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Retinal ganglion cell survival is enhanced by gabapentin-lactam in vitro: evidence for involvement of mitochondrial K_{ATP} channels

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Abstract *Background:* Recently, gabapentin-lactam (GBP-L) was shown to be neuroprotective in vivo. It has been suggested that GBP-L may act by opening mitochondrial ATP-sensitive potassium (K_{ATP}) channels. We tested this hypothesis by quantifying the effect of GBP-L on the survival of purified retinal ganglion cells (RGCs). *Methods:* RGCs were purified from early postnatal rat retinae by immunopanning with antibodies against Thy1.1 and cultured in serum-free medium for 2 days. Cell survival was quantified by counting vital cells under phase-contrast optics. Results were normalized to controls. RGCs were treated with various concentrations (3.2–320 µM) of GBP-L with and without 1 µM glibenclamide, blocking both plasmalemmal and mitochondrial K_{ATP} channels, or 100 µM 5-hydroxydecanoate (5-HD), antagonizing selectively mitochondrial K_{ATP} channels. For comparison, additional cultures were treated with 32 µM gabapentin, the parent drug of GBP-L. A combination of the neurotrophic factors BDNF and CNTF (50 ng/ml each) served as a positive control. *Results:* GBP-L increased RGC survival to a maximum of 145±5% (mean ± SEM) in a concentration-dependent manner. The pEC_{50} was 5.0, CI95 [4.7, 5.3]. Preincubation with glibenclamide changed the dose-response of GBP-L, indicating that it acted as a competitive antagonist with a pA2 value of 6.8, CI95 [5.9, 7.5]. 5-HD completely blocked the survival-promoting effect of GBP-L. Gabapentin had no effect, whereas the combination of CNTF and BDNF enhanced survival to 177±9%. *Conclusions:* GBP-L, but not gabapentin, can promote the survival of cultured central nervous system neurons, possibly by opening mitochondrial K_{ATP} channels. These results suggest further testing of GBP-L as a potentially neuroprotective drug.

Introduction

Preventing degeneration of injured retinal neurons opens the way for the development of new therapies. A common feature of conditions such as glaucoma, optic nerve injury, or retinal ischemia is apoptotic retinal ganglion cell (RGC) death [17, 18, 27]. Many investigations have focused on interference in apoptotic and anti-apoptotic pathways with certain drugs. One focus is the role of ATP-sensitive potassium (K_{ATP}) channels in cell degeneration after neuronal injury [11, 12]. These channels are composed of pore-forming units (K_{ir} 6.1 or K_{ir} 6.2) and a sulfonylurea binding site. They open in states of ATP deficiency, hyperpolarizing cell membranes and thus rendering cells more resistant to stress [6, 24]. They occur in many different neuronal tissues, particularly in the hippocampus [5]. There is also evidence for the expression of K_{ATP} channel subunits in the rat retina, where they have been localized to the ganglion cell layer [8]. Hence, particular interest

has been focused on the examination of drugs acting at K_{ATP} channels.

Under ischemic conditions K_{ATP} channel openers have been found to support neuronal survival. In a model of cerebral ischemia different K_{ATP} channel openers reduced neuronal death, an effect that could be blocked by the K_{ATP} channel antagonist glipizide [11]. These findings were supported by investigations demonstrating that K_{ATP} channel agonists prevent ischemia-induced expression of the immediate early genes c-*fos* and c-*jun* [11]. Other studies showed that K_{ATP} channel openers mimic the effect of ischemic preconditioning [12].

Besides other factors, neuronal cell death is triggered by the excessive release or diminished uptake of the neurotransmitter glutamate [4]. In the course of investigations on glutamate excitotoxicity following ischemia, the anticonvulsant drug gabapentin and its derivative gabapentin-lactam (GBP-L) showed antiglutamatergic properties. In vitro, both gabapentin and GBP-L reduced oxygen and glucose deprivation-induced glutamate release in superfused rat hippocampal slices [16]. The antiglutamatergic effect of GBP-L could be reproduced by the K_{ATP} channel agonist minoxidil sulfate and could be blocked by the antidiabetic drug glibenclamide, which is a K_{ATP} channel antagonist. In vivo, GBP-L was neuroprotective in a rat model of pressure-induced retinal ischemia, in contrast to its parental substance gabapentin [23]. In a transgenic mouse model of Huntington's disease, GBP-L, but not gabapentin, had a beneficial effect on motor abilities. Moreover, a substantial reduction in the size and density of neuronal nuclear and cytoplasmatic inclusions was observed under GBP-L treatment [9].

These observations lead to the hypothesis that GBP-L promotes the survival of neuronal cells by opening K_{ATP} channels. In the present study, we used purified rat RGC cultures to quantify and pharmacologically characterize the survival-promoting effect of GBP-L. Interactions of GBP-L with K_{ATP} channels were analyzed by using the antagonists glibenclamide, which acts on both sarcolemmal and mitochondrial K_{ATP} channels [19], and with 5-hydroxydecanoate (5-HD), which is selective for mitochondrial K_{ATP} channels [25, 30].

Methods

Cell culture

All experiments were performed in accordance with the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985), the OPRR Public Health Service Policy on the Human Care and Use of Laboratory Animals (revised 1986) and the U.S. Animal Welfare Act, as amended, as well as our national and institutional guidelines for the care and use of animals in research. RGCs were purified by a modification [26] of immunopanning with antibodies against Thy 1.1 specific for RGCs and cultured in serum-free medium. In brief, retinae were dissected from 0- to 2-day-old Sprague Dawley rats and incubated at 37 °C for 20 min in 0.125% trypsin in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution. Enzyme treatment was stopped by washing the tissue twice with Dulbecco's minimal essential medium (DMEM) containing 10% horse serum, 2 mM L-alanyl-L-glutamine, 10 mM Hepes, 100 units/ml penicillin G (sodium salt) and 100 µg/ml streptomycin sulfate, followed by centrifugation at 140 *g* for 2 min. To yield a suspension of single cells, the retinal tissue was triturated with a flame-narrowed glass pipette in DMEM containing 10% horse serum. Cell culture reagents were obtained from Gibco (Invitrogen, Karlsruhe, Germany).

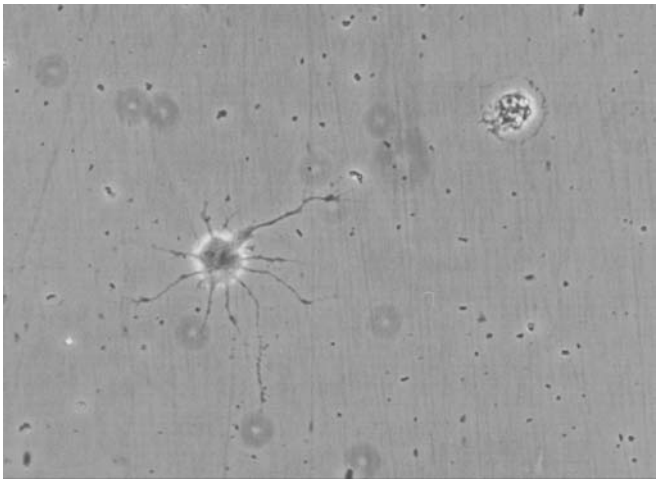

Fig. 1 Cell culture. RGCs fixed after 48 h in culture. Photograph obtained by phase-contrast microscopy at 400× magnification. The cell on the left shows intact neurites and was counted as vital, while the cell on the right is fragmented. The diffuse oval-shaped objects are due to dust particles outside the focal plane

Panning dishes (Falcon, Becton & Dickinson, NY, USA) were incubated with goat anti-mouse IgG antibodies (2 µg/ml, Sigma, Munich, Germany) in Tris-HCl buffer (pH 9.5) for 12 h at 4°C. Dishes were then washed three times with phosphate-buffered saline (PBS) prior to each of the subsequent steps. Incubation with antibodies against Thy1.1 (1.25–2.5 µg/ml, mouse anti rat CD90, Serotec, Düsseldorf, Germany) was performed for at least 2 h at 4°C in PBS. To prevent nonspecific binding of cells, dishes were then incubated with 2 mg/ml bovine serum albumin in PBS for 20 min at room temperature. Approximately 35×10^6 cells in 5 ml medium were added per dish and incubated for 20 min at 37°C. Dishes were gently swirled every 5 min to ensure access of all RGCs to the surface of the plate. To remove nonadherent cells, dishes were washed repeatedly with PBS and swirled moderately until only adherent cells remained. Washing was monitored under the microscope.

RGCs were mechanically removed from the panning dish in DMEM containing 10% HS by using a cell scraper. After centrifugation at 140°*g* for 5 min, purified RGCs were suspended in culture medium and their density was determined by counting an aliquot in a hemocytometer. The cells were seeded in 96-well plates (Falcon, Becton & Dickinson) at a density of 3,000 cells per well and incubated for 48 h at 37°C in a humidified atmosphere containing 5% CO_2 (Fig. 1). Plates had been previously coated with poly-L-lysine (0.1 mg/ml) followed by laminin (0.94 µg/cm^2) in DMEM. Culture medium consisted of DMEM (see above) supplemented with N2 (500 µg/ml insulin, 10 mg/ml human transferrin, 0.63 µg/ml progesterone, 1611 µg/ml putrescine, 0.52 µg/ml selenite) and 5 µM forskolin (Tocris Cookson, Avonmouth, UK).

After a culture period of 2 days, RGC cultures were fixed in 1% glutaraldehyde, rinsed with water and examined by phase-contrast microscopy at 400× magnification. The number of surviving RGCs was assessed by counting vital RGCs over the vertical and horizontal diameter of each well in a blinded fashion. Vital ganglion cells were morphologically identified by their phase-bright appearance, intact cell bodies with smooth membranes and neuritic processes.

Drug application

Cultures were exposed to various drugs. In a first set of experiments GBP-L (IBAM, Freiburg, Germany) was added at concentrations ranging from 3.2 µM to 320 µM. Control cultures received no drug treatment. In a second set, survival under GBP-L treatment was measured in the presence of 1 µM glibenclamide (Sigma, Deisenhofen, Germany) added 15 min prior to GBP-L application. In a third set, 100 µM 5-HD (Sigma, Deisenhofen) was added 15 min prior to application of 32 µM GBP-L. For comparison, additional cell cultures were treated with either 32 µM gabapentin (Pfizer, Ann Arbor, USA) or a combination of 50 ng/ml BDNF and CNTF (both from Peprotech/Tebu-Bio, Offenbach, Germany).

Statistics

All values were normalized to controls which received no drug treatment. Results are given as means ± standard error of the mean (SEM). An unpaired *t*-test (Stat View, SAS Institute, Cary, NC, USA) was used to assess the significance of differences between groups. A Bonferroni adjustment was performed to correct for multiple testing. The concentration-response relationships for GBP-L in the absence and presence of glibenclamide were analyzed by non-linear regression analysis of the concentration–response data, using the following transformation: normalized cell count=$1+E_{max}\times 10^{lg[GBP-L]}/(10^{-pEC50}+10^{lg[GBP-L]})$ with normalized cell count as the dependent and [GBP-L] as the independent variable, respectively. Parameters to be estimated were E_{max}, representing the maximal effect of GBP-L, and pEC_{50}, being the negative logarithm of the GBP-L concentration that yields the half maximal effect, i.e., the inflection point of a semilogarithmic concentration-response curve. Thus, the pEC_{50} indicates the potency of GBP-L. Comparison of the pEC_{50} in the absence or in the presence of glibenclamide serves to elucidate the pharmacologic interaction at the K_{ATP} channel receptor and thus yields information about the mode of antagonism. As a next step the pA_2 value was calculated, since it reflects the potency of an antagonist. It is defined as the antagonist concentration which leads to the same effect at doubled agonist concentration. The following formula was used: $pA_2=lg[10^{pEC50-pEC50*}-1]-lg[glibenclamide]$. pEC_{50} refers to the effect of GBP-L in the absence and pEC_{50}* to the effect of GBP-L in the presence of 1 µM glibenclamide. The pEC_{50} and the pA_2 values were within their 95% confidence intervals [1, 10].

Results

GBP-L increased survival of RGCs in a concentration-dependent manner (Fig. 2a). With low concentrations of GBP-L, RGCs resembled controls with a survival rate of 108±4% at 3.2 µM and 117±4% at 10 µM. Higher concentrations of 32 µM, 100 µM, and 320 µM GBP-L significantly increased survival of RGCs to 145±5%, 141±5%, and 140±5%, respectively. Preincubation with

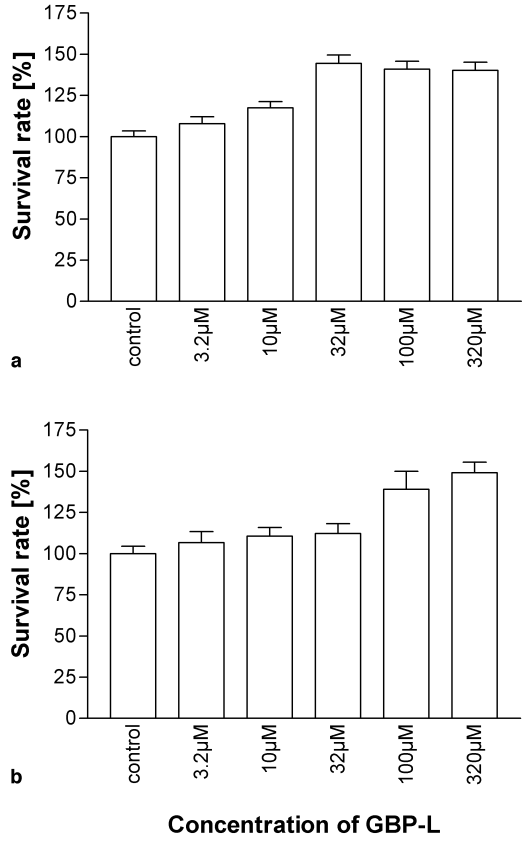

Fig. 2a, b Effect of GBP-L on survival of postnatal RGCs in vitro. **a** Effect of different concentrations of GBP-L ranging between 3.2 µM and 320 µM. Cell survival increases with the dose and reaches a plateau of 145% at 32 µM. **b** Same experiment performed in the presence of 1 µM glibenclamide. A tenfold concentration of GBP-L is needed to reach the survival rate observed without glibenclamide. Values are normalized to controls and are given as means ± SEM (*n*=18)

the K_{ATP} channel antagonist glibenclamide 15 min prior to GBP-L treatment inhibited the neuroprotective effect of GBP-L (Fig. 2b). Survival at 32 µM GBP-L decreased from 145% (see above) to 112±6%. Only high concentrations of 100 µM and 320 µM GBP-L still resulted in significantly increased cell survival of 139±11% and 149±7%, respectively. For GBP-L alone the pEC_{50} was 5.0, CI_{95} [4.7, 5.3] and for GBP-L in the presence of 1 µM glibenclamide it was 4.1, CI_{95} [3.5, 4.7]. The resulting pA_2 value for glibenclamide amounted to 6.8, CI_{95} [5.9, 7.5]. Since the E_{max} is almost identical without and with glibenclamide and the pEC_{50} differs significantly between the two conditions, competitive antagonism of GBP-L and glibenclamide can be assumed.

To investigate whether GBP-L acts at mitochondrial K_{ATP} channels, cell survival was quantified in the presence and absence of 100 µM 5-HD, which is a selective blocker of K_{ATP} channels located at the mitochondrial membrane. In the presence of 5-HD the survival rate un-

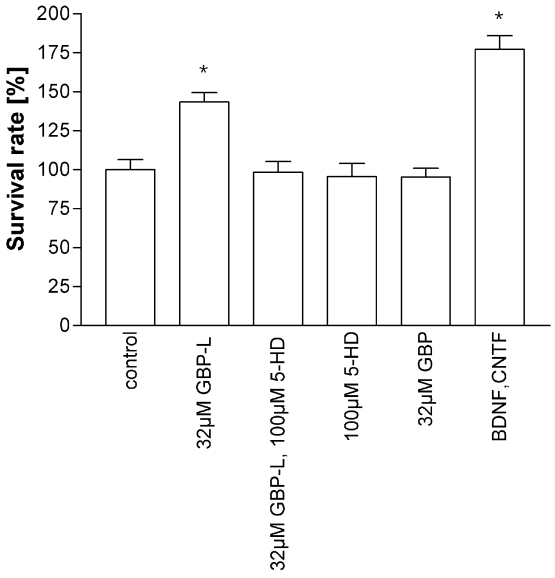

Fig. 3 Interaction of GBP-L and 5-HD. Compared with the control, 32 µM GBP-L increased cell survival to 143% ($P<0.05$). Preincubation with 5-HD decreased the survival rate to the level of the controls. Given alone, 5-HD had no effect on the survival rate. Gabapentin, the parental drug of GBP-L, at a concentration of 32 µM did not affect the survival rate. A combination of neurotrophic factors BDNF and CNTF in a concentration of 50 ng/ml each enhanced cell survival to 177% ($P<0.05$). Values are normalized to controls and are given as means ± SEM ($n\geq18$)

der 32 µM GBP-L decreased from 143±6% to 98±7% (Fig. 3). 5-HD alone did not affect survival (96±8%). In order to compare GBP-L with its parental substance gabapentin, equimolar concentrations of each were tested. 32 µM gabapentin was not effective (95±6%; Fig. 3). A combination of neurotrophic factors, 50 ng/ml CNTF plus 50 ng/ml BDNF, led to an increase to 177±9%.

Discussion

The present study is the first report on a survival-promoting effect of GBP-L in vitro. This phenomenon was tested under minimal essential conditions in purified rat RGC cultures without trophic tissue factors. At a saturating concentration of 32 µM, GBP-L enhanced the survival rate to 145% of the control rate. This finding is in agreement with an earlier study in which the GBP-L effect on RGC survival was tested in vivo [23]. When applied i.p. prior to a 1-h period of retinal ischemia in rats, GBP-L enhanced neuronal survival from 28% to 70%. The dose used in that experiment was 75 mg/kg. However, blood levels of GBP-L were not determined.

In order to assess GBP-L's efficacy, its effect was compared with that of a combination of CNTF and BDNF. RGCs depend on these factors and express the respective receptors [2, 3, 21, 29]. In purified rat RGC culture, the combination of BDNF and CNTF has been shown to be highly efficacious in promoting RGC survival [26]. The combination enhanced cell survival to 177% of the controls, thus being more efficacious than GBP-L alone.

The mechanism by which GBP-L exerts its action was obscure in the past. It has been suggested that GBP-L acts as an opener of K_{ATP} channels [16]. These channels are located in the plasma membrane but occur also in mitochondrial inner membranes [14, 15, 22]. K_{ATP} channels belong to the family of inwardly rectifying potassium channels (K_{ir}) and are composed of pore-forming units and sulfonylurea receptors [30]. ATP depletion leads to an opening of plasmalemmal channels, thus hyperpolarizing the cell membrane in states of energy deficiency or ischemia [19]. K_{ATP} channels are also essential for cardiac and cerebral ischemic preconditioning [12, 25, 31]. This phenomenon relates to the fact that a short period of ischemia renders tissue more resistant to subsequent longer periods of ischemia. It is transmitted by a release of adenosine, activation of adenosine A1 receptors and resultant activation of (mainly mitochondrial) K_{ATP} channels. Drugs opening K_{ATP} channels mimicked preconditioning, whereas antagonists decreased the protective effect. Additionally, it has been reported that neuronal death following ischemia was prevented by openers of K_{ATP} channels in the hippocampus, an effect which was blocked by the antagonist glipizide [11].

In order to test this hypothesis in our experimental setting, we applied substances blocking K_{ATP} channels. The first antagonist used was glibenclamide. It is known to act at both cell surface and mitochondrial channels [19]. Glibenclamide shifted the concentration response with a pA_2 value of 6.8, leaving the maximal effect of GBP-L unchanged. This indicates that glibenclamide acted as a competitive antagonist of GBP-L. Hence, it seems likely that GBP-L is an opener of K_{ATP} channels. Other experiments yielded corresponding pA_2 values for glibenclamide [7, 28]. The second substance tested was 5-HD, which is a specific inhibitor of mitochondrial K_{ATP} channels [13, 20, 25]. 5-HD blocked the effect of GBP-L completely, thereby supporting the hypothesis that GBP-L enhanced cell survival by a change in mitochondrial function. Mitochondria supply energy to the cell by synthesis of ATP. Respiring mitochondria transport H^+ into the cytoplasm, thus forming both the transmembrane potential and the pH gradient over the mitochondrial membrane. Influx of K^+ due to the opening of mitochondrial K_{ATP} channels diminishes only the electrical gradient, not the pH gradient, over the mitochondrial membrane. Consequently mitochondria no longer have to maintain two gradients and therefore become more resistant to stress by saving energy [20, 30].

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