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Nucleoside diphosphate kinase associated with membranes modulates μ-opioid receptor-mediated [^{35}S]GTPγS binding and agonist binding to μ-opioid receptor

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Abstract

The role of nucleoside diphosphate kinase (NDKP), which converts GDP to GTP, in the coupling of μ-opioid receptors to G protein was investigated in membranes of Chinese hamster ovary cells stably transfected with the cloned rat μ-opioid receptor (rmor). Endogenous NDPK activity in membranes was determined to be 0.60 ± 0.02 μmol/mg protein/30 min UDP (at 10 mM), a competitive substrate of NDPK for GDP with no effect on guanine nucleotide binding to G proteins, reduced basal [^{35}S]GTPγS binding and unmasked morphine-stimulated [^{35}S]GTPγS binding to pertussis toxin-sensitive G proteins, indicating that [^{35}S]GTPγS binding to NDPK accounts for part of its high basal binding. UDP increased the extent of morphine-induced increase in [^{35}S]GTPγS binding in the presence of GDP, most likely by reducing basal binding and inhibiting conversion of GDP to GTP. ATP greatly reduced morphine-induced increase in [^{35}S]GTPγS binding, whereas AMP-PCP (adenylyl-(β,γ-methylene)-diphosphoate tetralithium salt), which cannot serve as the phosphate donor for NDPK, did not, demonstrating that effects of ATP is mediated by the NDPK product GTP. In addition, GDP and ATP increased the K_d and lowered the B_{max} of the agonist [^{3}H]DAMGO ([D-Ala^2,*N*-Me-Phe^4,Gly^{5}ol]-Enkephalin) for the μ-opioid receptor and GDP alone increased K_d, most likely through their conversion to GTP by NDPK. Addition of exogenous NDPK enhanced the inhibitory effects of GDP and combined GDP and ATP on [^{3}H]DAMGO binding. Thus, NDPK appears to play a role in modulating signal transduction of and agonist binding to μ-opioid receptors.

Keywords: Nucleoside diphosphate kinase; μ-Opioid receptor; G protein; GTPγS binding; CHO (chinese hamster ovary) cell; Receptor binding

1. Introduction

Opioid receptors mediate pharmacological effects of opiate and opioid compounds. The presence of multiple opioid receptors (μ, δ, κ, ε) has been demonstrated based on pharmacological, binding, anatomical and molecular studies (for reviews, see Mansour et al., 1988; Pasternak, 1988; Knapp et al., 1995). Activation of opioid receptors leads to an inhibition of adenylate cyclase, an increase in inward-rectifying K^+ conductance and a decrease in L-type Ca^{2+} conductance (for a review, see Childers, 1991) and activation of mitogen-activated protein kinase pathway (Fukuda et al., 1996; Li and Chang, 1996). μ-Opioid receptors are closely associated with analgesic and euphoric actions of opiate and opioid compounds (Pasternak, 1988). Two splice variants of μ-opioid receptors have been cloned (Chen et al., 1993; Wang et al., 1993; Bare et al., 1994; Zimprich et al., 1995). Deduced amino acid sequences of these clones display the motif of putative seven α-helical transmembrane helices, that is characteristic of G protein-coupled receptors.

Events leading to activation of effectors following binding of an agonist are similar among all G protein-coupled receptors (for reviews, see Gilman, 1987; Birnbaumer et al., 1990). At the resting state, the guanine nucleotide binding site of Gα subunits is occupied by GDP. Binding of an agonist to the receptor leads to the coupling of the receptor to G proteins, in which GDP bound to the Gα subunits is replaced by GTP. GTP binding to the Gα subunits triggers dissociation of the G protein heterotrimers into Gα and Gβγ subunits and the transformation

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of the receptors from high affinity state for agonists to low affinity state. GTP-bound Gα subunits and Gβγ subunits, in turn, activate effectors. Hydrolysis of GTP by the intrinsic GTPase activity of the subunit terminates the action of GTP-bound Gα.

Nucleoside diphosphate kinase (EC 2.7.4.6, NDPK) is a family of enzymes that catalyze the transfer of the terminal phosphate group of 5′-nucleoside triphosphate to 5′-nucleoside diphosphate by the following general mechanism:

$$N_1TP + NDPK \leftrightarrow N_1DP + NDPK\text{-}P$$

$$N_2DP + NDPK\text{-}P \leftrightarrow N_2TP + NDPK$$

Where N_1 and N_2 are purine or pyrimidine ribo- or deoxyribonucleosides and NDPK-P is the phosphorylated NDPK intermediate. This is the common pathway for the synthesis of all the 5′-nucleoside triphosphates except ATP (for a review, see Parks and Agarwal, 1973). Because of its abundance, ATP is the primary phosphate donor. In addition to its enzymatic activity, NDPK has been characterized as inhibitors of metastasis, as a factor stimulating gene transcription, and as a protein kinase (for a review, see Lacombe and Jakobs, 1992). NDPK is widely distributed in various types of cells in animals and plants (for a review, see Parks and Agarwal, 1973). Within the cells, NDPK is present in several different compartments (for a review, see Parks and Agarwal, 1973), including membranes, cytosol and nucleus (Kraeft et al., 1996). While G proteins have much higher affinities for guanine nucleotides (GTP, GDP) than other nucleotides, NDPK has similar affinities for most nucleoside diphosphates or triphosphates as either donors or acceptors of the high-energy phosphate (for a review, see Parks and Agarwal, 1973).

Several reports suggest that NDPK is involved in the switch between GDP-bound and GTP-bound forms of G proteins, and thus plays a role as a modulator in transmembrane signal transduction mechanisms of G protein-coupled receptors (for reviews, see Otero, 1990; Piacentini and Niroomand, 1996). Through its action of converting GDP to GTP, NDPK was shown to reduce agonist binding affinity of formyl peptide (*N*-formyl-Met-Leu-Phe), the complement component C5a, cAMP and cholecystokinin receptors (Wieland et al., 1991; Wieland and Jakobs, 1992; Bominaar et al., 1993; Blevins et al., 1994, 1996). Physical association of NDPK with the α subunits of G_s and transducin and RAS protein has been reported (Ohtsuki et al., 1985, 1986; Kimura and Shimada, 1988; Orlov et al., 1996; Orlov and Kimura, 1998). Evidence supporting the notion that NDPK-dependent formation of GTP regulate effectors of G proteins has been provided for increase in K^+ conductance (Otero et al., 1988; Heidbuchel et al., 1993), stimulation and inhibition of adenylate cyclase (Kimura and Shimada, 1983; Shimada and Kimura, 1983; Jakobs and Wieland, 1989; Wieland and Jakobs, 1989; Niroomand et al., 1997), activation of phospholipase C (Bominaar et al., 1993; Zhang and Chang, 1995), stimulation of phospholipase D (Fan et al., 1994) and reduction in neuronal Ca^{2+} currents (Gross et al., 1990).

The role of NDPK in the regulation of opioid receptors and their coupling to G proteins has not been reported. Activation of μ-, δ- and κ-opioid receptors increased [^{35}S]GTPγS binding to G proteins in membrane preparations, which was blocked by pertussis toxin (Traynor and Nahorski, 1995; Befort et al., 1996; Zhu et al., 1997; Szekeres and Traynor, 1997). Stimulation of [^{35}S]GTPγS binding by agonists provides a useful functional measure for interaction between opioid receptors and pertussis toxin-sensitive G proteins. In this study, we examined whether NPDK modulates agonist-stimulated [^{35}S]GTPγS binding and agonist binding to μ-opioid receptors. Chinese hamster ovary (CHO) cells stably transfected with the rat μ-opioid receptor (rmor) (Chen et al., 1993) were used in this study.

2. Materials and methods

2.1. Materials

[^{3}H]DAMGO ([D-Ala^2,*N*-Me-Phe^4,Gly^{5}ol]-Enkephalin) (55.3 Ci/mmol), [^{35}S]GTPγS (~1200 Ci/mmol) and [γ-^{32}P]ATP (6000 Ci/mmol) were obtained for NEN Life Sciences (Boston, MA). Morphine and diprenorphine were provided by National Institute on Drug Abuse. DAMGO was purchased from Peninsula Laboratories (Belmont, CA). ATP, GDP, UDP, GTP, GTPγS and purified bovine liver NDPK were purchased from Sigma (St. Louis, MO).

2.2. Stable expression of the rmor in CHO cells

CHO cell lines stably expressing the cloned rat μ-opioid receptor (CHO-rmor cells) were established as described previously (Chen et al., 1995). One of the clones (μ66) was used in this study. Cells were grown in Dulbecco's Modified Eagle Medium-F12 HAM supplemented with 10% fetal calf serum/0.2 mg/ml Geneticin in humidified 5% CO_2/95% air at 37°C.

2.3. Measurement of NDPK activity in membranes

NDPK activity in CHO-rmor cell membranes was measured using a modification of the method of (Zhang and Chang, 1992). Briefly, membranes (4 μg protein/assay) were incubated for 30 min in 100 μl of [^{35}S]GTPγS binding buffer containing 100 μM [γ-^{32}P]ATP (5 mCi/assay) and 100 μM GDP at 30°C. Reaction was terminated by the addition of 50 μl 0.25 M EDTA. Reaction mixtures were centrifuged to pellet membrane, and 2 μl aliquot of each supernatant was spotted on polyethyleneimine-cellulose plastic sheets for thin layer chromatography. After

developing in 0.75 M KH_2PO_4 solution, the sheets were dried and exposed to X-ray films overnight for autoradiography films. The positions of ATP and GTP were determined by reference to the standards, which had been applied at the same time as the samples. The ATP and GTP spots were cut out and their radioactivities were measured.

2.4. Membrane preparation

Cells were harvested and membranes prepared using a procedure described previously (Zhu et al., 1997). Briefly, cells were washed twice with 100 mM PBS (phosphate buffered saline), harvested in Versene solution and centrifuged at $500 \times g$ for 3 min and washed once with PBS. The cell pellet was resuspended in 50 mM Tris–HCl buffer containing 1 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid, 5 μM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, sonicated and centrifuged at $46{,}000 \times g$ for 30 min. The pellet was resuspended in 50 mM Tris, pH 7.0 and centrifuged again. The membrane pellet was resuspended in 50 mM Tris, 0.32 M sucrose, pH 7.0, aliquoted at ~100 μg protein/ml, frozen in dry ice/ethanol and stored in −70°C until use. All procedures were performed at 4°C.

2.5. Morphine-stimulated [^{35}S]GTPγS binding

Determination of [^{35}S]GTPγS binding to G proteins in cell membranes was carried with our modified procedure (Zhu et al., 1997) of that of (Traynor and Nahorski, 1995). Binding was conducted in a buffer of 50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA with 1 mM dithiothritol and 0.1% bovine serum albumin freshly added with ~80 pM [^{35}S]GTPγS and 3 μM GDP in a total volume of 0.5 ml for 60 min at 30°C. Nonspecific binding was defined by incubation in the presence of 10 μM GTPγS. EC_{50} and maximal response values were calculated by use of the equation $y = E_{max}/[1 + (x/EC_{50})^s]$ + background, where y is the response at the dose x, E_{max} is the maximal response and s is a slope factor.

2.6. Opioid receptor binding

Receptor binding was conducted with [^{3}H]DAMGO. Binding was carried out in [^{35}S]GTPγS binding buffer at 30°C for 30 min in duplicate in a volume of 0.5 ml with 20 μg protein. Binding data were analyzed with the EBDA and LIGAND programs (McPherson, 1983).

2.7. Protein assay

Protein contents of membranes were determined by the method of (Smith et al., 1985) with bovine serum albumin as the standard.

2.8. Statistical analysis

Experimental measurements were performed in duplicate, and data are expressed as means ± S.E.M. of n experiments. For comparison of multiple groups, data were analyzed with analysis of variance to determine if there were significant differences among groups. If so, Sheffe's F-test was performed to determine whether there was significant difference between the control and each treatment group. For comparison of two groups, Student's t-test was performed and $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Endogenous NDPK activity in CHO-rmor cell membrane

[γ-^{32}P]ATP (100 μM) was used as the donor of γ-phosphate and the formation of [γ-^{32}P]GTP in the presence of 100 μM GDP was used as the measure of NDPK activity in CHO-rmor membranes. An autoradiograph of TLC (thin layer liquid chromatography) separation of [γ-^{32}P]GTP and [γ-^{32}P]ATP is shown in Fig. 1. The rate of formation of [γ-^{32}P]GTP from GDP and [γ-^{32}P]ATP, calculated from radioactivities of [γ-^{32}P]GTP and [γ-^{32}P]ATP,

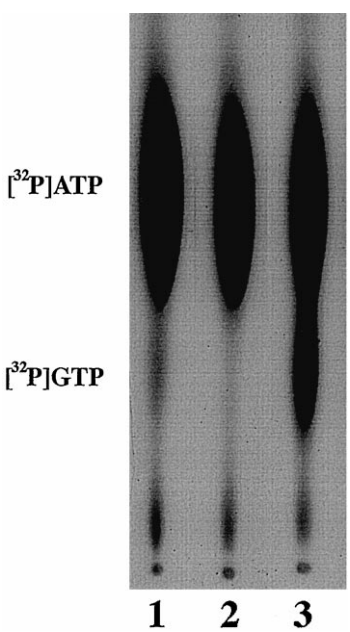

Fig. 1. NDPK activities in CHO-rmor cell membranes: Formation of [γ-^{32}P]GTP from [γ-^{32}P]ATP and GDP in the absence and presence of 10 mM UDP. NDPK activity was determined in the presence of 100 μM [γ-^{32}P]ATP, 100 μM GDP and 4 μg membrane proteins at 30°C for 30 min. TLC separation of [γ-^{32}P]GTP and [γ-^{32}P]GTP and autoradiography was performed as described in Section 2. Lane 1: membranes + [γ-^{32}P]ATP without GDP. Lane 2: [γ-^{32}P]ATP without membranes or GDP. Lane 3: membranes + [γ-^{32}P]ATP + GDP. This figure represents one of the two experiments performed with the same results.

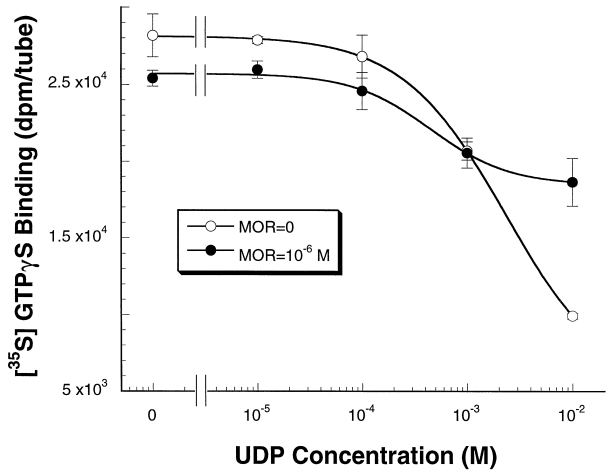

Fig. 2. Effect of UDP on morphine-stimulated [^{35}S]GTPγS binding to CHO-rmor membranes. Basal and morphine (1 μM)-stimulated [^{35}S]GTPγS binding was performed in the presence or absence of various concentrations of UDP with 7 μg membrane protein at 30°C for 60 min as described in Section 2. Each value represents mean ± S.E.M. of three independent experiments in duplicate.

was determined to be 0.60 ± 0.02 μmol/mg protein/30 min ($n = 3$, mean ± S.E.M.). These data indicate the presence of endogenous NDPK activities in CHO-rmor cell membranes. Activation of μ-opioid receptors by morphine or DAMGO did not increase NDPK activities (data not shown). UDP in mM range, which forms abortive NDPK-UDP complexes, has been used to inhibit binding of other nucleotides to NDPK (for a review, Parks and Agarwal, 1973). UDP (10 mM) reduced [^{32}P]GTP formation from [^{32}P]ATP and GDP by CHO-rmor membranes to 0.25 μmol/mg protein/30 min ($n = 2$), which represented a reduction of 58%. These results are consistent with the findings that UDP (1–10 mM) inhibited GTP formation from ATP and GDP in membranes of human leukemia HL60 cells (Wieland et al., 1991), human and rodent pancreatic β-cells (Kowluru and Metz, 1994) and rat hepatocytes (Kimura and Shimada, 1983).

3.2. Role of NDPK in the morphine-stimulated [^{35}S]GTPγS binding

Like G proteins, NDPK binds guanine nucleotides. K_d values of [^{35}S]GTPγS binding to NDPK and to pertussis toxin-sensitive G proteins were determined to be 5.7 nM and 0.34 nM, respectively (Zhang and Chang, 1995; Zhu et al., 1997). NDPK has similar affinities to many nucleoside diphosphates and triphosphates including ATP, GTP, GDP, UTP and UDP (Zhang and Chang, 1992, 1995). In contrast, G proteins have much higher binding affinities for guanine nucleotides than for uracil, adenine and cytosine nucleotides. The IC_{50} values of ATP, CTP and UTP for inhibition of [^{35}S]GTPγS binding to G_o are greater than 1 mM (Sternweis and Robishaw, 1984). UDP in mM range has been used to inhibit binding of other nucleotides to NDPK (for a review, see Parks and Agarwal, 1973) with no significant effect on GTP binding to G proteins (Kimura and Shimada, 1983; Jakobs and Wieland, 1989). UDP at 1 mM had no effect on GTP-induced inhibition of forskolin-stimulated adenylated cyclase activity in human platelet membranes (Jakobs and Wieland, 1989). UDP at 10 mM had no effect on stimulation of adenylyl cyclase by GTP in the presence of glucagon (Kimura and Shimada, 1983). Thus, UDP in mM range has been used to inhibit NDPK for examination of the role of NDPK in the regulation of G proteins-mediated events (Kimura and Shimada, 1983; Jakobs and Wieland, 1989; Wieland et al., 1991).

For agonist-induced increase in [^{35}S]GTPγS binding to G proteins to be observed, a high concentration (1 μM to 10 μM) of GDP was required (Hilf et al., 1989; Lorenzen et al., 1993; Traynor and Nahorski, 1995; Zhu et al., 1997). Such concentrations represent about 1000 to 10 000 times of [^{35}S]GTPγS concentration (~ 0.1 nM). We examined whether high concentrations of UDP could decrease basal [^{35}S]GTPγS binding. As shown in Fig. 2, high levels of [^{35}S]GTPγS binding were found in the absence of GDP and UDP, but the stimulatory effect of morphine could not be detected under this condition. In the presence of 10 mM UDP, [^{35}S]GTPγS binding in the presence or absence of morphine was reduced and the stimulatory effect of morphine was evident in the absence of GDP (Fig. 2). Since UDP at 1–10 mM has negligible binding to G proteins (Kimura and Shimada, 1983; Jakobs and Wieland, 1989), part of high levels of basal [^{35}S]GTPγS binding is attributed to its binding to NDPK. An alternative interpretation is that 10 mM UDP binds to G protein, albeit at a very low level, reduced the basal [^{35}S]GTPγS binding.

We next examined effects of UDP on dose-effect relationship of GDP on [^{35}S]GTPγS binding. Without GDP or UDP, morphine had no effect on [^{35}S]GTPγS binding (Fig. 3). In the absence of UDP, GDP inhibited [^{35}S]GTPγS

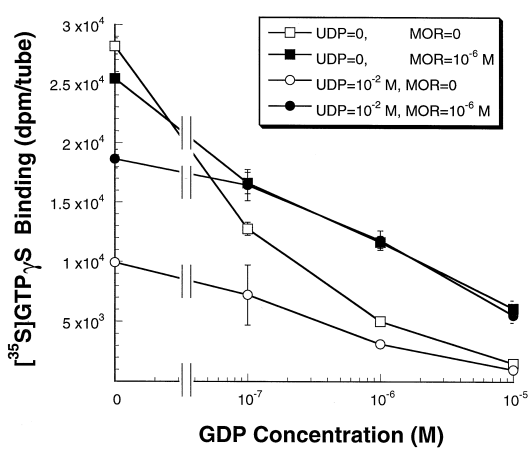

Fig. 3. Effect of GDP on morphine-stimulated [^{35}S]GTPγS binding to CHO-rmor membranes in the presence and absence of 10 mM UDP. Basal and morphine (1 μM)-stimulated [^{35}S]GTPγS binding was performed in the presence or absence of various concentrations of GDP with or without 10 mM UDP with 7 μg membrane protein at 30°C for 60 min as described in Section 2. Each value represents mean ± S.E.M. of three independent experiments in duplicate.

binding in a dose-dependent manner in the presence and absence of morphine and morphine-induced increase in [^{35}S]GTPγS binding could be observed at [GDP] $\geq 10^{-7}$ M (Fig. 3). This observation is similar to those of Traynor and Nahorski (1995) and Zhu et al. (1997). In the presence of 10^{-7} M or 10^{-6} M GDP, 10 mM UDP reduced the basal [^{35}S]GTPγS binding without affecting the binding in the presence of morphine, thus resulting in an increase in morphine-stimulated [^{35}S]GTPγS binding, with the increase being more pronounced at 10^{-7} M than 10^{-6} M of GDP (Fig. 3). To examine whether this increase in morphine-stimulated [^{35}S]GTPγS binding at 10^{-7} M and 10^{-6} M GDP represents binding to pertussis toxin sensitive G proteins and/or other GTP binding proteins, we examined effects of pertussis toxin pretreatment on morphine-induced increase in [^{35}S]GTPγS binding in the presence of 10 mM UDP (Table 1). Morphine-stimulated [^{35}S]GTPγS binding in the presence of 10^{-2} M UDP and 10^{-7} M or 10^{-6} M GDP was completely abolished by pertussis toxin pretreatment (Table 1), indicating that under such conditions, morphine-stimulated [^{35}S]GTPγS binding is mediated entirely through pertussis toxin-sensitive G proteins. Our finding that [^{35}S]GTPγS binding in the presence of 10^{-6} M morphine and different concentrations of GDP (from 10^{-7} to 10^{-5} M) was identical in the presence and absence of 10 mM UDP (Fig. 3) indicates that 10 mM UDP does not affect the binding of [^{35}S]GTPγS binding to G protein. These results are in accord with the observations that UDP at 1–10 mM had no effect on GTP-induced inhibition of forskolin-stimulated adenylated cyclase activity in human platelet membranes (Jakobs and Wieland, 1989) or stimulation of adenylyl cyclase by GTP in the presence of glucagon in rat hepatocyte membranes (Kimura and Shimada, 1983). Thus, the inhibitory effect on [^{35}S]GTPγS binding of UDP is not the result of nonspecific binding of high concentration UDP to G protein. Rather, these results are consistent with the notion that when the formation of GTP from GDP and ATP by NDPK is reduced by UDP, the guanine nucleotide binding sites of Gα subunits are more fully occupied by GDP, the basal [^{35}S]GTPγS binding is decreased and morphine-stimulated increased is augmented. In addition, pertussis toxin reduced basal [^{35}S]GTPγS binding, indicating that active coupling of unoccupied receptors to pertussis toxin-sensitive G proteins may occur. This finding is similar to those of Traynor and Nahorski (1995) and Zhu et al. (1997).

ATP is the primary phosphate donor of the phosphate transfer reaction catalyzed by NDPK. Effects of ATP on basal and morphine-stimulated [^{35}S]GTPγS binding were examined in the presence of 1 μM GDP (Fig. 4). ATP (10^{-5} M) essentially abolished morphine-stimulated [^{35}S]GTPγS binding. In contrast, AMP-PCP (adenylyl-(β,γ-methylene)-diphosphonate tetralithium salt) (10^{-5} M), an ATP analog that cannot serve as a phosphate donor, did not (Fig. 4). Furthermore, AMP-PCP increased morphine-stimulated [^{35}S]GTPγS binding. ATP and AppNHp up to 1 mM have no detectable effect on [^{35}S]GTPγS binding to purified G_i proteins (Bokoch et al., 1984). These results indicate that effects of ATP are due to its conversion to GTP by endogenous NDPK acting on GDP. GTP thus formed saturates the guanine nucleotide binding site of Gα subunits and, in turn, prevents [^{35}S]GTPγS binding. Due to its inability to serve as phosphate donor for NDPK reaction, AMP-PCP likely leads to a reduction of GTP in the vicinity of G proteins and hence favors GDP-bound G proteins, which in turn leads to an increase in morphine-stimulated [^{35}S]GTPγS binding.

The findings that ATP markedly reduced morphine-stimulated [^{35}S]GTPγS binding and UDP decreased basal [^{35}S]GTPγS binding in the presence of GDP suggest that GTP formed from GDP and ATP by NDPK appears to be closely associated with membranes and is efficient in binding to G proteins. Similar observations have been reported (Wieland et al., 1991; Wieland and Jakobs, 1992; Niroomand et al., 1997). By measuring [^{32}P]GTPγS formed from GDP and [^{32}P]ATPγS catalyzed by NDPK, Wieland and Jakobs (1992) found that endogenously formed GTPγS was about 10-fold more potent than exogenously added GTPγS in inhibiting [^{125}I]C5a binding. Since NDPK is localized in membranes (in addition to cytosol and nucleus) and there is a high rate of GTP hydrolysis on the intracellular side of membranes, it has been suggested that NDPK may act to modulate GTP/GDP ratio in the intracellular side of plasma membranes, possibly in the imme-

Table 1
Effect of GDP and UDP on morphine-induced increase in [^{35}S]GTPγS binding (in dpm/assay tube) to membranes of control and pertussis toxin-treated CHO-rmor cells. CHO-rmor cells were treated with or without 100 ng/ml pertussis toxin for 24 h. [^{35}S]GTPγS binding to membranes of control and pertussis toxin-treated cells was performed in the presence of 10^{-2} M UDP and 10^{-7} M or 10^{-6} M GDP with 7 μg membrane protein at 30°C for 60 min as described in Section 2. Each value represents the mean ± S.E.M. of two independent experiments in duplicate

	GDP = 10^{-7} M		GDP = 10^{-6} M	
	Control	+ pertussis toxin	Control	+ pertussis toxin
UDP = 10^{-2} M, Morphine = 0	4612 ± 68	3569 ± 118[b]	3446 ± 57	2861 ± 50[b]
UDP = 10^{-2} M, Morphine = 10^{-6} M	8053 ± 319[a]	3937 ± 149[a,c]	7982 ± 172[a]	2753 ± 65[c]

[a] $P < 0.01$, compare to the value determined with UDP = 10^{-2} M, Morphine = 0.
[b] $P < 0.05$, [c] $P < 0.01$, compare to the value determined in the absence of pertussis toxin under the same condition.

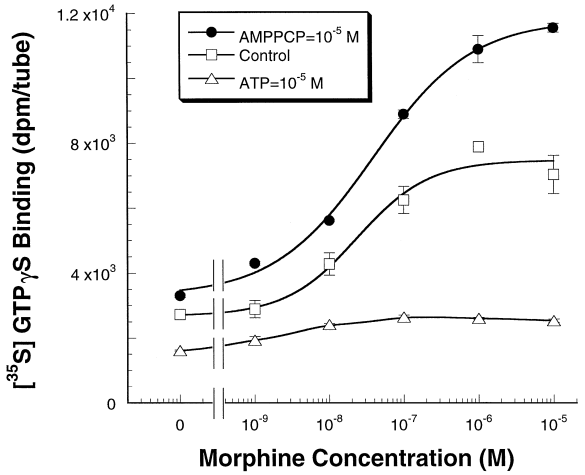

Fig. 4. Effects of ATP and AMP-PCP on dose–response relationships of morphine-stimulated [^{35}S]GTPγS binding. [^{35}S]GTPγS binding was performed in the presence of 1 μM GDP with or without 100 μM ATP or AMP-PCP (adenylyl-(β,γ-methylene)-diphosphonate tetralithium salt) and various concentrations of morphine with 7 μg membrane protein at 30°C for 60 min as described in Section 2. Each value represents mean ± S.E.M. of three independent experiments in duplicate.

diate vicinity of G proteins and receptors (Wieland et al., 1991; Wieland and Jakobs, 1992; Bominaar et al., 1993; Blevins et al., 1994). This action leads to channeling of GTP to G proteins. Because of the physical association of NDPK with α subunits of G proteins (Kimura and Shimada, 1988; Orlov et al., 1996; Orlov and Kimura, 1998), it was suggested that NDPK may catalyze phosphate transfer directly onto Gα subunit-bound GDP. However, whether NDPK has such actions has not been unambiguously demonstrated (Ohtsuki and Yokoyama, 1987; Kikkawa et al., 1990).

3.3. Role of NDPK in [^{3}H]DAMGO binding to the μ-opioid receptor

3.3.1. Effects of nucleotides on [^{3}H]DAMGO binding to the μ-opioid receptor

GTP profoundly reduced agonist binding to opioid receptors (Blume, 1978; Childers and Snyder, 1978, 1980). The selective μ agonist [^{3}H]DAMGO saturation binding to the μ-opioid receptor was performed to delineate effects of GDP, ATP and both on K_d and B_{max} values (Table 2). [^{3}H]DAMGO binding under all four conditions fit one-site model better than two-site model. ATP (10^{-4} M) did not significantly affect K_d or B_{max}. Although the changes in K_d and in B_{max} did not reach statistical significance in the presence of ATP alone (Table 2), there appears to be a trend of an increase in K_d and a decrease in B_{max}. GDP (10^{-7} M) increased the K_d value without changing the B_{max}. The combination of both ATP (10^{-4} M) and GDP (10^{-7} M) significantly increased the K_d value and reduced the B_{max} value.

Table 2
Effects of ATP and GDP on K_d and B_{max} values of [^{3}H]DAMGO binding to the μ receptor. [^{3}H]DAMGO saturation binding to the μ receptor was performed in the presence and absence of 10^{-4} M ATP and 10^{-7} M GDP and K_d and B_{max} values were determined as described in Section 2. Each datum is the mean ± S.E.M. of three independent experiments

Nucleotides	K_d (nM)	B_{max} (pmol/mg protein)
ATP = 0, GDP = 0	0.45 ± 0.05	3.00 ± 0.18
ATP = 10^{-4} M, GDP = 0	0.63 ± 0.02	2.84 ± 0.23
ATP = 0, GDP = 10^{-7} M	1.87 ± 0.05[a]	2.59 ± 0.08
ATP = 10^{-4} M, GDP = 10^{-7} M	2.33 ± 0.17[a]	1.93 ± 0.24[a]

[a] $P < 0.05$, compare to the value in the absence of both ATP and GDP.

3.3.2. Effect of exogenous NDPK on [^{3}H]DAMGO binding to the μ-opioid receptor

Effects of exogenous NDPK on 2.5 nM [^{3}H]DAMGO binding to the μ-opioid receptor were examined by addition of purified bovine liver NDPK to the assay medium. Lower concentrations of ATP and GDP were used to more easily detect effects of exogenous NDPK. As shown in Table 3, 0.01 unit of NDPK caused a small, yet statistically significant, reduction in [^{3}H]DAMGO binding in the absence of any exogenous nucleotides. The endogenous ATP and GDP in the membrane preparation are likely to be substrates for exogenous NDPK. Addition of ATP (10^{-5} M) alone reduced [^{3}H]DAMGO binding by 10%, which presumably is due to endogenous NDPK activity. Addition of NDPK enhanced the effect of ATP and reduced [^{3}H]DAMGO binding by 24%. GDP at 10^{-8} M significantly reduced [^{3}H]DAMGO binding by itself (Table 3) and effect of exogenous NDPK was not evident at this concentration of GDP. A combination of ATP (10^{-5} M) and GDP (10^{-8} M) decreased [^{3}H]DAMGO binding by 18% and the exogenous NDPK enhanced this effect of

Table 3
Effects of exogenous NDPK on [^{3}H]DAMGO binding to the μ opioid receptor. [^{3}H]DAMGO (2.5 nM) binding was performed with or without NDPK, 10^{-5} M ATP and 10^{-8} M GDP for 30 min at 30°C with 20 μg membrane proteins as described in Section 2. Each value represents the mean ± S.E.M. of three independent experiments

Nucleotides	[^{3}H]DAMGO binding (dpm/20 μg protein)	
	NDPK = 0	NDPK = 0.01 unit
ATP = 0, GDP = 0	8458 ± 48	7717 ± 275[b]
ATP = 0, GDP = 10^{-8} M	7223 ± 197[a]	7193 ± 95[a]
ATP = 10^{-5} M, GDP = 0	7611 ± 70[a]	6546 ± 53[a,b]
ATP = 10^{-5} M, GDP = 10^{-8} M	6982 ± 97[a]	5398 ± 225[a,b]

[a] $P < 0.05$, compared with the binding in the absence of ATP and GDP.
[b] $P < 0.05$, compared with the binding in the absence of NDPK.

ATP (10^{-5} M) and GDP (10^{-8} M) to 36%. Thus, exogenous NDPK enhanced effects of ATP or GDP and ATP, due to its converting GDP (endogenous and exogenous) to GTP.

The findings that ATP alone had slight effect on [^{3}H]DAMGO binding and GDP alone significantly reduced [^{3}H]DAMGO binding are consistent with those of Blume (1978) who demonstrated that while 50 μM ATP had no effect on [^{3}H]dihydromorphine binding to opioid receptors in the rat brain membranes, 50 μM GDP reduced the binding. The magnitude of reduction in [^{3}H]DAMGO binding to the μ-opioid receptor by ATP and GDP was not as profound as their effects in reducing receptor binding of [^{3}H]formyl-Met-Leu-Phe and [^{125}I]C5a in membranes of HL-60 cells (Wieland et al., 1991; Wieland and Jakobs, 1992). At least two reasons may account for these differences. Endogenous levels of NDPK activities may be quite different between membranes of CHO-rmor cells and HL-60 cells. In addition, sensitivity to nucleotides of agonist binding to its receptor may also vary among the receptors examined.

CHO-rmor cell membranes had NDPK activities and ATP and GDP significantly reduced binding of the μ-opioid selective agonist [^{3}H]DAMGO. Thus, GTP formed from GDP and ATP catalyzed by membrane-associated NDPK in the close vicinity of G proteins and receptors may modulate G protein and receptor activities. Addition of GTP or its nonhydrolyzable analogs has been found to decrease agonist affinity for opioid receptors (Chang et al., 1983; Law et al., 1985; Werling et al., 1988). These findings are consistent with several reports. NDPK was found to be present in membranes of HL-60 human leukemia cells and the combination of GDP and ATPγS caused a marked reduction in the binding of the chemotatic peptide [^{3}H]formyl-Met-Leu-Phe and the complement component [^{125}I]C5a to their respective receptors in membranes (Wieland et al., 1991; Wieland and Jakobs, 1992). Blevins et al. (1994) reported that pancreatic membranes possessed NDPK activity and GTPγS formed from ATPγS and GDP induced high- and low-agonist affinity states for CCK receptors and also elicited a significant concentration-dependent reduction in the total number of measurable CCK receptors. Similar results were observed in membranes of CHO-K1 cells stably transfected with the CCK type A receptor (Blevins et al., 1996). NDPK-mediated formation of GTPγS from ATPγS and GDP in membranes of the cellular mold *Dictyostelium discoideum* reduced agonist affinity for the cAMP receptor (Bominaar et al., 1993).

While ATP (10^{-5} M) markedly reduced morphine-stimulated [^{35}S]GTPγS binding (see Fig. 4), it decreased only slightly [^{3}H]DAMGO binding to the μ-opioid receptor (see Table 3). Effects of ATP are most likely via its conversion to GTP. The reason for this apparent discrepancy may be that a much lower concentration of GTP is needed to bind to G proteins than to affect agonist binding. K_d of morphine-stimulated [^{35}S]GTPγS binding to G proteins was about 0.3 nM (Traynor and Nahorski, 1995; Befort et al., 1996; Zhu et al., 1997) and the affinity of GTP is most likely similar to that of GTPγS. In contrast, ≥ 3 μM GTP was required to have detectable inhibition on agonist binding to opioid receptors and the IC_{50} value was about 10 μM (Blume, 1978; Childers and Snyder, 1978, 1980).

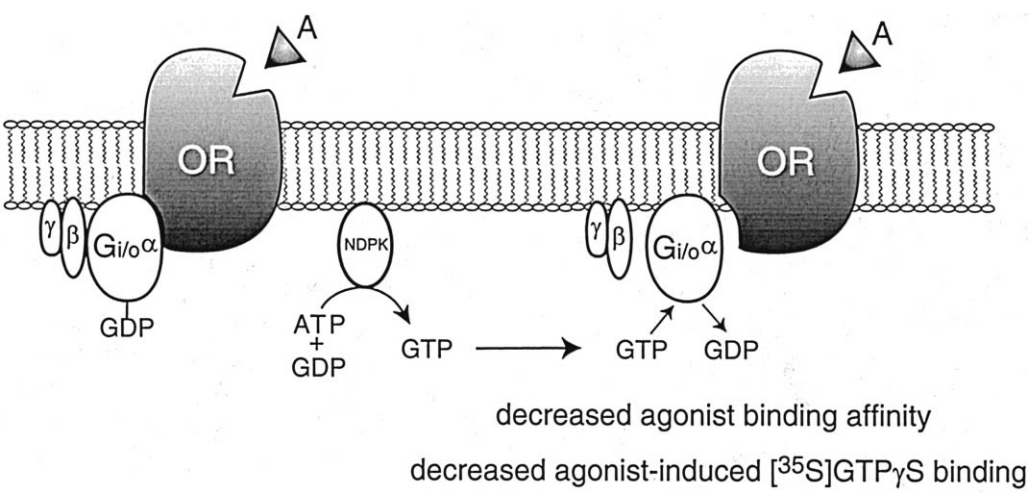

Fig. 5. The role of NDPK in the regulation of μ-opioid receptor. Mu-opioid receptors are coupled to pertussis toxin-sensitive G proteins, i.e., G_o or G_i proteins in CHO-rmor cells. In membranes of these cells, NDPK catalyzes the formation of GTP from ATP and GDP. GTP thus formed is likely to be channeled to $G\alpha_{o/i}$ subunits (Wieland et al., 1991; Wieland and Jakobs, 1992; Bominaar et al., 1993; Blevins et al., 1994). Binding of GTP to $G\alpha_{o/i}$ proteins reduces morphine-induced [^{35}S]GTPγS binding to $G\alpha_{o/i}$ proteins. Like many other G protein-coupled receptors, the affinity of μ-opioid receptor to its agonists is regulated by guanine nucleotide binding to $G\alpha_{o/i}$ proteins (Chang et al., 1983; Law et al., 1985; Werling et al., 1988). Binding of GTP, formed by NDPK from GDP and ATP, to $G\alpha_{o/i\alpha}$ subunits leads to uncoupling of G proteins from the receptor and uncoupled receptors have low affinity for agonists such as [^{3}H]DAMGO. OR, opioid receptor; A, agonist.

In conclusion, we demonstrated that NDPK activities were present in CHO-rmor cell membranes and through its conversion of GDP and ATP to GTP, NDPK modulated agonist binding to and signal transduction of μ-opioid receptors (Fig. 5).

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