Differential Regulation by cAMP-dependent Protein Kinase and Protein Kinase C of the μ Opioid Receptor Coupling to a G Protein-activated K⁺ Channel*

Yan Chen and Lei Yu†

From the Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, Indiana 46202

A μ opioid receptor and a G protein-activated K⁺ channel were coexpressed in Xenopus oocytes. Stimulation of the μ opioid receptor induced an inwardly rectifying current that was blocked by opioid antagonist naloxone, indicating that the μ opioid receptor is functionally coupled to the K⁺ channel. The coupling is mediated by G proteins, since pertussis toxin treatment reduced the K⁺ current and injection of GTP-γ-S (guanosine 5'-O-(thiotriphosphate)) enhanced it. Repeated stimulation of the μ receptor leads to desensitization, as the K⁺ current from the second stimulation was reduced to 70% of that from the first one. Both cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) regulate this process, but in opposite direction. Activation of PKC by treatment of the oocyte with phorbol ester potentiated the desensitization of the μ receptor-induced current. However, incubation of the cell with a membrane-permeable cAMP analog, 8-chlorophenylthio-cAMP, completely abolished the desensitization. The cAMP effect appears to be mediated by PKA, since injection of a PKA catalytic subunit showed the same effect as cAMP incubation. These results suggest that PKA and PKC differentially regulate the μ opioid receptor coupling to the G protein-activated K⁺ channel.

Opioids, both endogenous peptides and exogenous alkaloids, affect the functioning of the central nervous system by interacting with membrane receptors (1, 2). Pharmacological studies suggest the presence of three major types of opioid receptors in the brain and spinal cord; δ, κ, and μ, of which μ opioid receptor plays an important role in supraspinal analgesia and development of morphine tolerance and dependence (3, 4). Opioid receptor activation has been shown to mediate the inhibition of neuronal firing and neurotransmitter release in a variety of brain areas (5, 6). Stimulation of the μ opioid receptor results in a membrane hyperpolarization caused by an increase in an inwardly rectifying K⁺ conductance in both locus coeruleus and hippocampus neurons (7–9). The coupling of the μ opioid receptor to the brain inwardly rectifying K⁺ channel appears to be mediated by a pertussis toxin (PTX)-sensitive G protein(s) (5, 10). However, the molecular identity of the K⁺ channel that is coupled to the μ opioid receptor is still unclear, and little is known about the functional regulation of the coupling.

Recently, a μ opioid receptor and a G protein-activated K⁺ channel have been molecularly cloned (11–13). The μ opioid receptor was isolated from rat brain and is functionally coupled to G proteins and adenylyl cyclase (11). The G protein-activated K⁺ channel was originally isolated from the atrial cells, and RNAs analyses indicate that it is also abundantly expressed in the brain (12, 13). In addition, Dascal et al. (13) isolated an almost identical clone from the brain. The brain expression of this clone suggests that it may be a K⁺ channel identical or similar to that activated by the μ opioid receptor. To test this hypothesis, we expressed the μ opioid receptor and the G protein-activated K⁺ channel in Xenopus oocytes and investigated the modulation by PKA and PKC.

MATERIALS AND METHODS

Chemicals—[3H]DAMGO (35 Ci/mmol) was from the National Institute on Drug Abuse. DAMGO was from Bachem. Naloxone was from Research Biochemicals International. All other chemicals were from Sigma.

Complementary DNA Clones for the μ Opioid Receptor and the G Protein-ACTivated K⁺ Channel—A cDNA clone, MOR-1, containing the protein coding region of a rat μ opioid receptor has been described (11). Based on the cDNA sequence for the rat G protein-activated K⁺ channel (12, 13), two oligonucleotide primers were synthesized corresponding to the 5′- and 3′-untranslated regions, respectively: CTCGGATCCGTATCTGCTG and ATAGTCGACTAAAACTAAATC. PCR was performed in an air cycler (Idaho Technology) with 10 s at 94 °C, 20 s at 56 °C, and 1 min at 75 °C for 35 cycles, using the two primers and the purified lambda DNA from a rat brain cDNA library (14). A Deep Vent® DNA polymerase (New England Biolabs) was used to reduce PCR errors (15). The 1.7-kilobase pair polymerase chain reaction product was cloned into a TA-cloning vector (Invitrogen). Both cDNA clones were used to synthesize mRNA by in vitro transcription as described (16).

Oocyte Injection and Binding Assay—Xenopus oocytes were prepared as described (16). In vitro transcribed RNA (1–2 ng/oocyte) was injected into oocytes with a Drummond microinjector. Oocytes were incubated in L-15 medium supplemented with 0.8 mM glutamine and 10 μg/ml of gentamicin at 20 °C for 3–4 days before analysis. Binding of the injected oocytes was carried out in regular ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 1.5 mM CaCl₂) solution at 20 °C for 90 min, using 1 μM [3H]DAMGO. Binding was terminated by vacuum filtration through a Whatman GF/B filter pretreated with 1% polyethyleneimine. Three milliliters of ND96 was used to wash the oocytes, and nonspecific binding was determined using 1 μM naloxone. The radioactivity of the oocytes were determined in 6 ml of Scintiverse (Fisher) with a Beckman LS8001 scintillation counter.

Electrophysiology—Oocytes were voltage-clamped at −80 mV with two electrodes filled with 3 M potassium chloride and having a resistance of 0.5–2 megmohms, using an Axoclamp-2A and the pCLAMP software (both from Axon Instruments). Oocytes were superfused with either ND96 containing 6 mM CaCl₂ or a high potassium solution (96 mM KCl, 2 mM NaCl, 1 mM MgCl₂, and 1.5 mM CaCl₂). Analysis of variance and Student's t test were used to determine the statistic significance among different groups.

* This work was supported in part by National Institutes of Health Grant NS28190 (to L. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of National Institutes of Health Research Career Development Award NS01657 and a J. Alfred Prufrock investigation grant. To whom correspondence should be addressed: Dept. of Medical and Molecular Genetics, Indiana University School of Medicine, 975 W. Walnut St., Indianapolis, IN 46202. Tel: 317-274-5737; Fax: 317-274-2087.

‡ The abbreviations used are: PTX, pertussis toxin; DAMGO, [d-Ala²,MePhe³,Gly⁵]enkephalin; 8-CTP-cAMP, 8-chlorophenylthiocAMP; PMA, phorbol 12-myristate 13-acetate; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; GTP-γ-S, guanosine 5′-O-(thiotriphosphate).
Kinase Regulation of μ Opioid Receptor-K⁺ Channel Coupling

RESULTS

Coupling of the μ Opioid Receptor to the G Protein-activated K⁺ Channel—To determine whether the μ opioid receptor couples to the G protein-activated K⁺ channel, we expressed both proteins in Xenopus oocytes. Messenger RNAs of these two proteins were microinjected with each mRNA alone or both mRNAs. DAMGO plus 10 μM naloxone (right trace) as indicated. Inward current is downward. B, membrane currents with voltage steps ranging from -160 mV to +40 mV were recorded before and 1 min after DAMGO superfusion. The DAMGO-induced net currents were derived by subtracting the currents before DAMGO application from those after, and are shown in the left panel. The right panel shows the I-V curve of these currents.

Differential Regulation of the Coupling by PKA and PKC—To determine whether the coupling between the μ opioid receptor and the K⁺ channel in oocytes is affected by PTX, cells injected with both mRNAs were incubated with 0.5 μg/ml PTX for 24 h. PTX treatment reduced the DAMGO-induced membrane current by 60% (Fig. 2A), and this reduction was proportionally uniform across the voltage range (Fig. 2B). These data indicate that a PKA-sensitive G protein(s) is needed for the μ receptor activation of the K⁺ channel, accounting for at least 60% of the coupling.

The involvement of heterotrimeric G proteins in the coupling was further studied using GTPyS, a nonhydrolyzable GTP analog that interacts with G protein and keeps it in an activated state. After DAMGO-induced current reached a plateau, intra-cellular injection of GTPyS elicited an additional increase of the current (Fig. 3A). When the time course of the normalized current was plotted using the peak current value before GTPyS injection as the standard, GTPyS injection resulted in a gradual rise of the current which, after reaching the maximum, decreased toward the base line following a similar time course as that of control oocytes (Fig. 3B). However, injection of GTPyS itself without stimulation of the μ receptor by DAMGO did not induce appreciable membrane current change (data not shown), indicating that the GTPyS-mediated enhancement of the K⁺ conductance is dependent on the activation of the receptor.

G Protein Involvement in the Coupling—Previous studies in neurons suggested that the coupling of opioid receptors to the membrane K⁺ conductance involves a PTX-sensitive G protein (5, 10). To test whether the coupling between the μ opioid receptor and the K⁺ channel in oocytes is affected by PTX, cells injected with both mRNAs were incubated with 0.5 μg/ml PTX for 24 h. PTX treatment reduced the DAMGO-induced membrane current by 60% (Fig. 2A), and this reduction was proportionally uniform across the voltage range (Fig. 2B). These data indicate that a PKA-sensitive G protein(s) is needed for the μ receptor activation of the K⁺ channel, accounting for at least 60% of the coupling.

The involvement of heterotrimeric G proteins in the coupling was further studied using GTPyS, a nonhydrolyzable GTP analog that interacts with G protein and keeps it in an activated state. After DAMGO-induced current reached a plateau, intra-cellular injection of GTPyS elicited an additional increase of the current (Fig. 3A). When the time course of the normalized current was plotted using the peak current value before GTPyS injection as the standard, GTPyS injection resulted in a gradual rise of the current which, after reaching the maximum, decreased toward the base line following a similar time course as that of control oocytes (Fig. 3B). However, injection of GTPyS itself without stimulation of the μ receptor by DAMGO did not induce appreciable membrane current change (data not shown), indicating that the GTPyS-mediated enhancement of the K⁺ conductance is dependent on the activation of the receptor.
Kinase Regulation of µ Opioid Receptor-K⁺ Channel Coupling

Neurotransmitters modulate the excitability of neurons by affecting ion channels. K⁺ channel being one of the primary targets of such modulation. In fact, many neurotransmitters have been shown to couple to a K⁺ conductance in neurons (17–20). The effect of neurotransmitters on K⁺ channel involves a receptor-mediated mechanism, and opioids are no exception. In both locus coeruleus and hippocampus, µ opioid receptors have been shown to regulate K⁺ conductance, leading to membrane hyperpolarization and a decrease in neuronal firing rate (8–10). The recent cloning of a µ opioid receptor, as well as a G protein-activated K⁺ channel, provided the opportunity to examine the molecular mechanism of this coupling. The K⁺ channel was isolated from the heart atrial cells, where it is mainly involved in the heart beat regulation mediated by muscarinic receptors (21, 22). However, both RNA blot analysis and cloning effort suggested that the same channel also exists in the brain (12, 13); thus, it may mediate the neuronal effect of various neurotransmitters. In this report, we showed that the µ opioid receptor and the G protein-coupled K⁺ channel, when coexpressed in Xenopus oocytes, are functionally coupled. Although we cannot exclude the possibility that other K⁺ channels may be involved in the coupling to the µ opioid receptor, our data suggest that this G protein-coupled inward rectifier may be the long-sought K⁺ channel that is linked to the µ opioid receptor and other neurotransmitter receptors. Kubo et al. (12) have shown that this channel can be activated by injection of purified G protein Gαs, β1, and γ2 subunits. Our experiments with PTX (Fig. 2) and GTPγS (Fig. 3) suggested the involvement of a PTX-sensitive G-protein in the coupling. It has been shown that opioid receptors are associated with G proteins of the G1 and G2 subtypes (23–25). Therefore, it is not surprising that similar PTX-sensitive G proteins of the G1 and/or G2 subtypes in Xenopus oocyte can mediate the coupling between the µ opioid receptor and the K⁺ channel. However, the fact that PTX treatment did not completely block the DAMGO-induced K⁺ current (Fig. 2) suggests that other G proteins not sensitive to PTX may also be involved in the µ receptor coupling.

Phosphorylation by kinases is one of the most important mechanisms for functional regulation of many cellular proteins including neurotransmitter receptors and ion channels, and PKA and PKC are two of the most widely studied kinases (26, 27). Phosphorylation of β2-adrenergic receptor by either PKA or PKC leads to its uncoupling from G proteins, resulting in desensitization to further agonist stimulation (28, 29). In the case of voltage-dependent Ca²⁺ channels such as the endogenous oocyte Ca²⁺ channel, PKA- and PKC-mediated phosphorylation is able to potentiate channel activity (30, 31). Cystic fibrosis transmembrane conductance regulator, a Cl⁻ channel associated with cystic fibrosis, is also regulated by cAMP through PKA pathway (32, 33). Regulation of the inwardly rectifying K⁺ channels by either PKA or PKC, however, is not clear. Molecular cloning has shown that the µ opioid receptor and the G protein-activated K⁺ channel possess multiple putative sites for PKA and PKC phosphorylation (11–13). In this study, we found that the coupling of the µ opioid receptor to the K⁺ channel desensitizes upon repeated stimulation by the µ opioid agonist DAMGO, as the peak current by the second DAMGO ap-

---

**DISCUSSION**

The illustration of K⁺ channel regulation by PKA and PKC is shown in the diagram. The diagram includes a bar graph illustrating the differential regulation of the coupling by PKA and PKC. The bar graph shows the change in current (µA) over time (min) for different treatment groups: Control, PMA, and 8-CPT-cAMP. The experiment was performed in X. laevis oocytes, and the results indicate that PKA and PKC can differentially regulate K⁺ channel activity, with PKA increasing the current and PKC decreasing it. The diagram also includes a graph showing the time course of current change in response to DAMGO stimulation. The current change is measured in pA over time (ms), with peaks observed at various time points indicating the transient nature of the coupling. The results suggest that PKA and PKC can modulate K⁺ channel activity, possibly through a cAMP-mediated mechanism, which is consistent with previous studies in other systems.
Kinase Regulation of μ Opioid Receptor-K⁺ Channel Coupling

Complication is reduced to 70% of that by the first one (Fig. 4). Treatment of the cells with phorbol ester enhanced this desensitization (Fig. 4B), suggesting PKC-mediated phosphorylation. Surprisingly, treatment with 8-CPT-cAMP or injection of the catalytic subunit of PKA completely abolished the desensitization (Fig. 4B). Thus, PKA and PKC appear to exert opposite effects on this μ receptor-induced K⁺ current. Our results, however, do not reveal the molecular entities of PKA- and PKC-mediated phosphorylation. Further studies using mutagenesis are needed to determine the correlation between specific phosphorylation sites on these membrane proteins and the regulation by PKA and PKC.

Acknowledgments—We thank Yuhuan Wang for the catalytic subunit of PKA and Mingting Tian for technical assistance.

REFERENCES
1. Pasternak, G. W. (1988) The Opiate Receptors, Humana Press, Totowa, NJ
2. Goldstein, A. (1987) Trends Pharmacol. Sci. 8, 456–459
3. Pasternak, G. W., Childers, S. R., and Snyder, S. H. (1980) Science 208, 514–516
4. Chaillot, P., Coulaud, A., Zajac, J. M., Fournier-Zaluski, M. C., Costentin, J., and Roques, B. P. (1989) Eur. J. Pharmacol. 161, 85–90
5. Attulli, B., Sayla, D., Nah, S., and Vogel, Z. (1989) J. Biol. Chem. 264, 347–353
6. Caude, R. M., and Chavkin, C. (1990) J. Pharmacol. Exp. Ther. 283, 1361–1369
7. North, R. A., Williams, J. T., Surprenant, A., and Christie, M. J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5487–5491
8. Miyake, M., Christie, M. J., and North, R. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3419–3422
9. Winpey, T. L., and Chavkin, C. (1991) Neuron 6, 281–289
10. Williams, J. T., North, R. A., and Tokimasa, T. (1988) J. Neurosci. 8, 4299–4306
11. Chen, Y., Mestek, A., Liu, J., Hurley, J. A., and Yu, L. (1993) Mol. Pharmacol. 44, 8–12
12. Kubo, Y., Reaveny, E., Slesinger, P. A., Jan, Y. N., and Jan, L. Y. (1993) Nature 364, 892–896
13. Dascal, N., Lim, N. F., Schweikmayer, W., Wang, W., Davidson, N., and Lester, H. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6596–6600
14. Snutch, T. P., Leonard, J. P., Gilbert, M. M., Lester, H. A., and Davidson, N. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3391–3395
15. Cariello, N. F., Swenneberg, J. A., and Sopcke, T. R. (1991) Nucleic Acids Res. 19, 4193–4196
16. Yu, L., Nguyen, H., Le, H., Bloem, L. J., Kozak, C. A., Hoffman, B. J., Snutch, T. P., Lester, H. A., Davidson, N., and Lubbert, H. (1991) Mol. Brain Res. 11, 143–149
17. Einhorn, L. C., Gregerson, K. A., and Oxford, G. S. (1991) J. Neurosci. 11, 3727–3737
18. Kurtz, A., and Penner, R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3423–3427
19. Mihara, K., North, R. A., and Surprenant, A. (1987) J. Physiol. 390, 335–355
20. Shen, K. Z., North, R. A., and Surprenant, A. (1992) J. Physiol. 445, 581–599
21. Noma, A., and Trustwein, W. (1978) Pflogers Arch. 377, 193–200
22. Noma, A., Peper, K., and Trustwein, W. (1979) Pflogers Arch. 381, 255–262
23. Carter, B. D., and Medzihradsky, F. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4062–4066
24. Offermanns, S., Schultz, G., and Rosenthal, W. (1991) J. Biol. Chem. 266, 3365–3368
25. Ueda, H., Harada, H., Nozaki, M., Katada, T., Ui, M., Satoe, M., and Takagi, H. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7013–7017
26. Krebs, E. G. (1989) J. Am. Med. Assoc. 262, 1815–1818
27. Shearmun, M. S., Sekiuchi, K., and Nishizuka, Y. (1989) Pharmacol. Rev. 41, 211–237
28. Lefkowitz, R. J., and Caron, M. G. (1988) J. Biol. Chem. 263, 4993–4996
29. Sibley, D. R., and Lefkowitz, R. J. (1985) Nature 317, 124–129
30. Bourinet, E., Fournier, F., Nargeot, J., and Charnet, P. (1992) FEBS Lett. 314, 197–202
31. Chen, Y., Mestek, A., Liu, J., Hurley, J. A., and Yu, L. (1994) FEBS Lett. 336, 191–196
32. Anderson, M. P., Berger, H. A., Rich, D. P., Gregory, R. J., Smith, A. E., and Welsh, M. J. (1991) Cell 67, 775–784
33. Bear, C. E., Dupuy, F., Nasmith, A. L., Kartner, N., Hanahan, J. W., and Riordan, J. R. (1991) J. Biol. Chem. 266, 19142–19145