ORIGINAL ARTICLE

Histamine via histamine H1 receptor enhances the muscarinic receptor-induced calcium response to acetylcholine in an enterochromaffin cell model

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Abstract

As a prerequisite for serotonin secretion, the P-STS ileal enterochromaffin cell line responds to acetylcholine (ACh) stimulation with an increase in intracellular calcium mediated by the muscarinic ACh receptor M3 (M3R). Histamine increases intracellular calcium via histamine H1 receptor (H1R) in P-STS cells and pre-incubation with histamine specifically augments the response to ACh but not to epinephrine or nicotine. We aimed to elucidate whether histamine receptors are involved in this synergism. Astonishingly, HEK-293 T cells—known to express M3R, but only a very low amount of histamine receptor messenger RNA—showed a similar enhancement of the calcium response to ACh by pre-incubation with histamine. Despite the much lower level of H1R protein detected in HEK-293 T cells as compared to P-STS cells, in both cell lines pre-treatment with H1R antagonists inhibited the synergism between histamine and ACh. No indication for an involvement of histamine H2 or H4 receptors in the synergism was found. Furthermore, pre-incubation with the cAMP-inducing compound forskolin had no influence on the intracellular calcium response to ACh. Serotonin secretion from P-STS cells was increased after challenge with ACh and histamine added simultaneously compared to ACh alone, suggesting that histamine increases ACh-induced serotonin secretion from enterochromaffin cells. In conclusion, our data suggest that histamine enhances the M3R-mediated intracellular calcium response to ACh via activation of H1R. This probably increases serotonin secretion from enterochromaffin cells and thereby affects intestinal motility in histamine intolerance, food allergies and irritable bowel syndrome.

KEYWORDS
acetylcholine, enterochromaffin, histamine, irritable bowel disease, JNJ7777120, muscarinic acetylcholine receptor M3

1 | INTRODUCTION

Enterochromaffin cells are serotonin-secreting neuroendocrine cells located in the intestinal epithelium in close proximity to afferent nerve endings of the enteric nervous system. They respond to acidic pH, nutrients and chemical mediators as well as to mechanical stimulation.[1,2] Serotonin increases gastrointestinal motility and promotes diarrhoea, therefore, enterochromaffin

cells are likely to contribute to symptoms in gastrointestinal hypersensitivities.

The P-STS ileal enterochromaffin cell line[3,4] responds to ACh with an increase in intracellular calcium ($[Ca^{2+}]_i$), but contains only very low amounts of serotonin.[5] Mobilization of $[Ca^{2+}]_i$ is mediated by M3R in enterochromaffin cells[6] and is a prerequisite for secretion.[7] Histamine added before or together with ACh synergistically enhances this calcium response.[8] Histamine may be ingested as a food
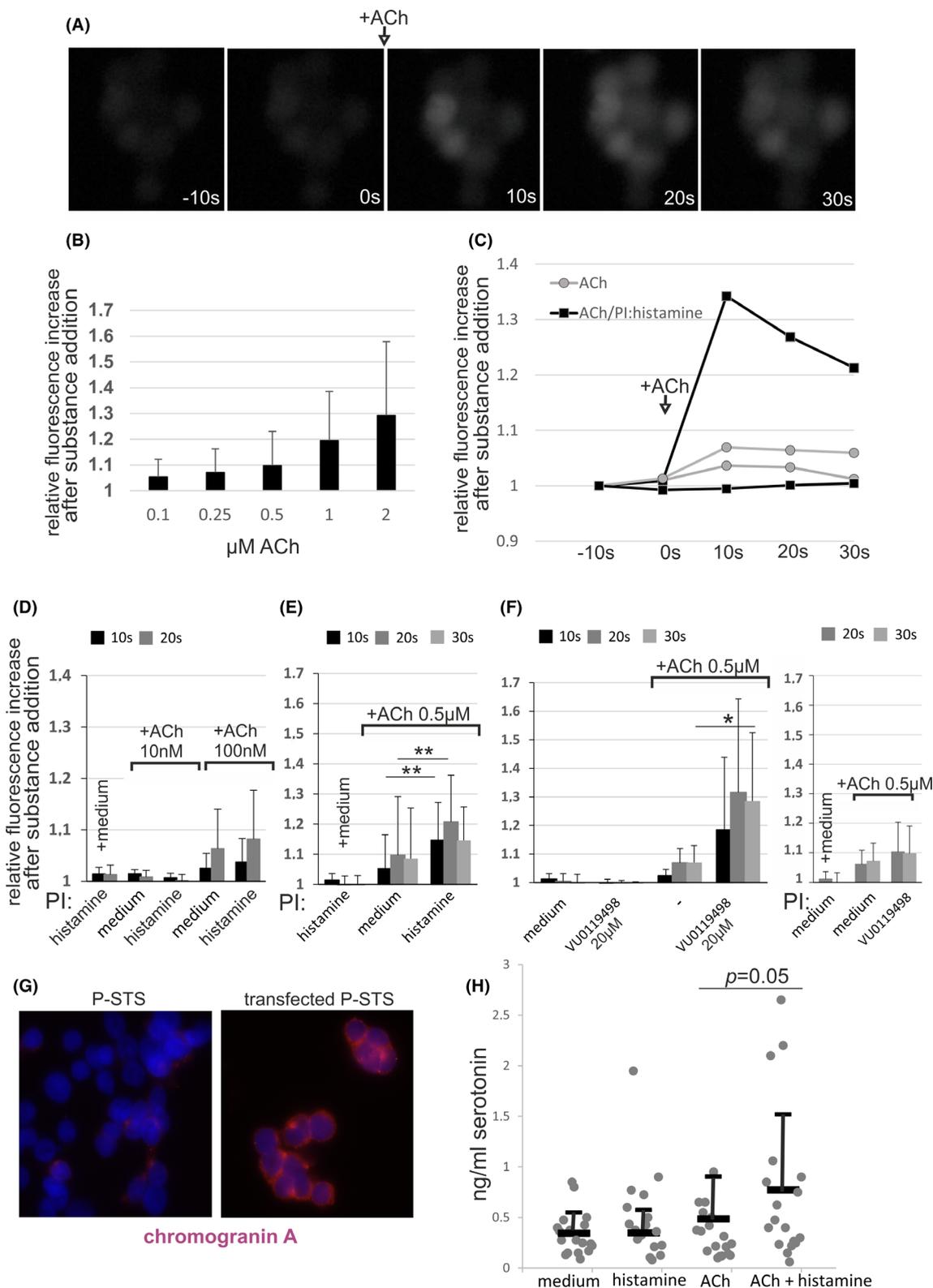

FIGURE 1 Legend on next page.

constituent or may reach enterochromaffin cells after secretion from mast cells, the mediators of intestinal allergic inflammation in food allergy. Increased numbers of mast cells in intestinal tissues have been implicated in diarrhoea-type irritable bowel disease and celiac disease.[9]

The $[Ca^{2+}]_i$ response of P-STS cells to histamine alone is largely mediated by H1R, as shown by the strong inhibitory effect of the H1R antagonist mepyramine, whereas the H2R antagonist ranitidine had no effect.[8] The presence of functional histamine H3 receptor (H3R) in P-STS cells was indicated by a clear inhibitory effect of the H3R agonist

FIGURE 2 Histamine does not enhance the $[Ca^{2+}]_i$ response of P-STS cells to nicotine or adrenaline. PI: pre-incubation (10 min). A, $[Ca^{2+}]_i$ response to nicotine with or without pre-incubation with 10 μM histamine (6 experiments). B, $[Ca^{2+}]_i$ response to histamine (10 μM) and adrenaline (10 μM) alone and added simultaneously (left panel, 7 experiments) and effect of pre-incubation with histamine (10 μM) on the $[Ca^{2+}]_i$ response to adrenaline (right panel, 8 experiments). PI, pre-incubation
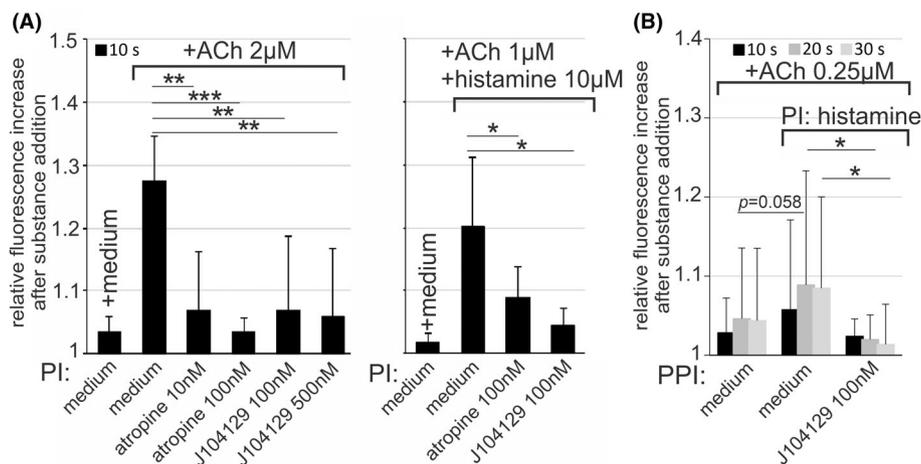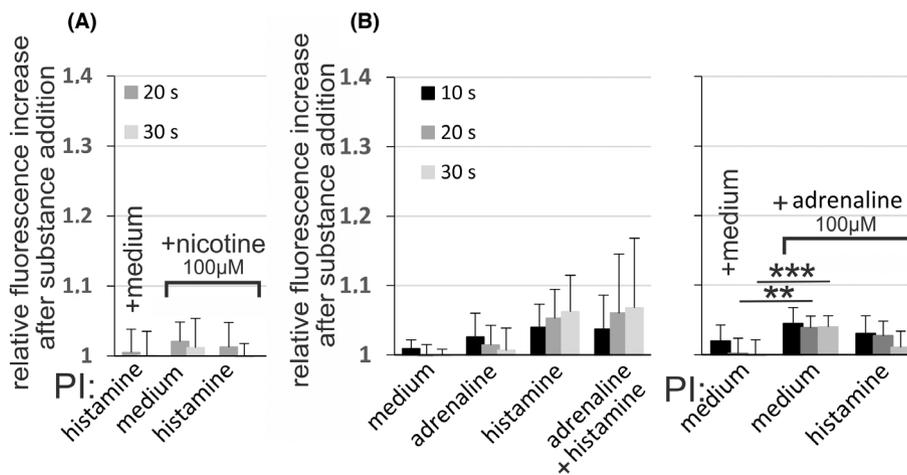

FIGURE 3 The $[Ca^{2+}]_i$ response of P-STS cells to ACh is mediated by M3R. *$p \leq 0.05$, **$P \leq .01$, ***$P \leq .001$, PI: pre-incubation (10 min), PPI: pre-pre-incubation, e.g. pre-incubation (5 min) before pre-incubation with histamine). A, Inhibition of the $[Ca^{2+}]_i$ response to ACh (left panel) and ACh added simultaneously with histamine (right panel) by pre-incubation with atropine and the specific M3R antagonist J104129 (6 experiments each). B, Inhibitory effect of the specific M3R antagonist J104129 on the $[Ca^{2+}]_i$ response to ACh after pre-incubation of the cells with 10 μM histamine (20 experiments). ACh, acetylcholine; MER3, muscarinic ACh receptor M3;

FIGURE 1 Histamine and the allosteric muscarinic agonist VU0119498 enhance the $[Ca^{2+}]_i$ response to ACh in P-STS cells to similar extents. *$P \leq .05$, **$P \leq .01$, PI: pre-incubation (10 min). A, Imaging of Fluo4-AM fluorescence in P-STS cells after addition of ACh. B, Relative fluorescence increase 20 s after addition of increasing concentrations of ACh (compilation from the experiments conducted, the number of samples used for calculation ranges from 22 for 0.1 μM ACh to 143 for 0.5 μM ACh). No significance tests were performed. C, Examples of a typical time course of Fluo4-AM fluorescence in P-STS cells of four samples treated in parallel. The cells were challenged with ACh (0.5 μM) with or without pre-incubation with 10 μM histamine. One of the samples with histamine pre-incubation shows no reaction, as is often observed irrespective of the pre-incubation or the agonist applied. This causes a non-normal distribution of the increases in fluorescence as well as large standard deviations. D, Effect of pre-incubation with 20 μM histamine on the $[Ca^{2+}]_i$ response to low concentrations of ACh (0 or 10 nM: 6 experiments, 100 nM: 16 experiments). E, Enhancement of the $[Ca^{2+}]_i$ response to 0.5 μM ACh by pre-incubation with 20 μM histamine (15 experiments). F, The $[Ca^{2+}]_i$ response to ACh is enhanced by simultaneous addition of VU0119498 (left panel, 14 experiments), but pre-incubation with VU0119498 has less effect (right panel, 13 experiments). G, Immunofluorescence microscopy of P-STS cells transfected with the chromogranin A expression plasmid (chromogranin A detected as described before[5]). H, Serotonin secretion by P-STS cells (transfected with the chromogranin A expression plasmid) in response to 1 μM ACh, 10 μM histamine or both agonists added simultaneously (18 experiments). ACh, acetylcholine

methimepip on the $[Ca^{2+}]_i$ response of P-STS cells to a mixture of ACh and histamine, as expected from the inhibitory role of this receptor in nerve cells.[10]

The aim of this work was to further investigate the mechanism of synergism between histamine and ACh. It has been shown that activation of H1R or H2R can exert an influence on the signalling in response to activation of other G-protein coupled receptor (PCR) proteins.[11] In P-STS cells, histamine receptor antagonists had not shown

any obvious effect on the response to ACh added together with histamine. However, in these experiments the concentration of ACh was relatively high and evoked a strong calcium response, maybe too strong to be overcome by these inhibitors. To better reflect the in vivo situation, we used lower concentrations of ACh, causing on average only a weak calcium response. Pre-incubation with histamine instead of simultaneous addition with ACh facilitated the interpretation of results. It may represent a better model for the in vivo
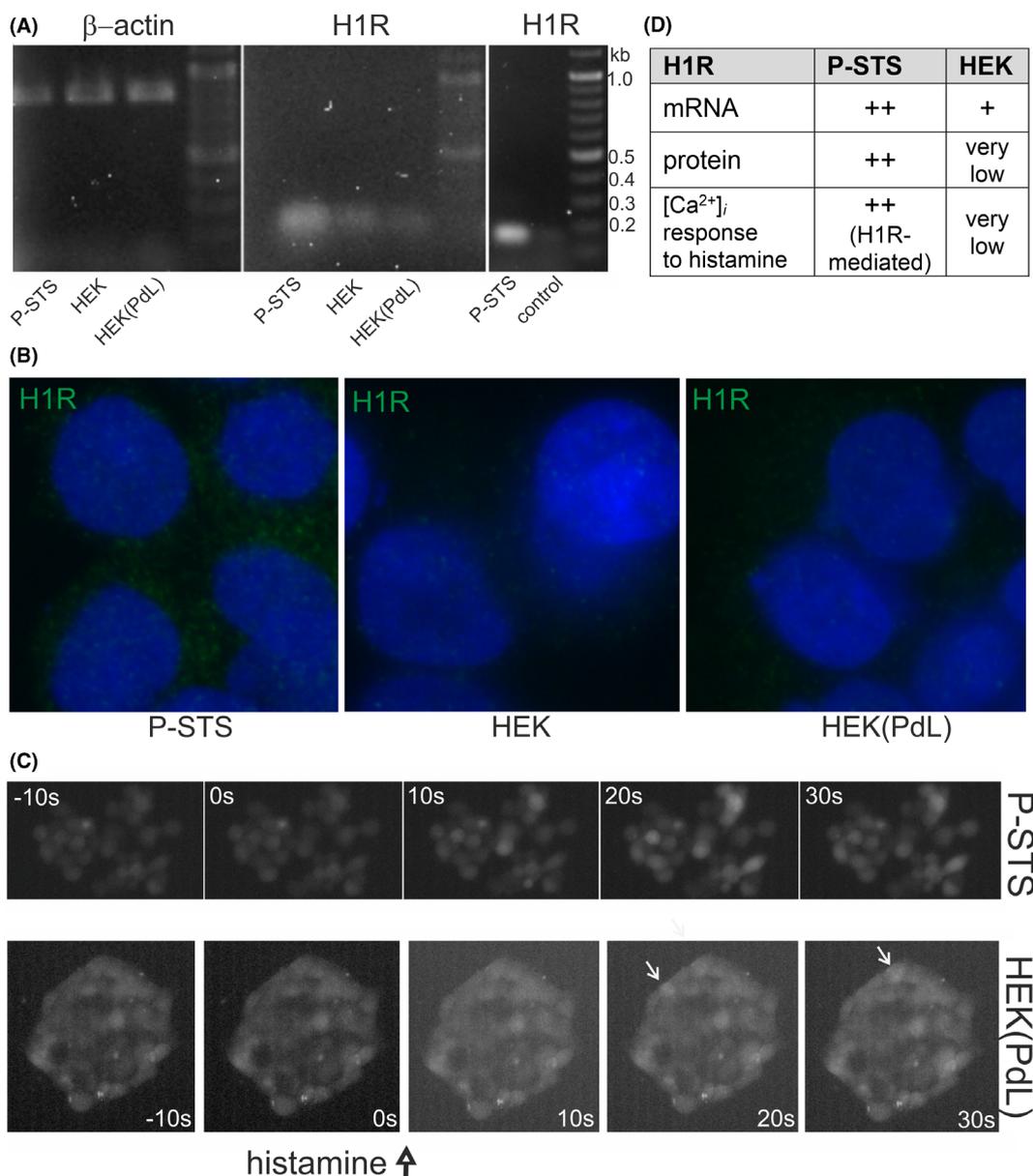

H1R	P-STS	HEK
mRNA	++	+
protein	++	very low
$[Ca^{2+}]_i$ response to histamine	++ (H1R-mediated)	very low

FIGURE 4 Expression of H1R in P-STS and HEK-293 T cells. A, Expression of H1R and ß-Actin mRNA in P-STS, HEK-293 T cells grown on untreated cell culture plastic (HEK) and HEK-293 T cells grown in wells coated with poly-D-lysine (HEK[PdL]). The results were confirmed with an independent reverse amplification from the same cDNA. Control: PCR with P-STS RNA without reverse transcription. B, H1R protein of P-STS cells and HEK-293 T cells detected by immunofluorescence microscopy as described.[8] C, $[Ca^{2+}]_i$ response of P-STS and HEK-293 T cells to histamine (10 µM) visualized by Fluo-4 AM staining. As described in the text, HEK-293 T cells in most experiments did not show any detectable $[Ca^{2+}]_i$ response to histamine. White arrows indicate individual HEK-293 T cells showing an increase in $[Ca^{2+}]_i$ in the experiment shown. D, Summary of the results for H1R mRNA and protein in the two cell lines and their $[Ca^{2+}]_i$ response to histamine, which in P-STS cells is largely mediated by H1R.[8] H1R, histamine H1 receptor; mRNA, messenger RNA; cDNA, complementary DNA; PCR, polymerase chain reaction

situation, assuming that enterochromaffin cells bathed in histamine from neighbouring histamine secreting mast cells are reached by waves of quickly degraded ACh[12] emitted from nearby nerve endings of the enteric nervous system.

2 | RESULTS

2.1 | Histamine appears to specifically enhance the M3R-mediated $[Ca^{2+}]_i$ response of P-STS cells to ACh

P-STS cells respond with an increase in $[Ca^{2+}]_i$ to ACh (Figure 1A,B). As shown in previous work,[8] this response to ACh is significantly enhanced by pre-incubation with histamine or simultaneous addition of histamine and ACh. Examples of typical time courses of Fluo4-AM fluorescence in P-STS cells of four samples treated in parallel with ACh with or without pre-incubation with histamine are shown in Figure 1C. ACh at very low concentration (10 nM) did not evoke a detectable $[Ca^{2+}]_i$ response even after pre-incubation with 20 μM histamine (Figure 1D). The $[Ca^{2+}]_i$ response to 100 nM ACh was detectable and appeared to be enhanced by histamine. This enhancement by pre-incubation with histamine became significant with 0.5 μM ACh (Figure 1E). We conducted most experiments for which synergism by pre-incubation with histamine was expected with 0.25 or 0.5 μM ACh to obtain a 1.05- to 1.15-fold increase in fluorescence with ACh alone, leaving extensive room for enhancement. To evaluate its physiological significance, the increase in $[Ca^{2+}]_i$ response to ACh mediated by histamine in P-STS cells was compared to the increase mediated by the allosteric muscarinic agonist VU0119498.[13] MIN6-K8 mouse pancreatic β-cells and glucose-perfused human pancreatic islets reacted with an about 1.5- to 2-fold increase of $[Ca^{2+}]_i$ and insulin secretion to pre-incubation with VU0119498 before challenge with ACh and its use as an anti-diabetic was suggested.[14] In P-STS cells, the effect of VU0119498 on the $[Ca^{2+}]_i$ response to ACh was high when added simultaneously with ACh (Figure 1F, left panel). Like in MIN6-K8 cells, pre-incubation with VU0119498 appeared to have some enhancing effect on the response to ACh, but this effect did not reach statistical significance in P-STS cells (Figure 1F, right panel).

Because only low amounts of serotonin were detected in lysates of P-STS cells,[5] the cells were transfected with the chromogranin A expression plasmid RC200492 in the hope to increase the serotonin content and excretion of the cells. Although chromogranin A expression was much higher in the transfected cells (Figure 1G), the serotonin content of these cells—as determined by a serotonin enzyme-linked immunosorbent assay (ELISA)—was only increased ∼2-fold (not shown). Nevertheless, a significant enhancement of serotonin secretion into the medium in response to a mixture of 1 μM ACh and 10 μM histamine compared to ACh alone was detected (Figure 1H). Because serotonin secretion in this experiment is the sum of the responses to histamine and to ACh, no conclusion about synergism between ACh and histamine can be drawn. This experiment shows, however, that histamine increases ACh-induced serotonin secretion from enterochromaffin cells.

P-STS cells only weakly reacted to 100 μM nicotine (Figure 2A), confirming that ACh primarily acts via muscarinic receptors. Histamine pre-incubation did not increase this weak response. As shown
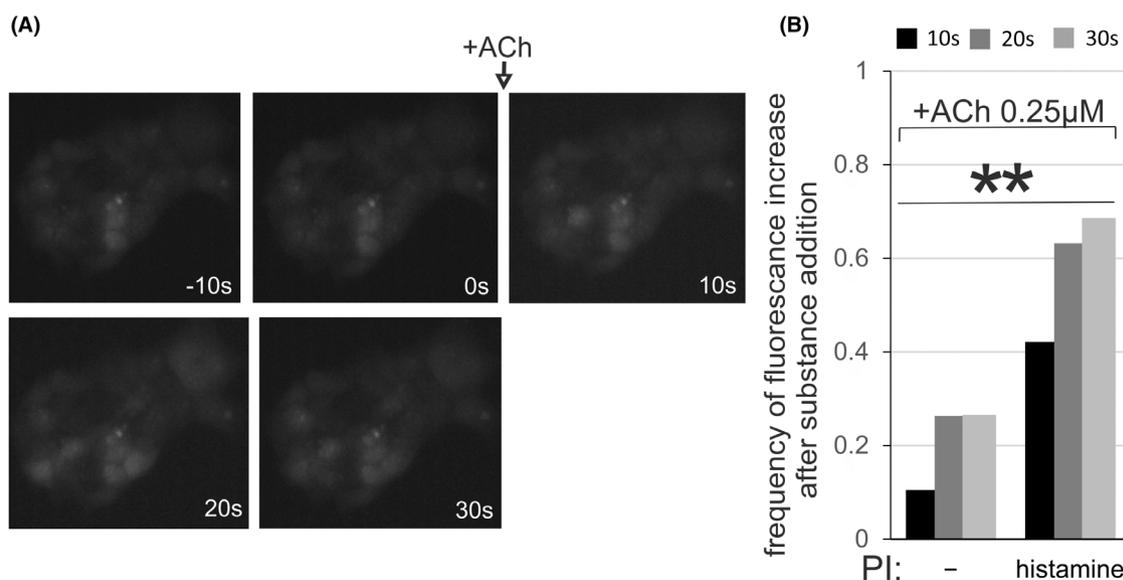

FIGURE 5 Pre-incubation with histamine enhances the $[Ca^{2+}]_i$ response of HEK-293 T cells to ACh. **$P \leq .01$, PI: pre-incubation (10 min). A, Positive $[Ca^{2+}]_i$ response to ACh (1 μM) visualized by Fluo-4 AM staining. Addition of ACh caused an increase in background fluorescence impeding quantification of intracellular Ca^{2+} by Image J, therefore, another method for analysis had to be applied (see Materials and Methods). B, Enhancement of the $[Ca^{2+}]_i$ response to ACh by pre-incubation with 10 μM histamine (19 experiments, wells coated with poly-D-lysine). ACh, acetylcholine

previously, P-STS cells show a weak, but significant increase in $[Ca^{2+}]_i$ when challenged with 100 μM adrenaline.[8] Simultaneous addition of adrenaline and histamine (Figure 2B, left panel) or pre-incubation with histamine (Figure 2B right panel) had no synergistic effect, however. These data suggest that the enhancing effect of histamine on the $[Ca^{2+}]_i$ response to ACh is specific and not because of a general increase in the excitability of the cells.

Secretion in enterochromaffin cells is known to be induced by binding of ACh to M3R.[7] The $[Ca^{2+}]_i$ response to ACh was inhibited by the muscarinic ACh receptor antagonist atropine as well as by J104129, an antagonist more specific for M3R (Figure 3A, left panel). J104129 also strongly inhibited the response to ACh added

simultaneously with histamine (Figure 3A, right panel) as well as to a challenge with ACh after pre-incubation with histamine (Figure 3B).

2.2 | Pre-incubation with histamine also enhances the $[Ca^{2+}]_i$ response of HEK-293 T cells to ACh

Like in P-STS cells, the $[Ca^{2+}]_i$ response to muscarinic agonists in HEK-293 cells is mediated by M3R.[15] The strength of their response to ACh has been shown to be low or intermediate at 0.25 μM ACh and to plateau at 1.5 μM.[16] In our HEK-293 T cells, there was no
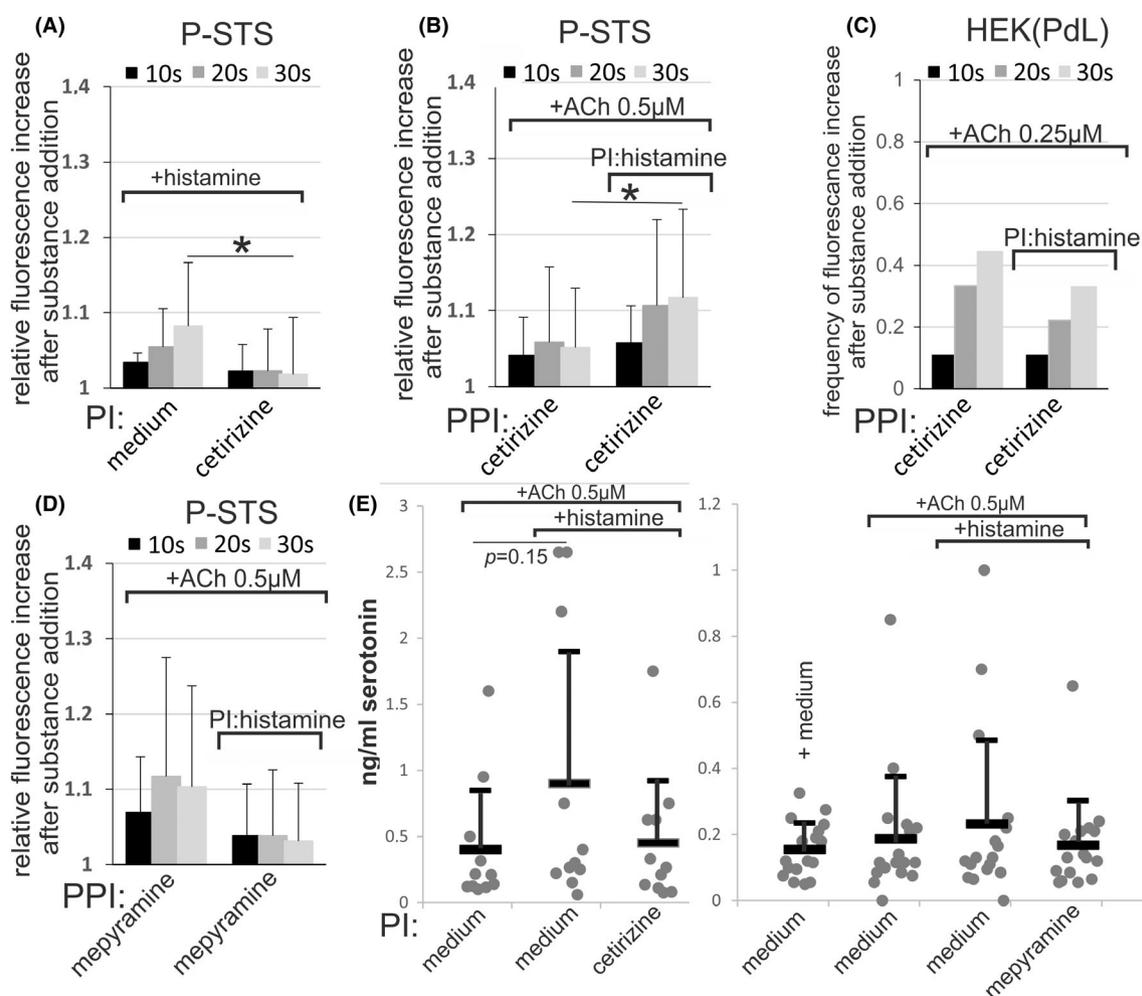

FIGURE 6 Effects of H1R antagonists on the response of P-STS and HEK-293 T cells to histamine (10 μM) and ACh. *$P \le .05$, PI: pre-incubation (10 min), PPI: pre-pre-incubation, e.g. pre-incubation (2 min) before pre-incubation with histamine. A, Pre-incubation with cetirizine (10 μM) inhibits the $[Ca^{2+}]_i$ response to histamine (15 experiments). B, Pre-incubation with cetirizine (10 μM) does not prevent the synergism of ACh with histamine in P-STS cells (18 experiments). D, No enhancing effect of pre-incubation with histamine is seen in HEK-293 T cells in the presence of cetirizine (10 μM, 9 experiments), although samples treated in parallel in the absence of cetirizine showed synergism (not shown, these samples are part of Figure 5B). D, No enhancing effect of pre-incubation with histamine is seen in P-STS cells in the presence of mepyramine (1 μM, 14 experiments). E, Pre-incubation with cetirizine (10 μM, left panel, 11 experiments) or mepyramine (1 μM, right panel, 17 experiments) appears inhibitory for serotonin secretion in response to ACh and histamine added simultaneously to P-STS cells. P-STS cells transfected with the chromogranin A expression plasmid were used in these experiments, but—as confirmed retrospectively—chromogranin A overexpression had been lost before the experiments conducted with mepyramine, explaining the lower serotonin secretion in these experiments. H1R, histamine H1 receptor; ACh, acetylcholine

$[Ca^{2+}]_i$ response to 100 μM nicotine (six experiments each, not shown). HEK-293 cells express only very low levels of histamine receptor messenger RNA (mRNA).[17] This was confirmed for H1R in our HEK-293 T cells, which—compared to β-actin mRNA—expressed significantly less H1R mRNA than P-STS cells (Figure 4A). H1R was not detected in immunofluorescence microscopy in HEK-293 T cells grown in uncoated or poly-D-lysine coated culture dishes (Figure 4B). In accordance with this and in contrast to P-STS cells, a $[Ca^{2+}]_i$ response to histamine was only sporadically seen in single HEK-293 T cells (Figure 4C). The data from Figure 4A-C are summarized in Figure 4D. Despite their almost undetectable expression of H1R, the $[Ca^{2+}]_i$ response of HEK-293 T cells to ACh (Figure 5A) was enhanced by pre-incubation with histamine like in P-STS cells (Figure 5B), suggesting that H1R was either not involved or a very low amount of receptor was sufficient to evoke the synergism of histamine and ACh.

2.3 | Inhibition of the synergism of histamine and ACh by H1R antagonists

We have shown previously—using the H1R antagonist mepyramine—that the $[Ca^{2+}]_i$ response of P-STS cells to histamine is mediated by H1R.[8] The H1R antagonist cetirizine was also able to inhibit the $[Ca^{2+}]_i$ response to histamine (Figure 6A). To control for the anti-muscarinic effects of these H1R antagonists, not only cells pre-incubated with histamine before challenge with ACh, but also cells challenged with ACh alone, were pre-incubated with H1R antagonist for evaluation of the participation of H1R in the synergistic response. Pre-incubation with cetirizine did not completely prevent

the synergistic effect of pre-incubation with histamine on the $[Ca^{2+}]_i$ response to ACh (Figure 6B). In HEK-293 T cells, on the contrary, no enhancing effect of pre-incubation with histamine on the $[Ca^{2+}]_i$ response to ACh was detected in the presence of cetirizine (Figure 6C), although in a series of control experiments conducted in parallel in the absence of cetirizine the expected synergism was observed (not shown). A likely explanation for these inconsistent results is that cetirizine reduced the very low H1R activity in HEK-293 T cells to a level not allowing synergism, whereas the higher H1R activity in P-STS cells could not be blocked to a sufficient extent by cetirizine. In contrast, mepyramine prevented the synergism of ACh and histamine also in P-STS cells (Figure 6D). This discrepancy is probably because of our short per-incubation time with the inhibitors. Although mepyramine quickly binds to the receptor, cetirizine has a more than 100-fold lower association constant for H1R binding and it's IC_{50} for mepyramine binding keeps decreasing for hours to less than one tenth of the value obtained after 10 min of incubation with the inhibitor.[18] Serotonin secretion after simultaneous addition of histamine and ACh to P-STS cells appeared to be higher than secretion in response to ACh alone and to be prevented by cetirizine (Figure 6E, left panel) and mepyramine (Figure 6E, right panel). For the interpretation of this result we refer to the discussion.

2.4 | Effect of the H2R antagonist ranitidine and the cAMP-inducing substance forskolin

The H2R antagonist ranitidine appeared to have some inhibitory effect on the synergism between ACh and histamine in the pre-
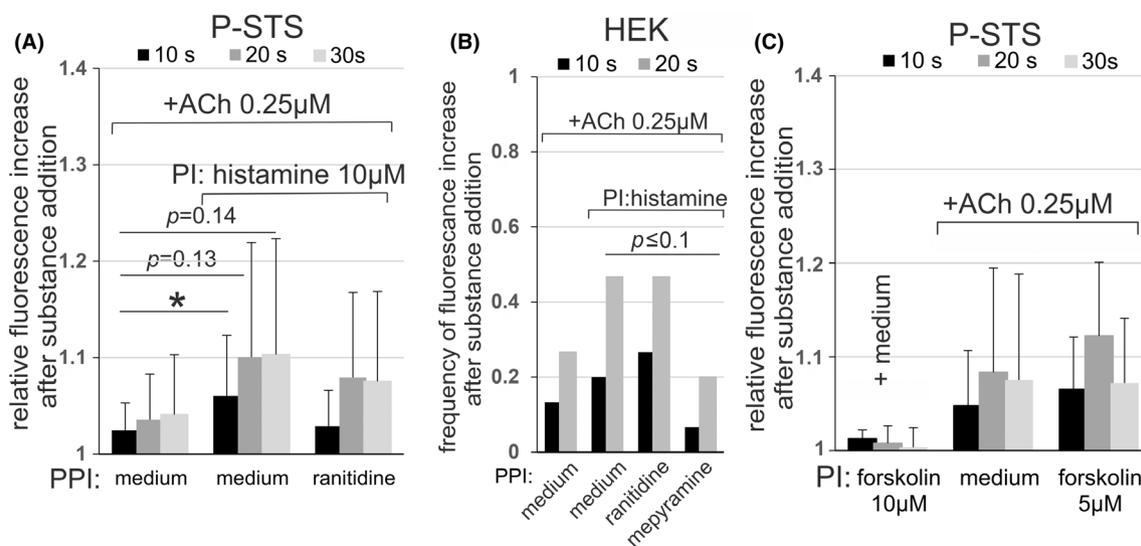

FIGURE 7 Effects of the H2R antagonist ranitidine and the cAMP-inducing substance forskolin. *$P \leq .05$, PI: pre-incubation (10 min), PPI: pre-pre-incubation, for example pre-incubation 2 min) before pre-incubation with histamine). A, Pre-incubation with the H2R antagonist ranitidine (10 μM) only transiently (for 10 s) inhibited the synergism between histamine (20 μM) and ACh in P-STS cells (16 experiments). B, Pre-incubation with the H2R antagonist ranitidine (10 μM) did not inhibit the synergism between histamine (10 μM) and ACh in HEK-293 T cells grown on uncoated cell culture plastic (15 experiments, mepyramine: 1 μM). (c) Pre-incubation with the cAMP-inducing substance forskolin did not enhance the $[Ca^{2+}]_i$ response to ACh in P-STS cells (12 experiments). H2R, histamine H2 receptor; ACh, acetylcholine

(B)

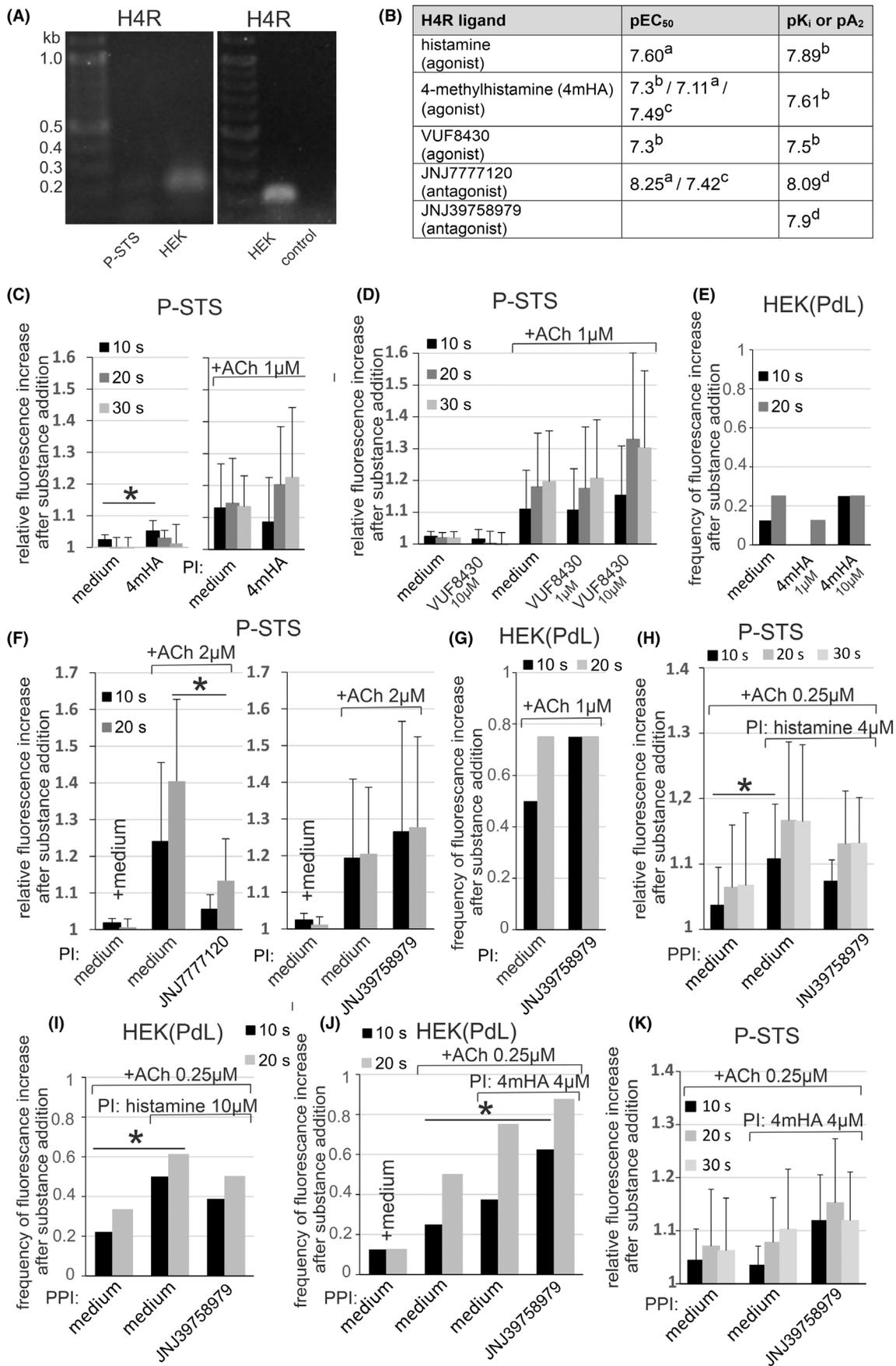| H4R ligand	pEC$_{50}$	pK$_i$ or pA$_2$
histamine (agonist)	7.60[a]	7.89[b]
4-methylhistamine (4mHA) (agonist)	7.3[b] / 7.11[a] / 7.49[c]	7.61[b]
VUF8430 (agonist)	7.3[b]	7.5[b]
JNJ7777120 (antagonist)	8.25[a] / 7.42[c]	8.09[d]
JNJ39758979 (antagonist)		7.9[d]

FIGURE 8 Legend on next page.

incubation medium, however this effect was only short-lived and not visible any more after 20 s (Figure 7A). A similar pattern of inhibition by ranitidine was observed after challenge of the cells with ACh alone, suggesting that this transient inhibition did not involve histamine (not shown). Furthermore, ranitidine did not show any significant inhibitory effect on the response to ACh in the presence of histamine in HEK-293 T cells (Figure 7B). Signal transduction after binding of histamine to H2R is known to proceed primarily via formation of cAMP.[11,19] We, therefore, tested whether incubation of P-STS cells with the cAMP-inducing substance forskolin would have an enhancing effect on the $[Ca^{2+}]_i$ response to ACh, substantiating a role of H2R in this process. However, pre-incubation with 5 μM forskolin was without significant effect (Figure 7C). These results obtained with ranitidine and forskolin argue against a role of H2R in the effect of histamine pre-incubation on the $[Ca^{2+}]_i$ response to ACh.

2.5 | Effects of H4R agonist and antagonists on the $[Ca^{2+}]_i$ response of P-STS cells to ACh

P-STS cells had very low expression of H4R mRNA even compared to HEK-293 T cells, which have already been shown to express very little histamine receptor mRNA by others[17] (Figure 8A). As a low level of H1R appeared sufficient to cause synergism of H1R and M3R in HEK-293 T cells and therefore, a contribution of a low level of H4R to the synergism could also not be excluded, we used H4R agonists and antagonists to investigate a possible role of H4R. The published pEC50 values and affinities for these H4R ligands are shown in Figure 8B. P-STS cells showed a weak, but significant $[Ca^{2+}]_i$ response to the H4R agonist 4-methylhistamine (Figure 8C, left panel). However, unlike pre-incubation with histamine, pre-incubation with 4-methylhistamine did not have any clear effect on the response to ACh in P-STS cells (Figure 8C, right panel). Furthermore, VUF8430,[20] another H4R agonist, had only a weak, but not significant enhancing effect when added simultaneously with ACh (Figure 8D). It therefore, seems likely that the observed $[Ca^{2+}]_i$ response to 4-methylhistamine in P-STS cells—that was not seen in HEK—293 T cells (Figure 8E)

despite their higher expression of H4R mRNA—was some unspecific reaction not mediated by H4R.

In previous experiments, the H4R antagonist JNJ7777120 inhibited the $[Ca^{2+}]_i$ response to ACh alone.[5] Here, we re-evaluated the effect of JNJ7777120 on the $[Ca^{2+}]_i$ response to ACh in independent experiments. Again, pre-incubation with JNJ7777120 inhibited the $[Ca^{2+}]_i$ response to ACh (Figure 8F, left panel). In contrast, JNJ39758979,[21] another H4R antagonist with similar affinity for H4R as JNJ7777120,[22] did not show any inhibitory effect on the $[Ca^{2+}]_i$ response to ACh (Figure 8F, right panel), indicating that the inhibitory effect of JNJ7777120 on the $[Ca^{2+}]_i$ response to ACh is not mediated by H4R. JNJ39758979 (10 μM) alone did not cause any significant $[Ca^{2+}]_i$ response in P-STS cells (not shown). Like in P-STS cells, in HEK-293 T cells no inhibitory effect of 1 μM JNJ39758979 on the $[Ca^{2+}]_i$ response to ACh alone was seen (Figure 8G). This antagonist, which appears to have similar affinity to H4R as JNJ7777120,[21,23] was therefore, used in further experiments. Although P-STS cells expressed only a very low amount of H4R mRNA and did not show any significant synergism of H4R agonists with ACh, JNJ39758979 appeared slightly inhibitory for the $[Ca^{2+}]_i$ response to ACh after pre-incubation with 4 μM histamine in P-STS cells (Figure 8H), a histamine concentration supposed to be low enough to reveal an inhibitory effect of 1 μM JNJ39758979.[21] In HEK-293 T cells, the effect of pre-incubation with 1 μM JNJ39758979 on the synergism of ACh with histamine appeared similar as in P-STS cells (Figure 8I). In these experiments, 10 μM histamine had to be used for pre-incubation, because with 4 μM histamine no significant synergism with ACh was seen (not shown). In P-STS as well as HEK-293 T cells, pre-incubation with the H4R agonist 4-methylhistamine did not significantly enhance the $[Ca^{2+}]_i$ response to ACh (Figure 8J,K). Although JNJ7777120 has been shown to inhibit the agonist activity of 4-methylhistamine,[24] JNJ39758979 had rather an enhancing than an inhibitory effect on the $[Ca^{2+}]_i$ response to ACh after pre-incubation with 4 μM 4-methylhistamine in both cell lines in our experiments. This suggests that any putative enhancing effect of 4-methylhistamine on the response to ACh was not mediated by H4R.

FIGURE 8   H4R mRNA expression and effects of H4R ligands on the $[Ca^{2+}]_i$ response of P-STS and HEK-293 T cells to ACh. *$P \leq .05$, PI: pre-incubation (10 min), PPI: pre-pre-incubation, for example, pre-incubation (2 min) before pre-incubation with histamine. A, Expression of H4R mRNA in P-STS and HEK-293 T cells evaluated by amplification from the same cDNA used for H1R mRNA detection in Figure 4. The results were confirmed with an independent amplification from the same cDNA. Control: PCR with HEK-293 T RNA without reverse transcription. B, Published pEC50 and pKi or pA2 values of the H4R ligands used in this study (an[37], b,[20] c,[38] d[19]). D, $[Ca^{2+}]_i$ response of P-STS cells to 10 μM 4-methylhistamine (4mHA, left panel, 12 experiments) and to ACh with or without pre-incubation with 10 μM 4mHA (right panel, 8 experiments). D, $[Ca^{2+}]_i$ response of P-STS cells to VUF8430 alone or added simultaneously with ACh (8 experiments). E, $[Ca^{2+}]_i$ response of HEK-293 T cells to 10 μM 4-methylhistamine (8 experiments). F, Pre-incubation with the H4R antagonist JNJ7777120 (1 μM) inhibits the $[Ca^{2+}]_i$ response to ACh in P-STS cells (left panel, 7 experiments), whereas pre-incubation with JNJ39758979 (1 μM), another H4R antagonist, is not inhibitory (right panel, 15 experiments). G, Pre-incubation with JNJ39758979 (1 μM) does not inhibit the $[Ca^{2+}]_i$ response to ACh in HEK-293 T cells (8 experiments). H, Effect of pre-incubation with JNJ39758979 (1 μM) on the synergism of ACh with histamine in P-STS cells (8 experiments). I, Effect of pre-incubation with JNJ39758979 (1 μM) on the synergism of ACh with histamine in HEK-293 T cells (18 experiments). J, JNJ39758979 (1 μM) does not inhibit the $[Ca^{2+}]_i$ response to ACh after pre-incubation with the H4R agonist 4-methylhistamine in HEK-293 T cells (8 experiments). K, JNJ39758979 (1 μM) does not inhibit the $[Ca^{2+}]_i$ response to ACh after pre-incubation with the H4R agonist 4-methylhistamine in P-STS cells (8 experiments). H4R, histamine H4 receptor; mRNA, messenger RNA; ACh, acetylcholine

3 | DISCUSSION

Secretion of serotonin from enterochromaffin cells is thought to be mediated by M3R, because this receptor plays a major role in hormone secretion.[25] By using the specific M3R inhibitor J104129 we confirmed that the $[Ca^{2+}]_i$ response to ACh in our P-STS enterochromaffin cell line was largely mediated by M3R in the presence as well as in the absence of histamine. There was only a very weak response to nicotine in P-STS cells, excluding a substantial role of nicotinic ACh receptors in cell activation by ACh. Neither with nicotine nor with adrenaline, which both appear to evoke a slight $[Ca^{2+}]_i$ response at high concentrations, any synergism with histamine was observed, indicating that the enhancing effect of histamine is specific for ACh.

In P-STS cells, pre-incubation with histamine had an equally strong enhancing effect on the $[Ca^{2+}]_i$ response to ACh as simultaneous addition,[8] although the mechanisms of these synergisms might be different. Histamine might almost instantaneously increase the excitability of the cells by interaction of the histamine receptor G-protein or an early second messenger formed after histamine receptor ligation with an ion channel,[26] causing an increased $[Ca^{2+}]_i$ response to simultaneously added ACh. In P-STS cells, voltage-gated T-type channels mediate most of the increase in $[Ca^{2+}]_i$ caused by ACh in the presence or absence of histamine.[8] These channels are not expressed in HEK-293-T cells,[27] however, and cannot be involved in the synergism between histamine and ACh seen in these cells. Alternatively, incubation with histamine might inhibit the membrane potential-stabilizing outward leak current mediated by some potassium channel or modulate some other ion channel involved in signalling after M3R activation.[28] In contrast to simultaneous addition with ACh, pre-incubation with histamine for several minutes gives intracellular signal transduction following activation of histamine receptors time to proceed and activate proteins of the signalling cascade and their products may be an additional cause for synergism with ACh.

Supporting its physiological relevance, the $[Ca^{2+}]_i$ response elicited by pre-incubation with histamine before challenge with ACh was comparable in extent to the effect of the positive allosteric MR-modulator VU0119498[13] added simultaneously with ACh to P-STS cells. Interestingly, pre-incubation with this compound significantly reduced its effect compared to simultaneous addition with ACh. This suggests that the risk of gastrointestinal side effects of VU0119498 may be low. The difference between the effect of pre-incubation and simultaneous addition of VU0119498 on the response to ACh might be because of partial inhibition of ACh-binding to the orthosteric site during pre-incubation. A total of 30 µM VU0119498 inhibited binding of the orthosteric muscarinic ACh receptor ligand $[^3H]$-N-methylscopolamine to muscarinic ACh receptor 5 by 50%[13] and might similarly inhibit binding of ACh to M3R.

Activation of H1R or H2R has been found to exert an influence on signalling in response to activation of other GPCR proteins.[11] To elucidate the mechanism of the synergism between histamine and ACh, we, therefore, tested whether the effect of histamine on the $[Ca^{2+}]_i$ response to ACh could be inhibited by antagonists of these receptors. Activation of H2R by histamine normally leads to an elevation of cAMP,[19] which might synergistically enhance the $[Ca^{2+}]_i$ response caused by ACh.[29] However forskolin, which causes an elevation of cytoplasmic cAMP, did not enhance the $[Ca^{2+}]_i$ response to ACh. In accordance with this, the H2R antagonist ranitidine induced at best a slight inhibition of the synergistic effect of ACh and histamine in P-STS cells and was without effect in HEK-293 T cells, indicating that H2R is not involved in the observed synergism. The H1R antagonist cetirizine did not abolish the enhancing effect of histamine on the $[Ca^{2+}]_i$ response to ACh in P-STS cells, but was able to prevent this synergistic response in HEK-293 T cells, which express only a very low amount of H1R. Complete inhibition of the synergism between histamine and ACh in P-STS was achieved by the H1R antagonist mepyramine. This suggests that activation of a low number of H1Rs is sufficient to cause the enhancing effect of histamine pre-incubation on the $[Ca^{2+}]_i$ response to ACh and that this synergism is mediated by H1R in both cell lines. Serotonin secretion from P-STS cells in response to histamine added together with ACh was higher than secretion in response to ACh alone, showing that histamine augments serotonin secretion from enterochromaffin cells (Figure 1H). However, no conclusion about synergism between ACh and histamine can be drawn from this experiment. The apparent inhibition of secretion in response to ACh plus histamine by cetirizine as well as mepyramine (Figure 6E) may have been entirely because of inhibition of secretion in response to H1R activation by histamine. In addition, a slight anti-muscarinic effect of the H1R antagonists may also have contributed.

P-STS cells express only a very low amount of H4R mRNA. Although the H4R agonist 4-methylhistamine elicited a weak, but significant $[Ca^{2+}]_i$ response in P-STS cells when added at the rather high concentration of 10 µM, pre-incubation with this agonist before challenge with ACh did not mimic the response to pre-incubation with histamine. Another H4R agonist, VUF8430, did not elicit any $[Ca^{2+}]_i$ response itself and did not increase the response to ACh. A role of H4R in the synergism between histamine and ACh in P-STS cells is, therefore, unlikely. HEK-293 T cells expressed some H4R mRNA, but—unlike P-STS cells—did not show any notable $[Ca^{2+}]_i$ response to 4-methylhistamine.

Additional experiments confirmed our previous observation that in P-STS cells the H4R antagonist JNJ7777120 inhibited the $[Ca^{2+}]_i$ response to ACh alone.[5] JNJ7777120 has been extensively used as a reference compound to study the role of H4R antagonism.[19] At a concentration of 1 µM it showed <10% competition with binding of the orthosteric ligand 4-DAMP to M3R in a screening assay.[23] The significant inhibition of the ACh-induced increase in $[Ca^{2+}]_i$ observed in our experiments was, therefore, surprising and remains unexplained. JNJ7777120 was also unexpectedly found to reduce ACh-induced sweating in mice.[30] JNJ39758979, a newer H4R antagonist with similar IC_{50},[21] had no inhibitory effect on the ACh-induced $[Ca^{2+}]_i$ response and in this respect was a better choice for testing the effect of H4R antagonism in our experiments. This H4R antagonist was, therefore, used to test whether inhibition of low levels of H4R might counteract the synergism between Ach and histamine. Data from literature suggest that H4R-mediated effects of 4 µM histamine should be significantly inhibited by 1 µM JNJ39758979.[21] Inhibition of the synergism between 4 µM histamine and ACh in P-STS cells was too weak to draw any conclusions. Although HEK-293 T cells showed no $[Ca^{2+}]_i$ response to

4-methylhistamine alone, pre-incubation with 4 μM 4-methylhistamine appeared to have an enhancing effect on the response of HEK-293 T cells to ACh. JNJ39758979 did not inhibit this response, arguing against an involvement of H4R in the synergism between histamine and ACh in HEK-293 T cells. However, because H4R agonists and antagonists had to be used at a much higher concentrations than their pK_i, they may have reacted unspecifically. It is also possible that JNJ39758979, as has been shown for JNJ7777120,[31] has complex pharmacological effects making it an agonist under special circumstances.

Synergism of histamine with ACh, the main secretagogue of enterochromaffin cells, might contribute significantly to serotonin secretion. The fact that not only P-STS cells, but also non-excitable cell types like HEK-293 T, is synergistically activated by histamine and ACh suggests that this could be a more general phenomenon. Enhancement of the M3R-mediated response to ACh by histamine could have effects on a range of glandular and respiratory tissues, where M3R is primarily expressed[32] and enhances secretion and bronchoconstriction, respectively. Elevated M3R activation in the presence of histamine might also promote colon neoplasia, which has been found to depend on M3R in mouse models.[33]

In conclusion, our data show that histamine synergistically enhances the M3R-mediated $[Ca^{2+}]_i$ response of P-STS enterochromaffin and HEK-293 T cells to ACh and probably also serotonin secretion by P-STS cells. From studies with HEK-293 T cells with very low H1R expression as well as with H1R antagonists it appears that even low activity of H1R is sufficient to cause this synergism. Surprisingly, the H4R antagonist JNJ7777120 inhibited the ACh-induced $[Ca^{2+}]_i$ response, suggesting that it should be used with caution for evaluation of the effects of H4R inhibition when a reduction in M3R activity could play a role. Our data derive from a secretory and a non-secretory cell line, which naturally express M3R as predominant muscarinic receptor and therefore are likely to reflect the behaviour in native receptor environment. In this way, histamine ingested with food or produced by mast cells or gut bacteria may modulate gut motility and faecal water content in histamine intolerance, food allergies and irritable bowel syndrome.

4 | METHODS

4.1 | Cell culture

The P-STS midgut neuroendocrine tumour cell line was originally isolated from a WHO III neuroendocrine tumour of the terminal ileum.[3] P-STS cells were grown in a 1:1 mixture of medium M199 and Ham's F12 nutrient mixture supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U mL^{-1} penicillin G and 100 μg mL^{-1} streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. HEK-293 T human embryonic kidney cells (ATCC, CRL-3216) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g l^{-1} glucose and the same supplements. To enhance the adherence to the cell culture plates, for most experiments HEK-293 T cells were grown on plates coated with 50 μg mL^{-1} poly-D-lysine (HEK[PdL]). Both cell lines were tested for *Mycoplasma* contamination by staining with Hoechst dye 33342.

4.2 | Reagents

Mepyramine maleate, ranitidine hydrochloride, JNJ7777120 dihydrobromid and JNJ39758979 dihydrochloride were from Tocris; ACh chloride, histamine dihydrochloride, VUF8430 dihydrobromid, cetirizine dihydrochloride, J104129 fumarate and L-adrenaline were from Sigma Aldrich; VU0119498 was from Abcam, forskolin was from Cayman Chemical, Fluo-4 AM and poly-D-lysine were from Thermo Fisher Scientific, and goat anti-chromogranin A as well as mouse anti-human monoclonal H1R antibody (G11) were from Santa Cruz Biotechnology.

4.3 | $[Ca^{2+}]_i$ imaging

$[Ca^{2+}]_i$ imaging with Fluo-4 AM in P-STS cells and quantification with Image J were done as described previously.[5] The cells were grown in 24-well plates to a low density of 100 to 1000 cells per well, because higher cell densities appeared to increase the likelihood of increases in baseline fluorescence before substance addition. The cells were stained 45 min at room temperature with Fluo-4 AM in serum-free medium. The medium was exchanged for pure medium and the plate was placed on an inverted microscope Axio Observer Z1 equipped with a high resolution AxioCam MRc 5 camera (Carl Zeiss). After 25 min of incubation at room temperature photographs were taken (program Axiovision) before and after careful addition of 100 or 200 μL of medium control or agonists in pure medium to a total volume of 400 μL. The $[Ca^{2+}]_i$ response of Fluo-4 AM-stained P-STS cells to addition of the indicated substances was determined as fluorescence enhancement every 10 s after the start of substance addition in relation to baseline fluorescence. A total of 2 min before challenge with agonist or addition of histamine for 10 min pre-incubation, muscarinic or histamine receptor antagonists were added to the indicated final concentration in the pre-incubation medium. Pre-incubation with H4R agonists, forskolin and VU0119498 was done in the same manner for 10 min.

$[Ca^{2+}]_i$ imaging in HEK-293 T cells was done as described for P-STS cells at a cell density of 10 000 to 30 000 cells per well. Because of an abrupt increase in background fluorescence even on addition of pure medium, the increase in $[Ca^{2+}]_i$ could not be quantified with Image J as in P-STS cells. Instead, all samples with individual cells showing an increase in $[Ca^{2+}]_i$ within the observation period of 20 or 40 s after histamine addition were counted as positive and given the numeric value 1 for graphic representation and statistics. Samples showing no reaction were given the value 0.

4.3.1 | Transfection of P-STS cells with a chromogranin A expression plasmid

P-STS cells were transfected with the plasmid RC200492 (Origene) for expression of Myk-DDK-tagged human chromogranin A using Lipofectamine 3000 Reagent (Thermo Fisher Scientific) following the manufacturers' instructions. The cells were cultivated with 200 μg/mL G418 sulfate.

4.4 | Measurement of serotonin secretion

For serotonin secretion experiments chromogranin A-transfected P-STS were grown in 24-well plates to a density of about 50% in culture medium containing 200 μg/mL G418 sulfate. The cells were washed with 300 μL culture medium without G418 and FCS and then incubated for 10 min in 300 μL of the same medium. This medium was the replaced by 100 μL fresh medium containing no antagonist or 10 μM cetirizine. After 3 min of incubation the agonist's histamine and/or acetylcholine were added in a volume of 10 μL to reach final concentrations of 1 μM acetylcholine and/or 10 μM histamine to two wells in parallel. After incubation for 5 min the supernatants were collected, combining the two wells treated in parallel, and kept at 4°C for 1 h to allow sedimentation of cells that might have been detached and collected. A total of 45 μL of the supernatants without cells were then added to 5 μL 10% stabilizer of the ImmuSmol Serotonin Research ELISA kit (ImmuSmol SAS) and 20 μL were analysed with this ELISA kit as described by the manufacturer, adding 20 μL medium with stabilizer also to the standards.

4.5 | Statistical analysis of $[Ca^{2+}]_i$ imaging and serotonin secretion experiments

It was obvious that increases in fluorescence calculated from the $[Ca^{2+}]_i$ imaging experiments were not normally distributed for P-STS cells, but rather bimodal (Pfanzagl et al).[5] Therefore, statistical significance of the difference of two mean values could not be calculated directly with a parametric test, but was calculated by the unpaired two-tailed t test assuming unequal variances after ranking the data of the two groups tested, as described.[5] The same method was applied to serotonin secretion experiments. Ranking the data reduced the power of the t test to detect significance, because it caused an underestimation of the highest fluorescence values obtained. To increase statistical reliability and strength, in every experiment the different treatments were conducted in parallel, resulting in equal sample numbers in each treatment group. Up to 3 such experiments were combined (e.g. conducted together on the same cell culture plate). As shown in Figure 1C with data from 2 such experiments, it was appropriate to consider each well as independent data point for the statistics. Significant differences are indicated by stars (*$P \leq$ .05, **$P \leq$ .01, ***$P \leq$ .001). Means $+/-$ standard deviation are displayed. For HEK-293 T cells statistical significance was calculated with the two-tailed binomial test using the BINOM.INV function of Microsoft Excel as described before for HeLa cells.[34]

4.6 | RNA isolation, reverse transcription and polymerase chain reaction

RNA from 50% confluent P-STS or HEK-293 T cells was isolated, purified and quantified with TRIzol (Thermo Fisher Scientific) following the manufacturer's instructions. In a total volume of 20 μL, complementary DNA (cDNA) was synthesized from 0.7 μg RNA using the iScript Select cDNA Synthesis Kit (Bio-Rad) using random primers as described by the manufacturer and kept at −20°C until use. Polymerase chain reaction (PCR) amplifications were performed with 0.1 μL cDNA. *Thermus aquaticus* Taq DNA polymerase and deoxynucleotides were from Sigma Aldrich. The MgCl₂ concentration was 1.5 mM. The amplification process was started with incubation at 95°C for 5 min. After 22 cycles for β-actin and 36 cycles for H1R and H4R (30 s at 95°C, 30 s at 60°C, 1 min at 72°C) elongation was completed for 7 min at 72°C. With these cycle numbers the PCR reaction was still in the exponential phase. The primers used were the following: human β-actin forward 5′-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3′and reverse 5′-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3′ (these primers are from different exons, product length 838 nucleotides); human H4R forward 5′-CCG TTT GGG TGC TGG CCT TCT TAG-3′ and reverse 5′-GAT CAC GCT TCC ACA GGC TCC AAT-3′ (product size 204 bp)[35] and human H1R PrimerBank[36] primer pair ID 149158708c2 (forward 5′-CTG AGC ACT ATC TGC TTG GTC-3′ and reverse 5′-AGG ATG TTC ATA GGC ATG ACG A-3′, product size 158 bp).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

TRANSPARENCY PEER REVIEW STATEMENT

Transparency Peer Review available on request from the authors.

DATA AVAILABILITY STATEMENT

The data supporting the figures in this study are available from the corresponding author on request.

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