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Activation of the Canonical β-Catenin Pathway by Histamine*

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Histamine signaling is a principal regulator in a variety of pathophysiological processes including inflammation, gastric acid secretion, neurotransmission, and tumor growth. We report that histamine stimulation causes transactivation of a T cell factor/β-catenin-responsive construct in HeLa cells and in the SW-480 colon cell line, whereas histamine did not effect transactivation of a construct containing the mutated response construct FOP. On the protein level, histamine treatment increases phosphorylation of glycogen synthase kinase 3-β in HeLa cells, murine macrophages, and DLD-1, HT-29, and SW-480 colon cell lines. Furthermore, histamine also decreases the phosphorylated β-catenin content in HeLa cells and murine macrophages. Finally, pharmacological inhibitors of the histamine H1 receptor counteracted histamine-induced T cell factor/β-catenin-responsive construct transactivation and the dephosphorylation of β-catenin in HeLa cells and in macrophages. We conclude that the canonical β-catenin pathway acts downstream of the histamine receptor H1 in a variety of cell types. The observation that inflammatory molecules, like histamine, activate the β-catenin pathway may provide a molecular explanation for a possible link between inflammation and cancer.

Histamine, a biogenic amine formed by decarboxylation of the amino acid L-histidine (1), is found in large quantities in different kinds of tissue, such as mast cell granules, although numerous other cell types are capable of histamine synthesis as well (2). Histamine controls a multitude of physiological functions by activating specific receptors on the target cells. Four types of receptors for histamine have already been described. These receptors are distinguished by their sensitivity to specific pharmacological agonists and antagonists and are named H1–H4 receptors (3–5). In general, the H1 receptor is involved in inflammatory responses, mediating blood vessel and bronchial constriction, vascular permeabilization, and the synthesis of other inflammatory agents (6, 7). The H2 receptor is involved in gastric acid secretion (8), and the H3 receptor is implicated in autoinhibition of histamine synthesis and release (9). The newly discovered H4 receptor has many similarities with the H3 receptor and is able to bind histamine and pyrilamine with high affinity, but tissue distribution is different from the H3 receptor (4). Although anti-histamines are among the most prescribed drugs in the western world, signal transduction and the molecular mechanisms by which histamine receptor activation induces changes in gene transcription and expression remain largely unresolved.

Several groups have reported that cAMP and Ca^{2+} signaling is induced in different cell types upon histamine stimulation (10, 11). We decided to determine the transcriptional responses of HeLa cells upon histamine stimulation by using specific reporter constructs for different transcription factors. We did this by using a panel of different transcription factor-sensitive reporter constructs, and we observed a histamine-dependent activation of Myc, a target of β-catenin signaling. The molecular details of the activation of β-catenin-dependent signal transduction are well investigated with respect to the canonical Wnt signaling pathway. Secreted Wnt glycoproteins bind to the frizzled receptor to activate Dishevelled. In turn, Disheveled phosphorylates and inhibits a complex containing glycogen synthase kinase 3-β (GSK3-β)[1], axin, and adenomatous polyposis coli. When there is no Wnt signal, unphosphorylated GSK3-β phosphorylates β-catenin, leading to the ubiquitination and degradation of β-catenin by the proteasome (12). Thus, activation of the Wnt pathway inhibits phosphorylation and subsequent degradation of β-catenin, allowing its nuclear transport and gene induction by means of binding to T cell factor (TCF) (13, 14).

Histamine receptors are G protein-coupled receptors that are able to activate $G\alpha_q/G\alpha_{11}$ similarly to the frizzled receptor (3, 15, 16), but a connection between histamine and the β-catenin pathway has not yet been described. Activation of both histamine receptors and the TCF/LEF family are associated with the regulation of T cell development (17–20). In addition, it is widely accepted that the development of colon cancer requires activation of the TCF/LEF/β-catenin pathway (21) and that anti-inflammatory drugs inhibit colon cancer development (22). Cooper *et al.* (23) proposed a link between cancer and inflammation in an animal colitis model. They reported a positive correlation between inflammation and cancer in their colitis model, and an early event in this process is the nuclear translocation of β-catenin. Histamine is well known for it pro-inflammatory characteristics, and histamine antagonists have been reported to inhibit tumor growth in the colon (24–27). Other studies have implicated histamine as a growth factor in a mammary carcinoma cell line (28), whereas the TCF/LEF/β-catenin pathway is also implicated in the growth of these cells (29, 30). Thus, a role for the TCF/LEF/β-catenin pathway downstream of histamine receptors would not be inconsistent with existing literature data. These considerations prompted us to include the TCF/LEF/β-catenin pathway in a screen for

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[1] The abbreviations used are: GSK3-β, glycogen synthase kinase 3-β; TCF, T cell factor; LEF, lymphoid enhancer factor; SEAP, secreted alkaline phosphatase; SRE, serum responsive.

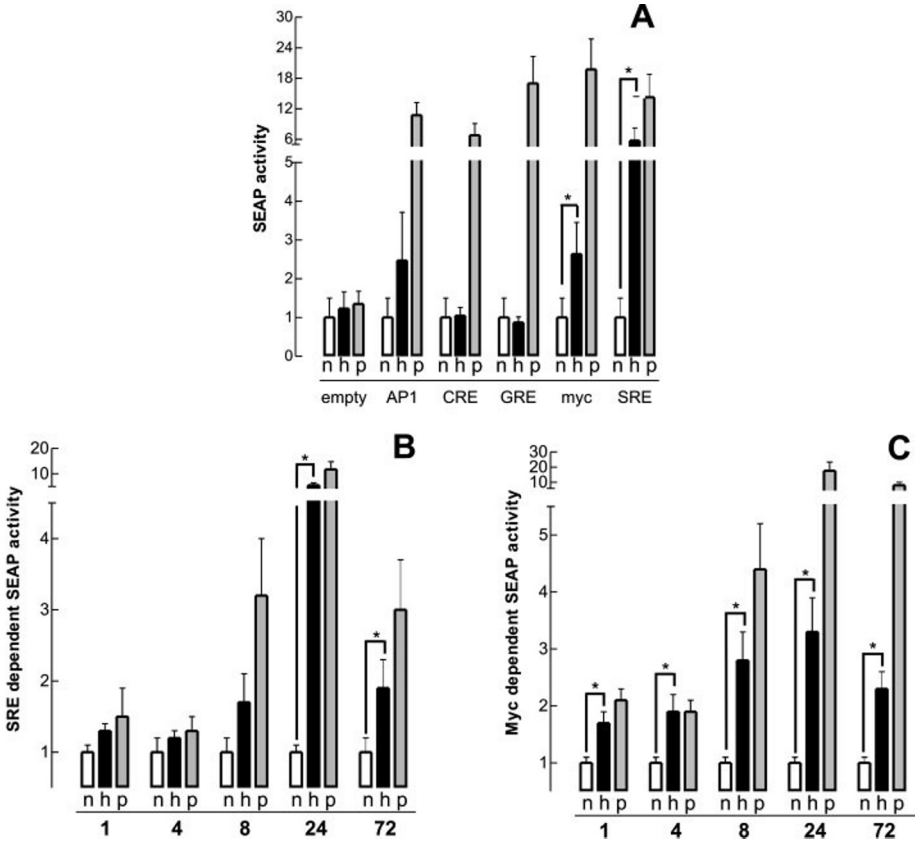


FIG. 1. **Changes in transcriptional activity in HeLa cells after a 24-h, 100 μM histamine stimulation.** *A*, the first white bar (*n*) represents the unstimulated state of the cells and is set to 1; the black histamine (*h*) and grey positive (*p*) bars are relative stimulations compared with the unstimulated cells. The response element activity of the unstimulated negative control (2.5% serum) is standardized, and the other stimulations are relative to the negative control. AP1 is the principal response element for the mitogen-activated protein kinase signal transduction pathways, and the positive control for this response element is activation with 20% serum. cAMP responsive construct (*CRE*) is the response element for the cAMP/Ca^{2+}-dependent signal transduction pathways, and the positive control employed for this response element is 10 μM forskolin. Glucocorticoid responsive construct (*GRE*) is a general glucocorticoid response element, and 100 μM dexamethasone is used as a positive control for this response element. *myc* and *SRE* are prolific response elements, and the positive controls are activation by growth factors (1 μg/ml EGF) and 20% serum, respectively. *B* and *C*, a time-dependent increase in histamine-dependent SEAP production of the SRE and Myc response element is depicted. The black bars are significantly elevated ($p < 0.05$) compared with the unstimulated control.

transcriptional changes induced by histamine receptors. We observed that histamine stimulation causes transactivation of a TCF/β-catenin-responsive construct in HeLa cells and a colon cell line. On the protein level, histamine treatment increases phosphorylation of GSK3-β in HeLa cells, murine macrophages (4–4 Mϕ), and several colon cell lines. Furthermore, histamine also decreases the phosphorylated β-catenin content in these cells. We conclude that the canonical Wnt pathway acts downstream of a pyrilamine-sensitive histamine receptor in a variety of cell types. Together with recent data on prostanoid receptor signaling, we conclude that activation of the canonical β-catenin pathway is not restricted to frizzled receptors but may also be induced by pro-inflammatory G protein-coupled receptors (31). The observation that inflammatory molecules, like prostanoids and histamine, activate the β-catenin pathway may provide a molecular explanation for another link between inflammation and cancer.

EXPERIMENTAL PROCEDURES

Reagents—Histamine, cimetidine, and pyrilamine were purchased from Sigma. The Mercury Pathfinder System was purchased from Clontech (Becton Dickinson). The phospho-specific β-catenin (Ser41/45), GSK3-α/β (Ser9/21), and PKB/Akt (Ser473) anti-bodies were purchased from Cell Signaling Technology. The nonphospho-β-catenin anti-body (clone 8E4) was purchased from Upstate Biotechnology. The TCF-responsive constructs were kindly provided by Dr. H. C. Clevers (Departments of Immunology and Cell Biology, University Medical Center, Utrecht, The Netherlands).

Cell Culture—The different cell lines were grown in their recommended media supplemented with 10% fetal calf serum. HeLa, DLD-1, HT-29, and SW-480 were grown in Dulbecco's modified Eagle's medium supplemented with 5 mM glutamine, 1:500 antibiotics/antimitotics, and 10% fetal calf serum, according to routine procedures. The murine 4–4 macrophages were grown in RPMI 1640 medium supplemented with 5 mM glutamine, 1:500 antibiotics/antimitotics, and 10% fetal calf serum.

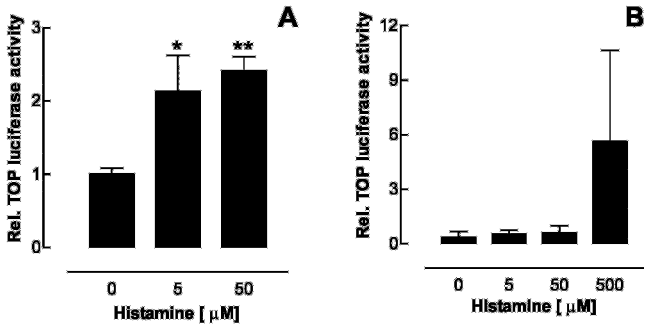
Activity of Transcription Factors Using Secreted Alkaline Phosphatase—HeLa cells were seeded in 96-wells plates at 20% confluence and transfected using Effectene (Qiagen) according to the supplier's protocol, with the different transcription factor-responsive constructs (AP1, cAMP responsive construct, glucocorticoid responsive construct, Myc, and serum responsive (SRE) construct) coupled to a secreted alkaline phosphatase (SEAP) as the reporter protein. The following day, the medium was changed for growth medium containing 2.5% serum. After 24 h, the cells were stimulated with histamine (100 μM) or a positive control for the specific transcription factor. After different time points, a sample of the medium was assayed for the presence of SEAP, as previously described (32).

Activation of the TCF/β-Catenin-responsive Construct Using Luciferase—Activation of the TCF/β-catenin mediated gene expression by histamine. HeLa cells were seeded in 24-well plates and transfected with the plasmids CMV-Renilla Luciferase and FOP- or TOP-FLASH with the ratio 1:10 using the transfection reagent Effectene (Qiagen) according to the standard protocol. SW-480 cells were electroporated with 11 μg of the plasmids CMV-Renilla Luciferase and FOP- or TOP-FLASH with the ratio 1:10, in 400 μl of electroporation medium (culture medium with 10 mM dextrose and 1 mM dithiothreitol). After 24 h, the medium was refreshed with medium containing 2.5% fetal calf serum, and after 24 h, different concentrations of histamine were added with or without the H1 receptor antagonist pyrilamine (20 μM). A dual-luciferase assay kit (PROMEGA) was used to detect luciferase activity after 24 h of incubation.

Western Blotting—Different cell lines were seeded in 6-well plates to be at 80% confluence at the time of stimulation. The cells were changed to their medium containing 2.5% serum 24 h prior to stimulation. After the stimulation, the cells were washed in ice-cold phosphate-buffered saline, taken up in 100 μl of sample buffer, and heated to 95 °C for 5 min. 30 μl of the cell lysate were separated on a 10% SDS-PAGE gel and blotted onto a polyvinylidene difluoride membrane. Antibody probing was performed according to the supplier's protocol. The blots were developed and, when necessary, quantified using software from Syngene.

RESULTS

Histamine-induced Alterations in Gene Expression in HeLa Cells—For investigating histamine signal transduction, we used HeLa cells, which express functional H1 receptors (10, 33). We transfected these cells with a variety of transcription factor reporter constructs driving protein expression of SEAP. No effect of histamine was detected on the transacti-



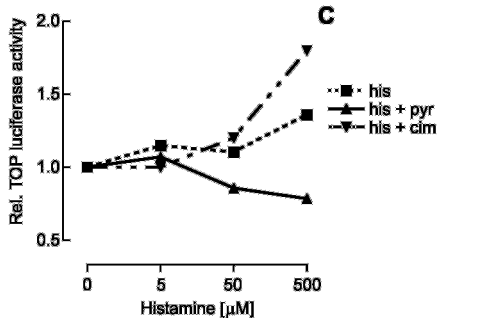


FIG. 2. **Up-regulation of TCF/β-catenin reporter constructs by histamine in HeLa cells.** HeLa cells respond to histamine by significantly increasing the TCF/β-catenin-dependent generation of luciferase (*TOP*) in these cells. This effect is already visible at low concentrations of histamine (*A*); this increase is also detectable in SW-480 colon cells, but at greater concentrations of histamine (*B*). The effects of pharmacological histamine receptor inhibitors on TOP transactivation in HeLa cells are shown in *C*. The H1 receptor antagonist pyrilamine is able to inhibit this effect (*C*), whereas cimetidine, an H2 antagonist, is not.

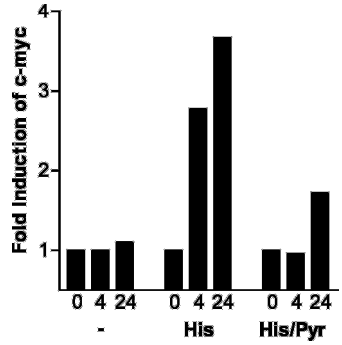


FIG. 3. **Protein levels of Myc, a target of the β-catenin/TCF transcription factor complex, is up-regulated upon histamine stimulation, and this up-regulation can be down-regulated with pyrilamine.** The protein levels were normalized for equal loading, and the fold increase is depicted in the graph.

vation of the cAMP responsive construct and glucocorticoid responsive construct (Fig. 1*A*). However, histamine did result in an enhanced transactivation of AP1, an SRE construct, and a Myc-responsive construct (Fig. 1*A*). In temporal terms, SRE- and Myc-dependent transcription in time also showed a gradual increase peaking after 24 h (Fig. 1, *B* and *C*). The diminished SEAP enzymatic activity after 72 h could be caused by increased degradation of SEAP in combination with lowered histamine-dependent production of SEAP.

Histamine Induces Wnt-responsive Gene Expression—As Myc is an established target for Wnt-signaling (34), we decided to investigate the possibility that the β-catenin pathway is a target for histamine signal transduction. Indeed, a TCF/β-catenin responsive construct (TOP-FLASH), which has been shown to be a useful tool for studying β-catenin-dependent transactivation (35), was induced by histamine treatment of HeLa cells (Fig. 2*A*). This increase is also detectable in the colon cancer cell line SW-480; however, this occurs only at high concentrations (Fig. 2*B*). The relatively high concentration of histamine required for transactivation of the TOP-Flash construct in SW480 cells might be caused by the differences in expression levels of histamine receptors on these cells and because of the fact that these cells already have an activated Wnt pathway. Histamine, therefore, can only further alter the transactivation of the TCF/LEF construct at higher concentrations, thereby overcoming the activated state of the Wnt pathway by the mutations already present in the genome of the cell. CMV-driven luciferase expression was not influenced by histamine, excluding aspecific effects in mRNA and protein synthesis (data not shown). This effect of histamine on transactivation was sensitive to pyrilamine, a pharmacological inhibitor of the H1 receptor, which is prominently expressed in HeLa cells (Fig. 2*C*; Ref. 36), demonstrating that transactivation of this construct by the neurohormone requires functional pyrilamine-sensitive histamine receptors. The histamine receptor 2 antagonist, cimetidine, even has a positive effect on the transactivation of the TCF/β-catenin-responsive construct by histamine (Fig. 2*C*). The fact that cimetidine even enhances the transactivation of the TOP-Flash construct might be caused by a cimetidine-mediated block of binding of histamine to the H2 type of histamine receptors; therefore, more histamine is available to bind the H1 type of histamine receptors, leading to a higher transactivation of the TOP construct. A construct containing a scrambled TCF/β-catenin-binding site (FOP-Flash) was not induced by histamine, and thus the effect of histamine on the TCF/β-catenin-responsive construct does not represent an aspecific effect on mRNA transcription (data not shown). Thus, induction of TCF-dependent transcription is a general feature of histamine signal transduction at physiological concentrations in HeLa cells and at supraphysiological concentrations in SW-480 cells.

Histamine Induces Myc Expression—Next, we verified whether the expression of Myc is also increased in HeLa cells after stimulation of these cells with histamine. When HeLa cells were stimulated with histamine in combination with pyrilamine, the amount of Myc was increased in a histamine-dependent manner, and the increase in Myc expression was inhibited or at least delayed by pyrilamine (Fig. 3). An explanation for the delay in the increase in Myc expression might be that it was caused by degradation and/or deactivation of pyrilamine, which lead to a lower concentration of functional pyrilamine in the medium. When the active concentration of pyrilamine dropped below the inhibitory threshold, the activation of the canonical β-catenin pathway by histamine occurred.

Histamine Lowers β-Catenin Phosphorylation—To investigate whether histamine-induced activation of TCF-dependent transcription is mediated by the canonical β-catenin pathway, changes in the phosphorylation status of Ser41 and Ser45 in β-catenin were studied, as it is well established that dephosphorylation of these residues leads to β-catenin stabilization and subsequent activation of TCF-dependent transcription. We observed that histamine treatment caused a fast, concentration-dependent dephosphorylation of Ser41 and Ser45 in a cervix cell line (HeLa), which is well known for its functional histamine type 1 receptors (10, 37). We also verified the effect of histamine stimulation in murine macrophages (4–4 Mϕ) at three different time points and at two different concentrations (Fig. 4*A*). Furthermore, this effect on β-catenin phosphoryla-

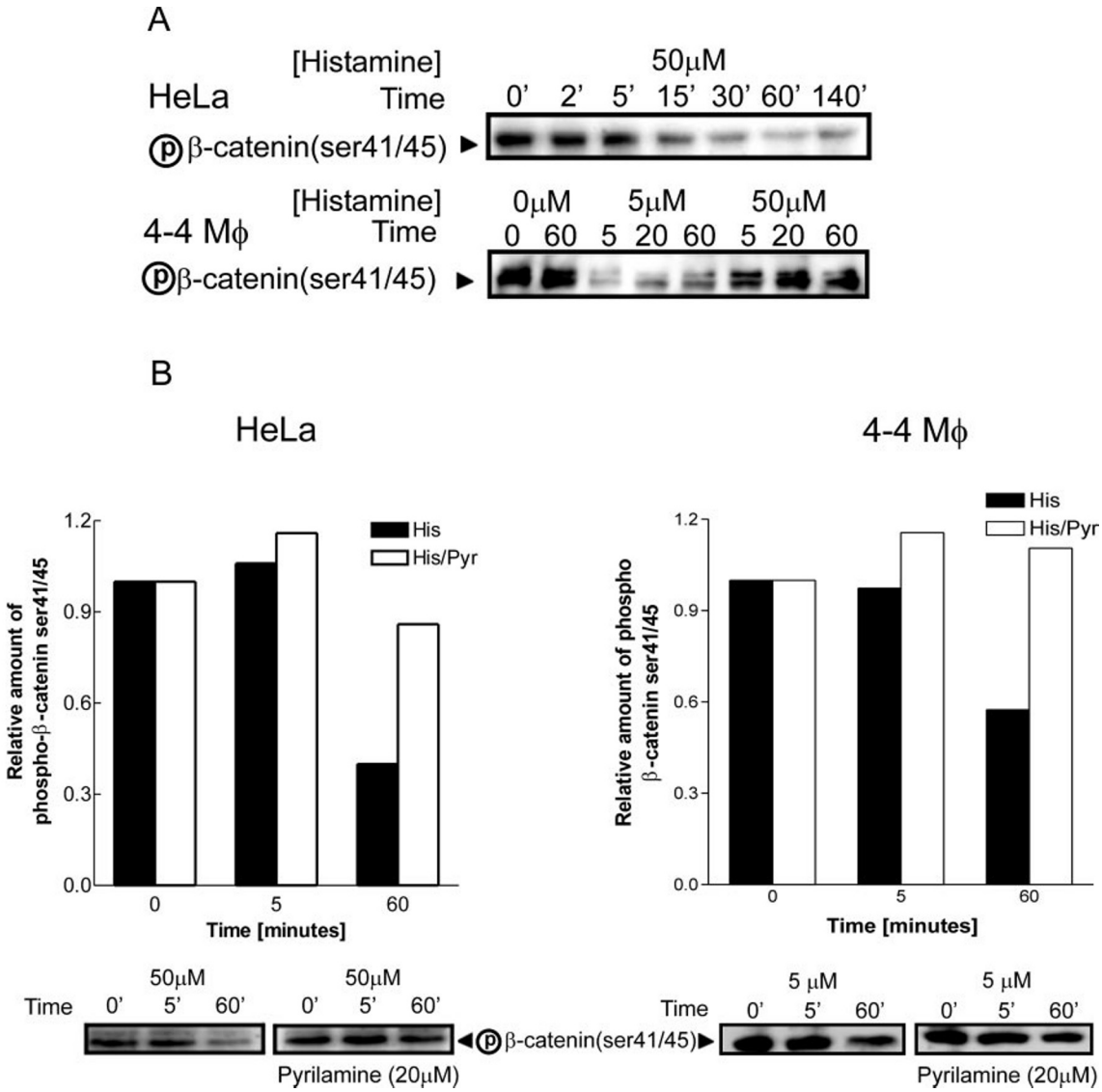


FIG. 4. **Changes in the phosphorylation state of β-catenin upon stimulation of different cell lines with histamine.** Histamine causes dephosphorylation of β-catenin on the Ser41 and Ser45 in different cell lines and elevates the amount of non-phosphorylated β-catenin (*A*). The different cell lines were kept at 2.5% serum for 24 h prior to histamine stimulation. Addition of pyrilamine during stimulation with histamine abolished the decrease in phosphorylation of β-catenin in HeLa cells and in 4–4 Mϕ, which has also been quantified (*B*).

tion was sensitive to pharmacological inhibition of histamine receptors (Fig. 4*B*). Strikingly, the decrease in phosphorylated β-catenin in 4–4 Mϕ is more clearly visible at much lower concentrations of histamine compared with the concentrations used in HeLa cells. This diminished decrease in β-catenin phosphorylation at higher concentrations of histamine might be caused by the presence of a negative feedback loop. The fact that, in HeLa cells, the addition of cimetidine even increases the transactivation of the TOP construct upon histamine stimulation indicates that the cimetidine-sensitive pathway might be involved in a negative feedback of the canonical β-catenin pathway.

Phosphorylation of GSK3-β but Not PKB/Akt by Histamine—Further experiments addressed the question as to whether the upstream activator of β-catenin, GSK3-β, was phosphorylated upon histamine stimulation. Normally, in the Wnt signaling pathway, inhibition of GSK3-β is achieved by means of an inhibitory phosphorylation of Ser21 or Ser9 in GSK3-α and GSK3-β, respectively (38–40). However, the phosphorylation of GSK3-β is normally associated with insulin signaling, but in specific occasions, it is also involved in Wnt signaling (41–43). Similarly, histamine also caused time- and concentration-de-

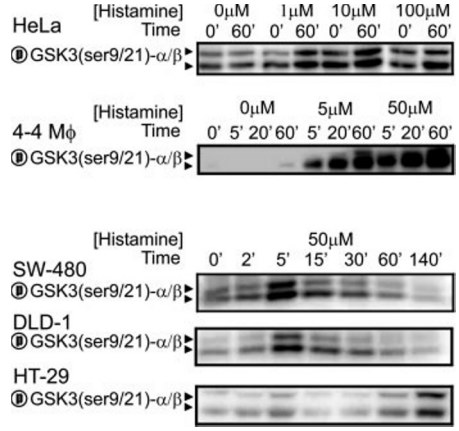


FIG. 5. **Histamine is able to phosphorylate GSK3-β in a time- and concentration-dependent manner in different cell lines.** The different cell lines were kept at 2.5% serum for 24 h prior to histamine stimulation. The phosphorylation of GSK3-α/β upon histamine stimulation was even observed in three different colon cell lines, DLD-1, HT-29, and SW-480. However, the kinetics of this increase in phosphorylation differs between the three different cell types, probably because of differences in activity of the Wnt pathway.

pendent phosphorylation of these residues in HeLa cells and murine 4–4 macrophages (Fig. 5*A*). Furthermore, this effect of histamine was also detectable in the colon cell lines DLD-1, HT-29, and SW-480 (Fig. 5*B*). Thus, phosphorylation of GSK3-β is a specific and general phenomenon in histamine signal transduction. However, phosphorylation of GSK3-β may be brought about in a PKB/Akt-dependent fashion (38) or, as is the case in the canonical β-catenin pathway, in a PKB/Akt-independent fashion (44). The PKB/Akt-independent activation of GSK3-β is mediated by FRAT (frequently rearranged in advanced T-cell lymphomas) and not by PKB/Akt (45–47). Hence, we also investigated PKB/Akt activation by studying the effects of histamine on the phosphorylation of residue Ser473 in this protein (48). However, we did not detect any effect of histamine on PKB/Akt phosphorylation in HeLa, HT-29, and murine macrophages (data not shown). The fact that histamine is not able to phosphorylate PKB/Akt has already been reported by Thors *et al.* (49). Therefore, we concluded that histamine-induced TCF-dependent transactivation is mediated by the canonical β-catenin pathway.

DISCUSSION

Although the importance of histamine-induced signaling in physiology and pathophysiology is widely recognized, the signal transduction pathways mediating long-term effects of histamine on gene transcription remain remarkably poorly characterized. The present study has shown that histamine inactivates GSK3-β, stabilizes β-catenin, and activates TCF-dependent transcription in a variety of cell types. Together, these data indicate that activation of Wnt/frizzled-like signaling cascade is a general feature of histamine-H1 receptor interaction. It must be noted that, for example, the increase in transcriptional activity caused by histamine stimulation of endogenous H1 receptors is somewhat smaller than that observed by Fujino and Regan (31), who used a prostanoid-dependent stimulation in a prostanoid receptor-overexpressing cell line. However, the more modest increase of transcriptional activity might be due to the fact that these cells do not overexpress a histamine receptor, and, therefore, the increase in luciferase expression is more indicative of a more *in vivo*-like cellular response.

Alteration of the activity of the β-catenin pathway can be mediated through mechanisms other than the frizzled-induced Dishevelled-mediated inhibitory phosphorylation of GSK-3β. In addition, enhanced activity of PP2A and subsequent reduced β-catenin phosphorylation may account for activation of this pathway (50). Next, PKC-dependent mechanisms that activate β-catenin-dependent transcription have also been reported (51, 52), again showing that multiple pathways influence the activity of β-catenin-dependent transcription. Our observation that histamine is not capable of inducing enhanced PKB phosphorylation in our experimental systems argues against an important role for this kinase in the activation of the β-catenin pathway by histamine. Furthermore, the fact that the histamine (H1) receptor and the frizzled 1 both use the G protein $G\alpha_q$, and that suppression of $G\alpha_0$ and $G\alpha_q$ abolishes Wnt signaling, also indicates that a link between histamine receptor and β-catenin stability is possible (16). An interesting observation was also reported by Meigs *et al.* (53) who showed that Gα proteins can liberate β-catenin from cadherins and activate β-catenin-dependent transcription. The strict correlation seen between GSK3-β phosphorylation and β-catenin dephosphorylation does not argue in favor of GSK3-β-independent mechanisms leading to diminished β-catenin phosphorylation. Thus, we assume that the canonical β-catenin pathway is the valid mediator of the histamine-induced TCF-dependent transcription. However, until experiments are performed in which the contribution of Dishevelled or Dishevelled-like proteins is assessed directly, other possibilities like PKC should be kept in mind.

Nevertheless, a link between inflammation and colon cancer is well recognized: patients with inflammatory disorders of the bowel are a high-risk population for the development of colon cancer, and it is widely recognized that the regular use of anti-inflammatory drugs reduces the risk of mortality of colon cancer, both with respect to familial adenomatous polyposis coli and sporadic colon cancer (6, 54–57). The observation that histamine activates the β-catenin pathway provides an obvious connection between inflammation and the induction of colorectal cancer. Histamine is well known for its pro-inflammatory action, whereas the β-catenin pathway is almost invariably associated with the induction of colorectal cancer (14, 58–64). In agreement, several studies have documented that histamine antagonists inhibit tumor growth (24–27). Thus, our observation that inflammatory molecules, like histamine, activate the β-catenin pathway may provide a molecular explanation for the link between inflammation and cancer.

In conclusion, our data indicate that additional mechanisms exist to activate β-catenin signaling besides Wnt. These interactions might be involved in the successful fine-tuning of cellular responses during differentiation. Furthermore, histamine-dependent activation of the β-catenin pathway may be an important constituent of the inflammatory response. Thus, it should prove interesting to address the relevance of histamine-induced activation of the β-catenin pathway *in vivo*. Experiments addressing this question are currently being performed in our laboratory.

The *in vivo* role for this effect is still not clear, but histamine might be able to facilitate the sensitivity of cells to become more prone to cancerous insults by elevating the activity of the TCF/LEF-dependent transcription.

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