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Inactivation of 5-HT_{1A} receptors in hippocampal and cortical homogenates

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Abstract

5-HT_{1A} receptor function can be assessed in rat hippocampal and cortical membrane preparations as agonist-stimulated [^{35}S]GTPγS binding. Membranes were preincubated in vitro with *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). *R*(+)-8-hydroxy-2-(di-*n*-propylamino)tetralin [*R*(+)-8-OH-DPAT]-stimulated [^{35}S]GTPγS binding and [^{3}H]8-OH-DPAT binding assays were used to assess 5-HT_{1A} receptor function and density, respectively. EEDQ decreased both *R*(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS and [^{3}H]8-OH-DPAT binding in hippocampal and cortical membranes. The E_{max} but not the EC_{50} of *R*(+)-8-OH-DPAT to stimulate [^{35}S]GTPγS binding was decreased by EEDQ in both preparations. Additionally, the IC_{50} for EEDQ to reduce *R*(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS and [^{3}H]8-OH-DPAT binding was the same for both brain regions in both assays. In contrast to EEDQ alone, agonist-stimulated [^{35}S]GTPγS binding was not reduced in hippocampal membranes preincubated with EEDQ and the 5-HT_{1A} receptor antagonist *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinyl-cyclohexanecarboxamide maleate (WAY 100,635), suggesting that EEDQ acts directly on the receptor. Due to parallel reductions in receptor density and maximal functional response, it is concluded that there is little or no reserve for 5-HT_{1A} receptor coupling to G_{α} in these preparations. In addition, the sensitivity of hippocampal and cortical 5-HT_{1A} receptors to inactivation by EEDQ in vitro is the same.

Keywords: 5-HT_{1A} receptor; EEDQ; [^{35}S]GTPγS binding assay; Hippocampus; Cortex; Receptor reserve; Spare receptor

1. Introduction

We recently described the application of agonist-stimulated [^{35}S]GTPγS binding to assess the function of 5-HT_{1A} receptors in membranes of rat hippocampus and cortex (Alper and Nelson, 1998). Full 5-HT_{1A} receptor agonists were distinguished from partial agonists based on maximal stimulation of [^{35}S]GTPγS binding in the hippocampus, suggesting lack of receptor reserve (Hoyer and Boddeke, 1993). This came as no particular surprise as others have presented similar findings when looking at the ability of 5-HT_{1A} receptor agonists to inhibit forskolin-stimulated adenylyl cyclase in the hippocampus (De Vivo and Maayani, 1986; Schoeffter and Hoyer, 1988). However, agonist-stimulated [^{35}S]GTPγS binding theoretically reflects receptor-mediated activation of all G proteins associated with the 5-HT_{1A} receptor, not only those that are inhibitory to adenylyl cyclase. Thus, it was thought that receptor inactivation by the in vitro exposure to the irreversible alkylating agent *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) would not necessarily present the same findings in agonist-stimulated [^{35}S]GTPγS binding assays as in other functional models. Furthermore, it has been shown that the selective 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) behaves like a full agonist in the mouse hippocampus but as a partial agonist in the cortex (Dumuis et al., 1988).

Experiments were designed to assess the effects of EEDQ in vitro on 5-HT_{1A} receptor function and density in rat hippocampal and cortical membranes. In both brain areas, EEDQ produced concentration-dependent parallel decreases in 5-HT_{1A} receptor E_{max} and density without altering the affinity. Furthermore, the effect of EEDQ on *R*(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS binding in hippocampal membranes was prevented by the selective 5-HT_{1A} receptor antagonist WAY 100,635. It is concluded

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that there is little or no reserve for 5-HT_{1A} receptor-mediated activation of G_{α} in hippocampal and cortical membranes. In addition, there is no difference in the sensitivity of hippocampal and cortical 5-HT_{1A} receptors to inactivation by EEDQ in vitro.

2. Materials and methods

2.1. Tissue preparation

Male rat hippocampus and cortex were obtained as frozen tissue from Pel-Freez Biologicals (Rogers, AR) stored at −70°C. Upon defrosting the brains were homogenized using a Tekmar tissue homogenizer in cold Tris buffer (50 mM Tris base, pH 7.4). The homogenate was centrifuged (39,800 × *g*, 4°C for 10 min), the supernatant decanted and the remaining pellet resuspended in the same Tris buffer. After homogenization the suspension was incubated at 37°C for 10 min in a shaking water bath to remove endogenous 5-HT (Nelson et al., 1978). The suspension was then centrifuged, the supernatant decanted and the pellet again washed with cold Tris buffer. After one final centrifugation, the remaining pellet was homogenized in the same Tris buffer at a final concentration of ~100 mg tissue/ml. This suspension was frozen in aliquots at −70°C for later use.

On the day of the experiment, the frozen tissue suspension was defrosted, resuspended in ~35 ml Tris buffer and centrifuged as above. The pellets were resuspended in 4.2 ml Tris buffer; aliquots of 2 ml containing sufficient membrane protein for both the [^{35}S]GTPγS assay and [^{3}H]-8-OH-DPAT radioligand binding assay were transferred to 40 ml centrifuge tubes on ice. Additions of EEDQ, WAY 100,635 or their respective solvents were 20 μl aliquots (1:100 dilution). The tubes were then transferred to a shaking water bath (37°C) for 15 min. This preincubation was stopped by the addition of ~35 ml ice cold Tris buffer immediately followed by centrifugation

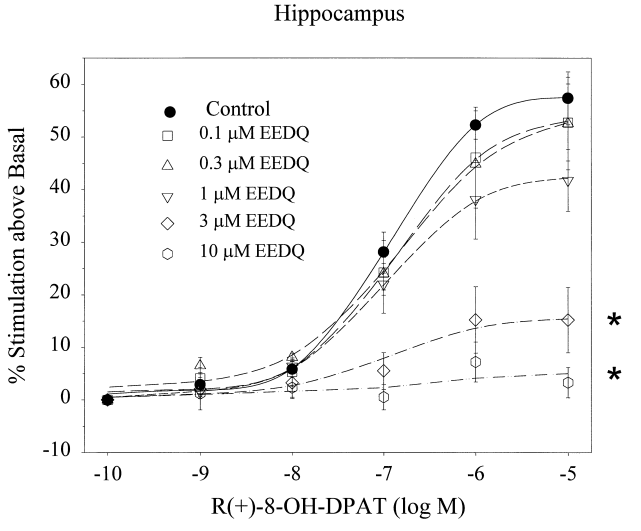

Fig. 1. Effect of graded concentrations of EEDQ on *R*(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS binding in rat hippocampal membranes. Membranes (2 ml) prepared from rat hippocampus were preincubated in vehicle (20 μl of 100% ethanol; Control) or EEDQ (0.1 to 10 μM) as described in Section 2. Symbols represent the mean ± S.E.M. as determined from triplicates in three separate experiments. The lines are the best fit as determined by nonlinear regression analysis. $^{*}E_{max}$ is significantly different from control as determined by one-way ANOVA followed by Dunnett's post-hoc test ($P < 0.05$).

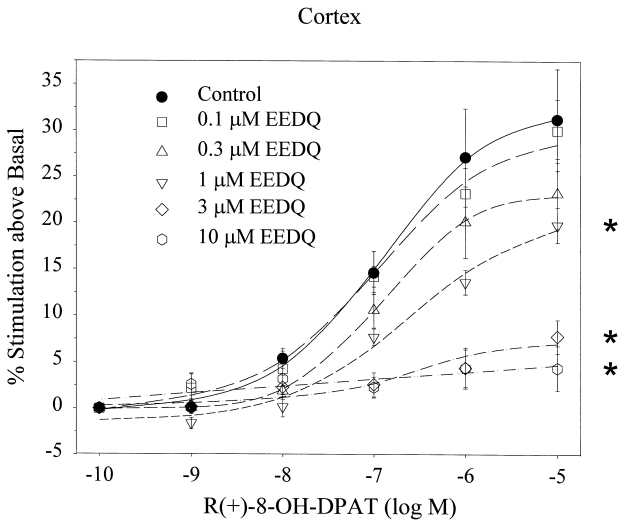

Fig. 2. Effect of graded concentrations of EEDQ on *R*(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS binding in rat cortical membranes. Membranes (2 ml) prepared from rat cortex were preincubated in vehicle (20 μl of 100% ethanol; control) or EEDQ (0.1 to 10 μM) as described in Section 2. Symbols represent the mean ± SEM as determined from triplicates in three separate experiments. The lines are the best fit as determined by nonlinear regression analysis. $^{*}E_{max}$ is significantly different from control as determined by one-way ANOVA followed by Dunnett's post-hoc test ($P < 0.05$).

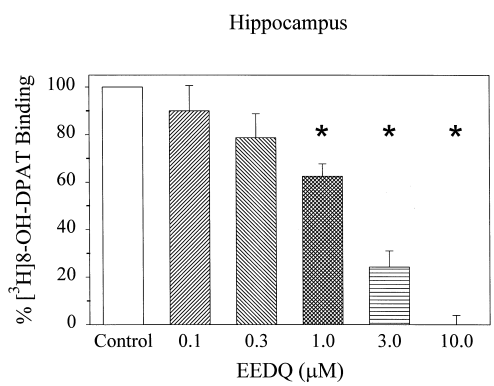

Fig. 3. Effect of graded concentrations of EEDQ on [^{3}H]8-OH-DPAT binding in rat hippocampal membranes. See legend to Fig. 1 for details. The radioligand binding data are expressed as percent of control for each of the three separate experiments. The specific binding at 1.0 nM radioligand was 132.1 ± 15.5 fmol [^{3}H]8-OH-DPAT/mg protein in the control preincubations. *Significantly different from control as determined by one-way ANOVA followed by Dunnett's post-hoc test ($P < 0.05$).

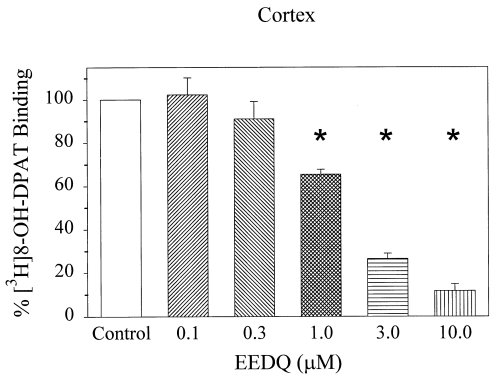

Fig. 4. Effect of graded concentrations of EEDQ on [^{3}H]8-OH-DPAT binding in rat cortical membranes. See legend to Fig. 2 for details. The radioligand binding data are expressed as percent of control for each of the three separate experiments. The specific binding at 1.0 nM radioligand was 33.6 ± 4.4 fmol [^{3}H]8-OH-DPAT/mg protein in the control preincubations. *Significantly different from Control as determined by one-way ANOVA followed by Dunnett's post-hoc test ($P < 0.05$).

(39,800 × *g*, 4°C for 10 min). In the protection experiment (Fig. 5) the membranes were washed with ~35 ml ice cold Tris buffer a second time. The final pellets were suspended in appropriate volumes of [^{35}S]GTPγS binding assay buffer (4 nM $MgCl_2$, 160 mM NaCl, 0.267 mM EGTA, 67 mM Tris base, pH 7.4) to produce a protein concentration of ~200 μg/ml. Following removal of the required amount of membrane for the [^{35}S]GTPγS binding assay, the remaining suspension was diluted in 50 mM Tris buffer (pH 7.4) and centrifuged. The pellet was then resuspended in radioligand binding assay buffer (described below) and stored on ice for [^{3}H]8-OH-DPAT binding assay to follow later that same day.

2.2. [^{35}S]GTPγS binding assay

R(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS binding was performed as described previously (Alper and Nelson, 1998). Briefly, water or *R*(+)-8-OH-DPAT (10^{-10}–10^{-5} M), 0.4 nM [^{35}S]GTPγS in ligand buffer (assay buffer containing 1200 μM GDP), and tissue homogenate (~30–50 μg protein) were incubated at a final volume of 800 μl for 20 min in a shaking water bath at 37°C. The reaction was terminated by rapid vacuum filtration through Whatman GF/B filters pre-wet with 20 mM $Na_4P_2O_7 \cdot 10\ H_2O$ and pre-cooled with 3 washes of 2 ml cold Tris buffer. Following 3 subsequent washes with 1 ml cold Tris buffer, the filters were placed into 7 ml scintillation vials and 5 ml scintillation cocktail added. The samples were shaken by inversion, allowed to sit for a minimum of 2 h, shaken again immediately prior to counting (2 min/sample) in a scintillation counter. Nonspecific binding was determined as the amount of [^{35}S]GTPγS bound in the presence of 10 μM unlabeled GTPγS and was subtracted from all samples. Basal (agonist-independent) [^{35}S]GTPγS binding was defined as the specific binding when water containing no drug was added to the assay tube.

2.3. Radioligand binding assay

Membranes were suspended in the radioligand binding assay buffer; 67 mM Tris base, 12 mM $CaCl_2$, 0.04 mM pargyline, pH 7.4. Specific [^{3}H]8-OH-DPAT binding was determined at 1.0 nM radioligand and was calculated by subtracting total from nonspecific binding (defined by the addition of 10 μM 5-HT) as described previously (Taylor et al., 1986). All samples from each experiment were run in triplicate determinations in one radioligand binding assay.

2.4. Data analysis

The protein concentration of each tissue homogenate was determined using the standard Bradford protein assay (Bradford, 1976). The [^{35}S]GTPγS binding data are presented as % stimulation over basal:

$$\left[\left(\frac{\text{agonist-stimulated specific [}^{35}\text{S]GTP}\gamma\text{S bound (fmol/mg protein)}}{\text{agonist-independent specific [}^{35}\text{S]GTP}\gamma\text{S bound (fmol/mg protein)}}\right) \times 100\%\right] - 100\%$$

Specific [^{3}H]8-OH-DPAT binding was calculated by subtracting nonspecific binding from total binding. The radioligand binding data are presented as % of control, the specific binding determined in membranes preincubated with the addition of the appropriate drug solvents (see below). The absolute values (in fmol [^{3}H]8-OH-DPAT bound/mg protein) are presented in the figure legends.

Values represent the mean ± S.E.M. from triplicate determinations in 3–4 separate experiments. The EC_{50}, IC_{50}

Table 1
EEDQ does not alter the potency (EC_{50}) of *R*(+)-8-OH-DPAT to stimulate [^{35}S]GTPγS binding in rat hippocampal and cortical membranes
Values represent the mean ± S.E.M. ($n = 3$ for each brain region) calculated by nonlinear regression analysis from the 6-point *R*(+)-8-OH-DPAT concentration-response curves presented in Figs. 1 and 2. The values in parentheses are the pEC_{50} ± S.E.M. N.D. means that EC_{50} values were not able to be determined from the concentration-response curves (see Figs. 1 and 2). There were no significant differences among the values within a brain region ($P > 0.05$ by one-way analysis of variances).

	EEDQ (μM)					
	Control	0.1	0.3	1.0	3.0	10.0
	EC_{50} (nM)					
Hippocampus	113 ± 14 (5.95 ± 0.05)	192 ± 65 (5.78 ± 0.18)	162 ± 33 (5.81 ± 0.01)	99 ± 5 (6.01 ± 0.02)	109 ± 9 (5.97 ± 0.03)	N.D.
Cortex	123 ± 28 (5.94 ± 0.11)	116 ± 14 (5.94 ± 0.05)	123 ± 10 (5.91 ± 0.04)	332 ± 179 (5.61 ± 0.24)	190 ± 58 (5.77 ± 0.15)	N.D.

and E_{max} (maximal response) values were calculated in each individual experiment by nonlinear regression analysis using a four parameter model (SigmaPlot v. 4.01, SPSS, Chicago, IL).

2.5. Materials

R(+)-8-hydroxy-2-(di-*n*-propylamino)tetralin HBr [*R*(+)-8-OH-DPAT] and EEDQ were purchased from RBI (Natick, MA). 5-HT creatinine sulfate complex (5-HT) was purchased from Sigma (St. Louis, MO). WAY 100,635 (*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinyl-cyclohexanecarboxamide trihydrochloride) was a gift from Wyeth-Ayerst Research (Princeton, NJ). [^{35}S]GTPγS (1,250 Ci/mmol) and [^{3}H]-8-OH-DPAT (154.3 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA).

R(+)-8-OH-DPAT, WAY 100,635 and 5-HT were dissolved in deionized water; EEDQ was dissolved in 100% ethanol. All drugs were prepared fresh daily.

3. Results

The in vitro preincubation of rat hippocampal or cortical membranes with the irreversible alkylating agent EEDQ produced concentration-dependent decreases in both maximal *R*(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS responses (Figs. 1 and 2) and [^{3}H]8-OH-DPAT binding (Figs. 3 and 4). In neither region did EEDQ affect the affinity of the receptor assessed in the functional [^{35}S]GTPγS binding assay (Table 1). Regression analysis of percent maximal [^{35}S]GTPγS stimulation vs. percent maximal [^{3}H]8-OH-DPAT binding revealed a very strong correlation for hippocampus ($r^2 = 0.90$) and cortex ($r^2 = 0.93$; $n = 15$ for each region) with slopes of 0.86 for both brain areas (data not presented). In addition, the IC_{50} values (i.e., the potency) of EEDQ to inhibit maximal [^{35}S]GTPγS binding by *R*(+)-8-OH-DPAT and [^{3}H]8-OH-DPAT binding were ~2 μM in both assays and did not differ between hippocampus and cortex (Table 2).

Table 2
Potency (IC_{50}) of EEDQ to inhibit *R*(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS and [^{3}H]8-OH-DPAT binding in rat hippocampal and cortical membranes

Values represent the mean ± S.E.M. ($n = 3$ for each brain region) calculated by nonlinear regression analysis using 5 concentrations of EEDQ and: (1) the maximal stimulation as calculated from the 6-point *R*(+)-8-OH-DPAT concentration-response curves presented in Figs. 1 and 2 for the [^{35}S]GTPγS binding data, and (2) the percent of control presented in Figs. 3 and 4 for the [^{3}H]8-OH-DPAT binding data. There were no significant differences among the values ($P > 0.05$ by two-way analysis of variances).

Binding assay	IC_{50} (μM)	
	Hippocampus	Cortex
[^{35}S]GTPγS	2.0 ± 0.6	2.3 ± 0.7
[^{3}H]8-OH-DPAT	2.2 ± 0.7	1.4 ± 0.3

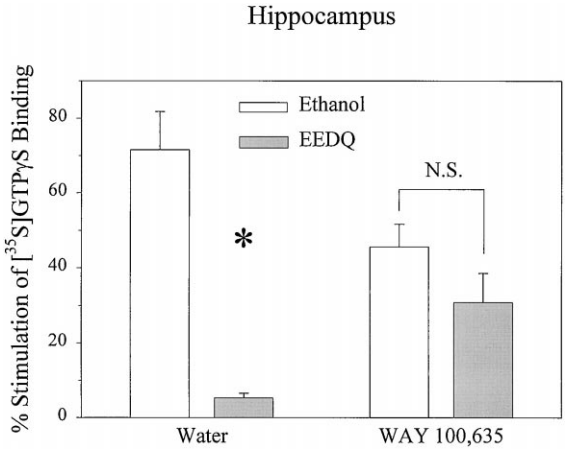

Fig. 5. Protection of EEDQ-induced decrease in *R*(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS binding by WAY 100,635 in rat hippocampal membranes. Membranes (2 ml) prepared from rat hippocampus were preincubated in 20 μl water or WAY 100,635 (0.1 μM) plus either 20 μl 100% ethanol (open columns) or EEDQ (3 μM, filled columns). Values represent the mean ± S.E.M. as determined from triplicates in 4 separate experiments. The data were initially analyzed by two-way analysis of variances and a significant ($P < 0.05$) interaction term was calculated. * Significantly ($P < 0.05$) different from water/ethanol preincubation by Tukey's post-hoc test for all pair wise multiple comparisons. N.S., not significantly different from each other or from water/ethanol preincubation ($P > 0.05$).

The ability of the selective 5-HT_{1A} receptor antagonist WAY 100,635 to protect from EEDQ-induced receptor inactivation was demonstrated in a second set of experiments (Fig. 5). Preincubation of hippocampal membranes with 3 μM EEDQ significantly reduced 10 μM *R*(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS binding by greater than 90% (open vs. filled columns, left panel), whereas preincubation with 0.1 μM WAY 100,635 followed by 2 washes in Tris buffer did not significantly alter R(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS binding (open columns, left vs. right panels). Furthermore, EEDQ did not inhibit *R*(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS binding when membranes were preincubated along with WAY 100,635 (open vs. filled columns, right panel) rather than water.

4. Discussion

In these experiments, we present data consistent with the hypothesis that there is little or no 5-HT_{1A} receptor reserve in membranes prepared from rat hippocampus or cortex when the initial functional biochemical process (i.e., G protein activation) is compared to receptor binding. Qualitative evidence for this is: (1) a near perfect correlation between the ability of the alkylating agent EEDQ to affect functional and radioligand binding assays in both brain regions, (2) exposure of membranes causes reductions in maximal functional responses to *R*(+)-8-OH-

DPAT without a shift in the EC_{50}, consistent with classical receptor occupancy theory (Furchgott and Bursztyn, 1967; Ruffolo, 1982), and (3) the IC_{50} of EEDQ to decrease [^{3}H]8-OH-DPAT binding and maximal *R*(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS binding were indistinguishable (Table 2). Had there been receptor reserve, as the number of receptors was reduced by EEDQ the affinity of *R*(+)-8-OH-DPAT to stimulate [^{35}S]GTPγS binding in membranes would decrease until the reserve was exhausted; further decreases in receptor number would result in decreased efficacy, contrary to what was observed in our studies.

EEDQ has been used extensively in vivo to examine 5-HT_{1A} receptor reserve (Emerit et al., 1985; Meller and Bohmaker, 1994; Meller et al., 1990; Meller et al., 1992; Cox et al., 1993) and turnover (Bolaños-Jiménez et al., 1991; Gozlan et al., 1994; Keck and Lakoski, 1996a,b; Raghupathi et al., 1996). However, to the best of our knowledge there is but one report demonstrating alkylation of 5-HT_{1A} receptors ([^{3}H]-8-OH-DPAT binding sites) in vitro which employed a substituted analog of 8-OH-DPAT, 8-methoxy-2-(*n*-2′-chloropropyl, *n*-propylamino)tetralin (8-methoxy-2′-chloro-PAT), rather than EEDQ (Emerit et al., 1985). There do not appear to be any reports describing the use of EEDQ in vitro to investigate 5-HT_{1A} receptor function.

The demonstration of a large receptor reserve in the raphé nuclei suggests that there are many spare somatodendritic 5-HT_{1A} autoreceptors (Meller et al., 1990; Bohmaker et al., 1992; Cox et al., 1993). The postsynaptic 5-HT_{1A} receptor in the hippocampus does not demonstrate receptor reserve as defined by inhibition of forskolin-stimulated adenylyl cyclase (Meller et al., 1992; Yocca et al., 1992). However, some events presumably mediated by activation of postsynaptic 5-HT_{1A} receptors do appear to have receptor reserve (Meller and Bohmaker, 1994). This points out that ''reserve'' depends, in part, on the response being studied. To that extent, the current experiments are novel in two manners: (1) they are the initial reports describing inactivation of 5-HT_{1A} receptor-mediated responses using EEDQ in vitro, and (2) they suggest lack of 5-HT_{1A} receptor reserve in hippocampal or cortical membrane preparations as assessed by the first step of G protein activation, the binding of [^{35}S]GTPγS to the α subunit following receptor stimulation. As such, this model directly assesses the reserve between the 5-HT_{1A} receptor and its coupling to G protein prior to modulation of downstream second messenger systems such as adenylyl cyclase or K^+ channels.

EEDQ binds to and irreversibly alkylates many G protein-coupled receptors including α_2-adrenergic (Adler et al., 1985), muscarinic (Norman et al., 1989a), dopaminergic (Henry and Roth, 1984), $GABA_A$ (Miller et al., 1991), and several serotonergic receptor subtypes (Emerit et al., 1985; Bolaños-Jiménez et al., 1991; Bohmaker et al., 1992; Adham et al., 1993; Gozlan et al., 1994; Keck and Lakoski, 1996a,b). Inasmuch as it has been shown that EEDQ can also inhibit [^{3}H]forskolin binding in striatal homogenates with an IC_{50} of ~200 μM (Norman et al., 1989b), our protection experiment was very important. Our data suggest that EEDQ binds directly to 5-HT_{1A} receptors in homogenates thereby decreasing their function based on two observations. First, the ability of EEDQ to inhibit *R*(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS binding in hippocampal membranes was prevented by the concomitant preincubation with the potent, highly selective 5-HT_{1A} receptor antagonist WAY 100,635 (Fletcher et al., 1996) at 0.1 μM. At this concentration WAY 100,635 antagonizes the [^{35}S]GTPγS response elicited by 1 μM *R*(+)-8-OH-DPAT (Alper and Nelson, 1998). Second, the IC_{50} for EEDQ to inhibit 5-HT_{1A} receptor function was found to be ~2 μM. This is ~100-fold more potent then its IC_{50} for inhibition of [^{3}H]forskolin binding, further supporting our conclusion of direct alkylation of the 5-HT_{1A} receptor by EEDQ rather than alkylation of the α subunit of G proteins. Although we did not conduct a similar protection experiment in cortical membrane preparations, we have no reason to suspect that the results would differ from the hippocampus.

Subjectively, it appears that the *R*(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS response was reduced in homogenates preincubated with WAY 100,635 as compared to control (open columns, Fig. 5). Although this difference did not attain statistical significance, it cannot be completely ignored or discounted. Due to the high affinity of WAY 100,635 for the 5-HT_{1A} binding site (Fletcher et al., 1996) and receptor (Fletcher et al., 1996; Alper and Nelson, 1998) two washes might not have been sufficient to eliminate the antagonist from the membrane suspension. In preliminary studies it was found that suspensions preincubated with WAY 100,635 and washed only once in Tris buffer were barely responsive to stimulation by *R*(+)-8-OH-DPAT; following two washes in Tris buffer the response returned to be statistically indistinguishable from control levels.

It has been reported that a single dose of EEDQ in vivo causes a greater reduction in 5-HT_{1A} receptor binding in hippocampus than in cortex (Keck and Lakoski, 1996b; Nénoné et al., 1996; Raghupathi et al., 1996). This could be interpreted to suggest that there is a difference at the cellular or molecular level in the 5-HT_{1A} receptor found in these two brain areas. Our data suggest, however, that this is not true since there was no difference in the sensitivity of the 5-HT_{1A} receptor in the hippocampus and the cortex to the alkylating effects of EEDQ in vitro. One possible explanation for the in vivo data is that the pharmacokinetics are such that more EEDQ accumulates in the hippocampus than in the cortex. A second more plausible explanation for the regional difference reported in vivo might be due to differential receptor occupancy by the endogenous neurotransmitter 5-HT. The effects of EEDQ are dependent on its binding to unoccupied receptors and

thus are inhibited when any ligand (agonist or antagonist) is bound to the receptor. The membrane preparations utilized in these studies include a 10-min incubation at 37°C specifically to eliminate endogenous 5-HT (Nelson et al., 1978). We find that the subsequent in vitro EEDQ exposure of membranes prepared this way results in a complete elimination of 5-HT_{1A} receptor binding and function. Such a complete effect has never been reported in vivo where there is a certainty that a percentage of the receptors will be occupied by endogenous 5-HT at any given time. Thus, it may be that the regional-specific effect of EEDQ reported in vivo is neither a difference in the receptor molecular biology nor drug distribution, but may be instead due to greater occupancy of the 5-HT_{1A} receptor by endogenous 5-HT in the cortex than in the hippocampus.

One final question arises regarding the specificity of *R*(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS binding as an assessment of 5-HT_{1A} receptors. Although the pharmacology of the 5-HT_{1A} and 5-HT_7 receptors is similar, their associated G proteins are different. The inclusion of a high concentration of GDP (300 μM) in the assay favors the binding of GTP to the α subunit of $G_{i/o}$ but not to G_s, G_q or other G_α subunits (Weiland and Jakobs, 1994). As the 5-HT_{1A} receptor is coupled to $G_{i/o}$ and the 5-HT_7 receptor is coupled to G_s (Bard et al., 1993), it is likely that *R*(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS binding reflects 5-HT_{1A} receptor function (Alper and Nelson, 1998).

In summary, we have presented data suggesting that EEDQ can be used in vitro to irreversibly inactivate 5-HT_{1A} receptor function in brain homogenates. Furthermore, it appears that virtually all 5-HT_{1A} binding sites (i.e., [^{3}H]8-OH-DPAT binding sites) in membranes prepared from male rat hippocampus and cortex are functionally coupled to G protein; there is no apparent receptor reserve at the initial level of intracellular signaling. In addition, there are no inherent differences between the 5-HT_{1A} receptors in these two brain regions regarding their sensitivity to inactivation by the alkylating agent EEDQ.

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