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Regulation of phospholipase C activation by the number of H_2 receptors during Ca^{2+}-induced differentiation of mouse keratinocytes

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Abstract

We have reported previously that the histamine H_2 receptor (H_2R) can stimulate the phospholipase C (PLC) signaling pathway in mouse keratinocytes. In the present work, we examined the physiological mechanisms involved in this activation by studying histamine metabolism and H_2R expression and coupling during mouse keratinocyte differentiation. Ca^{2+}-induced differentiation decreased histidine decarboxylase (HDC) mRNA, the enzyme responsible for histamine synthesis, by $68.9 \pm 5.0\%$. Concomitantly, intracellular histamine content and its release into the extracellular medium were reduced significantly by 68.2 ± 2.0 and $74.1 \pm 1.7\%$, respectively. Binding of [^{3}H]tiotidine to H_2Rs present on the surface of whole cells was also decreased by cellular differentiation [$(18.17 \pm 2.1) \times 10^4$ vs. $(6.27 \pm 0.87) \times 10^4$ sites/cell, undifferentiated and differentiated cells, respectively], without affecting H_2R affinity. Northern blot and reverse transcriptase–polymerase chain reaction (RT–PCR) analysis of the H_2R mRNA showed that the expression was also down-regulated at the transcriptional level. Moreover, the inhibition of H_2R expression strongly affected the ability of the receptor to induce PLC activation. Our findings suggest that H_2R signaling through the PLC second messenger system is inhibited during keratinocyte differentiation by an autocrine loop involving down-regulation of H_2R expression and inhibition of histamine metabolism.

Keywords: Histamine receptors; Signal transduction; Phospholipase C; Differentiation; Keratinocytes

1. Introduction

The presence of histamine in the epidermis has been widely reported [1]. Histamine is released in the skin from mast cells and keratinocytes themselves. Both cellular types contain histidine decarboxylase, the enzyme responsible for histamine synthesis in most mammalian tissues [2]. It has been suggested that the functions of histamine in the skin may be related to inflammation and itching, and, more recently, to other physiological functions such as the control of keratinocyte proliferation, differentiation, and function; however; the mechanisms by which histamine could influence these processes are not clear [3,4].

The actions of histamine on the epidermis are mediated by H_1 and H_2 receptor subtypes, present on the surface of keratinocyte extracellular membrane. Binding of histamine to these receptors results in the activation of adenylyl cyclase and PLC-mediated signal transduction pathways [5,6]. These signaling pathways can affect keratinocyte metabolism in different ways. cAMP-elevating agents have shown effects on keratinocyte proliferation, while PLC-mediated activation of PKC has been proven to be relevant for keratinocyte differentiation [7,8].

Nevertheless, the effects of histamine and histaminergic ligands on keratinocyte growth seem to be variable and

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Abbreviations: PLC, phospholipase C; HDC, histidine decarboxylase; PKC, protein kinase C; IBMX, 3-isobutyl-1-methylxanthine; RT–PCR, reverse transcriptase–polymerase chain reaction; S-MEM, minimum essential medium; TGk, keratinocyte transglutaminase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGE_2, prostaglandin E_2; InsP, total inositol phosphates; and cAMP, adenosine 3′,5′-cyclic monophosphate.

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dependent upon the method used for the study [9]. Some authors have reported that histamine can inhibit keratinocyte proliferation *in vitro* [10,11], while others have reported stimulation of keratinocyte proliferation [4]. The functional and molecular diversity of histamine receptors has been suggested as a possible explanation for similar controversial observations in other tissues and cell lines [12,13].

We and others have reported previously that H_2 receptors can be functionally coupled to different intracellular mediators [14,15]. In keratinocytes, the stimulation of H_2 receptors can induce the activation of cAMP [5] and PLC/intracellular Ca^{2+} signaling pathways [16,17]. This multiple signaling is common among G_s-coupled receptors and has been suggested to be dependent upon receptor density [18]. According to this hypothesis, the regulation of the receptor number on the cell surface can strongly affect the receptor functional coupling and, thus, its biological effects.

It has been suggested that H_2 receptor functionality could be regulated by histamine in an autocrine or paracrine loop [19]. Based on these observations, we decided to investigate histamine metabolism, the expression of histamine H_2 receptor mRNA, [^{3}H]tiotidine binding sites, and H_2 receptor functional coupling during Ca^{2+}-induced differentiation in a murine keratinocyte-derived cell line, to clarify the mechanisms of histamine action on keratinocytes.

2. Materials and methods

2.1. Materials

[^{3}H]Tiotidine, [^{3}H]mepyramine, [^{3}H]*myo*-inositol, and [^{3}H]cAMP were purchased from New England Nuclear. Histamine dihydrochloride, mepyramine hydrochloride, *myo*-inositol, cAMP, IBMX, bovine serum albumin, bicinchoninic acid method reagents, and ethidium bromide were purchased from the Sigma Chemical Co. Dowex AG-1X8 (formate form) and Chelex 100 resins were obtained from Bio-Rad Laboratories. The H_1 agonist, 2-(3-trifluoromethylphenyl)histamine dihydrogenmaleate, and the H_2 agonists, dimaprit, arpromidine, and BU-E-75, were provided by Dr. W. Schunack from Freie Universität and Dr. A. Buschauer from Regensburg Universität. Tiotidine and famotidine were purchased from Tocris Cookson, Inc., Shephadex G 75 was obtained from Pharmacia Fine Chemicals, and S-MEM, fetal bovine serum, agarose, TRIzol reagent, and antibiotics were purchased from Gibco BRL, Life Technologies, Inc. Forskolin was purchased from Calbiochem. *Taq* DNA polymerase was obtained from the Promega Corp.

2.2. Cell culture

PB cells, a mouse epidermal keratinocyte-derived cell line [20], donated by Dr. F. Larcher (CIEMAT), were grown in monolayers on plastic dishes (Falcon Labware) in S-MEM containing 8% fetal bovine serum, 50 μg/mL of gentamicin, 0.05 mM Ca^{2+} in an atmosphere of 95% air and 5% CO_2 at 37°. Calcium was removed previously from the fetal bovine serum by ion-exchange chromatography using Chelex 100 resin. Proliferating PB cells at 60–80% confluence were induced to differentiate by elevating the extracellular Ca^{2+} concentration from 0.05 mM (low) to 1.2 mM (high) for 72 hr [20].

2.3. Histaminergic ligand binding assays

Radioligand binding assays were performed as previously described [14,19]. For radioligand binding to intact cells, the reactions were carried out in 24-well plastic plates at 4° for 60 min in S-MEM containing 8% fetal bovine serum; 2×10^5 cells/well were incubated in triplicate in the presence of different concentrations of [^{3}H]tio-[^{3}H]tiotidine or [^{3}H]mepyramine, ranging from 1 to 70 nM, in a final volume of 200 μL. After the incubation, cells were washed three times with ice-cold PBS and removed from the plates in 0.5% SDS; radioactivity was measured in a Wallac 1410 liquid scintillation counter. Nonspecific binding was determined in the presence of 10^{-5} M unlabeled tiotidine or mepyramine. Specific binding was calculated by subtracting nonspecific binding from total binding, using GraphPad Prism 3.00 for Windows, GraphPad software. Binding parameters were confirmed by Scatchard plot, performed with the same software.

2.4. Second messenger measurements

2.4.1. cAMP production

PB cells were seeded in 24-well plates and cultured for 48 hr (60–80% confluence) in S-MEM. Then the cells were washed, and the medium was replaced with serum-free S-MEM and cultured for 24 hr before treatment with either histaminergic agonists or histamine. Cells were incubated for 3 min in Hanks' balanced salt solution in the presence of 1 mM IBMX to inhibit phosphodiesterase activity and then were stimulated for 10 min with different concentrations of histamine, H_1, or H_2 agonists (0.01 to 10 μM) in a final volume of 300 μL. The reaction was stopped by rapid aspiration of the culture medium and the addition of 500 μL of ice-cold ethanol. The cells were kept on ice and disrupted by sonication. The resulting supernatant was dried, and the residue was resuspended to determine the amount of cAMP, by a protein-binding assay, according to the method of Nordstedt and Fredholm [21]. Protein content was determined by the bicinchoninic acid method, and results were expressed as picomoles of cAMP per milligram of protein.

2.4.2. InsP production

PB cells were seeded in 24-well plates and cultured for 48 hr (60–80% confluence) in S-MEM. Cells were then

washed, the medium was replaced with serum-free S-MEM containing [^{3}H]*myo*-inositol (2 μCi/mL), and the cells were cultured for an additional 24 hr. Thereafter, the medium was aspirated from the cultures and replaced with serum-free S-MEM containing 10 mM LiCl, and the cells were incubated for 10 min. Cells were then stimulated for 20 min with histamine, H_1, or H_2 agonists in concentrations ranging from 0.01 to 10 μM in a final volume of 300 μL. The incubation was stopped by the addition of 900 μL of chloroform:methanol (1:2, freshly prepared), and phases were split by the addition of 300 μL water and 300 μL chloroform. Next the mixture was centrifuged at 1500 *g* for 10 min at 4°, and the water-soluble inositol phosphate fraction was purified by anion exchange chromatography [22]. Radioactivity of eluted fractions was measured using a Wallac 1410 liquid scintillation counter. Results were expressed as the ratio obtained when total [^{3}H]inositol phosphate activity was normalized to total [^{3}H]inositol recovered from the initial water wash of the columns [23].

2.5. Isolation of total RNA

Total RNA was extracted from PB cells cultured under low or high extracellular Ca^{2+} concentrations, using the TRIzol reagent, following the instructions of the manufacturer. Briefly, cells were grown on plastic dishes for 72 hr under the selected Ca^{2+} concentration and then lysed by the addition of TRIzol reagent. The cell lysate was passed several times through a pipette and incubated for 5 min at room temperature. The homogenates were mixed with chloroform and centrifuged at 12,000 *g* for 15 min at 4°. RNA was precipitated from the aqueous phase by the addition of isopropanol. Samples were then centrifuged at 12,000 *g* for 10 min at room temperature.

The RNA pellet was washed with 70% ice-cold ethanol, air-dried, dissolved in 20 μL of diethyl pyrocarbonate-treated water and stored at −70° until used. The purity of total RNA was determined by the measurement of optical density at 260 and 280 nm using a Shimadzu UV-210A spectrophotometer. The integrity of the 18S and 28S ribosomal RNA was tested by gel electrophoresis and ethidium bromide staining.

2.6. Hybridization probes

The rat cDNA fragment used as a probe for hybridization to mouse TGk mRNA was donated by Dr C. Conti (MD Anderson Cancer Center, University of Texas). This 2.2-kb rat cDNA fragment has been used successfully by other authors to probe for mouse TGk mRNA in cultured murine keratinocytes [24].

The probe used for hybridization to H_2 receptor mRNA was a 468 bp cDNA fragment synthesized by PCR from mouse genomic DNA. The sense and antisense oligonucleotides for PCR amplification were designed from a mouse sequence obtained from GenBank (Accession No. D50096), using OLIGO primer analysis software. These oligonucleotides were: sense primer 5′-CGTCTGCCTG-GCTGTCAGCTTG-3′ corresponding to nucleotides 248–269, and antisense primer 5′-AGAGGCAGGTAGAAGGT-GACCA-3′ complementary to nucleotides 694–715 of the published sequence. The PCR was composed of 1 μL of a 2 ng/μL mouse DNA solution and 49 μL master mix containing 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3), 2.5 mM $MgCl_2$, 50 pmol of each primer, and 2 units of *Taq* DNA polymerase). The reaction was overlaid with mineral oil and subjected to 30 cycles of PCR amplification in a Biometra Personal Cycler (Biometra). After an initial denaturation step at 94° for 2 min, a 30-cycle profile of denaturation for 30 s at 94°, annealing for 30 s at 55°, and elongation for 2 min at 72° was performed. After the final cycle, the temperature was maintained for 10 min at 72° to allow for complete synthesis of the amplified products.

The probe for hybridization to GAPDH mRNA, used as a control housekeeping gene, was a mouse GAPDH cDNA fragment obtained from Ambion, Inc.

Histidine decarboxylase mRNA was detected by hybridization to a 440-bp cDNA probe amplified from mouse genomic DNA by PCR as described previously [25]. Two 20-mer oligonucleotides (5′-AGAGATGGTGGATTA-CATCT-3′, sense primer, and 5′-TACTTGTGCTTGACC-CAGAA-3′, antisense primer) were used as primers for the PCR reaction. Briefly, 1 μL of a 2 ng/μL mouse DNA solution was incubated with 19 μL of a master mix containing 1× PCR buffer, 1.5 mM $MgCl_2$, 400 μM of each dNTP, 1 μM of each sense and antisense primer, and 1 unit of *Taq* DNA polymerase. After an initial step of denaturation at 94° for 2 min, a 30-cycle profile of denaturation at 94° for 1 min, primer annealing at 55° for 2 min, and extension at 72° for 3 min was performed in a Biometra Personal thermal cycler.

The probes were labeled by random priming (Ready-to-Go, Pharmacia Biotech) using [α^{32}P]dCTP (3000 Ci/mmol, NEN Life Science Products, Inc.).

2.7. Northern hybridization

Poly(A)$^+$ RNA was prepared using the PolyATtract mRNA isolation system (Promega Corp.). Poly(A)$^+$ RNA was electrophoresed in 1% agarose gels containing 0.22 M formaldehyde and transferred to Zeta-Probe GT nylon membranes (Bio-Rad Laboratories).

The nylon membranes were prehybridized in hybridization buffer [5× sodium saline phosphate EDTA (SSPE), 50% formamide, 0.1% SDS, 150 μg/mL of denatured salmon sperm DNA and 1× Denhardt's solution] at 42° for 2 hr. Hybridization was performed using 10^7 counts min^{-1} mL^{-1} of heat denaturated ^{32}P-labeled probes in the same buffer for 24 hr at 42°. Membranes were washed sequentially, two times at room temperature with 2× standard saline citrate (SSC), 0.1% SDS for 30 min, two times

at room temperature with 1× SSC, 0.1% SDS for 30 min, and two times with 1× SSC, 0.1% SDS for 30 min at 60°. Using an intensifying screen, hybridization signals were detected on Kodak X-OMAT AR film, following exposure for 72 hr at −80°. To ensure that equivalent amounts of RNA were electrophoresed and transferred, the level of GAPDH mRNA was measured in each lane. A densitometric analysis was performed using the image analysis software Scion Image (Scion Corp.), and the results were expressed as means ± SEM of three independent experiments.

2.8. DNA sequencing

The identity of the PCR-derived mouse DNA probes for histamine H_2 receptor mRNA was verified by sequencing both strands by the dideoxynucleotide chain-termination method, using the *fmol* DNA Sequencing System (Promega Corp.), following the instructions of the manufacturer. The obtained sequences were compared to the published sequence of the mouse histamine H_2 receptor [26].

2.9. RT–PCR

Samples of 1 μg of total RNA were treated with 1 unit of amplification grade DNAase I (Gibco BRL, Life Technologies, Inc.) in 1× DNAase I reaction buffer, in the presence of 20 units of RNAase inhibitor (Promega Corp.) in a final volume of 10 μL and incubated for 15 min at room temperature. The reaction was stopped by heating the samples for 10 min at 65° in the presence of 25 mM EDTA (pH 8.0) and cooled on ice immediately afterwards.

DNAase-treated RNA samples were reverse transcribed to cDNA in a final volume of 20 μL in the presence of 1× PCR buffer, 5 mM $MgCl_2$, 1 mM deoxyribonucleoside triphosphate (dNTP), 50 pmol of random hexamer primers, and 50 units of MMLV reverse transcriptase (Promega Corp.). The samples were incubated subsequently at room temperature for 10 min, and at 42° for 45 min; the enzyme was inactivated at 99° for 5 min and cooled on ice immediately afterwards.

Following cDNA synthesis, PCR was performed from 10 μL of a cDNA mixture using the same oligonucleotides and conditions described above for the PCR reaction performed to synthesize the mouse genomic DNA-derived cDNA probe for H_2 receptor mRNA in a final volume of 30 μL.

In parallel with each sample, a reverse transcriptase-negative reaction was performed where the enzyme was replaced by RNase-free water, to verify that the amplification products were derived exclusively from RNA. To establish the optimal number of cycles for the PCR, control experiments were performed with cycle numbers ranging from 20 to 40. Reaction cycle–PCR product yield curves of each reaction were plotted, and, from the plot, the number of cycles to which the sample could be exponentially amplified was chosen. From these experiments, an optimal number of 33 cycles was determined.

As internal control, mouse GAPDH mRNA was amplified from each sample and used to semi-quantify the expression of the histamine H_2 receptor [27]. Two 21-mer oligonucleotides (5′-CAGTATGACTCCACTCACGGC-3′, sense primer, and 5′-TTCTTGATGTCATCATACTTG-3′, antisense primer) were used to amplify a 638-bp fragment of cDNA by PCR. Briefly, 10 μL of the cDNA mixture were incubated with 20 μL of a master mix containing 1× PCR buffer, 2.5 mM $MgCl_2$, 1 μM of each sense and antisense primer, and 2 units of *Taq* DNA polymerase. Cycling was performed in the same conditions and number of cycles as those for the H_2 receptor.

2.10. Electrophoretic analysis

Each product (10 μL) was analyzed electrophoretically in one single 2% agarose gel containing 0.5 mg/mL of ethidium bromide in 1× TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.0) at 5 V/cm for 2 hr. The intensity obtained from the ethidium bromide luminescence was digitalized and analyzed using Scion Image software. To semi-quantify the levels of expression of H_2 receptor mRNA, the optical density values were transformed to integrated area units and normalized against GAPDH expression by calculating the H_2 receptor/GAPDH ratio of integrated area units.

2.11. Southern hybridization

The identity of the RT–PCR products obtained from PB cell cultures was confirmed by southern blot analysis [28], using a genomic DNA-derived probe for mouse H_2 receptor. Briefly, 10 μg of each PCR sample was electrophoresed in 2% agarose gels and transferred to Zeta-Probe GT nylon membranes. Hybridization to the mouse ^{32}P-labeled H_2 receptor cDNA probe was performed at 40° in 50% formamide containing 150 mM NaCl, 5 mM EDTA, 50 mM sodium phosphate (pH 7.5), and 1% SDS. Washes were performed sequentially, two times at room temperature with 2× SSC, 0.1% SDS for 30 min, two times at room temperature with 1× SSC, 0.1% SDS for 30 min, two times at room temperature with 0.1× SSC, 0.1% SDS for 30 min, and two times with 0.1× SSC, 0.1% SDS for 30 min at 62°. Using an intensifying screen, hybridization signals were detected on Kodak X-OMAT AR film following exposure for 72 hr at −80°. The nucleotide sequence of the RT–PCR products was determined further by the dideoxynucleotide chain-termination method.

2.12. Histamine metabolism

PB cells were cultured for 72 hr under the conditions described above, and the intracellular histamine content and the histamine released into the extracellular medium

were evaluated by a radioimmunoassay (Immunotech). Briefly, the culture medium from 5×10^6 cells was removed and stored at −20° for the determination of histamine. The cells were lysed and homogenized in a 0.1 M $HClO_4$ solution, centrifuged at 8000 *g* for 10 min at 4°, and the supernatant was neutralized with buffer provided by the manufacturer. For the radioimmunoassay, samples were diluted with the same buffer, so as to fit the valid range of the standard curve. Results were expressed as picomoles of histamine/10^6 cells.

2.13. Cholera toxin pretreatment

PB cells were preincubated with 2 μg/mL of cholera toxin for 2 hr before the effect of the histaminergic agonists was determined.

2.14. Statistical analysis

Data were analyzed statistically with either Student's *t*-test when two groups were compared or one-way ANOVA when multiple comparisons were performed. In all cases, *P* values less than 0.05 were considered statistically significant. Non-linear regression and concentration–response curves were generated and analyzed using GraphPad Prism software.

3. Results

3.1. Effect of extracellular [Ca^{2+}] on differentiation of PB cells

As previously described by other authors, PB cell differentiation can be modulated by extracellular [Ca^{2+}]. In a low [Ca^{2+}] medium (0.05 mM), PB cells display a basal cell-like phenotype, whereas in a high [Ca^{2+}] medium cells display a suprabasal cell-like phenotype [20]. This was further confirmed here by studying the expression of TGk mRNA, the enzyme responsible for the assembly of cornified envelopes, a late marker of keratinocyte terminal differentiation [29]. As shown in Fig. 1, TGk mRNA was detectable only in PB cells grown under high [Ca^{2+}]. The expression of TGk mRNA was accompanied by morphological changes, such as cell flattening, enlargement, stratification, and higher release of cornified envelopes into the culture medium, typically described for differentiating keratinocytes (not shown).

3.2. Effect of Ca^{2+}-induced differentiation on the expression of HDC mRNA and histamine metabolism

Northern blot analysis using the cDNA probe for HDC, amplified from mouse genomic DNA by PCR, confirmed the expression of a 2.7 kb mRNA species in PB cells similar to that described for other mouse-derived cell lines

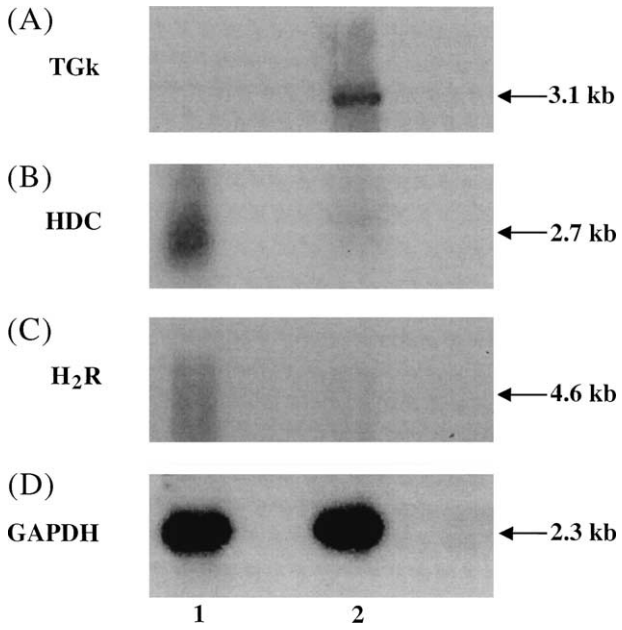

Fig. 1. Northern blot analysis of the expression of different mRNAs in PB keratinocytes cultured under different concentrations of extracellular [Ca^{2+}]. The mRNA levels were detected in poly(A)$^+$ RNA samples obtained from PB keratinocytes cultured under [Ca^{2+}] = 0.05 mM (lane 1) or [Ca^{2+}] = 1.2 mM (lane 2). The same blots were sequentially hybridized to different probes. (A) Transglutaminase k (TGk) expression was detectable only in cells cultured in high calcium conditions; (B) histidine decarboxylase (HDC) expression was higher in low calcium conditions; (C) histamine H_2 receptor (H_2R) expression produced a faint band in both culture conditions; the expression was significantly higher in low calcium conditions; and (D) GAPDH expression, used as an internal control, was similar in both lanes. Densitometric measurements were made from the autoradiograms. Each measurement of TGk, HDC, or H_2 receptor was normalized to measurements of GAPDH in the same sample. Results described in the text are means ± SEM of three independent experiments. Statistical differences between both culture conditions were analyzed using Student's *t*-test.

[30]. The culture of the cells in high [Ca^{2+}] conditions for 72 hr resulted in a marked decrease in HDC mRNA in the differentiated stage (Fig. 1). Densitometric analysis of the data revealed that HDC mRNA expression was significantly higher in undifferentiated PB cells. The ratio obtained for the normalized amounts of HDC mRNA in undifferentiated/differentiated cells was 2.69 ± 0.5. Concomitantly, the intracellular histamine content and the release into the extracellular medium were reduced significantly in differentiated PB cells when compared with cells grown under low [Ca^{2+}] (Table 1).

3.3. Histaminergic ligand binding studies

The cell-surface expression of histamine H_1 and H_2 receptors was studied by whole cell binding experiments with specific histaminergic ligands. The expression of H_1 and H_2 receptors was evaluated using [^{3}H]mepyramine and [^{3}H]tiotidine, respectively. The histaminergic ligand binding sites on the surface of whole PB cells were specific and saturable (Fig. 2). The dissociation constant (K_d) values

Table 1
Effect of Ca^{2+}-induced differentiation on histamine metabolism

	Histamine release (pmol/10^6 cells)	Histamine content (pmol/10^6 cells)
Undifferentiated PB keratinocytes	11.2 ± 2.3*	13.5 ± 1.4*
Differentiated PB keratinocytes	2.9 ± 1.7	4.3 ± 2.0

PB keratinocytes were cultured in 0.05 mM Ca^{2+} (undifferentiated) or were induced to differentiate by raising the extracellular [Ca^{2+}] to 1.2 mM (differentiated). Intracellular histamine content and histamine release to the extracellular medium were measured by radioimmunoassay. Data are expressed as means ± SEM of triplicate determinations from a single experiment performed three independent times.

* Significantly different from differentiated PB keratinocytes ($P < 0.01$, Student's t-test).

obtained for both [^{3}H]mepyramine and [^{3}H]tiotidine were similar to those reported in other systems [31,32] (Table 2).

Interestingly, Ca^{2+}-induced differentiation of PB cells was associated with a significant reduction of [^{3}H]tiotidine binding sites on the cell surface. As we have reported previously in preliminary form [33], no changes were observed in either the number of [^{3}H]mepyramine binding sites or the affinity of H_1 or H_2 ligand binding sites (Table 2).

3.4. Expression of H_2 receptor gene by northern hybridization

To establish if the differences observed in the cell-surface expression of the H_2 receptor protein were correlated

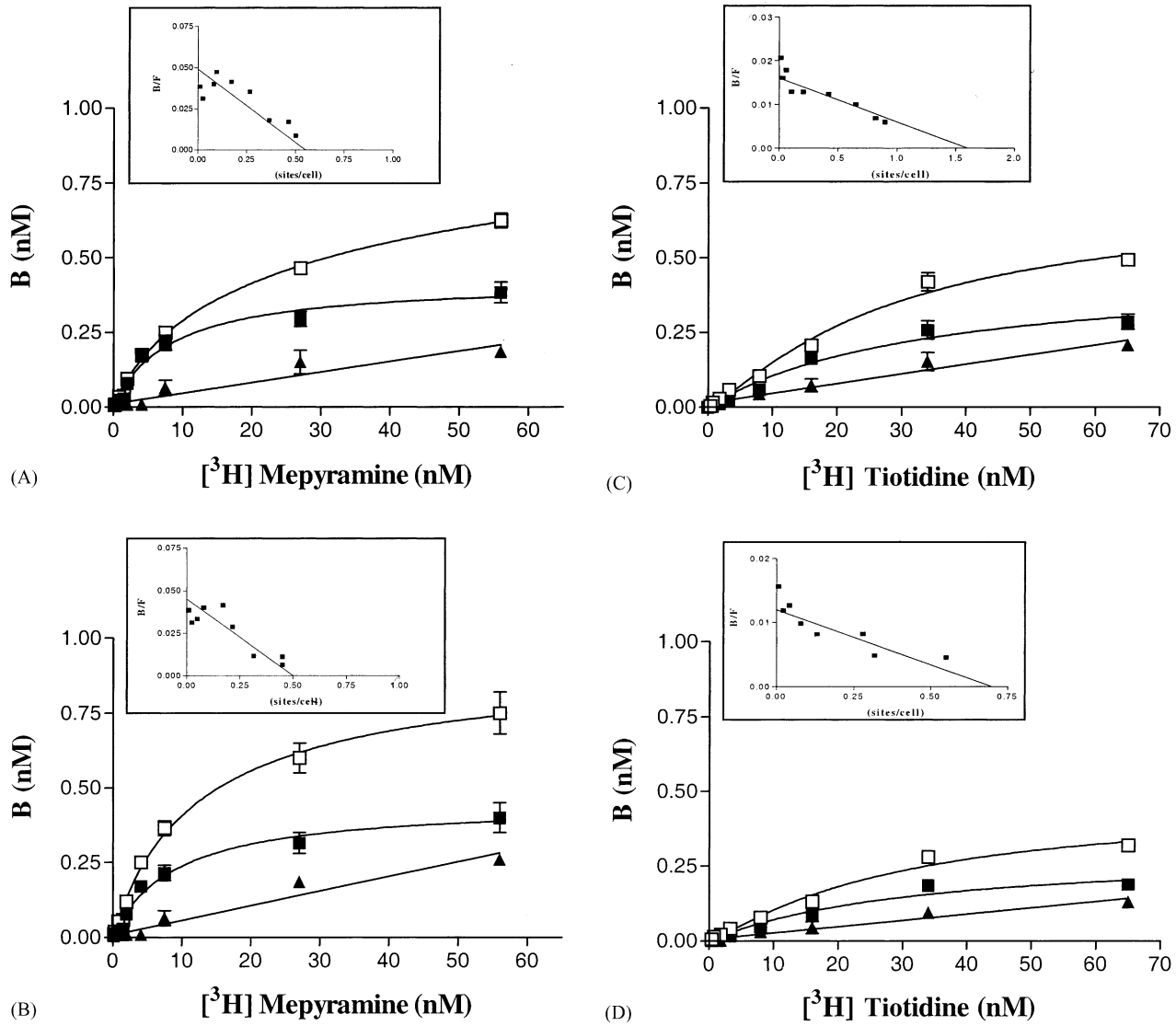

Fig. 2. Saturable binding studies of radiolabeled histaminergic ligands to undifferentiated and differentiated PB keratinocytes. A saturation isotherm was obtained using whole cells cultured under each Ca^{2+} concentration. (A) [^{3}H]Mepyramine binding to undifferentiated cells, (B) [^{3}H]mepyramine binding to differentiated cells, (C) [^{3}H]tiotidine binding to undifferentiated cells, and (D) [^{3}H]tiotidine binding to differentiated cells. Key: total binding (□), specific binding (■), and unspecific binding (▲). Insets: Scatchard analysis of the data revealed single binding sites for [^{3}H]mepyramine and for [^{3}H]tiotidine, with binding parameters similar to those obtained using non-linear regression analysis. Each point represents the mean ± SEM of three independent experiments performed with triplicate determinations.

Table 2
Binding characteristics of H_1 and H_2 histamine receptors on PB keratinocytes

	[^{3}H]Mepyramine		[^{3}H]Tiotidine	
	No. of sites/cell	K_d (nM)	No. of sites/cell	K_d (nM)
Undifferentiated PB keratinocytes	$(5.33 \pm 0.34) \times 10^4$	7.0 ± 1.5	$(18.17 \pm 2.1) \times 10^{4*}$	20.8 ± 3.2
Differentiated PB keratinocytes	$(4.91 \pm 0.76) \times 10^4$	6.6 ± 11.3	$(6.27 \pm 0.87) \times 10^4$	18.2 ± 4.1

Binding parameters were obtained after non-linear regression analysis of saturation assay data. The results are expressed as means ± SEM of triplicate determinations from a single experiment performed three independent times.
* Significantly different from differentiated PB keratinocytes ($P < 0.01$, Student's t-test).

with changes at the mRNA level during Ca^{2+}-induced differentiation of PB cells, we studied H_2 receptor mRNA expression by northern hybridization. Fig. 1 shows the results of a 72-hr film exposure at −80°. A faint band of approximately 4.6 kb was observed for the H_2 receptor in both lanes. A statistically significant lower normalized amount of H_2 receptor mRNA was found in differentiated PB cells (1.45 ± 0.3 undifferentiated/differentiated cells) from the densitometrical analysis. We also performed northern hybridization for H_2 receptors on poly(A)$^+$ RNA isolated from primary cultured murine keratinocytes, which exhibited expression of the same 4.6-kb faint band (data not shown).

3.5. Expression of histamine H_2 receptor gene by RT–PCR

To further study the regulation of the expression of the H_2 receptor at the mRNA level during Ca^{2+}-induced differentiation of PB cells, we measured the levels of expression of the specific mRNA by applying a more sensitive method, RT–PCR. Agarose gel electrophoresis of the PCR products, obtained from PB cells cultured under low or high [Ca^{2+}], confirmed products of the expected size corresponding to H_2 receptors (468 bp) (Fig. 3A). Samples from mouse stomach were used as a positive control for the RT reaction and from mouse genomic DNA as a positive control of the PCR reaction. DNAase I treatment was successful in eliminating any contaminating DNA, since no bands were detected in the negative controls in the absence of reverse transcriptase. The amplified PCR products were sequenced and the results compared with the published sequence of mouse histamine H_2 receptor, showing 100% homology (not shown). Southern blot analysis of these RT–PCR products further confirmed that they were derived from the H_2 receptor gene (Fig. 3B). Our experiments showed that the amount of H_2 receptor mRNA was significantly lower in differentiated PB cells, i.e. cultured under high [Ca^{2+}] (Fig. 3A, lane 4), than that present in undifferentiated PB cells, i.e. cultured under low [Ca^{2+}] (Fig. 3A, lane 2). The densitometric analysis of the bands revealed that the differentiation of PB cells was accompanied by a significant decrease in the amount of H_2 receptor mRNA (Table 3).

3.6. Effects of Ca^{2+}-induced differentiation on functional coupling to second messenger production

3.6.1. cAMP production

The application of histamine or H_1 or H_2 agonists in concentrations ranging from 0.01 to 10 μM, in the presence

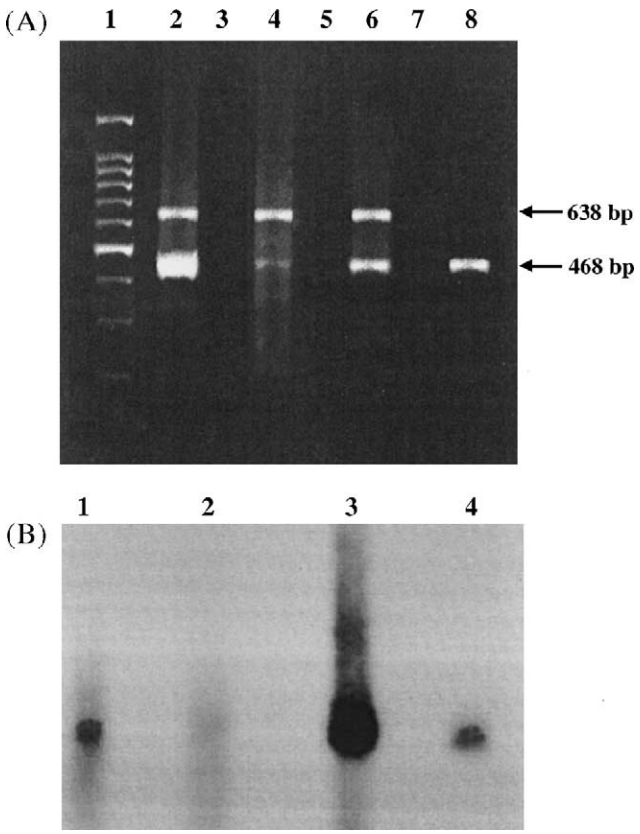

Fig. 3. RT–PCR products corresponding to histamine H_2 receptor and GAPDH mRNA. (A) RT–PCR reactions were performed using total mRNA extracted from undifferentiated (lane 2) or differentiated (lane 4) PB keratinocytes. Products of the expected size for the H_2 receptor (468 bp) and for GAPDH (638 bp) were obtained in both lanes. As positive controls for RT and PCR reactions, mouse stomach mRNA (lane 6) and mouse genomic DNA (lane 8) were used. As a negative control, no amplification products were observed when reverse transcriptase was omitted in the first-strand cDNA synthesis (lanes 3, 5, and 7). In lane 1, a 100-bp DNA ladder molecular size marker was run to confirm the size of the amplification products. (B) Southern blot analysis of RT–PCR products from the top panel using a mouse genomic DNA-derived probe. Products from undifferentiated (lane 1) or differentiated (lane 2) keratinocytes, mouse stomach (lane 3), and mouse genomic DNA (lane 4) are shown.

Table 3
Effect of PB keratinocyte differentiation on H_2 receptor mRNA expression measured by RT–PCR

	GAPDH (IAU)	H_2 receptor (IAU)	H_2 receptor/GAPDH
Undifferentiated PB keratinocytes	221.7 ± 13.3	248 ± 7.5	1.12 ± 0.02*
Differentiated PB keratinocytes	226.3 ± 5.6	115.0 ± 9.1	0.50 ± 0.09

The level of expression of the H_2 receptor was semi-quantitated by using Scion Image software (see "Section 2"). The values of integrated area units (IAU) obtained for the 468-bp band corresponding to the H_2 receptor were normalized against the values of IAU for the 638-bp band corresponding to GAPDH run in the same gel. Results are expressed as means ± SEM of six independent RT–PCR reactions.

* Significantly different obtain from differentiated PB keratinocytes ($P < 0.01$, Student's t-test).

of 1 mM IBMX did not result in an increase of the cellular cAMP level in either undifferentiated or differentiated PB cells (Fig. 4). Yet, stimulation of the endogenous PGE_2 receptor with 1 μM PGE_2 resulted in an increase of 442 ± 11% of the basal levels of cAMP. Moreover, sodium fluoride, an unspecific G-protein activator, and forskolin, an adenylyl cyclase activator, demonstrated the functionality of the signaling pathway at different levels (Fig. 4). These results were confirmed by a more sensitive radioimmunoassay using 2′-*O*-mono-succinyladenosine-3′,5′-cyclic monophosphate tyrosil methyl ester radioiodinated with $Na^{125}I$ and the corresponding antibody (data not shown). Pretreatment of the cells with 2 μg/mL of cholera toxin potentiated the stimulatory effect of PGE_2 on adenylyl cyclase activity as was expected for a G_s-coupled receptor such as the prostaglandin EP_2 receptor present on keratinocytes [7] (Fig. 4).

3.6.2. InsP production

In undifferentiated PB cells, InsP production induced by H_1 and H_2 receptor agonists in the presence of LiCl was concentration-dependent ($EC_{50} = 0.38 \pm 0.05$ μM for 2-(3-trifluoromethylphenyl)histamine and 1.06 ± 0.24 μM for BU-E-75) (Fig. 5A). These effects were inhibited by the corresponding selective antagonists, mepyramine (H_1) or tiotidine or famotidine (H_2), indicating a specific receptor-mediated activation of the intracellular signaling pathway (Fig. 5A). Histamine induced a concentration-dependent production of InsP ($EC_{50} = 0.234 \pm 0.02$ μM) (Fig. 5A) in the same culture conditions. This induction was insensitive to the H_1 antagonist mepyramine but was blocked completely by the H_2 antagonists tiotidine or famotidine (Fig. 5A).

In differentiated PB cells, cultured at 1.2 mM Ca^{2+}, the H_1 receptor agonist-mediated InsP production was detectable, concentration-dependent ($EC_{50} = 0.31 \pm 0.10$ μM), and blocked by H_1 receptor-specific antagonists (Fig. 5B). Interestingly, under these culture conditions, the H_2 receptor agonists did not increase InsP production above basal values. The effect of histamine on InsP production was concentration-dependent ($EC_{50} = 0.302 \pm 0.05$ μM) but weaker than that detected in undifferentiated PB cells

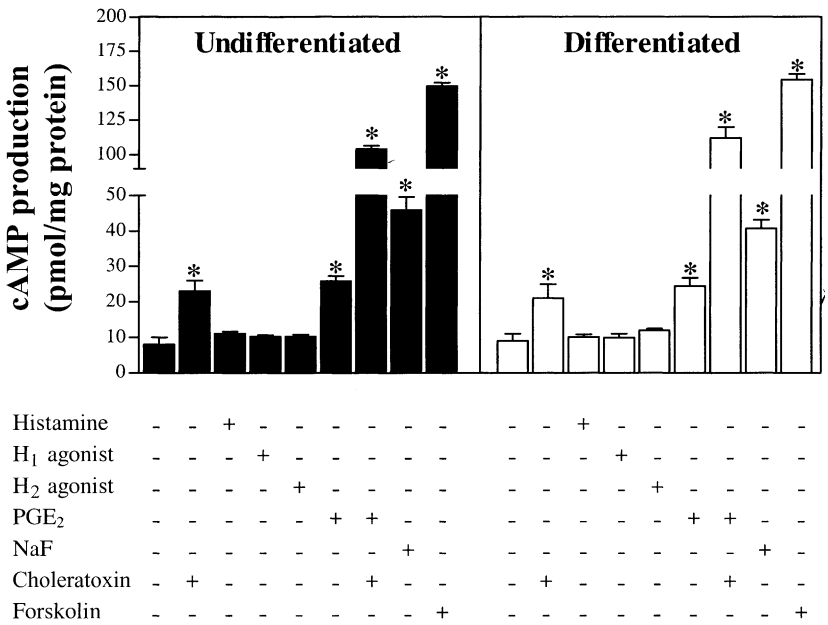

Fig. 4. Effect of diverse agents on the production of cAMP in undifferentiated and differentiated PB keratinocytes. The effects of 10 μM histamine, 10 μM 2-(3-trifluoromethylphenyl)histamine (H_1 agonist), 10 μM BU-E-75 (H_2 agonist), 1 μM PGE_2, 2 μg/mL cholera toxin, 10 mM sodium flouride, and 10 μM forskolin, in the presence of 1 mM IBMX, are shown. Data are presented as normalized cAMP concentration (pmol/mg protein). Each bar represents the mean ± SEM of triplicate determinations from a single experiment performed three independent times. Key: (*) significantly different as compared with basal levels of cAMP ($P < 0.05$, one-way ANOVA test).

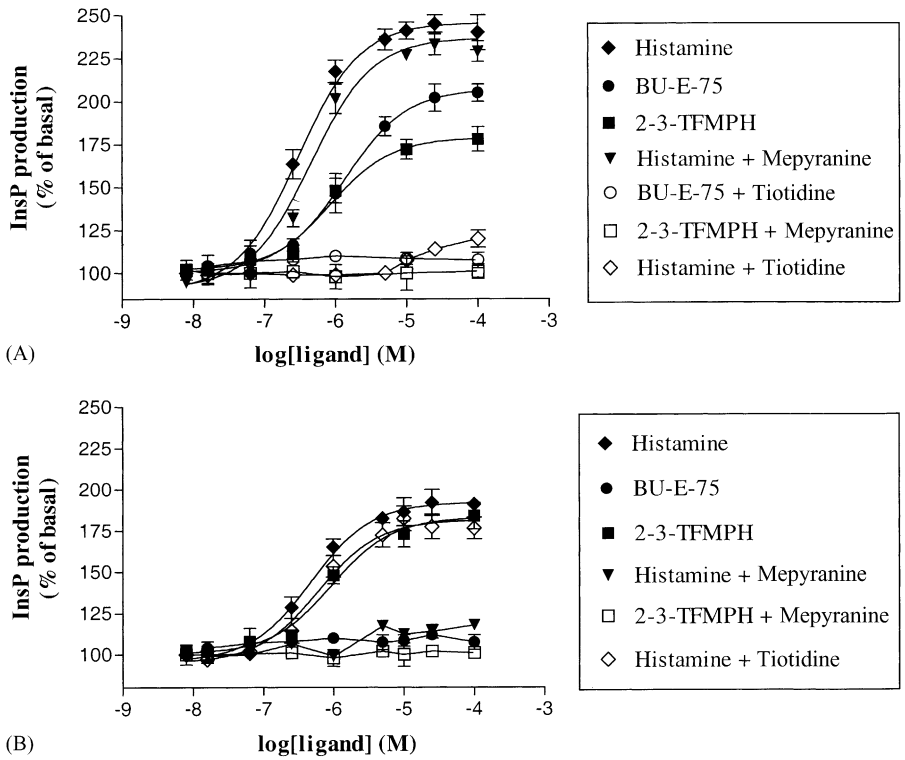

Fig. 5. Effects of histamine and histaminergic ligands on PLC activity in (A) undifferentiated and (B) differentiated PB keratinocytes. Cells were treated with different concentrations of histamine, 2-(3-trifluoromethylphenyl)histamine (2-3-TFMPH, H_1 agonist), and BU-E-75 (H_2 agonist). The H_1 antagonist mepyramine and the H_2 antagonists tiotidine and famotidine (not shown) were used at a 10 μM concentration in (A) undifferentiated PB keratinocytes (100% = 0.290 ± 0.011 [^{3}H]InsP/[^{3}H]Ins, see Table 4) and (B) differentiated PB keratinocytes (100% = 0.315 ± 0.025 [^{3}H]InsP/[^{3}H]Ins). Each point represents the mean ± SEM of triplicate determinations from a single experiment performed four independent times.

Table 4
Effect of cholera toxin or forskolin pretreatment on basal levels of PLC activity

	Basal PLC activity ([^{3}H]InsP/[^{3}H]Ins)	Basal PLC activity after cholera toxin pretreatment ([^{3}H]InsP/[^{3}H]Ins)	Basal PLC activity after forskolin ([^{3}H]InsP/[^{3}H]Ins)
Undifferentiated PB keratinocytes	0.290 ± 0.011	0.300 ± 0.020	0.310 ± 0.030
Differentiated PB keratinocytes	0.315 ± 0.025	0.346 ± 0.023	0.300 ± 0.06

Cells were incubated for 2 hr with either cholera toxin (2 μg/mL) or forskolin (10 μM). Basal levels of PLC activity were measured without any exogenous agonist stimulation and expressed as the ratio obtained when total [^{3}H]inositol phosphate (InsP) radioactivity was normalized against total intracellular [^{3}H]inositol (Ins) radioactivity. Neither cholera toxin nor forskolin affected PLC basal levels in any of the culture conditions. Results are (expressed as means ± SEM of triplicate determinations from a single experiment performed three independent times and analyzed by one-way ANOVA. No significant differences were detected between groups.

(154 ± 11% above basal levels) and blocked by the H_1 antagonist mepyramine (Fig. 5B). Pretreatment of the cells with 2 μg/mL cholera toxin or 10 μM forskolin did not affect PLC basal activity in any of the culture conditions (Table 4).

4. Discussion

We have investigated how the functional coupling of the histamine H_2 receptor to the PLC signaling pathway could be influenced by the levels of expression of the receptor. Previously, Mitra *et al*. [34] had shown the presence of the H_2 receptor on keratinocytes by binding experiments. In the present work, we demonstrated by binding experiments and RT–PCR that the expression of the H_2 receptor is down-regulated during differentiation of the PB murine keratinocyte cell line, without affecting the affinity (K_d) of the H_2 receptor. Results obtained by northern blotting further support this conclusion, even when the signal obtained for H_2 receptor mRNA was rather weak. Interestingly, many studies have reported difficulties in detecting the H_2 receptor transcript by northern blotting in tissues other than brain and stomach [26,27]. The K_d values for [^{3}H]tiotidine in PB cells were similar before and after differentiation. These values were in agreement with those previously reported for [^{3}H]tiotidine binding to H_2 receptors in mouse skin and cell lines derived from other peripheral tissues [17,32,35]. In contrast, values for K_d and maximal binding capacity (B_{max}) for the H_1 antagonist

mepyramine were unchanged during the differentiation of PB cells, suggesting a regulatory mechanism specific for the H_2 subtype.

In PB keratinocytes, Ca^{2+}-induced differentiation into a granular cell-like phenotype was also associated with a significant reduction in HDC mRNA levels, intracellular histamine content, and histamine release into the extracellular medium. This reduction could be ascribed to transcriptional regulation of HDC, since it has been reported that the amount of HDC mRNA correlates well with the enzyme activity [25]. The observed reduction in intracellular histamine content and release to the extracellular medium further points to this conclusion. These results suggest that in keratinocytes, like in other cellular systems, histamine and the H_2 receptor could be involved in an autocrine/paracrine loop regulating histamine metabolism and functions. Moreover, some observations in human mononuclear cells, showing that secretion of histamine-releasing factors is able to stimulate autocrine histamine release, suggest that this might be a more general mechanism [36].

The regulation of expression of the H_2 receptor at the protein and mRNA level has been demonstrated in other cell lines during the cellular differentiation process [19]. To further elucidate if the regulation of the expression of the H_2 receptor could affect histamine metabolism and functions in endogenous expression systems, we decided to study the functional coupling of histamine receptors during Ca^{2+}-induced differentiation of PB keratinocytes. The early effects of histamine on keratinocytes include adenylyl cyclase activation, PLC activation, and intracellular Ca^{2+} increase and arachidonic acid release [5,6,17]. These effects are thought to be mediated by H_1 and H_2 but not H_3 histamine receptor subtypes [37]. Typically, H_1 receptors have been described as coupled to PLC activation and H_2 to cAMP production in many tissues and cell lines, but more recently some authors have reported the functional diversity of histamine receptors [12,13]. Histamine-mediated activation of the H_2 receptor subtype, in particular, can result in the activation of both adenylyl cyclase and PLC second messenger systems [15]. The structural components of the human H_2 receptor required to activate adenylyl cyclase and PLC systems have been elucidated recently, but the physiological significance of H_2 receptor coupling to PLC activation has not been totally clarified [38]. However, several reports have suggested that the H_2 receptor-mediated activation of PLC and intracellular calcium mobilization could influence cell proliferation [39,40]. Here, we demonstrate that in undifferentiated PB keratinocytes InsP production induced by H_2 receptor agonists was concentration-dependent and blocked by the correspondent selective antagonists, indicating a specific receptor-mediated activation of the intracellular signaling pathway. In differentiated PB keratinocytes, where the levels of receptor expression are significantly lower, the H_2 agonists were not able to stimulate InsP production in any of the concentrations tested. In the same experimental conditions, H_1 receptor-mediated PLC activation was detectable, concentration-dependent, and blocked by H_1 receptor-specific antagonists, suggesting that the functional regulation is specific for the histamine H_2 receptor subtype.

Histamine itself induced a concentration-dependent increase in InsP production in PB keratinocytes. This induction was insensitive to the H_1 antagonist mepyramine but was blocked completely by the H_2 antagonist tiotidine, implying that activation of the PLC signaling pathway may occur mainly through H_2 receptors in undifferentiated mouse keratinocytes. Recently, others [17] have reported similar observations using the H_2 antagonist cimetidine on human cultured keratinocytes, suggesting that our results are not attributable to some unexpected effect but to a general consequence of H_2 receptor blockage. On differentiated PB keratinocytes, the effects of histamine on PLC activation were significantly weaker and insensitive to H_2 antagonists but blocked by the addition of the H_1 antagonist mepyramine. These observations suggest that the final action of histamine on the PLC/intracellular Ca^{2+} signaling cascade may be a coordinated event involving both H_1 and H_2 receptor subtypes and could be affected by the levels of expression of the H_2 subtype and the cellular differentiation stage in endogenous expression systems. Strengthening this last conclusion, progressive loss of histamine H_2 receptor functional activity has been shown to parallel spontaneous differentiation of human peripheral monocytes to macrophages [19].

In accordance with the results reported herein, some accumulating evidence indicates that the activation of the H_2 receptor may not increase cAMP accumulation in keratinocytes [41,42]. Neither histamine nor H_1 or H_2 agonists could induce cAMP accumulation in PB keratinocytes, although sodium fluoride, forskolin, and PGE_2 demonstrated the functionality of the pathway at different levels at both Ca^{2+} concentrations in the culture medium. Pretreatment of PB cells with cholera toxin did not affect basal PLC activity levels, but potentiated the cAMP accumulation induced by PGE_2. These observations seem to exclude the possibility that the stimulation of PLC by the H_2 receptor could depend on the activation of the adenylyl cyclase-mediated signaling cascade in PB keratinocytes, but further experiments are required to verify this hypothesis [43].

The ability of keratinocytes to synthesize, store, and release histamine has been reported previously to be related to some physiological functions of histamine in the skin [44]. These functions are thought to be mediated mainly by the activation of the H_1 receptor subtype [42]. The expression of the H_2 receptor on keratinocytes has also been reported, but its physiological importance is less clear [34]. Recent observations have suggested that the activation of this receptor may have a role beyond the usually described response to inflammation [39]. We found that

histamine metabolism is reduced during the differentiation of murine keratinocytes. This reduction is followed by a substantial down-regulation in the expression of the H_2 receptor that influences the functional coupling to the PLC signaling pathway. Our results suggest that histamine may have paracrine or autocrine effects on the regulation of keratinocyte growth. In accordance with this suggestion, histamine could stimulate the production of interleukin-6 and other cytokines by keratinocytes, thereby enhancing cell proliferation [42]. Furthermore, strong evidence suggests that histamine participates in epithelial cell differentiation in an autocrine manner [45]. In view of these observations, we suggest that histamine metabolism is down-regulated in an autocrine loop during keratinocyte differentiation. Activation of the H_2 receptor in undifferentiated keratinocytes leads to signaling through the PLC/PKC second messenger system and could therefore stimulate the expression of the HDC gene, that has PKC-responsive elements [46]. Consequently, the presence of HDC and the accumulation of histamine in the undifferentiated phenotype point to a broader function for histamine and the H_2 receptor on the regulation of keratinocyte growth.

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