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Research Article

Morphine 6 glucuronide stimulates nitric oxide release in mussel neural tissues: evidence for a morphine 6 glucuronide opiate receptor subtype

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Abstract. We have previously demonstrated that *Mytilus edulis* pedal ganglia contain opiate alkaloids, i.e., morphine and morphine 6 glucuronide (M6G), as well as mu opiate receptor subtype fragments exhibiting high sequence similarity to those found in mammals. Now we demonstrate that M6G stimulates pedal ganglia constitutive nitric oxide (NO) synthase (cNOS)-derived NO release at identical concentrations and to similar peak levels as morphine. However, the classic opiate antagonist, naloxone, only blocked the ability of morphine to stimulate cNOS-derived NO release and not that of M6G. CTOP, a mu-specific antagonist, blocked the ability of M6G to induce cNOS-derived NO release as well as that of morphine, suggesting that a novel mu opiate receptor was present and selective toward M6G. In examining a receptor displacement analysis, both opiate alkaloids displaced [^{3}H]-dihydromorphine binding to the mu opiate receptor subtype. However, morphine exhibited a twofold higher affinity, again suggesting that a novel mu opiate receptor may be present.

Key words. Nitric oxide; morphine 6 glucuronide; morphine; mu receptor; CTOP; nervous tissue; invertebrate; mussel.

Morphine 6 beta-glucuronide (M6G), a metabolite of morphine, is an opiate agonist that plays a role in the clinical effects of morphine [1]. Additionally, the relative efficacy of M6G is higher than that of morphine in locus caeruleus neurons [2], suggesting fundamental differences between these endogenous opiate alkaloid signal molecules [3].

Recently, we identified both morphine and M6G by nanoflow electrospray ionization double quadrupole orthogonal acceleration time-of-flight mass spectrometry (Q-TOF MS) in *Mytilus edulis* pedal ganglia [4]. This organism's nervous tissue also expresses a mu opiate receptor transcript that exhibits high sequence similarity to that found in mammals [5]. Thus, *Mytilus* neural tissue may provide insight into whether or not morphine and M6G are using the same type of mu opiate receptor.

In the present study, we demonstrate that M6G can stimulate constitutive nitric oxide (NO) synthase (cNOS) in a naloxone insensitive and D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP; a selective mu receptor antagonist [6]) -antagonizable manner, whereas both mu opiate receptor antagonists block NO release induced by morphine. Thus, M6G appears to exert cNOS-derived NO release via a novel mu opiate receptor subtype.

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Material and methods

M. edulis was collected directly from the seashore in Mattituck, Long Island, New York, and kept under laboratory conditions as previously described [7]. *C*ontrol animals (600) were kept in artificial seawater (Instant Ocean, Boston, Mass.) at room temperature (25 °C). For the biochemical receptor studies, the number of animals was increased to 800. Each experiment was repeated three times.

Binding analysis

Eight hundred pedal ganglia of *Mytilus* were dissected at room temperature and stored on ice. The ganglia were incubated at 9 °C for 30 min in the incubation medium containing antibiotics (50 mg streptomycin, 30 mg penicillin, 50 mg gentamicin in 100 ml, pH 7.5). The ganglia were washed three times in *Mytilus* saline and processed for binding as described in detail elsewhere [8]. Briefly, before homogenization, they were washed in saline [9] and then homogenized in 5 ml of 0.32 M sucrose, pH 7.4, at 4 °C, using a Brinkmann polytron (30 s, setting no. 5). The crude homogenate was centrifuged at 900 g for 100 min at 4 °C, and the supernatant was reserved on ice. The whitish crude pellet was resuspended by homogenization (15 s, setting no. 5) in 30 vol of 0.32 M sucrose/ Tris-HCl buffer, pH 7.4, and centrifuged at 900 g for 10 min. The resulting supernatants (S_1') were used immediately.

Prior to the binding experiment, the S_1' supernatant was centrifuged at 30,000 g for 15 min and the resulting pellet (P_2) was washed once by centrifugation in 100 µl of sucrose/Tris-HCl. The P_2 pellet was then resuspended with a Dounce hand-held homogenizer (ten strokes) in 100 vol of buffer. Binding analysis was then performed on the cell membrane suspensions. To demonstrate μ_3 binding, all experiments were performed in the presence of D-ala^2-Met^5-enkephalinamide (DAMA, 1 µM) to occupy the previously reported delta binding sites (δ^1 and δ^2) in these tissues [8, 10–12]. This ensured that the novel μ_3 site would remain free, since it is opioid peptide insensitive [12].

Aliquots of membrane suspension (0.2 ml, 0.12 mg of membrane protein) were incubated at 4 °C for 90 min with the appropriate radiolabeled ligand in the presence of dextrorphan (10 µM) or levorphanol (10 µM) in 10 mM Tris-HCl buffer, pH 7.4, containing 0.1% bovine serum albumin (BSA) and 150 mM KCl. Free ligand was separated from membrane-bound labeled ligand by filtration under reduced pressure through GF/B glass fiber filters (Whatman); filters were presoaked (45 min, 4 °C) in buffer containing 0.5% BSA. The filters were rapidly washed with 2.5-ml aliquots of the incubation buffer (4 °C), containing 2% polyethylene glycol 6000 (Baker). The filters were assayed by liquid scintillation spectrometry for bound [^{3}H]-agonists (Packard 460). Stereospecific binding was defined as binding in the presence of 10 µM levorphanol. Protein concentration was determined in membrane suspensions (prepared in the absence of BSA).

For the displacement assay, aliquots of ganglionic membrane suspension were incubated with nonradioactive opioid/opiate compounds or extracts at six concentrations for 10 min at 22 °C and then with [^{3}H]-dihydromorphine ([^{3}H]-DHM) for 60 min at 4 °C. One hundred percent binding is defined as bound [^{3}H]-DHM in the presence of 10 µM dextrorphan minus bound [^{3}H]-DHM in the presence of 10 µM levorphanol. The IC_{50} is defined as the concentration of drug or extract which elicits half-maximal inhibition of specific binding. The mean ± SE for three experiments are reported for each compound tested. [^{3}H]-DHM was obtained from New England Nuclear, Boston, Mass. Opioid peptides and CTOP were obtained from Sigma (St. Louis, Mo.).

Monitoring of NO release

Central nervous system (ten pedal ganglia) of *M. edulis* were bathed in an artificial saline (Instant Ocean) incubation medium [13]. NO release was monitored with an NO-selective microprobe manufactured by World Precision Instruments (Sarasota, Fla). The redox current was detected by a current-voltage converter circuit and continuously recorded. The tip diameter of the probe (200 µm) permitted the use of a micromanipulator (Zeiss-Eppendorff) attached to the stage of an inverted microscope (Nikon Diaphot) to position the sensor 20 µm above the respective tissue surface. Calibration of the electrochemical sensor was performed using different concentrations of a nitrosothiol donor S-nitroso-N-acetyl-DL-penicillamine (SNAP), as previously described [13]. The NO detection system was calibrated daily. The probe was allowed to equilibrate for 12 h in the incubation medium free of tissue before being transferred to vials containing the ganglia for another 30 min. Manipulations/handling of the ganglia were only performed with glass instruments. Each experiment was repeated four times and the NO mean values every 2 min were graphed to represent the actual NO release (± SE). Each experiment was performed simultaneously with a control (vehicle minus drug) from the same animal. Thus, the experiment was performed with three probes measuring the different experimental preparations (control, drug exposed, and drug plus antagonist). This strategy eliminated the probability of 'noise' drift.

The data obtained were then evaluated by Student's t test. Data acquisition was by computer-interfaced DUO-18 software (World Precision Instruments). The experimental values were then transferred to Sigma-Plot and -Stat (Jandel, Calif.) for graphic representation and evaluation. Data gatherers (undergraduate research assistants) were unaware of the experimental conditions.

Results

Figure 1 and table 1 demonstrate that morphine, as noted in earlier reports [14, 15], and M6G stimulate pedal ganglia cNOS-derived NO release at identical concentrations and to similar peak levels. Both morphine and M6G, at lower concentrations (10^{-7} M), exert an additive effect on ganglionic NO release, suggesting they are operating via separate receptors (table 1). The classic opiate antagonist, naloxone, only blocked the ability of morphine to stimulate cNOS-derived NO release and not that of M6G. CTOP, a mu-specific antagonist, did block the M6G ability to induce cNOS-derived NO release as well as that of morphine, suggesting that a novel mu opiate receptor was present and selective towards M6G (table 1, fig. 1). Furthermore, morphine 3 glucuronide does not stimulate cNOS-derived NO release.

In examining the displacement analysis, both opiate alkaloids displaced [^{3}H]-DHM binding to the μ_3 opiate receptor subtype (table 2). However, in this regard, morphine exhibited a two-fold higher affinity, again suggesting a novel mu opiate receptor may be present, since both ligands stimulated NO release to the same level and at the same dose (table 1).

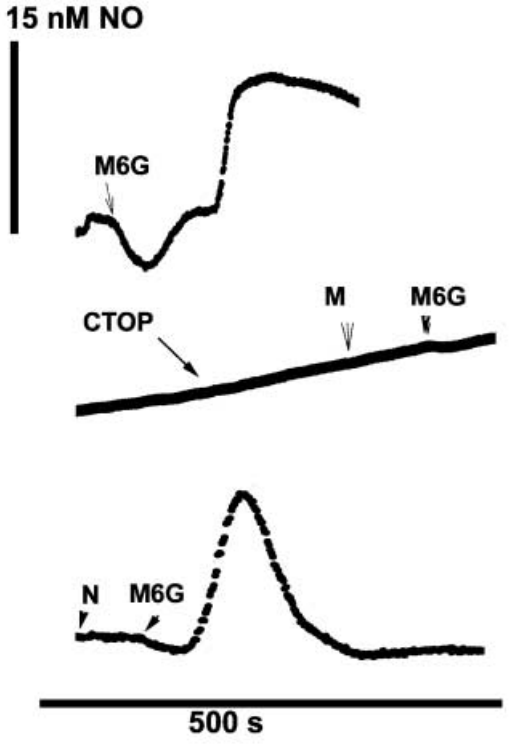

Figure 1. Representative MGG-stimulated (10^{-7} M) NO release from *Mytilus* ganglionic tissues (top). CTOP (10^{-7} M) antagonism of morphine (M, 10^{-7} M) and M6G (10^{-7} M) stimulated release of pedal ganglia NO release (middle). Naloxone (10^{-7} M) does not antagonize M6G ganglionic NO release.

Table 1. Differential effects of opiate antagonists on the ability of opiate alkaloids to stimulate ganglionic NO release.

Agent	Concentration (M)	Peak NO Levels (nM ± SE)
Control		0.8 ± 0.2
M6G	10^{-9}	0.9 ± 0.3
M6G	10^{-7}	12.3 ± 2.7
M6G	10^{-6}	27.4 ± 4.5
M	10^{-9}	0.8 ± 0.3
M	10^{-7}	14.1 ± 3.2
M	10^{-6}	31.8 ± 5.1
M + M6G	10^{-7}, 10^{-7}	24.4 ± 2.1
M + M6G + N	10^{-7}, 10^{-7}, 10^{-7}	11.4 ± 1.3
M6G + N	10^{-6} M (both)	26.3 ± 3.9
M6G + CTOP	10^{-6} M (both)	2.7 ± 0.9
M + N	10^{-6} M (both)	3.6 ± 1.2
M + CTOP	10^{-6} M (both)	2.8 ± 1.4

M, morphine; M6G, morphine 6 glucuronide; N, naloxone. Each experiment was performed and replicated as indicated in the text.

Table 2. Displacement of ^{3}H-dihydromorphine by opioid and opiate ligands in membrane suspensions of ganglionic homogenates of *Mytilus edulis*.

δ	IC_{50} (nM ± SE)
DPDPE	>1000
DAMA	>1000
μ	
DAGO	>1000
Dihydromorphine	33.4 ± 7.2
Morphine	35.6 ± 5.7
Morphine 6 glucuronide	79.5 ± 6.1
Morphine 3 glucuronide	>1000
Antagonists	
Naloxone	51 ± 7.3
CTOP	43.5 ± 5.1

Aliquots of membrane ligands were incubated with nonradioactive compounds at five concentrations for 10 min at 22 °C and then with [^{3}H]-dihydromorphine for 60 min at 40 °C. One hundred percent binding is defined as bound [^{3}H]-dihydromorphine in the presence of 1 μM [^{3}H]-DAMA. IC_{50} is defined as the concentration of drug which elicits half-maximal inhibition of specific binding. Statistical significance of morphine and M6G binding is at the $p < 0.01$ level of confidence (ANOVA). DPDPE, (D-Pen^2, D-pen^5) enkephalin; DAGO, D-ala^2. N-MePhe^4, Gly(ol)^5)-enkephalin; DAMA, D-ala^2 methionine^5 enkephalinamide; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2.

Discussion

In the present report, we have provided evidence for the existence of a novel mu opiate receptor subtype that is naloxone insensitive and CTOP selective. Both morphine and M6G stimulate ganglionic cNOS-derived NO release. CTOP, a mu-selective antagonist, inhibits this action of both opiate alkaloids, whereas naloxone, the classic, non selective opiate receptor subtype antagonist, only inhibits morphine action. Both ligands, at the same

concentration, release similar peak levels of NO and at low concentrations exert an additive effect on NO release peak levels, suggesting that they may be acting through a different mu receptor. An analysis of the ganglionic displacement data reveals that morphine has a twofold higher affinity for the naloxone-sensitive receptor compared to M6G, and that CTOP exhibits a similar affinity as morphine. We surmise that M6G may exert its actions via a novel mu opiate receptor, one selective to CTOP antagonism and M6G actions.

There is literature supporting a separate and selective mu opiate receptor for M6G. Rossi and colleagues [16, 17], using an antisense probe targeting Gi alpha 1 in mammals, found that it blocked both heroin and M6G analgesia, but not that of morphine. Their results indicated that heroin, 6-acetylmorphine, fentanyl, and etonitazine can all produce analgesia through a novel mu analgesic system which is similar to that activated by M6G [17]. Antisense mapping studies on exons 1, 2 and 3 of MOR-1 in mice suggested the presence of a novel mu receptor subtype responsible for M6G analgesia that may represent a splice variant of MOR-1 [18]. In locomotor activity in C57BL/6JBom mice, there was a dose-dependent development of tolerance to daily injections of morphine or M6G [19]. These investigators also found that there was a lack of cross-tolerance toward M6G after 1 day of morphine pretreatment, whereas cross-tolerance to M6G was observed after 7 days of exposure to morphine pretreatment. They concluded that the main part of the effect caused by M6G was mediated through a specific M6G receptor. We previously demonstrated that in three murine macrophage cell lines (J774.2, RAW 264.7, and BAC1.2F5), the mu opiate receptor subtype is μ_3 because it binds morphine, its active metabolite M6G, and certain other alkaloids, excluding morphine 3 glucuronide and any of the opioid peptides tested [20] as also noted in the current report. Additionally, the study also noted that the affinity of M6G for this receptor was not as strong as that of morphine, suggesting once more that it may not be the selective M6G receptor. Thus, the two endogenous opiate alkaloids would appear to have separate receptor-mediated processes, as suggested by our data as well.

The significance of these studies is underlined by our recent finding of both morphine and M6G by Q-TOF MS in *M. edulis* pedal ganglia [4]. Thus morphine and the M6G are endogenous signal molecules [3]. Additionally, this organism's nervous tissue also expresses a mu opiate receptor transcript that exhibits high sequence similarity to that found in mammals [5]. We surmise the existense of a novel mu opiate receptor splice variant to account for the novel finding of a separate M6G receptor that is CTOP antagonizable in *Mytilus* neural tissues.

In conclusion, opioid processes appear to have evolved much earlier than previously thought. Opioid peptide and opiate alkaloid signaling are evolutionarily conserved, as demonstrated by the high sequence identity of the actual signaling molecules as well as the mu opiate receptor. We surmise that this signaling 'family' is quite important in evolution based on this high level of conservation. Furthermore, the significance of this signaling family can be ascertained by the fact that it can simultaneously coordinate, in a timedependent manner, both immune and neural signaling appropriate to a noncognitive and/or cognitive threat in animals [21–23].

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