Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) 38 and PACAP4–6 Are Neuroprotective through Inhibition of NADPH Oxidase: Potent Regulators of Microglia-Mediated Oxidative Stress

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ABSTRACT

Microglial activation is implicated in the progressive nature of numerous neurodegenerative diseases, including Parkinson’s disease. Using primary rat mesencephalic neuron-glia cultures, we found that pituitary adenylate cyclase-activating polypeptide (PACAP) 38, PACAP27, and its internal peptide, Gly-Ile-Phe (GIF; PACAP4–6), are neuroprotective at 10^{-13} M against lipopolysaccharide (LPS)-induced dopaminergic (DA) neurotoxicity, as determined by [3H]DA uptake and the number of tyrosine hydroxylase-immunoreactive neurons. PACAP38 and GIF also protected against 1-methyl-4-phenylpyridinium (MPP+) neurotoxicity with the number of tyrosine hydroxylase-immunoreactive neurons. PACAP38 and GIF ameliorated the production of microglia-derived reactive oxygen species (ROS), where both LPS- and phorbol 12-myristate 13-acetate-induced superoxide and intracellular ROS were inhibited. The critical role of NADPH oxidase for GIF and PACAP38 neuroprotection against LPS-induced DA neurotoxicity was demonstrated using neuron-glia cultures from mice deficient in NADPH oxidase (PHOX^{-/-}), where PACAP38 and GIF reduced tumor necrosis factor α production and were neuroprotective only in PHOX^{-/-} cultures and not in PHOX^{-/+} cultures. Pretreatment with PACAP6–38 (3 μM; PACAP-specific receptor antagonist) was unable to attenuate PACAP38, PACAP27, or GIF (10^{-13} M) neuroprotection. PACAP38 and GIF (10^{-13} M) failed to induce cAMP in neuron-glia cultures, supporting that the neuroprotective effect was independent of traditional high-affinity PACAP receptors. Pharmacophore analysis revealed that GIF shares common chemical properties (hydrogen bond acceptor, positive ionizable, and hydrophobic regions) with other subpicomolar-acting compounds known to inhibit NADPH oxidase: naloxone, dextromethorphan, and Gly-Gly-Phe. These results indicate a common high-affinity site of action across numerous diverse peptides and compounds, revealing a basic neuropeptide regulatory mechanism that inhibits microglia-derived oxidative stress and promotes neuron survival.

Parkinson’s disease (PD) is characterized by the progressive and selective degeneration of dopaminergic (DA) neurons in the substantia nigra, but the etiology and the precise mechanisms underlying the selective destruction of the nigrostriatal dopaminergic pathway are unknown. Recently, increasing evidence from clinical (McGeer et al., 1988) and animal studies (Castano et al., 1998; Sriram et al., 2002) suggests the involvement of inflammation in the pathogenesis of PD. Inflammation in the brain is characterized by the activation of microglia (Aloisi, 1999), the resident innate immune cells in the central nervous system. Microglia are activated by a diverse list of environmental (particulate matter, pesticides, heavy metals) and endogenous triggers (α-synuclein, β-amyloid) to produce reactive oxygen species (ROS) and/or proinflammatory factors, which are toxic to

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ABBREVIATIONS: PD, Parkinson’s disease; DA, dopaminergic; ROS, reactive oxygen species; PACAP, pituitary adenylate cyclase-activating polypeptide; VIP, vasoactive intestinal peptide; LPS, lipopolysaccharide; GGF, Gly-Gly-Phe; GIF, Gly-Ile-Phe; TH, tyrosine hydroxylase; DCF-DA, 2',7'-dichlorofluorescin diacetate; DA, dopamine; TNF, tumor necrosis factor; NO, nitric oxide; SOD, superoxide dismutase; HBSS, Hank’s balanced salt solution; MPP+, 1-methyl-4-phenylpyridinium; PMA, phorbol-12-myristate 13-acetate; PAC1, PACAP receptor; VPAC, VIP/PACAP receptor; WST, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium; DCFH, 2',7'-dichlorodihydrofluorescein.
neurons (Block and Hong, 2005). DA neurons are more vulnerable to oxidative damage compared with other cell types (Jenner, 1996), and microglia are the predominant source of inflammation and oxidative stress. Thus, inhibition of microglial activation and production of extracellular superoxide are critical to DA neuron survival. There is increasing support that neuropeptides are components of a critical homeostatic mechanism regulating the inflammatory milieu in the brain (Block et al., 2006). However, the details of the homeostatic mechanisms regulating microglial function are poorly defined.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is widely distributed in the peripheral and central nervous system, where PACAP release is reported to serve as a neuronal survival factor (Arimura et al., 1994; Arimura, 1998). PACAP is a member of the secretin/glucagon/vasoactive intestinal polypeptide (VIP) family and has the highest sequence similarity to VIP. However, PACAP is 1000 to 10,000 times more potent than VIP in pituitary cells, neurons, and astrocyte cultures (Arimura et al., 1994). PACAP exists in two amidated forms with 38 and 27 amino acid residues, designated PACAP38 and PACAP27, respectively (Miyata et al., 1989, 1990). In tissues, PACAP38 is the predominant form, whereas PACAP27 constitutes less than 10% of the total PACAP (Arimura et al., 1991). PACAP is reported to have diverse functions because PACAP38 has been shown to act as a neurotransmitter/neuromodulator (Kozicz et al., 2000). PACAP acts as a neuronal survival factor (Arimura et al., 1994; Arimura, 1998) and has diverse functions because PACAP38 has been shown to exert its effects through anti-inflammatory properties (Kong et al., 1999). Although PACAP is often reported to exert its effects through the PAC1 and VPAC2 receptors (Vaudry et al., 2000; Yamada et al., 1999), PACAP receptors are critical to DA neuron survival. There is increasing support that neuropeptides are components of a critical homeostatic mechanism regulating the inflammatory milieu in the brain (Block et al., 2006). However, the details of the homeostatic mechanisms regulating microglial function are poorly defined.

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The potential therapeutic benefits of biologically active compounds at subpicomolar concentrations (Gozes et al., 1999; Wilkemeyer et al., 2003) is receiving wide attention. Recently, we identified a class of compounds that are neuroprotective at subpicomolar concentrations through the inhibition of NADPH oxidase (Qin et al., 2005a) and the consequent reduction of extracellular superoxide production. Previously, we reported that Gly-Gly-Phe (GGF), a tripeptide from dynorphin, is chemically and functionally similar to other previously reported subpicomolar-acting compounds, such as dextromethorphan (Li et al., 2005) and naloxone (Qin et al., 2005a). Although the detailed mechanisms are still being investigated, the high-affinity receptor mechanism is likely to be independent of traditional opiate receptors because both opiate agonists (dynorphin) and antagonists (naloxone) elicit anti-inflammatory and neuroprotective effects. However, other opiate peptides containing the GGF peptide sequence, such as leucine enkephalin and des-tyrosine leucine enkephalin, are also neuroprotective at subpicomolar concentrations through the inhibition of NADPH oxidase (Qin et al., 2005b), suggesting that this high-affinity site of action may be a common mechanism of microglia regulation.

In the current study, we tested whether subpicomolar concentrations of PACAP and the internal PACAP4-6 tripeptide Gly-Ile-Phe (GIF) fall into the class of neuropeptides that inhibit microglial activation at subpicomolar concentrations through NADPH oxidase. Here, we report for the first time that PACAP and its peptide fragment GIF are neuroprotective through inhibition of NADPH oxidase and consequent reduction of microglia-derived ROS. Furthermore, using pharmacophore analysis, we show that GIF has chemical similarities to other previously reported subpicomolar-acting compounds, suggesting a common high-affinity site of action and mechanism of microglia regulation for several neuropeptides.

Materials and Methods

Animals. Timed pregnant Fisher F344 rats were obtained from Charles River Laboratories (Raleigh, NC). Eight-week-old (25–30 g) male and female B6.129S6-Cybβtm1Din (PHOX^{+/+}) and C57BL/6J (PHOX^{+/-}) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in a strict pathogen-free environment. The PHOX^{+/-} mice lack the functional catalytic subunit of the NADPH oxidase complex gp91. NADPH oxidase is an inducible electron transport system in phagocytic cells that is responsible for the generation of the respiratory burst. PHOX^{+/-} mice are unable to generate extracellular superoxide in response to LPS or other immunological stimuli. The PHOX^{+/-} mutation is maintained in the C57BL/6J background; thus, the C57BL/6J (PHOX^{+/-}) mice were used as control animals. Breeding of the mice was designed to achieve accurate timed pregnancy at 13 ± 0.5 days. Housing, breeding, and experimental use of the animals were performed in strict accordance with the National Institutes of Heath guidelines and were approved by the Institute’s Animal Care and Use Committee.

Reagents. PACAP38 was purchased from American Peptide Company (Sunnyvale, CA). PACAP27, PACAP6-38, and GIF were purchased from Phoenix Pharmaceuticals, Inc. (Belmont, CA). Cell culture materials were obtained from Invitrogen (Grand Island, NY). The polyclonal anti-tyrosine hydroxylase (TH) antibody was a generous gift from Dr. John Reinhard (GlaxoSmithKline, Research Triangle Park, NC). The Vecta stain ABC kit and biotinylated secondary antibodies were purchased from Vector Laboratories (Angels Island, CA). The polyclonal anti-tyrosine hydroxylase (TH) antibody was a generous gift from Dr. John Reinhard (GlaxoSmithKline, Research Triangle Park, NC). The Vecta stain ABC kit and biotinylated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA). WST-1 was purchased from Dojindo Laboratories (Gaithersburg, MD). Lipopolysaccharide (strain O111:B4) and 2,7'-dichlorofluorescein diacetate (DCF-DA) were obtained from Calbiochem (La Jolla, CA). 5HIDA (28 Ci/mmol) was purchased from NEN Life Science (Boston, MA). All other reagents came from Sigma (Minneapolis, MN).

Primary Mesencephalic Neuron-Glia Cultures. Neuron-glia cultures were prepared from the ventral mesencephalic tissues of embryonic days 13 to 14 rat and mice, as described previously (Liu and Hong, 2003). In brief, midbrain tissues were dissected from day 14 Fisher 344 rat or mouse embryos (PHOX^{+/+} or PHOX^{+/-}). Cells were dissociated via gentle mechanical trituration in minimum essential medium and immediately seeded (5 × 10^6/well) in poly-D-lysine (20 μg/ml)- precoated 24-well plates. Cells were seeded in maintenance media and exposed to the treatment medium described previously (Liu et al., 2001). Three days after seeding, the cells were replenished with 500 μl of fresh maintenance medium. Cultures were exposed 7 days after seeding. At the time of treatment, cells were pretreated for 30 min with vehicle or indicated concentration of PACAP/GIF in treatment media, followed by exposure to 2.5 ng/ml LPS.

Microglia-Enriched Cultures. Primary microglia-enriched cultures were prepared from the whole brain of 1-day-old Fisher 344 rat...
pups, using a previously described procedure (Liu et al., 2001). Cells were treated 24 h after seeding the microglia.

**Microglia-Depleted Cultures.** Primary microglia-depleted cultures were prepared as described previously (Wang et al., 2006) by adding 1 μM leucine methyl ester into the primary neuron-glial cultures 24 h after the initial seeding. The medium was changed 6 days later. Seven-day-old cultures were used for treatment. At the time of treatment, ICC analysis indicated that the microglial composition was <0.1%.

**Dopamine Uptake Assay.** DA uptake assays were performed as previously described (Liu et al., 2000b). In brief, after washing twice with warm Krebs-Ringer buffer (16 mM NaH2PO4, 119 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, and 1.3 mM EDTA, pH 7.4), cultures were incubated for 20 min at 37°C with 1 μM [3H]DA in Krebs-Ringer buffer for DA uptake. Afterward, cultures were washed three times with ice-cold Krebs-Ringer buffer, and cells were then dissolved in 1 N NaOH. Radioactivity was determined by liquid scintillation counting. Nonspecific [3H]DA uptake determined in the presence of 10 μM mazindol was subtracted.

**Immunostaining.** DA neurons were recognized with the anti-TH antibody, as described previously (Liu et al., 2000a). In brief, formaldehyde (3.7%)-fixed cultures were treated with 1% hydrogen peroxide followed by sequential incubation with a blocking solution (30 min), the primary antibody (overnight, 4°C), a biotinylated secondary antibody (2 h), and ABC reagents (40 min). Color was developed with 3,3′-diaminobenzidine. For morphological analysis, images were acquired using an inverted microscope (Nikon, Tokyo, Japan) connected to a camera (DAGE-MTI, Michigan City, IN) operated with MetaMorph software (Universal Imaging Corporation, Downingtown, PA). To quantify cell numbers, nine representative areas per well in the 24-well plate were counted under the microscope at 100× magnification by two individuals. The average of these two scores was reported.

**Tumor Necrosis Factor α Assay.** Culture supernatants were collected after 3 h of stimulation with LPS. The concentration of tumor necrosis factor (TNFα) was measured with a mouse TNFα enzyme-linked immunosorbent assay kit from Genzyme (Cambridge, MA). The threshold for detection with this assay was approximately 15 pg/ml. The antisera did not have detectable cross-reactivity with any other cytokines.

**cAMP Assay.** At 30 min after treatment with PACAP peptides, cells were lysed, and cAMP was measured, as described in the cAMP Parameter Assay Kit (R&D Systems, Minneapolis, MN).

**Nitrite Oxide Assay.** The production of nitric oxide (NO) was assessed as the accumulation of nitrite in the culture supernatants, using a colorimetric reaction with the Griess reagent. The culture supernatants were collected after 24 h of stimulation with LPS and mixed with equal volumes of the Griess reagent (0.1% N-[1-naphthalyl] ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H3PO4). The absorbance at 540 nm was measured with a UV MAX Plus microtiter plate spectrophotometer (Molecular Devices).

**Pharmacophore Analysis.** The Catalyst version 4.9.1 software (Accelrys, San Diego, CA) was used for conformational and pharmacophore analysis. All molecules were built within the Catalyst Modeling software, and peptides were constructed as linear chains. The maximum number of conformers generated for each molecule was 250. Conformers with relative energy >20 kcal/mol were removed. The best (most rigorous and time-consuming) conformer generation method was used. The following chemical features were included in the development of the pharmacophore model: hydrogen bond acceptor, hydrogen bond donor, hydrophobic, negative charge or negative ionizable, positive ionizable, aromatic ring, and hydrophobic aromatic.

Small molecules and peptides previously determined to be neuroprotective through inhibition of NADPH oxidase at submicromolar concentrations (naloxone, GGF, and dextromethorphan) were used to generate the 10 best pharmacophores. Each of the 10 best pharmacophores was evaluated based on the fit for naloxone, GGF, and dextromethorphan. The pharmacophore with the highest score and best fit for all three molecules was used as the reference for comparison to GIF.

**Statistical Analysis.** The data are expressed as the mean ± S.E.M., and statistical significance was assessed with an analysis of variance followed by Bonferroni’s multiple comparisons. A value of P < 0.05 was considered statistically significant.

**Results**

**Subpicomolar Concentrations of PACAP38, PACAP27, and GIF Are Neuroprotective against LPS-Induced DA Neurotoxicity.** Neuron-glia cultures treated with PACAP38 (10−10 to 10−13 M) alone showed no effect on [3H]DA uptake at 7 days post-treatment, but there was a minor decline of DA uptake shown with 10−7 M PACAP38 (Fig. 1A). These results indicate the absence of a PACAP38-induced trophic effect and emphasize the importance of the anti-inflammatory effect evident in our model. Mesencephalic neuron-glia cultures were pretreated with PACAP38, PACAP27, or GIF for 0.5 h and then stimulated with LPS for 7 days. Mesencephalic neuron-glia cultures were pretreated with PACAP38 for 0.5 h and then stimulated with LPS for 7 days. The diminution of DA neurons was then determined by [3H]DA uptake assay. The DA uptake assay showed that LPS reduced the capacity of the cultures to take up DA to approximately 40% of the vehicle control. LPS-induced reduction of DA uptake was prevented by pretreatment with PACAP38, PACAP27, and GIF at 10−7, 10−10, 10−13, and 10−14 M (Fig. 1, B–D). However, the neuroprotective effect...
against LPS was absent at concentrations below $10^{-15}$ M. Consistent with earlier reports (Kong et al., 1999), the lower concentrations of PACAP ($10^{-13}$ and $10^{-14}$ M) showed greater neuroprotection, compared with the higher concentrations of $10^{-7}$ and $10^{-10}$ M (Fig. 1B). Thus, further analysis of neuroprotection and the associated mechanisms focused on the most effective concentration of $10^{-13}$ M.

To identify whether GIF is an active neuroprotective peptide sequence of its parent peptide PACAP38, we determined the ability of subpicomolar concentrations of both peptides to protect against LPS-induced reduction of DA uptake and loss of DA neurons. Figure 2A shows that GIF ($10^{-13}$ M) has similar neuroprotective effects on DA neurons against LPS-induced neurotoxicity in neuron-glia cultures compared with PACAP38 ($10^{-13}$ M). GIF and PACAP38 ($10^{-13}$ M) also showed a similar neuroprotection against the LPS-induced loss of TH-immunoreactive neurons (Fig. 2B). Morphological analysis revealed that both PACAP38 and GIF also protected DA neurons against LPS-induced morphological damage (Fig. 2C), such as the loss of dendrites, axon disintegration, and loss of DA neurons. However, IGF, a scrambled PACAP4–6 tripeptide, failed to protect DA neurons against LPS-induced neurotoxicity in neuron-glia cultures for either DA uptake or morphological damage compared with $10^{-13}$ M PACAP38 (data not shown). Taken together, these data indicate that the GIF tripeptide possesses the same neuroprotective efficacy as its parent peptide, PACAP38, at $10^{-13}$ M.

Microglia Are Critical for PACAP38 and GIF Neuroprotection at Subpicomolar Concentrations. Microglia become activated in response to neuron damage to contribute to progressive neurotoxicity (McGeer et al., 2003), where a component of MPP+–induced DA neuron damage can be attributed to microglial activation (reactive microgliosis) (Gao et al., 2003b). We compared the ability of PACAP38 and GIF ($10^{-13}$ M) to protect DA neurons against MPP+–induced neurotoxicity in mixed neuron-glia cultures and in microglia-depleted cultures (containing only neurons and astrocytes). PACAP38 and GIF were only able to protect against MPP+ in the presence of microglia (Fig. 2D), supporting that these peptides attenuate microglial activation and reactive microgliosis.

PACAP38 and GIF Inhibit Microglial Activation. LPS-induced microglial activation and the consequent production of proinflammatory factors has been linked to DA neurotoxicity (Gao et al., 2002). To discern the effect of PACAP and GIF on the release of proinflammatory factors from microglia, the LPS-induced production of TNF$\alpha$ (3 h) and NO (24 h) was measured in mesencephalic neuron-glia cultures pretreated for 30 min with GIF or PACAP38. The results show that pretreatment with GIF or PACAP38 at $10^{-13}$ M reduces both TNF$\alpha$ and NO production in response to LPS, compared with LPS (Fig. 3, A and B). Together, these results suggest that subpicomolar concentrations of PACAP and GIF are neuroprotective through inhibition of microglial activation.

To test the ability of PACAP38 and GIF to attenuate the production of microglial extracellular superoxide, microglia-enriched cultures were pretreated with PACAP38 or GIF and then exposed to LPS or phorbol-12-myristate 13-acetate (PMA). Both $10^{-13}$ M PACAP38 and $10^{-13}$ M GIF reduced PMA-induced extracellular superoxide production by approximately 35% (Fig. 4A) and reduced LPS-induced extracellular superoxide production by approximately 55% (Fig. 4B). Both PACAP and GIF were shown to have no effect on extracellular superoxide alone (data not shown).

Intracellular ROS are critical for the microglial inflammatory response and are components of a signaling pathway.
regulating proinflammatory gene expression in multiple cell types, including microglia (Gorlach et al., 2002). To test the effect of PACAP38 and GIF on the microglial generation of ROS, microglia-enriched cultures were pretreated with PACAP38 or GIF (both at $10^{-13}$ M) for 30 min before the addition of LPS (2.5 ng/ml); then DA neurotoxicity was measured 7 days later with the [3H]DA uptake assay (A). B, loss of DA neurons was determined 7 days later by counting the number of TH-immunoreactive neurons. C, immunocytochemical analysis demonstrates the ability of GIF and PACAP38 to protect DA neurons from LPS-induced morphological damage. Representative images were shown from three separate experiments. D, ability of PACAP38 and GIF to protect against MPP+ (0.5 μM)-induced loss of DA neuron function was compared in neuron-glia and in microglia-depleted cultures. Graphs show the results expressed as percentage of the control cultures and are the mean ± S.E.M. from three independent experiments in triplicate. *, $P < 0.05$; and **, $P < 0.01$, compared with treatment with LPS.

**PACAP38 and GIF Are Neuroprotective through Inhibition of NADPH Oxidase.** Although the effect of PACAP38 and GIF on intracellular ROS and extracellular superoxide was evident, it was unclear how these subpicomolar concentrations of peptides attenuated microglial activation and whether the neuroprotective effects were due to the anti-ROS qualities. To identify the mechanism through which subpicomolar concentrations of PACAP38 and GIF are inhibiting the microglial inflammatory response, the ability of PACAP38 and GIF to protect DA neurons from LPS-in-
duced neurotoxicity in mesencephalic neuron-glia cultures from NADPH oxidase-deficient mice (PHOX\(^{-/-}\)) was determined. PHOX\(^{-/-}\) mice are unable to produce extracellular superoxide in response to LPS due to the lack of functional NADPH oxidase. Compounds that protect through reduction of NADPH oxidase activation are ineffective in PHOX\(^{-/-}\) cultures. Both PACAP and GIF failed to show neuroprotection in PHOX\(^{-/-}\) cultures as determined by DA uptake (Fig. 5A), supporting that inhibition of this enzyme is critical for their neuroprotective mechanism of action.

The production of TNF\(\alpha\) was also measured in response to LPS in PHOX\(^{-/-}\) and PHOX\(^{+/+}\) mesencephalic neuron-glia cultures pretreated for 30 min with GIF or PACAP38. Again, pretreatment with either neuroprotective peptide failed to show any reduction of TNF\(\alpha\) in response to LPS in PHOX\(^{-/-}\) cultures, whereas pretreatment with GIF or PACAP38 (10\(^{-13}\) M) attenuated the LPS-induced TNF\(\alpha\) production in PHOX\(^{+/+}\) cultures (Fig. 5B), demonstrating that these subpicomolar-acting compounds also inhibit the ROS-induced

Fig. 4. Subpicomolar concentrations of GIF and PACAP reduce microglial activation in rat primary microglia-enriched cultures. Microglia were pretreated for 30 min with PACAP38 or GIF (both at 10\(^{-13}\) M) followed by the addition of PMA (25 nm; A) or LPS (10 ng/ml; B). Extracellular superoxide was measured by the SOD-inhibitable reduction of the tetrazolium salt WST-1. Intracellular ROS were determined by DCFH oxidation (C and D). The results are expressed as the mean ± S.E.M. of three experiments performed in triplicate. *, \(P < 0.05\); and **, \(P < 0.01\), compared with treatment with LPS.

Fig. 5. NADPH oxidase mediates PACAP38 and GIF DA neuroprotection through inhibition of microglial activation in mouse primary mesencephalic mixed neuron-glia cultures. The effects of GIF and PACAP38 on LPS-induced DA neurotoxicity were compared in primary mesencephalic mixed neuron-glia cultures from PHOX\(^{-/-}\) and PHOX\(^{+/+}\) mice. The cultures were pretreated with PACAP38 or GIF (both at 10\(^{-13}\) M) followed by the addition of LPS (10 ng/ml). A, DA neurotoxicity was measured 7 days later with the \(^{[3]H}\)DA uptake assay. Data are expressed as the percentage of the control cultures. B, TNF\(\alpha\) production was measured 3 h later with a commercially available enzyme-linked immunosorbent assay kit. The results are the mean ± S.E.M. **, \(P < 0.01\), compared with treatment with LPS.
amplification of TNFα production. Together, these results support the conclusion that subpicomolar concentrations of PACAP and GIF afford neuroprotection through inhibition of microglial NADPH oxidase.

**PACAP6–38 Fails to Attenuate PACAP38, PACAP27, and GIF Neuroprotection.** In an effort to understand how PACAP38, PACAP27, and GIF were exerting their effects, we pretreated neuron-glia cultures with PACAP6–38 (PAC1 receptor antagonist) before exposing the cells to PACAP38, PACAP27, or GIF, followed by LPS treatment. PACAP6–38 (3 μM) by itself did not protect against LPS-induced loss of DA uptake (data not shown) and failed to attenuate the neuroprotective effects of PACAP38, PACAP27, or GIF (10−13 M) (Fig. 6).

**Subpicomolar Concentrations of PACAP Peptides Fail to Increase cAMP.** Activation of PAC1 and VPAC receptors results in increased intracellular cAMP levels (Brenneman et al., 2003), but not all effects of VIP or PACAP are mediated through these traditional pathways (Hill et al., 1999; Brenneman et al., 2002, 2003). Consistent with previous reports (Brenneman et al., 2003), we show that the addition of PACAP27 (5 μM) to neuron-glia cultures resulted in the production of cAMP, expressed as picomolar per milliliter of cell lysate (mean = 211; S.D. = 21; P < 0.01). However, subpicomolar concentrations of PACAP38, PACAP27, and GIF (10−13 M) showed no significant effect on cAMP, compared with control. The data are the mean of cAMP levels from three separate experiments. Together, this suggests that PACAP38, PACAP27, and GIF are neuroprotective through a receptor mechanism independent of traditional PACAP receptors.

**Chemical Similarities of Subpicomolar-Acting Neuroprotective Compounds.** Pharmacophore modeling and analysis were performed on the compounds experimentally identified as exhibiting neuroprotective activity: naloxone; dextromethorphan; and dynorphin A (2–4), GGF. This reference pharmacophore aligned with GIF is shown in Fig. 7. We found that the highest scoring pharmacophore contained a hydrogen bond acceptor (3.21/3.11), a hydrophobic region (2.11), and a positive ionizable region (1.11). This pharmacophore illustrates the common chemical properties that are shared among naloxone, dextromethorphan, GGF, and GIF with a common relationship in three-dimensional space. The shared common properties identified by pharmacophore analysis that are necessary to elicit neuroprotection at subpicomolar concentrations were: a hydrogen bond acceptor, a hydrophobic region, and a positive ionizable region.

**Discussion**

Increasing evidence supports that microglia-derived ROS is a common denominator across numerous neurodegenerative diseases. Microglia-derived oxidative stress is critical in the pathogenesis of PD because DA neurons are particularly vulnerable to oxidative insult. However, the protective homeostatic mechanisms opposing microglial overactivation in the brain are poorly defined. Here, we are the first to report that the PACAP38 neuropeptide inhibits microglia-derived superoxide. Furthermore, we also demonstrate that GIF and its parent peptide, PACAP38, are neuroprotective through NADPH oxidase inhibition, implicating a novel mechanism of PACAP neuroprotection and inhibition of microglial activation that is independent of the traditional G protein-coupled VPAC and PAC1 receptors.

Several biological effects of PACAP have been reported previously at a wide dose range, where the detailed mechanisms and the receptors responsible are often unclear. It has been traditionally accepted that there are two classes of PACAP binding sites that are characterized by their relative affinities for PACAP and VIP. Type 1 PACAP receptors are predominantly located in the pituitary and the hypothalamus with a reported Kd of approximately 0.5 nM for PACAP (Laburthe and Couvineau, 2002). Type 2 receptors were reported to be localized in other tissues, such as the lung, duodenum, and thymus with a reported Kd of approximately 0.5 nM for both PACAP and VIP (Laburthe and Couvineau, 2002). However, the reported Kd values for both the type 1 and type 2 receptors are unable to explain the effects of PACAP at lower concentrations, such as 10−13 M, suggesting the existence of an additional high-affinity site of action that is independent of these receptors. Furthermore, PACAP (14–
which lacks the GIF sequence, is still able to stimulate PAC1 receptors, suggesting that GIF is not necessary to stimulate cyclic adenylyl cyclase activity of PACAP (Vandermeers et al., 1992). In fact, several domains are required for PACAP to interact with its traditional receptors (Vaudry et al., 2000).

In the current study, we demonstrate that GIF, an internal sequence of PACAP (from a region that is not necessary for the activation of PAC1 or VIP receptors), is able to attenuate microglial activation and associated neurotoxicity with an efficacy similar to its parent compound, PACAP38. The activation of PAC1/VPAC receptors is well documented to cause an increase in cAMP levels (Nowak et al., 2003), and we could find no evidence of cAMP production with subpicomolar concentrations of the PACAP peptides (10⁻¹³ M). Furthermore, the PAC1 antagonist was unable to attenuate PACAP38, PACAP27, or GIF (10⁻¹³ M) neuroprotection. Although it is clear that many of the effects exerted by PACAP work through traditional receptor-mediated mechanisms, the current data support that 10⁻¹³ M GIF and PACAP exert their low-dose neuroprotective and anti-inflammatory effects through a mechanism independent of traditional PACAP receptor mechanisms. Although the detailed mechanism of this alternative site of action for PACAP is under investigation, there are striking similarities among PACAP, GIF, and other neuroprotective compounds studied in our laboratory that may provide valuable insight.

Previously, we identified a subset of compounds (GGF, dynorphin, leucine enkephalin, des-tyrosine leucine enkephalin, dextromethorphan, and naloxone) that were neuroprotective through NADPH oxidase inhibition (Li et al., 2005; Qin et al., 2005a,b). These subpicomolar-acting compounds share chemical, functional, and mechanistic similarities, indicating that these ligands exert their similar effects through a common high-affinity site of action. Specifically, this group of subpicomolar-acting compounds was shown to be neuroprotective against LPS-induced DA neurotoxicity through inhibition of NADPH oxidase and shared structural and chemical similarities, as determined by pharmacophore analysis (Qin et al., 2005a). The pharmacophore generated from this analysis is a graphical representation of the common three-dimensional chemical features of ligands that are neuroprotective through NADPH oxidase inhibition at subpicomolar concentrations. Noting that the primary structure of GIF is similar to the neuroprotective peptide GGF discovered in our laboratory, we sought to determine whether PACAP38 and GIF were neuroprotective through the same mechanism and chemically similar to the previously defined pharmacophore for subpicomolar-acting molecules. In the current study, we demonstrate that at subpicomolar concentrations, both GIF and PACAP are neuroprotective through the attenuation of microglial activation and NADPH oxidase inhibition. In addition, pharmacophore analysis revealed that PACAP and GIF fit the subpicomolar-acting pharmacophore, whereas the ineffective scrambled peptide (IGF) did not. Together, these data demonstrate that PACAP and GIF join a class of subpicomolar-acting compounds that are neuroprotective through a high-affinity site of action common to many subpicomolar-acting compounds.

These findings have broad implications because the potential therapeutic utility of this class of subpicomolar-acting neuroprotective compounds is extensive and provides great hope for the treatment of neurodegenerative disease. First, compounds exerting their effects at low concentrations through high-affinity receptors offer a low potential for unwanted side effects. Second, inflammation-mediated neurodegeneration is a characteristic of multiple neurodegenerative diseases, where microglial NADPH oxidase has been implicated as a common mechanism of microglia-mediated neurotoxicity (Block and Hong, 2005). Interestingly, previous reports from our laboratory have indicated that microglial activation is regulated by neutrophpeptide control of NADPH oxidase at subpicomolar concentrations (Block et al., 2006). The attenuation of microglial NADPH oxidase both inhibits the production of neurotoxic ROS and reduces microglial signaling leading to the production of proinflammatory factors (Qin et al., 2005a), making the inhibition of this enzyme an effective and ideal therapeutic target.

In summary, here we demonstrate that PACAP4–6 (GIF) shares a similar neuroprotective dose-response curve and mechanism of action with its parent peptide PACAP38 that is independent of traditional receptor pathways. In addition, we show that PACAP joins a growing list of subpicomolar-acting neuropeptides with mechanistic, chemical, and neuroprotective similarities, suggesting that attenuation of microglial activation may be regulated by multiple neuropeptides at subpicomolar concentrations.

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References


Kong LY, Maderuril JL, Jhun GH, and Hong JS (1999) Reduction of lipopolysac-


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