Regulation of Opioid Antagonist and Mu, Kappa or Delta Agonist Binding by Guanine Nucleotide and Sodium

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Abstract—Effects of 5'-guanylylimidodiphosphate (Gpp(NH)p) and sodium on the inhibition by various opioids of [3H]-naloxone binding to guinea-pig brain membrane preparations were studied. The ratio of the concentration required to produce a 50% inhibition of [3H]-naloxone binding in the presence of both Gpp(NH)p and sodium to that in the absence of both Gpp(NH)p and sodium (IC50 ratio) was less than 1 for antagonists, from 3 to 10 for mixed agonist-antagonists, from 16 to 85 for either kappa, delta, or peptide mu agonists, and more than 200 for morphine-like non-peptide mu agonists. Exceptionally, the IC50 ratio of N,N-diallyl-[D-Ala2, D-Leu5]-enkephalin, an opioid which had been shown not to have an agonist activity in guinea-pig ileum but to have a naloxone-reversible agonist activity in mouse vas deferens, was less than 1. The significance of the different IC50 ratio among opioids employed in the present study was discussed.

The different pharmacological profiles of various opioids in neurophysiological and behavioral tests in the chronic spinal dog indicated the presence of three types of receptors, designated mu, kappa and sigma, for which the prototype agonists were morphine, ketocyclazocine and SKF 10,047 (N-allylnormetazocine), respectively (1). After the discovery of the endogenous opioid peptides, it was suggested that there were additional two types of receptors, delta and epsilon, on the basis of both the different rank order of potencies of opioid peptides and the different effectiveness of an antagonist, naloxone, against opioids in in vitro isolated preparations (2, 3). Among these five subtypes of opioid binding sites, three sites, mu, kappa and delta, have been analyzed well enough to make possible some correlation between the binding sites and the pharmacological responses. However, among three subclasses of opioid receptors, differences in the biochemical sequential events, which must occur between the receptor binding and the pharmacological response, have not been studied well.

Guanine nucleotides have been suggested to control the responses to hormones and neurotransmitters by affecting the affinity of the receptor to the agonist and the coupling of a recognition site of the receptor and a factor that translates the binding into biochemical events which ultimately lead to a biological response (4). Opioids have been shown to inhibit the activity of the adenylate cyclase of intact mouse neuroblastoma rat glioma hybrid cells (5). Moreover, the inhibition of the adenylate cyclase activity in the hybrid cells by an opioid peptide has been reported to require the presence of GTP as well as sodium ion (6). Finally, guanine nucleotides and sodium have been shown to decrease the receptor binding of opioid agonists in an additive fashion, but not antagonists (7).

At present, however, it is not clear whether or not each subclass of opioid agonists is susceptible to inhibition by guanine nucleotide and sodium to the same extent. In the present investigation, therefore, the effects of guanine nucleotide and sodium on the receptor binding of opioid mu, kappa and
Materials and Methods

Chemicals: Gifts of compounds which were gratefully received were naloxone-HCl and pentazocine from Sankyo Company (Tokyo); Mr. 2266 [(-)-(3-furylmethyl)-5,9-diethyl-2'-hydroxy-6,7-benzomorphan]. FW 34-569 (MeTyr-D-Ala-Gly-MePhe-Met(0)ol). FK 33-824 (Tyr-D-Ala-Gly-MePhe-Met(0)ol) and tifluadom from Dr. D. Römer, Sandoz Ltd. (Basle, Switzerland); ICI 154,129 (N,N-diallyl-Tyr-Gly-Gly-[CH2S]-Phe-Leu) (where CH2S signifies replacement of the amide CO-NH bond by CH2S) and U-50,488 (trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzenecetamide) from Dr. J. W. Holaday, Walter Reed Army Inst. Res. (Washington, D.C., U.S.A.); bis-[N,N-diallyl-[D-Ala2, Leu5]-enkephalin]-cystine, [D-Ser2, Leu5]-enkephalin-Thr6, deltakethalin, [D-Ala2, D-Leu5]-enkephalin and N,N-diallyl-[D-Ala2, D-Leu5]-enkephalin from Dr. M. Ueki, Science University of Tokyo (Tokyo); SKF 10,047 (N-allylnormetazocine), ketocyclazocine and ethylketocyclazocine from Dr. W. R. Martin, University of Kentucky (Lexington, Kentucky, U.S.A.); cyclazocine from Sterling-Winthrop Res. Inst. (Rensselaer, New York, U.S.A.); nalorphine-HCl and methadone-HCl from Dr. T. Muraki, Keio University (Tokyo); syndyphalin-25 (Tyr-D-Met(0)-Gly-MePheol) from Dr. Y. Kiso, Kyoto College of Pharmacy (Kyoto); levorphanol from Takeda Chemical Ind., Ltd. (Osaka); and dynorphin-(1–13) from Dr. Y. Kiso, Kyoto College of Pharmacy (Kyoto); levorphanol from Takeda Chemical Ind., Ltd. (Osaka); and dynorphin-(1–13) from Dr. M. Fujino, Takeda Chemical Ind., Ltd. Levallorphan tartrate and morphine-HCl were purchased from Takeda Chemical Ind., Ltd.; pethidine-HCl from Tanabe Seiyaku Co., Ltd. (Osaka); and [3H]-naloxone (38.6 Ci/mmole) from New England Nuclear Corp. (Boston, Massachusetts, U.S.A.).

Brain membrane preparations: Male Hartley guinea-pigs weighing 400-600 g were used for this study. After decapitation, brains were rapidly dissected out. After removal of the cerebellum, each brain was homogenized in 10 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4 at 25°C) for 20 sec with a Kinematica Polytron PT (10/35) at maximum setting. After centrifugation at 4°C for 15 min at 800×g, the supernatant fluid was collected. The following procedure was repeated twice: the pellet was resuspended in the original Tris buffer, centrifuged as stated above and the supernatant fluid was collected. The collected supernatant fluid was combined and centrifuged at 4°C for 10 min at 49,000×g. The resulting pellets were resuspended in the original Tris buffer. The suspension was diluted with the original Tris buffer to the final protein concentration of 0.5 mg/ml. Protein concentrations were measured by the method of Lowry et al. (8), with bovine serum albumin as a standard. Brain membrane preparations could be stored at −70°C for three weeks without significant loss of [3H]-naloxone binding.

[3H]-naloxone binding assay: Triplicate samples were prepared in plastic tubes containing 0.87 ml of brain membrane preparations, 1 nM [3H]-naloxone and various concentrations of unlabeled opioids with and without both 50 μM Gpp(NH)p and 100 mM NaCl. The tubes were incubated at 25°C for 30 min and filtered rapidly through Whatman GF/B glass-fiber filters with three 5 ml washes of ice-cold original Tris buffer. Radioactivity bound to filters was determined by liquid scintillation spectrometry at 55±2% efficiency. Specific binding was defined as the difference in binding in the absence and presence of 1 μM naloxone. The concentration required to produce a 50% inhibition of [3H]-naloxone binding (IC50) was calculated by log-probit regression analysis. The preliminary experiment on characteristics of [3H]-naloxone binding to guinea-pig brain membrane preparations in the original Tris buffer showed that the Bmax (maximal saturable binding) and KD values (reciprocal indices of affinity), which were determined from Scatchard plots of the data for 11 ligand concentrations (0.25–8 nM), were 100 fmol/mg protein and 2.93 nM, respectively.

Results

Effects of Gpp(NH)p and sodium on opioid antagonist binding: Effect of sodium and Gpp(NH)p, a nonhydrolyzable derivative of GTP, on the inhibition by four opioid antagonists of [3H]-naloxone binding to
guinea-pig brain membrane preparations were examined. Among four antagonists employed for this study, naloxone has high affinity to mu receptors and relatively low affinity to both kappa and delta receptors (2, 9), while Mr 2266 has high affinity to both mu and kappa receptors and relatively low affinity to delta receptors (9). In contrast to naloxone and Mr 2266, both ICI 154,129 (10) and bis-[N,N-diallyl-[D-Ala²,Leu⁵]-enkephalyl]-cystine (11) have significantly higher affinity to delta receptors than mu and kappa receptors.

The IC50 values of the four opioid antagonists in the presence of either sodium alone or both Gpp(NH)p and sodium was not higher than that in the absence of both Gpp(NH)p and sodium (Table 1). Childers and Snyder, who employed [3H]-diprenorphine instead of [3H]-naloxone, reported that the ratio of the IC50 value of naloxone in the presence of either sodium alone or both GTP and sodium to that in the absence of both GTP and sodium was 0.9 or 0.79, respectively (7), being consistent with the present result. Thus, the present study confirmed the previous findings by Childers and Snyder (7) and extended their observations.

The ratios of the IC50 values of the two delta antagonists in the presence of both Gpp(NH)p and sodium to that in the absence of both Gpp(NH)p and sodium were approximately one half (Table 1), indicating that the affinity of delta antagonists to [3H]-naloxone binding sites was increased 2-fold in the presence of both Gpp(NH)p and sodium.

Effects of Gpp(NH)p and sodium on opioid mixed agonist-antagonist binding: Opioid mixed agonist-antagonists became 3–10 times as weak in competing with [3H]-naloxone binding in the presence as in the absence of both Gpp(NH)p and sodium (Table 2). The reduction of the affinity of mixed agonist-antagonists to [3H]-naloxone binding sites was more pronounced in the combination of Gpp(NH)p and sodium than sodium alone (Table 2).

Effects of Gpp(NH)p and sodium on opioid mu agonist binding: Opioid peptide mu agonists and morphine-like non-peptide mu agonists became 20–35 and 210–300 times, respectively, as weak in inhibiting [3H]-naloxone binding in the presence as in the absence of both Gpp(NH)p and sodium (Table 3). An exception was pethidine. Its IC50 ratio in the presence to in the absence of both Gpp(NH)p and sodium was not determined due to the low affinity of pethidine to [3H]-naloxone binding sites even in the absence of both Gpp(NH)p and sodium. The decrease in potencies of both peptide and non-peptide mu agonists was more prominent in the combination of Gpp(NH)p and sodium than sodium alone (Table 3). It should be noted that the magnitude of the decrease in potencies in the presence of both Gpp(NH)p and sodium of a morphine-like non-peptide mu agonist was approximately 10 times higher than that of a peptide mu agonist (Table 3).

Effects of Gpp(NH)p and sodium on opioid kappa agonist binding: Opioid kappa agonists became 16–85 times as weak in competing with [3H]-naloxone binding in the presence as in the absence of both Gpp(NH)p and sodium (Table 4). The magnitude of the decrease in potencies in the presence of both Gpp(NH)p and sodium of kappa agonists was higher than that of mixed agonist-antagonists, lower than that of non-peptide mu agonists, and similar to that of peptide mu agonists (Tables 2, 3 and 4). Again, the decrease in potencies of kappa agonists was more significant in the combination of Gpp(NH)p and sodium than sodium alone (Table 4). It should be noted that the reduction of potencies among kappa agonists was the largest for U-50,488 in the presence of sodium alone, while it was the largest for tifluadom in the presence of both Gpp(NH)p and sodium (Table 4). Additionally, the reduction of the potency of dynorphin-(1–13) was quite small in the presence of sodium alone, while it was large in the presence of both Gpp(NH)p and sodium (Table 4). Interestingly, the chemical structure of either U-50,488, dynorphin-(1–13) or tifluadom is quite different from that of traditional benzomorphan kappa agonists such as ketocyclazocine and ethylketocyclazocine (12–14).
Table 1. The effects of 5'-guanylylimidodiphosphate (Gpp(NH)p) and sodium on the potencies of opioid antagonists in competing with the binding of [3H]-naloxone to guinea-pig brain membranes

| Opioid antagonists                      | IC50 (nM) | IC50 Ratios |
|-----------------------------------------|-----------|-------------|
|                                         | Tris\(^a\) | Tris + NaCl\(^b\) | Tris + NaCl + Gpp(NH)p\(^c\) | Tris + NaCl\(^b\) | Tris + NaCl + Gpp(NH)p\(^c\) |
| ICI 154,129                             | 24900     | 18400       | 10300       | 0.74         | 0.41         |
| Bis-[N,N-di(2-[(2-alanyl-D-Ala\(^2\),Leu\(^b\)])-enkephalin]-cystine | 5520      | 5290        | 3200        | 0.96         | 0.58         |
| Naloxone                                | 1.84      | 1.68        | 1.55        | 0.91         | 0.56         |
| Mr 2266                                 | 0.53      | 0.48        | 0.51        | 0.91         | 0.96         |

Guinea-pig brain membranes were incubated in 50 mM Tris-HCl buffer as described in Materials and Methods either \(^a\) without both Gpp(NH)p and sodium, \(^b\) with 100 mM NaCl, or \(^c\) with both 50 μM Gpp(NH)p and 100 mM NaCl. Each value represents the mean of triplicates with standard errors varying less than ±10%.

Table 2. The effects of 5'-guanylylimidodiphosphate (Gpp(NH)p) and sodium on the potencies of opioid mixed agonist-antagonists in competing with the binding of [3H]-naloxone to guinea-pig brain membranes

| Opioid mixed agonist-antagonists | IC50 (nM) | IC50 Ratios |
|----------------------------------|-----------|-------------|
|                                  | Tris\(^a\) | Tris + NaCl\(^b\) | Tris + NaCl + Gpp(NH)p\(^c\) | Tris + NaCl\(^b\) | Tris + NaCl + Gpp(NH)p\(^c\) |
| SKF 10,047                       | 1.94      | 3.14        | 6.82        | 1.62         | 3.52         |
| Levalloporphan                    | 0.34      | 0.52        | 1.32        | 1.53         | 3.88         |
| Cyclazocine                       | 0.31      | 0.58        | 2.45        | 1.87         | 7.90         |
| Pentazocine                       | 19.2      | 54.0        | 192         | 2.81         | 10.0         |
| Nalorphine                       | 0.96      | 2.22        | 9.92        | 2.31         | 10.3         |

Guinea-pig brain membranes were incubated in 50 mM Tris-HCl buffer as described in Materials and Methods either \(^a\) without both Gpp(NH)p and sodium, \(^b\) with 100 mM NaCl, or \(^c\) with both 50 μM Gpp(NH)p and 100 mM NaCl. Each value represents the mean of triplicates with standard errors varying less than ±7%.
Table 3. The effects of 5'-guanylylimidodiphosphate (Gpp(NH)p) and sodium on the potencies of opioid mu agonists in competing with the binding of [³H]-naloxone to guinea-pig brain membranes

| Opioid mu agonists | IC50 (nM) | IC50 Ratios |
|--------------------|-----------|-------------|
|                    | Tris<sup>a</sup> | Tris+NaCl<sup>b</sup> | Tris+NaCl+Gpp(NH)p<sup>c</sup> | Tris+NaCl<sup>b</sup> | Tris<sup>a</sup> |
| Syndyphalin-25     | 5.74      | 38.4        | 118                      | 6.69                    | 20.6                 |
| FW 34–569          | 4.32      | 13.0        | 125                      | 3.01                    | 28.9                 |
| FK 33–824          | 3.59      | 13.6        | 124                      | 3.79                    | 34.5                 |
| Levorphanol        | 0.31      | 3.23        | 65.2                     | 10.4                    | 210                  |
| Methadone          | 4.91      | 42.5        | 1400                     | 8.66                    | 285                  |
| Morphine           | 2.91      | 33.1        | 856                      | 11.4                    | 294                  |
| Pethidine          | 443       | 4890        | >10000                   | 11.0                    | >22.6                |

Guinea-pig brain membranes were incubated in 50 mM Tris-HCl buffer as described in Materials and Methods either <sup>a</sup> without both Gpp(NH)p and sodium, <sup>b</sup> with 100 mM NaCl, or <sup>c</sup> with both 50 µM Gpp(NH)p and 100 mM NaCl. Each value represents the mean of triplicates with standard errors varying less than ±7%.

Table 4. The effects of 5'-guanylylimidodiphosphate (Gpp(NH)p) and sodium on the potencies of opioid kappa agonist in competing with the binding of [³H]-naloxone to guinea-pig brain membranes

| Opioid kappa agonists | IC50 (nM) | IC50 Ratios |
|-----------------------|-----------|-------------|
|                       | Tris<sup>a</sup> | Tris+NaCl<sup>b</sup> | Tris+NaCl+Gpp(NH)p<sup>c</sup> | Tris+NaCl<sup>b</sup> | Tris<sup>a</sup> |
| U-50,488              | 263       | 1790        | 4380                     | 6.81                    | 16.7                 |
| Ketocyclazocine       | 3.84      | 7.74        | 80.4                     | 2.02                    | 20.9                 |
| Dynorphin-(1–13)      | 20.9      | 27.2        | 494                      | 1.30                    | 23.6                 |
| Ethylketocyclazocine  | 1.15      | 4.48        | 33.4                     | 3.90                    | 29.0                 |
| Tifluadom             | 6.05      | 14.0        | 512                      | 2.32                    | 84.6                 |

Guinea-pig brain membranes were incubated in 50 mM Tris-HCl buffer as described in Materials and Methods either <sup>a</sup> without both Gpp(NH)p and sodium, <sup>b</sup> with 100 mM NaCl, or <sup>c</sup> with both 50 µM Gpp(NH)p and 100 mM NaCl. Each value represents the mean of triplicates with standard errors varying less than ±8%.
**Table 5.** The effects of 5'-guanylylimidodiphosphate (Gpp(NH)p) and sodium on the potencies of opioid delta agonists in competing with the binding of [³H]-naloxone to guinea-pig brain membranes

| Opioid delta agonists | IC50 (nM) | IC50 Ratios |
|-----------------------|-----------|-------------|
|                       | Tris⁰     | Tris+NaCl^b | Tris+NaCl+Gpp(NH)p^c | Tris+NaCl^b/Tris⁰ | Tris+NaCl+Gpp(NH)p^c/Tris⁰ |
| N,N-Diallyl-[D-Ala⁵,D-Leu⁶]-enkephalin | 5090 | 4550 | 5000 | 0.89 | 0.98 |
| [D-Ser², Leu⁶]-Enkephalin-Thr⁶ | 65.6 | 309 | 2150 | 4.71 | 32.8 |
| Deltakephalin          | 44.0 | 179 | 1490 | 4.07 | 33.9 |
| [D-Ala²,D-Leu⁶]-Enkephalin | 19.2 | 96.5 | 855 | 5.03 | 44.5 |

Guinea-pig brain membranes were incubated in 50 mM Tris-HCl buffer as described in Materials and Methods either ^a without both Gpp(NH)p and sodium, ^b with 100 mM NaCl, or ^c with both 50 nM Gpp(NH)p and 100 mM NaCl. Each value represents the mean of triplicates with standard errors varying less than ±5%.
Effects of Gpp(NH)p and sodium on opioid delta agonist binding: Opioid delta agonists became 32–45 times as weak in inhibiting [3H]-naloxone binding in the presence as in the absence of both Gpp(NH)p and sodium (Table 5). The reduction of potencies of delta agonists was more prominent in the combination of Gpp(NH)p and sodium than sodium alone (Table 5). The magnitude of the decrease in potencies in the presence of both Gpp(NH)p and sodium of delta agonists was higher than that of mixed agonist-antagonists, lower than that of morphine-like non-peptide mu agonists, and similar to that of both peptide mu agonists and kappa agonists (Tables 2, 3, 4 and 5). A quite interesting exception was N,N-diallyl-[D-Ala2,D-Leu5]-enkephalin which had been shown not to have an agonist activity in guinea-pig ileum, but to have a naloxone-reversible agonist activity in mouse vas deferens (11). The IC50 ratio of N,N-diallyl-[D-Ala2,D-Leu5]-enkephalin in the presence to in the absence of both Gpp(NH)p and sodium was below 1 and similar to that of antagonists (Tables 1 and 5).

Discussion

The data in the present investigation, confirming and extending the previous observations (6, 7, 15–20), show that both Gpp(NH)p and sodium do not decrease the binding of opioid antagonists, but decrease that of opioid agonists to [3H]-naloxone binding sites. Moreover, the present study reveals that there are a few very interesting exceptions to this general rule. Additionally, the data in the present study show that the ratio of the concentration required to produce a 50% inhibition of [3H]-naloxone binding in the presence of both Gpp(NH)p and sodium to that in the absence of both Gpp(NH)p and sodium is from 3 to 10 for mixed agonist-antagonists, from 16 to 85 for either kappa, delta, or peptide mu agonists, and more than 200 for morphine-like non-peptide mu agonists.

The prominently high similar IC50 ratio observed among morphine-like non-peptide mu agonists indicates that the IC50 ratio may be employed as a marker of morphine-like compounds. The significantly different IC50 ratio between peptide mu agonists and morphine-like non-peptide mu agonists may be caused by the different chemical structure; the former has a flexible structure while the latter has a rigid structure. It is possible, therefore, that although peptide mu agonists have the highest affinity to mu receptors, they may interact with receptor subclasses other than mu receptors due to their flexible structure when the affinity of mu receptors to their agonists is significantly reduced by the presence of both Gpp(NH)p and sodium. Another possible interpretation is the existence of subtypes (isoreceptors) of the mu receptors such as μ1 or μ2 receptors (21), with either of which peptide or non-peptide mu agonists selectively interact. The observation that the relative potency of FK 33–824 to morphine in rat vas deferens is significantly higher than that in guinea-pig ileum (T. Oka et al., unpublished observation) indicates that FK 33–824 has an ability to interact with an opioid-receptor subtype(s) on which morphine can not act. More detailed experiments to reveal the difference between peptide and non-peptide mu agonists, however, are required to explain the different IC50 ratio between peptide and non-peptide mu agonists.

Anyway, the significantly different IC50 ratio that existed between the two groups of mu agonists suggests that some of the responses produced after the administration of peptide mu agonists may be different from those of non-peptide mu agonists.

The fact that IC50 ratio of N,N-diallyl-[D-Ala2,D-Leu5]-enkephalin in the presence to in the absence of both Gpp(NH)p and sodium was less than 1, indicates the quite interesting possibility that opioid delta receptors are not coupled to adenylyl cyclase, since the fact that N,N-diallyl-[D-Ala2,D-Leu5]-enkephalin has been shown not to have an agonist activity in guinea-pig ileum but to have a naloxone-reversible agonist activity in mouse vas deferens (11) strongly suggests that N,N-diallyl-[D-Ala2,D-Leu5]-enkephalin is a pure delta agonist having no mu and kappa agonist activities. Moreover, other delta agonists, [D-Ser2,Leu5]-enkephalin-Thr6, deltakephalin and [D-Ala2, D-Leu5]-enkephalin, act on guinea-pig ileum as strong mu agonists (T. Oka et al., unpublished
data), showing that these delta agonists are not pure delta agonists and act on mu as well as delta receptors. Therefore, the IC50 ratios of these delta agonists may reflect their mu component. However, more detailed experiments are apparently needed to determine whether or not delta receptors are coupled to adenylate cyclase.

Another interesting finding observed in the present study is the fact that the IC50 ratio in the presence to in the absence of sodium of dynorphin-(1–13), which had been shown to be a strong kappa agonist (13), was less than those of mixed agonist-antagonists and significantly low when it was compared to those of other opioid agonists. This character of dynorphin-(1–13) may relate to its peculiar antagonistic action (22). Interestingly, the IC50 ratio in the presence to in the absence of sodium of ketocyclazocine, which had been reported to behave as an effective antagonist as well as a potent agonist in the guinea-pig ileum (23), was also low. The fact that the chemical structure of either U-50,488, dynorphin-(1–13) or tifluadom is quite different from that of traditional benzomorphan kappa agonists such as ketocyclazocine and ethylketocyclazocine (12–14) may be a cause of the broad range of IC50 ratio among kappa agonists. However, the cause of the significant difference in the magnitude of the influence by Gpp(NH)p and sodium among kappa agonists remains to be elucidated.

Gpp(NH)p as well as GTP has been suggested to produce a lower-affinity form of receptors to agonists by interacting with the nucleotide regulatory component of adenylate cyclase (4). Additionally, negative heterotropic effects of Gpp(NH)p on agonist binding have been shown to be augmented by sodium (4). Therefore, the receptors which are changed to lower-affinity form by the presence of both Gpp(NH)p and sodium can be considered to be coupled with adenylate cyclase. Thus, the data in the present study indicate that both mu and kappa receptors are, more or less, coupled to adenylate cyclase. However, the coupling of delta receptors to adenylate cyclase remains to be elucidated.

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