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N-Methyl-D-aspartate Receptor Stimulation Activates Tyrosinase and Promotes Melanin Synthesis in the Ink Gland of the Cuttlefish *Sepia officinalis* through the Nitric Oxide/cGMP Signal Transduction Pathway

A NOVEL POSSIBLE ROLE FOR GLUTAMATE AS PHYSIOLOGIC ACTIVATOR OF MELANOGENESIS*

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The tyrosinase-catalyzed conversion of L-tyrosine to melanin represents the most distinctive biochemical pathway in the ink gland of the cuttlefish *Sepia officinalis*; however, the molecular mechanisms underlying its activation have remained so far largely uncharted. In this paper we demonstrate for the first time that L-glutamate can stimulate tyrosinase activity and promote melanin synthesis in *Sepia* ink gland via the *N*-methyl-D-aspartate (NMDA) receptor/NO/cGMP signal transduction pathway. Incubation of intact ink glands with either L-glutamate or NMDA resulted in an up to 18-fold increase of tyrosinase activity and a more than 6-fold elevation of cGMP levels. Comparable stimulation of tyrosinase was induced by an NO donor and by 8-bromo-cGMP. An NMDA receptor antagonist, NO synthase (NOS) inhibitors, and a guanylate cyclase blocker suppressed NMDA-induced effects. Immunohistochemical evidence indicated that enhanced cGMP production was localized largely in the mature part of the ink gland. Increased *de novo* synthesis of melanin was demonstrated in NMDA- and NO-stimulated ink glands by a combined microanalytical approach based on spectrophotometric determination of pigment levels and high performance liquid chromatography quantitation of pyrrole-2,3,5-tricarboxylic acid, a specific melanin marker, in melanosome-containing fractions. These results fill a longstanding gap in the understanding of the complex biochemical mechanisms underlying activation of melanogenesis in the mature ink gland cells of *S. officinalis* and disclose a novel physiologic role of the excitatory neurotransmitter glutamate mediated by the NMDA receptor/NO/cGMP signaling pathway.

Inking is a characteristic behavior adopted by nearly all coleoid cephalopods, *i.e. Sepia*, *Loligo,* and *Octopus*, to confuse predators and alert conspecifics to danger while retreating. It occurs in two distinct stages as follows: first, production of a "pseudomorph," a puff of ink and mucus supposed to attract the predator by virtue of its deceptive resemblance to the cephalopod shape; and second, the ejection of a large cloud of ink, providing a smokey screen covering the retreat (1–3).

Biochemically, the production of ink by cephalopods shares many features in common with the process of melanogenesis in epidermal melanocytes (4, 5) (Scheme 1).

Both processes are triggered by the oxidation of L-tyrosine promoted by tyrosinase, the rate-limiting enzyme in melanogenesis, leading to the formation of dopaquinone. This unstable *o*-quinone suffers rapid intramolecular cyclization to afford an orange aminochrome (λ_{max} = 480 nm) termed dopachrome, which may undergo rearrangement with or without decarboxylation to give 5,6-dihydroxyindole (DHI)[1] or 5,6-dihydroxyindole-2-carboxylic acid (DHICA), respectively. In the oxidative environment of the melanosome, the 5,6-dihydroxyindoles can co-polymerize via the corresponding quinones to give eventually the insoluble melanin granules responsible for the typical black appearance of the ink (5).

Despite considerable advances in the elucidation of the origin and structure of *Sepia* melanin (6), the biochemical mechanisms involved in regulation of tyrosinase activity and ink production still remain to be elucidated. To keep the animal prepared to face a dangerous situation, the mature ink gland cells regularly produce the melanin that accumulates into the ink sac, the typical effector organ delegated to the storage and ejection of the ink. This entails the existence of a specific, as yet unknown, signaling system that keeps the biochemical machinery for ink production active, so to ensure that sufficient amounts of melanin are available when necessary.

Clues to potential candidate mechanisms for regulation of melanogenesis in *Sepia* have recently derived from the demon-

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This paper is dedicated to the memory of Prof. Giovanna Misuraca, an esteemed colleague and friend who is dearly missed by all those who had the fortune to share her interest in the field of melanogenesis.

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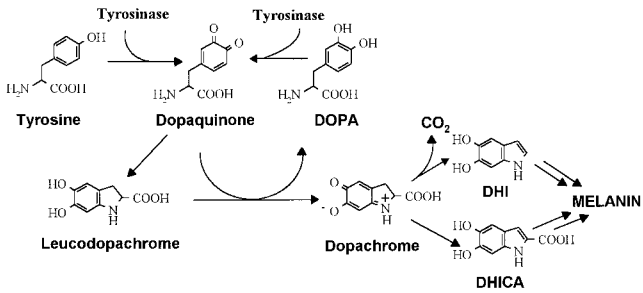
[1] The abbreviations used are: DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; NOS, NO synthase; NMDA, *N*-methyl-D-aspartate; D-AP5, D(−)-2-amino-5-phosphonopentanoic acid; D-NA, N^G-nitro-D-arginine; L-NA, N^G-nitro-L-arginine; L-NAME, N^G-nitro-L-arginine methyl ester; 8-Br-cGMP, 8-bromo-cGMP; LY 83583, 6-anilino-5,8-quinolinedione; DEA/NO, 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide; H-89, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide; Bisindolylmaleimide I, 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl) maleimide; IBMX, 3-isobutyl-1-methylxanthine; DOPA, L-3,4-dihydroxyphenylalanine; PTCA, pyrrole-2,3,5-tricarboxylic acid; HPLC, high performance liquid chromatography.



SCHEME 1. **Schematic outline of the early stages of the biosynthesis of melanin highlighting the main rate-determining steps, *i.e.* the tyrosinase-catalyzed conversion of tyrosine to dopaquinone and the rearrangement of dopachrome to DHI and DHICA.**

stration in the immature ink gland cells of a Ca^{2+}/calmodulin-dependent NO synthase (NOS) as well as of glutamate *N*-methyl-D-aspartate (NMDA) R1 receptor subunits (7). Glutamate and NMDA receptors and a Ca^{2+}/calmodulin-dependent NOS have also been detected by chemical and immunohistochemical techniques in certain neural pathways of *Sepia* central nervous system relating to the ink defense system (8). These lines of evidence hinted at a possible role of the glutamate/NO/cGMP pathway in the rapid transduction of the stimuli derived from alarming inputs to those neural pathways controlling contraction of the ink sac sphincters and wall muscles in the ejection mechanism.

In this paper, we report experimental evidence demonstrating for the first time that stimulation of NMDA receptors in the ink gland of *Sepia officinalis* induces a marked elevation of tyrosinase activity and increases melanin synthesis through the NO/cGMP pathway.

EXPERIMENTAL PROCEDURES

Materials—D(−)-2-Amino-5-phosphonopentanoic acid (D-AP5) was from Tocris Cookson Ltd. (Bristol, UK). 8-Bromo-cGMP (8-Br-cGMP), 6-anilino-5,8-quinolinedione (LY 83583), KT5823, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide) (H-89), and 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl) maleimide (Bisindolylmaleimide I) were from Calbiochem. 2-(*N*,*N*-Diethylamino)-diazenolate-2-oxide (DEA/NO) and N^G-nitro-D-arginine (D-NA) were obtained from Alexis Italia (Vinci, Italy). L-[3,5-^{3}H]Tyrosine (52.0 Ci/mmol, 1 mCi/ml) was from Amersham Pharmacia Biotech. All other chemicals were from Sigma. Pyrrole-2,3,5-tricarboxylic acid (PTCA) was prepared by oxidative degradation of DHICA (9). Rabbit polyclonal antiserum raised against cGMP was obtained from Chemicon International Inc.(Temecula, CA). EnVision+ was obtained from Dako Corp. (Carpinteria, CA).

Animals—Speciments of *S. officinalis* were collected in the bay of Naples, kept in a controlled environment for 2 days, and dissections were performed using cold anesthesia. The ink glands were removed and used immediately.

Ink Gland Stimulation—Single intact ink glands were incubated at 20 °C in seawater (2 ml) or in Ca^{2+}-free artificial seawater (10) (2 ml) with gentle agitation in the presence of additives as indicated under "Results." When necessary, before stimulation with 1.5 mM NMDA, ink glands were preincubated for 30 min with one of the following additives: 10 mM N^G-nitro-L-arginine methyl ester (L-NAME), 10 mM N^G-nitro-L-arginine (L-NA), 10 mM D-NA, 100 μM LY 83583, 1 mg of puromycin, 1 mg of cycloheximide, 100 nM H-89, 20 nM bisindolylmaleimide I, 30 μM KT5823. For determination of melanin content, ink glands were incubated at 20 °C in seawater (2 ml) for 6 h with 0.06 mM L-3,4-dihydroxyphenylalanine (DOPA) and in the presence of the additives as indicated under "Results." After incubation, the tissues were immediately frozen in liquid nitrogen and then examined for enzyme assays, cGMP determination, and melanin content.

Enzyme Assays—Ink glands were homogenized in ice-cold 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM dithiothreitol buffer supplemented with leupeptin (10 μg/ml), pepstatin A (10 μg/ml), and phenylmethylsulfonyl fluoride (100 μg/ml). All subsequent manipulations were carried out at 4 °C. After centrifugation at 3,000 rpm for 10 min, the pellet was re-extracted with the same buffer and centrifuged. The combined supernatants were centrifuged at 25,000 rpm for 20 min, and the supernatant was used for enzyme assays. Tyrosinase activity was determined by the Pomerantz method as described by Kuzumaki *et al.* (11). Peroxidase activity was determined essentially as described previously (12). Total protein concentration in tissues was determined using a Bio-Rad Protein Assay Reagent (Bio-Rad) with bovine serum albumin as a standard.

cGMP Determination—Ink glands were homogenized in cold 6% (w/v) trichloroacetic acid at 4 °C to give a 10% (w/v) homogenate that was centrifuged at 5,000 rpm for 15 min. The supernatant was washed 4 times with 5 volumes of water-saturated diethyl ether. The aqueous extracts were lyophilized, and cGMP levels were determined by an enzyme immunoassay system under non-acetylation conditions according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Melanin Content—After centrifugation of the ink gland homogenates at 25,000 rpm for 20 min, the melanosome-containing pellet was subjected to sucrose-density gradient ultracentrifugation (30, 25, 20, and 15%) at 28,000 rpm for 60 min. The brown melanosomal fraction at the bottom of 30% sucrose was extensively washed with water, dried on silica gel, and equilibrated in the presence of saturated $CaCl_2$ (13). For spectrophotometric determination of melanin content, the melanosomal fraction (1–2 mg) was stirred with 0.03% H_2O_2 in 1 M NaOH (1 ml) at 80 °C for 30 min (14). After cooling, the sample was centrifuged, and the absorbance was determined at 350 nm. For PTCA quantitation, a modification of the degradative method previously reported (9) was used. Briefly, the melanosomal fraction was suspended under stirring in 1 M NaOH (1 mg/ml) containing 30% H_2O_2 (0.1 ml/mg) at room temperature for 3 h. After treatment with sodium bisulfite to destroy excess H_2O_2, the reaction was acidified to pH 0–1 and extracted five times with ethyl acetate. The organic phase was evaporated and analyzed for PTCA content by high performance liquid chromatography (HPLC). HPLC was carried out on a Gilson model 302 instrument, using a Sphereclone 5-μm ODS (2) column (250 × 4.60 mm) (Phenomenex, Chemtek Analytica, Bologna, Italy). The mobile phase was 0.1 M phosphoric acid, pH 2.5, containing 7.5% methanol. The flow rate was maintained at 1 ml/min. Detection of PTCA was carried out at 280 nm with a Gilson model 116 UV detector. Measurements of retention times and peak areas and comparison with external calibration curve for PTCA allowed quantitative analysis of the reaction mixture.

Data Presentation—Assays were carried out in triplicate. Each set of experiments was repeated at least three times with similar results.

Immunohistochemical Staining—Ink glands were preincubated in seawater for 15 min at 4 °C with 1 mM 3-isobutyl-1-methylxanthine (IBMX) to inhibit endogenous phosphodiesterases. The ink glands were then incubated for 30 min with fresh 1 mM IBMX and 1.5 mM NMDA. Control experiments were performed omitting NMDA. The ink glands were then fixed overnight in 4% paraformaldehyde in phosphate-buffered saline and embedded in sucrose by gradually increasing its concentration up to 30%. Dry cryostat sections (25 μm), collected on slides pretreated with Biobond (British Biocell International, Cardiff, UK), were incubated with 10% normal goat serum for 30 min at room temperature in a moist, humid chamber to reduce background staining. After a 30-min treatment with 3% H_2O_2 to block the endogenous peroxidase, the sections were incubated overnight with rabbit polyclonal antiserum raised against cGMP (1:500) (15). The sections were subsequently incubated in En/Vision+ (anti-rabbit immunoglobulins conjugated to peroxidase labeled-dextran polymer) for 30 min at room temperature. En/Vision+ is a recently introduced enhancement system (16), and the labeled cells were visualized by using diaminobenzidine as chromogen. The enzyme reaction was visually monitored and was blocked with several washes in phosphate-buffered saline. Sections were dehydrated in ethanol series, cleared in xylene, and mounted by using Biomount (British Biocell International). Sections were viewed and photographed through Nikon Eclipse E400. Images of ink gland sections were analyzed using a CCD video camera and screen measurements LUCIA analysis software image (Laboratory Imaging). For control of nonspecific cGMP-like immunoreactivity, experiments were performed by omitting primary antibody or by using a preabsorbed antibody obtained by incubation with 10^{-5} M cGMP for 1 h at room temperature.

RESULTS

Effects of Glutamate Receptor Stimulation on Tyrosinase Activity in Sepia Ink Gland—Incubation of fresh collected intact ink glands in seawater in the presence of various concentrations of L-glutamate resulted in an up to 18-fold increase in tyrosinase activity, as determined by the Pomerantz assay (11) (Fig. 1). The effect was time-dependent, plateauing after about

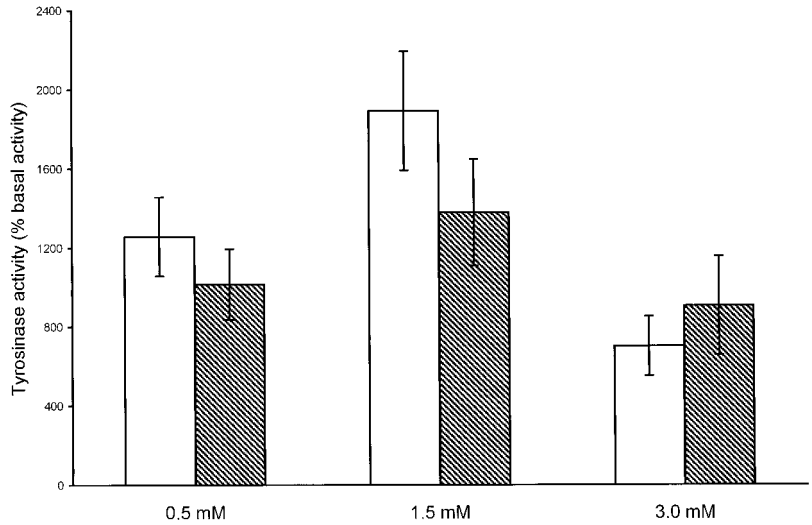


FIG. 1. **Stimulation of tyrosinase activity by L-glutamate and NMDA in *Sepia* ink glands.** Ink glands were incubated in seawater for 4 h in the presence of various concentrations of L-glutamate (*open bars*) and NMDA (*hatched bars*). Tyrosinase activity was assayed as described under "Experimental Procedures." Each value represents the mean ± S.D. (*bars*) of triplicates of one representative experiment.

4 h incubation (data not shown), and concentration-dependent, being maximum with an L-glutamate concentration of 1.5 mM. Of the ionotropic glutamate receptors, *i.e.* α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)[2] and NMDA, only the latter receptors were previously shown to be present in *Sepia* ink gland cells (7) by immunohistochemical and Western blot analyses. Accordingly, the effects of NMDA on tyrosinase activity were investigated. At 1.5 mM concentration, NMDA caused a 13-fold increase of tyrosinase activity after 4 h incubation (Fig. 1). The NMDA-induced effects exhibited time-dependent (data not shown) and concentration-dependent profiles similar to those observed in the case of L-glutamate. At a concentration of 1 mM, D-AP5, an NMDA receptor antagonist, completely inhibited the stimulatory effect of 1.5 mM NMDA on tyrosinase activity (data not shown).

Based on these results, an NMDA concentration of 1.5 mM was preferably used throughout this paper. At concentrations up to 2 mM, NMDA had no detectable effect on peroxidase, another enzyme putatively involved in melanogenesis (data not shown) (17–21).

Role of NO in NMDA-induced Stimulation of Tyrosinase—NMDA-induced activation of tyrosinase was completely suppressed by two NOS inhibitors, L-NAME and L-NA, at 10 mM concentration, whereas the inactive enantiomer D-NA at the same concentration was ineffective (data not shown). Moreover, exposure of intact ink glands to 1.5 mM DEA/NO, a widely used chemical NO donor with $t_{1/2}$ = 2–4 min, at pH 7.4 and 37 °C (22), markedly enhanced tyrosinase activity, whereas an aged solution of DEA/NO, used as a control, failed to stimulate tyrosinase to any appreciable extent (Fig. 2). These data indicated that NO was involved in NMDA-induced stimulation of tyrosinase.

Involvement of cGMP in the NMDA-elicited Stimulation of Tyrosinase via NO—Stimulation of ink glands with 1.5 mM NMDA caused a more than 6-fold elevation of cGMP levels compared with basal levels (Fig. 3). The effect was transient, since cGMP levels peaked after 1 min incubation and dropped down to basal after 10 min (data not shown), and was suppressed by LY 83583, a specific inhibitor of guanylate cyclase, as well as by the NOS inhibitors L-NAME and L-NA but not by D-NA (Fig. 3).

In Ca^{2+}-free artificial seawater, L-glutamate (1.5 mM) and NMDA (1.5 mM) failed to stimulate guanylate cyclase and cGMP synthesis in intact ink glands, whereas NO donors were able to induce elevation of cGMP levels, indicating that cell functionality was maintained under such conditions (data not shown).

Exposure of intact ink glands to 7.5 mM 8-Br-cGMP, a membrane-permeable, phosphodiesterase-resistant active analog of cGMP, resulted in an about 36-fold increase in the levels of

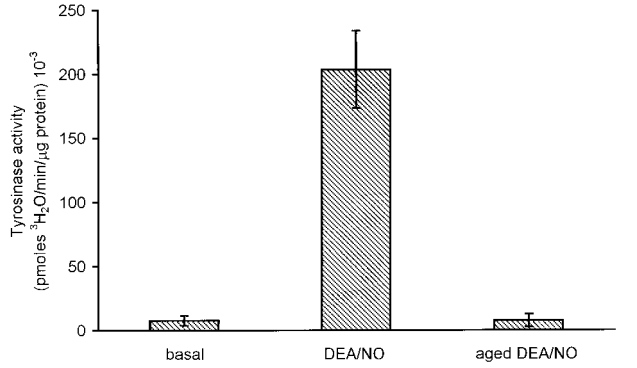


FIG. 2. **Stimulation of tyrosinase activity by the NO donor DEA/NO.** Ink glands were incubated in seawater with or without 1.25 mM DEA/NO for 4 h. As a control, aged DEA/NO, obtained by incubating a solution of DEA/NO, pH 7.4, at 37 °C for 60 min, was used. Tyrosinase activity was assayed as described under "Experimental Procedures." Each value represents the mean ± S.D. (*bars*) of triplicates of one representative experiment.

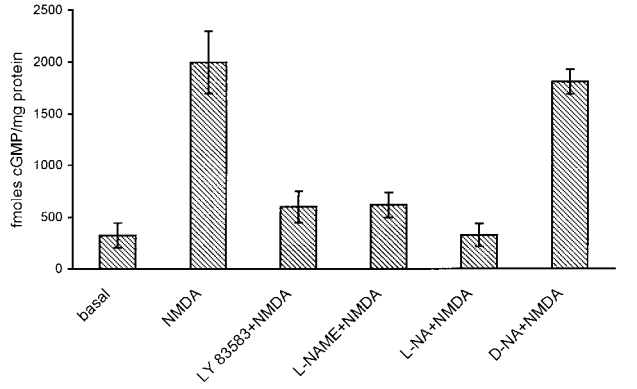


FIG. 3. **Production of cGMP by ink glands in response to NMDA stimulation and the effect of guanylate cyclase and NOS inhibitors.** Ink glands were incubated with 1.5 mM NMDA in seawater and then cGMP was measured after 1 min. When indicated, glands were preincubated for 30 min with 100 μM LY 83583, 10 mM L-NAME, 10 mM L-NA or with 10 mM D-NA before NMDA stimulation. Each value represents the mean ± S.D. (*bars*) of triplicates of one representative experiment.

[2] A. Di Cosmo, unpublished observations.

tyrosinase (280×10^{-3} pmol of ^3H_2O/min/μg of protein *versus* 7.8×10^{-3} pmol of ^3H_2O/min/μg of protein), indicating that cGMP is able to mimic the stimulating effects of NMDA.

cGMP Immunohistochemistry—After incubation of whole excised *Sepia* ink gland in the presence of NMDA and IBMX, a strong immunoreactive reaction could be observed throughout the trilayer of the outer glandular epithelium. Staining became detectable in the cytoplasm of the cells in which coated vesicles had completed the inversion process and the melanization was at the onset (see stage 4, Ref. 12) and was also intense in the cytoplasm of the cells containing mature melanosomes (Fig. 4, *A* and *B*). A weak staining was observed throughout the inner glandular epithelium (Fig. 4, *A* and *B*). Incubation of *Sepia* ink gland in the absence of NMDA gave no evidence of immunostaining. Staining was also completely abolished when the primary antibody was omitted or the antibody was preabsorbed with cGMP (Fig. 4*C*).

Involvement of cGMP-dependent Kinase in NMDA-induced Stimulation of Tyrosinase—To investigate whether stimulation of melanogenesis involved *de novo* synthesis of tyrosinase, intact ink glands were preincubated with the protein synthesis inhibitors puromycin or cycloheximide prior to treatment with NMDA. In none of these cases, however, could a decrease in the extent of stimulation of tyrosinase activity be observed (data not shown).

In further experiments, the effects of different protein kinase inhibitors on the NMDA-induced stimulation of tyrosinase were investigated. 100 nM H-89 and 20 nM bisindolylmaleimide I, specific inhibitors of protein kinases A and C, did not affect NMDA-induced stimulation of tyrosinase to any significant extent (data not shown), whereas 30 μM KT5823, a protein kinase G inhibitor, caused a moderate, poorly reproducible yet significant decrease in tyrosinase activity ($50 \pm 25 \times 10^{-3}$ pmol of ^3H_2O/min/μg of protein *versus* NMDA value of $108 \pm 20 \times 10^{-3}$ pmol of ^3H_2O/min/μg of protein, $n = 4$, $p < 0.05$). It should be noted that in two out of six different experiments the impairment of tyrosinase activity with KT5823 was not statistically significant.

Effects of NMDA-Receptor Stimulation and NO Exposure on Melanin Synthesis—In a final set of experiments, the effects of NMDA receptor stimulation and NO exposure on melanin synthesis in intact ink glands were investigated in the presence of L-DOPA, an excellent substrate of tyrosinase and a widely used melanin precursor (23). To ensure complete removal of additives, including autoxidized L-DOPA, and to overcome analytical problems connected with the intrinsically low pigment content, after the appropriate incubation time the ink glands were extensively washed, homogenized, and the melanosome-containing fractions were partially purified prior to analysis by an *ad hoc* sequential centrifugation protocol. Two complementary microanalytical methods for pigment quantitation in the melanosome-containing fractions were used, involving (*a*) spectrophotometric determination of the absorbance at 350 nm after mild solubilization with alkaline H_2O_2 (14), and (*b*) HPLC quantitation of PTCA, a characteristic marker of melanins produced by H_2O_2 degradation of the samples (9). With these methods, fairly reproducible A_{350} values and PTCA yields were determined on the partially purified melanosome-containing fractions from different groups of unstimulated ink glands, as controls (data not shown).

When ink glands were stimulated with either NMDA or DEA/NO in the presence of L-DOPA, a marked increase in the melanin content of the melanosome-containing fractions was observed (Table I).

These data indicated that the extents of the stimulatory effects were slightly different depending on the analytical method used but were consistently similar for NMDA and DEA/NO at the same concentrations. Control experiments carried out in the absence of ink glands, but with all other conditions as described, indicated less than 10% autoxidation of L-DOPA during the incubation time (data not shown).

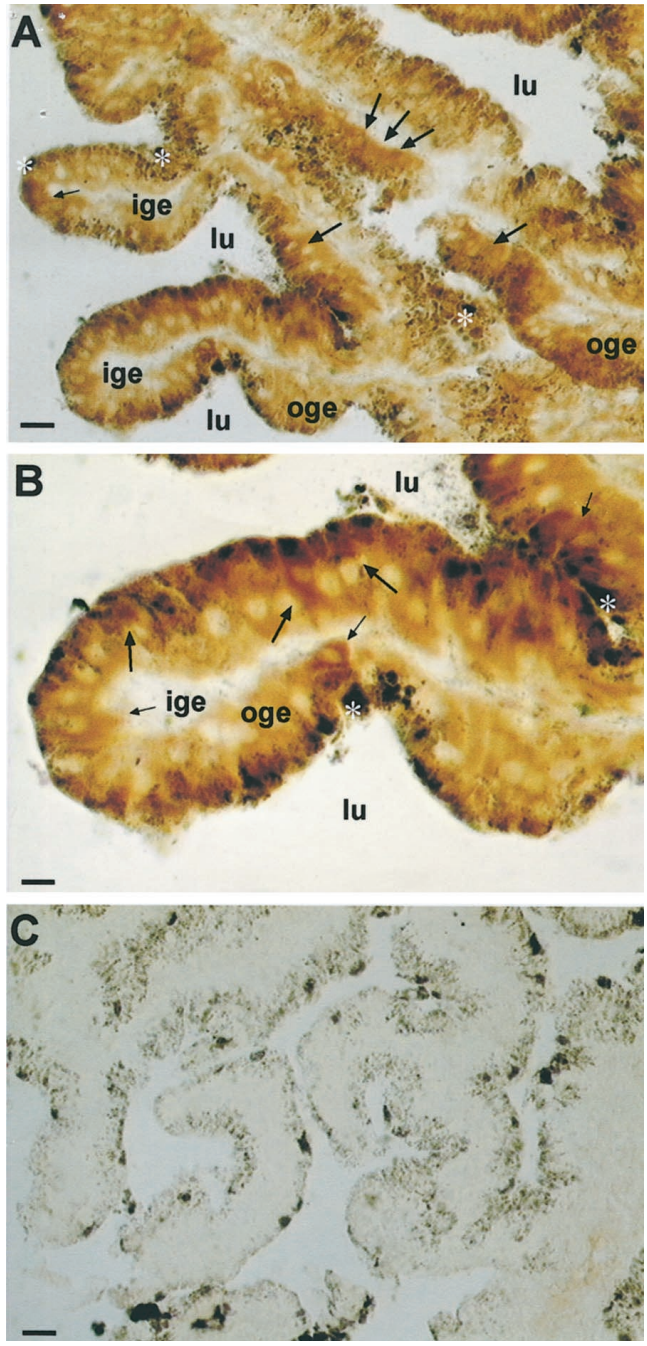


FIG. 4. **Immunostaining analysis of the cellular distribution of cGMP in ink gland.** Frozen sections were incubated with rabbit polyclonal antibody specific for cGMP. Immunoreactive reaction was observed throughout the trilayer of the outer glandular epithelium (*A*, *arrows*). The staining was intense both in the cytoplasm of the glandular cells containing premelanosomes (*small arrows*) and in the cytoplasm of the cells containing mature melanosomes (*large arrows*) (*B*). *Asterisk* indicates melanin granules in mature melanosomes. Immunoreactivity was completely blocked when primary antibody was previously preabsorbed with 10^{-5} M cGMP (*C*). Immunoreactivity was visualized using En/Vision+, and the labeled cells were visualized by using diaminobenzidine as chromogen. *B* is higher magnification image of a region of *A*. *Scale bar,* 50 μm in *A* and *C*; 20 μm in *B*. *ige,* inner glandular epithelium; *oge,* outer glandular epithelium; *lu,* lumen.

TABLE I

Effect of NMDA and NO on melanin synthesis in Sepia ink glands

Ink glands were incubated with 1.5 mM NMDA or 1.5 mM DEA/NO for 6 h in seawater containing 0.06 mM L-DOPA. Melanin content was determined by the combined use of spectrophotometric (A_{350}) and PTCA assays as described under "Experimental Procedures."

Sample	A_{350}/mg[a]	PTCA[a]
		nmol/mg
Control	2.47 ± 0.36	6.9 ± 0.9
NMDA-stimulated	4.16 ± 0.63[b]	15.2 ± 2.5[b]
DEA/NO stimulated	4.33 ± 0.51[b]	16.2 ± 2.2[b]

[a] Data refer to melanosome-containing fractions. Values are averages of triplicates ± S.D. of one representative experiment.

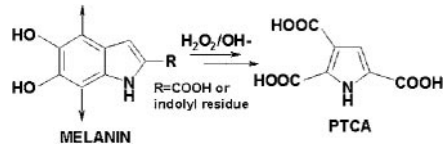
[b] $p < 0.01$ with respect to control.

DISCUSSION

The activation of tyrosinase and the appearance of black melanin granules within melanosomes are the culminating and most peculiar events in the maturation of epithelial cells in the ink gland of *S. officinalis* (5, 12, 24). During this process, it is reasonable to envisage the existence of a specific transmitter and/or signaling agent promoting activation of tyrosinase at the appropriate stage of maturation and maintaining the enzyme in an active state. Based on the results of the present study, it is proposed that the excitatory neurotransmitter L-glutamate subserves this role, acting via NMDA receptors to stimulate NOS to produce NO, which in turn activates its downstream target guanylate cyclase to elevate cGMP and to increase the catalytic activity of tyrosinase. The comparable activation of tyrosinase induced by NMDA and NO indicated a fast signal transduction mechanism that is compatible with the involvement of a constitutive, Ca^{2+}/calmodulin-dependent NOS (7). The crucial role of Ca^{2+} mobilization through ionotropic receptors could also be inferred from the observed inability of NMDA to cause elevation of cGMP levels in Ca^{2+}-free artificial seawater as the incubation medium.

The observed selective localization of NOS-like immunopositivity in the immature cells would warrant also speculations about a possible role of NO as the key messenger controlling the process of maturation of ink gland cells. In these cells, NO could act by switching the program from proliferation to melanogenesis, a differentiated function of mature ink gland cells and, more in general, of pigment cells. This is very apparent in human epidermal melanocytes, in which the melanin content is inversely related to the rate of proliferation (25). If confirmed in further studies, these observations would lead support to the currently emerging view of NO as a general regulator of cell proliferation and differentiation during organ development and morphogenesis (26, 27). The specific localization of NOS-like and NMDA R1 receptor-like immunopositivity in the non-pigmented region of the gland, coupled with detection of enhanced cGMP immunopositivity in active melanizing cells, suggests moreover that NO contributes to a paracrine regulation of basal melanogenesis in the inner melanized cell layer and that NOS expression is suppressed once the maturation process is complete.

The NO-cGMP pathway has recently been shown to mediate the stimulatory effects of UVB radiation on melanogenesis in cultured human melanocytes (28). Elucidation of this signaling pathway, in particular, disclosed the ability of NO to serve as both autocrine and paracrine chemical messenger increasing tyrosinase activity after UV irradiation through elevation of cGMP and activation of protein kinase G. That study, however, was mainly relevant to the specific conditions associated with UV irradiations, where cell homeostasis may be affected or disrupted as a result of more or less severe inflammation, and no relationship has so far been demonstrated between the NO signaling pathway and the basal melanogenic activity under physiologic conditions.



SCHEME 2. **Reaction scheme showing the formation of PTCA by oxidative degradation of DHI- and DHICA-derived melanin units.**

With regard to the mechanism(s) of the NO and cGMP-mediated enhancement of tyrosinase activity, the observed lack of effect of the protein synthesis inhibitors puromycin and cycloheximide would argue in favor of a specific response occurring at the post-translational level. Although experimental difficulties due to the use of whole intact ink glands hampered a more detailed insight, the reported effects of a protein kinase G inhibitor pointed to protein kinase G as a plausible downstream target of cGMP, possibly causing a direct phosphorylation of tyrosinase (29).

In human melanocytes, stimulation of melanogenesis by UV irradiation or diacylglycerol treatment has been demonstrated to occur through the action of the β isoform of protein kinase C, which activates tyrosinase by phosphorylation of the serine residues in its cytoplasmic domain (30, 31). The observed failure of protein kinase C inhibitors to affect the NMDA-induced stimulation of tyrosinase would imply that the protein kinase C activation pathway of tyrosinase, which is critical for stimulation of melanogenesis in human melanocytes (31), is not the primary downstream regulator of NO-induced melanogenesis in *Sepia* ink gland cells. The possibility remains, however, that protein kinase C activates tyrosinase in *Sepia* in response to other stimuli.

Consistent with the observed activation of tyrosinase, exposure of ink glands to NMDA or NO resulted also in an increased melanin synthesis in the mature cells. The extent of enhanced melanin synthesis, however, was not proportional to the degree of tyrosinase activation. This is an important point since tyrosinase is the main but not the sole enzyme responsible for pigment production in the melanosomes (5, 32), and its activation does not necessarily imply that the whole system can sustain an enhanced rate of melanin synthesis to a proportional extent (31, 33, 34). Whether the glutamate receptor/NO/cGMP pathway can affect other melanogenic enzymes in *Sepia* in addition to tyrosinase remains an important focus for future studies.

Combined use of the spectrophotometric assay and PTCA quantitation on partially purified melanosomal fractions allowed reliable determination of the extents of melanin synthesis in NMDA- and NO-stimulated *versus* unstimulated ink glands. This analytical approach was deemed superior to the direct scintillation counting of the insoluble pigment after incubation with [^{14}C]L-DOPA or other radiolabeled precursors, a method commonly used to measure melanin synthesis (28, 35), because it would minimize possible pitfalls derived from physical adsorption of the label, as well as from non-enzymatic oxidation of the precursor on the surface of the tissue in the incubation medium. In particular, HPLC determination of the PTCA produced by oxidative degradation of carefully separated melanosome-containing fractions provided unambiguous indications for *de novo* synthesis of authentic melanin within the melanosomes of mature cells via the 5,6-dihydroxyindole route (Scheme 2).

PTCA may in principle arise from oxidative breakdown of both DHICA-derived units and DHI-derived units substituted on the 2-position of the indole ring (Scheme 2). However, recent

studies indicated that DHICA-derived units contribute to a much greater extent to the total PTCA formed from melanin degradation and account for as much as 75% of the overall monomer units in sepiomelanin (6, 36, 37). Such a conclusion seems to hold also for the newly synthesized melanin in stimulated ink glands, as judged from the large increases in the PTCA yields compared with the corresponding changes in A_{350} values. The slight difference in the relative increases in the A_{350} and PTCA values after NMDA and NO stimulation of ink glands conceivably reflects the different specificities of the two analytical methods. It is possible, for instance, that the lower increases in melanin levels determined with the spectrophotometric assay depend at least in part on the greater susceptibility of the less polymeric and newly synthesized pigment to hydrogen peroxide-induced bleaching (5).

In conclusion, the demonstration that glutamate NMDA receptor stimulation substantially elevates tyrosinase activity and melanin synthesis in the ink glands of *S. officinalis* through the NO/cGMP pathway not only reinforces current appreciation of NO as a key modulator of melanogenesis but hints for the first time at a possible role of L-glutamate as the neurotransmitter deputed to stimulate melanin synthesis in *Sepia* in response to alarming inputs.

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