Chromogranin A Induces a Neurotoxic Phenotype in Brain Microglial Cells*

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Chromogranin A (CGA) belongs to a multifunctional protein family widely distributed in secretory vesicles in neurons and neuroendocrine cells. Within the brain, CGA is localized in neurodegenerative areas associated with reactive microglia. By using cultured rodent microglia, we recently described that CGA induces an activated phenotype and the generation of nitric oxide. These findings led us to examine whether CGA might affect neuronal survival, expression of neurofilaments, and high affinity γ-aminobutyric acid uptake in neurons cultured in the presence or absence of microglial cells. We found that CGA was unable to exert a direct toxic effect on neurons but provoked neuronal injury and degeneration in the presence of microglial cells. These effects were observed with natural and recombinant CGA and with a recombinant N-terminal fragment corresponding to residues 1–78. CGA stimulated microglial cells to secrete heat-stable diffusible neurotoxic agents. CGA also induced a marked accumulation of nitric oxide and tumor necrosis factor-α by microglia, but we could not establish a direct correlation between the levels of nitric oxide and tumor necrosis factor-α and the neuronal damage. The possibility that CGA represents an endogenous factor that triggers the microglial responses responsible for the pathogenesis of neuronal degeneration is discussed.

Chromogranin A (CGA) is a polypeptidic chain of 431–445 amino acid residues corresponding to a 48–52-kDa glycoprotein, widely distributed in endocrine and nervous tissue (1, 2). In neuroendocrine secretory granules, CGA is proteolytically processed (3) leading to the formation of various small peptides, some of them with a defined biological activity. For example, CGA is the precursor of pancreastatin, a peptide that negatively modulates secretion from exocrine and endocrine pancreas (4, 5), parastatin, a peptide that inhibits parathyroid cell secretion (6), and catestatin, a peptide controlling catecholamine secretion from chromaffin cells (7). Vasostatins, which exhibit a vasoinhibitory activity when applied to blood vessels (8), contain the N-terminal domain of CGA. In addition, several chromogranin A-derived peptides have been found to exhibit an antibacterial activity (9), suggesting a potential role in the host defense against microbial infections.

Both CGA and its corresponding mRNA have been localized in a variety of neurons and glial cells in the central nervous system (10, 11) indicating a major role for CGA in the brain. Indeed, injection of CGA into the rostral ventrolateral medulla of rat brain induces changes in blood pressure and renal nerve activities (12), and injection into the nucleus accumbens modifies locomotor activity (13). Treatment with anti-psychotic drugs affects the mRNA levels of various neuropeptides and chromogranins (14). Dehydration of rats, which stimulates the secretion of vasopressin and oxytocin, induces a parallel change of CGA immunoreactivity in the hypothalamo-neurohypophyseal system (15). However, the precise function(s) of CGA in the brain remains elusive. CGA may directly modulate synaptic activity, since an inhibition of dopamine release has been reported (13). Alternatively, by analogy with peripheral neuroendocrine cells, CGA may play a role in the biosynthesis and stabilization of large dense core secretory vesicles in central neurons (15). Because CGA is secreted during synaptic activity, the protein may also have a functional significance in the extracellular space. This idea is supported by the observation that CGA accumulates in brain areas with neuronal degeneration. CGA is concentrated in the Levy bodies of substantia nigra in Parkinson’s disease, in the Pick bodies, and swollen neurons in Pick’s disease (16, 17) and tends to accumulate in the senile and pre-amyloid plaques in Alzheimer’s disease (18).

In addition, high levels of CGA have been measured in the cerebrospinal fluid of patients with Alzheimer’s disease (19).

Neurodegenerative diseases are characterized by a progressive loss of neurons, but the underlying mechanisms are not well understood. Several lines of evidence indicate that neurons and microglial cells cooperate in the initiation and development of toxic signaling pathways. Both cell-surface interactions and released molecules seem to be involved in the process of neuronal degeneration. The presence of CGA in brain lesions encouraged us to examine whether CGA may modulate the functional activity of brain microglial cells and thereby contribute to neuronal degeneration. We found that both recombinant human CGA and natural CGA purified from bovine chromaffin granules were able to induce an activated phenotype in rat microglial cells (20). CGA induced a drastic morphological change in cultured microglia that was accompanied by a complete reorganization of the actin cytoskeleton and the accumulation of nitrite, a stable metabolite of nitric oxide (NO), in the...
cortex medium. To probe further the idea that CGA might be one of the neurotoxic signaling molecules in neurodegenerative diseases, in the present study we examine the effect of CGA on neurons. For this purpose, we used highly purified neurons from rat brain cerebral hemispheres either in culture alone or in co-culture with microglial cells. The effect of CGA on neuronal survival and proliferation, on the expression of neurofilaments, and on the uptake of GABA was analyzed. Our results demonstrate that CGA impairs the function and survival of neurons, and this toxic effect requires the presence of microglia.

**MATERIALS AND METHODS**

**Purification of Bovine Chromogranin A (bCGA) and Recombinant Human Chromogranin A (rHCGA)—**bCGA was purified from bovine adrenal medullary chromaffin granules as described by Simon et al. (21) with an additional purification step on a column of Ultrasphercogel SEC 2000 (7.5 × 30 mm, Beckman). Stock solutions usually containing 50 μM bCGA were aliquoted and stored at −20 °C. The final bCGA preparation was composed of the native 70-kDa CA (60% of the total proteins) and a set of smaller processed components (60 to 43 kDa) together representing 40% of the total proteins as estimated from scanned monodimensional electrophoretic profiles. Immunodetection on nitrocellulose sheets indicated that all protein bands were immunoreactive with specific anti-native CA antibodies (21) and anti-CAg173–194 rabbit immunoglobulins (21, 22). Sequence analysis (by automatic Edman degradation on an Applied Biosystems 473A microsequencer) demonstrated that the final CGA preparation contained only CAg-derived sequences with 80% of the sequence material containing the N-terminal sequence of CA (LPVNS).

rHCGA was expressed in Escherichia coli strain BL21 (DE3) and purified in our laboratory using the multistep procedure previously described (23). VS-1 (NH2–Ser–Thr–Ala–rhCGA1–78) was cloned, purified, and characterized as described elsewhere (24). The synthetic SG173–194 peptide (YPGPQQAKEDSEGSQGPPASREK) and GB161–165 peptide (QKIAEKFSGTRG) were synthesized in our laboratory (42A Peptide Synthesizer SYNERGY, Applied Biosystems, Warrington, UK). Purity was checked on high performance liquid chromatography. Sequence analysis was performed by Edman degradation on an automated gas phase protein sequencer (Applied Biosystems, Warrington, UK), and mass spectrometry was used to assess the structure of the final products.

The content of endotoxin in the CGA preparations was determined at the Institut d’Hygiène et de Médecine Préventive (Strasbourg, France) with the chromogenic Limulus amoebocyte lysate test (Coamatic Endotoxin Chromogenex, Biogenic, Maurin, France). At 10 ng, the CA preparations contained less endotoxin than the culture medium alone, which itself is unable to trigger the production of NO in microglial cells. CGA was usually dissolved at the indicated final concentrations in serum-free defined culture medium and directly applied to cultures by centrifugation.

The activity of the CA preparations was systematically controlled by measuring the production of NO in microglial cell cultures. Acid hydrolysis of the CA preparations (6 N HCl, 18 h) totally abolished the NO2− accumulation in the culture medium.

**Neuronal Cultures—**Neuronal cell cultures were established after mechanical dispersion of cerebral cortices from Wistar rats (day 14 of gestation) rat brain as described previously (25) with minor modifications. Briefly, cerebral cortices cleaned from meninges were forced through a nylon sieve (pore size 48 μM) in nutrient medium consisting of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. The cell suspension was centrifuged and cell viability determined with trypan blue. Viable cells were plated at a density of 2.5 × 10^5 cells per well in 24 multiwell Falcon plates coated with poly-L-ornithine in DMEM supplemented with 10% fetal calf serum. For immunocytochemistry, each well contained a 12-mm glass coverslip. Cells were subsequently incubated for 1 h at 37 °C in 5% CO2 humidified atmosphere. The serum-containing medium was then replaced by serum-free defined medium consisting of DMEM supplemented with transferrin (100 μg/ml), insulin (5 μg/ml), albumin (100 μg/ml), progesterone (6 ng/ml), and sodium selenite (5.2 ng/ml).

**Microglial Cultures—**Microglial cells were isolated from high density glial cell cultures as described previously (20). Briefly, cells dissociated from cerebral hemispheres of neonatal rat brain (Wistar strain) were plated at density of 5 × 10^6 cells/cm2 in culture medium consisting of DMEM supplemented with 10% fetal calf serum. Culture medium was changed after 5 days and then twice a week. After 2 weeks, cultures contained glial cells including amoeboïd microglia mostly localized on the top of the cellular layer. The loosely adherent microglial cells were recovered by shaking. After centrifugation (1000 rpm/5 min), cell viability was determined by trypan blue exclusion, and viable cells were then plated at a final density of 2.5 × 10^6 cells per well on 24 multiwell Falcon plates coated with 10% fetal calf serum. Non-adherent cells were removed 30 min after plating by changing the medium for serum-free defined medium. Amoeboid microglia isolated from neonatal rat brain cultures consist of flat, round, or spindle-shaped, highly adherent cells. They respond to bacterial endotoxin (26) and express FC (27) and CR3 complement receptors (20).

Conditioned medium was prepared by incubating microglial cell cultures for 48 h in serum-free defined medium. Medium was subsequently collected and cleared by centrifugation (4000 rpm/20 min). To probe the heat stability of neurotoxic factors, medium conditioned by CGA-treated microglial cells was boiled for 15 min and centrifuged (12,000 rpm/20 min at 4 °C) to remove the precipitate. The supernatant was then diluted 1/1 with fresh medium and added to cultured neurons.

Neuronal/Microglial Cell Co-cultures—Co-cultures of neurons and microglial cells were prepared by plating a suspension of isolated microglia (2.5 × 10^6 cells) on neurons maintained 4 days in culture. Cells were incubated for 30 min in DMEM containing 10% fetal calf serum. Serum-free defined medium with or without additives was then added, and co-cultures were grown for at least 6 days.

**Immunocytochemical and Confocal Laser Scanning Microscopy—**Cells grown on coverslips were fixed for 5 min in methanol at −20 °C followed by brief immersion in acetone at −20 °C. Alternatively, fixation with 4% paraformaldehyde for 10 min, followed by 5 min permeabilization with 0.02% Triton X-100 in 4% paraformaldehyde was used and produced identical results. Fixed cells were incubated at room temperature for 30 min in phosphate-buffered saline containing 10% bovine serum albumin to inhibit nonspecific binding of antibodies and with propidium iodide (PI, Sigma, St. Quentin, Fallavier, France) at 25 μg/ml for 20 min to visualize nuclei before incubation for 1 h with antibodies. Rabbit polyclonal antibodies against glial fibrillary acidic protein (Dako S. A., Trappes, France) were used at 1/100 dilution, monoclonal antibodies to neurofilaments (SMI 31 Sternberger Monoclonals Inc., Baltimore, MD), and monoclonal antibodies to microtubule-associated protein 2 (SMI 52 Sternberger Monoclonals Inc.) at 1/500 dilution. After washing with phosphate-buffered saline (5 changes, 30 min), the coverslips were incubated for 1 h with the secondary antibodies which consisted of either fluorescein (FITC)-conjugated goat anti-rabbit immunoglobulins (Sigma) used at 1/100 dilution or FITC-conjugated affinity purified Fab fragment of goat anti-mouse class G F(ab′)_2 fragment (Jackson Immunoresearch Laboratories, West Grove, PA). Coverslips were subsequently washed with phosphate-buffered saline (5 changes, 30 min) and mounted in Moviol (Calbiochem, Meudon, France).

Mounted coverslips were examined with a Zeiss Axioskop microscope equipped with an epifluorescence system and appropriate filters. Sequential through-focus images of labeled cells were obtained using a confocal microscopy (LSM 410 invert) and an argon 488-nm and a helium/neon 543-nm lasers. The emission signals were filtered with a 515–565-nm filter (FITC) or with a long pass 595-nm filter (rhodamine). The images were recorded using identical laser power, wavelength, and photomultiplier tube voltage. They were recorded digitally in a 768 × 556 pixel format and saved on an magneto optical disk. Digital data were downloaded to a color video printer, Sony UP-1800EPM.

**Cell Viability Assays—**To estimate the effect of CGA on neuronal survival, living cells were incubated at 37 °C for 20 min with PI (25 μg/ml) in culture medium before fixation. Under these conditions, PI stains only the nuclei of damaged cells having a permeable membrane. Cells were then fixed and immunolabeled with anti-neurofilament antibody revealed with FITC-conjugated secondary antibodies.

The viability of cells in neuronal cultures and in neuronal/microglial co-cultures was also estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a technique that is based on the reduction of MTT by mitochondrial enzymes (28). Cultures were incubated for 6 h in MTT (1 mg/ml) which is converted into an insoluble blue formazan product by mitochondrial enzymes. The blue formazan solubilized by the addition of isopropl alcohol, 0.08 % HCl, and the optical density of the mixture was measured within 1 h at 570 and 630 nm. The value at 630 nm was subtracted from the value at 570 nm.

**Uptake of [3H]GABA—**Neuronal cell cultures and neuronal/microglial co-cultures in 24 multiwell Falcon plates were washed three times with 500 μl of DMEM without serum and incubated for 30 min at 37 °C.
with 1 mM β-alanine (Sigma) to inhibit the uptake of GABA by astroglial cells (29, 32). No uptake of GABA was found in microglial cultures without neurons. The incubation medium was then discarded, and the cultures were further incubated for 10 min at 37 °C with 100 nM [3H]GABA (92 Ci/mmol; Amersham Pharmacia Biotech, France) in the presence of β-alanine. Control values were obtained by the addition of [3H]GABA to cell-free wells. Cells were subsequently washed with DMEM (three times) and extracted with NaOH (0.1 N, 400 μl per well). After neutralization with HCl (1 N, 40 μl per well), the radioactivity present in the extracts was estimated by scintillation counting with Rotiszint (Roth, Karlsruhe, Germany).

NO2− Determination—NO2− levels, measured with the Griess reagent, were taken as an estimate of NO generation (30). Griess reagent consists of an equal volume of (i) 0.1% (w/v) N-1-naphthylethenediamine dichloride (Sigma) in water and (ii) 1% (w/v) sulfanilamide (Sigma) plus 5% (v/v) H3PO4 in water. NO2− was measured in the supernatants of cells grown on 24-multiwell Falcon plates. Briefly, a volume of 350 μl of supernatant was mixed with an equal volume of Griess reagent. After 10 min of incubation at room temperature, the optical density of the mixture was read at 546 nm. A standard curve was established using NO2− (Merck, Darmstadt, Germany) in a concentration range from 0 to 50 μM. Data were corrected for the background levels of nitrite in the cell-free medium.

RESULTS

Characteristics of Brain Cortical Neurons Cultured in Serum-free Medium—Neurons derived from rat embryonal brain cortex maintained in serum-free medium formed small aggregates and within 4 days of culture extended neuritic processes, immunoreactive with anti-neurofilament antibodies. Since in the presence of cytosine arabinoside, an inhibitor of cellular proliferation, neurons showed morphological damage and marked reduction in neuronal specific [3H]GABA uptake (data not shown); the present study was performed without mitotic inhibitors. Instead, the presence of glial cells was systematically controlled by staining parallel cultures with anti-glial fibrillary acidic protein antibodies. Glial fibrillary acidic protein-positive cells usually represent 3–5% of the total cell population. Cultures were discarded when a higher percentage was detected. The extension of neuronal processes and the expression of neurofilaments was complete within 4 days in culture. Furthermore, up to 14 days in culture in serum-free medium, neurons showed no sign of degeneration such as the retraction of processes and loss of cell adhesion. Therefore, we chose day 4 in culture to establish the co-cultures with microglial cells.

Effects of Chromogranin A on Neurons in the Absence of Microglial Cells—To examine the effect of natural bovine CGA on neuron survival and neurofilament expression, neurons after 4 days in culture were incubated with 10 nM bCGA for 6 days. We previously demonstrated that the maximal effect of natural and recombinant CGA on microglia is obtained at 1 nM (20). Fig. 1 illustrates a double-staining experiment with anti-neurofilament antibodies and with the DNA-specific fluorochrome PI. Cells were exposed to PI before fixation. Under these conditions, PI labels only extracellular DNA fragments and the nuclei of damaged cells displaying a permeable membrane. Treatment with bCGA did not modify the nuclear staining in cells expressing neurofilaments, indicating that CGA apparently did not alter neuronal viability. Note the presence of Schwann cells, a contaminating cell population in the absence (circles) or presence (squares) of 10 μg/ml bCGA and exposed to [3H]thymidine for the indicated times. The incorporated radioactivity was then determined (open symbols). The neuronal uptake of GABA (closed symbols) was estimated in parallel experiments by incubating control and CGA-treated neurons for 10 min with 100 nM [3H]GABA. Values are means of six determinations ± S.E. Similar results were obtained in three separate experiments.

FIG. 1. Effect of chromogranin A on survival, expression of neurofilaments, [3H]thymidine incorporation, and [3H]GABA uptake in cultured neurons. Neurons in culture were incubated for 6 days in the absence (A) or in the presence (B) of 10 nM bCGA. Cells were then incubated with propidium iodide and after fixation stained with anti-neurofilament antibodies revealed with fluorescein. Confocal images obtained in the rhodamine (propidium iodide) and fluorescein channels were recorded simultaneously in the same optical section by a double-exposure procedure. Bar = 25 μm. C, neurons were incubated in the absence (circles) or presence (squares) of 10 nM bCGA and exposed to [3H]thymidine for the indicated times. The incorporated radioactivity was then determined (open symbols). The neuronal uptake of GABA (closed symbols) was estimated in parallel experiments by incubating control and CGA-treated neurons for 10 min with 100 nM [3H]GABA. Values are means of six determinations ± S.E. Similar results were obtained in three separate experiments.
of some prominently stained nuclear fragments reflecting cell debris which are common features of neurons cultured in the absence of glial cells (Fig. 1, A and B).

To test further a possible direct toxic effect of CGA on neurons, we examined the effect of bCGA on neuronal proliferation assessed by the incorporation of [3H]thymidine. As shown in Fig. 1C, neurons in culture displayed mitotic activity, as demonstrated by the significant incorporation of [3H]thymidine at day 1, which progressively declined with time in culture. Exposure of neuronal cultures to 10 nM bCGA did not change the pattern of [3H]thymidine incorporation, clearly indicating that the protein does not interfere with the capacity of neurons to proliferate. We also used the high affinity neuronal [3H]GABA uptake as an index of functional behavior, since it has been previously reported that at least 50% of the cortical neurons are GABAergic when maintained in culture (31). Indeed, we found that the uptake of [3H]GABA in rat brain cortical neurons progressively increased up to 3–4-fold between days 1 and 6 in culture (Fig. 1C). Incubation of cortical neurons with 10 nM bCGA (Fig. 1C) or 10 nM recombinant human CGA (data not shown) did not significantly affect the maximal values of [3H]GABA uptake, further indicating that CGA does not provoke neuronal cell injury.

Effect of Chromogranin A on Neuronal/Microglial Cell Co-cultures—We previously reported that CGA is able to induce an activated phenotype in microglia (20). Since activated microglia release cytotoxic substances potentially responsible for neuronal degeneration (32–34), we examined the effect of CGA on neurons co-cultured with microglia. Fig. 2 illustrates a neuronal/microglial co-culture double-labeled with PI and anti-neurofilament antibodies. In the absence of CGA, the survival of neurons as judged by the density of the neurofilament network was not affected by a 6-day co-culture with microglial cells (Fig. 2A). However, addition of 10 nM bCGA to the co-culture medium for 6 days markedly reduced the expression of neurofilaments and increased the number of compacted PI-labeled nuclei, indicating that neurons progressively degenerated (Fig. 2B). The remaining neuronal processes appeared abnormally dilated, and almost all cells were damaged in CGA-treated co-cultures. The microglial cell density required to produce maximal toxic effect in response to 10 nM bCGA was 2.5 × 10⁶ microglia per well, representing a 1/1 neuron/microglia cell ratio (Fig. 2B). Neuronal damage and disruption of neurofilaments were also evident in a co-culture with a 2/1 neuron/microglia cell ratio (data not shown).

To confirm further the effect of CGA on neuronal viability in neuronal/microglial cell co-cultures, we performed the MTT assay in control and 10 nM bCGA-treated co-cultures. MTT assay is a sensitive indicator of mitochondrial damage preceding cell death (35). Compared with untreated co-cultures, treatment with 10 nM bCGA provoked a 50% decrease in MTT reduction after 6 days of incubation (Fig. 2C), an observation which is consistent with the decrease in cell viability detected by microscopic observation.

The functional activity of cortical neurons cultured with microglial cells was estimated by measuring [3H]GABA uptake. As shown in Fig. 3, addition of bCGA to the co-culture produced a dramatic dose-dependent decrease of the neuronal uptake of GABA. At 10 nM, bCGA decreased the [3H]GABA uptake after 3 and 6 days of treatment by 70 and 95%, respectively. Similarly, rhCGA at 10 nM inhibited the neuronal uptake of GABA after 6 days of incubation (see Fig. 5C), indicating that the recombinant protein was as effective as the natural protein in promoting neuronal injury and that CGA displayed no difference in activity when used across species.

Chromogranin A Stimulates the Production of TNF-α by
Microglial Cells—Recent studies suggested that TNF-α is involved in the activation of microglia induced by β-amyloid protein and may thereby contribute to the pathogenesis of neuronal degeneration observed in aging and in Alzheimer’s disease (36). Therefore, we investigated the effect of CGA on TNF-α production by microglial cells cultured alone or together with neurons. In the absence of CGA, the level of TNF-α in the culture medium was near the limit of detection (Fig. 4), indicating that microglia constitutively produce only very small amounts of this cytokine. CGA triggered a dramatic increase in the levels of TNF-α within 1 day of incubation. In the presence of 10 nM bCGA, the concentration of TNF-α reached approximately 50 and 30 ng/ml in the culture medium of microglial cells and neuronal/microglial co-cultures, respectively (Fig. 4).

To examine the possible direct neurotoxic effect of TNF-α, cultured neurons were exposed for 6 days to recombinant rat TNF-α. Table I shows that TNF-α at 50 and 100 ng/ml did not significantly modify the uptake of [3H]GABA. Accordingly, pre-incubation of neuronal/microglial co-cultures with a polyclonal anti-TNF-α antibody could not prevent the CGA-induced decrease in neuronal [3H]GABA uptake (data not shown), indicating that TNF-α by itself was unable to alter the functional activity of neurons.

Chromogranin A-induced Neurotoxicity in Neuronal/Microglial Cell Co-cultures Is Not Mediated by Nitric Oxide—CGA triggers the production of nitrite (NO) in microglia by a mechanism involving the inducible form of nitric oxide synthase (iNOS) (20). In order to determine whether nitrite is involved in the CGA-induced neuronal cell injury observed in neuronal/microglial co-cultures, we examined the effect of 1-[N^ω-(1-imino-ethyl)ornithine hydrochloride (L-NIO), one of the most selective inhibitors of iNOS (37), on the CGA-induced accumulation of NO_2^−, a stable derivative of NO. Fig. 5A shows that, in microglial cultures, 10 nM bCGA triggered an important accumulation of NO_2^− over a 6-day incubation period. In neuronal/microglial co-cultures, both natural and recombinant CGA also increased the level of NO_2^− after 6 days of treatment (Fig. 5B). Addition of 100 μM L-NIO to the culture medium completely abolished the CGA-evoked increase of NO_2^− in microglial cultures (Fig. 5A) and in neuronal/microglial co-cultures (Fig. 5B).

We then examined the effect of L-NIO on [3H]GABA uptake in neuronal/microglial co-cultures incubated with either 10 nM bCGA or 10 nM rhCGA. No significant effect on CGA-evoked inhibition of [3H]GABA uptake was found with 100 μM L-NIO (Fig. 5C). Similarly, 1 mM N-[ω-nitro-l-arginine methyl ester, another competitive inhibitor of l-arginine on NO_2^− production, strongly reduced the CGA-evoked accumulation of nitrite in neuronal/microglial co-cultures without affecting the CGA-induced inhibition of [3H]GABA uptake (data not shown). These observations suggest that the high levels of nitrite present in CGA-treated co-cultures do not apparently mediate neuronal cell injury.

The accumulation of reactive oxygen species has been described as a potent factor of neurotoxicity (38). To examine whether the combination of NO with reactive oxygen species formed by microglia in response to CGA is neurotoxic, neuronal/microglial cell co-cultures were exposed to CGA in the absence of the H_2O_2-scavenging enzyme catalase in combination with superoxide dismutase. Even at high concentration, catalase (520 units/ml) with or without superoxide dismutase (300 units/ml) was unable to prevent the reduction of neuronal [3H]GABA uptake triggered by a 6-day treatment with 10 nM bCGA (data not shown). Furthermore, staining with 2,7-dichlorofluorescein diacetate indicated that CGA did not stimulate the formation of intracellular peroxides in neurons (data not shown), making the involvement of peroxynitrite in the observed neurotoxic effect unlikely.

**FIG. 3.** Effect of chromogranin A on the high affinity uptake of [3H]GABA in neurons cultured with microglial cells. Neuronal/microglial co-cultures were incubated for the indicated times with 1 or 10 nM bCGA, and the uptake of [3H]GABA was subsequently determined. For each incubation period, results are expressed relative to the uptake measured in control cells. Values are the means of six determinations ± S.E. Similar results were obtained in three separate experiments. 1 nM CGA (open columns) significantly inhibited the uptake of GABA after 6 days of treatment. Exposure of co-cultures to 10 nM bCGA (closed columns) strongly reduced the GABA uptake after 3 days (70% inhibition) and nearly abolished it after 6 days of treatment.

**FIG. 4.** Effect of chromogranin A on the release of TNF-α from rat microglia. TNF-α was determined in the culture medium from microglial cultures (M) or neuronal/microglial co-cultures (N + M) using an enzyme-linked immunosorbent assay kit with a detection limit of 30 pg/ml. Cells were incubated for 1 day in the absence (open columns) or presence (filled columns) of 10 nM bCGA. Values are means of eight determinations performed in the same experiment ± S.E.

| TABLE I | Effect of TNF-α on neuronal [3H]GABA uptake |
|---------|-----------------------------------------|
| Cultured neurons were incubated for the indicated times with either 50 or 100 ng/ml rat recombinant TNF-α, and the uptake of [3H]GABA was subsequently determined. Values are the means of six determinations ± S.E. Similar results were obtained in three separate experiments. |
| [3H]GABA uptake | 1 day | 2 days | 3 days | 4 days | 5 days | 6 days |
|------------------|-------|-------|-------|-------|-------|-------|
|                   | fmol/min | % control | fmol/min | % control | fmol/min | % control | fmol/min | % control | fmol/min | % control |
| Control           | 211 ± 11 | 100 | 354 ± 27 | 100 | 491 ± 18b | 100 | 645 ± 22b | 100 |
| TNF-α 50 ng/ml    | 215 ± 9 | 102 ± 4 | 419 ± 23 | 118 ± 6 | 471 ± 31 | 96 ± 6 | 645 ± 22b | 100 |
| TNF-α 100 ng/ml   | 219 ± 45 | 101 ± 21 | 368 ± 64 | 104 ± 18 | 435 ± 12b | 88 ± 2 | 645 ± 22b | 100 |

*Days of treatment.

* p > 0.01 when tested by Student’s t test.
CGA Induces Neurotoxicity in Microglia

In order to obtain more information on the nature of the neurotoxic factor(s) responsible for the impairment of neuronal function in CGA-exposed co-cultures, we collected culture medium from microglial cells treated for 2 days with 10 nM bCGA and examined its effect on neurons. Fig. 6 shows that culture medium from CGA-untreated microglia did not change the uptake of [3H]GABA in neuronal cultures. In contrast, medium conditioned by CGA-treated microglia provoked a marked reduction in neuronal [3H]GABA uptake (Fig. 7A). VS-1 produced a similar reduction in neuronal functional activity since 10 nM VS-1 inhibited [3H]GABA uptake by approximately 50%, possibly reflecting the lower concentration of the toxic factors. However, the boiled medium conditioned by CGA-treated microglia reduced its inhibitory effect on [3H]GABA uptake by approximately 50%, possibly reflecting the lower concentration of the toxic factors. Therefore, medium collected from CGA-stimulated microglia was maintained at 37 °C or boiled at 100 °C for 15 min. Since boiling of culture medium usually triggers a 20% reduction in neuronal [3H]GABA uptake, conditioned media were first diluted with fresh culture medium to obtain a 1 to 1 ratio prior to incubation with neurons. As illustrated in Fig. 6, diluting the medium conditioned by CGA-treated microglia reduced its inhibitory effect on [3H]GABA uptake by approximately 50%, possibly reflecting the lower concentration of the toxic factors. However, the boiled medium conditioned by CGA-treated microglia and diluted to a 1 to 1 ratio retained a comparable toxic effect since it reduced the [3H]GABA uptake by 40% (Fig. 6). These observations suggest that the neurotoxic effect exerted by microglia in response to CGA is due to diffusible heat-stable factors rather than to close contact between microglial cells and neurons.

A Recombinant Chromogranin A N-terminal Fragment Promotes Neurotoxicity in Neuronal/Microglial Cell Co-cultures—In the search for the CGA domain which stimulates microglial cells and promotes neuronal injury, we tested a previously described recombinant N-terminal fragment of human CGA, VS-1 corresponding to residues 1–78 (24, 39). VS-1 was recently found to increase adhesion and spreading of fibroblasts at concentrations in the 10–50 μM range (39), suggesting that VS-1 is a valuable tool for investigating the biological activity of CGA N-terminal domain. Since fetal calf serum was apparently required for VS-1 adhesive activity (39), the effect of VS-1 on neuronal/microglial co-cultures was assessed in the presence of 1% fetal calf serum. Under these conditions, exposure to 10 nM bCGA for 6 days inhibited the neuronal [3H]GABA uptake by 51% (Fig. 7A). VS-1 produced a similar reduction in neuronal functional activity since 10 μM VS-1 inhibited [3H]GABA uptake by 53% (Fig. 7A). For comparison, we also examined the effects of synthetic peptides corresponding in sequence to residues 173–194 of CGA and residues 614–626 of CGB. At 10 μM, neither CGA(173–194) nor CGB(614–626) significantly inhibited the neuronal [3H]GABA uptake (Fig. 7A). We then investigated the effects of VS-1,
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CGA173–194 and CGB(614–626) on nitrite production by microglia. In contrast to full-length CGA, the N-terminal VS-1 fragment or the synthetic CGA(173–194) and CGB(614–626) peptides were unable to stimulate the accumulation of NO2 in the cell incubation medium (Fig. 7B). These observations reinforce the idea that NO does not mediate the neuronal injury produced by CGA-stimulated microglial cells. Furthermore, these findings reveal that the release of neurotoxic factors by microglia is an activity due at least partially to the N-terminal domain of the CGA molecule.

**DISCUSSION**

Microglial cells provide the nervous system with its first line of defense against damage and infection (33). The pathophysiology of Alzheimer’s disease is intimately associated with local inflammatory processes, and activated microglia are frequently present, close to diffuse and dense core neuritic amyloid deposits (40, 41). Although the activation of microglia represents a beneficial physiological response in host defense, sustained activation of microglial cells may be harmful and lead to the extensive damage of neighboring cells (34). Thus, by releasing neurotoxic agents (reactive oxygen and nitrogen intermediates, proteases, pro-inflammatory cytokines), activated microglia are likely to contribute to the progression of neuronal dysfunction. The factors that trigger microglial activation in Alzheimer’s disease remain elusive. Besides β-amyloid protein, various inflammatory factors and their receptors have been described in the extracellular deposits (40, 42, 43), and the possible combination of these factors could well cause gradual chronic and sustained immune responses in microglia. CGA has also been found in senile and pre-amyloid plaques, where the protein co-localizes with β-amyloid protein (17, 18, 44) and has been detected in large dystrophic neurites containing the amyloid precursor protein (45). These observations suggest that CGA might be one of the natural inflammatory factors that trigger microglial activation. In agreement with this hypothesis, we previously described that CGA purified from bovine chromaffin granules as well as human recombinant CGA induce an activated phenotype in rat microglia in culture (20). Both natural and recombinant CGA elicited calcium transients preceding the reorganization of the actin cytoskeleton and stimulated the production of NO in microglia (20).

In the present report, we focused on the possible capacity of CGA to trigger toxic effects on neurons. The potential neurotoxicity of CGA was first assessed in pure neuronal cultures, using a neuron-specific marker (neurofilaments) and the high affinity GABA uptake which is a sensitive indicator of the functional activity of neurons from brain cortex (31, 36). Our results indicate that CGA by itself does not appear to affect the survival and expression of neurofilaments of rat cortical neurons. In contrast, CGA-induced cell injury and degeneration in neurons cultured in the presence of microglial cells, as seen by the drastic reduction in both neurofilament expression and GABA uptake in neuronal/microglial co-cultures exposed to CGA. The microglial component of the co-culture was important since the neurotoxic effects produced by 10 nM CGA were never observed in the absence of microglia and were maximal at a neuronal/microglial cell ratio of 1/1. The development of neuronal cell injury required several days of treatment with CGA. This rather slow time course of the CGA-evoked response is consistent with the fact that CGA does not interfere directly with neurons but is likely to induce an immune-mediated pathway through the activated phenotype of microglia (20).

CGA triggers the production of NO in microglia by a mechanism that involves the induction of iNOS (20). A sustained production and high levels of NO have been suspected to be the main neurotoxic factors responsible for neuronal degeneration (33). In co-cultures of neurons with microglia, CGA was also able to induce an increase of NO, but we could not establish a direct correlation between the levels of nitrite in the culture medium and the impairment of GABA uptake evoked by CGA. It has been recently suggested that NO may not have a toxic effect on its own but may contribute to the generation of peroxynitrite, a toxic oxidant formed in a rapid and diffusion-limited manner from NO superoxide (37, 38). However, treatment with free radical scavengers was unable to protect neurons in co-culture with microglial cells, suggesting that the neurotoxic factor induced by CGA may be different. Furthermore, incubation of neurons with culture medium conditioned by CGA-activated microglia provoked a similar neuronal injury, indicating that the toxic factors secreted by CGA-activated microglia are active despite the spatial segregation of microglia and neurons. These observations suggest that short-lived radical intermediates are probably not involved, since such agents require close proximity between secretory and target cells.

The neurotoxic factors recovered from medium conditioned by CGA-activated microglia are at least in part heat-stable. A number of cytotoxic factors have been reported to participate in the β-amyloid protein-induced neurotoxicity, including a heat-stable neurotoxin recovered from activated human microglia.
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