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THERMODYNAMIC PARAMETERS OF OPIOID BINDING IN THE PRESENCE AND ABSENCE OF G-PROTEIN COUPLING

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ABSTRACT

We have investigated the thermodynamic parameters of various opioid ligands interacting with their receptors in rat brain membranes. Affinity constants (K_a), enthalpy and entropy values were obtained from homologous displacement experiments performed at 0, 24 and 33°C. It was found that all the opioid agonists tested ([^{3}H]dihydromorphine (DHM) μ alkaloid; [^{3}H]DAMGO μ peptide; [^{3}H]deltorphin-B δ peptide) display endothermic binding accompanied with a large entropy increase, regardless of their chemical structure (alkaloid or peptide), or of their μ or δ receptor selectivity. In contrast, binding of the antagonist naloxone is exothermic, mainly enthalpy driven. Na^+ or Mg^{2+} results only in quantitative changes of the thermodynamic parameters. In the presence of the GTP-analog Gpp(NH)p; or Gpp(NH)p + Na^+; or Gpp(NH)p + Na^+ + Mg^{2+} the affinity of DHM binding dramatically decreases which might reflect functional uncoupling of the receptor-ligand complex and G-proteins. This altered molecular interactions are also indicated by curvilinear van't Hoff plot and entropy increase. It is concluded that the thermodynamic analysis provides means of determining the underlying driving forces of ligand binding and helps to delineate its mechanism.

INTRODUCTION

The opioid receptor system is a fairly complex object. The existence of multiple opioid receptor types, the most widely accepted ones being nominated as μ, δ and κ is well documented by pharmacological, biochemical (1, 2) and more recently by molecular cloning experiments (3 and references cited therein). The observation that opioids can activate various second messenger systems provides further heterogeneity. Opioid receptors have been shown to interact with G_i and G_o types of guanine nucleotide binding regulatory proteins (G-proteins), and trigger signals via adenylyl cyclase, ion channels or inositol phosphate metabolism (4). Opioid ligands themselves also show heterogeneity in their receptor selectivity (μ, δ or κ), pharmacological profile (agonist or antagonist) as well as chemical structure (alkaloid or peptide).

As for other receptor systems, radioligand binding studies have provided a great deal of information on the interaction of opioid ligands with their receptors. It is thought that only agonist binding leads to activation of the receptor followed by conformational changes and information transfer. Antagonist binding in contrast would not elicit a biological response. Thermodynamic analysis provides means of determining the underlying driving forces of binding and intermolecular interactions which information can not be easily obtained by other techniques. Thus conformational changes or protein-protein associations should provoke characteristic thermodynamic behaviour. In their pioneering work Weiland et al. found that antagonist binding to β-adrenergic receptors was largely entropy-driven, whereas the binding of agonists occurred with a decrease in enthalpy sufficient to overcome an unfavourable decrease in entropy (5).

In contrast, the binding of the opiate agonist, β-endorphin is accompanied with positive values of ΔH^+ (standard enthalpy) and ΔS^+ (standard entropy) (6). In subsequent papers binding of a general agonist and antagonist (7), or a μ-agonist and a general antagonist (8) was analyzed. All these studies were conducted in rat brain membranes and gave similar results, namely that opioid agonist binding is mainly entropy driven, while opioid antagonist binding is exothermic thus enthalpy driven. Temperature dependence of the binding of receptor selective opioid ligands have been

examined in guinea pig brain membranes (9). The recent attempt of Wild et al. to employ thermodynamic analysis to differentiate delta opioid receptor subtypes is most intriguing (10).

Opioid binding is modulated by a number of reagents. Na^+ and GTP decrease agonist binding without affecting antagonist binding. Divalent cations also differentiate agonist and antagonist binding (11, 12 and references therein). These three agents are also known to be required for functional coupling of opioid receptors to inhibitory G-proteins (4, 13, 14). With thermodynamic inquiry additional information can be expected also about this signal transduction step. Therefore, in the present paper we studied the temperature dependence of opioid binding in the presence or absence of Na^+, guanine nucleotide and Mg^{2+}. Alkaloid ([^{3}H]DHM) and peptide ([^{3}H]DAMGO) μ agonists were compared with the δ-selective peptide deltorphin-B ([^{3}H]DT-B) and the nonselective opioid antagonist ([^{3}H]naloxone). Characteristic thermodynamic parameters ($\Delta G^{o'}$, $\Delta H^{o'}$ and $\Delta S^{o'}$) of the receptor-ligand interaction were calculated from the binding affinity constants, K_a values obtained at various temperatures.

MATERIALS AND METHODS

Chemicals

[^{3}H]naloxone ([^{3}H]NX, 72 Ci/mmol), [^{3}H]dihydromorphine ([^{3}H]DHM, 47 Ci/mmol), [^{3}H]deltorphin-B (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2, [^{3}H]DT-B, 24.55 Ci/mmol) were synthesized by Dr. G. Tóth and associates (15, 16, 17). [^{3}H]-DAMGO (Tyr-D-Ala-Gly-MePhe-Gly-ol, 60 Ci/mmol) was purchased from Amersham. Sodium chloride, magnesium chloride, 5`-guanylylimidodiphosphate, Gpp(NH)p were from Sigma. All other chemicals were of analytical grade.

Membrane preparation

Particulate membrane fractions were prepared as in (18), with minor modifications. Briefly, rats (PVG/C strain) were killed by decapitation, whole brains without cerebella

were excised and homogenized in 20 volumes (w/v) of ice-cold 50 mM Tris-HCl, pH 7.4 buffer with a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 20 min at 40,000 x g and the supernatant discarded. Pellets were suspended in 20 volumes of fresh buffer and incubated for 30 min at 37°C to remove endogenous opioids. Centrifugation was then repeated as described above. Finally, pellets were suspended in 5 volumes of 50 mM Tris-HCl pH 7.4 buffer containing 320 mM sucrose, and stored at -70°C. Membranes were thawed before use, diluted with buffer and spun at 40,000 g to remove sucrose. The resulting pellet was suspended in 80 volumes of buffer to give a protein concentration of about 300 μg/ml and was used for binding experiments. Protein content was determined according to Bradford (19).

Binding assay

Routinely, the binding assay was performed in a total volume of 1 ml containing 700 μl membrane suspension (≈250 mg protein), [^{3}H]-labelled ligands at 1 nM concentration with or without appropriate concentrations (10^{-5}-10^{-11} M) of displacers, ions (2 mM $MgCl_2$, 100 mM or 25 mM NaCl for the antagonist and agonists, respectively) and nucleotide ($5x10^{-5}$ M Gpp(NH)p). Incubation was started by the addition of the membrane protein and continued until equilibrium was achieved (1 h at 0 °C, 1 h at 24°C, 30 min at 33°C in the case of [^{3}H]NX or [^{3}H]DHM; and 90 min at 0° C, 40 min at 24 °C and 33 °C for [^{3}H]DT-B, respectively). In case of the heterologous displacements additional temperatures were also assayed, 14°C and 18°C for 60 min and 28°C for 40 min. The reaction was stopped by filtration through Whatman GF/B ([^{3}H]NX) or GF/C filters ([^{3}H]DHM, [^{3}H]DT-B and [^{3}H]DAMGO) with Brandel M 24-R cell harvester. Filters were rapidly washed twice with 10 ml ice-cold 50 mM Tris-HCl pH 7.4 buffer, dried and counted in a toluene-based scintillation cocktail in a Beckman LS 5000TD counter.

Data analysis

Displacement experiments in the presence of 1 nM radioligand and 11 concentrations of competing ligands were performed at different temperatures.

Untransformed binding data were analyzed with the nonlinear least-squares regression computer program LIGAND (20). From the resulting affinity constants, K_a the following thermodynamic parameters were calculated:

Gibb's free energy: $\Delta G^{\circ\prime} = - R T \ln K_a$

standard free enthalpy: $\Delta H^{\circ\prime} = - R m$

standard free entropy: $\Delta S^{\circ\prime} = (\Delta H^{\circ} - \Delta G^{\circ}) / T$

where R is the gas constant (8.31 J mol^{-1} K^{-1}), T is the absolute temperature in degree Kelvin, m is the slope of the van't Hoff plots (ln K_a vs. 1/T) which was fitted by the computer program 'Microstat' of Ecosoft, 1984).

In the case of curvilinear van't Hoff plots a non-linear regression analysis of the $\Delta G^{\circ\prime}$ values as a function of temperature was performed according to the following equation (21):

$$\Delta G^{\circ\prime} = a + bT + cT^2 \qquad (1)$$

The coefficients a, b, c were calculated from the curve which fitted best the experimental results of $\Delta G^{\circ\prime}$ versus T. $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ were evaluated from the mathematical derivatives of the above equation using the calculated a, b, c values:

$$\Delta H^{\circ\prime} = d(\Delta G^{\circ} / T) / d(1 / T) = a - cT^2 \qquad (2)$$

$$\Delta S^{\circ\prime} = d(\Delta G^{\circ}) / dT = -b - 2\,cT \qquad (3)$$

RESULTS

The effect of temperature on the affinity of four opioid ligands have been studied under equilibrium conditions at 0, 24 and 33°C in homologous displacement experiments. Computer analysis of the curves with the LIGAND program gave a one site fit for all ligands at all temperatures under the conditions tested. (In the case of

[^{3}H]NX binding some experiments resulted in a 2-site fit, but with pooled data the 2-site fit was not significantly better than the 1-site fit.) The resulting K_D (dissociation constant) values in the presence or absence of Na^+ or Mg^{2+} are shown in Table 1. In the case of agonists, the affinity slightly increased as the temperature increased, but decreased for the antagonist [^{3}H]NX. Na^+ enhanced the affinity of the antagonist [^{3}H]NX at all temperature tested, but an opposite effect, namely an attenuation of affinity was observed for μ and δ agonists alike which results agree with previous data in the literature (7, 8).

Transformation of the equilibrium K_D values according to the van't Hoff equation (22) resulted in linear plots (Fig. 1). The slope of the plot was positive for the antagonist [^{3}H]NX binding, but negative for all the agonists tested, independent of their receptor preference or chemical structure. The calculated thermodynamic parameters ($\Delta H°'$, $\Delta G°'$ and $\Delta S°'$) of [^{3}H]NX, [^{3}H]DHM, [^{3}H]DAMGO and [^{3}H]DT-B binding in the absence and presence of regulators are given in Fig. 2 and Table 2. Changes of the standard free energy ($\Delta G°'$) values were negative in all cases indicating that the binding of opioids to their receptors is exergonic, thus spontaneously occurring. Binding of the antagonist [^{3}H]NX seems to be enthalpy-driven. In contrast, binding of all the agonists is endothermic ($\Delta H°>0$). The entropy changes are positive thus thermodynamically favourable for all the ligands tested.

A widely used experimental paradigm for measuring GTP-shift of agonist affinity is to measure the displacement of a radiolabelled antagonist binding by unlabelled agonist in the absence or presence of guanine nucleotide. In our experiments where 10^{-5} - 10^{-11} M unlabelled DHM was tested as displacer of 1 nM [^{3}H]NX analysis of the data with LIGAND failed to show significantly better fit to a two-site than to a one-site model either in the absence (control) or in the presence of regulators. The van't Hoff plots resulted in straight lines with negative slopes in the absence of regulators, or in the presence of Na^+ or Mg^{2+} (Fig.3.) However, the presence of 50 mM Gpp(NH)p (a hydrolysis-resistant GTP analog), the simultaneous presence of Gpp(NH)p + Na^+, or Gpp(NH)p + Na^+ + Mg^{2+} changed the temperature dependence of the system which was reflected as a break in the plot (Fig.3). Such non-linear van't Hoff plots can reflect

TABLE 1.

Temperature dependence of the K_D values of the binding of opioid ligands to rat brain opioid receptors.

	K_D (nmol l^{-1})		
	0°C	24°C	33°C
[^{3}H]NX			
control	2.67±1.06	7.11±0.33	12.32±0.60
Na^+	0.87±0.42	1.02±0.25	1.81±0.34
Mg^{2+}	2.26±0.92	9.45±3.51	18.50±2.95
[^{3}H]DHM			
control	1.06±0.39	0.82±0.04	0.74±0.33
Na^+	9.50±6.39	3.11±0.21	2.24±0.02
Mg^{2+}	7.00±4.30	1.50±0.16	0.56±0.23
[^{3}H]DAMGO			
control	2.83±0.56	2.12±0.46	2.15±0.83
Na^+	4.31±1.17	3.14±0.62	2.41±0.89
Mg^{2+}	7.06±1.04	1.71±0.29	2.17±0.93
[^{3}H]DT-B			
control	5.33±1.20	4.00±0.59	2.66±0.78
Na^+	25.90±7.60	5.37±0.58	4.33±1.03
Mg^{2+}	46.20±10.1	3.35±0.69	1.72±0.45

Homologous displacement experiments were performed with 1 nM of the radioligands and 11 concentrations (10^{-5}-10^{-11} M) of the same ligand in unlabelled form in the absence (control) or in the presence of 2.5 mM Mg^{2+}, and 100 mM or 25 mM NaCl for the antagonist and agonists, respectively. K_D values were obtained by means of computer analysis with the LIGAND program and presented as mean±S.E.M. of 3-5 independent experiments each run in duplicate.

complex binding interactions involving more than one step, or conformational change. As shown in Table 3, the thermodynamic parameters, especially $\Delta H^{o\prime}$ and $\Delta S^{o\prime}$, undergo major changes when an uncoupling of the receptor from G-proteins is assumed to take place. Thus increased entropy and enthalpy changes were detected when Gpp(NH)p, Na^+ and Mg^{2+} were simultaneously present in the incubation mixture (Table 3.).

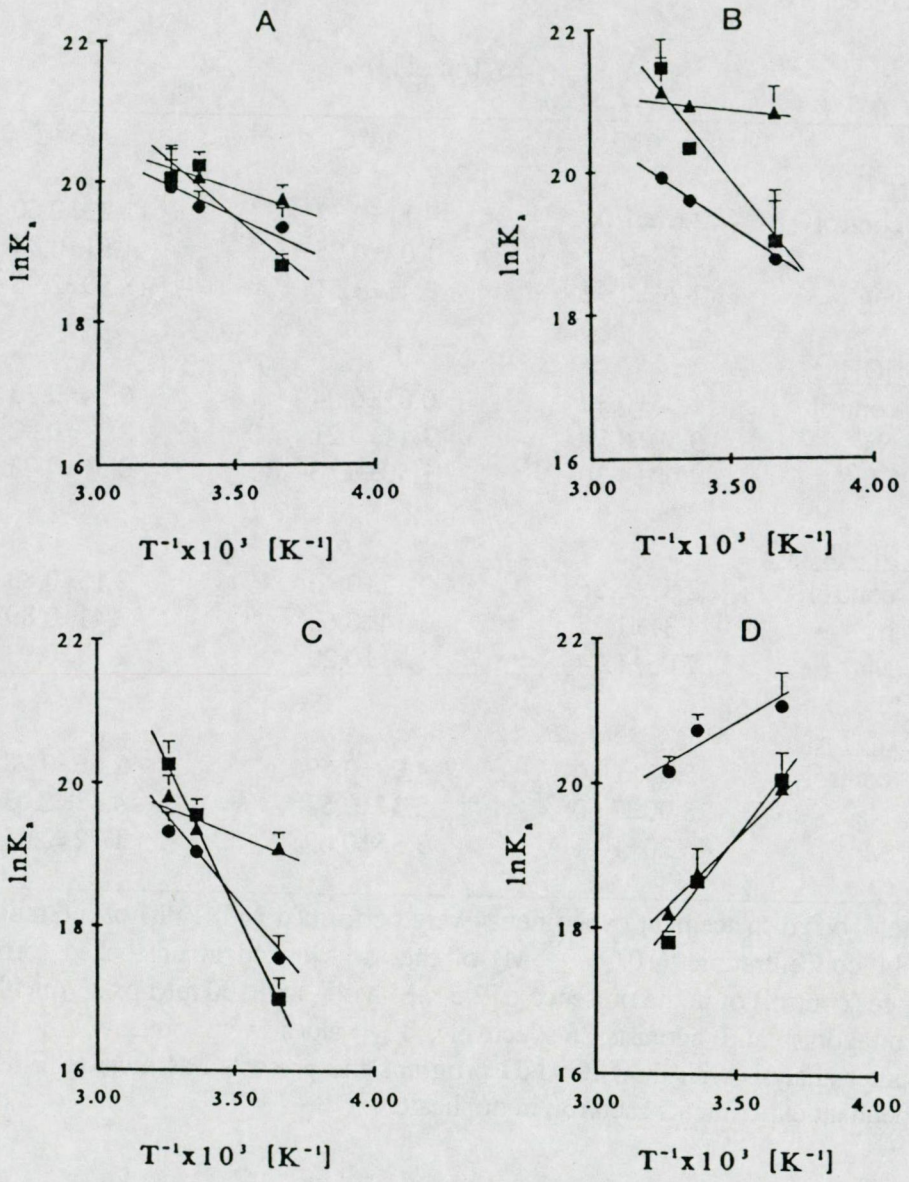

FIG. 1.

Van't Hoff plots of the binding of various opioid ligands to rat brain membranes in the presence or absence of NaCl or $MgCl_2$. A:[^{3}H]DAMGO, B:[^{3}H]DHM, C:[^{3}H]DT-B, D:[^{3}H]NX. The values shown are the mean±S.E.M. of lnK_a of at least 3 independent determinations at each temperature. The symbols are: ▲ - control, ● - in the presence of Na^+ and ■ - in the presence of Mg^{2+}. The regression coefficient of the linear regression analysis $r>0.9$ in all cases.

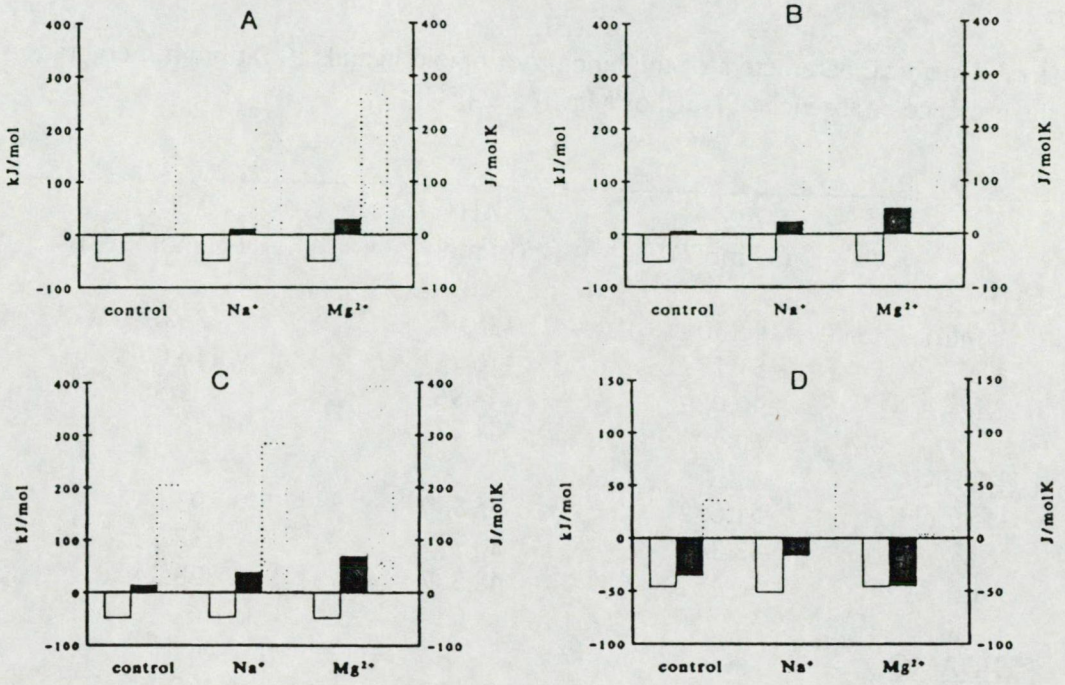

FIG. 2.

Thermodynamical parameters of the binding of opioid ligands to rat brain membranes in the presence or absence of NaCl or $MgCl_2$ at 24°C. A:[^{3}H]DAMGO, B:[^{3}H]DHM, C:[^{3}H]DT-B, D:[^{3}H]NX. ▭ : $\Delta G^{o'}$ (kJ/mol), ■ : $\Delta H^{o'}$ (kJ/mol), ▨ : $\Delta S^{o'}$ (kJ/molK).

DISCUSSION

In this paper the temperature dependence of opioid binding affinity in the presence of guanine nucleotides, Na^+ and Mg^{2+} is investigated, and a comparative study of the thermodynamic parameters characterizing the binding of various opioid ligands to rat brain membranes is presented. It was found that the binding of the opioid antagonist naloxone is an exothermic reaction, mainly driven by a negative enthalpy change (Fig. 2. and Table 2.). In contrast, all the opioid agonists tested here displayed endothermic binding accompanied with a large entropy increase regardless of their chemical structure. The results presented here with μ and δ receptor specific ligands confirm and extend previous observations obtained with less specific opioids (7-9). Although we

TABLE 2.
Thermodynamical parameters of the binding of opioid ligands to rat brain membranes in the presence or absence of NaCl or $MgCl_2$ at 24 °C.

	ΔG°' (kJ mol^{-1})	ΔH°' (kJ mol^{-1})	ΔS°' (kJ mol^{-1} K^{-1})
[^{3}H]NX			
control	-46.300	-35.408	36.67
Na^+	-51.187	-16.711	116.08
Mg^{2+}	-46.029	-44.965	3.58
[^{3}H]DHM			
control	-51.632	5.880	193.64
Na^+	-48.349	20.775	232.74
Mg^{2+}	-50.151	48.367	328.34
[^{3}H]DAMGO			
control	-49.484	6.999	190.17
Na^+	-48.423	11.118	200.47
Mg^{2+}	-49.879	29.051	265.75
[^{3}H]DT-B			
control	-47.781	13.387	205.95
Na^+	-46.992	37.568	284.77
Mg^{2+}	-48.250	68.615	393.48

See legend of TABLE 1. Thermodynamic parameters were calculated as in Methods.

measured our binding data just at 3 different temperatures transformation of these and the subsequent calculations resulted very similar parameters for [^{3}H]DHM and [^{3}H]NX as in Ref. (8), where six different temperatures were assayed. One interesting finding of the present work is that the same alterations of the binding energetics for the alkaloid μ agonist [^{3}H]DHM and the peptide μ agonist [^{3}H]DAMGO were detected, although the first ligand possesses a rigid, while the second one a much more flexible conformation. There are also experimental indications that the binding of the two ligands display some differences (23). Other data represent the ability of the δ opioid receptor to discriminate between opioid peptides and opiate alkaloids (24).

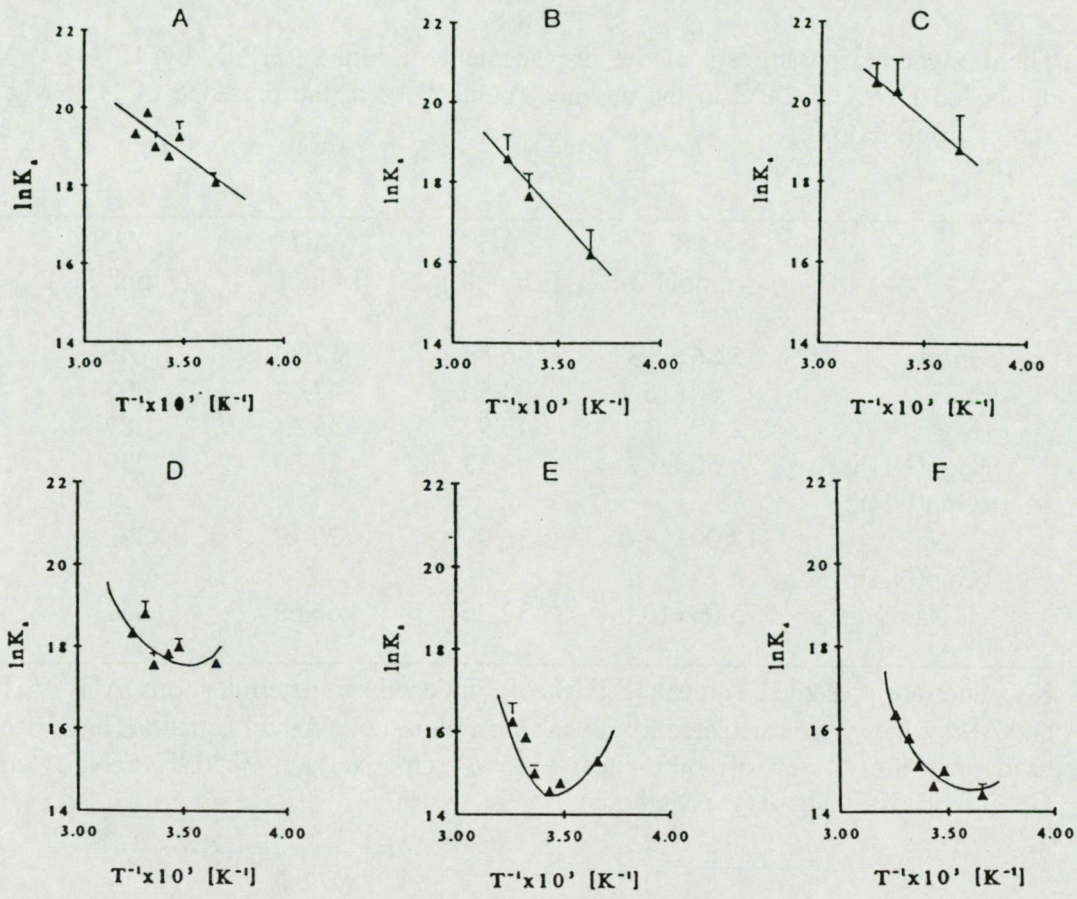

FIG. 3.
Van't Hoff plots of unlabelled DHM (10^{-10}-10^{-5} M) competition against 1 nM [^{3}H]NX with no addition (A), in presence of Na^+ (B), Mg^{2+} (C), Gpp(NH)p (D), Gpp(NH)p+Na^+ (E), or Gpp(NH)p+Na^++Mg^{2+} (F). Values shown are the mean±S.E.M. of lnK_a of at least 3 independent determinations at each temperature.

Another intriguing observation is that thermodynamically very similar behaviour was noted for μ ([^{3}H]DHM, [^{3}H]DAMGO) versus δ selective ligands ([^{3}H]DT-B) (Fig..4.) To our knowledge this is the first report dealing with the regulation and thermodynamic characterization of the binding of [^{3}H]deltorphin-B, which was described as one of the most specific and potent delta ligand by Búzás et al. (15). If enthalpy and entropy changes can be taken as indication of the mechanism, then our results suggest a substantial similarity for μ and δ binding.

TABLE 3.

Thermodynamic parameters of the displacement of 1 nM [^{3}H]NX by 10^{-5}-10^{-11} M unlabelled DHM at 24°C in the absence (control) or in the presence of Na^+, Mg^{2+} and/or Gpp(NH)p.

	K_D (nmol l^{-1})	$\Delta G°'$ (kJ mol^{-1})	$\Delta H°'$ (kJ mol^{-1})	$\Delta S°'$ (J $mol^{-1}K^{-1}$)
control	5.86± 1.5	-46.84	17.80	246
+Na^+	29.80±16.9	-43.56	47.68	307
+Mg^{2+}	2.47± 1.1	-49.97	35.94	287
+Gpp(NH)p	24.50± 6.7	-43.33	26.50	239
+Gpp(NH)p+ Na^+	313.00±59.0	-36.99	70.19	362
+Gpp(NH)p+ Na^++Mg^{2+}	275.00±10.0	-37.26	66.62	352

K_D values are presented as mean±S.E.M. of 3 independent determinations. $\Delta G°'$, $\Delta H°'$ and $\Delta S°'$ values were calculated from van't Hoff plots, but different equations have been used in the case of non-linear plots (Gpp(NH)p, Gpp(NH)p+Na^+ and Gpp(NH)p+Na^++Mg^{2+}) (see Methods).

Importance of charged residues in TM II, III and VI for different functions are being delineated for opioid receptors (23). Electrostatic interactions with complementary polar residues of the receptor would lead to negative or slightly positive values of $\Delta H°'$. While the former is true for [^{3}H]NX binding, the latter is the case for all the agonists tested (Fig. 2. and Table 2.). The distinct thermodynamic parameters detected for opioid agonist and antagonist ligands most probably reflect rather dissimilar binding mechanisms. Such clear distinction is not always possible for other receptors (25, 26), e.g. in the case of the D_2 dopamine receptors hydrophobicity of the ligand might be the main factor to determine the thermodynamic profile (27). The relatively small entropy increase and the opposite direction of the enthalpy changes (Fig. 2. and Table 2.) might indicate different thermodynamic forces of [^{3}H]NX binding compared to that of agonists. This assumption is supported by recent molecular engineering data (23) which show that mutation of the cloned rat μ opioid receptor at

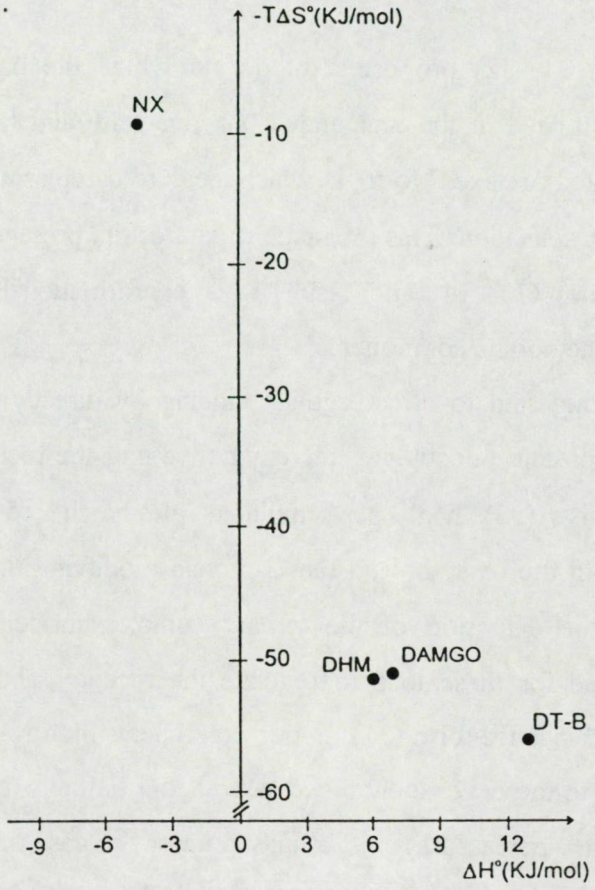

FIG.4.

Plot of the entropic term ($-T\Delta S^{o'}$) vs. the enthalpic term ($\Delta H^{o'}$) for the binding of opiate agonists (DHM, DAMGO, DT-B) and antagonist (NX). Data are shown at 0°C.

Asp^{114} in the putative second transmembrane domain reduced the binding of [^{3}H]DAMGO (agonist) to a much larger extent than that of [^{3}H]NX (antagonist).

The entropy increase observed might indicate hydrophobic interactions, and since it is present in all cases, it might be one of the first steps of the ligand binding, when ordered water molecules on the surface of the ligand and the receptors have to be removed (28, 29). Another source of the entropy increase might be a conformational change of the receptor upon ligand binding. Recent theories based on point mutation experiments of the cloned β_2-adrenergic receptor resulting in constitutively active

receptor mutants (30, 31, 32) propose a model, in which the native receptor is conformationally constrained in the unliganded, inactive state and hormone binding drives it into the activated, "relaxed" form, in which the third cytoplasmic loop becomes available for G-protein activation. This interaction requires the presence of Na^+, Mg^{2+} and guanine nucleotides (13, 14) and results in a conformational change and/or rearrangement of the membrane components.

Na^+ has been suggested to affect agonist binding by directly interacting with receptors to cause G-protein uncoupling, thereby converting the receptor into a low affinity state for agonists (33). Mg^{2+} plays multiple roles in the course of receptor function acting on both the receptor and the G-protein modifying their function and interaction (34). In the extension of the ternary complex model Samama et al. suggested a direct role for these ions to regulate the receptor ability to isomerise between the active and inactive form (31). In our experiments monovalent and divalent cations acted similarly to these, i.e. sodium decreased agonist affinity, but increased that of antagonist; and Mg^{2+} increased agonist affinity primarily in case of DT-B at higher temperatures (Table 1). Nevertheles, presence of Na^+ or Mg^{2+} results only in quantitative changes of $\Delta S^{o'}$ and $\Delta H^{o'}$ values.

The presence of Gpp(NH)p in heterologous displacement experiments (DHM competing with [^{3}H]NX) resulted in curvilinear van't Hoff plots (Fig.3.). We fitted the data without the ln K_a value obtained at the lowest temperature and this also gave better fit for polynomial than for linear regression. The simultaneous presence of Gpp(NH)p, Na^+ and Mg^{2+} increased the enthalpy more than 3 times compared to the control value (66.62 vs. 17.80 kJ mol^{-1}), and at least doubled compared to the presence of a single regulator (Table 3.). Entropy likewise increased from 246 to 352 J mol^{-1} K^{-1}. We suggest that these alterations are the consequences of the molecular rearrangement in the membrane due to uncoupling of the receptor-ligand complex from G-proteins which results in "free" protein molecules. This hypothesis is strengthened by the low affinity of agonist binding measured in this case (275 nM vs. 5.86 nmol l^{-1} of the control, Table 3), indicating the utilisation of the binding energy of receptor-ligand to

drive G-proteins to the open form, thus weakening the observed binding of the receptor for the ligand.

Physicochemical interpretations of thermodynamic parameters are more or less speculative for such a complex system. Some molecular events, however, can be postulated. It is becoming more and more feasible to validate these as our understanding of receptor structure-function increases due to recent molecular biology approaches.

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Receptor-mediated activation of G-proteins by κ opioid agonists in frog (*Rana esculenta*) brain membranes

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ABSTRACT: This study delineates the heterotrimeric guanine nucleotide binding regulatory protein (G-protein) types in frog (*Rana esculenta*) brain membranes and their activation by κ opioid agonists. Ethylketocyclazocine (EKC), trans-(±)-3,4-dichloro-*N*-methy-*N*-(2-[1-pyrrolidinyl]cyclohexyl)benzeneacetamide (U-50,488) and bremazocine displayed dose-dependent, norbinaltorphimine-reversible stimulation of guanosine-5′-O-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding in crude membrane preparations. G-proteins were identified by Western-blotting using previously characterized specific antisera that were generated against mammalian G-protein α-subunits and β-subunits. A photoreactive guanosine 5′-triphosphate (GTP) analog, [α-^{32}P]GTP azidoanilide ([α-^{32}P]AA-GTP) irreversibly labeled four proteins in the molecular weight range of 39–43 kDa. Ethylketocyclazocine and U-50,488 stimulated photolabelling of these proteins among which the 39 kDa band comigrated with the protein specifically labelled with the α_{i2} antibody and the 40 kDa band was identified as α_{o1}. The other two bands were also stained with the α_{common} antibody, but were not further identified. These results suggest that the endogenously expressed κ opioid receptors that are present in frog brain interact with multiple G-proteins *in situ*. Furthermore, the structure of most G-proteins seems to be well preserved during phylogenesis. © 1998 Elsevier Science Inc.

KEY WORDS: [^{35}S]GTPγS binding, Western-blotting, κ opioid agonist, Functional coupling.

INTRODUCTION

Many hormones and neurotransmitters transmit their signals to appropriate effector systems via heterotrimeric guanine nucleotide binding proteins (G-proteins) [20]. A body of evidence suggested, and recent molecular cloning experiments have further supported, that opioid receptors also function via G-proteins [16,26,44]. Therefore, it was demonstrated that μ, δ, and κ opioid binding sites were regulated by guanosine 5′-triphosphate (GTP) and its analogues [13]. The sensitivity of κ binding to guanine nucleotides was, however, somewhat lower than that of the μ and δ opioid receptors [8,31,59]. Opioids were shown to stimulate the high affinity GTPase that is an inherent property of the α-subunits of G-proteins or [^{35}S]guanosine 5′-O-(3-thiotriphosphate) ([^{35}S]GTPγS) binding [3,15,27,54,61] in several systems. Photoaffinity labelling of G-proteins was also enhanced by μ and δ opioid agonists [30,37]. The latter reports also provide strong support for the hypothesis that members of the G_i and G_o family are the mediators for opioid receptors. Activation of opioid receptors leads to inhibition of the enzyme adenylyl cyclase [17,27,33], Ca^{2+}-channels [2,53], or induce the opening of certain types of K^+-channels [1,14,36].

The signalling pathways for κ opioid receptors, especially for the still ill-determined hypothetical κ_1–κ_4 subtypes [65], are less well understood. Molecular cloning experiments so far have identified a single gene encoding κ receptor proteins from various sources [43,44]. When the cloned κ receptor was stably transfected in Chinese hamster ovary (CHO) cells it appeared to interact with multiple G-proteins [42], a phenomenon that was also noticed for recombinant μ and δ opioid receptors [12,41]. However, no data are available on the specificity of G-protein activation of endogenously expressed κ opioid receptors, partly due to the heterogeneity of opioid receptors in most mammalian tissues. However, frog brain is a rich source of the kappa opioid receptors [6,35,50,55] and were poposed to represent the κ_2 subtype [4–9,32]. Previous binding experiments indicated that κ and μ agonist binding in frog brain membranes are inhibited by guanine nucleotides [7,35,48], and therefore, implied that these receptors might also be working through heterotrimeric G-proteins.

The aim of the present study was to elucidate the interactions between frog brain opioid receptors and G-proteins. To this end, G-proteins of frog brain membranes were labelled with [^{35}S]GTPγS or photolabelled with [α-^{32}P]GTP azidoanilide ([α-^{32}P]AA-GTP), and their activation by opioid agonists was tested. The G-proteins were identified with specific antipeptide antibodies generated against known sequences of G-proteins in mammals. It was concluded that κ opioid agonists activate multiple G-proteins in frog brain membranes.

EXPERIMENTAL PROCEDURES

Materials and Methods

[α-^{32}P]nicotinamide adenine dinucleotide ([α-^{32}P]NAD; specific activity 800 Ci/mmol) was purchased from New England Nuclear, Boston, MA, USA; [^{35}S]GTPγS (37-42 TBq/mmol) was from Isotope Institute Ltd., (Budapest, Hungary); ethylketocylazo-

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cine (EKC) and bremazocine was provided by Sterling Winthrop Research Institute, Reninselaer, NY, USA, U-50,488 was from Research Biochemicals International (Natick, MA, USA); adenosine 5'-triphosphate (ATP), guanosine 5'-diphosphate (GDP), GTP, GTPγS, bacitracin, DL-dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulphonyl fluoride (PMSF), thymidine, and pertussis toxin (PTX) were products of Sigma Chemicals, St. Louis, MO, USA; sucrose was from Boehringer (Mannheim, Germany); trasylol (Gordox, aprotinin) was purchased from Gedeon Richter Pharmaceutical Company (Budapest, Hungary); and norbinaltorphimine was from Alkaloida Chemical Company Ltd (Tiszavasvári, Hungary). Sources of other materials have been cited [38,47].

Membrane Preparation

Frog brain membranes were prepared as described [55]. Adequate measures were taken to minimize pain or discomfort of the experimental animals. Briefly, whole brains were homogenized in 50 mM Tris-HCl buffer (pH 7.4), containing 1 mM EDTA, 0.1 mM PMSF, 10 mg/ml bacitracin, and 40 kIU aprotinin. The homogenate was centrifuged (20 min, 25,000 × *g*, 4°C). The resulting pellet was resuspended in the same buffer containing 0.32 M sucrose and stored at −70°C. Protein concentrations were measured with the method of Bradford [10], using bovine serum albumin as a standard.

[^{35}S]GTPγS Binding Assay

Frozen membranes (≈ 10 μg of protein) were thawed and washed free of sucrose by centrifugation and suspension in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EGTA (ethyleneglycol-bis(B-aminoethyl ether)-N,N,N',N'-tetraacetic acid) and 3 mM $MgCl_2$. Incubation of membrane proteins was performed with 0.05 nM [^{35}S]GTPγS and increasing concentrations (10^{-9} – 10^{-5} M) of opioid ligands in the presence of 100 μM GDP in a total volume of 1 ml for 60 min at 30°C according to Sim et al. [54] and Traynor et al. [61]. Nonspecific binding was determined with 10 μM GTPγS and subtracted. Bound and free [^{35}S]GTPγS were separated by vacuum filtration through Whatman GF/B filters with a Millipore manifold. Filters were washed with 3 × 5 ml ice-cold buffer, and radioactivity of the dried filters was detected in a toluene based scintillation cocktail in a Searle liquid scintillation counter.

Photolabelling of G-Protein α Subunits

[α-^{32}P]GTP azidoanilide [α-^{32}P]AA-GTP was synthesized and photolabelling was performed according to Offermanns et al. [38,39]. The final assay volume was 60 μl in an incubation buffer of the following composition (final concentrations in brackets): HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (30 mM, pH 7.4); EGTA (0.1 mM); benzamidine (1 mM); $MgCl_2$ (5 mM); NaCl (20 mM); GDP (100 μM). For photolabelling of G-proteins, membranes were thawed, centrifuged, and resuspended in two-fold concentrated incubation buffer. For each sample, 50 μg protein in 30 μl volume was pipetted into an Eppendorf tube. In order to study the potency of opioid receptor agonists to modulate [α-^{32}P]AA-GTP binding to G-proteins, samples were preincubated with 10 μl of EKC or U-50,488 in a final concentration of 10 μM or water for 3 min at 30°C, followed by the addition of [α-^{32}P]AA-GTP (approximately 0.4 mCi/tube in 20 μl of water) and further incubated at 30°C. After 3 min, samples were transferred to ice to stop the reaction, and all consequent steps were carried out at 4°C. Membranes were spun down in a benchtop centrifuge for 5 min and the supernatant, which contains the excess radiolabelled substrate, was removed. The pellet was then resuspended in 60 μl of GDP-free incubation buffer including 2 mM DTT to reduce nonspecific labelling. The samples were pipetted onto a Parafilm-covered metal plate in a refrigerator and irradiated as drops for 10 s, using a 150 W UV lamp (λ = 254 nm) that was placed at a constant distance of 3 cm above the plate. Upon photoactivation, samples were recollected into the tubes and pelleted by another centrifugation step. The supernatants were removed and 35 μl of electrophoresis sample buffer was added to each pellet. Samples were vortexed thoroughly and kept at a temperature of −80°C until they were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Adenosine 5'-Diphosphate (ADP)-Ribosylation

The protocol for adenosine 5'-diphosphate (ADP) ribosylation of membrane proteins was developed by Ribeiro-Neto et al. [46]. Pertussis toxin was preactivated by incubation with 62.5 mM DTT at 37°C. Per sample, approximately 180 μg membrane protein was subjected to ADP-ribosylation with 1 × 10^7 dpm [α-^{32}P] nicotinamide adenine dinucleotide (NAD) and 60 μM unlabelled NAD.

Electrophoresis and Immunoblotting

Pertussis toxin or photolabelled samples as well as unlabelled, acetone-precipitated membrane proteins were analyzed by SDS-PAGE [28] and immunoblotting according to Rosenthal et al. [47], with the following modifications. If not indicated otherwise, a 6 M urea, 9% acrylamide, 1.5 mm thick, and 11 cm long gel was employed as a separating gel to achieve a sufficient electrophoretic resolution. Pharmacia Low Molecular Weight Markers (#17-0446-01) were used as standards. After electrophoresis, the separating gels were either immunoblotted or stained with 1% Coomassie blue G-250. Stained gels were dried and exposed to X-ray films, starting with a sensitive Kodak X-OMAT AR for one night, followed by a 3M R and CTR7 for 4 days and 2 weeks, respectively. Prior to immunostaining with various antibodies against G-protein subunits, blots of [α-^{32}P]AA-GTP labelled samples were autoradiographed. For the visualization of bound antibodies, a goat antirabbit antibody, coupled to horseradish-peroxidase (Sigma, Deisenhofen, Germany; #A 6154), was used in a dilution of 1:1000, with the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham, Braunschweig, Germany).

The anti-G-protein peptide antibodies used were α_{common}-antiserum AS8 [23], α_s-antiserum AS348 [56], $\alpha_{i\ common}$-antiserum AS266 [52], α_{i2}-antiserum AS269 [52], $\alpha_{o\ common}$-antiserum AS6 [23], α_{o1}-antiserum AS248 [58], α_{o2}-antiserum AS201 [57], β_{common}-antiserum AS11 [24], β_1-antiserum AS28 [24], and β_2-antiserum AS36 [24]. In some experiments, anti-α_o antibodies donated by Prof. J. Bockaert [24] were also tested and gave similar results.

RESULTS

In order to assess the functional coupling of frog brain opioid receptors to G-proteins, the ability of opioid agonists to activate G-proteins was measured with [^{35}S]GTPγS binding assay. Ethylketocylazacine, a prototypic κ opiate ligand, which however, also binds to μ and δ sites at least in rat brain [19], was the most effective among the ligands tested achieving about 60% stimulation over the basal activity at 10 μM concentration (Fig. 1). Bremazocine, another κ-preferring ligand with crossreactivity to μ and δ sites at high concentrations, was less efficient in activating G-proteins. The κ_1 selective U-50,488 [63] displayed very similar activation to the latter; the maximal stimulation was 20%–30% at the highest concentrations tested (Fig. 1). When the κ antagonist norbinaltorphimine at 10 μM was also included, it completely

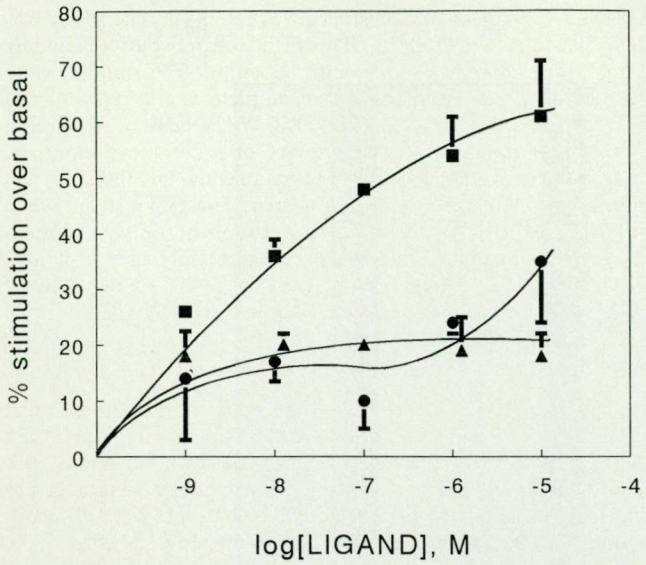

FIG. 1. Effect of opioid agonists on [^{35}S]GTPγS binding in frog brain membranes. Proteins were incubated with 0.05 nM [^{35}S]GTPγS and 10 μM GDP in Tris-EGTA pH 7.4 buffer in the absence (basal activity), or in the presence of various concentrations of EKC (■), U-50,488 (●), or bremazocine (▲). Basal binding was 84.5 ± 7 fmol/mg protein. Data are mean ± S.E.M. of two to six experiments each performed in triplicate.

blocked the stimulating effect of all three ligands tested at 1 μM, implying that their effect is due to activation of κ opioid receptors (data not shown).

Experiments were also performed to describe the G-protein composition of frog brain membranes. To this end, proteins were separated by SDS-PAGE and blotted onto nitrocellulose filter membranes. The blots were then incubated with antibodies against various G-protein α- and β-subunits, and the bound antibodies were visualized by ECL, a sensitive chemiluminescence detection system (Fig. 2). The α_{common} antibody strongly reacted with proteins of about 39–41 and 44–45 kDa molecular weights. The $\alpha_{i\ common}$ antibody weakly stained a band at ≈ 44 kDa, but strongly a band at approximately 39 kDa that also reacted with the α_{i2} antibody. This band disappeared when the blot was incubated with the α_{i2} antiserum that was preincubated with an excess of the peptide that was used to generate the antibody, therefore, demonstrating the specificity of the labelling. The α_s antibody gave a strong signal at 43 kDa that disappeared when the antibody was preincubated with α_s peptide. The affinity-purified $\alpha_{o\ common}$ and α_{o1} antibodies both strongly reacted with a protein of ≈ 40 kDa. Immunoblots were also tested for β-subunits of G-proteins. The β_{common} antibody immunoreacted with a doublet of bands of about 35 kDa that resembled very much those found with the β_1 antibody (Fig. 2).

In another set of experiments, attempts were made to identify those G-proteins that were capable of interacting with kappa opioid receptors of frog brain membranes. The α-subunits of

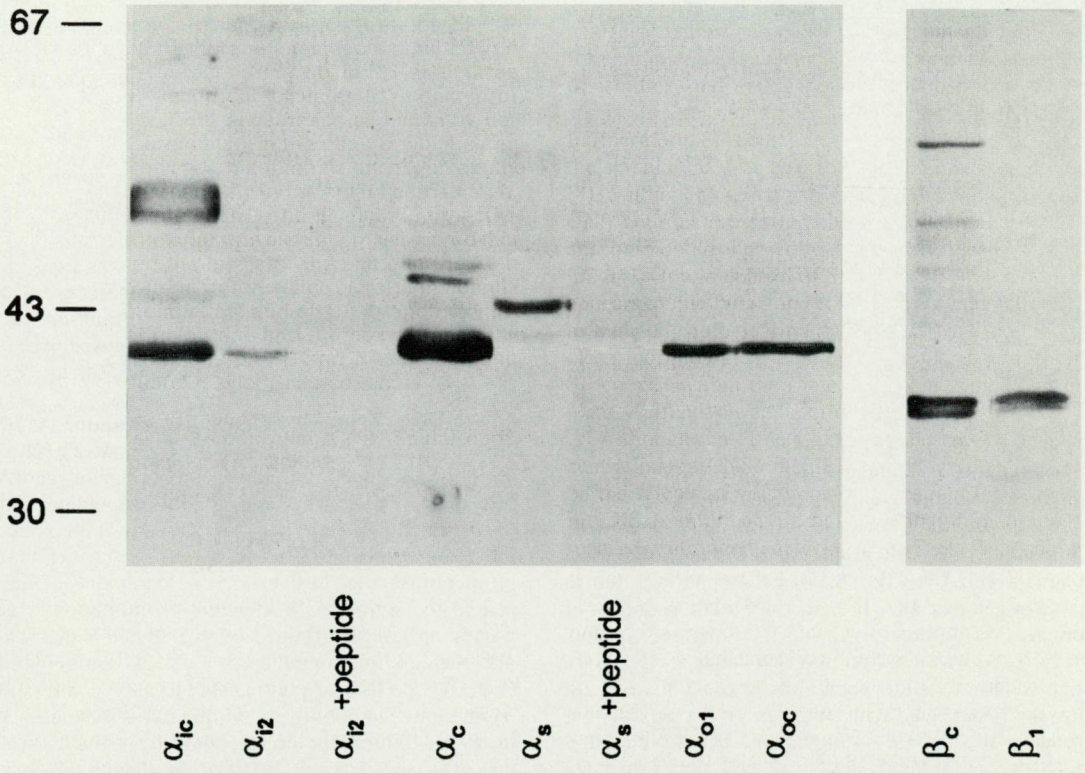

FIG. 2. Immunoblots of G-proteins in frog brain membranes. Proteins were separated on a 10% acrylamide SDS-gel, then blotted, and subsequently stained using G-protein subunit-specific antibodies, as described under "Experimental Procedures." The G-protein peptide antibodies used were α_c: α_{common}-antibody, α_s: α_s-antibody, α_{ic}: $\alpha_{i\ common}$-antibody, α_{i2}: α_{i2}-antibody, α_{oc}: $\alpha_{o\ common}$-antibody, α_{o1}: α_{o1}-antibody, β_c: β_{common}-antibody, and β_1: β_1-antibody. "+peptide" designates the presence of an excess of the peptide to which the particular antibody was raised. Values on the left indicate the migration of molecular weight markers. This is a representative experiment out of three to five others giving similar results.

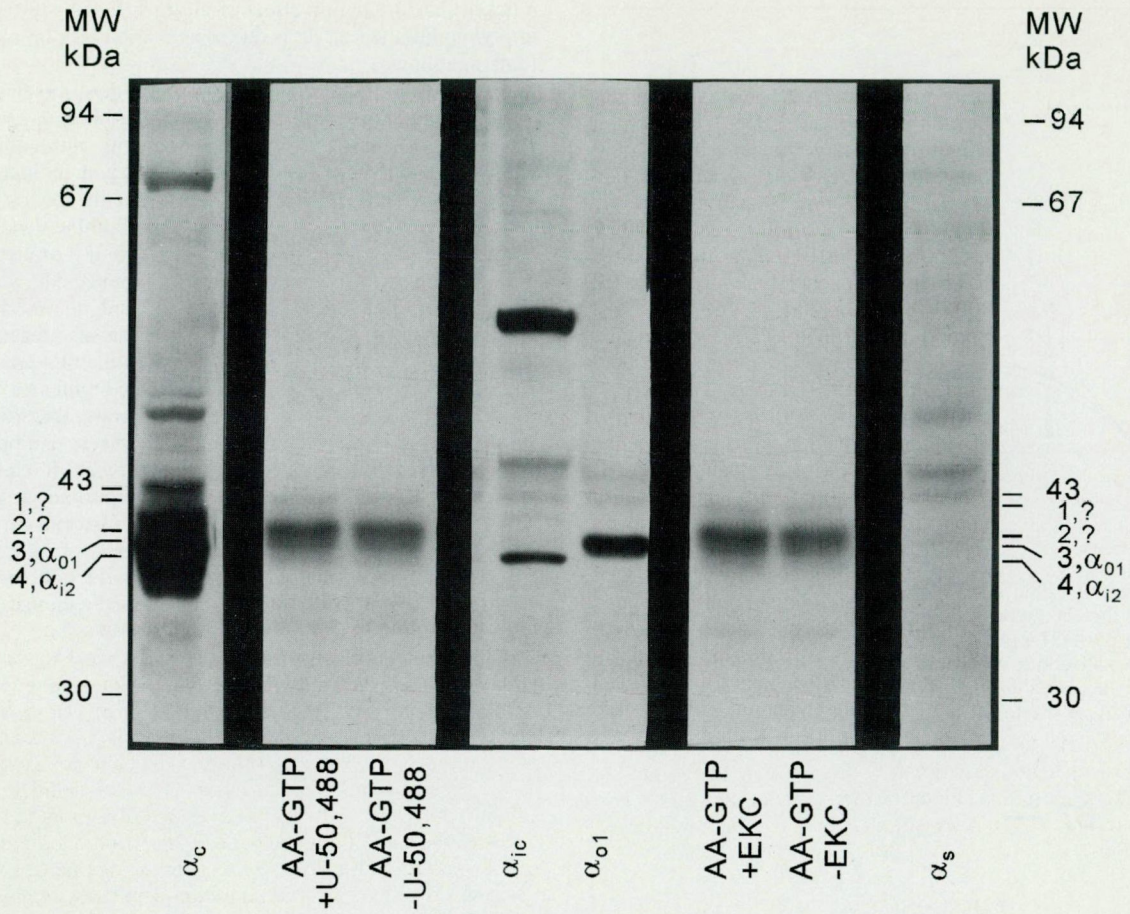

FIG. 3. Photoaffinity labelling and subsequent immunoblotting of G-proteins in frog brain membranes. G-protein α subunits of frog brain membranes (50 μg/sample) were photolabelled with [α-^{32}P]AA-GTP, separated on a 6 M urea, 9% acrylamide SDS-gel, blotted and visualized by autoradiography followed by Western-blotting of the same immunoblot with G-protein antisera. The autoradiography presented in lanes 2–3, and 6–7 show photoaffinity labelling in the presence (+) and in the absence (−) of 10 μM U-50,488 or EKC, respectively. Lanes 1, 4, 5, and 8 display the subsequent immunoblots with α_c: α_{common}-antibody, α_{ic}: $\alpha_{i\ common}$-antibody, or α_{o1}: α_{o1}-antibody, α_s: α_s-antibody. Also shown are the molecular weights of marker proteins in kDa on both sides of the figure. This is a representative experiment out of four others giving similar results.

G-proteins were photolabelled with [α-^{32}P]AA-GTP, and subsequently separated by SDS-PAGE containing urea. Proteins were blotted onto nitrocellulose membranes and subjected to autoradiography followed by staining with antibodies specific for G_α subunits (Fig. 3, lanes 1, 4, 5, 8). Urea was included because it improves the resolution of closely migrating G-protein subunits in SDS-polyacrylamide gels [51,45]. This effect is possibly due to conformational changes that alter the electrophoretic mobility of G-protein subtypes to a different extent [52]. Compared to blots without urea (Fig. 2) α_s was resolved into two bands at 43 and 47 kDa (Fig. 3) that were not visible when blots were incubated with antiserum that was preincubated with an excess of the peptide that was used to generate the antibody (not shown). The protein band stained by the $\alpha_{o\ common}$ antibody in Fig. 2 was not further resolved and, interestingly, mobility of the β subunits were dramatically reduced on urea containing gels without improved separation (not shown). As shown in Fig. 3, [α-^{32}P]AA-GTP was photoincorporated into multiple bands in the molecular weight range of 39–42 kDa; a bulky major one that could be separated into a doublet (2, 3) under careful titration of the exposition time, a faint band (1) above, and another weak one (4) under the main band (Fig. 3, lanes 2–3, 6–7). This pattern of photolabelling resembled the picture obtained by PTX catalyzed ADP-ribosylation (data not shown), and also corresponded with appropriate bands stained with the α_{common} antibody (Fig. 3, lane 1). Whereas the upper two bands labelled by [α-^{32}P]AA-GTP (1, 2) could not be identified, the third band (the lower one of the doublet) appeared to migrate with identical electrophoretic mobility as the protein specifically stained by α_{o1}. Band 4, that was very faintly photolabelled, comigrated with the α_{i2} antibody stained protein (Fig. 3). The lack of photolabelled bands comigrating with the G α_s immunoreactivity is not unique to our system. It was published in several tissues that the amount of α_s is much lower compared to that of α_i and α_o, and visualization of such labelled bands would require much longer exposition time, or alternatively, higher specific activity of [α-^{32}P]AA-GTP [38].

Activation of G-proteins by opioid agonists was assayed in the presence of 100 μM GDP, which suppressed the basal photolabelling to a greater extent than photolabelling of receptor activated G-proteins. Densitometric analysis of the data shown in Fig. 3

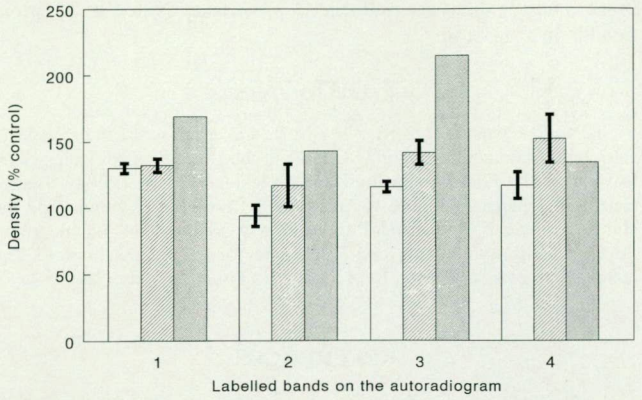

FIG. 4. Densitometric analysis of photoaffinity labelling by [α-^{32}]AA-GTP. Lanes 2–3 and 6–7 of Fig. 3, and four similar experiments were scanned by laser densitometry. The values shown represent mean ± S.E.M. of those calculation except with morphine when a single determination was performed. Lane 1, 2, 3, and 4 are the appropriate bands designated as in Fig. 3. The effect of EKC is shown by open bar, U-50,488 by hatched bar, and morphine by dotted columns.

revealed that EKC and U-50,488 slightly but significantly stimulated the photoincorporation into band 1 (the identity of which is not known), band 3 (α_{o1}), and band 4 (α_{i2}). There was no statistically significant stimulation by either EKC or U-50,488 into band 2 (n = 3–5 independent experiments). It should be noted that the μ opioid agonist morphine elicited higher stimulation than the two κ agonists tested, and it was significant at all the four bands separated (Fig. 4).

DISCUSSION

This study characterizes the G-proteins present in frog brain membranes, and furthermore, their activation by κ opioid agonists. In order to typify the G-proteins we utilized antibodies raised against peptides with either common or subtype-specific sequences of given G-protein subunits deduced from mammalian G-protein sequences as published previously [23,24,52,56–58], since no sequence data of G-proteins in *Rana esculenta* have been available. Therefore, the reactivity and subtype selectivity of the peptide antibodies had to be examined in frog. The immunolabelling pattern with α_{common} and α_o were compared in rat and frog brain tissues in preliminary experiments. It was concluded that these antisera were able to recognize appropriate G-protein subunits in frog brain with identical or slightly different molecular weights than in rat brain membranes (data not shown). Published data concerning the G-protein composition in amphibian tissues such as *Xenopus laevis* oocytes [40], frog skeletal muscle [11], rod photoreceptors [62], and even in neuronal tissues of different vertebrates including frogs [18,25,60] have indicated a high degree of homology of the G-proteins of these tissues with their mammalian counterparts.

The G-protein composition of frog brain membranes was further elucidated by using more specific antisera (Figs. 2 and 3). Our conclusions concerning the identity of a given immunostained protein band were based on: (1) its comigration with proteins that were reactive to other peptide antibodies in the absence and in the presence of 6 M urea in the separating gels; and that its mobility was in accordance with the molecular range labelled (2) by [α-^{32}P]AA-GTP; or (3) PTX-sensitive proteins. (This latter requirement obviously was not applicable to proteins immunoreactive to α_s or β antibodies.)

On the basis of our results one might speculate that β-subunits emerge as two different isoforms of β_1 (Fig. 2), as the comigration of β_1-immunoreactive and β_{common}-immunoreactive proteins and the absence of β_2-reactive material (not shown) would suggest. The two G_s α forms shown by peptide-reversible anti-α_s immunostaining after running on a urea-containing gel were also reactive to the α_{common} antibody (Figs. 2 and 3) that recognized α_i, α_o, α_s, α_t, and to a lesser extent α_z in mammalian cells [52]. Testing for α_i subunits led to the detection of an α_{i2}-like protein of about 39 kDa that was immunostained with the α_{i2} affinity purified antibody and also coincided with the appropriate anti-$\alpha_{i\ common}$ reactive band. The $\alpha_{i\ common}$ peptide antibody that recognized the α_{i1}, α_{i2}, and α_{i3} proteins [52] did not however, show immunolabelling in the region where α_{i1} would be expected under the experimental conditions in frog brain (Figs. 2 and 3). This result was confirmed with an antibody specific for α_{i1} that also showed no labelling in frog (data not shown). The other, higher molecular weight band stained by the $\alpha_{i\ common}$ antibody (Figs. 2 and 3) might correspond to α_{i3}, which protein was also detected in frog skeletal muscle [11]. The affinity-purified α_{o1} recognized a protein band at 40 kDa that was also seen with the $\alpha_{o\ common}$ antiserum (Figs. 2 and 3). An antibody specific for α_{o2} showed no detectable signal (data not shown). These observations suggest that there is only one type of G_o protein in frog brain, and that G_{o1} seems to play a role in κ opioid receptor signalling in frog brain (to be elaborated on later).

Evaluation of agonists stimulation of [^{35}S]GTPγS binding offers an opportunity to study the direct coupling of a receptor to the activation of G-proteins regardless of the types of G-proteins and effector systems involved. In this respect it is similar to agonist stimulation of the high K_m GTPase activity. Evidence was provided for the coupling of kappa, besides μ and δ, opioid receptors to rat brain GTPase [15]. In frog brain membranes, κ opioid ligands resulted in a concentration-dependent stimulation of [^{35}S]GTPγS binding (Fig. 1), which was fully inhibited by the κ-specific antagonist norbinaltorphimine (data not shown). The rank order of potency was EKC > U-50,488 ≈ bremazocine. The EC_{50} values defined as the concentration of the ligand producing 50% of the maximal response was in the nanomolar range for all three agonists tested (Fig. 1), which agrees well with the equilibrium dissociation constants of these ligands obtained in receptor binding experiments [5–9,35,50]. While U-50,488 is considered to be a selective ligand for κ_1 opioid receptors [63] that represent only 20%–30% of the kappa receptor pool in frog brain [6,7], the benzomorphan EKC and bremazocine bind to both κ_1 and κ_2 subtypes [65] besides their well-documented crossreactivity with μ and δ sites [19]. Although the potency of the latter two ligands is similar in most mammalian tissues, this is not necessarily the case in frog brain. Previous experiments revealed unique characteristics of opioid sites of frog brain, among them the antagonistic like binding pattern of these two ligands *in vitro* [5,48,66], and the ability of EKC to antagonize morphine-induced antinociception *in vivo* [4]. The observation that EKC was more potent than bremazocine in stimulating [^{35}S]GTPγS binding will require future work, nevertheless it agrees with recent results of Benyhe et al. [9], who studied the binding of [^{3}H] Met-enkephalin-Arg^6-Phe^7, the proposed endogenous ligand of the κ_2 receptors [65]. In their study bremazocine and EKC displaced about 50% and 80% of the specific binding of the radioligand in frog brain membranes, respectively [9]. We have also evaluated the potency of Met-enkephalin-Arg^6-Phe^7 in [^{35}S]GTPγS binding. This full agonist of the κ_2 sites displayed about 120% stimulation over the basal activity at 1 μM concentration (results to be published). The main conclusion that can be drawn from [^{35}S]GTPγS binding experiments is that kappa receptors including κ_1 and κ_2 subtypes do

interact with G-proteins in frog brain. This observation is also supported by previous ligand binding experiments where the binding of [^{3}H]EKC, [^{3}H]dihydromorphine, [^{3}H]etorphine, and [^{3}H]U-50,488 was shown to be regulated by guanine nucleotides [7,35,48].

Photolabelling of G-protein α subunits by [α-^{32}P][α-^{32}]AA-GTP in the absence and presence of opioid agonists provided another means to assay the functional coupling of opioid receptors to G-proteins *in situ* (i.e., in the native frog brain membranes). As shown on Fig. 3, [α-^{32}P]AA-GTP was photoincorporated into multiple bands in the molecular weight range of 39–42 kDa. The 39 kDa band that showed very faint labelling comigrated with the protein specifically labelled with the α_{i2} antibody. The 40 kDa band was identified as α_{o1}. The other two bands were also stained with the α_{common} antibody, but were not further identified. Photoincorporation of the label was slightly but significantly stimulated by EKC and U-50,488 into three bands, including proteins identified as α_{o1}, α_{i2}, and the unknown α-subunit with higher molecular weight. The ligands were applied in 10 μM in this assay to achieve maximal stimulation. Therefore, their potency at distinct α-subunits was not evaluated. However U-50,4888 elicited higher increase than EKC of photolabelling α-subunits of 39–42 kDa (Fig. 4), while the latter was more potent in stimulating [^{35}S]GTPγS binding (Fig. 1). This discrepancy can be explained by the difference in the assay conditions of the two techniques. Labelling of [α-^{32}P]AA-GTP was performed in the presence of Na^+ and GDP, which both decrease the affinity of opioid agonists, and increase that of opioid antagonists [13]. As discussed above and demonstrated before, EKC displays antagonistic profiles, while U-50,488 is a full agonist in frog brains [4,5,48]. Based on the observation that the kappa ligands enhanced photolabelling of multiple proteins by [α-^{32}P]AA-GTP, it seems likely that multiple types of G-proteins are able to interact with κ sites in frog brain (Fig. 3). Likewise, this was shown to be the case for the cloned rat κ receptors [29,42]. Prather et al. found that G_{i2}, G_{i3}, and a not identified G-protein were able to interact with recombinant κ_1 receptors expressed in Chinese hamster ovary (CHO) cells [42]. Similar results were obtained for the δ opioid receptors by Offermanns et al in N × G 108-15 cells [37]. Laugwitz et al. [30] found in human neuroblastoma SH-SY5Y cells that [D-Ala^2, MePhe^4, gly-ol^5] enkephalin (DAMGO), a μ specific peptide ligand preferentially stimulated photolabelling of α_{i3}, but [D-Pen^2, D-Pen^5]enkephalin (DPDPE) (δ agonist) activated G_{i1}. In contrast, both opioids equally facilitated the incorporation of radioactivity into α_{i2}. Therefore, multiple types of G-proteins might seem to couple to all three opioid receptors.

The complexity of the coupling of opioid receptors to effector systems via G-proteins requires a broad consideration when specific physiological responses elicited by selective opioid agonists are examined. Adenylyl cyclase is inhibited by G_{i2} following opioid activation in N × G 108-15 cells [33]. The G_o protein has been shown to inhibit the voltage-dependent Ca^{2+} channel [21,22,34,49]. Weisskopf et al. related modulation of long-term potentiation by dynorphin, the proposed endogenous ligand for κ opioid receptors, to inhibitory action on N-type Ca^{2+} channels in hippocampal mossy fibres [64].

The physiological function of κ opioid receptor activation in frog brain is not known yet. There is some evidence that it might mediate analgesia [4] like kappa sites in mammals [63]. Since frog brain is a rich source of this receptor type, possibly even the κ_2-subtype, it is a convenient source to elucidate the molecular components participating in its signalling. Results of the present work suggest that (1) the structure of most G-protein types is well preserved during phylogenesis; (2) that κ opioid receptors and G-proteins interact *in situ* (i.e., within the native plasma membrane; and (3) there are multiple G-proteins activated by κ opioid ligands in frog brain.

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Expression of G-protein subtypes in cultured cerebral endothelial cells

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Abstract

This paper describes Western-blotting evidence for the presence of various guanine nucleotide binding proteins, G-proteins in cultured rat cerebral endothelial cells (CECs) and two immortalized cerebral endothelial cell lines, RBE4 and GP8. By using specific antibodies raised against known sequences of appropriate G-protein types that were previously characterized, we demonstrated the presence of $G_s\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, $G_{q/11}\alpha$, $G_o\alpha$ and $G\beta$ in cell lysates of primary cultures of CECs, and plasmamembranes of RBE4 and GP8 cells. The appearance of $G_o\alpha$ proteins in CECs might be of special importance, since they were not detected in peripheral endothelial cells in previous studies. Isoproterenol and bradykinin displayed significant, dose-dependent stimulation of [^{35}S]GTPγS binding above basal values. This assay, reflecting the GDP-GTP exchange reaction on Gα-subunits by receptor agonists, suggested that there were functional, G-protein coupled β-adrenergic and bradykinin receptors in these systems. No significant stimulation of [^{35}S]GTPγS binding was noted with serotonin under our experimental conditions. Since stimulation of [^{35}S]GTPγS binding by isoproterenol and bradykinin was additive, it was concluded that different Gα proteins were activated by these two ligands. In analogy to other systems, activation of G_s is most likely by isoproterenol, while G_i and/or Gq/11 proteins might be activated by bradykinin receptors. The possible significance of the receptors and G-proteins detected is being discussed in the functioning of cerebral endothelium, and thus the blood-brain barrier.

Key words: Cerebral endothelial cells; G-proteins; Bradykinin; β-adrenergic receptors; [^{35}S]GTPγS binding

1. Introduction

By forming the morphological substrate of the blood-brain barrier (BBB), cerebral endothelial cells (CECs) play a crucial role in transporting various substances between the blood and brain, and in maintaining the homeostasis of the central nervous system (CNS). In order to accomplish this complex task, CECs should be equipped with specific receptors and proper signal transduction pathways. Among others, β-adrenergic (Durieu–Trautmann et al., 1991), bradykinin (Homayoun and Harik, 1991; Revest et al., 1991), histamine type H_1 and H_2 (Karnushina et al., 1980; Spatz et al., 1989), P_2 purino (Revest et al., 1991; Nobles et al., 1995), dopamine (Bacic et al., 1991), endothelin (Vigne et al., 1990; Stanimirovic et al., 1994), V_1 vasopressin (Hess et al., 1991), atrial natriuretic peptide (Smith et al., 1988) receptors have been demonstrated in brain endothelium.

In recent years considerable progress has also been made in the identification of the effector molecules that are present in CECs including adenylyl cyclase (Joó et al., 1975), protein kinase C (PKC) family members (Krizbai et al., 1995), calcium/calmodulin-stimulated protein kinase II (CaM-PK II) (Deli et al., 1993), nitric oxide synthase (NOS) (Durieu–Trautmann et al., 1993), phospholipase A_2 (Paglan et al., 1993). However, little is known about the pathways leading to the activation of these effectors. One group of the membrane receptors exerts its intracellular effects via coupling to heterotrimeric G-proteins that, when activated, will act to modulate different intracellular signalling molecules and

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Abbreviations—BBB, blood-brain barrier; CECs, cerebral endothelial cells; CNS, central nervous system; PLC, phospholipase C; 7TM receptors, 7 transmembrane domain containing receptors; G-protein, heterotrimeric guanine nucleotide binding regulatory protein; GTPγS, guanosine-5′-O-(3-thio)triphosphate; GDP, guanosine 5′-diphosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamid gelelectrophoresis.

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enzymes (Offermanns and Schultz, 1994; Gudermann et al., 1996). G-proteins form a permanently growing family of proteins composed of three subunits, α, β and γ of which at least 23 α, 6 different β and 11 γ isoforms are known to date (for recent reviews see Helmreich and Hofmann, 1996; Kalkbrenner et al., 1996). The α subunits can be further classified into subfamilies of which the G_s and G_i types regulate, above all but not exclusively, the enzyme adenylyl cyclase. $G_{q/11}$ types in most cases are linked to phospholipase C (PLC), whereas G_o types are believed to couple to ion channels.

Owing to their strategic role in signal transduction, the expression and role of different G-proteins have been investigated in various types of endothelial cells in a large number of studies (Gil-Longo et al., 1993; Day et al., 1995; Liao and Homey, 1993a and 1993b). It has been shown for example that bovine aortic endothelial cells express a complex set of G-proteins such as $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, $G_{q/11}\alpha$, $G_s\alpha$, but no $G_o\alpha$ (Gil-Longo et al., 1993). The aim of our work was to describe the presence of various types of G-proteins in CECs, a highly specialized endothelium, and to find possible ways of their activation by diverse receptor agonists.

2. Experimental procedures

2.1. Primary cultures of rat CECs

Cultures were prepared as described in details earlier (Deli et al., 1997), from 2-week-old rat brains. All CFY rats used for the primary cultures of CECs were acquired from the local animal house and were cared for in accordance with NIH guidelines. Animal experiments were approved by the ethical committee of the Biological Research Center, Szeged. Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% plasma derived bovine serum (PDS), 2.0 mM glutamine and antibiotics at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. To further purify cultures of CECs, a selective cytolysis of contaminating cells by specific anti-Thy 1.1 antibody and complement was performed (Risau et al., 1990). After 7 days in vitro, endothelial cells developed continuous monolayers which were used for the experiments. The cultures were regularly tested for Factor VIII-related antigen, and over 98% of the cells expressed positivity.

2.2. Endothelial cell lines

GP8 cells (Greenwood et al., 1996) were cultured in DMEM supplemented with 20% PDS, 1 ng/ml basic fibroblast growth factor (bFGF) and 300 μg/ml G418 and used between passages 15–20. RBE4 cells (Roux et al., 1994) were cultured in DMEM plus 10% fetal calf serum, 1 ng/ml bFGF and 300 μg/ml G418 and used between passages 32–47. Both cell lines expressed general endothelial as well as specific cerebral endothelial features as published (Greenwood et al., 1996; Roux et al., 1994).

2.3. Cell membrane preparation

Cell cultures were washed twice with phosphate buffered saline (PBS); the monolayers were detached from the plastic by tissue scraper and suspended in PBS (pH 7.4). Samples were sedimented for 10 min at $3000 \times g$ (4°C) and the pellets were then suspended in 10 ml lysis buffer (5 mM Tris-HCl, 50 μM $CaCl_2$, 0.5 mM dithiothreitol, and 0.1 mM phenylmethyl-sulfonyl fluoride, pH 8.1) followed by homogenization in a glass teflon potter at 4°C. The homogenate was centrifuged for 5 min at $3000 \times g$ and the resulting supernatant spun at $20{,}000 \times g$ for 30 min. Membrane pellets obtained were suspended in 50 mM Tris-HCl (pH 7.4) to yield about 0.4–1 mg protein/ml, and either freshly used for [^{35}S]GTPγS studies or kept frozen at −70°C until used for immunoblotting. Protein content was determined with bovine serum albumin as a standard according to Bradford (Bradford, 1976).

2.4. Gel electrophoresis and immunoblotting

SDS-PAGE was performed according to Laemmli (Laemmli, 1970) with modifications. Membrane samples of RBE4 and GP8 cells were delipidated in methanol:chloroform:water mixture (4:1:3), spun down in an Eppendorf centrifuge, washed with methanol and the pellet resuspended in sample buffer. Cells from the primary culture were lysed in sample buffer, sonicated for 1 min and boiled for 5 min. Equivalent amounts of proteins (30–80 μg) were loaded onto a 16 cm long 10% slab gel containing high purity, deionized urea (6 M), which improved the resolution of Gα and Gβ subunits with molecular weights close to each other (Ribeiro-Neto and Rodbell, 1989; Hinsch et al., 1989). Electrophoresis was performed at room temperature at a constant voltage of 150 V for 6 h. Proteins resolved by SDS-PAGE were transferred onto nitrocellulose membranes at a constant current of 200 mA for 1 h in a three buffer component semi-dry system (Kyhse-Andersen, 1984). Transfer buffer 1 contained 0.3 M TRIS-HCl, 20% methanol; transfer buffer 2 and 3 consisted of 25 mM TRIS-HCl, 20% methanol; and 25 mM TRIS-HCl, 20% methanol, 40 mM ε-amino-caproic acid, respectively. Nitrocellulose membranes were stained with amido-black for evaluation of the protein loading and transfer, blocked for 1 h in 3% ovalbumin in TRIS-buffered saline (TBS), pH 7.4. Thereafter, filters were cut into stripes and incubated for 1 h with various anti-G-protein antisera which were diluted in TBS (pH 7.4) supplemented with 1% (w/v) BSA (fr. V., protease free) and sodium azide. After washing 4 times with TBS supplemented with 0.05% (v/v) Tween-

20 (TBS/Tween) for 15 min filters were blocked again as above and subsequently incubated with the second antibody (goat anti-rabbit IgG, peroxidase conjugated, diluted 1:1000) in TBS/BSA for 1 h. The extensive washing was repeated and labelled protein bands were visualized by ECL reagent on Kodak X-OMAT AR film. Films were analyzed by an LKB Ultroscan XL Enhanced Laser Densitometer and GelScan XL Laser Densitometer Program computer software.

2.5. [^{35}S]GTPγS binding assay

Endothelial cell membranes (≈ 10 μg of protein) were incubated in Tris-EGTA (50 mM TRIS, 1 mM EGTA, 3 mM $MgCl_2$ pH 7.4) buffer containing [^{35}S]GTPγS (0.05 nM) and increasing concentrations (10^{-8}–10^{-4} M) of stimulating ligands in the presence of 100 μM GDP in a total volume of 1 ml for 60 min at 30°C, according to Sim et al., 1995 and Traynor and Nahorski, 1995. Isoproterenol was dissolved and diluted in 10 mM TRIS-HCl, pH 7.4 containing 1.1 mM ascorbic acid. Non-specific binding was determined with 10 μM GTPγS and subtracted. Bound and free [^{35}S]GTPγS were separated by vacuum filtration through Whatman GF/B filters with a Millipore manifold. Filters were washed with 3×5 ml ice-cold buffer, and radioactivity was detected after drying in a toluene based scintillation cocktail in a Searle liquid scintillation counter.

2.6. Materials

Urea was purchased from Merck, ion-exchange resin for urea (AG-501 X8) was from Bio-Rad, Low Molecular Weight markers were from Pharmacia, nitrocellulose (Hybond) and ECL were from Amersham. Anti-G-protein antibodies As/7 (anti-$G_{i1,2}\alpha$; 1:500), RM/1 (anti-$G_s\alpha$; 1:500) and GC/2 (anti-$G_o\alpha$; 1:500) were from Du Pont-NEN. AS 11 (anti-Gβ-common; 1:300) (Hinsch et al., 1989), AS 369 (anti-$G_{q/11}\alpha$; 1:1000) (Offermanns et al., 1994), AS 269 (anti-$G_{i2}\alpha$; 1:150) (Laugwitz et al., 1993) and AS 86 (preferentially anti-$G_{i3}\alpha$; 1:500) (Nürnberg et al., 1994) were characterized and kindly donated by Prof. G. Schultz and Dr K. Spicher (Freie Universität Berlin, Germany). [^{35}S]GTPγS (37-42 TBq/mmol) was obtained from IZINTA (Budapest, Hungary). All other chemicals were purchased from Sigma.

3. Results

3.1. Western-blots

G-protein composition of primary cultures of rat brain endothelial cell lysates and that of plasma membranes of immortalized rat brain endothelial cell lines, RBE4 and GP8, was studied with immunoblotting using specific antisera against different G-protein subtypes. SDS-PAGE separation of proteins was performed in the presence of urea to improve resolution of the Gα subunits of closely similar molecular weights (39-43 kDa) (Ribeiro-Neto and Rodbell, 1989; Hinsch et al., 1989). Basically, all the Gα subunits tested were present in the three types of CECs studies with only slight quantitative differences (Fig. 1). Antiserum AS 369 (anti-$G_{q/11}\alpha$) detected a protein band which, however, was in many experiments resolved into two distinct bands. Even in cases where the resolution was not so clear, the densitometric analysis of the films revealed the presence of two peaks very close to each other. With antiserum RM/1 (anti-$G_s\alpha$) heavy

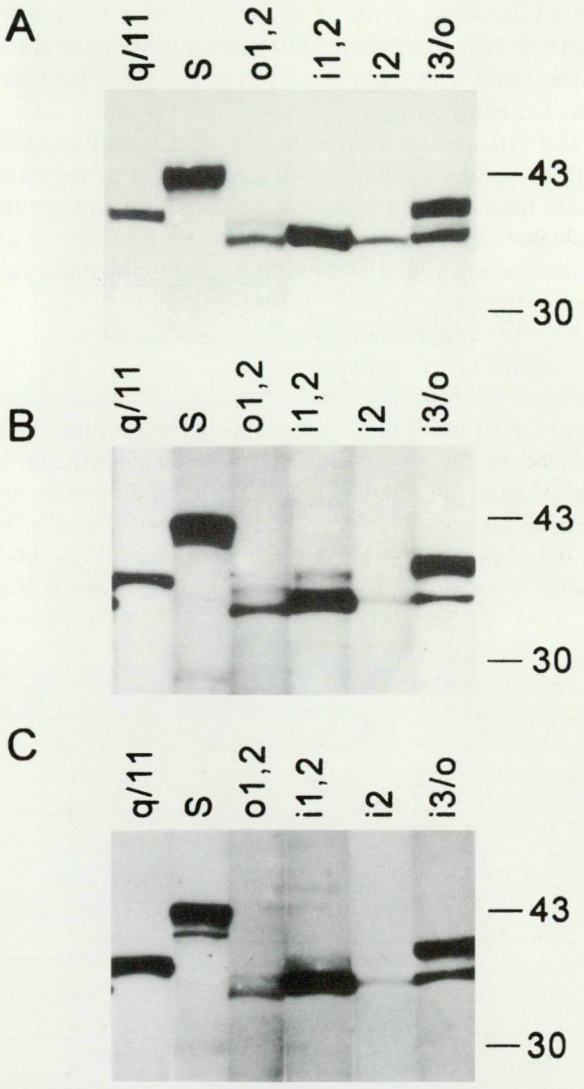

Fig. 1. Immunoblot analysis of G-protein α subunits in primary cultured rat brain endothelial cell lysates (A), or crude membranes of RBE4 (B) and GP8 (C) cell lines. Samples were run on SDS-PAGE containing urea and transferred onto nitrocellulose membranes. Immunoblotting was performed with specific anti-peptide antisera which recognise G-protein α subunits indicated on the top of each lane. Filter-bound antibodies were detected by ECL system. Molecular weights in kDa are indicated on the right.

staining of proteins of 43 kDa was seen. A minor band of about 42 kDa was also detected mainly in GP8 cell membranes (Fig. 1, panel C). This latter protein was sometimes also seen in the other two CECs tested. Antiserum GC/2 (anti-$G_o\alpha$) detected a faint band at approximately 41 kDa, and a more intense staining at about 39 kDa. Antiserum AS/7 (anti-$G_{i1}\alpha$ and $G_{i2}\alpha$) recognised one bulky band which comigrated with the $G_{i2}\alpha$ labelled band obtained by incubation of the membranes with AS 269 (anti-$G_{i2}\alpha$). In RBE4 and GP8 cell membranes a faint band of approx. 42 kDa was also detected with AS7 (Fig. 1, B and C) which was not seen in lysates of primary cell cultures (Fig. 1, A) and might be $G_{i1}\alpha$. AS 86 (preferentially anti-$G_{i3}\alpha$) detected 2 bands of which the upper band might be $G_{i3}\alpha$ and the lower one $G_o\alpha$ based on the known cross-reactivity of AS 86 with $G_o\alpha$ (Simonds et al., 1989; Nürnberg et al., 1994), and also the comigration of this band with the lower band of the GC/2 (anti-$G_o\alpha$) detected bands (Fig. 1).

The $G\beta$-specific antiserum AS 11 detected a doublet with molecular weights of 40 and 41 kDa in the tissues tested (data not shown) that are somewhat higher than the values obtained in most tissues, i.e. 35 resp. 36 kDa (Hinsch et al., 1989), and might be due to the presence of urea in the gel.

3.2. [^{35}S]GTPγS binding

In order to see which receptors might be functionally coupled to the G-proteins in rat brain endothelial cell lines, ligand stimulated [^{35}S]GTPγS binding was measured in plasmamembranes of RBE4 and GP8 cells (Fig. 2). The low yield of the primary CECs cultures, giving about 1 mg protein of cell homogenate from cultures started from ten rat brains (Deli et al., 1997), which yield was further reduced to approximately 100 μg of total protein during membrane preparation prompted us to use only the immortalized cell lines in the [^{35}S]GTPγS binding studies. It has been shown in previous studies that GDP is necessary to observe significant stimulation of [^{35}S]GTPγS binding by most agonist ligands (Sim et al., 1995). Thus, our assays were performed with GDP in μM concentrations. Basal activities were similar in both cell lines (RBE4: 22 ± 1.7 fmol/mg protein; GP8: 20 ± 1.9 fmol/mg protein, mean $\pm$S.E.M.). Highest stimulation was achieved with the β-adrenergic receptor agonist isoproterenol in both endothelial cells (Fig. 2). Bradykinin, a bradykinin receptor agonist stimulated [^{35}S]GTPγS binding to about the same degree ($\approx$20–30%) in both cell lines. No significant stimulation of [^{35}S]GTPγS binding was noted using serotonin. To exclude the possibility that the absence of serotonin stimulation might be due to a different basal activity of the serotonin-coupled G-proteins, the concentration of GDP was varied between 1–100 μM and the effect of serotonin was evaluated (data not shown). The results confirmed our previous observations, namely that highest stimulation can be achieved at 30–100 μM GDP which however was not statistically different from the values obtained in the absence of serotonin.

In the next experiment the additivity of the effect of isoproterenol and bradykinin was tested in GP8 cells (Fig. 3). It was speculated that if different Gα proteins are activated by these receptor agonists (as expected), then the resulting [^{35}S]GTPγS binding should be the sum of those measured in the presence of each ligand alone. This was exactly the case, 10^{-5} M isoproterenol alone elevated the enzyme activity above basal values by 80%

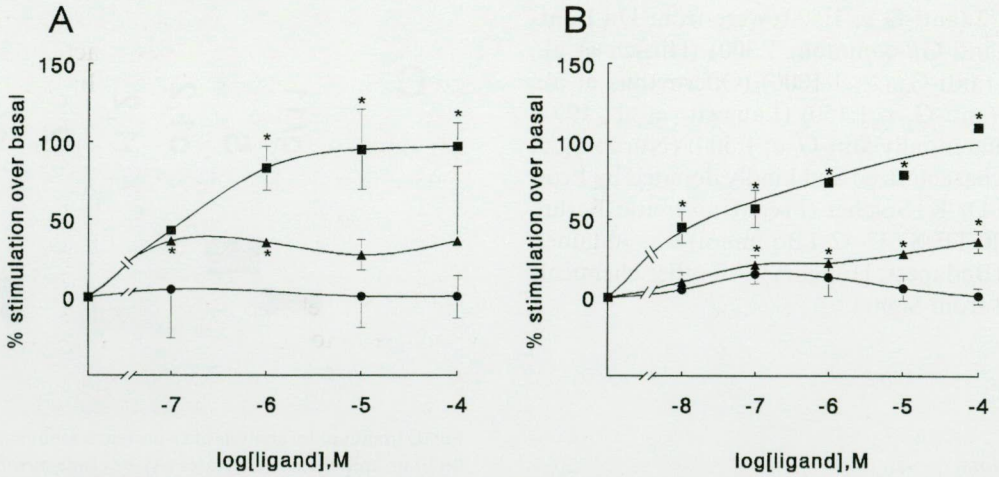

Fig. 2. Ligand stimulated [^{35}S]GTPγS binding in crude membranes of RBE4 (A) and GP8 (B) cell lines. Proteins ($\approx$10 μg) were incubated with [^{35}S]GTPγS (0.05 nM) and various concentrations (10^{-8}–10^{-4} M) of isoproterenol (■), serotonin (●), or bradykinin (▲) in the presence of 100 μM GDP for 60 min at 30°C. Nonspecific binding was determined with 10 μM GTPγS and subtracted. Basal activity, i.e. binding in the absence of stimulating ligands was 22.9 ± 1.7 and 20.0 ± 1.9 fmol $\times$ mg protein^{-1} in RBE4 and GP8 membranes, respectively. Points are means $\pm$ S.E.M. from 3–7 independent experiments, each performed in triplicate. Where error bars are not shown, the SEM was smaller than indicated by the symbol. *$P < 0.05$.

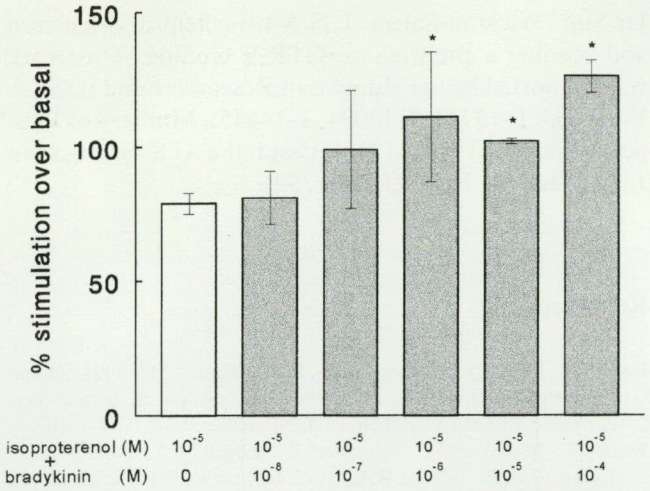

Fig. 3. Additivity of the effect of isoproterenol and bradykinin on [^{35}S]GTPγS binding in GP8 cell membranes. Isoproterenol in 10^{-5} M was probed alone (open bar), or in the presence of 10^{-8}–10^{-4} M bradykinin (filled bars). * denotes those points where the latter are significantly different from the former, $*P < 0.05$. Values are means ± S.E.M. from 3 independent experiments.

(Fig. 3, open bar). This value was further increased by bradykinin depending on its concentration, highest stimulation being seen when 10^{-4} M bradykinin was also present together with 10^{-5} M isoproterenol (Fig. 3, filled bars).

4. Discussion

In this work we identify the G-proteins that are present in cerebral endothelial cells. Highly purified primary cultures of CECs were studied to avoid possible astrocytic and pericytic contamination (Risau et al., 1990). By using specific antibodies raised against various G-protein subtypes and immunoblot techniques, we were able to demonstrate the presence of a complex set of G-proteins such as $G_s\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, $G_{q/11}\alpha$, $G_o\alpha$ and $G\beta$ in CECs. The same proteins were also present in the two immortalized cerebral endothelial cell lines tested, i.e. RBE4 and GP8 which showed similar morphological and functional characteristics to primary CECs (Roux et al., 1994; Greenwood et al., 1996).

The presence of the principal stimulatory G-protein, $G_s\alpha$ in CECs shown in our study (Fig. 1) may constitute an important element in coupling different 7TM receptor proteins to intracellular signalling pathways. Previously certain 7TM receptors, known to be coupled to $G_s\alpha$ in other systems, like β-adrenoceptors (Durieu-Trautmann et al., 1991), histamine H-2 receptors (Karnushina et al., 1980), and dopamine D-1 receptors (Bacic et al., 1991) have been shown to be expressed by the cerebral endothelium. Activation of these receptors may lead to increased intracellular cAMP levels. Increased endothelial cAMP levels in turn have been shown to regulate BBB permeability in vivo (Joó et al., 1975) and in vitro (Rubin et al., 1991; Deli et al., 1995), and to inhibit endothelial cell proliferation (Sexl et al., 1995).

Inhibitory G-proteins have also been detected on the cerebral endothelium (Fig. 1). To our knowledge this is the first report describing the presence of G-proteins in cells of endothelial origin. This might be characteristic to brain endothelial cells since no detectable amount of $G_o\alpha$ was expressed in peripheral endothelial cells (Gil-Longo et al., 1993), and might support the highly specialized function of brain endothelial cells, namely, the maintenance of BBB. Previous studies have demonstrated the expression of different G_i types ($G_{i2}\alpha$ and $G_{i3}\alpha$) in peripheral endothelial cells with the predominance of $G_{i2}\alpha$, and their role in stimulating endothelial cell proliferation and angiogenesis (Sexl et al., 1995; Bauer et al., 1992). In this respect the CECs have similar characteristics expressing significant amounts of $G_{i2}\alpha$ and $G_{i3}\alpha$ (Fig. 1). Activation of these G-proteins have been shown to decrease intracellular cAMP levels and activate K^+ channels in other systems.

G-proteins possibly coupling membrane receptors to phospholipase C (PLC) turned out to be present in CECs as well (Fig. 1). Similarly to its demonstrated role in peripheral endothelium, $G_{q/11}\alpha$ may be involved in mediating the effect of potent vasoactive substances like bradykinin (Liao and Homey, 1993b), histamine (Mancusi et al., 1996), endothelin (Eguchi et al., 1993), thrombin (Stasek and Garcia, 1992) and fibrin (Chang et al., 1995). Moreover, G-proteins may be involved in the regulation of ion channels as well.

Our results obtained with ligand stimulated [^{35}S]GTPγS binding experiments using isoproterenol and bradykinin suggested that β-adrenergic and some bradykinin receptors were present in membrane fractions prepared from RBE4 and GP8 cells, and activation of these receptors resulted in functional activation of some (but unknown) G-proteins (Fig. 2). In agreement with our results, functional β-adrenoceptors have also been demonstrated by other techniques in CECs (Durieu-Trautmann et al., 1991) and also in RBE4 cells (Durieu-Trautmann et al., 1993).

Bradykinin, another receptor agonist only weakly ($\approx$20–30%), but significantly elevated [^{35}S]GTPγS binding in both cell lines tested (Fig. 2). High affinity bradykinin receptor of B_2 type on rat cerebral microvessel was described (Homayoun and Harik, 1991). Moreover bradykinin increased the intracellular free Ca^{2+} in cultured rat CECs (Revest et al., 1991). Activation of bradykinin receptors may be coupled to the regulation of vascular tone and nitric oxide release via cholera toxin sensitive G-proteins as it has recently been demonstrated in peripheral endothelium (Gil-Longo et al., 1993). The possible coupling of bradykinin receptors to PLC via G_q may lead to activation of phospholipid signalling.

Bradykinin enhances the permeability of endothelial monolayers in the periphery (DeFouw et al., 1993; Ehringer et al., 1996). It also causes a rapid and irreversible decrease in transendothelial electrical resistance in pial microvessels of rats acting on the basal membranes (Butt, 1995). These data strengthen a role for bradykinin in brain oedema formation.

Serotonin augmented the extravasation of macromolecules across peripheral venules (Feng et al., 1996), while its effect on the permeability of the BBB is controversial. In frog pial venules, enhanced reversible ionic permeability was observed after administration of the mediator (Olesen and Crone, 1986) while in pial microvessels of rats no effect was seen (Butt, 1995). The lack of stimulation of [^{35}S]GTPγS binding by serotonin in GP8 and RBE4 cells (Fig. 3) might be due to the conditions used in our experiments, although it was tested at various GDP concentration which did not improve the effect measured (data not shown). Literature data, namely that while bradykinin, ATP and histamine elevated the intracellular free CA^{2+} in primary cultures of rat CECs, serotonin caused no detectable effect (Revest et al., 1991), support our observation. The possibility of altered responsiveness to vasoactive mediators in specific segments of the cardiovascular system (periphery vs BBB, large vessels vs capillaries etc.), or species differences should also be considered.

This work was not aiming at identifying the type of Gα proteins that have been activated by the receptor ligands tested in [^{35}S]GTPγS binding experiments. However, from the observation that the stimulating effect measured when both isoproterenol and bradykinin were present in the assay was the sum of activation measured with each ligand alone (Fig. 3), it was concluded that different Gα proteins were activated by these two ligands. In analogy to other systems, activation of G_s is most likely by isoproterenol, while G_i and $G_{q/11}$ proteins might be activated by bradykinin receptors. The validity of this speculation, as well as the pharmacological characterization of these receptors will require further investigations. Also, in order to demonstrate definitively that these various receptors and Gα proteins are expressed by endothelial cells, Northern-blots, in situ hybridization and so on, will be required. Nevertheless, to our knowledge this is the first study to identify the G-protein types in cerebral endothelial cells, and thus it may prepare the ground for future work.

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