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Binding Kinetics of δ Opioid Receptors Differ for Microsomal and Synaptic Sites

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SUMMARY

Earlier, we demonstrated that agonist binding to synaptic plasma membranes involves a multi-step association process. In this study, high affinity binding kinetics of an agonist, [^{3}H]D-Ala^2-D-Leu^5-enkephalin (DADLE), to δ sites on bovine hippocampal microsomal and synaptic plasma membranes (SPM) were compared. δ Site selectivity of DADLE was ensured by suppressing undesirable μ site binding with 20 nM unlabeled D-Ala^2-MePhe^4-Glyol^5-enkephalin. The kinetics of receptor binding to microsomal δ sites are generally more rapid than those of SPMs. Furthermore, the association time-dependent rate of dissociation, which is readily observed with SPMs, was not detected for microsomal binding sites. Although the apparent K_D of DADLE did not differ significantly from that in SPMs, kinetic analysis indicated that little or no formation of the high affinity, slowly dissociating, complex occurred with microsomes. The absence of this complex, shown previously in SPMs to be most sensitive to guanine nucleotides, appeared to account for the attenuated effect of guanyl 5′-yl-imidodiphosphate [Gpp(NH)p] on dissociation from microsomes. Nevertheless, the presence in microsomes of inhibitory guanine nucleotide binding proteins was demonstrated by specific ^{32}P-labeling by pertussis toxin of bands at 39 and 41 kDa, attributable to the α subunit of G_o and G_i, respectively.

The action of 100 mM Na^+ to increase the off-rate is similar for both preparations. In contrast, addition of Mn^{2+} reduced the rates of association and dissociation for both subcellular fractions. The off-rate in the presence of Mn^{2+} is similar for SPMs and microsomes, displaying association time-dependent rates of dissociation for both. To determine whether Mn^{2+} promotes coupling in microsomes, the effect of Gpp(NH)p was examined. After a 60-min association, Gpp(NH)p did not affect microsomal kinetics but increased the off-rate from SPMs. The actions of both Na^+ and Mn^{2+} appear to be mediated at early steps in the association process.

There is evidence to suggest that opioid receptors exist primarily in two general subcellular pools, SPM and microsomes (1–8). These membrane fractions can be distinguished by several criteria, including electron microscopy, marker enzyme analysis, and density.The SPM fraction is enriched with synaptic junctions and plasma membrane whereas the microsomal fractions have been shown to contain smooth vesicles generally associated with smooth endoplasmic reticulum and Golgi fractions. The functional significance of the light membrane binding sites, however, remains unclear despite their relative high concentration. In our studies, the apparent B_{max} (per mg of protein) and K_D of several opioid ligands were comparable for SPM and microsomal membranes. Although similar, we have found that opioid receptor binding characteristics for purified SPMs and microsomes differ with respect to the effects of guanine nucleotides, microsomes being less sensitive to the guanine nucleotide analog Gpp(NH)p (6, 9). These results suggested the possibility that microsomal sites may have only limited coupling to a G protein.

We previously demonstrated in kinetic studies that agonist binding to μ and δ opioid receptors on SPMs display a multistep association, as indicated by slow transformation of low affinity binding to a high affinity slowly dissociating state. In contrast, antagonists appear to undergo simple bimolecular association (10). Indeed, the occurrence of high and low affinity states for opioid agonist binding was subsequently corroborated by others (11, 12). We postulated that the high affinity state is indicative of receptor-effector coupling in signal-transduction. Potential effector systems for δ opioid receptors are thought to be adenylate cyclase and ion channels. Inhibition of adenylate cyclase (13–18) and ion channel gating (19) via an opioid receptor-mediated process that utilizes G proteins for signal transduction have been shown in both brain and neuroblastoma glioma hybrid cells. This mechanism is common to several neurotransmitters (for reviews, see Refs. 20 and 21). Opioid inhibition of adenylate cyclase activity can be blocked by pertussis toxin treatment (18), which ADP-ribosylates both G_o and G_i proteins (21).

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ABBREVIATIONS: SPM, Synaptic plasma membranes; G protein, guanine nucleotide binding protein; DADLE, D-Ala^2-D-Leu^5-enkephalin; DAMGE, D-Ala^2-MePhe^4-Glyol^5-enkephalin; Gpp(NH)p, guanyl 5′-yl-imidodiphosphate.

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Divalent cations (Mn^{2+}, Mg^{2+}, and Ca^{2+}) have also been shown to potentiate δ agonist (but not antagonist) binding to opioid receptors (22–27), suggesting a role in the coupling process. Although similar in effect, Mn^{2+} has proven to be the most potent of the three ions (27). In contrast, Na^+ has long been thought to favor an inactive conformation of a receptor (28–30), as evidenced by its selective reduction of agonist affinity. We now report that microsomal receptors form a cation-sensitive, more rapidly associating and dissociating, agonist high affinity state, but not the slowly dissociating, high affinity agonist state previously described for SPMs.

Materials and Methods

Materials. [^{3}H]DADLE (49.3 Ci/mmol) was obtained from Amersham Inc. (Arlington Heights, IL). The stability and initial purity of the radioligand were monitored by thin layer chromatography as previously described (31). Unlabeled DADLE and DAMGE were purchased from Bachem AG (Philadelphia, PA). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Membrane preparation and binding assay. Subcellular fractions were prepared from bovine hippocampal/dentate gyrus by a modified floatation-sedimentation sucrose density gradient centrifugation method as previously described (6) for rat brain. All experiments were performed on fresh brain tissue except for studies of ^{32}P-labeling of G proteins, in which purified membranes were frozen at −70° in 100 mM potassium phosphate buffer, pH 7.0, before use. Rapid filtration by a Brandel Cell Harvester was used to quantitate bound ligand. In all experiments, δ site selectivity for 1 nM [^{3}H]DADLE was ensured by a 40-min preincubation of membranes with 20 nM DAMGE before addition of radioligand (31, 32). In dissociation experiments, membranes were incubated with radioligand for the indicated time period before the initiation of dissociation with 1 μM unlabeled DADLE. For assessment of nonspecific binding, 1 μM DADLE was included before addition of radioligand. Each point was measured in duplicate except the zero minute and nonspecific points, which were quadruplicate. Addition of assay components was timed to permit simultaneous filtration of all time points. When appropriate, either 50 μM Gpp(NH)p or 100 mM NaCl was added coincident with the unlabeled DADLE, which initiated dissociation. Addition of $MnCl_2$ (20 mM), when indicated, was performed 10 min before the addition of radioligand.

Labeling with [^{32}P]NAD. The procedure used was a modification of that used by Wong *et al.* (33). Pertussis toxin was preactivated by 50 mM dithiothreitol during a 1-hr incubation at 25°. Concurrently, membranes (50–70 μg of protein) were preincubated for 45 min in 100 mM potassium phosphate buffer (pH 7.0), 20 mM GTP, 10 mM thymidine, 1 mM ATP, and 1 μM NAD with a 50-μl total volume. After preincubation, pertussis toxin was mixed with membranes plus 1 mM ATP and [^{32}P]NAD ($1–2 \times 10^6$ cpm) in phosphate buffer (final volume, 100 μl). The reaction was stopped after a 45-min incubation at 30° by the addition of 300 μl of 20% trichloroacetic acid. Samples were spun down (13,000 × *g*, 10 min) and pellets were washed twice with acetone by centrifugation. Dried pellets were solubilized in 50 μl of solubilizing buffer and proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (34) on 14% acrylamide gels.

Data analysis. Binding affinities and capacities were estimated from equilibrium homologous displacement curves as previously described (32) using the LIGAND program, which is a weighted, nonlinear regression receptor binding analysis program (35). The lines drawn in the dissociation plots were generated by nonlinear curve fit of the equation for a bi-exponential decay, which in all cases was statistically superior to the fit of a mono-exponential decay.

Results

We previously demonstrated that microsomal membranes possess 20–30% of the total rat brain membrane-bound opioid receptors (6). Homologous displacement of [^{3}H]DADLE in the presence of 20 nM DAMGE from bovine hippocampal SPMs and microsomes (Fig. 1), and subsequent analysis by the LIGAND computer program, demonstrated high affinity binding with apparent K_D values below 1 nM and the same number of sites. Furthermore, the data did not fit a two-site model better than a one-site model, suggesting that under these conditions DADLE binds to a single receptor type in each membrane fraction. In bovine hippocampus, δ receptors are the predominant opioid receptor type observed (10).

If the homologous displacement of [^{3}H]DADLE was repeated in the presence of 50 μM Gpp(NH)p, curves similar to those in Fig. 1 were obtained for both SPMs and microsomes. However, analyses with the LIGAND program revealed a two-site model fit the data better than one-site. For SPMs the K_D of the higher affinity site had become 0.29 ± 0.058 nM (three experiments), whereas for microsomes it was 0.43 ± 0.15 nM (three experiments). The low affinity sites for both membrane populations could only be estimated approximately but were closer to the micromolar range. The B_{max} of the high affinity sites decreased 37% for SPMs and 34% for microsomes. The suggestion by computer analysis that both low and high affinity sites occur in the presence of Gpp(NH)p prompted the kinetic studies.

Binding kinetics of [^{3}H]DADLE to the δ site in bovine hippocampal SPMs display characteristics that suggest a multistep association (10, 31). During association, there is a time-dependent formation of a high affinity slowly dissociating state, i.e., the off-rate after a brief association period is significantly more rapid than after a prolonged association. However, this association time-dependent dissociation was not observed for

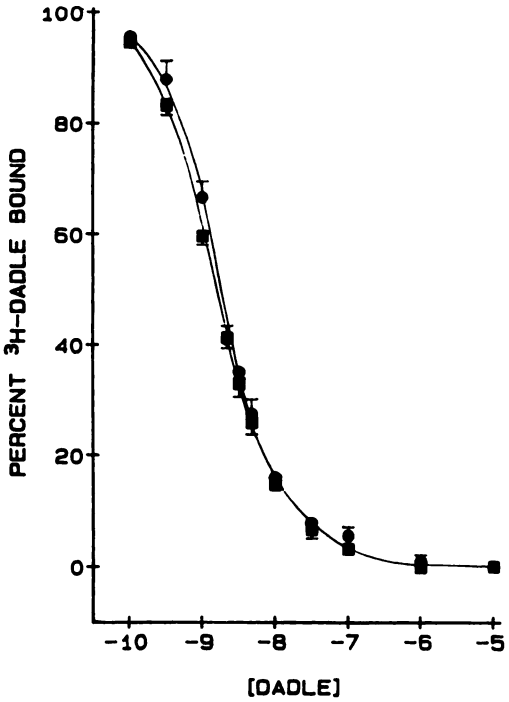

Fig. 1. Homologous displacement of [^{3}H]DADLE by unlabeled DADLE from bovine hippocampal SPMs (■) or microsomes (●). For SPMs K_D = 0.70 ± 0.08 nM and B_{max} = 170 ± 4 fmol/mg of protein whereas for microsomes, K_D = 0.86 ± 0.09 nM and B_{max} = 185 ± 6 fmol/mg of protein. The *abscissa* is the log of the molar concentration of unlabeled DADLE. *Points* represent the mean of seven (SPM) or five (microsome) experiments performed in duplicate. Where no standard error *bar* is seen, the error was less than the diameter of the point.

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microsomal receptors (Fig. 2), suggesting a less complex association process. The off-rate of microsomes, regardless of association time, was similar to that observed (31) from SPMs after only a brief association period (7 min). The rate of dissociation from microsomal δ receptors was not greatly affected by 50 μM Gpp(NH)p (Fig. 2).

The [^{3}H]DADLE association rate for microsomes was also faster than for SPMs (Fig. 3). The time required for SPM specific binding to reach 50% of its 60-min value was approximately twice that required for microsomes. A log transformation of the association data revealed that SPMs have a distinctly biphasic association profile (Fig. 3, *inset*). The fact that both off- and on-rates are increased for microsomes (Figs. 2 and 3) explains the similarity of their K_D to that of SPMs. Because the dissociation constant is a function of both rates, the differences produce no net effect on K_D.

Introduction of 20 mM $MnCl_2$ reduced the on-rate (Fig. 3) and off-rate (Figs. 4 and 5) for both SPMs and microsomes. Moreover, the off-rates for microsomes and SPMs after a 60-min association became similar and each demonstrated an association time-dependent rate of dissociation. Addition of Gpp(NH)p after a brief association period decreased the off-rate for both whereas the addition after a 60-min association increased the off-rate for SPMs (Fig. 5) to a much greater extent than for microsomes (Fig. 4).

Addition of 100 mM NaCl at the onset of dissociation resulted in a release of bound ligand (Fig 6). Despite the above-cited differences between SPMs and microsomes, the effect of Na^+ was similar for these membrane fractions (31). In both cases, the resultant dissociation curves are distinctly biphasic and consistent with earlier results (10, 11).

ADP-ribosylation of bovine hippocampal SPMs and microsomal proteins by pertussis toxin and [^{32}P]NAD demonstrated in both membrane preparations the presence of bands migrating in the same regions as the α subunit of G_o and G_i (39 and 41 kDa, respectively; Fig. 7). For both fractions, the 39-kDa band appeared to predominate.

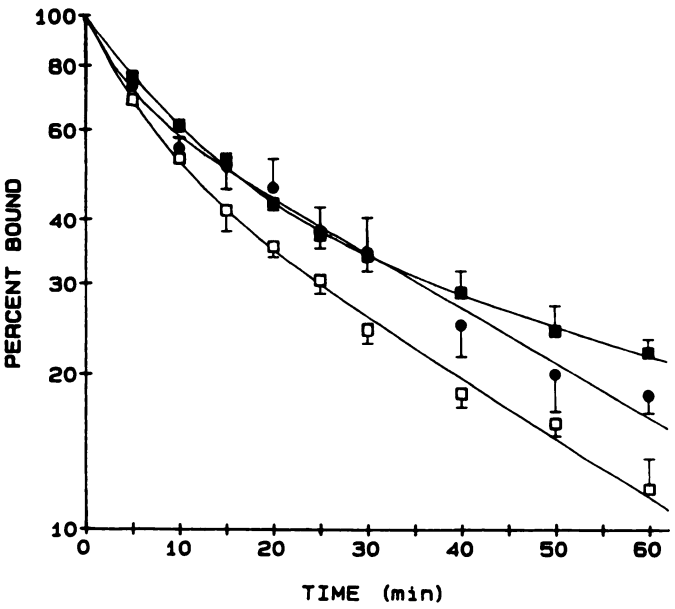

Fig. 2. The effect of association time on the dissociation of [^{3}H]DADLE from bovine hippocampal microsomes. Membranes were incubated with 1 nM [^{3}H]DADLE for 6 min (●) or 60 min (■), followed by displacement with 1 μM DADLE. Binding at 6 min was 69 ± 9% of steady state binding (60 min, 652 ± 100 specific dpm/tube). Gpp(NH)p (50 μM) was added (□) with the unlabeled DADLE after a 60-min association. *Points* represent the mean of three or four experiments performed in duplicate.

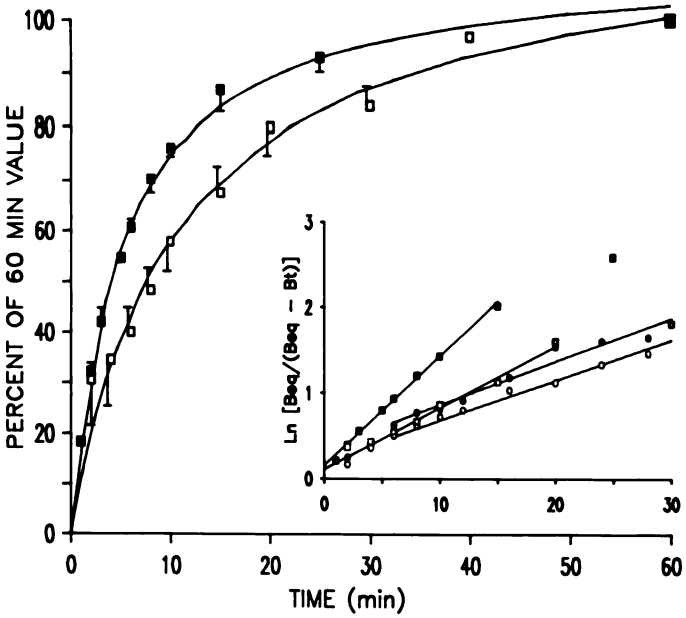

Fig. 3. The association of [^{3}H]DADLE to bovine hippocampal microsomes. □, The association in the presence of 20 mM Mn^{2+}; ■, association in its absence. *Points* are the mean of three or four experiments performed in duplicate. Where no standard error *bar* is seen, the error was less than the diameter of the point. *Inset*, log transformation of the data, in which B_t is the amount bound at a specific time and B_{eq} is that bound at 60 min. Also included are data showing the association to SPM (●) and SPM in the presence of Mn^{2+} (○). Linear regression of points from 5 to 30 min (SPM) or 0 to 20 min (microsomes) was used to generate lines.

Discussion

Steady state binding studies reveal the presence of large amounts of high affinity binding in the smooth microsomal fraction. For bovine hippocampal membranes, neither apparent B_{max} nor K_D for [^{3}H]DADLE binding to SPMs and microsomes is significantly different (Fig. 1). Nevertheless, it is clear from the above kinetic analysis that SPM δ opioid receptors can form a slowly dissociating high affinity ligand-receptor complex that is not formed by microsomal receptors. Calculations of K_D assume that the phenomenon measured is a bimolecular interaction, yet agonist binding has been shown to be a multi-step process for several receptor types (Ref. 10 and references therein). It should be noted that B_{max} and K_D were calculated in a conventional way by a state-of-the-art computer program that unfortunately makes the assumption that the binding phenomenon is a bimolecular interaction. This data analysis problem was recently pointed out by Colquhoun (36) and is illustrated in the present study.

Previously (31), we interpreted agonist binding to the δ opioid site on SPMs as follows. A slowly dissociating high affinity complex of receptor/ligand/G protein (LR*G) was preceded by the formation of more rapidly dissociating states, LR and LR*, which are of low and high affinity, respectively. The results of this study support the proposed model and suggest that microsomal δ sites undergo a less complex association process, forming the high affinity state, LR*, but not the slowly dissociating state, LR*G. This was demonstrated by the apparent lack of

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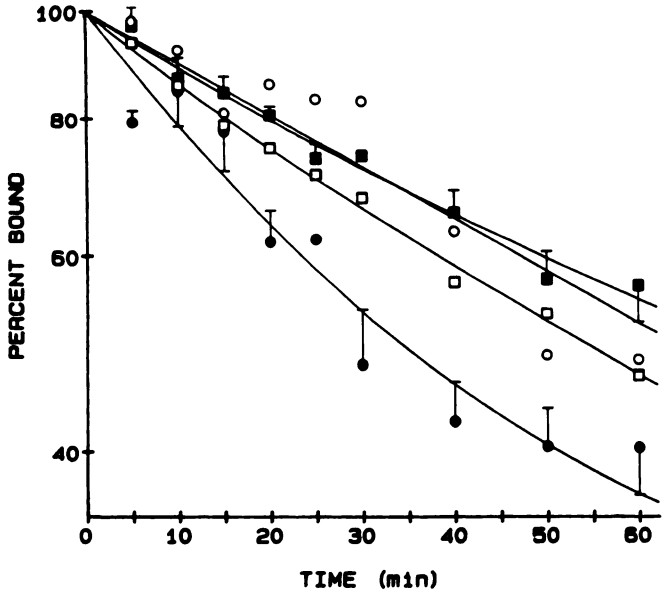

Fig. 4. Dissociation of [^{3}H]DADLE from bovine hippocampal microsomes in the presence of Mn^{2+}. Membranes were preincubated for 10 min with 20 mM Mn^{2+} then incubated with [^{3}H]DADLE for 6 min (●) or 60 min (■) before displacement with 1 μM DADLE. Gpp(NH)p (50 μM) was added with the unlabeled DADLE after a 6-min (○) or 60-min (□) association period. *Points* represent the mean of three experiments performed in duplicate. For clarity, standard error *bars* are not shown for points with Gpp(NH)p added but were within the same range as those shown for control.

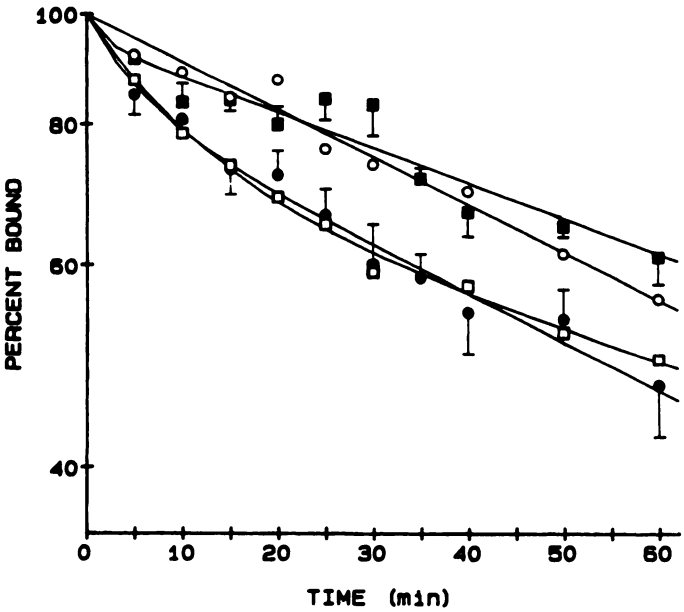

Fig. 5. Dissociation of [^{3}H]DADLE from bovine hippocampal SPMs in the presence of Mn^{2+}. Membranes were preincubated for 10 min with 20 mM Mn^{2+} then incubated with [^{3}H]DADLE for 7 min (●) or 60 min (■) before displacement with 1 μM DADLE. Gpp(NH)p (50 μM) was added with the unlabeled DADLE after the a 6-min (○) or 60-min (□) association period. *Points* represent the mean of three experiments performed in duplicate. For clarity, standard error *bars* are not shown for points with Gpp(NH)p added but were within the same range as those shown for control.

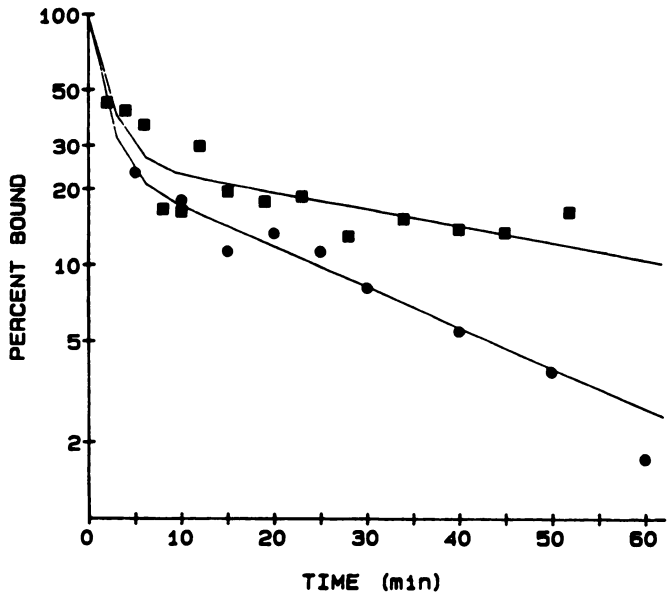

Fig. 6. Dissociation of [^{3}H]DADLE from bovine hippocampal SPMs (■) and microsomes (●), in the presence of sodium. After a 60-min association, dissociation was initiated with 1 μM DADLE and 100 mM NaCl. *Points* represent the mean of three experiments.

an association time dependency for the rate of dissociation and an off-rate similar to that seen in SPMs before the formation of the slowly dissociating high affinity complex (Fig. 2).

Past reports indicate that GTP and its analogs increase the rate of agonist dissociation (6, 37, 38). Apparently, the effect of guanine nucleotides on binding to SPMs is primarily to transform the slowly dissociating state to a rapidly dissociating state (31). This was demonstrated by the profound effect of 50 μM Gpp(NH)p on the off-rate from SPMs when added after a 60-min incubation whereas there is little effect on rate after a 7-min association (31). The guanine nucleotide-induced increase in the rate of dissociation from microsomes (Fig. 2) is modest when compared with the large increase evident with SPMs (31). Similar results with guanine nucleotides and glucagon receptors of rat liver plasma membrane or Golgi membranes were recently reported by Lipson *et al.* (39) which, with other evidence, supports the conclusion that these Golgi sites were not coupled.

In the presence of Mn^{2+}, the dissociations from either SPMs or microsomes were similar, raising the possibility of a role for this cation in the receptor-effector coupling process. Mn^{2+} was the divalent cation used because preliminary studies had shown it to have effects on binding that were similar to, but more profound than, those of either Mg^{2+} or Ca^{2+}. A divalent cation requirement for opioid-mediated inhibition of adenylate cyclase has been recognized for some time (16, 36). Divalent cations are essential for adenylate cyclase activity and they also appear to play a complex role in the interaction of G protein with both guanine nucleotides and receptors (20, 40). Recently it has been postulated that millimolar levels of Mg^{2+} facilitate the binding of G protein to desensitized β-adrenergic receptors in both light and heavy membranes of S49 lymphoma cells (40). Based on substantial evidence of high and low affinity Mg^{2+} interactions with G protein, it was proposed that divalent cation binds to G protein at an allosteric site that facilitates its interaction with receptor. As a result, even the desensitized receptor can bind to G protein in the presence of 10 mM Mg^{2+}. Such a possibility

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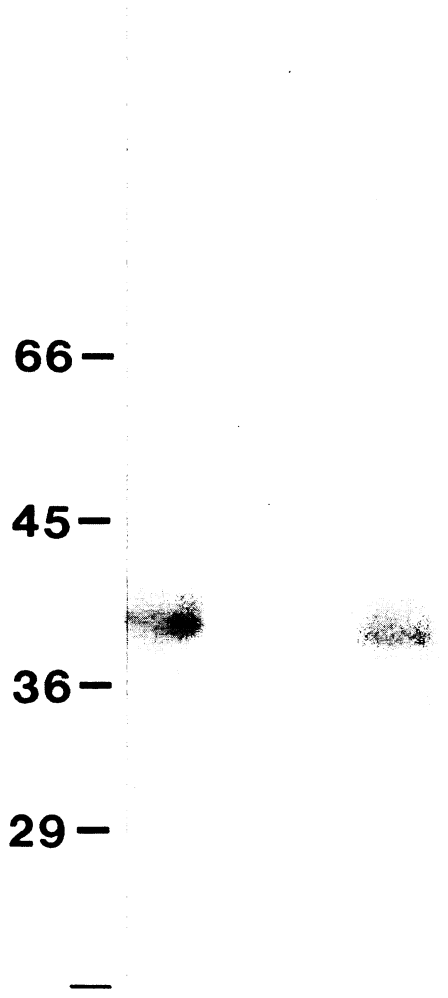

Fig. 7. Autoradiograph of sodium dodecyl sulfate-polyacrylamide gel electrophoresis-separated ^{32}P-labeled proteins. *Lanes* are designated left to right with *lanes 1* and *2* representing SPM specific and nonspecific labeling, respectively. *Lanes 3* and *4* represent microsomal specific and nonspecific labeling, respectively. The positions of molecular weight standards are shown.

would be compatible with our findings. We suggest that the association time-dependent rate of dissociation, which can be detected in the presence of Mn^{2+}, is due to the slow formation of receptor-G protein complex. Additional evidence in support of this hypothesis includes the presence of pertussis toxin ADP-ribosylatable G protein in microsomes (Fig. 7) and the similarity of the kinetics of SPMs and microsomes in the presence of Mn^{2+} (Figs. 4 and 5).

Recently, evidence for a three-step association of agonists to the turkey erythrocyte β-adrenergic receptor has been reported (41). Thermodynamic studies suggest that although agonist-mediated conformational changes occur in β-receptors, only a fraction become functionally coupled. This is in good agreement with the elegant studies of Lefkowitz and associates (42), which have shown that desensitization of β-receptors entails their phosphorylation on the cell surface prior to internalization, whereas dephosphorylation to re-activate receptor occurs at an intracellular site. Hence, both heavy and light membranes (analogous to brain SPMs and microsomes, respectively) would contain desensitized receptors with the higher proportion in the internal sites (Refs 40 and 42 and references cited therein). An analogous relationship for opioid receptors could explain why, in all of our steady state and kinetic binding studies (6, 9, 31), SPMs are more responsive to Gpp(NH)p than microsomes but the latter always demonstrate some sensitivity.

Studies on the δ opioid receptors in neuroblastoma × glioma hybrid cells are consistent with the above hypothesis (8, 11, 18, 43). Using intact cells, computer modeling (LIGAND) of agonist binding reveals the existence of two binding sites with, perhaps, one being the desensitized form of the receptor (12, 43). Furthermore, agonist binding to membranes from these cells appeared to fit single-site parameter estimates only in the presence of 10 mM Mg^{2+} (11). Because these membranes were obtained by centrifugation at 100,000 × *g*, they would contain both uncoupled internal sites (8) and cell surface sites. The fact that, in these membranes, 10 mM Mg^{2+} was used to obtain single-site binding with LIGAND analysis further supports the notion that Mg^{2+} promotes the coupling of receptors to G protein.

That Mn^{2+} slows the rate of dissociation, even after the 7-min association period (Figs. 4 and 5), supports previous evidence that divalent cations play an important role in the hormone-receptor interaction and suggests that Mn^{2+} stabilizes the initial steps in the association process, enhancing the affinity of agonists (22). The action of Gpp(NH)p to slow the off-rate after a brief association period in the presence of Mn^{2+} has not previously been described (Figs. 4 and 5), however. It is opposite to the accepted and frequently reported effect of Gpp(NH)p to increase the dissociation rate (6, 36). Retarded dissociation was only observed in the presence of Mn^{2+} and only during the early phase of association to either subcellular fraction. This phenomenon may be related to the putative existence of another guanine nucleotide regulatory site suggested by ourselves (31) and others (11, 14, 44, 45).

Na^+ ion, like divalent cation, is required for opioid-dependent inhibition of adenylate cyclase and has been implicated in GTP interaction with G_i protein (14–17, 46). Consistent with its well known inhibitory effect on opioid agonist binding (28–30), the action of Na^+ on binding kinetics is the opposite of Mn^{2+}, increasing the rate of dissociation (Fig. 6). As is the case for steady state binding, this Na^+ effect is slightly greater for microsomes than for SPMs. These data are consistent with previous investigations of its action on the rapidly formed high affinity intermediate in SPMs (31), and suggests Na^+ is acting on the early steps of association. The biphasic nature of both curves (Fig. 6) is consistent with the intermediate nature of the rapidly dissociating high affinity state, LR*. Nevertheless, due to the heterogeneity of δ opioid receptors even in discrete brain regions such as hippocampus (47), the possibility of independent binding sites cannot be ruled out.

In summary, the examination of the kinetics of opioid binding to bovine hippocampal microsomal sites has provided insight into the nature of its uncoupling from G protein. In addition, the results have demonstrated that this subcellular population is an interesting model system for the study of uncoupled receptors and the coupling phenomenon in general.

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