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GLIAL CELL LINE–DERIVED NEUROTROPHIC FACTOR FAMILY LIGANDS ENHANCE CAPSAICIN-STIMULATED RELEASE OF CALCITONIN GENE-RELATED PEPTIDE FROM SENSORY NEURONS

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Abstract—The glial cell line–derived neurotrophic factor (GDNF) family ligands (GFLs) are a group of peptides that have been implicated as important factors in inflammation, since they are released in increased amounts during inflammation and induce thermal hyperalgesia upon injection. Mouse isolated sensory neurons in culture and freshly dissociated spinal cord slices were used to examine the enhancement in stimulated-release of the neuropeptide, calcitonin gene-related peptide (CGRP), as a measure of sensitization. Exposure of isolated sensory neurons in culture to GDNF, neurturin, and artemin enhanced the capsaicin-stimulated release of immunoreactive calcitonin gene-related peptide (iCGRP) two- to threefold, but did not increase potassium-stimulated release of iCGRP. A similar profile of sensitization was observed in freshly dissociated spinal cord slices. Persephin, another member of the GFL family thought to be important in development, was unable to induce an enhancement in the release of iCGRP. These results demonstrate that specific GFLs are important mediators affecting sensory neuronal sensitivity, likely through modulation of the capsaicin receptor. The sensitization of sensory neurons during inflammation, and the pain and neurogenic inflammation resulting from this sensitization, may be due in part to the effects of these selected GFLs.

Key words: artemin, dorsal root ganglia, inflammation, neurturin, sensitization.

Inflammatory mediators, which are released in increased amounts in a number of diseases (Schaible et al., 2002; Yang et al., 2003), are implicated in hyperalgesia and sensory neuronal sensitization. Growth factors are one set of mediators found in higher concentrations during inflammation. Although growth factors previously were thought to be responsible only for the growth and maintenance of sensory neurons, they are postulated now to be responsible for inflammatory hyperalgesia (Mendell et al., 1999). Nerve growth factor (NGF) is one such mediator with an established role in inflammatory hyperalgesia and sensory neuronal sensitization (Lewin et al., 1993; McMahon, 1996; Shu and Mendell, 1999). Another set of molecules found in higher amounts during inflammation is the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs). The levels of GFLs in the joint capsule and plasma of patients with osteoarthritis, Crohn's disease, and interstitial cystitis are greatly increased compared to people without these diseases (Okragly et al., 1999; De et al., 2004; von Boyen et al., 2006). Induction of inflammation by injection of complete Freund's adjuvant (CFA) or lipopolysaccharide (LPS) results in increased levels of the GFLs, GDNF, neurturin (NTN), and artemin (ART; Amaya et al., 2004; Hashimoto et al., 2005; Malin et al., 2006).

The GFLs are a group of small peptides in the TGFβ super-family of molecules. They exist naturally as homodimers and include GDNF, NTN, ART, and persephin (PSP; Eigenbrot and Gerber, 1997; Wang et al., 2003). The actions of GFLs are initiated by binding to specific glial cell line-derived neurotrophic factor family receptor alpha subtypes (GFRα), glycosyl phosphatidylinositol (GPI)–linked surface receptors found in lipid rafts. The GFL-GFRα complex translocates to the receptor tyrosine kinase, Ret, to initiate intracellular signaling (reviewed by Saarma, 2001; Sariola and Saarma, 2003). Higher binding affinity is found for GDNF and GFRα1, NTN and GFRα2, ART and GFRα3 and PSP and GFRα4. Non-specific binding can occur between GDNF and GFRα2 or GFRα3 (Airaksinen and Saarma, 2002). Evidence also exists for non-specific binding of ART and NTN to GFRα1 (Airaksinen and Saarma, 2002).

There is a growing body of evidence that, through binding to and translocation of GFRα receptors, select GFLs (GDNF, NTN, and ART) play a role in the induction of hyperalgesia (Amaya et al., 2004; Malin et al., 2006; Vellani et al., 2006). Importantly, there is significant overlap in the expression of the GFRα1-3 receptors and the transient receptor potential vanilloid type 1 (TRPV1) receptor, a ligand-gated ion channel that is activated by noxious stimuli, including heat and acidic pH (Aoki et al., 2005; Malin et al., 2006). Application of GDNF to isolated sensory neurons increases their inward flux of calcium in response to capsaicin, a molecule used to activate TRPV1, and the number of TRPV1 channels present on the neurons (Anand et al., 2006). These same selected GFLs

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Abbreviations: ANOVA, analysis of variance; ART, artemin; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; GDNF, glial cell line-derived neurotrophic factor; GFL, glial cell line-derived neurotrophic factor family ligands; GFRα, glial cell line-derived neurotrophic factor family receptor alpha; IB4, isolectin B4; iCGRP, immunoreactive calcitonin gene-related peptide; NGF, nerve growth factor; NTN, neurturin; OE, over-expressed; PSP, persephin; RIA, radioimmunoassay; TG, trigeminal ganglia; TRPV1, transient receptor potential vanilloid type 1.

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enhance both the peak calcium current and area under the curve of the calcium current in response to capsaicin in isolated sensory neurons, presumably via activation of the TRPV1 channel (Malin et al., 2006). Additionally, increased expression of acid sensing ion channels (ASICs) has been observed in the skin in response to GDNF exposure (Albers et al., 2006).

While there is evidence that select GFLs increase TRPV1-mediated calcium influx in sensory neurons, the cellular consequences of this change have not been established. The neuropeptide calcitonin gene-related peptide (CGRP), found predominantly in small diameter, nociceptive sensory neurons, has a high degree of co-localization with TRPV1 (Aoki et al., 2005). CGRP functions as a mediator of neurogenic inflammation in the periphery and has been associated with potentiation of the pain signal from primary sensory neurons to second order neurons in the spinal cord (Brain et al., 1985; Miletic and Tan, 1988; Ryu et al., 1988). To determine if GFLs alter the integrative activity of the primary sensory neuron, the ability of GFLs to alter the release of immunoreactive calcitonin gene-related peptide (iCGRP) from dorsal root ganglia (DRG) neurons was examined. Increase in the release of iCGRP from DRG neurons, indicating peripheral sensitization, could account in part for the hyperalgesia induced by GFLs.

EXPERIMENTAL PROCEDURES

Materials

The mice used for all experiments, C57BL/6 mice, were purchased from Harlan Laboratories (Indianapolis, IN, USA) and/or bred and housed in the Indiana University Laboratory Animal Research Center (LARC). Mice were housed in group cages in a light-controlled room at a constant temperature of 22 °C. All mice were adults, between 3 and 6 months in age. Food and water were available at the convenience of the animals. Capsaicin was purchased from Sigma Chemical Company (St. Louis, MO, USA) and was first dissolved in 1-methyl,2-pyrrolidinone (Aldrich Chemical, Co., Milwaukee, WI, USA) to a concentration of 10 mM. It was then serially diluted to a concentration of 50–500 nM in the appropriate release buffer as noted below. Horse serum, F-12 medium, L-glutamine, and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). NGF was purchased from Harlan Bioproducts for Science, Inc. (Indianapolis, IN, USA). Collagenase, poly-D-lysine, laminin, 5-fluoro-2-deoxyuridine, uridine and standard laboratory chemicals were from Sigma. Antibody to CGRP was generously provided by Michael R. Vasko (Indiana University School of Medicine, Indianapolis, IN, USA and originally produced by Michael J. Iadarola, NIH, Bethesda, MD, USA). The GFLs were purchased from Peprotech (Rocky Hills, NJ, USA).

Preparation of DRG cultures

DRG from adult mice were used to establish sensory neuronal cultures. Briefly, the DRG were removed from adult mice in a manner similar to that previously published (Hingtgen et al., 2006). DRG were digested in 0.1% collagenase in two separate 30-min incubations at 37 °C. Additionally, cells were digested in DNase for 1 min at room temperature. Lastly, the preparation was dissociated by mechanical agitation. Cells were plated in wells of 24-well falcon culture dishes coated with poly-D-lysine and laminin at a density of 30,000–50,000 cells/well. Cultures were maintained at 37 °C in a 5% CO_2 atmosphere in F12 media supplemented with 2 mM glutamine, 50 μg/mL penicillin and streptomycin, 10% heat-inactivated horse serum and mitotic inhibitors (50 μM 5-fluoro-2-deoxyuridine and 150 μM uridine). NGF, at a concentration of 30 ng/mL, was added to this medium. Growth medium was changed every 2–3 days, and the added NGF removed 48 h prior to all experiments.

Stimulated-release of iCGRP

Measurement of stimulus-evoked release and content of iCGRP from isolated sensory neurons was accomplished as previously published (Hingtgen et al., 2006). After 5–7 days in culture, culture medium was removed from the sensory neurons in culture and the basal or resting release of iCGRP measured from cells incubated for 10 min in Hepes buffer consisting of (in mM): 25 Hepes, 135 NaCl, 3.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 3.3 dextrose, and 0.1% (w/v) bovine serum albumin, pH 7.4, and maintained at 37 °C. The cells were incubated in Hepes buffer containing stimulus (capsaicin or high potassium) for 10 min, and then incubated again with Hepes buffer alone to reestablish resting release levels. The concentrations of capsaicin and potassium were chosen because they lie on the low end of the highly sloped portion of the concentration response curve for iCGRP release (data not shown). The use of these concentrations allows for evaluation of enhancement in release of CGRP after exposure to sensitizing molecules. The amount of iCGRP released in each incubation was measured by radioimmunoassay (RIA). After the release protocol, the remaining peptide content in each well was determined by exposing the cells to 2 N acetic acid for 10 min. Aliquots of this incubation were diluted in Hepes and iCGRP was determined by RIA. The release of iCGRP during the 10-min incubation period is expressed as percent of the total content. GFLs were added in the basal incubation period (10 min) and in the stimulated incubation period (10 additional minutes). The neurons were exposed to GFLs for a total time of 20 min. A minimum of three different preparations was used for each condition, including growth factor application and stimulus.

Stimulus-evoked release and content of iCGRP from spinal cord slices was accomplished as previously published (Chen et al., 1996; Southall et al., 1998). Briefly, the entire spinal cord was removed from each animal. It was weighed and chopped into 300 μm cross-sections using a McIllwain Tissue Chopper. The chopped spinal cord from each animal was placed into its own individual chamber and perfused at a rate of 0.1 mL/min for 20 min with Hepes buffer supplemented with 200 mM ascorbic acid, 100 μM Phe–Ala, and 20 μM bacitracin (all used as peptidase inhibitors to prevent the breakdown of CGRP during the process; Chen et al., 1996). The perfusion buffer was aerated with 95% O_2/5% CO_2 and maintained at a pH of 7.4. Serial 10-min collections (1.0 mL of perfusate) were obtained from each spinal cord. Initially, the tissue was perfused with Hepes buffer alone or Hepes buffer containing 10 ng/mL growth factor for 30 min. The perfusate was changed to Hepes buffer containing 500 nM capsaicin±10 ng/mL growth factor for 30 min to measure stimulated release. For release experiments in spinal cord tissue, 500 nM capsaicin was used as a stimulus for peptide release. The higher concentration was used to ensure proper penetrance of the capsaicin into the tissue because of the lipophilic nature of capsaicin and the substantial density of the spinal cord tissue, and based on previous uses of this method (Chen et al., 1996; Southall et al., 1998). The tissue was perfused for 60 min with Hepes buffer after the stimulus exposure to allow a return to resting levels of peptide release. Aliquots from each 10-min collection period were assayed for iCGRP using RIA. After the protocol was completed, the remaining iCGRP content of the tissue was determined by homogenizing the spinal cord tissue in 0.1 N HCl and serially diluting the supernatant with Hepes buffer and 1.0 M MES. The content was added to the amount of iCGRP released during the entire perfusion to obtain the total peptide content. The release of iCGRP during

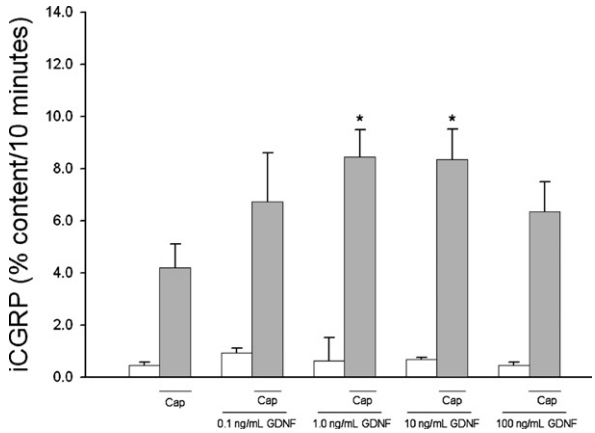

Fig. 1. GDNF enhances capsaicin-stimulated release of iCGRP from isolated sensory neurons. Peptide release elicited by a 10-min exposure to Hepes buffer alone (open bars) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well±SEM (n=9 wells per condition). GDNF (at concentrations from 0.1 ng/mL to 100 ng/mL) was included in the 10 min prior to and throughout capsaicin exposure. Total growth factor exposure time was 20 min. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no GFL condition using an ANOVA with Dunnett's post hoc test ($P<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

each 10-min period of perfusion is expressed as percent of the total iCGRP content for the tissue.

An RIA was used to quantify the amount of iCGRP released in the basal and stimulated conditions for both experiments with cells in culture and spinal cord tissue. The minimum amount of iCGRP detected by the RIA is 5 fmol with a 95% confidence interval (Chen et al., 1996). Additionally, the GFLs are peptides, which have the potential to affect the RIA. To that end, separate standard curves for the RIA were conducted using Hepes buffer containing each of the GFLs at the highest concentration used in experiments. None of the GFLs affected the sensitivity of the RIA (data not shown).

Statistical analyses

Results, represented as percent total content of iCGRP, are expressed as the mean±standard error of the mean (SEM). All differences in iCGRP release and total content were compared with analyses of variance (ANOVAs) and Dunnett's post hoc analysis or Student's t-tests, as indicated. A P-value of <0.05 was used to indicate statistical significance between treatment and non-treatment groups.

RESULTS

GFLs enhance the capsaicin-stimulated release of iCGRP from isolated sensory neurons

The levels of the GFLs are increased during inflammation (Aloe et al., 1992) and treatment of isolated sensory neurons with GFLs increases intracellular calcium levels in response to capsaicin (Malin et al., 2006). While the local levels of the GFLs near the sensory neurons in intact animals have not been established, levels in whole brain (Kirik et al., 2000) and in plasma (Onodera et al., 1999) are in the high pg/mL and low ng/mL range. Additionally, the concentrations of the GFLs used in previous experiments on freshly dissociated sensory neuronal preparations and sensory neurons in culture are between 1 ng/mL and 100 ng/mL (Malin et al., 2006; Price et al., 2005). These concentrations correspond to 0.0667–6.67 nM for GDNF, 0.0847–8.47 nM for NTN, 0.0833–8.83 nM for ART, and 0.0971–9.71 nM for PSP. To determine if the actions of GFLs on the TRPV1 receptor result in increased functional output, such as enhanced transmitter release, the ability of different GFLs to modulate the stimulated-release of iCGRP from isolated sensory neurons was measured. Studies of the effects of the GFLs on sensory neuronal sensitization were conducted with concentrations of the GFLs between 0.1 ng/mL and 500 ng/mL to remain in the physiological range and to correspond to concentrations used in similar experiments.

Individual preparations of isolated sensory neurons in culture were exposed to different concentrations of GDNF for 10 min prior to and during a 10-min capsaicin-stimulated period. In the absence of GDNF, basal release of iCGRP was 7.15±1.25 fmol/well and capsaicin-stimulated release was 78.84±6.21 fmol/well (mean±SEM). When expressed as the percent of the total content of iCGRP in the well, these values correspond to 0.45%±0.11% in the basal condition and 4.19%±0.93% in the capsaicin-stimulated condition (Fig. 1). When 1 or 10 ng/mL GDNF was added, capsaicin-stimulated release of iCGRP was significantly enhanced (no GFL: 4.19%±0.93%, 0.1 ng/mL GDNF: 6.74%±1.87%, 1.0 ng/mL GDNF: 8.45%±1.05%, 10 ng/mL GDNF: 8.35%±1.16%, 100 ng/mL GDNF: 6.34%±1.15%; Fig. 1). There was no change in the basal release of iCGRP with exposure to GDNF. In addition, and as seen in Fig. 2, 10 ng/mL NTN and ART also significantly enhanced the capsaicin-stimulated release of iCGRP (no GLF: 4.30%±0.58%, NTN: 10.60%±1.70%, ART: 10.00%±1.57%). Unlike the

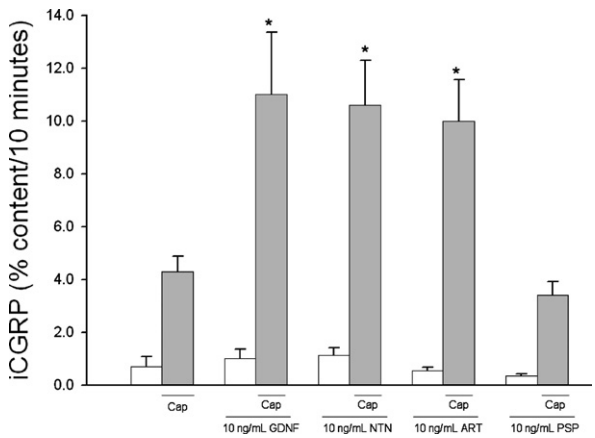

Fig. 2. GFLs enhance capsaicin-stimulated release of iCGRP from isolated sensory neurons. Peptide release elicited by a 10-min exposure to Hepes buffer alone (open bars) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well±SEM (n=9–22 wells per condition). GDNF, NTN, ART, or PSP, at 10 ng/mL, was included in the 10 min prior to and throughout capsaicin exposure. Total growth factor exposure time was 20 min. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no GFL condition using an ANOVA with Dunnett's post hoc test ($P<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

other GFLs, PSP did not alter the capsaicin-stimulated release of iCGRP (PSP: 3.40%±0.52%). This may result from a lack of functional GFRα4 in adult DRG neurons, and the fact that PSP binds specifically to GFRα4 (Enokido et al., 1998; Paveliev et al., 2004). Even at concentrations as high as 500 ng/mL, PSP was unable to enhance the capsaicin-stimulated release of iCGRP (Table 1). Additionally, to ensure that the GFLs did not directly alter the resting release of iCGRP, sensory neurons were exposed to the GFLs for two consecutive 10-min incubations in the absence of any stimulus. No enhancement in the release of iCGRP was observed with these treatments (Table 2). These data indicate that GDNF, NTN, and ART alter the sensitivity of sensory neurons to capsaicin stimulation, but do not directly evoke the release of CGRP.

When sensory neurons are exposed to the GFLs for several days, the levels of CGRP are increased (Ramer et al., 2003; Price et al., 2005). To ensure that the GFL-induced enhancement in capsaicin-stimulated release of iCGRP was not the result of an increase in the total content of iCGRP, we measured iCGRP content at the end of each experiment. There was no change in the total content of iCGRP after the 20-min exposure to the GFLs (no GFL: 1487±154 fmol/well, GDNF: 1322±108 fmol/well, NTN: 1500±128 fmol/well, ART: 1320±102 fmol/well, and PSP: 1518±177 fmol/well, n=9–22 wells per condition).

NGF treatment of sensory neuronal cultures increases the expression of TRPV1 (Xue et al., 2007) and increases the amount of TRPV1 insertion into the plasma membrane (Stein et al., 2006). Additionally, sensory neuronal exposure to NGF in culture increases the expression of CGRP and other neuropeptides (MacLean et al., 1989; Sango et al., 1994). Therefore, there is a possibility that some of the media components, specifically the NGF, are affecting the responses of the sensory neurons to either or both the capsaicin and the GFLs. To address this concern, we conducted CGRP release studies on neurons grown in the presence or absence of NGF to determine whether this change in the media components would alter the capsaicin-stimulated release and the GFL-induced enhancement in this release. The presence or absence of NGF in the culture media did not change the magnitude of capsaicin-stimulated release of iCGRP or the GDNF-induced enhancement of peptide release (Fig. 3). The absolute level of capsaicin-stimulated iCGRP released when NGF was omitted from the culture media was ~25% less than when NGF was present (no added NGF: 97.39±10.42 fmol/well, growth in 30 ng/mL NGF: 127.83±11.24 fmol/well). While the amount of capsaicin-evoked release was enhanced by 10 ng/mL GDNF in both conditions, the absolute level of iCGRP released was again ~25% less in the cells that were not exposed to NGF (no added NGF: 162.54±8.45, growth in 30 ng/mL NGF: 219.27±21.86 fmol/well). The reduction in absolute levels of iCGRP released from sensory neurons maintained in culture in the absence of added NGF, but an absence of change in the percent of the total content of iCGRP released is consistent with previous observations (Park et al., 2006).

Table 1. Persephin does not enhance the capsaicin-stimulated release of iCGRP

	Basal	50 nM capsaicin
No GFL	0.67±0.13	5.28±0.46
10 ng/mL PSP	0.68±0.18	5.94±0.88
100 ng/mL PSP	0.68±0.16	4.97±0.41
500 ng/mL PSP	1.23±0.16	5.59±0.64

All values are mean±S.E.M. % content iCGRP released; n=9 wells per condition.

Table 2. 20-min exposure to GFLs does not increase release of iCGRP

GFL	No GFL	10-min exposure to GFL	20-min exposure to GFL
No GFL	0.55±0.35	0.43±0.11	0.76±0.20
10 ng/mL GDNF	0.96±0.12	0.67±0.14	0.50±0.19
10 ng/mL NTN	0.41±0.06	0.44±0.04	0.70±0.15
10 ng/mL ART	0.91±0.30	0.68±0.20	0.54±0.16

All values are mean±S.E.M. % content iCGRP released; n=9 wells per condition.

GFLs do not enhance the potassium-stimulated release of iCGRP from isolated sensory neurons

Previous studies have focused primarily on GFL-induced changes in response to capsaicin in isolated DRG neurons. To determine whether responses to stimuli other than capsaicin could be enhanced by GFLs, a general depolarizing stimulus, high extracellular potassium, was used.

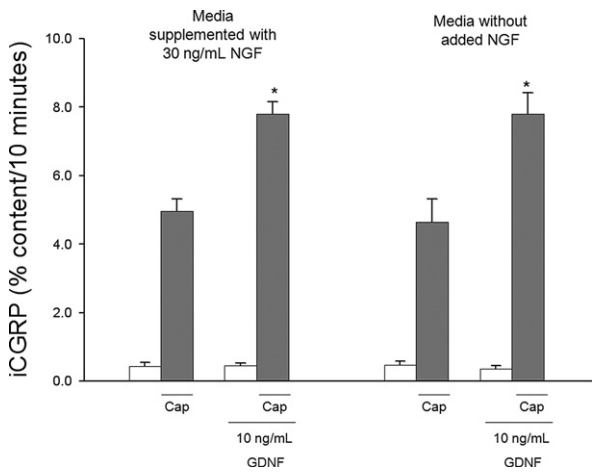

Fig. 3. NGF in culture media does not change the stimulated release of iCGRP and the GDNF-induced enhancement in release. Peptide release elicited by a 10-min exposure to Hepes buffer alone (open bars) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well±SEM (n=9 wells per condition). Ten nanograms/mL GDNF was included in the 10 min prior to and throughout capsaicin exposure. Total growth factor exposure time was 20 min. NGF was added in the culture medium at a concentration of 30 ng/mL or omitted from the culture medium. Asterisks (*) indicate statistically significant differences in iCGRP release between GDNF treatment group and the no GFL condition using an t-test (P<0.05). In all cases, release stimulated by capsaicin was significantly higher than basal release.

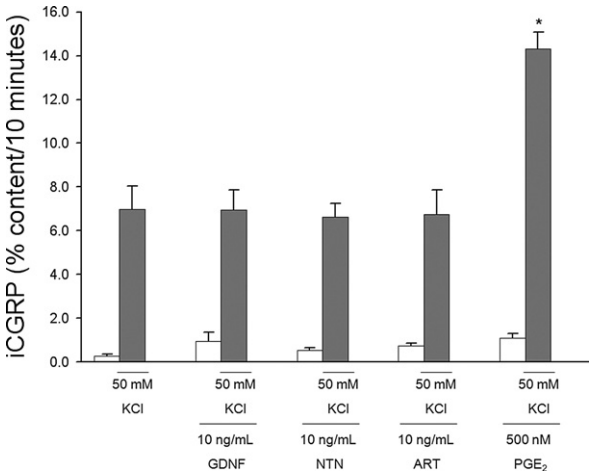

Fig. 4. GFLs do not enhance the potassium-stimulated release of iCGRP from isolated sensory neurons. Peptide release elicited by a 10 min exposure to Hepes buffer alone (open bars) or Hepes buffer containing 50 mM potassium (KCl; dark bars) is expressed as mean percent total peptide content of cells in each well±SEM (n=9–12 wells per condition). GDNF, NTN, or ART at 10 ng/mL, was included in the 10 min prior to and throughout potassium exposure. PGE_2 was present in the basal and stimulated conditions at a concentration of 500 nM. Total growth factor and PGE_2 exposure time was 20 min. There were no significant differences in iCGRP release between treatment groups and the no GFL condition. A significant enhancement in iCGRP release was observed with PGE_2 using ANOVA with Dunnett's post hoc test (P<0.05). In all cases, release stimulated by potassium was significantly higher than basal release.

Exposure to Hepes buffer containing 50 mM high extracellular potassium (KCl) for 10 min caused a release of iCGRP of 6.99%±1.06%. Treatment with GDNF, NTN, or ART (10 ng/mL) 10 min prior to and throughout the stimulus period did not alter KCl-stimulated iCGRP release (Fig. 4; no GFL: 6.99%±1.06%, GDNF: 6.95%±0.92%, NTN: 6.63%±0.61%, and ART: 6.74%±1.11%). In addition, treatment with 100 ng/mL GDNF was unable to enhance KCl-stimulated release of iCGRP (data not shown). PGE_2 is a well-established sensory neuronal sensitizing agent (Martin et al., 1987; Mense, 1981). It is known to sensitize sensory neurons to many stimuli, including high extracellular potassium (Southall and Vasko, 2000). Accordingly, PGE_2 enhanced the potassium-stimulated release of iCGRP by nearly twofold (Fig. 4; 14.3%±0.79%). These data suggest that GDNF, NTN, and ART, unlike PGE_2, sensitize sensory neurons through an interaction with TRPV1 and not by mechanisms independent of the stimulus type.

GFLs enhance the capsaicin-stimulated release of iCGRP from spinal cord slices

Sensitization of the central terminals of primary sensory neurons, which synapse onto second order neurons in the spinal cord, is also important during inflammation and propagation of the pain signal. The GFRα receptors are present on the central terminal of primary afferent neurons (Josephson et al., 2001), and GFLs are released by astrocytes in the spinal cord (Nosrat et al., 1996; Nomura et al., 2002). In order to examine the actions of GFLs in sensitization of the central terminals of sensory neurons, iCGRP release from spinal cord slices was measured.

Fig. 5A demonstrates a representative experiment comparing capsaicin-stimulated release of iCGRP in the absence or presence of 10 ng/mL GDNF. The fmol of iCGRP in each 10-min collection fraction was normalized to the total iCGRP content in the spinal cord, as described in Experimental Procedures. In the three basal fractions, iCGRP release was similar for both treatments. The capsaicin-stimulated release of iCGRP was significantly enhanced

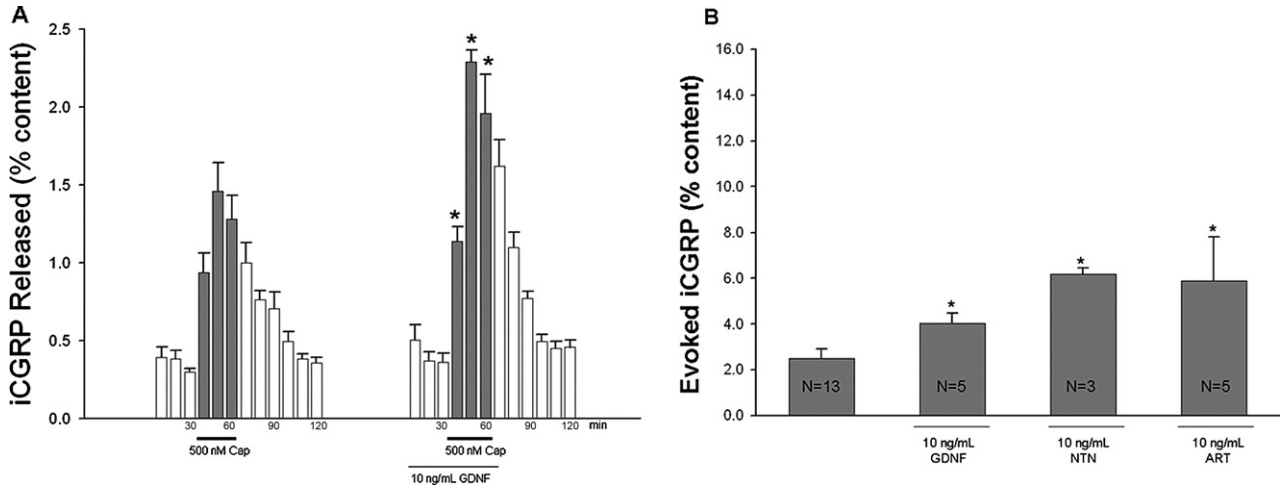

Fig. 5. GFLs enhance capsaicin-stimulated release of iCGRP from spinal cord slices. (A) Peptide release from spinal cord slices stimulated by three 10-min exposures to Hepes buffer alone (open bars) or Hepes buffer containing 500 nM capsaicin (dark bars) is expressed as mean percent total peptide content of iCGRP in the spinal cord slice±SEM (n=3–10 animals per condition). GDNF, NTN, or ART, at 10 ng/mL, was included in the six 10-min incubations indicated by lines with growth factor name below, for a total exposure time of 60 min (B) Evoked release, or release due to capsaicin stimulation alone, is compared between growth factor treatment and no GFL groups. The evoked release was obtained by subtracting peptide release during the three basal fractions from that during the three capsaicin-stimulated fractions in each treatment group. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no growth factor condition using an ANOVA with Dunnett's post hoc test (P<0.05). In all cases, release stimulated by capsaicin was significantly higher than basal release.

by exposure to GDNF for 30 min prior to and throughout the stimulus period (no GFL: 0.94±0.12%, 1.46±0.18%, and 1.28±0.15%; 10 ng/mL GDNF: 1.14±0.09%, 2.29±0.08%, and 1.96±0.25%). The profiles of increased release of iCGRP were similar when the spinal cord slices were exposed to NTN and ART (data not shown). Evoked release was determined by subtracting the three basal fractions of iCGRP release from the three capsaicin-stimulated fractions. As demonstrated in Fig. 5B, GDNF, NTN, and ART all were able to significantly enhance the capsaicin-evoked release of iCGRP from the spinal cord slices by two- to threefold (no GFL: 2.50%±0.42%, GDNF 4.05%±0.43%, NTN: 6.18%±0.28%, ART: 5.88%±1.94%). The total content of iCGRP per mg of protein in the spinal cord slices was not changed by exposure to GFLs (no GFL: 298.41±44.28 fmol/mg, GDNF: 275.46±25.13 fmol/mg, NTN: 220.71±84.84 fmol/mg, ART: 227.58±98.63 fmol/mg). These data indicate that the GFLs are able to sensitize the central terminals of the sensory neurons to capsaicin stimulation.

DISCUSSION

Previous studies have shown that the GFLs are potent modulators of the TRPV1 channel and that injection of these molecules induces hyperalgesia (Malin et al., 2006). However, whether this TRPV1 modulation results in a change in integrative functions of the sensory neurons, such as neurotransmitter release, and whether this change in function could be responsible for the GFL-induced hyperalgesia, is not clear. Here, direct evidence is provided, from both neuronal cultures and freshly dissociated neuronal tissues, that select GFLs sensitize sensory neurons, resulting in increased stimulated-release of iCGRP. This sensitization could be a contributing factor to the inflammatory hyperalgesia induced by GFLs.

Multiple GFLs were able to sensitize capsaicin-stimulated release of iCGRP. The selected GFLs were able to produce an enhancement in the capsaicin-stimulated release of iCGRP, not only in sensory neurons in culture but also in spinal cord slices. GFL-induced enhancement in the release of iCGRP from the spinal cord slices indicates that GFLs sensitize the central terminal of the primary sensory neurons, the terminal related to propagation of the nociceptive signal. GFRα1-3 are found throughout the CNS, and specifically in the dorsal horn of the spinal cord (Josephson et al., 2001; Quartu et al., 2007). It is possible that the GFLs are exerting their effects on the enhancement of release of iCGRP in sensory neurons through these GFRα receptors at this location during inflammation. There is direct evidence that CGRP is important in the propagation of the pain signal, since hyperalgesia due to both pancreatitis and inflammation induced by carrageenan is attenuated by a CGRP blocking antibody (Satoh et al., 1992; Wick et al., 2006). The GFRα receptors exist on motor neurons originating in the ventral horn of the spinal cord (Homma et al., 2003) and these neurons also can contain CGRP (Gibson et al., 1988; Kruger et al., 1988). Since the ventral half of the spinal cord is also present in the preparation used in these experiments, basal release levels of iCGRP in response to the GFLs could reflect release from both the dorsal and ventral neuronal terminals. However, motor neurons in the ventral horn are unlikely to significantly contribute to the stimulated release of iCGRP because motor neurons do not contain TRPV1 receptors (Lauria et al., 2006). Since CGRP from the central terminal of primary sensory neurons is important in propagation of the pain signal, modulation of release at this site may be a critically important component of pain processing and hyperalgesia.

These data add to the previous observation that GDNF, NTN, and ART are able to alter TRPV1 function directly (Malin et al., 2006). The modulation of TRPV1 channels has been observed with other sensitizers of sensory neurons, such as NGF (Shu and Mendell, 1999; Zhu and Oxford, 2007), which may provide some insight into the cellular mechanisms of GFL-induced sensitization. Specifically, the GFLs may sensitize sensory neurons by altering properties of TRPV1, perhaps by increasing the ion flow through the channel or rapidly increasing the membrane expression of TRPV1, similar to the mechanisms of NGF-induced sensitization (Zhang et al., 2005; Zhu and Oxford, 2007). Interestingly, at a concentration of 100 ng/mL, GDNF is unable to enhance the release of iCGRP. When the sensory neurons are exposed to this higher concentration of GDNF, compensatory pathways could be activated, which could alter its actions. This is not without precedence, since exposure of sensory neurons in culture to 10 ng/mL GDNF, but not 100 ng/mL GDNF, enhances the content and capsaicin-evoked release of CGRP (Price et al., 2005). Interestingly, the capsaicin concentration response curve is U-shaped (Bertelsen et al., 2003). Therefore, another possible mechanism for the inability of 100 ng/mL GDNF to induce sensitization is that the capsaicin concentration–response curve is shifted by GDNF. This could mean that 1 ng/mL and 10 ng/mL GDNF are at the peak of this curve, while 100 ng/mL is on the downward slopping portion of the curve.

Long term exposure to growth factors, specifically NGF, can change several properties of sensory neurons. NGF increases the amount of CGRP produced (Maclean et al., 1989; Sango et al., 1994) and the expression and membrane insertion of TRPV1 (Xue et al., 2007; Stein et al., 2006). These changes could affect the responses of sensory neurons to GFL-induced sensitization. However, while the absolute amount of iCGRP present and iCGRP released upon stimulation was reduced when the cultures lacked NGF, the sensitization profile of GFLs was unaffected (Fig. 3). This observation indicates that the NGF present in the cultures did not alter the sensitization phenotype of the sensory neuron, which is in line with previous studies (Park et al., 2006). In addition, when spinal cord tissue was exposed to the GFLs, there was a similar profile of enhanced release of iCGRP to that seen with isolated sensory neurons in culture (Fig. 5). Since this spinal cord tissue is freshly dissociated, and the responses of this tissue and the isolated sensory neurons in culture were identical, it is unlikely that the phenotype of the sensory neurons is changed by maintenance in culture.

While GDNF, NTN, and ART were able to sensitize capsaicin-stimulated release robustly, potassium-stimulated release was not enhanced by any of the GFLs. This is in contrast to previous observations in the trigeminal ganglia (TG) and neurons from transgenic mice (Price et al., 2005; Albers et al., 2006). The majority of previous studies have examined the role of GFLs in capsaicin-induced sensory neuronal changes. However, studies conducted on neurons from TG and transgenic mice have demonstrated that GDNF is able to alter the sensitivity of sensory neurons in ways other than through TRPV1. Potassium-stimulated release of CGRP from sensory neurons in the TG was sensitized by GDNF (Price et al., 2005). There could be critical physiological differences between the responses evoked in the TG and the DRG, which could be responsible for the observed differences in our studies. In fact, large differences exist between TG neurons and DRG neurons in the levels of CGRP, TRPV1, and isolectin B4 (IB4), as well as the co-localization of these proteins within the ganglia (Price and Flores, 2007). IB4 neurons are generally considered GDNF-responsive neurons (Kashiba et al., 2001). A much higher percentage of neurons in the DRG express both IB4 and TRPV1 than in the TG (Price and Flores, 2007), which may be one contributing factor to the differential responses seen with tissue from these two types of ganglia. Albers et al. (2006) used transgenic mice that over-expressed GDNF (GDNF-OE) in the skin and found that neurons from these mice exhibited enhanced electrical responses to mechanical stimuli. Mechanical hyperalgesia was also present in these GDNF-OE mice. The difference between the responses of the sensory neurons to the general stimulus (potassium) used in our studies and the results of studies conducted on the GDNF-OE mice described above could be the result of several factors. First, the amount of overlap between mechano-sensitive neurons and different TRP receptors is unclear (Lawson et al., 2008; Tender et al., 2008), which may mean that some of the mechanical hyperalgesia seen in these GDNF-OE mice is TRP-mediated. Since GDNF is necessary for the development of sensory neurons, the use of GDNF-OE mice may change certain properties of the neurons, thereby making them more likely to be sensitized to more types of stimuli by GFLs. What remains clear is that the GFLs sensitize sensory neurons through the TRPV1 channel. The mechanisms of this alteration in the TRPV1 channel, perhaps by changes in phosphorylation states of the channel, is not yet known and requires further investigation.

Unlike the other GFLs, PSP did not alter the capsaicin-stimulated release of iCGRP. Neurite outgrowth in adult DRG cultures, which can be efficiently induced by GDNF, NTN, and ART, is not caused by PSP (Lindahl et al., 2000; Paveliev et al., 2004). This inability of PSP to induce an enhancement in the capsaicin-stimulated release of iCGRP supports the current theory that adult mammalian sensory neurons are unresponsive to PSP. Even at concentrations as high as 500 ng/mL, PSP could not enhance the release of iCGRP. The use of these higher concentrations was necessary because the K_d value of PSP for its receptor, GFRα4 is ~6 mM (Enokido et al., 1998) which is much higher than the other GFLs for their preferred GFRα receptors (0.6 and 1 mM; Baloh et al., 1997; Klein et al., 1997; Trupp et al., 1998). Since PSP is not able to induce sensory neuronal sensitization at these high concentrations, it is unlikely to have significant binding or actions on the other GFRα receptors in our system.

We have demonstrated that the GFLs, GDNF, NTN, and ART, are sensitizers of neuropeptide release from sensory neurons. The level of enhancement of iCGRP release by the GFLs is similar to that produced in DRGs and TG by NGF, both in CGRP levels normalized to percent content and absolute levels of CGRP released (Hingtgen et al., 2006; Price et al., 2005). Additionally, inflammation, induced by an injection of a mixture of inflammatory mediators, noxious heat, and acidic shifts in pH enhanced the release of CGRP to similar levels as the GFLs (Eberhardt et al., 2008). The actions of the GFLs are specific to activation with capsaicin suggesting that the GFLs may induce sensitization through TRPV1-specific mechanisms. Since the levels of GFLs are greatly enhanced during inflammation, directly alter TRPV1 channel properties, and induce thermal hyperalgesia upon injection, they are clearly important mediators of sensory neuronal function. The cellular mechanisms responsible for this enhancement in release are not known. A number of candidate pathways exist, including the mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI-3K) pathways, since these pathways are activated by GFLs (Bron et al., 2003) and are known to be associated with sensory neuronal sensitization (Zhuang et al., 2004). Additionally, Ret-independent pathways may play a role in this sensitization. The Ret-independent pathways, initiated by integrin β1 and/or neural cell adhesion molecule, are a novel set of pathways induced by GFLs in sensory neurons (Cao et al., 2008a,b). It may be important to evaluate the role of these Ret-dependent and Ret-independent pathways in the GFL-induced sensory neuronal sensitization, as well as to determine how these pathways are specifically modulating TRPV1 channel function. With the GFL-induced increases in peptide release demonstrated here, we have provided a connection between the modulation of TRPV1 by GFLs and the hyperalgesia associated with the release of GFLs during inflammation.

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