Opioid peptides activate phospholipase D and protein kinase C- ϵ in chicken embryo neuron cultures

DIMITRA MANGOURA* AND GLYN DAWSON*†

Departments of *Pediatrics and †Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL 60637

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ABSTRACT The μ -opioid peptide morphiceptin stimulated a Ca^{2+} -independent protein kinase C (PKC- ϵ) that is expressed both in embryonic day 6 chicken telencephalon and in derived neuronal cultures. This activation was seen as a 2-fold increase in the activity and level of cytosolic PKC- ϵ and as a transient increase in membrane-associated PKC- ϵ following morphiceptin treatment. Morphiceptin did not activate phospholipase C-mediated phosphatidylinositol hydrolysis but did transiently activate (2- to 3-fold) phospholipase D (PLD), as measured by phosphatidylethanol formation in neuron cultures derived from embryonic day 6 or day 7 cerebral hemispheres. This PLD activation could provide an alternative source of diacylglycerol for the activation of PKC- ϵ and was naloxone-reversible and at least partially blocked by the tyrosine kinase inhibitor herbimycin A. Addition of phorbol 12-myristate 13-acetate stimulated both PLD and PKC- ϵ activities to a greater extent than opioids. The phorbol ester and insulin stimulation of PLD was also blocked by herbimycin. Both morphiceptin (in a naloxone-reversible manner) and phorbol ester increased phosphorylation of similar cytosolic proteins in intact cells, demonstrating a functional role for the PKC- ϵ activation by opioids. This is evidence that opioid receptors are transiently coupled to tyrosine kinase, PLD and PKC- ϵ activation and, by implication, to neuronal cell growth during brain morphogenesis.

Opioid receptors are expressed as early as the gastrula stage, throughout neuroembryogenesis, and in the adult nervous system (see references in ref. 1). Opioids act as neuromodulatory and neurotrophic agents early in development (2, 3) and promote analgesia, euphoria, dependence, and withdrawal in adults. Species, organism, tissue, and cellular differences in response to opioids suggest a complex, multifactorial, and developmentally regulated signal transduction machinery. Although the structure of opioid receptors remains unknown, it does appear that different opioid receptors are coupled to different signal transduction systems. Thus κ -opioid receptors have been shown to couple to the GTP-binding protein G_o (4, 5) and inhibit Ca^{2+} uptake through voltage-sensitive channels, whereas both μ - and δ -opioid receptors are coupled to G_{i1} (4) or G_{i2} (6), inhibition of adenylate cyclase, and activation of neuronal K^+ channels (7, 8). The decrease in intracellular free Ca^{2+} is not significant enough to affect overall Ca^{2+} stores, nor can it explain all the actions of opioids, especially early in development. At this stage, receptors have not assumed their final role as neurotransmitter release mediators (2, 9) and antinociception would not seem to be a likely function (1–3).

Neuron cultures derived from chicken embryonic day 6 cerebral hemisphere (E6CH cultures) synthesize opioid peptides and express both μ - and δ -opioid receptors (1–3). Opioids had no effect on carbachol- or bradykinin-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate by phos-

pholipase C (PLC) to produce the second messenger inositol 1,4,5-trisphosphate when administered acutely but, when administered for >24 hr to E6CH cultures, inhibited PLC activation by both muscarinic and bradykinin agonists (1). However, it is the activation of protein kinase C (PKC) by diacylglycerol, the other second messenger generated following PLC activation, that has an inhibitory action on phosphatidylinositol-specific PLC. In the present study, we examined the role of μ opioids as possible physiological regulators of signal-activated phospholipases whose products (diacylglycerols) could regulate PKC. The PKC family of isoforms is tentatively organized into the Ca^{2+} -dependent group A (α , β_1 , β_2 , γ) and the Ca^{2+} -independent group B (δ , ϵ , ϵ' , ζ , η). PKC- ϵ (10) is predominantly expressed in the brain (11, 12) and thus far is the only isoform known to be present in chicken embryo neuronal cultures from embryonic day 6 (12) or day 7 (13) forebrain. We report here on the Ca^{2+} -independent activation of phospholipase D (PLD) and PKC- ϵ in early embryonic neurons by μ -opioid receptor agonists and speculate on the role of opioids in early growth and development.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma. One PKC- ϵ -specific antibody (raised against residues 313–326 of PKC) was purchased from Upstate Biotechnology, Lake Placid, NY; and another polyclonal antibody, also specific for PKC- ϵ (residues 836–853) was a gift from K. Heidenrich, University of Colorado. The phosphate acceptor, epidermal growth factor (EGF) receptor peptide VRKRTLRLRG, was synthesized by G. Reddy, University of Chicago. Herbimycin A was a gift from L. Platanias, University of Chicago.

Neuron Cultures. Dissociated neurons derived from E6 or E7 cerebral hemispheres were set up on polylysine-coated culture dishes as described (14, 15). They were shown to be 95% neuronal, with a predominant opioidergic and cholinergic phenotype (1, 2, 14, 15). Treatments and experiments were done routinely after 5 days in culture unless otherwise indicated.

PKC Enzyme Assay. After treatment with morphiceptin (1 μM) or phorbol 12-myristate 13-acetate [“12-*O*-tetradecanoylphorbol 13-acetate” (TPA), (0.1 μM)], neurons were sonicated in 50 mM Tris-HCl/10 mM EGTA/1 mM EDTA/0.3% 2-mercaptoethanol containing protease inhibitors. The homogenate was then centrifuged at $300,000 \times g$ for 40 min to obtain cytosol (supernatant) and membrane (pellet) fractions. Ten micrograms of protein from each fraction was assayed for protein kinase activity in a final mixture of 25 mM Hepes, 10 mM MgCl_2 , 1 mM dithiothreitol, 25 μM ATP, 0.3

Abbreviations: E6CH and E7CH cultures, neuron cultures derived from embryonic day 6 and day 7 cerebral hemispheres; EGF, epidermal growth factor; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PtdEth, phosphatidylethanol; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; TPA, “12-*O*-tetradecanoylphorbol 13-acetate” (phorbol 12-myristate 13-acetate).

μCi of [γ - ^{32}P]ATP (1 μCi = 37 kBq), 20 μg of VRKRTLRLG peptide, 1 mM EGTA (to suppress any Ca^{2+} -dependent, non-PKC kinase activity, since the peptide is not absolutely specific), 5 μg of phosphatidylserine, and 0.3 μg of 1,2-diolein. After a 2-min incubation at 30°C, the reaction was stopped and radioactivity was determined with a scintillation counter. EGTA was replaced with CaCl_2 (0–15 mM) where indicated (e.g., Fig. 2B).

Immunoblot Analysis. The same fractions, standardized for protein concentration (16), were run in an SDS/8% polyacrylamide gel (17). After separation, proteins were transferred onto nitrocellulose membranes and blotted with two different polyclonal antibodies against PKC- ϵ or with monoclonal antibodies specific for PKC isoforms of group A. Primary antibodies were detected by ^{125}I -protein A or peroxidase-conjugated goat anti-rabbit antibodies. We have confirmed that the prevailing PKC isoform in E6CH cultures is PKC- ϵ (12).

PLD Assay. Neurons were exposed to [^3H]palmitate (2 $\mu\text{Ci}/\text{ml}$) for 18 hr, 0.2% ethanol for 60 min, and morphiceptin (1 μM), TPA (0.1 μM), or insulin (100 ng/ml) for up to 30 min. The tyrosine kinase inhibitor herbimycin A was added at 200 ng/ml for 18 hr prior to ethanol. PLD activity was measured as the formation of [^3H]phosphatidylethanol ([^3H]PtdEth), which was isolated by extraction of lipids and high-performance TLC (18).

Phosphoprotein Extraction. At day 1 or 8 of culture, E6CH cultures were labeled with [^{32}P]orthophosphate (296 MBq/ml) for 2 hr prior to 10 min of drug stimulation; proteins were then extracted (19) and samples were analyzed by SDS/8% PAGE and autoradiography.

Statistics. All experiments were performed at least three times, and data were analyzed by ANOVA. Standard errors for PKC assays were at the level of 5%.

RESULTS AND DISCUSSION

PKC- ϵ Activation by Morphiceptin in E6CH Cultures. The opioid peptide morphiceptin (1 μM), a μ -receptor-specific agonist, produced a rapid 2-fold stimulation of cytosolic PKC activity as determined by phosphorylation of a PKC substrate, the synthetic peptide VRKRTLRLG (Figs. 1 and 2A), which is believed to be specific for this kinase (11, 20). The activation was naloxone-reversible, suggesting that it was receptor-mediated. Activation of Ca^{2+} -dependent (group A) PKC isoforms typically results in translocation of PKC from cytosol to membrane (21) and a decrease in cytosolic PKC activity, as exemplified by the action of diacylglycerol mimics such as TPA. However, increased cytosolic activity

FIG. 1. Time course of activation of peptide kinase C activity in cytosol and membrane fractions following morphiceptin treatment of day 5 E6CH cultures. Cultures were treated with 1 μM morphiceptin (μ -opioid receptor agonist) for the times indicated. PKC- ϵ activity in the cytosol (●) and membrane fraction (○) is shown. Shaded area is the time period of translocation of the enzyme to the membrane.

of group B PKC isoforms has been reported for several activators, such as insulin, interferon α , and TPA, and in this case is the result of more enzyme protein being present in the cytosol rather than any increase in specific activity of PKC- ϵ (11, 13). In E6CH cultures treated with TPA (0.1 μM), an acute peak (<30 sec) in cytosolic PKC activity was observed, followed by a sharp decrease (Fig. 2B). Characteristic of the group B-type PKC- ϵ , both morphiceptin and TPA activated PKC in a Ca^{2+} -independent fashion (Fig. 2B).

We quantitated PKC- ϵ protein levels during the time of both TPA- and opioid-induced PKC activation in the cytosol by Western blot analysis with ^{125}I -protein A detection (Fig. 2C and D). Upon activation by morphiceptin, the major change in the intensity of the PKC- ϵ bands on the autoradiograms was an increase in a PKC- ϵ fragment of 60 kDa (Fig. 2C and D, lower arrow). The increase was dramatic for the TPA-treated neurons, an increase not seen with 1,2-dioleoyl-*sn*-glycerol and 1-oleoyl-2-acetyl-*sn*-glycerol. These two diacylglycerols are reported to have potencies 7- to 15-fold lower than phorbol esters for PKC- ϵ activation (22) and provided us with one more criterion for evaluating the morphiceptin-dependent PKC stimulation. The upper band of the 90/92-kDa PKC- ϵ also increased (upper arrow). This appears as a doublet, most probably due to phosphorylation of the upper band (best seen in Fig. 2D) (refs. 11 and 25 and D.M., unpublished data); the 70-kDa band is nonspecific, is unrelated to PKC, and acts to show that the same amount of protein was applied to each lane. The major PKC fragment in neuronal cultures was the 60-kDa form, whereas in crude cell homogenates from any central nervous system tissue of the same embryological age, the 90-kDa form predominated (12). Thus, at this point we consider the 60-kDa form of PKC- ϵ as an active form, on the basis of its lower profile in untreated cultures (Fig. 2C, lane 7, and Fig. 2D, lane 1) and the reversibility of its appearance when naloxone was coadministered with morphiceptin (Fig. 2D, lanes 3, 5, and 6). Further, the generation of an active, lipid-dependent, PKC- ϵ proteolytic fragment has also been reported in studies with recombinant PKC protein (23, 24) and with cell lysates in a small-cell lung carcinoma, which expresses PKC- ϵ as its major isoform (25).

There has been no previous evidence that opioids can act as stimulators of other signal transduction systems, with the possible exception of adenylate cyclase via the stimulatory GTP-binding protein G_s (26, 27). To our knowledge this is the first time that increased activity of a PKC isoform has been observed following treatment with an opioid peptide, a conclusion drawn by implication in previous studies (1, 28).

PKC- ϵ Translocation. Morphiceptin produced a rapid activation of membrane PKC- ϵ (Fig. 1), representing a 2-fold increase in specific activity (Fig. 2). This increase in amount of PKC- ϵ was transient but the peak (1 min) was sharper than the increase in cytosolic PKC activity. In addition to the increased PKC activity found in both cytosol and membrane fractions (Fig. 1) there was also an increase in the ratio of membrane to cytosolic PKC activity from 30% to almost 50% during the first 5 min of stimulation (Fig. 1, shaded area; Fig. 2A). This translocation is more typical of group A, Ca^{2+} -dependent PKC activation by phorbol ester (TPA). In E6CH cultures TPA mediated PKC translocation to the membrane (from 30% to 60% of total PKC activity), with a delayed and more sustained activation profile (for over 10 min; Fig. 2A) than that seen with morphiceptin. The distinction may be important in view of the fact that different dependence upon phospholipids and substrate specificity are likely for various PKC activators (10, 20, 22). The lack of PKC desensitization following TPA activation was expected, based on the absence in PKC- ϵ of the calpain proteolytic clip site (10, 11), found in group A PKC isoforms. The rapid return of PKC- ϵ activity back to control or lower levels in the membrane fraction

FIG. 2. Dependence of kinase (PKC- ϵ) activity of E6CH cultures on activators (morphiceptin and TPA) but independence from free Ca^{2+} . (A) Effects of morphiceptin ($1 \mu\text{M}$, $\circ$) and TPA ($0.25 \mu\text{M}$, $\bullet$) on subcellular distribution of PKC- ϵ with time of exposure, expressed as the percentage of PKC activity remaining in the cytosol at the time indicated. All homogenates were fractionated into membrane and cytosol and each fraction was assayed for PKC activity as measured by *in vitro* phosphorylation of VRKRTLRLG. (B) Dependence of PKC- ϵ activity of E6CH cultures on activators (morphiceptin and TPA for 5 min) but independence of free Ca^{2+} up to 15 mM. Results are expressed as the ratio of membrane to cytosol PKC activity measured with the *in vitro* assay. Points at zero Ca^{2+} concentration were results of assays in the presence of 5 mM EGTA. (C) Quantitative estimation of cytosolic PKC- ϵ . Photograph of a Western blot after exposure to ^{125}I -protein A shows the 90-kDa PKC- ϵ doublet (upper arrow), which increases in intensity following either morphiceptin or TPA treatment, and the active PKC- ϵ fragment at 60 kDa (lower arrow), which also increases with morphiceptin and TPA. The nonspecific band at 70 kDa is not PKC. Lanes: 1–3, increasing time of exposure (0.5, 2.0, 10 min) to morphiceptin (MO, $1 \mu\text{M}$); 4 and 5, diacylglycerols [1-oleoyl-2-acetyl-sn-glycerol (OAG, $1 \mu\text{M}$, 2 min) and 1,2-dioleoyl-sn-glycerol (DOG, $1 \mu\text{M}$, 2 min)]; 6, TPA ($0.1 \mu\text{M}$, 2 min); 7, untreated. (D) Reversibility of morphiceptin-mediated PKC- ϵ increases by the antagonist naloxone. Photograph of a representative Western blot shows both the 90-kDa and 60-kDa active forms of PKC- ϵ (arrows). Lanes: 1, untreated; 2, molecular weight (MW) standards of molecular mass 106, 80, and 49 kDa; 3, morphiceptin ($1 \mu\text{M}$) plus naloxone (NN, $1 \mu\text{M}$) for 10 min; 4, morphiceptin for 10 min; 5 and 6, morphiceptin plus naloxone for 5 min and 2 min, respectively; 7 and 8, morphiceptin for 5 min and 2 min, respectively.

following morphiceptin stimulation (Fig. 2A) may signify proteolytic cleavage by other types of opioid-activated proteases to inactive <50-kDa fragments (23) or opioid receptor down-regulation, leading to a shut off of the receptor-mediated supply of diacylglycerol.

PLD Activation by Morphiceptin. Since we have previously shown that opioid receptors are not linked to phosphatidylinositol-specific PLC in E6CH cultures (1), and there is no evidence for opioid activation of PLC in any other system, the possibility was investigated that the 1,2-diacylglycerol necessary for the activation of PKC could be provided by PLD. The hydrolysis of phosphatidylcholine (PtdCho) by PLD to form phosphatidate can be a source of the diacylglycerol (18, 29, 30) needed for PKC activation via the action of phosphatidate phosphohydrolase (29). Since PLD activation is most clearly demonstrated by the occurrence of transphosphatidyl, whereby choline is replaced by a primary alcohol, we measured the formation of PtdEth from phosphatidic acid after exposure of E6CH cultures to ethanol and either morphiceptin or TPA. Morphiceptin gave a 2- to 3-fold, naloxone-reversible activation of PLD in both E6CH (Fig. 3, solid line) and E7CH (dashed line) cultures, emphasizing the effect of the opioid agonist on two similar but not identical neuronal culture series. The profile was similar to that of PKC activation, with a peak at ≈ 1 min and then a rapid decline (Fig. 3). The decrease in PtdEth labeling after 2 min is attributable to its subsequent metabolism, although this is

much more rapid than is typically observed (e.g., in E15 chicken astrocyte cultures and see ref. 29). TPA ($0.1 \mu\text{M}$) gave a 4-fold stimulation of PLD after 10 min and this was

FIG. 3. Time course of morphiceptin-mediated stimulation of PLD activity in E6CH and E7CH cultures. Neurons were exposed to $[^3\text{H}]$ palmitate for 18 hr and to 0.2% ethanol and morphiceptin for the times indicated. PLD activity was measured as the formation of $[^3\text{H}]$ PtdEth. The stimulation was blocked by coaddition of the opioid antagonist naloxone ($1 \mu\text{M}$).

sustained for at least 30 min (Fig. 4B). PLD activation by TPA was slow in the first 2 min and increased rapidly thereafter, maintaining the maximum level for up to 1 hr. In contrast, morphiceptin effects lasted no more than 10 min. This might be relevant, since PLD exists as both a cytosolic form [acting on phosphatidylethanolamine (PtdEtn) and PtdCho] and a membrane form (acting on PtdCho selectively) (31). These two types of PLD have been shown to be activated preferentially by different agonists. Thus, EGF and insulin cannot stimulate PtdEtn hydrolysis, whereas TPA and platelet-derived growth factor stimulate hydrolysis of both PtdCho and PtdEtn (32). Our findings argue for a differential effect for TPA and morphiceptin. In both cases, PLC activation is not involved, but the time course of PtdEtn formation is consistent with the hypothesis that PtdCho hydrolysis is a prerequisite for PKC- ϵ activation by an opioid agonist, possibly comparable to that of a growth factor.

Agonist-mediated activation of PLD in the absence of activation of PLC has been reported in collagen-stimulated platelets treated with aspirin (33) and EGF-stimulated Swiss mouse 3T3 fibroblasts, where PLD activation appears to be mediated by tyrosine phosphorylation (34, 35). Furthermore, in Daudi cells, there is evidence that interferon- α binding results in (i) diacylglycerol production that does not derive from phosphatidylinositol hydrolysis and (ii) activation of PKC- ϵ preferentially over the α isoform (36). Our observation of such a phenomenon in the nervous system led us to investigate possible mechanisms by which PLD could be activated without prior PLC activation.

Role of Tyrosine Kinases in PLD Activation. The possibility of initial involvement of tyrosine kinase activation in PLD

activation was explored in E6CH cultures, since a known tyrosine kinase activator, insulin, also activated PKC- ϵ in these cultures (13, 37). In addition, other receptors which possess intrinsic tyrosine kinase activity (EGF receptors) can also stimulate PLD (32, 34), and tyrosine phosphatase inhibition by sodium orthovanadate has been reported to activate PLD (38).

A 2-fold PLD activation was observed after treatment of the neurons with insulin for up to 30 min (Fig. 4A), similar to that observed with morphiceptin. Our studies indicated that the tyrosine kinase inhibitor herbimycin A (39) was able to inhibit PLD activation by insulin (Fig. 3B), morphiceptin, and TPA, but with varying degrees of efficacy, suggesting a complex mechanism. Specifically, herbimycin prevented PLD activation by morphiceptin as for insulin (Fig. 4A) but spared 25% of the TPA-induced PLD activation (Fig. 4B). This suggests the operation of more than one mechanism for PLD activation by TPA.

The tyrosine phosphorylation activation of membrane-associated phospholipases has been shown for both PLC and PLD, with regulation by either phosphatases or tyrosine kinase inhibitors (38, 39). PLD activation was originally proposed to be dependent upon the Ca^{2+} increase resulting from prior PLC activation, but further studies have suggested two independent, parallel or overlapping processes. Involvement of tyrosine kinase in receptor coupling to PLD but not PLC has recently been reported in a number of cells, including neutrophils (38). Further, it appears that EGF can stimulate a small but sustained increase in diacylglycerol mass in the absence of Ca^{2+} fluxes and without inositol 1,4,5-trisphosphate formation in Swiss 3T3 cells via a coupled PLD/phosphatidate phosphohydrolase pathway (34). We believe that morphiceptin may be acting more like EGF, although the evidence to connect μ -opioid receptors, tyrosine kinase, and PLD activation with PKC- ϵ activation is only circumstantial. However, activation with morphiceptin did result in the phosphorylation and activation/mobilization of some specific PKC protein substrates.

Phosphorylation of Endogenous Neuronal Substrates. To study a functional result of PKC- ϵ activation, we looked at protein phosphorylation after 10 min of exposure to opioids, a time at which both *in vitro* peptide substrate phosphorylation and the amount of PKC- ϵ protein were maximum in the cytosolic fraction (Fig. 1A, Fig. 2 C and D). Both morphiceptin and TPA enhanced the phosphorylation of similar high (e.g., 125, 67, and 52 kDa) and low (e.g., 30 kDa) molecular mass proteins (Fig. 5). Inclusion of the antagonist naloxone reversed this phosphorylation. The similarities in PKC substrate specificity between morphiceptin- and TPA-treated cells are not unexpected, although TPA gives more intense responses. Differences have been reported between a number of other physiological activators and TPA (20). ^{32}P incorporation per mg of protein was 2- to 3-fold greater at culture day 1, which is a period of active neurite generation than at 8 days, when neurite maintenance predominates (12, 14, 15). The chicken 67-kDa myristoylated, alanine-rich C-kinase substrate (MARCKS-like protein) (40, 41) was always the common major substrate, and phosphorylation was even more enhanced in the presence of calyculin A, a phosphatase inhibitor (data not shown).

Conclusions. We suggest a hypothesis for the mechanism of action for μ -opioid peptides in the developing nervous system in which, after binding to the receptor, opioids can activate a tyrosine kinase-regulated PLD [possibly linked via a GTP-binding protein (42)] which hydrolyzes PtdCho to produce the 1,2-diacylglycerol needed to activate a specific PKC and specific protein phosphorylation. Although we have no knowledge of the nature or function of PKC- ϵ substrates at this stage of development, we do observe that a number of new protein phosphorylation events occur; we speculate that

FIG. 4. PLD activation by insulin and TPA in E6CH cultures. (A) Insulin mediated stimulation of PLD in a time-dependent manner (dark bars). Stimulation was reversed by the tyrosine kinase inhibitor herbimycin A (light bars). (B) TPA activation of PLD (hatched bars) is greater than that by insulin but is only partially reversed by herbimycin (open bars). Horizontal open rectangle on the ordinate shows the basal activity in A and B. Bars represent the mean $\pm$ SE of four samples.

FIG. 5. Stimulation of the phosphorylation of cytosolic PKC protein substrates by morphiceptin and TPA in E6CH cultures at day 1 (C1) and day 8 (C8) in culture. Arrows indicate major PKC substrates at 30, 52, 67 [chicken myristoylated, alanine-rich C-kinase substrate (MARCKS)], and 125 kDa. Lanes: MO, morphiceptin; Control, vehicle only; TPA, phorbol ester. Prestained molecular size markers of 110, 71, and 43 kDa were from Bio-Rad. At C1 more ^{32}P was incorporated when compared with the same number of cells at C8.

this phosphorylation has more to do with protooncogene (*c-fos*, *c-jun*, etc.) activation (43) than with secretion of neurotransmitters. This is quite different from the accepted role of opioids in the neonatal and mature nervous systems, in that it does not involve Ca^{2+} restriction. In the specific case of early embryonic neurons, which are the subject of this study, such signal transduction pathways could be prompting growth as well as responses (i.e., regulation of target genes) for both trophic actions (2) and differentiation processes (i.e., cytoskeleton build-up) (3), which constitute the molecular basis for later-emerging functions, such as memory. Growth factors such as EGF, with evident mitogenic potency, are observed in postmitotic neurons late in life. Their growth-promoting activity is obviously limited, but the second messengers they release may have different functions. Thus, opioids must be considered in a broader framework than just perception and pain, and these distinctly different functions should be sought in early embryonic tissue.

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