration of mepyramine.

Data are expressed as mean ± SEM of at least three experiments.

RESULTS

H_1-receptor stimulation in the smooth muscle cell line, DDT_1MF-2, results in a dose-dependent increase in intracellular Ca^{2+} which appeared to be biphasic in nature (Fig. 1a). Histamine (HA, 100 μM) increases basal $[Ca^{2+}]_i$ from 332 ± 28 nM (N = 19) to approximately 900 nM, within 20 sec of application (see Fig. 1a). The response is clearly well maintained with the plateau phase declining slowly to approximately 600 nM, 2 min after the initial peak. Histamine concentration–response curves for the initial peak (i.e. maximum response) and plateau phase (determined at 30, 60 and 90 sec after the initial peak) are shown in Fig. 1b, with their accompanying EC_{50} quoted in the figure legend. The EC_{50} values obtained for the peak and plateau phases show that both components are similarly sensitive to histamine.

To assess the involvement of extracellular calcium in the overall response to histamine, experiments were performed in nominally Ca^{2+}-free buffer. Figure 2a shows a profile obtained by stimulating

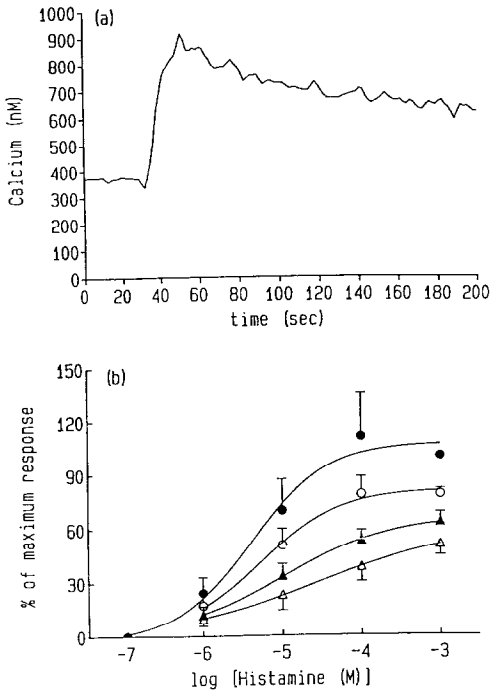

Fig. 1. (a) Effect of H_1-receptor stimulation on intracellular Ca^{2+} concentration in fura-2 loaded vas deferens smooth muscle cells. Histamine (HA; 100 μM) was added where indicated. Measurements of $[Ca^{2+}]_i$ were made every 1.6 sec as described in Materials and Methods. (b) Concentration–response curves for histamine-stimulated increases in $[Ca^{2+}]_i$ in DDT_1MF-2 cells. Profiles and accompanying EC_{50} values (μM) represent the peak ratio (F_{340}/F_{380}) response (●, 12.9 μM ± 7.1) and plateau phase at times 30 sec (○, 5.9 μM ± 1.5), 60 sec (▲, 8.2 μM ± 4.1), and 90 sec (△, 19.9 μM ± 10.1) after the maximum response. The data is expressed as a percentage of the peak response to 1 mM histamine (expressed as an increase in F_{340}/F_{380} ratio minus the basal fluorescence ratio), which was measured in each experiment. Data are means ± SEM of four experiments.

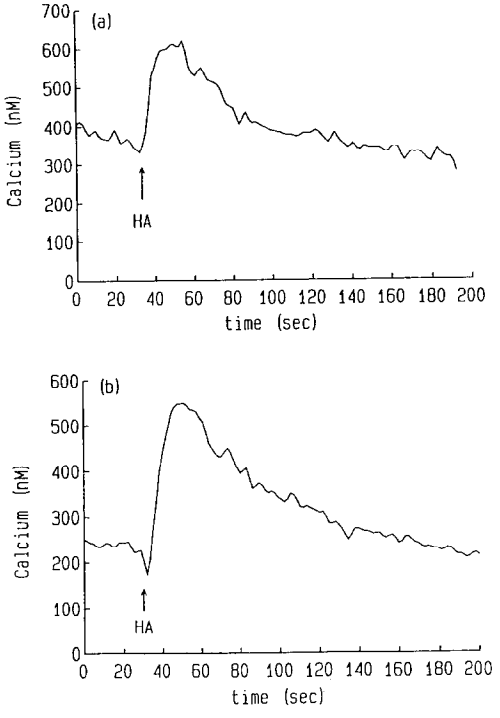

Fig. 2. Effect of removing extracellular Ca^{2+} on histamine stimulated $[Ca^{2+}]_i$ changes. (a) In the presence of nominally Ca^{2+}-free buffer; histamine (HA; 100 μM) was added where indicated. (b) In the presence of Ca^{2+}-free buffer and 0.1 mM EGTA; histamine (HA; 100 μM) was added where indicated. Similar results were obtained in three other experiments.

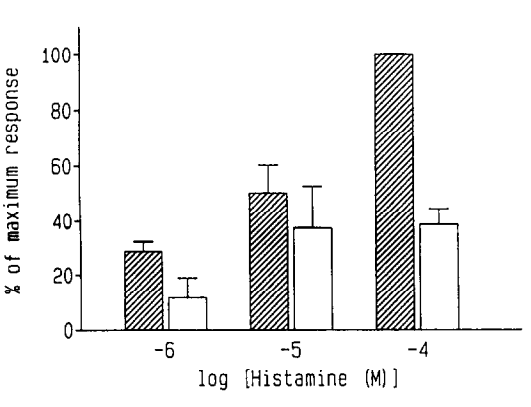

Fig. 3. The effect of removing extracellular calcium on the maximum peak response. Data were obtained in the absence (open bars) and presence (shaded bars) of 2 mM Ca^{2+}. The data are expressed as a percentage of the response to 100 μM histamine in the presence of 2 mM Ca^{2+}, which was taken as 100%. Data are means ± SEM of three experiments.

the H_1-receptors with 100 μM HA in the absence of added extracellular calcium. The most striking feature is the disappearance of a maintained plateau phase, with $[Ca^{2+}]_i$ returning to basal levels approximately 100 sec after stimulation. Similar results were obtained in nominally Ca^{2+}-free buffer, containing 0.1 mM EGTA (Fig. 2b). This would indicate that a calcium influx mechanism(s), across the plasma membrane, is responsible for the plateau phase. A further consequence of removing the extracellular calcium is the attenuation of the maximum response (Fig. 3). In the absence of extracellular calcium, the response to 100 μM HA was 38.7% ± 4.8 (N = 3) of that obtained in the presence of extracellular calcium.

The plateau phase and its requirement for extracellular calcium was examined further in a series of experiments in which extracellular calcium (2 mM) was reapplied, after the cells had been stimulated with 100 μM HA. Figure 4a clearly shows that reapplying calcium (2 mM) to the extracellular buffer, after stimulation with 100 μM HA, results in a rise in $[Ca^{2+}]_i$ which is attributed to the reappearance of the plateau phase. To dismiss the possibility that this rise in $[Ca^{2+}]_i$ is simply a consequence of fura-2 leakage into the extracellular

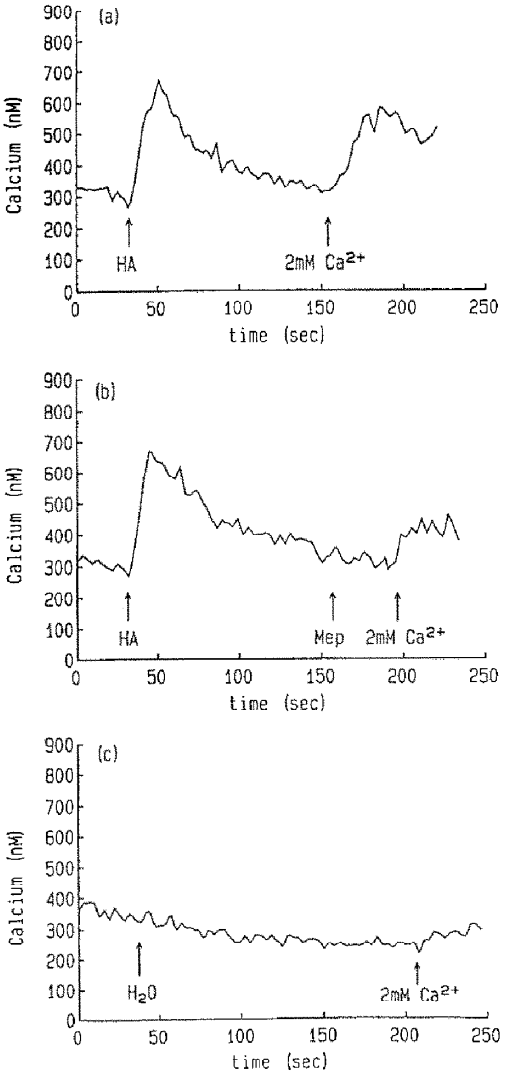

Fig. 4. The effects of reapplying extracellular Ca^{2+} (2 mM) in the absence and presence of mepyramine, during experiments performed in nominally Ca^{2+}-free buffer. All three experiments (a, b and c) were performed in nominally Ca^{2+}-free buffer. Histamine (HA; 100 μM), mepyramine (Mep; 100 nM), $CaCl_2$ or drug vehicle (H_2O; 10 μL) were added where indicated. Similar results were obtained in three other experiments.

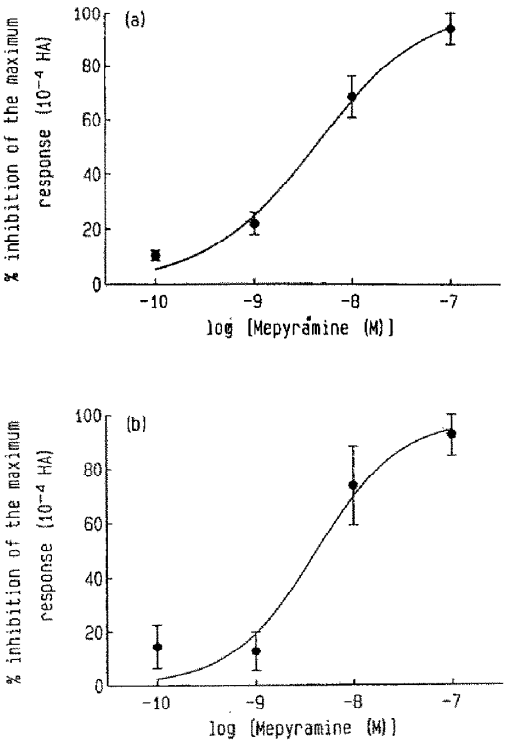

Fig. 5. Antagonism by mepyramine of the response produced by stimulation with 100 μM histamine. Cell suspensions were incubated with the relevant concentration of mepyramine for 15 min prior to the addition of histamine. The data are expressed as the percentage inhibition of the response to 100 μM histamine. The data represent inhibition of the peak (a) and plateau (b) phases of the response. The plateau phase measurements were taken at 100 sec after the maximum stimulation. Data are mean ± SEM of three experiments.

medium a control experiment was performed in which HA was replaced with water (Fig. 4c). There was no observable increase in basal calcium during this experiment, indicating that the rise in $[Ca^{2+}]_i$ (after reapplying 2 mM $CaCl_2$) shown in Fig. 4a is a result of calcium entry into the cell. The calcium entry (influx) that occurs during the plateau phase was shown to be dependent upon the continued presence of agonist, since removal of histamine from its receptor, using the H_1-antagonist mepyramine (100 nM, applied 40 sec prior to 2 mM $CaCl_2$), attenuates the observed rise in $[Ca^{2+}]_i$ seen when calcium (2 mM) was reapplied (Fig. 4b; compared with Fig. 4a).

The mepyramine sensitivity of the histamine response (both the peak and plateau phase) was explored further by producing mepyramine concentration–response curves under antagonist equilibrium conditions (10^{-7}–10^{-10} M, 15 min pre-incubation) in the presence of a constant HA concentration (100 μM). Two curves representing mepyramine antagonism of the peak and plateau phase are shown in Fig. 5a and b, respectively. The peak and plateau phase are equally sensitive to mepyramine with IC_{50} values of 6.6 ± 3.0 nM (N = 3) and 8.1 ± 3.4 nM (N = 3), respectively. This particular approach enabled apparent equilibrium dissociation constants (K_d) to be calculated (see Materials and Methods) for the peak and plateau phase. The K_d values obtained were 1.0 ± 0.4 nM (N = 3) and 1.2 ± 0.5 nM (N = 3) for the peak and plateau phase, respectively. It should be noted that pretreatment with the H_2-antagonist tiotidine (2×10^{-6} M; N = 2, data not shown) had no effect.

DISCUSSION

The present study set out to investigate whether

the reported low-affinity H_1-receptors found in the vas deferens smooth muscle cell line, DDT_1MF-2, regulate intracellular calcium release and calcium influx differently from the "classical" H_1-receptor detected in other cell lines. The data presented here show that when stimulated with histamine, suspensions of fura-2 loaded DDT_1MF-2 cells rapidly increase cytosolic $[Ca^{2+}]$. This histamine-stimulated increase in $[Ca^{2+}]_i$ comprises two distinct components: (1) release of Ca^{2+} from intracellular storage sites (mobilization), which is probably secondary to the production and action of IP_3 [11, 12], and (2) influx of extracellular Ca^{2+} through Ca^{2+} channels in the plasma membrane. A biphasic response to histamine can also be detected in DDT_1MF-2 cells on inspection of the data presented by Mitsuhashi and Payan [8]. The mechanism(s) underlying Ca^{2+} influx in this cell line remain to be established but it is clear from the present data that it requires the continued presence of histamine on the receptor site (see Fig. 4b). Thus, after activation of intracellular release by histamine under calcium-free conditions, subsequent calcium entry (after addition of exogenous calcium) can be reduced by the addition of the H_1-receptor antagonist mepyramine. It should be noted that the small rise in $[Ca^{2+}]_i$ observed in Fig. 4b (obtained after the reapplication of extracellular calcium (2 mM) in the presence of 100 nM mepyramine) is probably a consequence of the relatively short exposure time (40 sec) to the H_1-antagonist before the addition of 2 mM $CaCl_2$. These conclusions are similar to those recently reported in human airway smooth muscle [18] but differ from the data obtained in human umbilical vein endothelial cells, where continued H_1-receptor occupancy is not required for Ca^{2+} influx [26]. In this latter case it is thought that Ca^{2+} entry is driven by the state of the intracellular calcium pool rather than by direct receptor activation [26]. However, whether the histamine-activated Ca^{2+} channel involved in DDT_1MF-2 cells is a receptor-operated channel, a voltage-operated channel or a secondary messenger-activated Ca^{2+} channel, remains to be established [34].

The major finding of this study, however, concerns the sensitivity of the H_1-receptor-mediated calcium response to inhibition by mepyramine. The data reported here clearly shows that the rise in $[Ca^{2+}]_i$ is inhibited by concentrations of mepyramine that are indicative of the "classical" H_1-receptor i.e. K_d values in the order of 1 nM [1]. These experiments also revealed that the peak and plateau phase of the calcium response to histamine are equally sensitive to mepyramine (see Fig. 5) with K_d values of 1.0 ± 0.4 nM (N = 3) and 1.2 ± 0.5 nM (N = 3), respectively. This suggests that the same H_1-receptor is responsible for intracellular calcium release and influx of extracellular calcium, or that H_1-receptors coupled to different effector systems are involved, each with a high affinity for mepyramine. This is in contrast to the $[^3H]$mepyramine binding dissociation constant of 219 nM reported in this cell line by Mitsuhashi and Payan [8]. However, it is notable that these previous binding studies were undertaken at 4°, and in view of the marked temperature sensitivity of $[^3H]$mepyramine binding [35], this may partly account for the difference between the two sets of results.

The basal $[Ca^{2+}]_i$ observed during our experiments (332 ± 28.5 nM (N = 19)) is exceptionally high, compared with the generally accepted resting $[Ca^{2+}]_i$ of around 100 nM. Previous measurements of intracellular $[Ca^{2+}]$ in DDT_1MF-2 cells have revealed basal Ca^{2+} levels of 386 ± 50 nM [8] and 100–130 nM [36]. These values were obtained from suspensions of DDT_1MF-2 cells loaded with fura-2 and quin-2, respectively. The high basal $[Ca^{2+}]_i$ is not a consequence of dye leakage into the extracellular buffer (although dye leakage is a problem in this particular cell line), since cell suspensions were routinely centrifuged and resuspended in fresh buffer immediately before each experimental run. The suggestion that dye leakage, into the extracellular medium, was causing the high basal $[Ca^{2+}]_i$ can be further dismissed by the data shown in Fig. 4c, where re-addition of 2 mM $CaCl_2$ (experiment performed in nominally Ca^{2+}-free buffer and HA replaced with water) did not produce any rise $[Ca^{2+}]$, be it intracellular or extracellular. It is feasible that 300 nM is the correct basal $[Ca^{2+}]_i$ for this particular cell line. However, there are two possible explanations for the high basal $[Ca^{2+}]_i$, firstly, sequestration of fura-2 into cytoplasmic organelles is occurring [37] or secondly, disrupting the monolayer and placing the cells in suspension may have perturbed the cells in some way, increasing their permeability to Ca^{2+} and hence raising basal $[Ca^{2+}]_i$. This latter explanation would account for the lower basal $[Ca^{2+}]_i$ measured in Ca^{2+}-free EGTA conditions (compare Figs 1a and 2b). Preliminary $[Ca^{2+}]_i$ measurements on DDT_1MF-2 cells grown on glass coverslips produced basal $[Ca^{2+}]_i$ levels of around 100 nM (Dickenson and Hill; unpublished observations) supporting the view that the high basal $[Ca^{2+}]_i$ levels are due to cell suspension. If this explanation is correct, then the lower basal values detected using quin-2 [36] may be attributable to its greater buffering capacity.

In summary, the present study has shown that H_1-receptor stimulation in undifferentiated DDT_1MF-2 vas deferens smooth muscle cells evokes a rapid and sustained increase in $[Ca^{2+}]_i$. The H_1-receptor responsible displays a high affinity for the antagonist mepyramine, with a K_d of 1.3 nM. We conclude that the H_1-receptor on DDT_1MF-2 cells displays typical properties of the "classical" H_1-receptor and therefore does not represent a possible subclass of histamine H_1-receptor.

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