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Biphasic response to human galanin of extracellular acidification in human Bowes melanoma cells

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Abstract

The metabolic response of galanin GAL1 receptor subtype, endogenously expressed in human Bowes melanoma (HBM) cells, was investigated. Cytosensor microphysiometry was used to determine the extracellular acidification rate. A biphasic response, consisting of a rapid increase in the extracellular acidification rate followed by a decrease below the basal level, was observed after perfusion with human galanin. The magnitude and the rate of onset of both phases were dependent on the galanin concentration. The increase in the extracellular acidification rate (maximum of 25% of basal level; $-\log(EC_{50}) = 7.23 \pm 0.14$) was transient, whereas the following decrease (maximum of 40% of basal level; $-\log(EC_{50}) = 7.77 \pm 0.23$) was sustained. The EC_{50} values for the increase and decrease were in a similar range. After consecutive galanin administration, the magnitude of the response was the same as for the unexposed cells, indicating the absence of galanin receptor desensitization or internalization in HBM cells. Responses were blocked by pretreatment with pertussis toxin and phorbol-12-myristate-13-acetate (PMA), indicating a G-protein/protein kinase C signalling pathway. Our microphysiometry results show a biphasic response of the extracellular acidification rate mediated by the galanin receptor expressed in HBM cells which has not been described previously for any other endogenously expressed neuropeptide receptor.

Keywords: Galanin GAL1 receptor; Cytosensor microphysiometry; G-protein; Desensitization; HBM cell

1. Introduction

Galanin is a 29–30 amino acid peptide initially isolated from porcine intestine (Tatemoto et al., 1983). Subsequently, it has been shown to exhibit more widespread distribution and is present in the central and peripheral nervous system of vertebrate and invertebrate species (Kordower et al., 1992). In normal physiology, galanin has neuromodulatory, reproductive and endocrine functions (Iismaa and Shine, 1999).

Galanin stimulates different receptor subtypes which all belong to the G-protein-coupled, seven-transmembrane domain receptor family. The human galanin GAL1 receptor subtype was first cloned from cDNA derived from human Bowes melanoma (HBM) cell line (Habert-Ortoli et al., 1994; Heuillet et al., 1994). In recent years, molecular cloning has revealed the existence of at least two further human galanin receptor subtypes, galanin GAL2 and galanin GAL3 receptors (Fathi et al., 1998; Kolakowski et al., 1998). Galanin receptors activate multiple second messenger pathways including inhibition of cAMP (Amiranoff et al., 1991; Heuillet et al., 1994), activation of ion channels (De Weille et al., 1988; Dunne et al., 1989; Smith et al., 1998), stimulation of inositol phosphate turnover (Fathi et al., 1997; Sethi and Rozengurt, 1991), alteration of Ca^{2+} mobilization (Palazzi et al., 1991), and activation of phospholipase A_2 and mitogen-activated protein kinase (MAPK) (Mulvaney and Parsons, 1995; Seufferlein and Rozengurt, 1996; Wang et al., 1998b).

It has been demonstrated that G-protein-coupled receptors, such as the human neuropeptide Y Y1 receptor, the human secretin receptor, the human cholecystokinin B receptor, human tachykinin NK_3 receptor and the corticotrophin-releasing factor receptor, have the ability to alter the rate at which cells acidify their environment with products of their energy metabolism (Denyer et al., 1994;

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Jordan et al., 1998; Ng et al., 1999; Smart et al., 1999; Van Liefde et al., 1998). The cytosensor microphysiometer technique is a silicon-based biosensor system, which measures the extracellular acidification rate in real time in population of living cells (tissue culture or slice preparations). This in vitro system is particularly useful for the determination of signal transduction pathways. In addition, microphysiometry can be used for higher throughput screening of receptor agonists and antagonists than ligand binding and functional assays. Microphysiometry has already been successfully used for the analysis of the pharmacological profile of the rat galanin GAL2 receptor subtype stably expressed in the human embryonal kidney (HEK-293) cells (Ahmad et al., 1998). However, such a pharmacological profile of human galanin GAL1 receptor subtype has not yet been determined.

In the present study, we used microphysiometer technology to demonstrate changes in the extracellular acidification rate mediated through the galanin GAL1 receptor endogenously expressed in HBM cells.

2. Materials and methods

2.1. Materials

All cell culture reagents were obtained from Biowhittaker Europe (Verviers, Belgium). Human galanin, porcine galanin-(1–16), galanin-(1–13)-substance *P*-(5–11) amide (galantide), galanin-(1–13)-neuropeptide Y-(25–36) amide (galanin-neuropeptide Y) and galanin-(1–13)-spantide I (spantide) were from Bachem (Bubendorf, Switzerland). Stock solutions (100 μM) of the analogues were prepared in advance with double-distilled water and stored in aliquots at −70 °C to avoid repeated freezing and thawing. Pertussis toxin, phorbol-12-myristate-13-acetate (PMA), *N*-amidino-3.5-diamino-6-chloropyrazine boxamide (amiloride), forskolin and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma (St. Louis, USA).

2.2. Cell culture

HBM cells (ATCC Number: CRL-9607) were cultured in minimum essential medium with Earle's balanced salt solution (EMEM) supplemented with 10% fetal calf serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 0.25 μg ml^{-1} fungizone, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 1 × non essential amino acids. HEK-293 cells (ATCC Number: CRL-1573) were cultured in minimum essential medium with Hank's balanced salt solution (HMEM) supplemented with 10% fetal calf serum, 1 × penicillin/streptomycin fungizone mix, 2 mM L-glutamine and 0.75 g/l sodium bicarbonate. The cells were grown in an atmosphere of 5% CO_2 at 37 °C.

2.3. Measurement of extracellular acidification rate

After reaching confluence, the cells were harvested with 0.05% trypsin/0.02% EDTA solution and counted with a hemocytometer. Approximately 3×10^{-5} cells ml^{-1} were seeded into 3.0-μm pore transwells (Corning Costar, Cambridge, MA, USA) held in 12-well plates 1 day before the experiment. The cells were allowed to settle down in the EMEM medium described in Section 2.2 for about 7 h and cultured overnight in serum-free Dulbecco's modified Eagle's medium (DMEM) growth medium.

The extracellular acidification rate of HBM cells was determined by using a cytosensor microphysiometer (Molecular Devices, Gräfelfing, Germany). A 50-μm thick capsule spacer and a capsule insert were assembled in the transwells with the adherent cells, transferred to a sensor chamber and kept in DMEM (pH 7.25) without sodium bicarbonate containing 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 40 mM sodium chloride throughout the entire experiment at 37 °C.

The perfusion medium was pumped through each sensor chamber at 50 μl min^{-1}. A typical pumping cycle of 120 s consisted of a flow period of 90 s, followed by a flow-off period of 30 s. During flow-off periods, protons released from the cells accumulated in the sensor chamber, and the rate of proton release was quantified by fitting the sensor data to a straight line with the least-squares fit to the slope of the pH profile. Cells were allowed to equilibrate for 2–3 h to gain a stable baseline. Agonists or other agents were diluted into perfusion medium and perfused through a second fluid path.

2.4. Galanin receptor expression analysis by reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from HBM cells using TRI-Reagent™ (Molecular Research Center, Cincinnati, OH, USA) according to the instructions of the manufacturer. Total RNA was reverse transcribed into cDNA using SUPERSCRIPT II™ (Life Technologies, Gaithersburg, MD, USA).

The reaction mixes for galanin GAL1 receptor RT-PCR contained 1 × PCR buffer, 0.2 mM of each dNTP, 10 pmol of each primer (5′-CACTTGCATAAAAAGTTGAAG-3′; 5′-TTATCACACATGAGTACAATTGG-3′), 1 U of red hot DNA polymerase (Advanced Biotechnologies, Epsom, UK) and 100 ng cDNA. PCR was performed by an initial denaturation step at 95 °C for 5 min, followed by 38/44 cycles of 95 °C for 20 s, 58 °C for 30 s and 72 °C for 30 s. The RT-PCR reaction mixture for human galanin GAL2 receptor and galanin GAL3 receptor contained 1 × PCR buffer, 0.2 mM of each dNTP, 10 pmol of each primer (galanin GAL2 receptor: 5′-CTCATCTTCCTCGTGGGCACC-3′; 5′-AGGTTGGCCAGCTGCGACTG-3′; galanin GAL3 receptor: 5′-GTTCATCCTCAACCTGGCG-3′; 5′-GTAGCTGAGGTAGGGCGC-3′), 1 U of amplitaq gold DNA polymerase (Roche Molecular

Systems, Branchburg, NJ, USA) and 100 ng of cDNA. PCR was performed with a denaturation step at 95 °C for 8 min, followed by 38/44 cycles at 95 °C for 30 s, 65 °C for 35 s and 72 °C for 50 s for galanin GAL2 receptor, and 38/44 cycles at 95 °C for 20 s, 60 °C for 20 s and 72 °C for 30 s for galanin GAL3 receptor. A final extension step of 8 min at 72 °C was added. All primers were chosen in two different exons to exclude false positive PCR results arising from genomic DNA contamination.

2.5. Data analysis

The rate of acidification is given in microvolts per second. To reduce the scatter of the data induced by the variable rate of acidification measured under control conditions, the data were normalised using Cytosoft software (Molecular Devices, Sunnyvale, CA, USA). Five consecutive data points for the baseline extracellular acidification rate of each cell population were averaged. Normalised data are presented as percentages of basal rate (control rate). Data are presented as means ± S.E.M. unless otherwise stated. An overall analysis of variance (ANOVA) with Tukey's post-test was performed on individual time points to determine statistical significance, and curve fit-

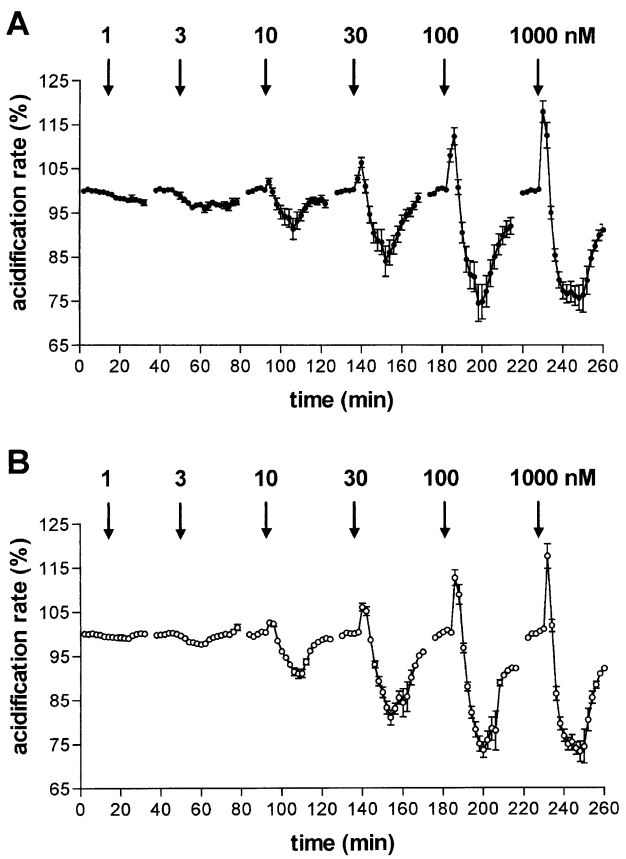

Fig. 1. Changes in extracellular acidification rate of HBM cells stimulated with human galanin. The extracellular acidification rate was measured using a Cytosensor microphysiometer when HBM cells were serially challenged (12-min exposure every 34 min) with increasing concentrations (1–1000 nM) of human galanin (A) and galanin-(1–16) (B). The change in the extracellular acidification rate is expressed as percent change over normalised baseline. Experimental values are means ± S.E.M., $n = 8$ (A) and $n = 7$ (B).

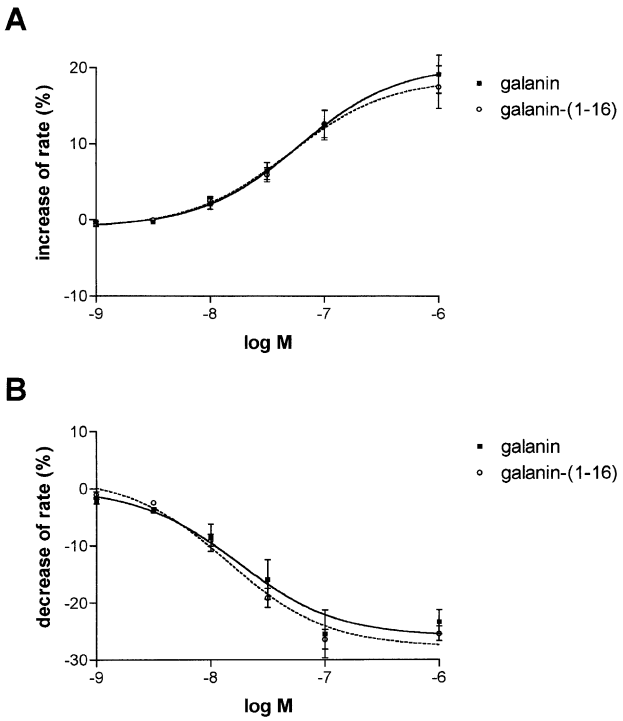

Fig. 2. Concentration–response curves for human galanin and galanin-(1–16). (A) Dose–response plots of peak of the transient increase in the acidification rate. (B) Dose–response plots of maximum, sustained decrease in the acidification rate. Data were normalised to the maximum response and given as means ± S.E.M., $n = 8$ (human galanin) and $n = 7$ (galanin-(1–16)).

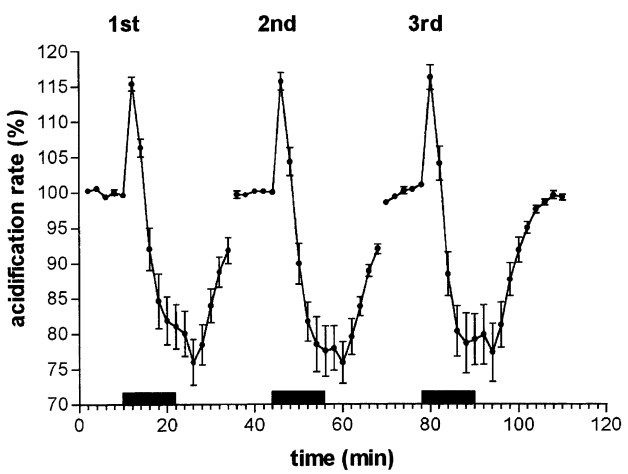

Fig. 3. Repeated ligand stimulation of extracellular acidification in HBM cells. HBM cells were stimulated by three consecutive exposures to 100 nM human galanin for 12 min. Between exposures cells were washed with perfusion medium for 22 min. Restimulation with galanin was performed after the acidification rates returned to basal levels. Values are means ± S.E.M. ($n = 3$) of the extracellular acidification rate from a single representative experiment.

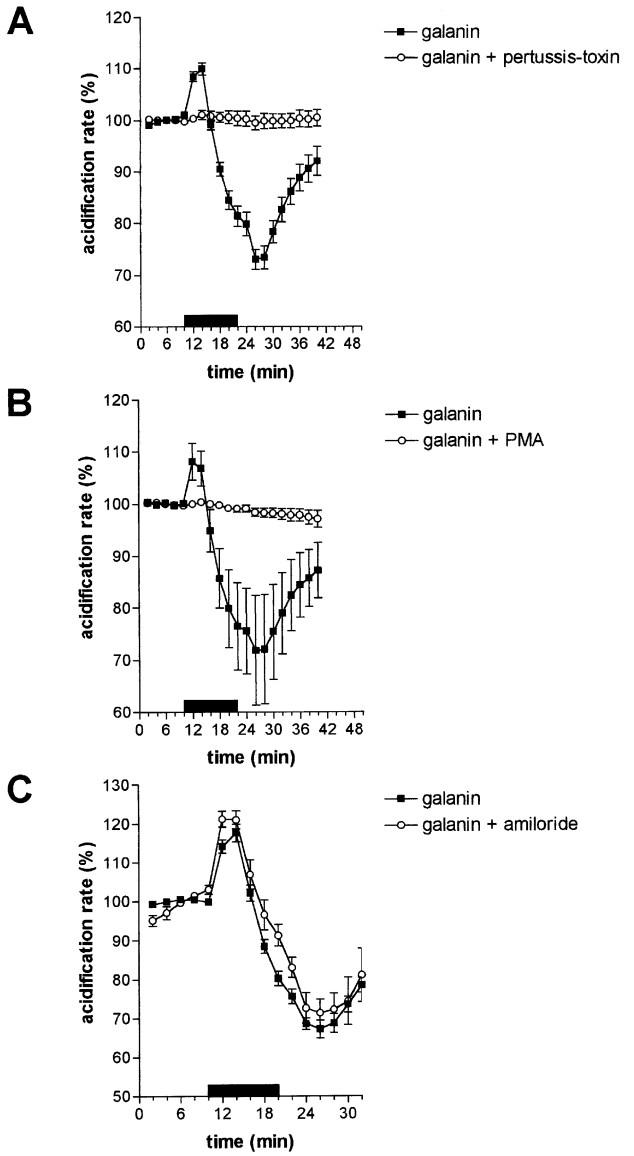

Fig. 4. Effects of (A) pertussis toxin (100 ng ml^{-1}, 6 h), (B) PMA (100 ng ml^{-1}, 14 h) pretreatment and (C) amiloride (100 μM) co-application on galanin-induced changes in the extracellular acidification rate compared to controls. Results are means ± S.E.M. from at least two independent experiments. Black horizontal bars indicate the time of galanin application.

ting was carried out using Prism™ 2.0 software (GraphPad Software, San Diego, CA, USA).

3. Results

The rate of extracellular acidification by HBM cells was determined using the cytosensor microphysiometer as described in Section 2. The extracellular acidification rate of 3×10^5 HBM cells/transwell in serum-free, low-buffered DMEM was 111.3 ± 10.1 μV s^{-1} (mean ± S.E.M., $n = 8$). Perfusion with human galanin caused a biphasic change in the extracellular acidification rate, consisting of a rapid increase followed by a decrease below baseline (Fig. 1A). The onset of the response was rapid (time to peak 2–4 min) and transient, decreasing to baseline within 2–4 min. The response rapidly decreased further to a sustained level below baseline. The negative plateau level persisted in the presence of 100 nM galanin for 30 min (data not shown). Both increase and decrease in the responses were dependent on the galanin concentration, with a threshold response of 1–3 nM and a maximum at about 1000 nM. Variations in the extracellular acidification rate, given as percentages of the basal level, in the presence of different concentrations of human galanin and galanin-(1–16) analogue are shown in Fig. 1. Short applications of galanin for only 2 min already developed the complete pattern of the changes in the extracellular acidification rate.

To determine the potency of galanin to alter the extracellular acidification rate in HBM cells, we used maximum positive and negative responses to make concentration–response curves. The $-\log(EC_{50})$ for the positive transient response (up to 25% of the basal level) in extracellular acidification was 7.23 ± 0.14 ($n = 8$) (Fig. 2A) and for the following negative sustained response in extracellular acidification (down to 40% of the basal level), it was 7.77 ± 0.23 ($n = 8$) (Fig. 2B). The intrinsic activity and functional potency of galanin analogue galanin-(1–16) (increase: $-\log(EC_{50}) = 7.30 \pm 0.15$; decrease: $-\log(EC_{50}) = 7.85 \pm 0.10$; $n = 7$) were similar to those of galanin (Fig. 2). The analogues galantide (100 nM), galanin-neuropeptide Y (100 nM) and spantide (100 nM) acted as agonists with regards to the extracellular acidification rate in HBM cells (data not shown).

To determine whether the effect of galanin on extracellular acidification in HBM cells was due to the presence of galanin receptors and not to an unspecific effect caused by pH changes in the perfusion medium as a result of the addition of galanin, we used the HEK-293 cell line, which does not bind galanin, as a negative control (Fathi et al.,

Table 1
Effects of galanin and/or modulators of intracellular cAMP on extracellular acidification of HBM cells

Drug	Increase (%)	Decrease (%)
10 μM forskolin	none	3.54 ± 0.40
10 μM IBMX	none	1.91 ± 0.75
10 μM forskolin + 10 μM IBMX	none	4.30 ± 1.29
100 nM galanin	12.82 ± 1.72	20.66 ± 2.02
100 nM galanin + 10 μM forskolin	12.17 ± 1.35[a]	21.47 ± 1.96[a]
100 nM galanin + 10 μM IBMX	11.89 ± 1.96[a]	20.53 ± 1.95[a]
100 nM galanin + 10 μM forskolin + 10 μM IBMX	12.25 ± 1.31[a]	21.78 ± 2.51[a]

Values are means ± S.E.M. ($n = 7$).
[a] Statistically not significant from galanin application by ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

1998). HEK-293 cells did not show changes in extracellular acidification after application of 100 nM galanin (data not shown).

In order to detect a potential desensitization of the response, we examined the effect of repeated application of human galanin on extracellular acidification rate. An initial response to a 12-min challenge with 100 nM galanin was followed by a 22-min washing period, during which the extracellular acidification rate returned to basal level. For three consecutive galanin administrations, the magnitude of the response was similar, indicating that desensitization did not occur at least on the time scale of applications used (Fig. 3).

To characterise the second messenger systems which led to the changes in the extracellular acidification rate in HBM cells in response to galanin, the cells were treated with agents which modulate different signal transduction pathways. Responses were completely blocked by pretreatment with 100 ng ml^{-1} pertussis toxin for 6 h (Fig. 4A) or with 100 nM PMA for 14 h (Fig. 4B). Basal acidification rates of pertussis toxin- and PMA-treated cells were within the range of untreated cells. Perfusion of HBM cells with 100 μM of the Na^+/H^+ exchange blocker amiloride altered neither the basal extracellular acidification rate of HBM cells nor the acidification response in the presence of 100 nM galanin (Fig. 4C).

Exposure of HBM cells to 10 μM forskolin produced a decrease in the extracellular acidification rate of 3% below basal level. IBMX (10 μM) had no significant effect on the extracellular acidification rates and a combination of 10 μM forskolin and 10 μM IBMX showed a 4% decrease. The extracellular acidification rate of HBM cells after perfusion of forskolin and IBMX in addition to 100 nM galanin was not significantly different from the rate for galanin alone (Table 1).

RT-PCR amplification of cDNA isolated from HBM cells revealed the presence of galanin GAL1 receptor, already showing a strong signal after 38 cycles, indicating a high level of expression (Fig. 5). For the galanin GAL2 receptor subtype, no signal could be detected after 44 cycles. In 9 out of 13 RT-PCR set-ups with 44 cycles, a weak signal for the galanin GAL3 receptor was obtained, indicating a very low level of expression of this receptor subtype.

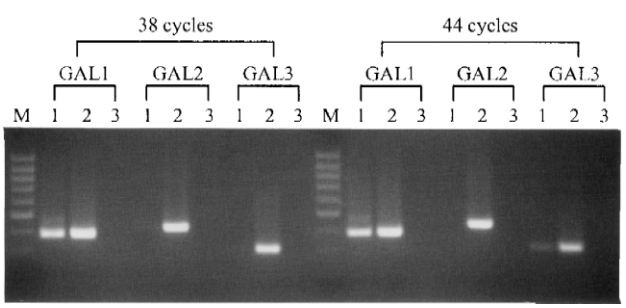

Fig. 5. RT-PCR analysis of galanin receptor subtype mRNA expression. Lane M: Marker; lane 1: cDNA isolated from HBM cells; lane 2: positive control for the galanin receptors GAL1, GAL2 and GAL3, respectively; lane 3: negative control (water).

4. Discussion

Our study confirms the utility of microphysiometry for the non-invasive investigation of the pharmacology of galanin receptors and shows the ability of the technique to detect a biphasic response in the HBM cell line following activation of the constitutively expressed galanin GAL1 receptor.

Since the specific cellular environment is critically important for receptor coupling to specific extracellular acidification responses (Chen and Tashjian, 1999), we chose the HBM cell line which endogenously expresses the galanin GAL1 receptor to detect alterations in extracellular acidification after galanin application. The EC_{50} of the biphasic extracellular acidification response was similar. This could indicate that both increasing and decreasing phases of the response are coupled and are due to a single galanin receptor subtype. This is supported by previous pharmacological analyses of galanin receptor binding in HBM cells which indicated the presence of a single class of high-affinity GAL1 binding sites (Habert-Ortoli et al., 1994; Heuillet et al., 1994). Although occasionally, a very low expression level of galanin GAL3 receptor mRNA could be detected by RT-PCR, we think that much higher level of galanin GAL1 receptor is responsible for the high amounts of galanin binding (Heuillet et al., 1994) and for the observed effects on the extracellular acidification rate in HBM cells. Receptor-mediated biphasic changes in the extracellular acidification rate have only been reported for rat pituitary F4C1 cells transfected with somatostatin subtype 2 receptor (Chen and Tashjian, 1999). However, the effect on the extracellular acidification of somatostatin subtype 2 receptor seems to be cell type-dependent since a biphasic response has not been observed for somatostatin subtype 2 receptor transfected into Chinese hamster ovary (CHO-K1) cells (Taylor et al., 1996). To date, no biphasic changes in extracellular acidification rate mediated by an endogenously expressed neuropeptide receptor have been described.

The high-affinity galanin analogues and chimeras were classified as antagonists in vivo in tissues such as hypothalamus, hippocampus, pituitary, spinal cord and pancreas (Bartfai et al., 1992; Kask et al., 1995), but in vitro they act as agonists for galanin GAL1, galanin GAL2 and galanin GAL3 receptors (Smith et al., 1997; Smith et al., 1998). The discrepancies may be explained by a system-dependent spectrum of partial agonist/antagonist activity, or the chimeric peptides may be degraded in vivo to fragments which have different properties from those of the original peptides (Smith et al., 1998). Alternatively, it may also indicate the existence of additional receptor subtypes not cloned to date (Wynick et al., 1993).

Changes in the extracellular acidification rate are related to energy consumption and ion transport across the plasma membrane. Receptor-mediated increases in extracellular acidification have been linked to Na^+/H^+ exchange (Chio et al., 1994; Pitchford et al., 1995). Proton extrusion by this exchanger could account for the rapid increase in the extracellular acidification rate, whereas depletion of ion stores might account partly for the decrease in the extracellular acidification rate, giving a possible explanation for the observed biphasic effect. However, in contrast to dopamine D4 receptor-mediated signalling (Chio et al., 1994), the changes in the extracellular acidification rate in HBM cells were not found to be sensitive to the Na^+/H^+ exchange blocker amiloride. There has to be an extrusion of bicarbonate ions, hydroxyl ions, or some other alkaline species to explain such a large and sustained reduction in the acidification rate. Previously, it has been shown that galanin receptors activate ATP-sensitive K^+ channels and inwardly rectifying K^+ channels (Branchek et al., 1998). These channels have been shown to be pertussis toxin-sensitive (Smith et al., 1998), which may be in accordance with our observation that both increase and decrease in the extracellular acidification rate in response to galanin were inhibited by pertussis toxin.

Receptor desensitization has been shown for several G-protein-coupled receptors (Barker et al., 1995; Bristow et al., 2000; Dixon et al., 1997; Drake et al., 2000; Ubl et al., 2000; Zamani et al., 1995; Zamani and Bristow, 1993), but there is no information on whether the same occurs for human galanin GAL1 receptor. Interestingly, upon repeated stimulation with galanin, the acidification responses were sustained and of similar magnitude and kinetics, suggesting that the human galanin GAL1 receptor does not exhibit homologous desensitization. Using microphysiometry, a similar lack of desensitization has been observed for the neuropeptide Y Y1 receptor and human secretin receptor (Ng et al., 1999; Van Liefde et al., 1998). Our findings do not support galanin GAL1 receptor internalization as reported by Wang et al. (1998a) for the rat galanin GAL1 receptor expressed in Chinese hamster ovary cells, unless internalization and expression occur at the same rate.

The coupling of galanin GAL1 receptor in HBM cells was shown to be completely inhibited by pertussis toxin. This finding indicates that changes in the extracellular acidification rate are mediated through G_i- or G_o-type G-proteins. The involvement of G_i-proteins in galanin GAL1 receptor-mediated extracellular signalling has also been reported for galanin-stimulated inhibition of forskolin-evoked intracellular cyclic AMP (cAMP) production (Heuillet et al., 1994; Wang et al., 1998b). Furthermore, galanin-induced changes in the extracellular acidification rate were completely inhibited by PMA, suggesting that galanin GAL1 receptor-mediated extracellular acidification rate is dependent on protein kinase C activity. The involvement of protein kinase C in intracellular signalling has been shown for galanin GAL2 receptor subtype-mediated MAPK activation but to our knowledge not for any signal transduction pathway of the galanin GAL1 receptor (Wang et al., 1998b). To analyse whether the changes in extracellular acidification rate were due to activation of the cAMP second messenger pathway, the cells were exposed to membrane-permeable drugs that increase intracellular cAMP levels. Since no significant effect was detected on extracellular acidification rate in response to galanin in the presence of forskolin and/or IBMX, we conclude that activation of cAMP signalling pathway is not involved in galanin-stimulated extracellular acidification in HBM cells.

The EC_{50} values for the acidification response to galanin or galanin-(1–16) were about 100-fold higher than their potencies in radioligand binding studies with HBM cells (Heuillet et al., 1994). A similar feature has been observed for rat galanin GAL2 receptor stably expressed in HEK-293 cells (Ahmad et al., 1998). Other neuropeptide receptors, like the somatostatin subtype 2 receptor, corticotrophin-releasing factor receptor and human tachykinin NK_3 receptor, show extracellular acidification rates consistent with the potencies of the ligand in binding assays (Jordan et al., 1998; Smart et al., 1999; Taylor et al., 1996).

In summary, this is the first report showing that endogenously expressed galanin receptors respond to ligand stimulation with an alteration of the extracellular acidification rate and that this type of response occurs in a biphasic manner. We further provide evidence that endogenously expressed galanin receptor signalling in HBM cells is mediated via a G-protein/protein kinase C transduction cascade. Microphysiometry of galanin receptors, therefore, provides a useful tool for screening novel galanin receptor agonists and antagonists.

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