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No evidence for G-protein-coupled epsilon receptor in the brain of triple opioid receptor knockout mouse

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Abstract

Pharmacological approaches have defined the epsilon receptor as a β-endorphin-preferring opioid receptor, described in rat vas deferens and in brain of several species. Only three opioid receptors—mu, delta and kappa—have been cloned and the existence of this additional subtype as a distinct protein remains controversial. Recently, the mouse brain epsilon receptor was detected in a G protein activation assay, as mediating residual β-endorphin activity following pharmacological blockade of mu, delta and kappa receptors. To clarify whether this site is independent from mu, delta and kappa receptors, we performed β-endorphin-induced [^{35}S]GTPγS binding using mice lacking these three receptors (triple knockout mice). We tested both pons–medulla and whole brain preparations. β-Endorphin strongly stimulated [^{35}S]GTPγS binding in wild-type membranes but had no detectable effect in membranes from triple knockout mice. We conclude that the brain epsilon site involves mu, delta and/or kappa receptors, possibly coupled to nonclassical G proteins.

Keywords: Epsilon receptor; Opioid receptor; Knockout mice; [^{35}S]GTPγS binding assay

1. Introduction

Opioid drugs exert their biological effects following binding to opioid receptors. Molecular cloning has evidenced three genes encoding mu (MOP), delta (DOP) and kappa (KOP) receptors (Kieffer, 1995), as previously predicted by pharmacological data. Opioid pharmacology however is complex and subtypes have been postulated within each mu, delta and kappa opioid receptor class (references in Kieffer, 1995), possibly resulting from distinct ligand–receptor interactions (Befort and Kieffer, 1997), variable cellular contents or receptor oligomerization (Devi, 2001). Intriguingly, the existence of another opioid receptor type, named epsilon, has been proposed and remains controversial.

The existence of the epsilon receptor was originally postulated by Wüster et al. (Wüster et al., 1979) studying opioid-induced inhibition of electrically evoked contractions of several smooth muscle preparations. Epsilon sites were proposed to account for unique characteristics of opioid responses in the rat vas deferens as compared to the classical mu and delta responses observed in the guinea pig ileum and the mouse vas deferens, respectively. Experimentally, the postulated epsilon site displays a high preference for β-endorphin, as compared to other endogenous opioid peptides or prototypical mu and delta receptor agonists (Sanchez-Blazquez et al., 1984; Wüster et al., 1979). Besides, benzomorphan drugs (ethylketocyclazocine, bremazocine) appear to be antagonists at this site whilst agonists at mu and delta receptors (Gillan et al., 1981). However, the existence of a non-mu, non-delta, non-kappa type of opioid receptor in the rat vas deferens was not consensual and several studies proposed alternative explanations for rationalizing experimental data (Schulz et al., 1979; Sheehan et al., 1988; Smith and Rance, 1983).

Interestingly the existence of epsilon sites was suggested in the rat brain also, on the basis of binding studies. [^{3}H]β-Endorphin labeling showed a unique regional distribution (Akil et al., 1980; Goodman et al., 1983; Johnson et al., 1982) and distinct sensitivity to bivalent cations (Law et al., 1979) as compared to other tritiated opioid ligands. Later,

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studies aiming at characterizing the so-called "benzomorphan sites" in rat brain revealed strong similarities with properties of the epsilon receptor described in the rat vas deferens. In particular, the binding affinity of several opioid compounds for this site labeled by tritiated benzomorphans in presence of excess mu, delta and kappa 1 blockers correlated well with their potency in the rat vas deferens bioassay (Chang et al., 1984; Nock et al., 1990). Noticeably, benzomorphans behaved as antagonists at this site as they are in rat vas deferens (Nock et al., 1990). Further investigation established an extensive pharmacological profile of the epsilon site in the brain of several species (Nock et al., 1993). This G-protein-coupled site shows highest affinity for benzomorphans (bremazocine, ethylketocyclazocine), oripavines (etorphine, diprenorphine, buprenorphine), other non-selective alkaloid antagonists (naloxone, naltrexone) and β-endorphin fragments including residues 1–21. It has low affinity for enkephalins and dynorphins and virtually no affinity for prototypical mu, delta and kappa 1 ligands. No selective compounds for this receptor could be identified.

What could be the functional role of central epsilon receptors? Tseng and co-workers investigated the possibility that brain epsilon receptors could mediate analgesia produced by supraspinal β-endorphin, in rats as well as in mice (see Narita and Tseng, 1998; Tseng, 2001 for reviews). Their findings supported the notion that supraspinal β-endorphin would act at a receptor distinct from mu, delta and kappa, supposedly epsilon. Furthermore, i.c.v. injection of bremazocine and etorphine, considered putative epsilon ligands, seemed to activate the same descending pathways as β-endorphin (see Narita and Tseng, 1998). It is however puzzling that these compounds behaved as agonists in analgesia studies, but were described as antagonists in the rat vas deferens bioassay and brain binding studies.

More recently, the ability of β-endorphin to activate a receptor distinct from mu, delta and kappa types was assessed in mouse brain, using the [^{35}S]GTPγS binding assay. β-Endorphin was able to induce significant G protein activation in pons–medulla membranes, under conditions where binding to mu, delta and kappa receptors was blocked by selective antagonists (Mizoguchi et al., 2000). Further, a significant stimulation of [^{35}S]GTPγS binding by β-endorphin also remained in pons–medulla membranes from mu receptor knockout mice in presence of excess delta and kappa blockers (Mizoguchi et al., 2002). In both cases, the authors proposed that epsilon receptors would mediate the residual β-endorphin activity.

The present study aims at clarifying whether the mouse brain epsilon site is independent from mu, delta and kappa receptors, using genetic invalidation instead of pharmacological blockade. We thus used tissue from mice lacking mu, delta and kappa receptors (triple knockout mice) to perform [^{35}S]GTPγS binding experiments. We tested pons–medulla, where Mizoguchi et al. detected epsilon receptors, as well as whole brain (excepting cerebellum), in order to extend our analysis beyond this particular area.

2. Materials and methods

2.1. Chemicals

Human β-endorphin was purchased from Calbiochem (San Diego, CA). β-Fulnaltrexamine, naltrindole, nor-binaltorphimine and GDP were from Sigma (St Louis, MO). [^{35}S]GTPγS (1250 Ci/mmol) was from Perkin Elmer (Boston, MA).

2.2. Animals

Mice lacking mu, delta or kappa opioid receptors were generated by homologous recombination. Triple knockout mutants were produced by interbreeding of single-mutant mice and maintained on the original hybrid 50% 129Sv/50% C57Bl6J (129/Bl6) genetic background. These mice were characterized by binding studies (Simonin et al., 2001) and acute antinociceptive responses (Martin et al., 2003). There was no obvious alteration of growth and fertility in the mutant animals. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986.

2.3. Brain membrane preparation

Male and female mice, from 7 to 20 weeks old, were decapitated. Sex and age were matched between genotypes. Brains were excised, and whole brain (excluding cerebellum) or dissected pons–medulla were quickly frozen. Exclusion of the cerebellum was based on the localization of previously described epsilon receptors (forebrain membranes in Nock et al., 1993; brainstem structures in Narita and Tseng, 1998) as well as on the general scarcity of opioid receptors in this brain region. Membranes were prepared as previously described (Simonin et al., 2001). Briefly, frozen samples from two animals were homogenized using a Polytron in 10 volumes of ice–cold 0.25 M sucrose. The homogenate was centrifuged at 1350 × *g* for 10 min. The pellet was resuspended in 0.25 M sucrose and spun again at 1350 × *g* for 10 min. The two supernatants were pooled and centrifuged at 48,000 × *g* for 30 min. The resulting pellet was resuspended in 0.32 M sucrose and homogenized using a Potter. Protein content was determined using the Bradford assay and aliquots were stored at − 80 °C.

2.4. Agonist-stimulated [^{35}S]GTPγS binding assay

Membranes (5 μg) were incubated in assay buffer (50 mM Tris–HCl pH 7.4; 3 mM $MgCl_2$; 100 mM NaCl; 0.2 mM EGTA; 30 μM GDP; 0.05 nM [^{35}S]GTPγS) with ligands in a total volume of 200 μl, at 30 °C for 1 h. For pharmacological blockade assays, 10 μM β-endorphin was used together with a combination of antagonists consisting of β-fulnaltrexamine (10 μM), naltrindole (30 nM or 10 μM) and nor-binaltorphimine (100 nM or 10 μM). For

dose–response curves, 13 concentrations of β-endorphin, ranging from 0.2 nM to 10 μM, were used. Reaction was terminated by rapid filtration through Whatman GF/B filters with an ice-cold buffer containing 50 mM Tris–HCl pH 7.4; 50 mM NaCl and 5 mM $MgCl_2$ using a Brandel cell harvester (Gaithersburg, MD). Bound radioactivity was determined with a liquid scintillation analyzer (Beckman LS6500, Fullerton, CA). Nonspecific binding was measured in presence of 10 μM unlabeled GTPγS and subtracted from total binding for both basal and stimulated conditions to obtain specific binding values. Stimulated specific binding was converted in percentage of basal specific binding, defined as 100%. Data were analyzed using Prism Graphpad software. One to four independent assays were performed on each of three distinct membrane preparations. Averages of stimulation (%) and EC_{50} (nM) were calculated for each preparation and final results are expressed as means ± S.E.M. of these three values.

3. Results

3.1. Activation of G proteins by β-endorphin is partially blocked by selective mu, delta and kappa receptor antagonists

To determine whether, as for Mizoguchi et al. (2000), epsilon sites are detectable in the pons–medulla of our wild-type mice, we performed [^{35}S]GTPγS binding experiments with β-endorphin in the presence of selective mu, delta and kappa receptor antagonists. We employed the same combination of antagonists as Mizoguchi et al. and used identical concentrations to reverse [^{35}S]GTPγS binding induced by 10 μM β-endorphin. The antagonist combination consisted of 10 μM β-fulnaltrexamine, 30 nM naltrindole and 100 nM nor-binaltorphimine. We first verified that these concentrations of antagonists were able to fully inhibit activation of G proteins by 10 μM selective mu (DAMGO), delta (SNC80) and kappa (U-50844) receptor agonists, respectively, confirming that blockade of classical mu, delta and kappa sites was complete under these experimental conditions (data not shown). We then tested activation of G proteins by β-endorphin. Ten μM β-endorphin induced 302.8 ± 3.3% of basal [^{35}S]GTPγS binding and addition of the three antagonists decreased this activation to 127.5 ± 4.6% of basal level. This residual activation therefore represents the epsilon-mediated response described by Mizoguchi et al.

3.2. Activation of G proteins by β-endorphin is abolished in triple knockout mice brain

To determine whether epsilon sites are detectable in the absence of mu, delta and kappa opioid receptors, we established dose–response curves for the stimulation of [^{35}S]GTPγS binding by β-endorphin in membranes from pons–medulla of both wild-type and triple knockout mice (Fig. 1A). β-Endorphin increased [^{35}S]GTPγS binding in wild-type preparations with an EC_{50} of 292 ± 11 nM and a maximal effect of 254.3 ± 12.2%. In contrast, the peptide was almost ineffective in membranes from triple knockout mice, eliciting 110.6 ± 0.6% of basal [^{35}S]GTPγS binding at a 10 μM concentration. This value is significantly lower than the residual level of activation obtained in wild-type preparations in presence of the three antagonists (127.5 ± 4.%, Student's *t*-test, $p = 0.02$). This indicates that the absence of mu, delta and kappa receptors markedly diminishes the epsilon-mediated response.

To examine whether the epsilon activity in wild-type mice arises from incomplete blockade of β-endorphin binding sites by the three antagonists, we used a combination where the concentration of all three antagonists was raised to 10 μM. Under these conditions, the activation of G proteins was further reduced to 112.2 ± 0.6% of basal level. This value is comparable to the level of stimulation obtained in triple knockout mice (110.6 ± 0.6%, Student's *t*-test,

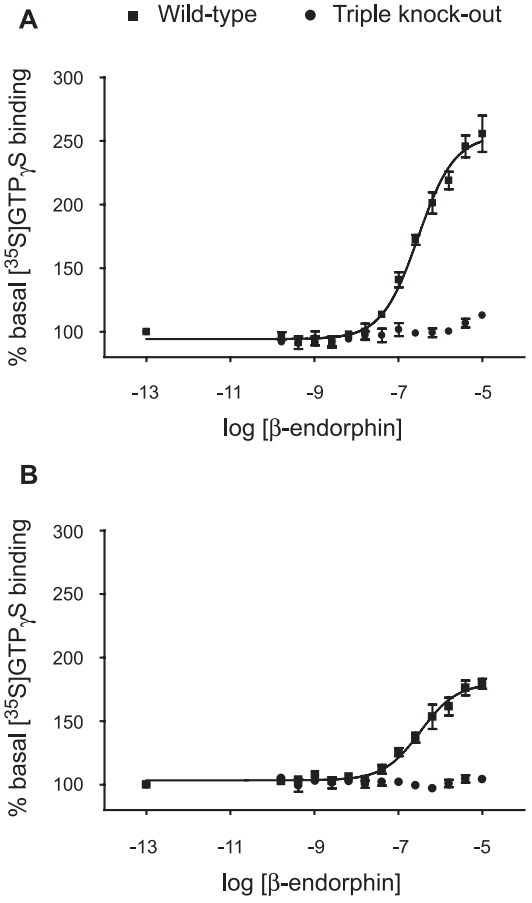

Fig. 1. Stimulation of [^{35}S]GTPγS binding to pons–medulla (A) and whole brain (without cerebellum) (B) membranes by β-endorphin. Membranes from wild-type and triple knockout mice were incubated with increasing concentrations of β-endorphin (see Materials and methods). Plotted values represent percentage of basal [^{35}S]GTPγS binding (without agonist). Results are mean ± S.E.M. of four (A) and seven (B) independent experiments performed in triplicate and conducted on three distinct membrane preparations.

$p = 0.2$). The epsilon-mediated response, therefore, is equally decreased either by high concentrations of selective mu, delta and kappa receptor antagonists, or invalidation of mu, delta and kappa receptor genes.

We finally tested β-endorphin activity in whole brain (minus cerebellum) membranes (Fig. 1B). Again, β-endorphin induced [^{35}S]GTPγS binding in wild-type preparations ($EC_{50} = 374 \pm 91$ nM, maximal stimulation = $180.6 \pm 4.9\%$), and this activation was almost undetectable in membranes from triple knockout mice ($104.2 \pm 1.6\%$ at 10 μM).

Noticeably, the slight activation left in the triple knockout mice was only detected with 10 μM β-endorphin. Both in pons–medulla and in whole brain without cerebellum, the stimulation was indistinguishable from basal level up to a 1.6 μM concentration ($100.4 \pm 1.4\%$ and $100.9 \pm 2.9\%$, respectively). The [^{35}S]GTPγS binding activity observed at a 10 μM concentration in the triple mutants therefore represents a low efficacy response whose significance remains to be clarified. Altogether in our study, both pharmacological blockade and genetic invalidation of mu, delta and kappa receptors ablate the epsilon component of β − endorphin activity.

4. Discussion

4.1. Genetic invalidation versus pharmacological blockade

Our data show that inactivation of mu, delta and kappa receptors essentially abolishes β-endorphin-stimulated [^{35}S]GTPγS binding in the pons–medulla, as well as in the entire mouse brain (excepting cerebellum). Our study follows previous investigations by Mizoguchi et al. These authors reported significant stimulation of [^{35}S]GTPγS binding by β-endorphin, either in wild-type mouse pons–medulla in the presence of blocking concentrations of mu, delta and kappa receptor antagonists (Mizoguchi et al., 2000), or in pons–medulla of mu receptor knockout mice in the presence of delta and kappa blockers (Mizoguchi et al., 2002). Specifically, this residual stimulation of [^{35}S]GTPγS binding by 10 μM β-endorphin was 83% (first study) and 105% (second study) above basal level, and was therefore considered to evidence the involvement of a non-mu, non-delta, non-kappa receptor, namely epsilon receptor. Consistent with their findings, we observed a residual stimulation in our wild-type mice under the same experimental conditions, although to a lower extent (27% above basal level). This epsilon-mediated activity, however, was abolished in our mutant mice lacking mu, delta and kappa receptors. A likely explanation for the apparent discordance between the two sets of data is that the residual G protein activation detected in wild-type mice arises from incomplete pharmacological blockade of mu, delta and kappa receptors by antagonists. Consistent with this assumption, raising the concentrations of antagonists further decreased the residual activation of G proteins to a level comparable to the level observed in triple knockout animals. Our data therefore do not support the existence of epsilon receptors that are independent from mu, delta and kappa receptors in mouse brain.

4.2. The epsilon site, a nonclassical form of mu, delta and kappa receptors?

There are several interpretations to the fact that β-endorphin does not stimulate [^{35}S]GTPγS binding to brain membranes in the absence of mu, delta and kappa receptors. First, the putative epsilon receptor may be a receptor distinct from the three known opioid receptors, and not coupled to G proteins. This is unlikely since epsilon receptors were early characterized as G-protein-coupled receptors (Nock et al., 1993). Second, the epsilon receptor may be a receptor distinct from the three known opioid receptors, but would activate G protein subtypes that are not well detected in the [^{35}S]GTPγS binding assay because of low basal activity (such as G_s or G_q/G_{11} G proteins, see Milligan, 2003). This receptor, however, would not have been detected in previous studies involving the [^{35}S]GTPγS binding assay. Third, and most likely, the epsilon site directly involves mu, delta and/or kappa receptors.

In the latter case, a possible explanation for the "epsilon" pharmacological profile of β-endorphin could be that mu, delta and/or kappa opioid receptors preferentially interact with this peptide when coupled to transduction pathways distinct from $G_{i/o}$ protein-mediated pathways. In accordance, epsilon-mediated analgesia was reported to be pertussis toxin resistant (see Narita and Tseng, 1998). This finding originally supported the notion that epsilon sites were distinct from mu, delta and kappa receptors, classically coupled to the pertussis toxin-sensitive $G_{i/o}$ proteins. However, there is evidence for opioid receptor coupling to pertussis toxin-insensitive G_z proteins, both in heterologous systems (see Connor and Christie, 1999) and in central nervous system (see, for example, Yang et al., 2000). It is conceivable that G_z-coupled forms of mu, delta and/or kappa receptors, for example, could represent privileged binding sites for β-endorphin. Such sites could be less sensitive to antagonist blockade and would be abolished in the triple knockout mice.

It is also tempting to speculate that the epsilon site results from opioid receptor dimerization or from heterodimerization of an opioid receptor with another partner. Again, these complexes could be less sensitive to opioid antagonists, but would be disrupted in the triple knockout mice. Supporting this hypothesis was the observation that opioid receptor heterodimers display unique binding, signalling and trafficking properties (Levac et al., 2002). Interestingly, mu-delta dimers were proposed to be coupled to pertussis toxin insensitive G proteins (George et al., 2000). There is also evidence for interaction of opioid receptors with β_2-adrenoceptors (Jordan et al., 2001) and chemokine CCR5 receptors (Suzuki et al., 2002), associated with altered

trafficking and signalling properties, respectively. However, none of these dimers has yet been proven to exist in brain neurons, and, in the future, experiments using single and double knockout mutants may clarify this issue.

Finally, it was previously proposed that the orphan G-protein-coupled receptor encoded by the GPR7 gene could represent the epsilon site (Narita and Tseng, 1998; O'Dowd et al., 1995). Some findings however are not consistent with this supposition. First, naloxone lacks affinity for the GPR7 receptor (O'Dowd et al., 1995) whereas it binds with nanomolar affinity to the epsilon site (Nock et al., 1993). Also, the distribution of GPR7 mRNA in rat brain (Lee et al., 1999) displays significant mismatches with the map of rat brain sites sensitive to epsilon-mediated antinociception (see Narita and Tseng, 1998). Recently, the GPR7 receptor has been desorphanized (see references in Tanaka et al., 2003) and the proposed endogenous ligands for this receptor, neuropeptides W and B, bear no homology with β-endorphin. Finally, GPR7 activation was shown to be pertussis toxin sensitive (Brezillon et al., 2003), contrary to epsilon-mediated antinociception (Narita and Tseng, 1998). Although many G-protein-coupled receptors still remain orphan, and some of them may indeed bind β-endorphin with high affinity, it is unlikely from our data that one of them indeed constitutes the epsilon receptor.

In conclusion, we propose that the putative epsilon receptor result from β-endorphin activity at a site involving mu, delta and/or kappa opioid receptors, possibly coupled to nonclassical G proteins. β-Endorphin otherwise is a complex peptide which, like the other opioid peptide Bovine Adrenal Medulla 22 (BAM22) (Lembo et al., 2002), has both opioid (naloxone sensitive) and non-opioid (naloxone insensitive) activities. Indeed β-endorphin is known to modulate immune cell function via its non-opioid C-terminus (Gilmore and Weiner, 1989; Hazum et al., 1979), and the molecular target for this activity remains to be identified.

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