Neuronal Differentiation of P19 Embryonal Carcinoma Cells Modulates Kinin B2 Receptor Gene Expression and Function*

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Kinins are vasoactive oligopeptides generated upon proteolytic cleavage of low and high molecular weight kininogens by kallikreins. These peptides have a well-established signaling role in inflammation and homeostasis. Nevertheless, emerging evidence suggests that bradykinin and other kinins are stored in the central nervous system and may act as neuromediators in the control of nociceptive response. Here we show that the kinin-B2 receptor (B2BKR) is differentially expressed during in vitro neuronal differentiation of P19 cells. Following induction by retinoic acid, cells form embryonic bodies and then undergo neuronal differentiation, which is complete after 8 and 9 days. Immunochemical staining revealed that B2BKR protein expression was below detection limits in nondifferentiated P19 cells but increased during the course of neuronal differentiation and peaked on days 8 and 9. Measurement of [Ca$^{2+}$]i, in the absence and presence of bradykinin showed that most undifferentiated cells are unresponsive to bradykinin application, but following differentiation, P19 cells express high molecular weight neurofilaments, secrete bradykinin into the culture medium, and respond to bradykinin application with a transient increase in [Ca$^{2+}$]i. However, inhibition of B2BKR activity with HOE-140 during early differentiation led to a decrease in the size of embryonic bodies formed. Pretreatment of differentiating P19 cells with HOE-140 on day 5 resulted in a reduction of the calcium response induced by the cholinergic agonist carbachol and decreased expression levels of M1–M3 muscarinic acetylcholine receptors, indicating crucial functions of the B2BKR during neuronal differentiation.

Bradykinin (BK) and kallidin are biological active peptides generated by the proteolytic cleavage of kininogens by serine proteases of the kallikrein family. High molecular weight kininogens are precursors of BK, whereas low molecular weight kininogens give origin to kallidin. Along with other kinins, BK and kallidin elicit a wide range of physiological responses, being classically involved in the control of cardiovascular homeostasis and inflammation. As a matter of fact, altered function of the kallikrein-kinin system has been implicated in the development of various pathological conditions such as arthritis, pancreatitis, and asthma (for a review see Refs. 1 and 2). Emerging evidence shows that kinins are stored in neuronal cells of the central nervous system and may act as neuromediators in various functions, including the control of nociceptive information (for a review see Ref. 3). Expression of kallikrein in developing rat brains (4) supports the notion that kinin-induced receptor activity might be required during neuronal development. BK has also been shown to enhance the release of neurotransmitters such as noradrenalin and neuropeptide Y by sympathetic neurons, chromaffin cells, and pheochromocytoma cells (5–8). Moreover, BK implication in the control of calcium homeostasis has already been demonstrated in adult sensory neurons (9).

Most of the biological actions of BK and kallidin are mediated by a serpentine receptor coupled to a G-protein, the kinin-B2 receptor (B2BKR), which is constitutively expressed and widely distributed throughout central and peripheral tissues under physiological conditions. However, there is evidence that expression of B2BKRs during development is regulated. For instance, B2BKR expression has been shown to be involved in the development of the urinary and cardiovascular systems (10). Inhibition of B2BKR activity in rat embryos resulted in animals with disturbed kidney development (11). Besides being regulated during the ontogenesis of cardiovascular and urinary systems, a large set of evidence exists showing that modulation of B2 receptor expression and function also appears during neuronal development. Thus, it has been detected in central and peripheral noradrenergic neurons, in the spinal cord, in neuronal differentiating PC12 pheochromocytoma cells, and in neuroblastoma and glia-derived cell lines (12–18). A cross-talk between the B2BKR and other hormone and neurotransmitter receptors involved in neuronal development and function has been reported, such as endothelin, serotonin, and purinergic receptors (19, 20).

P19 is an embryonic carcinoma cell line that has been extensively validated as a model of in vitro neuronal differentiation by retinoic acid (RA) induction (21, 22). In these cells, other kininogen; NFL, low molecular weight neurofilament; mACHr, muscarinic acetylcholine receptor; PBS, phosphate-buffered saline; RA, retinoic acid; HPLC, high performance liquid chromatography.

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neuropeptide receptors such as the head activator receptor, the endothelin B receptor, and the substance P receptor are expressed during neuronal differentiation (23–25). The signal transduction pathways triggered by these receptors include transient increases in cytosolic-free calcium concentration ([Ca\(^{2+}\)]), by the mobilization of intracellular calcium stores and also induction of calcium influx from the extracellular environment. BK, acting via B2BKRs, also induces a transient increase in [Ca\(^{2+}\)].

The modulation of B2BKR expression during the neuronal differentiation of cultured P19 cells was investigated herein at the levels of mRNA transcription, protein expression, and activity. Moreover, we have examined high and low molecular weight kininogen gene expression as precursors of the B2BKR agonists BK and kallidin as well as secretion of BK by differentiating P19 cells into the culture medium. The influence of the B2BKR antagonist HOE-140 has been tested on early and differentiating P19 cells into the culture medium. The influence of BK, acting via B2BKRs, also induces a transient increase in [Ca\(^{2+}\)].

**EXPERIMENTAL PROCEDURES**

**Materials**—If not otherwise indicated, all reagents were purchased from Sigma.

**Cell Culture and Neuronal Differentiation**—P19 embryonic carcinoma cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil), 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate, in a humidified incubator at 5% CO\(_2\) and 37 °C. To induce neuronal differentiation of P19 embryonic carcinoma cells, cell cultures were stimulated to form embryonic bodies (EBs) in bacterial grade Petri dishes by addition of 1 µM "all-trans-RA" at a density of 5 × 10\(^5\) cells/ml in defined medium (26). In order to avoid adhesion of the cell culture to plastic surfaces, dishes were coated with 0.2% agarose prior to cell plating. After 2 days, the EBs were plated in adherent culture flasks in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. After 48 h, the medium was replaced with defined medium to allow neuronal maturation. In order to prevent the increase of the percentage of glial cells in cell cultures older than 8 days, cultures were treated on day 8 with 50 µg/ml cytosine arabinoside to allow survival of only post-mitotic neurons.

**Reverse Transcription and PCR**—Total RNA was isolated using TRIzol (Invitrogen) from undifferentiated P19, EBs, and differentiated P19 neurons in different time periods. Integrity of the isolated RNA was assessed by separation on a 1% ethidium bromide-stained agarose gel. Contaminating DNA was removed using DNase I (Ambion Inc., Austin, TX) at a concentration of 1 unit/µg RNA in the presence of 20 mM Tris-HCl, pH 8.4, containing 2 mM MgCl\(_2\) and 50 mM KCl for 15 min at 25 °C.

Five µg of total RNA were used for cDNA synthesis in a total volume of 20 µl in the presence of 50 mM Tris-HCl, pH 8.3, 3 mM MgCl\(_2\), 10 mM dithiothreitol, 0.5 mM dNTPs, and 50 ng of random primers with 200 units of RevertAid\(^\text{TM}\) H Minus Moloney murine leukemia virus-reverse transcriptase (Fermentas Inc, Hanover, MD) for 50 min at 42 °C. For PCR amplifications, 5 µl of the reverse transcription reaction were used as a template. PCRs were performed in the presence of 2.5 mM MgCl\(_2\), 0.2 mM dNTPs, 20 µM each reverse and forward primers, and 0.5 units of Taq polymerase (Invitrogen). Primer sequences for amplification of B2BKR, β-actin, M1–M3 muscarinic acetylcholine receptor (mAChR), and high and low molecular weight kininogen cDNAs are listed in Table I.

For B2BKR cDNA amplification, the reaction was cycled 35 times (95 °C for 45 s, 56 °C for 45 s, and 72 °C for 105 s) plus a final extension at 62 °C, 30 s at 73 °C, followed by a final step for 7 min at 73 °C.

PCRs for amplification of kininogen cDNAs were one initial cycle for 3 min at 94 °C, 36 cycles of 1 min at 94 °C, 20 s at 55 °C, 45 s at 72 °C, and a final elongation cycle for 5 min at 72 °C (27). PCR products were sequenced and confirmed as high and low molecular weight kininogen cDNAs. The PCR products were electrophoresed on a 2% agarose gel, visualized by ethidium bromide staining under UV illumination, and verified for their expected size (see Table I). The gel was scanned, and the band intensity was measured by densitometry (NIAM software, Covance, North Yorkings, UK). Variations in cDNA concentrations were normalized with β-actin as an internal control, which is a constitutively expressed gene. Relative mRNA transcription was quantified as the x-fold increase of obtained densitometry values over threshold background levels.

**Real Time PCR**—Real time PCR was performed in order to quantify B2BKR mRNA transcription in embryonal P19 cells and P19 cells undergoing neuronal differentiation. Total RNAs (750 ng) were reverse-transcribed to cDNA as detailed above. The reaction product was amplifie by real time PCR on the 7000 Sequence Detection System (ABI Prism, Applied Biosystems, Foster City, CA) using the Taqman PCR core reaction kit (PerkinElmer Life Sciences). The thermal cycling conditions composed an initial denaturation step of 95 °C for 10 min and 50 cycles at 95 °C for 15 s and 60 °C for 1 min. Experiments were performed in triplicates for each data point. B2BKR mRNA abundance

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**Table I**

| Gene | GenBank\(^\text{TM}\) accession no. | Primer | Sequence | bp |
|------|-----------------------------------|--------|----------|----|
| B2BKR | NM009747 | Forward | 5'-ATCTCTACCCCTCTTGTGCT-3' | 627 |
| β-Actin | NM007393 | Reverse | 5'-AGAGGAGGATCCGAGTG-3'| 535 |
| M1 AChR | NM007698 | Forward | 5'-CAGGCGGGCAAGGAG-3' | 441 |
| M2 AChR | AF264049 | Reverse | 5'-TCTGACTGTTGGAAG-3'| 369 |
| M3 AChR | AF264050 | Forward | 5'-GACAAGTTTGGCCAGG-3'| 245 |
| Kinogen 1 | AY462056 | Forward | 5'-GCCAACCTCCACAGATGGAACAC-3'| 250 |
| | AY462057 | Reverse | 5'-CATTGGAGCTACCTAAGG-3'| 400 |
| | AY462058 | | | 752 |
| | AY462059 | | | 1000 |
| Kinogen 2 | AY462056 | Forward | 5'-GCATTAGAAGACTATCC-3'| 235 |
| | AY462057 | Reverse | 5'-CATTGGAGCTACCTAAGG-3'| 400 |
| | AY462058 | | | 854 |
| Kinogen L | AY462056 | Forward | 5'-GCGTTGAGCTACCTAAGG-3'| 214 |
| | AY462057 | Reverse | 5'-GCCAACCTCCACAGATGGAACAC-3'| 250 |
| | AY462059 | | | 250 |
was quantified as a relative value compared with an internal reference, β-actin, whose abundance was believed not to change between the varying experimental conditions (28). Primers used for real time PCR are as follows: murine B2BKR (GenBank™ accession number NM009747), forward primer 5′-CCCTCTCCTGGTTCTCTT-3′ and reverse primer 5′-GAAAGACCGTGAGCACAAGA-3′; 6-FAM probe, 5′-CCGCACTGAGGAA-3′; murine β-actin (GenBank™ accession number NM007393) forward primer 5′-CTGGCCTCAGTCGCCATT-3′ and reverse primer CGGACTCTGATGCTGCTT-3′; 6-FAM probe, 5′-CTGATCCACATGCTT-3′.

Quantitative values for murine B2BKR and β-actin mRNA transcription were obtained from the threshold cycle number where the increase in the signal associated with an exponential growth of PCR products begins to be detected. Melting curves were generated at the end of each run to ensure product uniformity. The data were analyzed according to the manufacturer’s instructions. B2BKR mRNA of undifferentiated and differentiated P19 cells were quantified as follows. The relative gene expression level was normalized on the basis of β-actin expression as an endogenous RNA control. ΔCt values of the samples were determined by subtracting the average Ct value of B2BKR mRNA from the average Ct value of the internal control β-actin.

**Immunohistochemical Staining and Confocal Laser-scan Microscopy—**For immunofluorescence detection undifferentiated P19 cells or EBs (100,000/1 ml PBS) of induced differentiation (31). Concentrations were calculated for cell populations containing at least each 10 cells. was diluted 1:150 in PBS (2%) rabbit serum. Then, the cells were incubated either with anti-NF-200 (Abcam, Cambridge, MA) or with anti-rabbit IgG-Cy3 (Abcam) for detection of the B2BKR and NF-200 are expressed by the same cells. Double-coupled to a Optiphot-2 epifluorescence microscope (Nikon, Melville, NY).

Confocal laser-scan microscopy was used to determine whether the B2BKR and NF-200 are expressed by the same cells. Double-immunofluorescence staining against the B2BKR and NF-200 was done as detailed above. Cells were observed under an inverted laser-scanning microscope (Zeiss LSM 510, Zeiss, Jena, Germany) using a Zeiss Plan-Apo 63×1.4 NA oil immersion objective lens. The Alexa-Fluor 488 (Molecular Probes) was excited by using a 488-nm argon laser and the Alexa-Fluor 594 (Molecular Probes) was excited by using a 594-nm argon laser, and the Alexa-Fluor 647 (Molecular Probes) was excited by using a 647-nm helium-neon laser, separated from the argon laser beam by a FT 510 beam splitter. B2BKR immunofluorescence emission above 655 nm was collected using a long pass filter (RG 665). Control experiments were conducted to exclude any unspecific fluorescence emission. Instead of double-excitation at 488 nm and 647 nm, the absence of fluorescence emission above 655 nm following excitation at 488 nm was verified. No fluorescence emission above 655 nm was detected when only NF-200 immunofluorescence had been excited (data not shown).

**Western Blot Analysis—**Partially purified membranes of P19 embryonic cells and EBs following induction to neuronal differentiation were prepared by cell sonication and differential centrifugation. One hundred million cells were collected from culture dishes, washed three times in PBS containing 100 μM phenylmethyl sulfonyl fluoride and 0.5 μg/ml leupeptin, and sonicated on ice until at least 95% of the cells were lysed. The cell extract was centrifuged at 600 × g for 10 min and 4 °C to remove cell debris. The supernatant was centrifuged at 100,000 × g for 1 h. The obtained plasma membranes were washed twice with PBS containing protease inhibitors and then resuspended in SDS-loading buffer at a concentration of 1 mg/ml protein. For Western blotting, membrane proteins (40 μg) and molecular weight markers were denatured by boiling for 5 min and then separated by 12% SDS-PAGE according to the Laemmli method (29). The proteins separated on the gel were electrophoretically transferred at 400 mA for 80 min onto a nitrocellulose membrane (Transblot, 0.45 μm, Bio-Rad). The nitrocellulose membrane was stained with 0.1% Ponceau solution in order to verify the transfer of the proteins and to localize the migration of the molecular weight markers. The nitrocellulose membrane was destained and blocked for 2 h with Tris-buffered saline with 0.03% Tween (TBS-T) containing 3% nonfat milk prior to the addition of a 1:200 dilution of a goat polyclonal anti-B2BKR antibody (Santa Cruz Biotechnology). Following 14 h of incubation with the primary antibody at 4 °C, the membrane was washed three times for 15 min with TBS-T. The membrane was blocked again for 30 min with TBS-T containing 3% nonfat milk and then incubated for 2 h at room temperature with a 1:1,000 dilution of a secondary anti-rabbit IgG antibody. The labeled membranes were detected by using the Super Signal West Pico Chemiluminescent Substrate (Pierce) according to the instructions provided by the manufacturer.

The anti-NF-200 (Abcam, Cambridge, MA) or with anti-rabbit IgG-Cy3 (Abcam) for detection of

**Calcium Measurements in Single Cells by Confocal Microscopy—**Measurements of [Ca2+]i, in undifferentiated and neuronal differentiated P19 (embryonic carcinoma) cells on days 7 and 8 following differentiation were made using Lanthascan Laser scanning Microscope (Zeiss, Jena, Germany). Two hundred fifty thousand cells were seeded and allowed to attach on cover slides in p60-mm culture dishes (Nalg Nunc International, Rochester, NY) 48 h before the measurements. Cells were loaded with 4 μM fluo-3AM (Sigma) in 0.5% MeSO and 0.1% of the nonionic surfactant pluronic acid F-127 for 30 min at 37 °C, washed three times with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, and transferred to defined medium. Fluo-3AM fluorescence was excited at 488 nm, and fluorescence was measured at 515 nm using a bandpass filter. [Ca2+]i, of undifferentiated cells was measured for 10 s. Then 1 μM BK or 1.5 mM carbamoylcholine (CCh) was added, and changes in [Ca2+]i were monitored for a further 2 min. Images were collected every 1.5 s. At the end of each experiment, 5 μM indocarbocyanine (4-Br-A23187) followed by 10 mM EGTA were used to determine maximal ([Ca2+]i) and minimal ([Ca2+]i) fluorescence values, respectively. The experimental intracellular calcium concentrations from relative fluorescence values (F) obtained upon BK addition were calculated using the following formulas: [Ca2+]i = F(Fmin − F)/Fmax − F, assuming a 450 nm Kd for fluo-3AM (31). The final concentration of each component was calculated as follows. The concentration of each component was calculated as follows.
Neuronal differentiation of P19 embryonal carcinoma cells. In vitro differentiation of P19 embryonal carcinoma cells resembles processes during early development in vivo. Pluripotent P19 cells (A) are treated with RA and plated into nonadherent culture dishes to form EBs (B). Forty-eight h after induction to neuronal differentiation, EBs are collected and replated in adherent cell culture dishes. On day 5 (C), neuronal precursors become post-mitotic and undergo end-terminal differentiation that is completed on day 8 (D). Beginning on day 5, the developing neurons express NF-200, reaching maximal expression on day 8. E and F, immunostaining of neuron-specific proteins: differentiating P19 cells on day 8, expressing NF-200 and β-III-tubulin, respectively. Cell nuclei were counterstained with DAPI. Images of immunostaining and DAPI staining were overlaid.

Treatment of P19 Cells in Later Differentiation (Days 5–8) with HOE-140—For determining the effect of B2BKR inhibition on expression of mAChRs, 1 μM HOE-140 was added to the cells on day 5 following induction to differentiation and applied again at intervals of 24 h. Control reactions were set up in the absence of HOE-140. On day 8, total RNA was isolated from the cell cultures, and M1, M2, and M3 mAChr mRNA transcription was quantified by semi-quantitative RT-PCR as detailed above.

For evaluation of HOE-140 (1 μM) pretreatment on day 5 on cholinergic signal transmission at days 7 and 8, HOE-140 was removed from the cell culture 6 h prior to calcium measurements, by medium change and washing the cell layers twice. Control cultures in absence of HOE-140 were treated the same way. Lys-des-Arg9-Leu8-BK, a kinin-B1 receptor antagonist, which does not bind to the B2BKR (33), was used as control at 1 μM concentration to confirm the specificity of HOE-140 action.

Determination of the Participation of Muscarinic Acetylcholine Receptor in Carbamoylcholine-induced Changes in [Ca2+]i—For studying muscarinic receptor-mediated intracellular Ca2+ mobilization upon carbamoylcholine stimulation, P19 cells on day 8 of neuronal differentiation were pretreated for 30 min with 180 ng/ml thapsigargin prior to calcium measurements in order to deplete the intracellular calcium stores and inhibit muscarinic receptor-induced calcium mobilization.

RESULTS

Expression of B2BKRs and Neuron-specific Proteins during RA-induced Differentiation of P19 Cells—Murine P19 embryonal carcinoma cells were induced to the neuroectodermal differentiation by culturing free floating EBs in defined medium containing 1 μM RA (26) (Fig. 1, A and B). After 2 days, EBs were plated into tissue culture flasks, whereupon extensive morphological differentiation occurred after RA treatment (Fig. 1C). After 7 days, cells with neuron-like morphology appeared (Fig. 1D), reaching maximum expression levels of neuron-specific proteins after 7–9 days as detected by staining with antibodies against NF-200 (Fig. 1E) and β-III-tubulin (Fig. 1F). In order to avoid contamination of elder P19 neuron cell cultures with glial cells that start to appear on day 8 after induction to neuronal stimulation (23), cells were treated on day 8 with the DNA synthesis inhibitor cytosine arabinoside (50 μg/ml) that eliminates glial cells in proliferation but not differentiated post-mitotic neurons.

P19 undifferentiated embryonal carcinoma cells and EBs express the B2BKR, as verified by Western blot analysis (Fig. 2A). Expression of the receptor is increased after cell aggregation following stimulation with RA and plating for morphological differentiation (Fig. 2B). The peak of maximal expression of B2BKR correlated with the expression of neuron-specific proteins as determined by immunofluorescence staining against NF-200. Double-immunostaining against the neuron-specific
Expression of High and Low Molecular Weight Kininogens and Secretion of Bradykinin by P19 Cells—High and low molecular weight kininogens were generated by alternative splicing from a single kininogen gene (27). The presence of mRNA transcription of high and low molecular weight kininogens during neuronal differentiation of P19, which give origin to bradykinin and kallidin, respectively, was detected by RT-PCR. High molecular weight kininogen 1 transcription could be detected throughout the course of neuronal differentiation (Fig. 4A). Three sizes of kininogen 1 mRNA, corresponding to sizes of 1000, 752, and 400 nucleotides, derived from alternative splicing were detected. Kallidin 2 mRNA, also derived from alternative splicing, was present in low quantities, beginning from day 2 of differentiation. Low molecular weight kininogen mRNA transcription, giving origin to the B2BKR agonist kallidin, was present in low quantities beginning from day 2.

Secretion of BK into the P19 cell cultures in neuronal differentiation was quantified by HPLC analysis. Undifferentiated P19 cells did not liberate any BK into the culture medium, whereas at the beginning from the stage of EB formation and during later neuronal differentiation the amount of BK detected in the culture medium increased (Fig. 4B). Maximal values of 0.7 ± 0.1 nmol BK/10^6 cells were detected on day 7 when cells undergo final neuronal maturation.

The Expression of Functional B2BKRs—The functionality of B2BKRs expressed in P19 cells was determined by measuring changes in [Ca^{2+}], following stimulation of the cells with bradykinin (1 μM) by confocal microscopy using the calcium-sensitive fluorophore fluo-3AM (Fig. 5). The undifferentiated embryonal P19 cells did not respond to BK application with a change in [Ca^{2+}] (Fig. 5, A and D). Following induction to neuronal differentiation and aggregation, the cells responded to BK application with an elevation of [Ca^{2+}]. Maximal responses were found in cells after 7 and 8 days of neuronal differentiation (Fig. 5, A and D). Fig. 5D shows a representative Ca^{2+} response of single undifferentiated and differentiated cells. We found that responsive cells showed an average increase in [Ca^{2+}] of 900 ± 200 nm (Fig. 5E).

Inhibition of Carbamoylcholine-induced Calcium Mobilization in Differentiating Neurons That Had Been Pretreated with HOE-140—P19 neuronal precursors stimulated to in vitro differentiation became post-mitotic after 4 or 5 days following RA addition (34) and underwent neuronal maturation and end-terminal differentiation, such as the formation of synapses and the expression of functional neurotransmitter and neuropeptide receptors. mAChR expression has been demonstrated in differentiated P19 neurons (35) and has been implicated in the regulation of final neuronal differentiation such as neurite outgrowth (36). Fig. 5B shows that the calcium response to CCh, which is already present in embryonal P19 cells, is largely increased following 7 and 8 days of neuronal differentiation (Fig. 5, B and D, lower panels).

In order to evaluate the effect of the B2BKR antagonist HOE-140 on final neuronal maturation, at the example of muscarinic receptor activity, P19 cells in neuronal differentiation were pretreated on day 5 with HOE-140 (1 μM). Control experiments were carried out in which P19 cells were differentiated in the presence of the kinin-B1 receptor antagonist Lys-des-Arg<sup>9</sup>-Leu<sup>8</sup>-BK (33). Following removal of the B2BKR antagonist HOE-140 or the control peptide Lys-des-Arg<sup>9</sup>-Leu<sup>8</sup>-BK 6 h prior to the calcium measurements, P19 cells were tested for B2BKR and muscarinic receptor activity on days 7 and 8 of differentiation (Fig. 5). P19 cells differentiated in the presence of HOE-140 responded to BK application (1 μM) with a smaller increase in [Ca^{2+}], than did the untreated control cells, suggesting a down-regulation of B2BKRs because of the prolonged
presence of the antagonist (Fig. 5A). When in control experiments, differentiated P19 cells were treated with HOE-140 and then washed for 75 min with cell culture medium, the BK-induced calcium response was fully restored (Fig. 5C).

The calcium response to the cholinergic agonist CCh was decreased by 90 and 50% on days 7 and 8, respectively, when cells had been differentiated in the presence of HOE-140 (1 μM) (Fig. 5, B and D). The CCh-induced rise in [Ca$^{2+}$], was confirmed to result from muscarinic, and not nicotinic, receptor activation, as the response was inhibited following pretreatment of the cells with thapsigargin (Fig. 5C).

Fig. 5E gives a quantification of Ca$^{2+}$ responses upon stimulation of HOE-140-pretreated and control P19 cells on day 7 by BK or CCh. The calcium responses upon stimulation of Lys-des-Arg$^9$-Leu$^8$-BK-pretreated P19 cells with CCh compared with responses of untreated control cells were largely unchanged (Fig. 5E), demonstrating that HOE-140-induced inhibition of muscarinic receptor function is because of the B2BKR blockade and not to possible toxic effects of a high concentration of the antagonist.

Down-regulation of mACHR mRNA Transcription Following Pretreatment of Differentiating P19 Cells with HOE-140—On day 5 following stimulation to neuronal differentiation by RA, B2BKR activity was blocked by addition of HOE-140 (1 μM). HOE-140 pretreatment resulted in a decrease of B2BKR activity, as shown by calcium measurements in the presence of BK. Pretreatment with HOE-140 also resulted in a decrease of M1–M3 muscarinic receptor mRNA transcription on day 8 of differentiation as determined by semi-quantitative RT-PCR (Fig. 6A). The analysis of intensities of the bands of the ethidium bromide-stained RT-PCR products of RNA isolated from P19 cells, which had been pretreated with HOE-140, revealed a decrease of M1 and M2 mACHR and M3 mACHR transcription levels by 96 and 93% compared with M1–M3 mACHR mRNA transcription levels of untreated control cells, respectively (Fig. 6B). The decrease in mACHR activity following HOE-140 pretreatment correlated with a down-regulation of gene expression of M1–M3 receptor subtypes. In the absence of HOE-140, gene expression levels of muscarinic receptors increase until day 8 of differentiation, as shown by the example of the M3 mACHR (Fig. 6C).

Inhibition of Formation of EBs in the Presence of the B2BKR Antagonist HOE-140—In order to evaluate the implication of the B2BKR during early neuronal determination and differentiation, embryonic P19 undifferentiated cells were plated in Petri dishes in the absence and in the presence of 10 nM to 1 μM HOE-140 and stimulated with 1 μM RA for EB formation. The sizes of EBs were compared after 2 days of treatment with the control experiment, where only RA was used for stimulation of neuronal differentiation (Fig. 7). Fig. 7A shows the results of a representative experiment. The percentage of EBs with sizes less than 1 mm increased in the presence of HOE-140, whereas large EBs mostly disappeared. Fig. 7B demonstrates that in presence of HOE-140 the overall diameters of EBs are reduced by an average of 20% as compared with the uninhibited controls. The differences in sizes of treated and untreated EBs were statistically significant at HOE-140 concentrations of 10 nM to 1 μM (p < 0.05) (Fig. 7C). The treatment of undifferentiated, proliferating cells for 48 h with 1 μM HOE-140 did not result in a reduction of cell number, indicating that HOE-140 did not have an unspecific toxic effect on the cell population (data not shown).

**DISCUSSION**

BK was originally characterized as a peptide with vasodilatory action and as a mediator during inflammatory processes. Besides its function in cardiovascular regulation, BK was also shown to elicit mitogenesis and the synthesis of DNA and is involved in the regulation of mitogenesis in arterial smooth muscle cells and in the proliferation of tumors (38, 39). In some tumors, such as in prostate cancer, BK acts as a growth factor (40), whereas in other tumor cells the inhibition of the B2BKR by the specific antagonist HOE-140 resulted in proliferation (41), leaving the physiological role of bradykinin during proliferation and differentiation processes undefined.

B2BKRs have been shown to be expressed in neuronal determined cell cultures and also in the brain (42, 43). The existence of a mechanism of regulation of B2BKR gene expression...
by trans-acting factors to a silencer motif in the B2BKR promoter has been shown in the neuronal cell line NG108-15 (44). B2BKRs are expressed in the growth cones of differentiating PC12 pheochromocytoma cells and also in brain regions such as dorsal root ganglia. B2KR expression was up-regulated following nerve injury or treating of primary cultures with neurotrophins (45), implicating a crucial role of B2BKRs in the repair and differentiation processes of neurons. A possible function of the B2BKR in neuronal development was found by Schelb et al. (12), who observed the postnatal expression of B2BKRs in sympathetically innervated tissues that modulate neurotransmitter release. The regulation of transmitter release, considered to be crucial in the development of the nervous system, was studied in PC12 cells and chromaffin tissue (5–8). Modulation of the B2BKR is implicated in neurodegeneration occurring during aging and Alzheimer disease (46). The suggestion that dysregulation of B2BKR function and expression could be implicated in the etiology of neurodegeneration and brain diseases is supported by the work of Arganaraz et al. (47) who detected an alteration of B2BKR expression in the hippocampus of a rat model of epilepsy.

The cell line P19 (21) was established previously as a model for in vitro neuronal differentiation. P19 cells constitute those

Fig. 5. Imaging of changes in \([\text{Ca}^{2+}]\), in P19 following stimulation by bradykinin and carbamoylcholine. Four \(\mu\text{M}\) fluo-3AM in 0.5% of Me\(_2\)SO and 0.1% of pluronic acid were used to load the cells 30 min before addition of 1 \(\mu\text{M}\) BK or 1.5 \(\text{mM}\) CCh. Calcium imaging was performed by confocal microscopy. Following imaging of variations in \([\text{Ca}^{2+}]\), of an unstimulated cell population, agonist was added, and stimulation was recorded for 3 min by collecting images in time intervals of 3 s. \(F_0\) = Fluo-3AM fluorescence following addition of the agonist; \(F_\text{a}\) = Fluo-3AM fluorescence of the unstimulated cell population. Arrows mark the time point of ligand addition. A, changes in \([\text{Ca}^{2+}]\), following stimulation by BK. Curve 1, P19 cells on day 8 of differentiation; curve 2, P19 cells on day 8 following pretreatment with HOE-140 (1 \(\mu\text{M}\)) on day 5; curve 3, undifferentiated, embryonal P19 cells. B, Changes in \([\text{Ca}^{2+}]\), following stimulation by CCh. Curve 1, P19 neurons at day 8 of differentiation; curve 2, undifferentiated P19 cells; curve 3, P19 cells on day 8 following pretreatment with HOE-140 (1 \(\mu\text{M}\)) on day 5. Curve 4, P19 neurons at day 8 of differentiation pretreated for 30 min with 180 ng/ml thapsigargin in order to deplete internal calcium stores. C, recovery of BK-induced calcium response following washout of HOE-140. Curve 1, changes in \([\text{Ca}^{2+}]\), of P19 cells at day 8 of neuronal differentiation following addition of BK (1 \(\mu\text{M}\)). Curve 2, recovery of the calcium response induced by BK following addition of HOE-140 and wash-out for 75 min. Arrows indicate time points of BK injection. D, representative images of calcium responses following stimulation by BK and CCh: Left panels, embryonal carcinoma cells; middle panels, P19 neurons on day 7 of differentiation. Right panels, P19 cells on day 7 of differentiation, which had been pretreated with HOE-140 on day 5. −, unstimulated cells; +, maximal response observed following stimulation. In upper and lower panels, BK (1 \(\mu\text{M}\)) or CCh (1.5 \(\text{mM}\)) was used for stimulation of the cells. E, the average maximal increases in \([\text{Ca}^{2+}]\), ± S.D. following BK or CCh addition to a control P19 cell population on day 7 were compared with BK- or CCh-stimulated P19 cells that had been pretreated on day 5 with HOE-140 (1 \(\mu\text{M}\)) or with the control peptide Lys-des-Arg⁹-Leu⁸-BK (leu 8) (1 \(\mu\text{M}\)).
cells of the inner cell mass of the blastocyst during the initial period of development, and the differentiation of P19 cells is believed to closely mimic critical events during early embryogenesis. RA treatment induces P19 cells to develop a neuronal phenotype characterized by the expression of neurotransmitters, the formation of neurites with synaptic specializations, and the expression of the neuronal markers β-III-tubulin and low (NFL), medium (NFM), and high (NFH) molecular weight neurofilament subunits (48–50). The NFL and NFM subunits are expressed during the early stages of neuronal differentiation, whereas NFHs such as neurofilament 200 (NF-200) appear later on days 5 and 6 when neurons are already postmitotic, reaching maximal expression following neuronal maturation on day 8. We have detected B2BKR expression and mRNA transcription during the course of neuronal differentiation. The highest levels of receptor expression and mRNA transcription were detected on days 8 and 9 of differentiation. P19 neurons responded to BK application with a large increase in [Ca^{2+}], whereas undifferentiated cells did not respond to BK stimulation (Fig. 5). The absence of a visible response of undifferentiated P19 cells to BK application may be explained by a low concentration of B2BKR expression on the cell surface. The absence of a response to BK application may also result from the existence of post-translational modifications that regulate B2BKR configuration and activity (51). As B2BKRs could be expressed by glial cells that appear during RA-induced differentiation, it was important to show that B2BKRs are expressed by P19 neurons. Double-staining immunofluorescence studies showed that during neuronal differentiation in vitro, B2BKRs are expressed by neurons and the BK-induced effect is not due to activation of B2BKRs expressed on glial cells, which may be present in the differentiating culture (Fig. 2) (52).

It was found that inhibition of B2BKR action at the early stage of differentiation during the formation of EBs counteracts differentiation as determined by the reduced size of EBs (20% versus control) (Fig. 7). Supposedly, B2BKR action is needed to induce calcium transients during early development, known to be necessary for following differentiation processes.

The effect of B2BKR blockade has also been examined on later differentiation events, such as the expression and activity of the mAChR (Figs. 5 and 6) whose functions are implicated in neurite outgrowth and formation of functional synapses. P19 cells have been proposed to serve as an in vitro neuronal differentiation model for studying mAChR subtypes in a proper neuronal context, as differentiated P19 neurons express high concentrations of cholinergic markers as well as subtypes of the muscarinic receptor family (35, 53). Cholinergic function has been suggested to play a key role in cell-cell communication during migration and differentiation of neural precursor cells. Acetylcholine is released by the growth cone in the process of fiber elongation, and fiber outgrowth in several systems is modulated by acetylcholine (55). Stimulation and inhibition of mAChR activity led to a higher and lower ability of differentiating neuroblastoma cells to induce neurite outgrowth and synapsin I expression, indicating its importance in neuronal maturation (55). Muscarinic and other hormone and neurotransmitter receptor expression is regulated during in vitro neuronal differentiation. For instance, RA induction of neuroblastoma cells to neuronal differentiation results in an increase of muscarinic receptor expression (56). We have shown that the M3 receptor gene expression increases during the course of neuronal differentiation (Fig. 6C). P19 cells on day 8 of differentiation responded with a higher increase in [Ca^{2+}], to CCh stimulation than cells on day 5 did (data not shown). Similar results were obtained from cells on days 5 and 8 of differentiation that were stimulated with N-methyl-D-aspartic acid or endothelin-B (data not shown). In accordance with these data, P19 cell cultures in neuronal differentiation showed increased responsiveness to application of γ-aminobutyric acid when they were maintained in differentiation for more than 5 days (57). Synaptotagmin, indicating the development of functional synapses, was detected on day 9 of differentiation of P19 and was absent on day 6 (53).

It was herein found that pretreatment of P19 cells in neuronal differentiation showed increased responsiveness to application of γ-aminobutyric acid when they were maintained in differentiation for more than 5 days (57). Synaptotagmin, indicating the development of functional synapses, was detected on day 9 of differentiation of P19 and was absent on day 6 (53).
nal differentiation with the B2BKR antagonist HOE-140 at 1 μM concentration resulted in less gene expression of M1–M3 mAChR on day 8 and a partial loss of mAChR function on days 7 and 8 of neuronal differentiation. These observations indicate that P19 cells at the time point of HOE-140 addition on day 5 of differentiation, although already expressing neuronal markers such as high molecular weight neurofilaments, are yet in the stage of neuronal precursors undergoing final maturation, which is inhibited in the presence of HOE-140.

Our data indicate that B2BKR function is needed for termination of neuronal differentiation and the formation of functional synapses. Control experiments were carried out at identical conditions in the presence of the kinin B1 receptor antagonist Lys-des-Arg9-Leu8-BK, which reportedly does not affect B2BKR activity in order to verify HOE-140 specificity in inhibiting neuronal differentiation and to exclude the possibility of unspecific effects caused by micromolar peptide concentrations. Pre-treatment of P19 cultures on day 5 with Lys-des-Arg9-Leu8-BK did not affect differentiation as determined by measuring mAChR activity on day 8 (Fig. 5E). For obtaining complete inhibition of B2BKR function, HOE-140 has been employed at similar concentrations in other in vitro assays, such as reversion of kinin-induced anion secretion in porcine ileum (58).

Previously published work supports the hypothesis of B2BKR participation in neuronal differentiation. In PC12 cells, BK signaling resulted in translocation of protein kinase C, an enzyme implicated in regulation of neurite outgrowth, and therefore BK was suggested to participate in neuronal differentiation of this cell line (59). The BK-induced signal transduction closely resembles inositol 1,4,5-trisphosphate accumulation followed by calcium release from intracellular stores, which was characterized during differentiation of the human neuroblastoma cell line SH-SY5Y (60, 61). Calcium transients have also been observed in the growth cones of developing neurons (62). It seems likely that B2BKR function is needed during neuronal maturation of P19 cells in vitro. This hypothesis is supported by published work (63, 64) showing BK-induced neurotransmitter release in differentiating PC12 and neuroblastoma cells. Buah et al. (64) observed that short and long term treatment of PC12 cells with nerve growth factor increased the potency of BK to raise intracellular calcium concentration [Ca2+]i 10- and 100-fold, respectively, implicating a crucial function of BK in inducing fast calcium waves in differentiating PC12 neurites (65).

We have shown that gene expression of kininogens, the precursors of the B2BKR agonists BK and kallidin, is induced following stimulation of P19 cells by RA and secretion of BK in the culture medium increases during the course of neuronal differentiation (Fig. 4). These results suggest the presence of an autocrine loop involving secretion of BK by P19 cells in order to activate B2BKRs on their cell surface and functional B2BKRs as prerequisites for the progress of neuronal differentiation.

FIG. 7. HOE-140 treatment on EBs (48 h). P19 undifferentiated cells were plated in Petri dishes at a concentration of 10⁵ cells/ml defined medium with 1 μM of RA or 1 μM each of RA and B2BKR antagonist HOE-140 (100 nM to 1 μM). After 48 h, 30 randomly chosen areas of each cell culture were photographed using a digital camera coupled to the microscope, and the size of EBs was measured and compared with EBs of cell cultures in the absence of HOE-140. A, the figure shows the results of a representative experiment. The left panel shows cells without HOE-140 treatment, and the right panel shows cells treated with 1 μM HOE-140. The scale bar indicates a size of 500 μm. B, the sizes of EBs were measured and compared with the control that had not been pretreated with HOE-140. p values of concentrations of 10 nM to 1 μM versus control were smaller than 0.05 using the unpaired Student’s t test. C shows the percentages of relative sizes of untreated EBs compared with the sizes of EBs formed in the presence of HOE-140. The data are plotted as mean values ± S.E.
Both neuronal maturation and survival of P19 neurons are directly correlated with B2BKR activity. When differentiating P19 were treated over 3 days with the B2BKR antagonist HOE-140, cells survived until day 8 (data not shown).

Despite the differential expression of various neuropeptide, neurotransmitter, and neurotrophphin receptors (NTRs), such as the endothelin-B receptor, glutamate receptors, and the p75 NTR in differentiating P19 cells (24, 66, 67), none has been shown directly to be essential during their neuronal differentiation. Fig. 8 shows a scheme that illustrates the results reported herein. BK is generated by cleaving of kininogen precursors and is liberated into the cell culture medium, where it acts on B2BKRs, implicating that P19 cells use an autocrine loop system for triggering neuronal differentiation. As the half-time of BK in plasma is only 15 s (54), this peptide can activate receptors at specific time points without causing long-lasting receptor desensitization. Inhibition of B2BKR action at the initial stage of differentiation negatively affects formation of EBs as prerequisites for later differentiation. Blocking of B2BKR activity during later neuronal differentiation results in a decrease in muscarinic receptor activity and M1–M3 subtype expression. Thus, it is proposed that B2BKR-induced modulation of gene expression, at least of mAChR M1–M3 subtypes, is important for the acquisition of a specific neuronal phenotype.

In summary, the B2BKR is the first neuropeptide receptor whose function was directly related to neuronal differentiation by a functional assay. We have presented data showing that both BK secretion and B2BKR expression and activity are augmented during the course of neuronal differentiation. The fact that blockade of B2BKR function by the specific B2BKR antagonist HOE-140 inhibits the progress of early and later neuronal differentiation is taken as evidence that B2BKRs have a crucial role in neuronal differentiation rather than only being a marker of differentiation states. The presented study may encourage further investigations to understand the molecular mechanism implicated in the B2BKR activation and its role during neuronal differentiation, neuroregeneration, and neuroregeneration following brain injury.

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