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The histamine H_1 receptor in GT1-7 neuronal cells is regulated by calcium influx and KN-62, a putative inhibitor of calcium/calmodulin protein kinase II

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1 In GT1-7 cells, histamine stimulated the initial $[Ca^{2+}]_i$ transient in a dose-dependent manner with a best-fit EC_{50} value of 4.2 ± 4.2 μM (mean $\pm$ s.e.mean, $n=4$) and a best-fit maximal effect of 138 ± 56 nM ($n=4$) increase above basal calcium levels.

2 Pretreatment of cells with 30 μM histamine for 30 min desensitized the population mean peak calcium signal by 53% to 75 ± 9 nM, ($n=3$, $P<0.04$). Analysis of the individual cells revealed that $39 \pm 7\%$ ($n=94$ cells from 8 experiments) of pretreated cells exhibited desensitized histamine-stimulated $[Ca^{2+}]_i$ transients of $\leqslant 1$ standard deviation below the control cells mean calcium transient level.

3 The desensitization induced by histamine was prevented ($P<0.01$) by KN-62 (10 μM), a putative inhibitor of the calcium/calmodulin-dependent protein kinase II (CaMKII). KN-62 (10 μM) alone did not induce $[Ca^{2+}]_i$ mobilization, nor did it antagonize the histamine-stimulated $[Ca^{2+}]_i$ signal. In addition, KN-62 did not appear to have its effect by hastening the rate of recovery from desensitization.

4 Histamine pretreatment in nominal (zero calcium + 0.2 mM EGTA) or in low (0.3 mM) extracellular calcium did not induce histamine receptor desensitization, supporting a role for extracellular calcium in the homologous H_1 receptor desensitization process.

5 Histamine (30 μM) stimulated at least four different types of $[Ca^{2+}]_i$ signals in GT1-7 cells. The majority (61%) were of single spikes with the remaining cells showing some form of calcium oscillatory behaviour. The proportion of GT1-7 cells showing histamine-induced calcium oscillations was histamine concentration-dependent and significantly reduced after acute desensitization. KN-62, when present during histamine pretreatment, prevented this fall in calcium oscillation. Under the conditions of nominal or 0.3 mM extracellular calcium the proportion of cells exhibiting histamine-stimulated calcium oscillations was not significantly different from the controls.

6 Bradykinin stimulated a $[Ca^{2+}]_i$ transient in GT1-7 cells with a population mean peak response of 147 ± 8 nM ($n=5$) over basal levels. The bradykinin-induced $[Ca^{2+}]_i$ signal was without any calcium oscillatory activity. Histamine pretreatment caused the heterologous desensitization of the bradykinin $[Ca^{2+}]_i$ signal (44% reduction, $P<0.007$), which was unaffected by KN-62.

7 The results presented here suggest that the histamine-mediated homologous H_1 receptor desensitization process involves extracellular calcium and can be blocked by KN-62, a putative inhibitor of CaMKII. In contrast, KN-62 does not appear to prevent the histamine-mediated heterologous desensitization cascade. These findings suggest fundamental differences in the mechanisms underlying homologous and heterologous H_1 receptor desensitization pathways in GT1-7 neuronal cells.

Keywords: Histamine H_1 receptor; homologous desensitization; intracellular calcium; calcium oscillation; calcium/calmodulin-dependent protein kinase II; GT1-7 neuronal cells; bradykinin; heterologous desensitization

Introduction

It is well known that most neurotransmitter-induced signalling pathways are desensitized after prolonged agonist stimulation. The resulting attenuation of cellular responsiveness to continued or subsequent receptor activation plays an important role in regulating biological signalling (Lohse, 1993). In addition, *in vivo* receptor desensitization has clinical implications, being purported to have a role in the aetiology of several diseases and suggested to be responsible for the tolerance to certain drugs after long-term treatment (Brodde & Michel, 1989).

The most comprehensive information on the molecular mechanisms underlying receptor desensitization has emerged from the study of the β-adrenoceptor system (Lohse, 1993). Thus, it has been established that the desensitization process is multifaceted and can generally be divided into two phases, homologous and heterologous desensitization. Homologous desensitization involves the selective loss of response to the stimulated receptor only, whereas, heterologous desensitization in addition to a reduced responsiveness of the stimulated receptor, also involves an attenuation of responses induced by other receptor systems. There are also clear distinctions between the mechanisms underlying desensitization, which include receptor affinity changes, receptor sequestration, receptor-effector uncoupling and receptor down-regulation, and are activated, perhaps sequentially, by increasing severity (acute to chronic, min–days) of receptor stimulation. A role for protein phosphorylation as the biochemical process associated with several (most convincingly with the acute agonist-induced effects) of the molecular mechanisms of desensitization is now accepted (Huganir & Greengard, 1990). In comparison, the studies undertaken to understand the molecular basis for agonist-induced desensitization of phosphoinositidase C (PIC)-coupled receptors are in their infancy, but have nonetheless highlighted some similarities with the β-adrenoceptor system (reviewed by Wojcikiewicz *et al.*, 1993). In particular, there is evidence that receptors coupled to PIC desensitize by both homologous and heterologous mechanisms (Dillon-Carter & Chuang, 1989; Smit *et al.*, 1992; Bristow & Zamani, 1993;

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Dickenson & Hill, 1993), undergo a rapid (s–min) agonist-induced desensitization, possibly associated with protein phosphorylation (Tobin & Nahorski, 1993), that acute agonist treatment (min) can attenuate inositol phosphate production (Bristow & Young, 1991; Bristow & Zamani, 1993; Martin & Harden, 1989), and that agonist treatment (minutes–hours) can induce receptor internalisation (Eva *et al.*, 1990; Wojcikiewicz *et al.*, 1993).

Histamine is an established neurotransmitter in the mammalian CNS, where it is found in discrete pathways that project throughout the brain (Hill, 1990). It interacts with three pharmacologically distinct receptor subtypes in the CNS, H_1, H_2 and H_3, to regulate a plethora of physiological actions, including arousal state, brain energy metabolism, locomotor activity, feeding, drinking, sexual behaviour, and neural plasticity (Wada *et al.*, 1991). Much work in the brain has focused on the histamine H_1 receptor which is coupled, via a G-protein, to PIC and the breakdown of phosphatidylinositol 4,5-biphosphate, leading to the mobilization of intracellular calcium and production of diacylglycerol (Hill, 1990). The responses induced by H_1 receptors can be desensitized after prolonged agonist stimulation in both a homologous (Nakahata & Harden, 1987; Dillon-Carter & Chuang, 1989; Cowlen *et al.*, 1990; Bristow & Zamani, 1993) and heterologous (Brown *et al.*, 1986; McDonough *et al.*, 1988; Dickenson & Hill, 1993; Smit *et al.*, 1992) manner. Studies to understand the biochemical processes underlying the H_1 receptor desensitization phenomenon in neuronal and non-neuronal cells have ruled out a role for protein kinase C (PKC) in homologous (Smit *et al.*, 1992; Dickenson & Hill, 1993; Zamani & Bristow, 1993; Zamani *et al.*, 1995) and heterologous (Smit *et al.*, 1992; Dickenson & Hill, 1993) H_1 receptor desensitization. The characteristics of histamine H_1 receptor desensitization, such as the time course for onset (Bristow & Zamani, 1993) and recovery (Bristow *et al.*, 1993; Bristow & Zamani, 1993), the consensus sites for protein kinases in the H_1 receptor amino acid sequence (Yamashita *et al.*, 1991), and the histamine-mediated phosphorylation of proteins (Rayamond *et al.*, 1991; Levin & Santell, 1991), all imply a role for protein phosphorylation in the desensitization process. This study has therefore investigated the involvement of extracellular calcium and KN-62, a putative inhibitor of calcium/calmodulin-dependent protein kinase II (CaMKII), in the homologous and heterologous histamine H_1 receptor desensitization cascades.

Methods

Cell culture

GT1-7 cells were maintained in 75 cm^2 poly-D-lysine-coated tissue culture flasks containing 20 ml growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) and supplemented with foetal calf serum (5% v/v), horse serum (5%, v/v), glucose (25 mM), penicillin (5×10^4 iu l^{-1}), and streptomycin (50 mg l^{-1}) in a 5% (v/v) CO_2-incubator at 37°C as described elsewhere (Zamani *et al.*, 1994). At confluence (approximately 18×10^6 cells per flask), the cells were washed with phosphate buffered saline (PBS-EDTA, pH 7.5, in mM: NaCl 137, Na_2HPO_4 8.1, KCl, 2.7, KH_2PO_4 1.5 and EDTA 0.6) and dissociated with trypsin/EDTA (5 ml, 500–750 BAEE u ml^{-1} trypsin/0.6 mM EDTA, Sigma) for 5 min at 37°C. The action of trypsin was terminated by the addition of DMEM growth medium (20 ml, as above). Cells were centrifuged at 220*g* for 5 min, the resultant pellet resuspended in 5 ml DMEM growth medium (as above), and 100 μl taken into 3 ml OptiMem (Gibco) supplemented with penicillin (5×10^4 iu l^{-1}), streptomycin (50 mg l^{-1}), and glucose (2 g l^{-1}, Sigma); 500 μl aliquots of the new mixture were then transferred on to poly-D-lysine-coated glass coverslips (31 cm^2 diameter) and left overnight in the CO_2 incubator. The next day 1 ml of supplemented OptiMem was added and cells kept in small petri dishes for 2–4 days pending the experiments.

Pretreatment of the cells

GT1-7 cells were transferred to HEPES-Krebs-Henseleit medium (HEPES-KHM, pH 7.4, in mM: NaCl 116, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 5, $CaCl_2$ 2.5, HEPES, 25 and glucose 25) by first washing them twice in 1 ml HEPES-KHM medium. Fura-2AM (4 μM) was then added to the cells and left for 30 min (37°C, CO_2 incubator). Throughout the Fura-2AM loading time, cells to be desensitized were treated with histamine (30 μM) in the presence or absence of KN-62 (10 μM, added 5 min before histamine). During the desensitization protocol some cells were incubated with nominally calcium-free (with 0.2 mM EGTA) or 0.3 mM calcium-containing incubation medium (HEPES-KHM as above with altered calcium).

Agonist-mediated intracellular calcium mobilization

At the end of the pretreatment period, GT1-7 cells were washed (3×2 ml HEPES-KHM medium), transferred to a temperature controlled perfusion chamber (set to 25°C, Biophysica Inc.), 1.5 ml HEPES-KHM medium (25°C) was added, and the incubation continued for 35 min to allow for Fura-2AM hydrolysis. During the hydrolysis period the buffer was continuously gassed (95%O_2: 5%CO_2 v/v) and for the last 5 min the temperature was raised to 37°C. After the collection of basal calcium levels for 3 min, cells were stimulated (at 37°C) with either histamine (1–100 μM) or bradykinin (5 μM) for 10 min. During this time cells were excited with pairs of 340 and 380 nm light and images were collected at 2 s intervals through a 470–520 nm band-width filter using a Zeiss 135TV Axiovert microscope and an extended ISIS camera (Photonic Sciences). Digitised images were fed to Apple Macintosh computers utilising an image analysis software developed by Image Processing and Vision Company Ltd (ImproVision). Images were background-subtracted, ratioed and intracellular calcium was measured against a calibration graph made using standard calcium/EGTA solutions (Molecular Probes Inc.). Single neuronal cells to be analysed were defined by drawing and intracellular calcium concentration variations measured throughout the length of experiment. Data from the individual cells in each experiment were calculated, then pooled and the population mean values calculated. The data are normally given as the mean ± s.e. mean of the population means from the number of experiments performed. The number of cells in each experiment ranged between 6 and 26.

Analysis of data

Concentration-response data for the histamine-induced initial $[Ca^{2+}]_i$ transients were fitted to the Hill (logistic) equation and EC_{50} values were estimated using UltraFit software package with weighted curve fitting according to the reciprocal of the variance associated with each data point. The programme used the following equation:

$$\text{Response} = R_{max} \times S^n/(EC_{50}{}^n + S^n)$$

where R_{max} is the maximum response, S is the histamine concentration, and n is the Hill slope. Statistical analyses were carried out by use of Student's *t* test. Data are presented as means ± s.e. mean, unless stated otherwise.

Results

Histamine-mediated desensitization of histamine H_1 receptor-stimulated $[Ca^{2+}]_i$ mobilization is inhibited by KN-62

Histamine stimulated the initial $[Ca^{2+}]_i$ transient in a dose-dependent manner with a best-fit EC_{50} value of 4.2 ± 4.2 μM and a best-fit maximal effect of 138 ± 56 nM (4 experiments)

increase above basal calcium (Figure 1). At 30 μM, the histamine-stimulated $[Ca^{2+}]_i$ signal gave a population mean peak level of 158 ± 25 nM (4 experiments) increase above basal calcium (Figure 2). This concentration was subsequently used for desensitization and stimulation protocols. Pretreatment with 30 μM histamine desensitized the mean peak calcium signal to 75 ± 9 nM (3 experiments, $P < 0.04$, Student's t test). The desensitization induced by histamine was significantly inhibited ($P < 0.01$) by the inclusion of KN-62 (10 μM), an inhibitor of the calcium/calmodulin-dependent protein kinase II (Tokumitsu *et al.*, 1990) during the pretreatment period (Figure 2). The effect of 10 μM KN-62 was considered maximal since a similar magnitude of inhibition was attained with 3 μM (mean histamine-stimulated $[Ca^{2+}]_i$ of 124 ± 7 nM $n = 14$ cells from 1 experiment compared to 138 ± 8 nM with 10 μM). KN-62 (10 μM) alone did not induce $[Ca^{2+}]_i$ mobilization (0 ± 2 nM above basal calcium, $n = 26$ cells), nor did it antagonize the histamine-stimulated calcium signal (30 μM histamine + 10 μM KN-62 gave 167 ± 27 nM calcium peak above basal, $n = 16$ cells).

KN-62 did not appear to have its effect by hastening the rate of recovery from desensitization (Figure 3). In this series of experiments the cells were stimulated 5 min after the termination of the desensitizing procedure. Thus, KN-62 fully inhibited the histamine-induced desensitization even after 5 min recovery period (during which time the temperature was increasing from 25 to 37°C) giving a histamine signal which was not significantly different from those of control cells (Figure 3). The extent of desensitization using this shorter recovery period was very similar to our routine measurement of the desensitization phenomenon (35 min delay between the end of histamine pretreatment and histamine challenge, Figure 2).

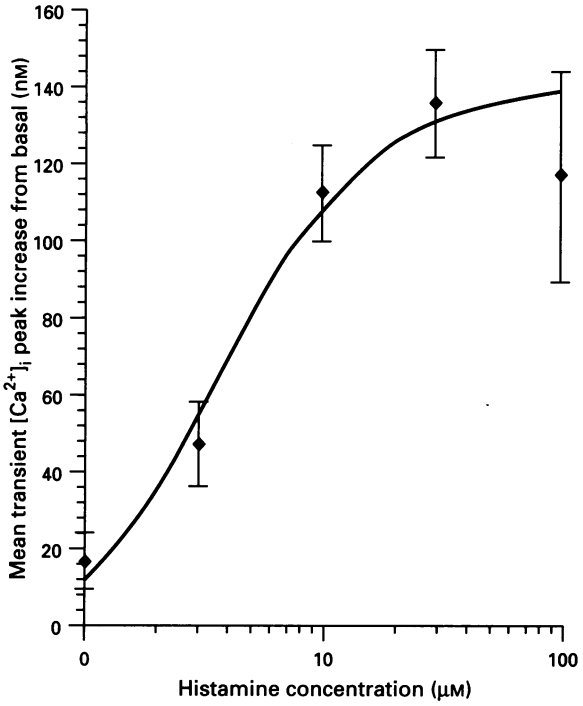

Figure 1 Histamine stimulated transient $[Ca^{2+}]_i$ peak in GT1-7 cells is concentration-dependent. GT1-7 cells were grown on coverslips, loaded with fura-2AM (37°C), the media changed, and the cells transferred to the temperature controlled perfusion chamber to allow for fura-2AM hydrolysis (25–27°C). The temperature of the incubation medium was raised to 37°C, and then the cells were stimulated with histamine (1–100 μM) and the individual cell $[Ca^{2+}]_i$ responses were recorded and analysed as described in methods section. The graph shows the best-fit curve for the data (as described in methods) and the values are presented as population mean ± s.e.mean of the histamine stimulated transient calcium peaks from 3–5 experiments. The mean population basal $[Ca^{2+}]_i$ level was 117 ± 3 nM ($n = 7$ experiments).

Desensitization of histamine-stimulated calcium mobilization is prevented by reducing extracellular calcium levels

Histamine pretreatment in the absence of extracellular calcium (zero calcium + 0.2 mM EGTA) did not induce desensitization (Table 1), since when they were stimulated with 30 μM histamine (in media containing the usual 2.5 mM calcium) the $[Ca^{2+}]_i$ peak was not significantly different from the control cells (which were deprived of extracellular calcium only during the corresponding pretreatment period) (Table 1). Similarly, no histamine-induced desensitization was observed in low (0.3 mM) extracellular calcium concentrations (Table 1).

All cells do not appear to express a desensitized histamine-stimulated initial $[Ca^{2+}]_i$ transient after histamine pretreatment

In order to establish whether all cells showed desensitization to a similar degree after histamine pretreatment the maximal initial $[Ca^{2+}]_i$ transients from the individual cells were analysed. From single cell analysis, $39 \pm 7\%$ ($n = 94$ cells from 8 experiments) of pretreated cells exhibited desensitized histamine-stimulated $[Ca^{2+}]_i$ transients. Desensitization was defined as a response of $\leqslant 1$ standard deviation below the control cells mean $[Ca^{2+}]_i$ transient level of 158 ± 50 nM (mean ± s.d., $n = 4$ experiments).

Desensitization of histamine receptors reduces the proportion of cells showing histamine-mediated calcium oscillations

Histamine stimulation caused a variety of $[Ca^{2+}]_i$ signals in GT1-7 cells (Figure 4). At 30 μM, histamine induced at least 4

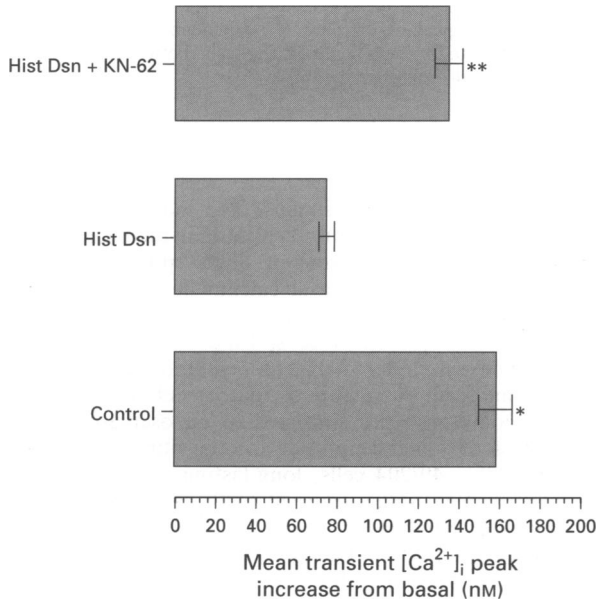

Figure 2 The role of CaMKII in the desensitization of the histamine H_1 receptor. GT1-7 cells were grown on coverslips, loaded with fura-2AM (37°C), the media changed, and the cells transferred to the temperature controlled perfusion chamber to allow for fura-2AM hydrolysis (25–27°C). During the loading period, cells to be desensitized were treated with either histamine (30 μM) or histamine and KN-62 (10 μM). Control samples were left in the incubation medium. The temperature of the incubation medium was raised to 37°C, then the cells were stimulated with histamine (Hist, 30 μM) and the $[Ca^{2+}]_i$ response recorded as described in methods section. Values are presented as population mean ± s.e.mean of the histamine stimulated transient calcium peaks from 3 to 8 independent treatments in each condition. *$P < 0.04$ and **$P < 0.01$, significantly different from histamine desensitized (Hist Dsn).

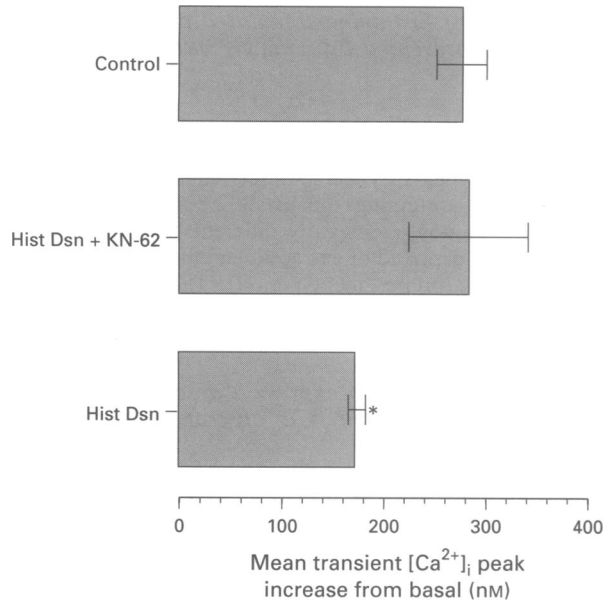

Figure 3 The effect of KN-62 on the rate of recovery from histamine-induced desensitization of the histamine H_1 receptor. GT1-7 cells were grown on coverslips, loaded with fura-2AM (37°C), the media changed, and the cells were transferred to the temperature controlled perfusion chamber to allow for fura-2AM hydrolysis (25–27°C). During the hydrolysis period they were treated with either histamine (30 μM) or histamine and KN-62 (10 μM). Control samples were left in the incubation medium. The temperature of the incubation medium was raised to 37°C and then the cells stimulated with histamine (30 μM) and the $[Ca^{2+}]_i$ response recorded as described in methods section. In these series of experiments the design allowed only 5 min (during which time the temperature was increasing from 25 to 37°C) to elapse between the termination of desensitization and the subsequent histamine stimulation. Values are presented as population mean ± s.e.mean of the histamine stimulated transient calcium peaks from 3 to 4 independent treatments in each condition. *$P<0.02$, significant difference between control and histamine-induced desensitized samples.

different types of calcium signals. The majority (61% of a total number of 204 cells) of histamine-mediated calcium responses in GT1-7 cells were of single spikes which either rapidly fell back to near basal calcium levels (25%, 51/204 cells, Figure 4a, fast re-equilibration) or showed a delayed re-equilibration (36%, 73/204 cells, Figure 4b, slow re-equilibration). The remainder of histamine-stimulated GT1-7 cells showed some form of calcium oscillatory behaviour, of which the most common types consisted of an oscillatory pattern without loss of signal amplitude for the duration of the experiment (24%, 49/204 cells, long-lasting oscillation, Figure 4c) and a classical single transient with oscillatory signals of decaying peak heights (15%, 31/204 cells, short-lived oscillation, Figure 4d). In addition a number of other calcium patterns were observed that were too infrequent to merit further classification. With increasing concentrations of histamine a greater proportion of GT1-7 cells exhibited calcium oscillations. At 1, 30 and 100 μM histamine 23% (5/23 cells), 39% (22/56 cells) and 56% (23/42 cells), respectively, showed oscillatory calcium responses.

The proportion of GT1-7 cells showing histamine-induced calcium oscillations was significantly reduced after histamine pretreatment (to 21 ± 5%, $P<0.05$, $n=119$ cells from 8 experiments, Figure 5), and, although a detailed analysis was not performed, the types of calcium behaviour did not appear to change (data not shown). KN-62, when present during histamine pretreatment, prevented the fall in the proportion of cells showing calcium oscillation. Under the conditions of zero or low (0.3 mM) extracellular calcium, which prevented histamine

Table 1 Desensitization of the histamine H_1 receptor in the presence of different concentrations of extracellular calcium

Condition	*Transient peak calcium level (mean increase from basal, nM)*
'Control' minus calcium	225 ± 46
HistDsn minus calcium	179 ± 16
'Control' in 0.3 mM calcium	198 ± 39
HistDsn in 0.3 mM calcium	170 ± 28

GT1-7 cells were grown on coverslips, loaded with fura-2AM (37°C), and then the media changed, and the cells transferred to the incubation chamber to allow for fura-2AM hydrolysis (25–27°C). During the loading period, cells to be desensitized were treated with histamine (HistDsn, 30 μM) either in the presence of 0.3 mM Ca^{2+} or in a nominally calcium free medium. Control cells ('Control') were incubated with medium containing the corresponding amounts of calcium. The temperature of the incubation medium was raised to 37°C and the cells were stimulated with histamine (Hist, 30 μM) in normal incubation medium and the $[Ca^{2+}]_i$ response recorded as described in methods section. Results are presented as population means ± s.e.mean of histamine stimulated $[Ca^{2+}]_i$ peak increase from basal levels and were obtained from 4 to 5 independent experiments. The cells for the control and desensitized treatments for each set of calcium concentrations were used on the same day and were derived from the same parent culture. None of the values from the desensitized treatments were significantly different from their corresponding control cells.

desensitization (Table 1), the proportion of cells exhibiting calcium oscillations was not significantly different from the control cells (Figure 5).

Histamine-induced heterologous desensitization of bradykinin-stimulated $[Ca^{2+}]_i$ is not prevented by KN-62

Bradykinin (5 μM) stimulated a $[Ca^{2+}]_i$ transient in GT1-7 cells with a population mean peak response of 147 ± 8 nM ($n=5$ experiments) over basal levels. The bradykinin-induced calcium pattern comprised of a transient, followed by a return to basal levels, and was without any calcium oscillatory activity (in 188 cells, $n=3$ experiments) (Figure 6a), and was similar to the histamine-stimulated fast re-equilibration pattern (Figure 4a). Histamine pretreatment caused the heterologous desensitization of the bradykinin $[Ca^{2+}]_i$ signal, resulting in a bradykinin stimulated $[Ca^{2+}]_i$ signal that was significantly reduced by 44% to 83 ± 15 nM ($n=5$, $P<0.007$, Figure 6b). KN-62, when present during the histamine desensitization protocol, was unable to prevent the heterologous desensitization cascade. The histamine-with-KN-62-pretreated cells showed a reduction in bradykinin-stimulated $[Ca^{2+}]_i$ signal of 40%, which was significantly different from the control $[Ca^{2+}]_i$ signal ($P<0.002$, $n=3$), but not significantly different from the histamine-mediated desensitization (Figure 6b).

Discussion

Histamine H_1 receptor desensitization has been characterized in a variety of cell lines (Taylor & Richelson, 1979; Brown *et al.*, 1986; Dillon-Carter & Chuang, 1989; Smit *et al.*, 1992; Bristow & Zamani, 1993; Dickenson & Hill, 1993) and in mammalian brain slices (Quach *et al.*, 1981; Bristow *et al.*, 1993). The studies into the mechanisms underlying H_1 receptor desensitization have observed both PKC-dependent and -independent pathways (Smit *et al.*, 1992; Dickenson & Hill, 1993; Zamani *et al.*, 1995). However, it appears that receptor-mediated homologous (Smit *et al.*, 1992; Zamani & Bristow,

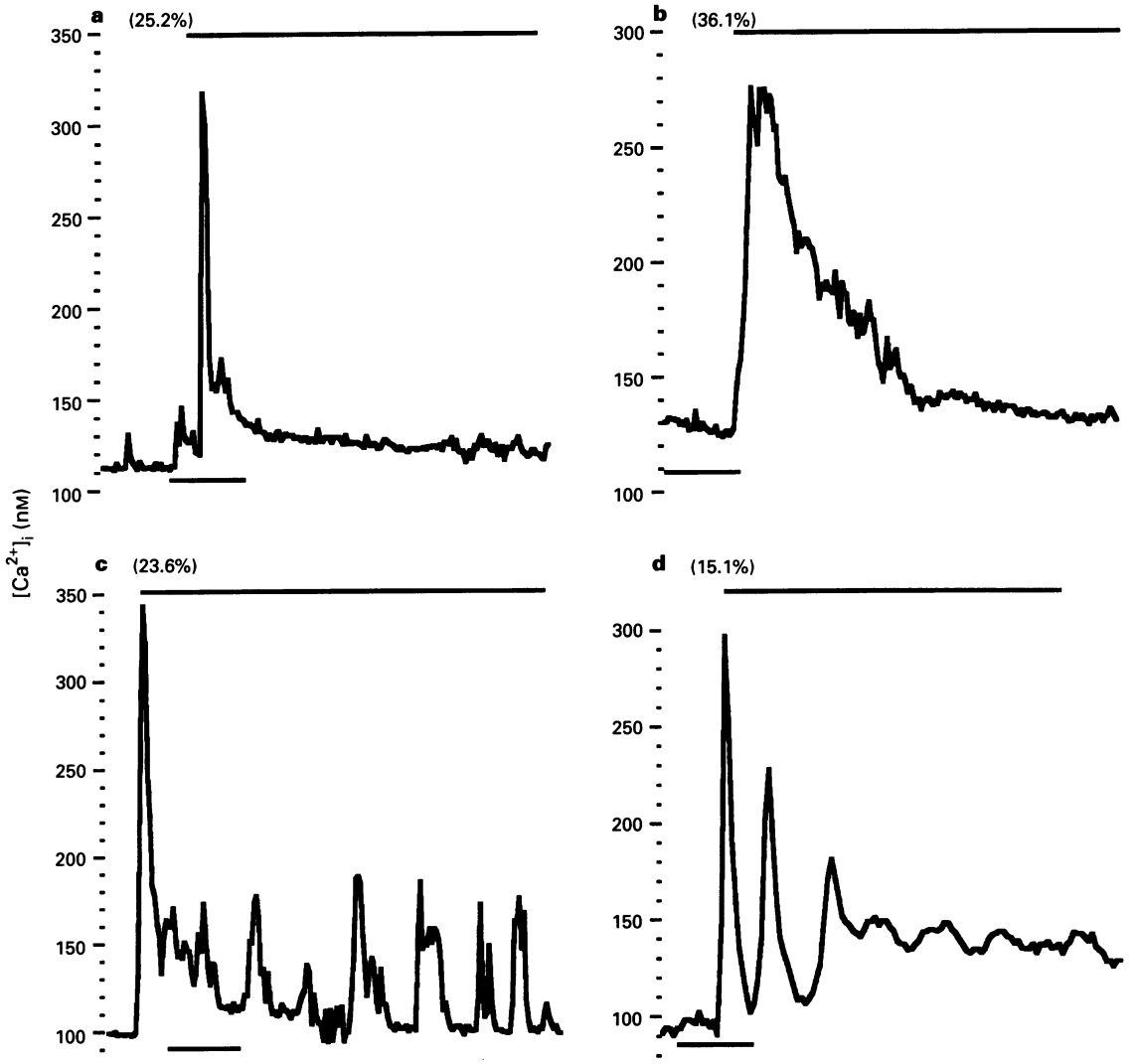

Figure 4 Patterns of $[Ca^{2+}]_i$ mobilization in single histamine-stimulated GT1-7 cells. GT1-7 cells were grown on coverslips, loaded with fura-2AM (37°C), the media changed, and cells were transferred to the temperature controlled perfusion chamber to allow for fura-2AM hydrolysis (25–27°C). The temperature of the incubation medium was raised to 37°C and the cells then stimulated with histamine (30 μM) and individual cell $[Ca^{2+}]_i$ responses were recorded and analysed as described in methods section. The graphs are representative measurements from single cells: (a) fast re-equilibration; (b) slow re-equilibration; (c) long-lasting $[Ca^{2+}]_i$ oscillation; (d) short-lived $[Ca^{2+}]_i$ oscillation. Numbers in parentheses on the top-left of each graph indicate the percentage of cells (from a total of 204 cells) exhibiting such types of oscillatory patterns. Horizontal lines above each graph indicate the presence of histamine in the medium. The scale bars at the bottom of the graphs are equivalent to 1 min.

1993; Dickenson & Hill, 1993; Zamani *et al.*, 1995) and heterologous H_1 receptor desensitization cascades (Dickenson & Hill, 1993; Staton, Nicholls & Bristow, unpublished) are both independent of PKC, and thus the mechanism underlying the agonist-mediated pathway remains unknown. Since H_1 receptor activation of PIC produces two intracellular second messengers, that of diacylglycerol which activates PKC, and IP_3 which raises $[Ca^{2+}]_i$, the possibility of a calcium-dependent pathway being responsible for H_1 receptor desensitization seemed plausible. This article presents evidence that extracellular Ca^{2+} and CaMKII are essential components of the agonist-mediated homologous H_1 receptor desensitization cascade. In addition, the results imply that CaMKII is not involved in the heterologous histamine-mediated desensitization cascade.

GT1-7 cells are a clonal line derived from specific tumours of gonadotropin-releasing hormone (GnRH)-secreting neurones (GT) from mouse hypothalamus (Mellon *et al.*, 1990). They exhibit a neuronal phenotype, including neurite outgrowth, growth cones, and make cell-cell contacts in culture (Mellon *et al.*, 1990). Research from this laboratory has established GT1-7 cells as a useful model neuronal cell system to study histamine H_1 receptor regulation (Zamani *et al.*, 1994; 1995).

The concentration-dependency of histamine-stimulated initial intracellular calcium transients and EC_{50} values in single cells shown here (Figure 1) were similar to our previous results in a study of populations of GT1-7 cells (Zamani *et al.*, 1995). From our single cell study it is also apparent that, within a given population, GT1-7 cells exhibited a range of $[Ca^{2+}]_i$ responses to the same histamine concentration, an effect also observed in HeLa cells (Sauvé *et al.*, 1991). This behaviour persisted in histamine pretreated cells. The cause of these cell-to-cell variations in the amplitude of the histamine-stimulated calcium spikes has not been established, but may conceivably result from differences in the size of IP_3-sensitive calcium pools or due to the cells being at different stages of the cell division cycle.

Acute histamine pretreatment of GT1-7 cells induced a desensitization of the histamine-stimulated $[Ca^{2+}]_i$ mobilization (Figure 2; Zamani *et al.*, 1995). The effect was manifest as a 53% attenuation of the mean histamine-induced $[Ca^{2+}]_i$

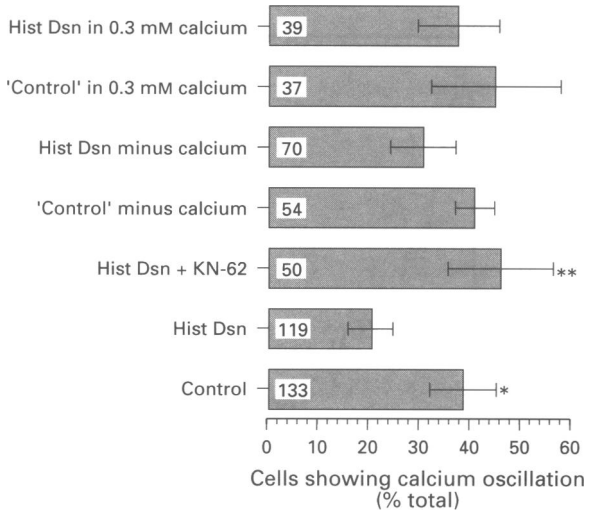

Figure 5 Measurement of number of GT1-7 cells showing $[Ca^{2+}]_i$ oscillation under various conditions. GT1-7 cells were grown on coverslips, loaded with fura-2AM (37°C), the media changed, and the cells were then transferred to the temperature controlled perfusion chamber to allow for fura-2AM hydrolysis (25–27°C). During the loading period, cells to be desensitized were treated with either histamine (30 μM) or histamine and KN-62 (10 μM). During the desensitization period some samples were incubated in normal incubation medium, some in 0.3 mM calcium, and some in the absence of extracellular calcium (nominal calcium + 0.2 mM EGTA). Control cells ('Control') were incubated in medium containing the corresponding calcium concentrations. The temperature of the incubation medium was raised to 37°C, then the cells were stimulated with histamine (Hist, 30 μM) in normal incubation medium and the $[Ca^{2+}]_i$ response recorded as described in methods section. Values are presented as population mean ± s.e.mean of the histamine stimulated transient calcium peaks from 5 to 9 independent treatments in each condition. Values inside each bar represent the total number of cells examined in each condition. Asterisks denote statistically different results between control and histamine desensitized samples (*$P<0.05$), and histamine desensitized with KN-62 pretreatment and histamine desensitized samples (**$P<0.03$), respectively.

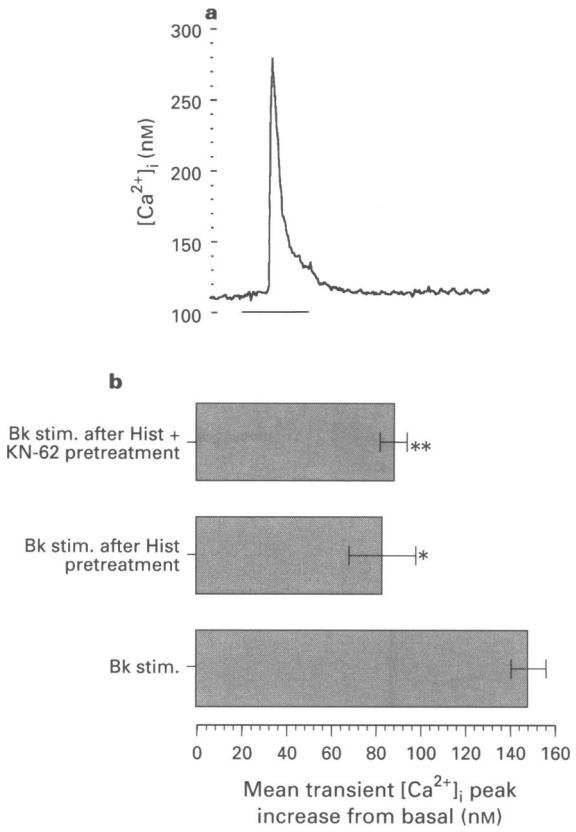

Figure 6 Heterologous desensitization of bradykinin-induced $[Ca^{2+}]_i$ mobilization by histamine pretreatment and the effect of KN-62. GT1-7 cells were grown on coverslips, loaded with fura-2AM (37°C), the media changed, and the cells were then transferred to the temperature controlled perfusion chamber to allow for fura-2AM hydrolysis (25–27°C). During the loading period, cells to be desensitized were treated with either histamine (30 μM) or histamine and KN-62 (10 μM). Control samples were left in the incubation medium. The temperature of the incubation medium was raised to 37°C, and then the cells stimulated with bradykinin (5 μM) and the $[Ca^{2+}]_i$ response recorded as described in methods section. (a) A typical response of a single GT1-7 cell to bradykinin, and (b) the effect of histamine (Hist) pretreatment on the subsequent response of the cells to bradykinin (Bk) stimulation (stim.) and the influence of KN-62. Results were obtained from 3 to 7 independent treatments in each condition and are expressed as population means ± s.e.mean of the bradykinin stimulated transient calcium peaks; *$P<0.007$ and **$P<0.002$. Scale bar in (a) is equal to 2 min.

transient peak compared to control cells. The involvement of CaMKII in the receptor-mediated desensitization process is supported by the observation that KN-62, a selective CaMKII inhibitor (Tokumitsu *et al.*, 1990), was able to inhibit the histamine-mediated desensitization. KN-62 was neither an H_1 receptor agonist nor an antagonist, nor did it have its action by increasing the rate of recovery from desensitization (Figure 3). Thus, assuming that KN-62 inhibits CaMKII in GT1-7 cells, it is concluded that the prevention of desensitization is due to the inhibition of CaMKII activity.

The presence of extracellular calcium at 2.5 mM is crucial for the induction of desensitization (Table 1). It is not known at present whether the intracellular calcium supplies, for example from the IP_3-sensitive or ryanodine-sensitive stores, are also involved in the desensitization process. It has previously been shown that in the absence of extracellular calcium the histamine-induced transient was unaffected but the sustained calcium phase was abolished (Zamani *et al.*, 1995). Taken together, these results infer an important role for the sustained calcium phase in histamine-mediated receptor desensitization. We believe that this is the first account of the effect of extracellular calcium on histamine H_1 receptor desensitization, but there are other examples of receptors that are regulated by extracellular calcium, including N-methyl-D-aspartate- (Clark *et al.*, 1990) and nicotinic acetylcholine- (Scubon-Mulieri & Parsons, 1977) receptors.

Histamine has been shown to induce calcium oscillations in HeLa cells (Suavé *et al.*, 1991; Zamani & Bristow, unpublished). BC3H-1 smooth muscle cells (Ambler *et al.*, 1988) and endothelial cells (Jacob *et al.*, 1988), and here we observe that histamine can induce a number of different calcium patterns in GT1-7 neuronal cells. The majority of histamine-mediated calcium responses in GT1-7 cells are single spikes which either rapidly fall back to near basal calcium levels (25%) or show a delayed re-equilibration (36%). The remainder of histamine-induced calcium patterns show some form of oscillatory behaviour, which do not exactly fit into the classes described by Berridge (1990), of which the most common types consist of a single transient and lower amplitude oscillatory pattern without significant loss of signal amplitude (24%) and a single transient with lower amplitude oscillatory signals of decaying transients (15%). We are unable to ascribe a role for these individual types of calcium behaviour, which is conceivably the result of cells being at different stages in the cell division cycle. Indeed, previous studies have shown differences between the histamine-stimulated sustained calcium signals in interphase and mitotic HeLa cells (Volpi & Berlin, 1988). Although our protocol of maintaining the cells for at least 48 hours in serum-free OptiMem media prior to experimentation does appear to slow cell division (Zamani & Bris-

tow, unpublished observation), and thus may to a degree have synchronized the cells, it is likely that some cells will be in different growth phases at the time of experimentation.

The significance of calcium oscillations is not yet established, and may vary in excitable and non-excitable cells, with agonist concentrations affecting either frequency and/or amplitude of oscillations. We did not observe any frequency modulation of the calcium signals with increasing agonist concentration, such as that observed, for example, in histamine-stimulated endothelial cells (Jacob *et al.*, 1988). Instead, in GT1-7 cells it appeared as though the amplitude of the initial (IP_3-sensitive) calcium transients were dependent on the histamine concentration (Figure 1). In addition, if we consider all types of oscillatory patterns together, then our results suggest that the proportion of cells expressing an oscillatory calcium response increases with agonist concentration up to a maximum of 56% of cells at maximal doses of histamine (100 μM).

In our hands the proportion of histamine-induced desensitized cells pretreated with KN-62 and showing Ca^{2+} oscillation was similar to that of control cells (Figure 5). Moreover, the proportion of cells exhibiting Ca^{2+} oscillation that were desensitized in either the absence or at low concentrations of extracellular Ca^{2+} was similar to that of control cells (Figure 5). These findings lead us to propose the sequence of events leading to desensitization as being intracellular Ca^{2+} increase and oscillation preceding the activation of CaMKII. Thus, the prerequisite for the desensitization cascade initiation in GT1-7 cells is the rise in intracellular Ca^{2+}, probably through the release of calcium from intracellular stores, followed shortly by Ca^{2+} entry from the extracellular medium. By inhibiting CaMKII activity, KN-62 blocks the initiation of desensitization regardless of the presence or absence of Ca^{2+} oscillations. This is why in spite of intracellular Ca^{2+} oscillations in KN-62 pretreated cells no significant attenuation of cellular response to subsequent histamine stimulation was observed (Figure 2). These cells did not desensitize and therefore, when stimulated with histamine, behaved like the control cells.

CaMKII has an important role as a mediator of calcium effects in a variety of intracellular pathways, including neuronal plasticity, gene expression, cell cycle, and neurotransmission (Braun & Schulman, 1995). Recently, it has been hypothesized that the activity of this enzyme can be regulated by repetitive calcium spiking (Hanson *et al.*, 1994). Since CaMKII may be involved in H_1 receptor desensitization and histamine can induce calcium oscillations, it is an intriguing possibility that in the GT1-7 cells that express histamine-induced calcium oscillations the CaMKII enzyme is able to decode the signals by gradually becoming activated. In these cells the H_1 receptors would therefore become desensitized. We are unable to confirm this hypothesis from our present studies because calcium responses were not measured in the same cells before and after the desensitization protocols. However, our results have shown an identical proportion of cells apparently showing desensitized histamine-induced $[Ca^{2+}]_i$ signals ($39 \pm 7\%$, mean $\pm$ s.e.mean, $n = 94$ cells from 8 experiments) to the number of control cells normally showing histamine-stimulated calcium oscillations ($39 \pm 7\%$, mean $\pm$ s.e.mean, $n = 56$ cells). In addition, H_1 receptor desensitization (Bristow & Zamani, 1993) and the proportion of cells expressing histamine-mediated calcium oscillatory patterns are both histamine concentration-dependent over the same range. Further in support of this idea, there is evidence illustrating the role of depolarization-induced oscillating $[Ca^{2+}]_i$ signals in the desensitization of prolactin release from lactotrophs from the rat anterior pituitary (Law *et al.*, 1989).

Bradykinin stimulated the elevation of $[Ca^{2+}]_i$ in GT1-7 cells and produced a calcium pattern of a simple transient rise in calcium and fall to basal levels (Figure 6a). This bradykinin-stimulated calcium behaviour was markedly attenuated after pretreatment with histamine, resulting in a significant reduction in the amplitude of the response (Figure 6b). Thus, histamine can induce both homologous and heterologous desensitization in GT1-7 cells, as is the case in a number of non-neuronal cells (Brown *et al.*, 1986; McDonough *et al.*, 1988; Smit *et al.*, 1992; Bristow & Zamani, 1993; Dickenson & Hill, 1993). It has already been shown that the histamine-induced heterologous desensitization pathway coupled to ADP receptors (Dickenson & Hill, 1993) is independent of PKC. The results presented here show that the histamine-mediated heterologous desensitization pathway in GT1-7 neuronal cells is unaffected by KN-62, suggesting that the mechanism is independent of CaMKII. Thus, the molecular mechanism underlying the heterologous desensitization pathway remains unresolved. It is possible that the heterologous pathway may be mediated not via the H_1 receptor, but through the H_2 (or in the CNS, the H_3) receptor. In support of this idea, preliminary studies in this laboratory (Nicholls, Sutch & Bristow, unpublished) have shown that the histamine-mediated heterologous desensitization of bradykinin-stimulated inositol phosphate accumulation in GT1-7 cells is prevented by ranitidine and thioperamide (100 μM and 1 μM, H_2 and H_3 antagonists respectively).

In conclusion, this study has shown that KN-62, a putative inhibitor of CaMKII, and lowering of extracellular calcium concentrations can regulate the histamine-mediated homologous H_1 receptor desensitization process. The bradykinin-stimulated calcium signal, which does not show an oscillatory pattern, was desensitized by a heterologous mechanism by histamine treatment, and this process was not prevented by KN-62. These findings suggest fundamental differences in the mechanisms underlying homologous and heterologous H_1 receptor desensitization pathways in GT1-7 neuronal cells. The results presented here infer a role for extracellular calcium and CaMKII in the mechanism underlying homologous histamine H_1 receptor desensitization.

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