Cellular and Molecular Neurobiology, Vol. 24, No. 3, June 2004 (© 2004)

Interaction of Serotonin$_{1A}$ Receptors From Bovine Hippocampus With Tertiary Amine Local Anesthetics

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Received September 19, 2003; accepted November 11, 2003

SUMMARY

1. We have examined the interaction of tertiary amine local anesthetics with the bovine hippocampal serotonin$_{1A}$ (5-HT$_{1A}$) receptor, an important member of the G-protein-coupled receptor superfamily.

2. The local anesthetics inhibit specific agonist and antagonist binding to the 5-HT$_{1A}$ receptor at a clinically relevant concentration range of the anesthetics. This is accompanied by a concomitant reduction in the binding affinity of the 5-HT$_{1A}$ receptor to the agonist. Interestingly, the extent of G-protein coupling of the receptor is reduced in the presence of the local anesthetics.

3. Fluorescence polarization measurements using depth-dependent fluorescent probes show that procaine and lidocaine do not show any significant change in membrane fluidity. On the other hand, tetracaine and dibucaine were found to alter fluidity of the membrane as indicated by a fluorescent probe which monitors the headgroup region of the membrane.

4. The local anesthetics showed inhibition of agonist binding to the 5-HT$_{1A}$ receptor in membranes depleted of cholesterol more or less to the same extent as that of control membranes in all cases. This suggests that the inhibition in ligand binding to the 5-HT$_{1A}$ receptor brought about by local anesthetics is independent of the membrane cholesterol content.

5. Our results on the effects of the local anesthetics on the ligand binding and G-protein coupling of the 5-HT$_{1A}$ receptor support the possibility that G-protein-coupled receptors could be involved in the action of local anesthetics.

KEY WORDS: bovine hippocampal 5-HT$_{1A}$ receptor; tertiary amine local anesthetics; G-protein coupling; fluorescence polarization; membrane fluidity; cholesterol.

INTRODUCTION

Local anesthetics suppress sensation within the area of their application in the body by reversibly blocking the action potential responsible for nerve transmission thereby relieving pain. A basic approach toward elucidating the mechanism of action of local anesthetics is to identify their possible molecular targets. Local anesthetics are amphiphilic in nature. The correlation between strength of an anesthetic and its solubility in olive oil, as described by the Meyer–Overton rule, gave rise to the

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0272-4340/04/0600-0403/0

general belief that anesthetics exert their actions by interacting with the hydrophobic sites of the membrane (Seeman, 1972). However, the molecular mechanisms associated with the process of anesthesia are not completely understood yet. There are two major models proposed for anesthetic action. The first model, known as the lipid hypothesis, ascribes the effect to alterations of the physical properties of the lipid bilayer. An underlying implication of this proposal is that perturbations in the physical properties of the membrane (such as membrane fluidity) ultimately affect the function of membrane proteins (De Paula and Schreier, 1996; Gutierrez-Merino *et al.*, 1989; Rehberg *et al.*, 1995). The second, called the protein hypothesis, explains anesthesia in terms of direct, specific interaction of anesthetics with membrane proteins so as to modulate their function (Arias, 1999; Mihic *et al.*, 1997). The question of whether anesthesia results from an indirect anesthetic–lipid environment effect or a specific anesthetic–protein interaction continues to be a major unresolved problem. In this overall scenario, understanding the action of anesthetics on the membrane as a system and not on its individual components taken separately assumes significance.

Among the membrane proteins, ligand-gated ion channels have emerged as the most probable targets of anesthetic action (Arias, 1999; Franks and Lieb, 1997). These ion channels belong to the superfamily of four transmembrane domain receptors (Ortells and Lunt, 1995). However, the role of the other important class of membrane receptors, the seven transmembrane domain G-protein-coupled receptors (GPCRs) which includes several neurotransmitter receptors (Bikker *et al.*, 1998; Pierce *et al.*, 2002; Strader *et al.*, 1995), in the action of anesthetics is an emerging area of research. Inhibition of G-protein signaling pathways is thought to be an important component of the action of both general (Pentyala *et al.*, 1999) and local (Xiong *et al.*, 1999; Zhou *et al.*, 2001) anesthetics. One of the ways signaling through G-proteins could be modulated is through interaction of the anesthetics with G-proteins (Hollmann *et al.*, 2001). However, evidence for direct interaction of certain inhaled anesthetics with GPCRs has recently been shown (Ishizawa *et al.*, 2002). Whether a similar mode of action is present in case of local anesthetics constitutes an interesting possibility. In view of the importance of G-protein-coupled receptors in intracellular signaling and their pharmacological significance (Shanti and Chattopadhyay, 2000), their role in the action of local anesthetics becomes crucial.

Serotonin receptors are members of a superfamily of seven transmembrane domain receptors (Pierce *et al.*, 2002; Strader *et al.*, 1995) that couple to GTP-binding regulatory proteins (G-proteins). Serotonergic signaling appears to play a key role in the generation and modulation of various cognitive and behavioral functions (Heath and Hen, 1995). Among the various types of serotonin receptors, the G-protein-coupled 5-HT_{1A} receptor subtype has been the most extensively studied for a number of reasons (Harikumar *et al.*, 2000; Harikumar and Chattopadhyay, 1998a). One of the main reasons for this is the availability of a selective ligand 8-OH-DPAT (8-hydroxy-2-(di-*N*-propylamino)tetralin) that allows extensive biochemical, physiological, and pharmacological characterization of the receptor (Gozlan *et al.*, 1983). The 5-HT_{1A} receptor is the first among all the serotonin receptors to be cloned and sequenced (Albert *et al.*, 1990; Fargin *et al.*, 1988). Furthermore, it was the first serotonin receptor for which polyclonal antibodies were obtained (Fargin *et al.*, 1988) allowing their visualization at the subcellular level in various regions of the brain.

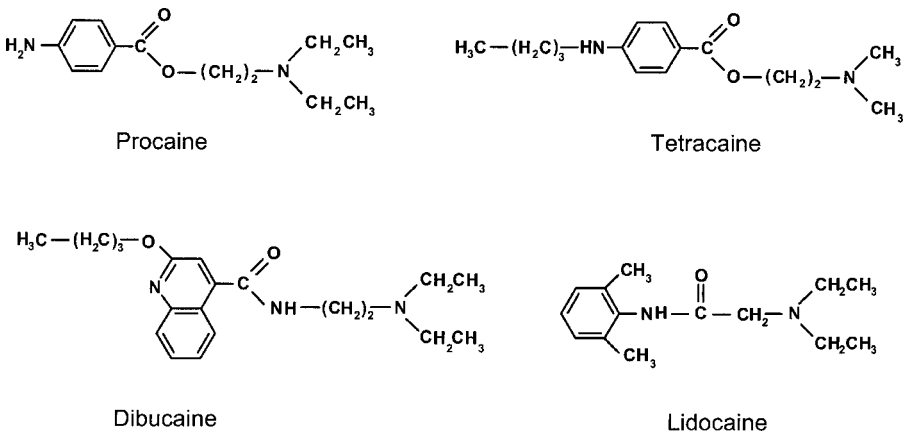

Fig. 1. Chemical structures of the tertiary amine local anesthetics used.

We have earlier partially purified and solubilized the 5-HT$_{1A}$ receptor from bovine hippocampus in a functionally active form (Chattopadhyay *et al.*, 2002; Chattopadhyay and Harikumar, 1996) and reported, for the first time, solubilization of 5-HT$_{1A}$ receptors stably expressed in Chinese Hamster Ovary (CHO) cells (Chattopadhyay *et al.*, 2004). We have shown modulation of ligand binding by metal ions, guanine nucleotides, alcohols, and covalent modifications of the disulfides and sulfhydryl groups (Harikumar *et al.*, 2000; Harikumar and Chattopadhyay, 1998a,b, 1999, 2000, 2001).

In this paper, we have investigated the interaction of four tertiary amine local anesthetics with the bovine hippocampal 5-HT$_{1A}$ receptor. Many clinically used local anesthetics are esters or amides of tertiary amines. The tertiary amine local anesthetics used are procaine, tetracaine, dibucaine, and lidocaine (see Fig. 1). Our results show that the local anesthetics interact with the serotonin$_{1A}$ receptor and this could be important in understanding the role of GPCRs in the action of the anesthetics.

MATERIALS AND METHODS

Materials

Procaine, tetracaine, dibucaine, lidocaine, serotonin, DPH (1,6-diphenyl-1,3,5-hexatriene), and MβCD (methyl-β- cyclodextrin) were obtained from Sigma Chemical Co. (St. Louis, MO). Cholesterol assay kit (Diagnostic Kit No. 139 050) and GTP-γ-S (guanosine-5′-*O*-(3-thiotriphosphate)) were purchased from Boehringer Mannheim (Germany). *p*-MPPI (4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-iodobenzamido]ethyl-piperazine) was from Research Biochemicals International (Natick, MA). [^{3}H]8-OH-DPAT (8-hydroxy-2-(di-*N*-propylamino)tetralin, sp. activity 127.0 Ci/mmol) and [^{3}H]*p*-MPPF (4-(2′-methoxy)-phenyl-1-[2′- (*N*-2″-pyridinyl)-*p*-fluorobenzamido]ethyl-piperazine, sp. activity 64.6 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA). TMA-DPH (1-[4-(trimethylammonio)

phenyl]-6-phenyl-1,3,5-hexatriene) and NBD-PE (*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine) were obtained from Molecular Probes (Eugene, OR). The purity of the fluorescent phospholipid NBD-PE was checked by thin layer chromatography on silica gel precoated plates (Sigma) in chloroform/methanol/water (65:35:5, v/v/v) and was found to be pure (gave one spot) when detected by its color or fluorescence. BCA (bicinchoninic acid) reagent kit for protein estimation was obtained from Pierce (Rockford, IL). All other chemicals used were of the highest purity available. GF/B glass microfiber filters were from Whatman International (Kent, UK). Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout. Fresh bovine brains were obtained from a local slaughterhouse within 10 min of death and the hippocampal region was carefully dissected out. The hippocampi were immediately flash frozen in liquid nitrogen and stored at -70°C till further use. Stock solutions of local anesthetics were prepared in 0.25% (v/v) ethanol.

Preparation of Native Hippocampal Membranes

Native hippocampal membranes were prepared as described earlier (Harikumar and Chattopadhyay, 1998a). Bovine hippocampal tissue ($\sim$100 g) was homogenized as 10% (w/v) in a polytron homogenizer in buffer A (2.5 mM Tris, 0.32 M sucrose, 5 mM EDTA, 5 mM EGTA, 0.02% sodium azide, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4). The homogenate was centrifuged at 900 $\times g$ for 10 min at 4°C. The supernatant was filtered through four layers of cheesecloth and the pellet was discarded. The supernatant was further centrifuged at 50,000 $\times g$ for 20 min at 4°C. The resulting pellet was suspended in 10 volumes of buffer B (50 mM Tris, 1 mM EDTA, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4) using a hand-held Dounce homogenizer and centrifuged at 50,000 $\times g$ for 20 min at 4°C. This procedure was repeated until the supernatant was clear. The final pellet (native membranes) was resuspended in a minimum volume of 50 mM Tris buffer (pH 7.4), homogenized using a hand-held Dounce homogenizer, flash frozen in liquid nitrogen and stored at -70°C.

Receptor Binding Assays of Hippocampal Membranes Treated With Local Anesthetics

Receptor binding assays for agonist and antagonist were carried out as described earlier (Harikumar and Chattopadhyay, 1998a) in the presence of varying concentrations of local anesthetics. Briefly, tubes in duplicate containing 1 mg of total protein in a total volume of 1 ml of buffer C (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, pH 7.4) for agonist-binding studies, or in buffer D (50 mM Tris, 1 mM EDTA, pH 7.4) for antagonist-binding assays were used. The contents of the tubes were mixed with appropriate amounts of local anesthetics by adding the local anesthetics from a stock solution made in 0.25% (v/v) ethanol. The final concentration of the local anesthetics in the assay tubes ranged from 10 to 1000 μM in case of procaine, tetracaine, and dibucaine, while in the case of lidocaine, it

ranged from 10 to 2000 μM. Tubes were incubated with the radiolabeled agonist [^{3}H]8-OH-DPAT (final concentration in the assay tube 0.29 nM) for 1 h at room temperature. Nonspecific binding was determined by performing the assay in the presence of 10 μM unlabeled 5-HT. The final concentration of ethanol in the assay tubes was maintained at 0.05% (v/v). Control experiments showed that ethanol at this concentration did not affect the ligand-binding properties of the receptor (Harikumar and Chattopadhyay, 1998b). The incubation was terminated by rapid filtration under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B (1.0 μm pore size) 2.5 cm diameter glass microfiber filters which were presoaked in 0.15% polyethylenimine for 3 h (Bruns *et al.*, 1983). The filters were then washed three times with 3 ml of ice-cold water, dried, and the retained radioactivity was measured in a Packard Tri-Carb 1500 scintillation counter using 5 ml of scintillation fluid.

Antagonist-binding assays in the presence of varying concentrations of local anesthetics were performed as above using [^{3}H]*p*-MPPF as the radioligand. The final concentration of local anesthetics in the assay tubes ranged from 10 to 1000 μM in all cases. The assay tubes contained 0.5 nM [^{3}H]*p*-MPPF in a total volume of 1 ml of buffer D. Nonspecific binding was determined by performing the assay in the presence of 10 μM unlabeled *p*-MPPI.

For experiments in which GTP-γ-S was used, the ligand-binding assays were performed in the presence of varying concentrations of GTP-γ-S as described earlier (Harikumar and Chattopadhyay, 1999) in the presence of local anesthetics. The concentrations of GTP-γ-S leading to 50% inhibition of specific agonist binding (IC_{50}) were calculated by nonlinear regression fitting of the data to a 4-parameter logistic function (Higashijima *et al.*, 1987):

$$B = \frac{a}{1 + (x/I)^S} + b$$

where B is the specific binding of the agonist normalized to control binding (in absence of GTP-γ-S), x denotes concentration of GTP-γ-S, a is the range ($y_{max} - y_{min}$) of the fitted curve on the ordinate (y-axis), I is the IC_{50} concentration, b is the background of the fitted curve (y_{min}), and S is the slope factor. Data were analyzed for statistical significance using one-way ANOVA (Microcal Origin Version 5.0, Microcal Software, Inc., USA).

In experiments involving cholesterol depletion using MβCD (see below), ligand-binding assays were carried out using cholesterol-depleted membranes in the presence of local anesthetics. To eliminate any complication in agonist binding due to the steps involved during the treatment of hippocampal membranes with MβCD, native hippocampal membranes were made to go through all steps of cholesterol depletion except that MβCD was not added to them. The agonist-binding activity of these "control" samples (and not that of native hippocampal membranes as such) was used to evaluate the consequence of cholesterol depletion on the effect of local anesthetics on agonist binding to the 5-HT$_{1A}$ receptor (Table IV). Protein concentration was determined using BCA reagent (Smith *et al.*, 1985).

Table I. IC_{50} Values for Inhibition of Specific [^{3}H]8-OH-DPAT and [^{3}H]*p*-MPPF Binding to 5-HT_{1A} Receptors From Bovine Hippocampal Membranes by Local Anesthetics[a]

	IC_{50} (μM)	
Local anesthetic	[^{3}H]8-OH-DPAT	[^{3}H]*p*-MPPF
Procaine	13	40
Tetracaine	200	170
Dibucaine	78	121
Lidocaine	624	174

[a] From Figs. 2 and 3.

Saturation Binding Assays

Saturation binding assays were carried out using varying concentrations (0.1–7.5 nM) of radiolabeled agonist ([^{3}H]8-OH-DPAT) using native membranes containing 1 mg of total protein in the presence of local anesthetics. Nonspecific binding was measured in the presence of 10 μM unlabeled 5-HT. Binding assays were carried out at room temperature as mentioned above. The specific local anesthetic concentration at which the assays were done was the half maximal inhibition concentration (IC_{50} value) of the respective anesthetic taken from Table I. Binding data were analyzed as described previously (Harikumar *et al.*, 2000). The concentration of bound ligand (RL^*) was calculated from the equation:

$$RL^* = \frac{10^{-9} \times B}{(V \times SA \times 2220)M}$$

where B is the bound radioactivity in disintegrations per minute (dpm) (i.e., total dpm—nonspecific dpm), V is the assay volume in ml, and SA is the specific activity of the radioligand. Scatchard plots (i.e., plots of $[RL^*]/[L^*]$ vs. $[RL^*]$) were analyzed using Sigma-Plot (version 3.1) on an IBM PC. The dissociation constants (K_d) were obtained from the negative inverse of the slopes, determined by linear regression analysis of the plots (in general, $r = 0.91 - 0.99$). The B_{max} values were obtained from the intercept on the abscissa. The B_{max} values reported in Table II have been normalized with respect to the amount of native membrane used. The binding parameters shown in Table II were obtained by averaging the results of three independent experiments, while the saturation binding data shown in Fig. 4 is from a representative experiment.

Measurement of Fluorescence Polarization

Stock solutions of the fluorescent probes (DPH, TMA-DPH, and NBD-PE) were prepared in methanol. Bovine hippocampal membranes containing 100 μg of total protein were resuspended in 1.5 ml of 50 mM Tris buffer (pH 7.4) in the presence of varying concentrations of local anesthetics. The samples were subsequently labeled with the appropriate fluorescent probe (DPH, TMA-DPH, or NBD-PE) to obtain optimal fluorescence intensity by incubating at room temperature

Table II. Effect of Local Anesthetics on Binding Affinity and Sites of [^{3}H]8-OH-DPAT to 5-HT$_{1A}$ Receptors From Bovine Hippocampal Membranes[a]

Condition	K_d (nM)	B_{max} (fmol/mg of protein)
Native membrane (control)[b]	0.73 ± 0.09	109.00 ± 14.3
Procaine	2.85 ± 0.24	115.66 ± 13.71
Tetracaine	1.90 ± 0.08	137.66 ± 12.39
Dibucaine	1.74 ± 0.26	111.00 ± 10.02
Lidocaine	1.70 ± 0.13	115.33 ± 8.51

[a] The binding parameters shown in the table represent the means ± SE from three independent experiments while saturation binding data shown in Fig. 4 is from a representative experiment. The local anesthetics were used at their respective IC_{50} values. See Materials and Methods section for other details.
[b] The control sample had no anesthetic but contained 0.05% ethanol (v/v).

(23°C) for 30 min in dark. The final probe concentration was 1 μM in all cases and the methanol content was always low (<1% (v/v)). In a control experiment, we found that this concentration of methanol did not affect ligand-binding properties of the receptor. Steady-state fluorescence measurements were performed with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes. Excitation and emission slits with a nominal bandpass of 5 nm were used for all experiments. The excitation/emission wavelengths used were 350/428 (for DPH), 355/428 (for TMA-DPH), and 461/530 nm (for NBD-PE), respectively. Excitation/emission wavelengths of 365/428 nm were used for DPH and TMA-DPH in case of samples containing dibucaine to avoid any interference from the anesthetic itself. Background intensities of samples in which fluorophores were omitted were negligible and were subtracted from sample intensities to cancel out any scattering artifacts. To reverse any photoisomerization of DPH, samples were kept in dark in the fluorimeter for 30 s before the excitation shutter was opened and fluorescence was measured (Chattopadhyay and London, 1984). Fluorescence polarization measurements were performed at 23°C using a Hitachi polarization accessory. Polarization values were calculated from the equation (Chen and Bowman, 1965):

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}$$

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is equal to I_{HV}/I_{HH}. All experiments were done with multiple sets of samples and average values of fluorescence polarization are shown in Fig. 7.

Cholesterol Depletion by Treatment of Native Membranes With MβCD

Native membranes were treated with MβCD for depleting cholesterol as described earlier (Klein *et al.*, 1995) with some modifications. Briefly, hippocampal membranes (containing 2 mg of total protein) were incubated in a total volume of 1 ml of 50 mM Tris buffer (pH 7.4) containing 40 mM MβCD by shaking for 1 h at room temperature (23°C). The treated membranes were then centrifuged at 50,000 $\times g$ for 10 min and the supernatant was discarded. The pellet was suspended in 1 ml of 50 mM Tris buffer (pH 7.4) and centrifuged at 50,000 $\times g$ for 10 min. The resulting pellet was resuspended in 1 ml of the same buffer and used for receptor binding assays.

Cholesterol Estimation in Native and Cholesterol-Depleted Membranes

Cholesterol estimation was done as described earlier by Gimpl *et al.* (1997). Lipids were extracted from bovine hippocampal native and cholesterol-depleted membranes with chloroform–methanol essentially according to the method of Bligh and Dyer (1959), with some modifications. Briefly, 300 μl of membranes (1 mg of total protein) and 1.125 ml of chloroform–methanol (1:2, v/v) were vigorously mixed for 10 min and centrifuged for 10 min at 21,000 $\times g$. The supernatant was mixed with 375 μl of chloroform and 375 μl of water and centrifuged for 30 min at 21,000 $\times g$. The lower lipid phase was evaporated under a nitrogen atmosphere and was then dissolved in isopropanol. Cholesterol was assayed spectrophotometrically by the cholesterol oxidase method using a kit supplied by Boehringer Mannheim (Diagnostic Kit No. 139 050).

RESULTS

Inhibition of Specific Agonist- and Antagonist-Binding Activity by Local Anesthetics

Figure 2 shows the effect of increasing concentrations of various local anesthetics on the specific 8-OH-DPAT binding to bovine hippocampal 5-HT_{1A} receptors in native membranes. Agonist binding is inhibited in a concentration-dependent manner in all cases with 50% inhibition in specific binding activity observed at concentrations <200 μM in all cases except lidocaine (see Table I). In case of lidocaine, the drop in specific binding activity of [^{3}H]8-OH-DPAT is more gradual with increasing concentrations which results in a higher IC_{50} value (Table I).

Two selective antagonists for the 5-HT_{1A} receptor, *p*-MPPI and *p*-MPPF, were introduced by Kung *et al.* (1995). Figure 3 shows the inhibition of specific *p*-MPPF binding to the 5-HT_{1A} receptor in native hippocampal membranes by increasing concentrations of local anesthetics. Antagonist binding is found to be inhibited in a concentration-dependent manner in all cases. Interestingly, similar to the results obtained with agonist binding, lidocaine shows a somewhat less sensitivity in inhibiting the specific [^{3}H]*p*-MPPF binding giving rise to a higher IC_{50} value. The half maximal inhibition concentration (IC_{50}) values for various local anesthetics for agonist and antagonist binding are shown in Table I.

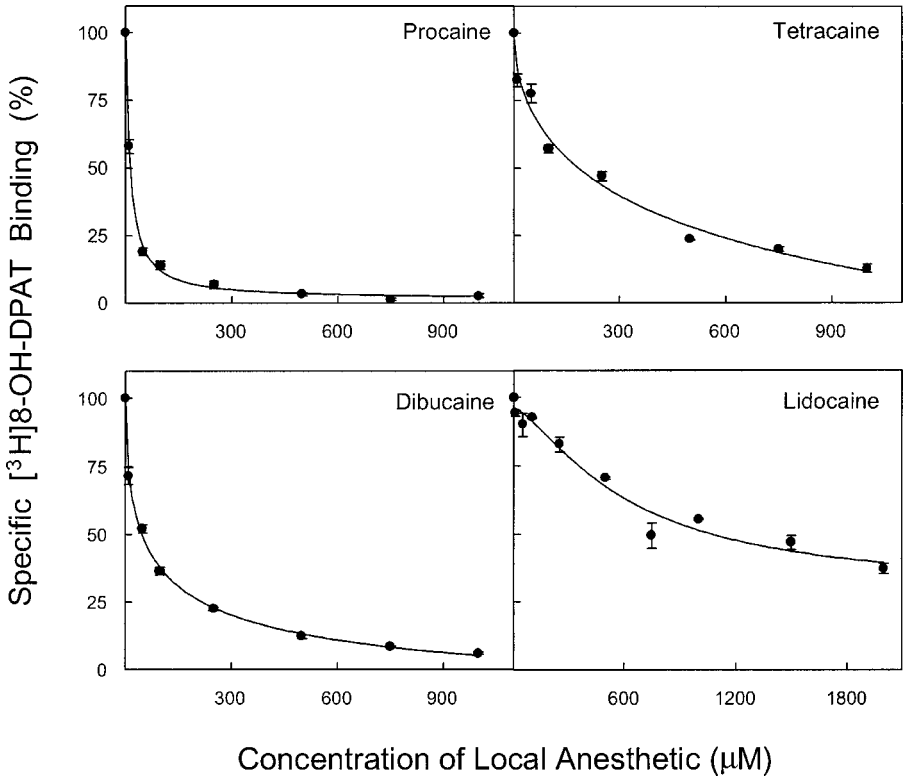

Fig. 2. Effect of increasing concentrations of local anesthetics on the specific binding of the agonist [^{3}H]8-OH-DPAT to the 5-HT$_{1A}$ receptor from bovine hippocampal membranes. Values are expressed as a percentage of the specific binding obtained in the absence of any local anesthetics. The data points represent means ± SE of duplicate points from three independent experiments. See Materials and Methods section for other details.

Changes in Binding Affinity and Sites due to the Action of Local Anesthetics by Saturation Binding Analysis

Figure 4 shows the Scatchard analysis of the specific binding of [^{3}H]8-OH-DPAT to 5-HT$_{1A}$ receptors in bovine hippocampal membranes in the presence and absence of local anesthetics. The binding parameters are shown in Table II. The binding affinity of [^{3}H]8-OH-DPAT is considerably reduced in the presence of local anesthetics with maximum reduction (~fourfold) observed in case of procaine. The values of B_{max}, on the other hand, show no significant change except in case of tetracaine where a slight increase is observed.

G-Protein Coupling to the 5-HT$_{1A}$ Receptor in the Presence of Local Anesthetics: Effect of GTP-γ-S

The 5-HT$_{1A}$ receptor is negatively coupled to adenylate cyclase system through G-proteins (Emerit *et al.*, 1990). Sensitivity of agonist binding to guanine nucleotides

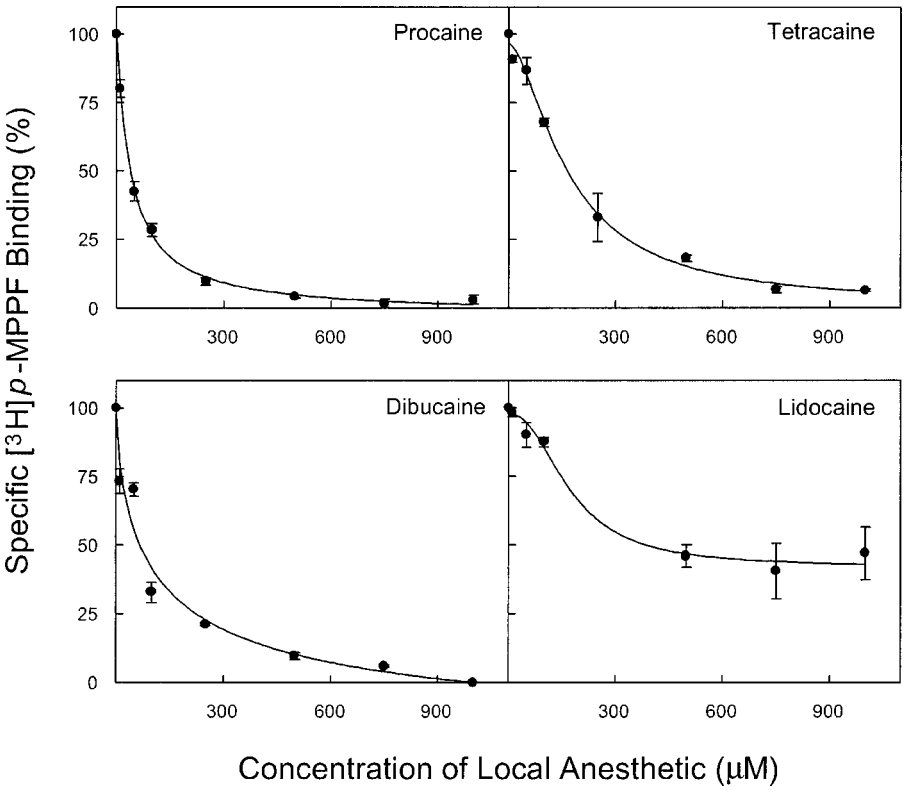

Fig. 3. Effect of increasing concentrations of local anesthetics on the specific binding of the antagonist [^{3}H]*p*-MPPF to the 5-HT_{1A} receptor from bovine hippocampal membranes. Values are expressed as a percentage of the specific binding obtained in the absence of any local anesthetics. The data points represent means ± SE of duplicate points from three independent experiments. See Materials and Methods section for other details.

can be monitored by performing the agonist-binding assay in the presence of GTP-γ-S, a nonhydrolyzable analogue of GTP. Figure 5 shows the inhibition of specific 8-OH-DPAT binding to the 5-HT_{1A} receptor in native membranes by GTP-γ-S in a characteristic concentration-dependent manner (shown as control curves in all four panels). This shows that, as reported earlier by us (Harikumar and Chattopadhyay, 1998a, 1999), the bovine hippocampal 5-HT_{1A} receptor is coupled to G-proteins and GTP-γ-S induces a transition of the receptor from a high-affinity to a low-affinity state. To investigate the effect of local anesthetics on G-protein coupling of the 5-HT_{1A} receptor, we studied the inhibition in agonist binding induced by GTP-γ-S in the presence of various local anesthetics. The results are shown in Fig. 5 and Table III. We observe a shift in the inhibition curves toward higher concentrations of GTP-γ-S in all cases indicating that the concentration of GTP-γ-S necessary to bring about the same change in agonist binding is more in the presence of local anesthetics. It is important to note that the IC_{50} values of GTP-γ-S obtained in presence of each of the anesthetics is higher than that of control which does not contain the anesthetics

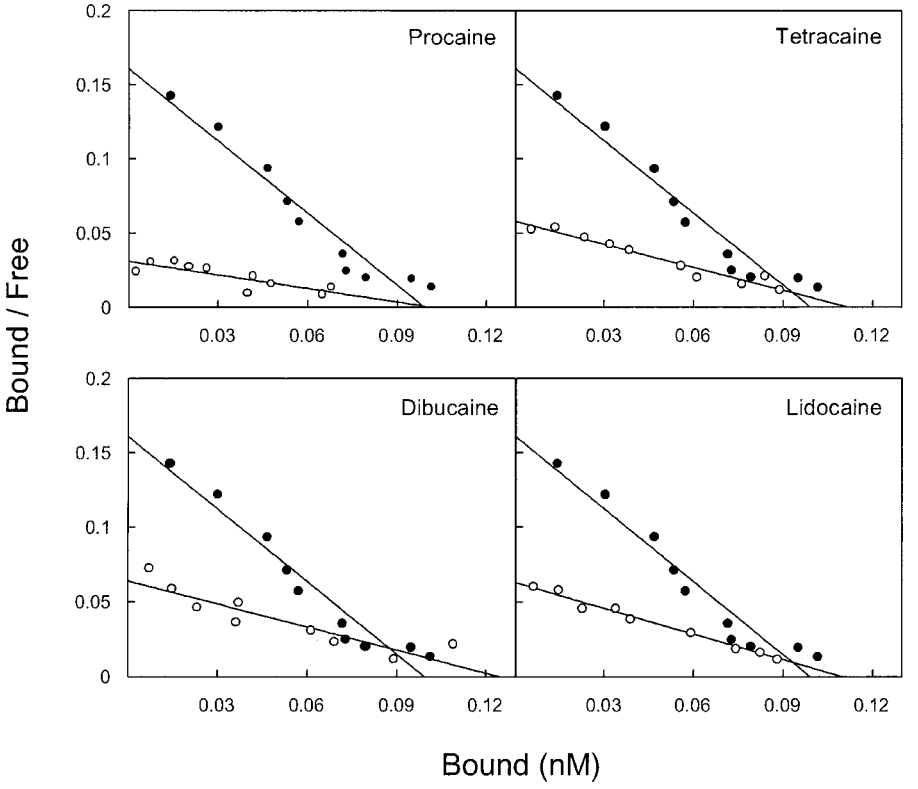

Fig. 4. Scatchard analysis of specific binding of the agonist [^{3}H]8-OH-DPAT to the 5-HT$_{1A}$ receptor from bovine hippocampal membranes in the presence (○) of local anesthetics. The Scatchard plot in the absence of any local anesthetics (●) is shown as a control in all the panels for comparison. The concentration of [^{3}H]8-OH-DPAT ranged from 0.1 to 7.5 nM. The concentrations of the anesthetics correspond to their respective IC$_{50}$ values (see Table I). Data shown are from a representative experiment and each point is the mean of duplicate determinations. See Materials and Methods section for other details.

(see Table III). In other words, the presence of local anesthetics effectively makes the system less sensitive to the effect of GTP-γ-S which shows that the extent of G-protein coupling is reduced by the anesthetics.

Effect of Local Anesthetics on Region-Specific Membrane Fluidity: Steady-State Fluorescence Polarization Measurements Using Fluorescent Probes Localized at Different Depths in the Membrane

Fluidity changes in membranes are generally monitored by measurement of steady-state fluorescence polarization of membrane-bound fluorescent probes. In previous studies of this type, the hydrophobic membrane probe DPH has been widely used (Lentz, 1989). DPH, which is a rod-like molecule, partitions into the interior of the bilayer. However, its precise orientation in the membrane interior is not known.

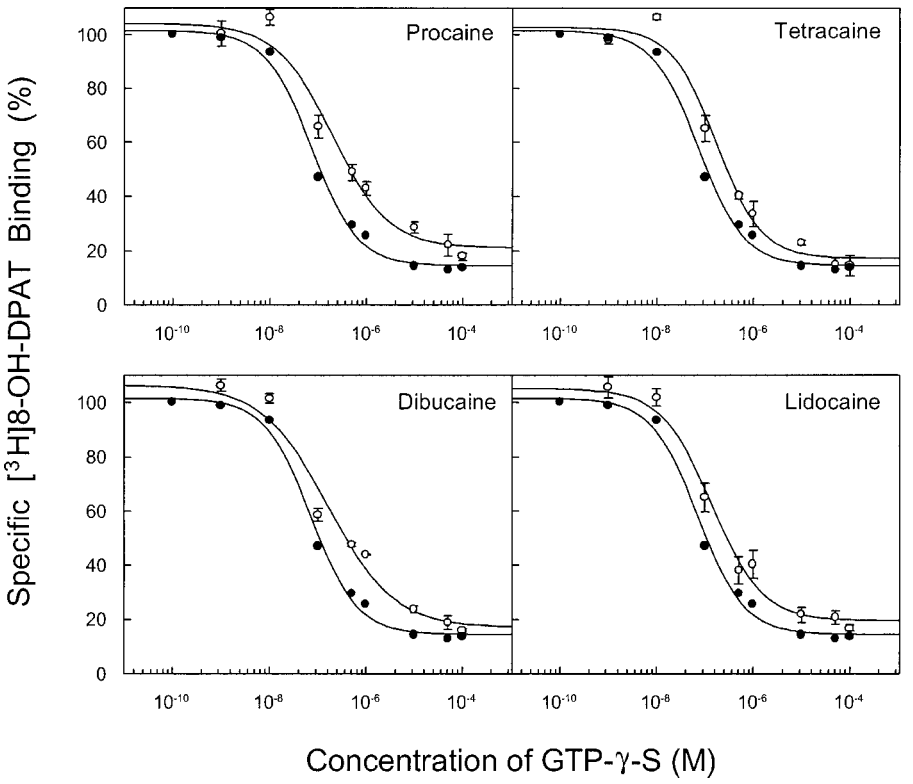

Fig. 5. Effect of increasing concentrations of GTP-γ-S on the specific binding of the agonist [^{3}H]8-OH-DPAT to the 5-HT$_{1A}$ receptor from bovine hippocampal membranes in the presence (○) of local anesthetics. The specific binding obtained with increasing concentrations of GTP-γ-S in the absence of any local anesthetics (●) is shown as a control in all the panels for comparison. Values are expressed as a percentage of the specific binding obtained in the absence of GTP-γ-S. The data points represent means ± SE of duplicate points from three independent experiments. See Materials and Methods section for other details.

Table III. Effect of GTP-γ-S on Specific [^{3}H]8-OH-DPAT Binding to 5-HT$_{1A}$ Receptors From Bovine Hippocampal Membranes in Presence of Local Anesthetics[a]

Local anesthetic	IC$_{50}$ (nM)
Native membrane (control)[b]	72 ± 14
Procaine	191 ± 71[c]
Tetracaine	167 ± 49[d]
Dibucaine	162 ± 76[d]
Lidocaine	157 ± 49[d]

[a]The binding parameters shown in the table represent the means ± SE from three independent experiments. The local anesthetics were used at their respective IC$_{50}$values. See Materials and Methods section for other details.
[b]The control sample had no anesthetic but contained 0.05% ethanol (v/v).
[c]Significant at $P < 0.05$ compared to native membranes.
[d]Significant at $P < 0.1$ compared to native membranes.

In addition, since the membrane is considered as a two-dimensional anisotropic fluid, any possible change in membrane fluidity may not be uniform and restricted to a unique location in the membrane. It is thus better to monitor the change in membrane fluidity at multiple sites in the membrane in order to obtain a comprehensive understanding of any change in membrane (lipid) dynamics induced by anesthetics. Moreover, one of us has previously shown that stress such as heat shock can induce anisotropic changes in membrane fluidity, i.e., the change in membrane fluidity was different when monitored in different regions in adult rat liver cells (Revathi *et al.*, 1994). Interestingly, such depth-dependent modulation of membrane fluidity induced by alcohols (Kitagawa and Hirata, 1992) and local anesthetics (Yun *et al.*, 2002) has also been reported. It is for this reason that we employed three fluorescent probes NBD-PE, TMA-DPH, and DPH to assess the fluidity changes induced by local anesthetics in this study. These probes differ in their orientation and location in the membrane (see Fig. 6). The NBD group in NBD-PE is localized ~19 Å from the center of the bilayer and reports the head group region of the membrane (Abrams and London, 1993; Chattopadhyay and London, 1987). TMA-DPH is a derivative of DPH with a cationic moiety attached to the *para* position of one of the phenyl rings (Prendergast *et al.*, 1981). While DPH is known to partition into the hydrophobic core of the membrane, the amphipathic TMA-DPH is oriented in the membrane bilayer with its positive charge localized at the lipid–water interface (Sojcic *et al.*,

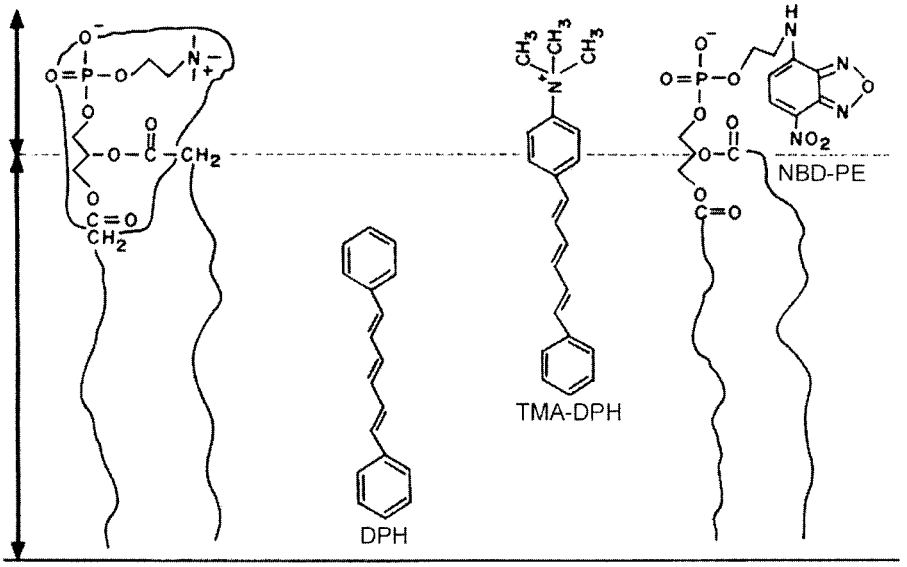

Fig. 6. A schematic representation of half of the membrane bilayer showing the chemical structures and locations of the fluorescent probes DPH, TMA-DPH, and NBD-PE. The location of the NBD group in NBD-PE was earlier deduced by fluorescence quenching measurements (Abrams and London, 1993; Chattopadhyay and London, 1987). The location of the DPH group in TMA-DPH and the average position of DPH in the membrane is shown according to Kaiser and London (1998). The horizontal line at the bottom indicates the center of the bilayer. See text for other details.

1992). Its DPH moiety is localized at ~11 Å from the center of the bilayer and reports the interfacial region of the membrane (Kaiser and London, 1998). In contrast to this, the average location of DPH has been shown to be ~8 Å from the center of the bilayer (Kaiser and London, 1998).

The fluorescence polarization of NBD-PE, TMA-DPH, and DPH in bovine hippocampal membranes in the presence and absence of local anesthetics are shown in Fig. 7. No significant change in polarization is detected by any of the probes in case of procaine and lidocaine. This could be indicative of the fact that the actions of these local anesthetics are probably not mediated through physical perturbations of the lipid bilayer. In case of dibucaine, however, the fluorescence polarization of NBD-PE shows a small increase. A more pronounced increase in fluorescence polarization of NBD-PE is observed in case of tetracaine, especially toward higher concentrations of the added anesthetics. This could indicate that in case of these two local

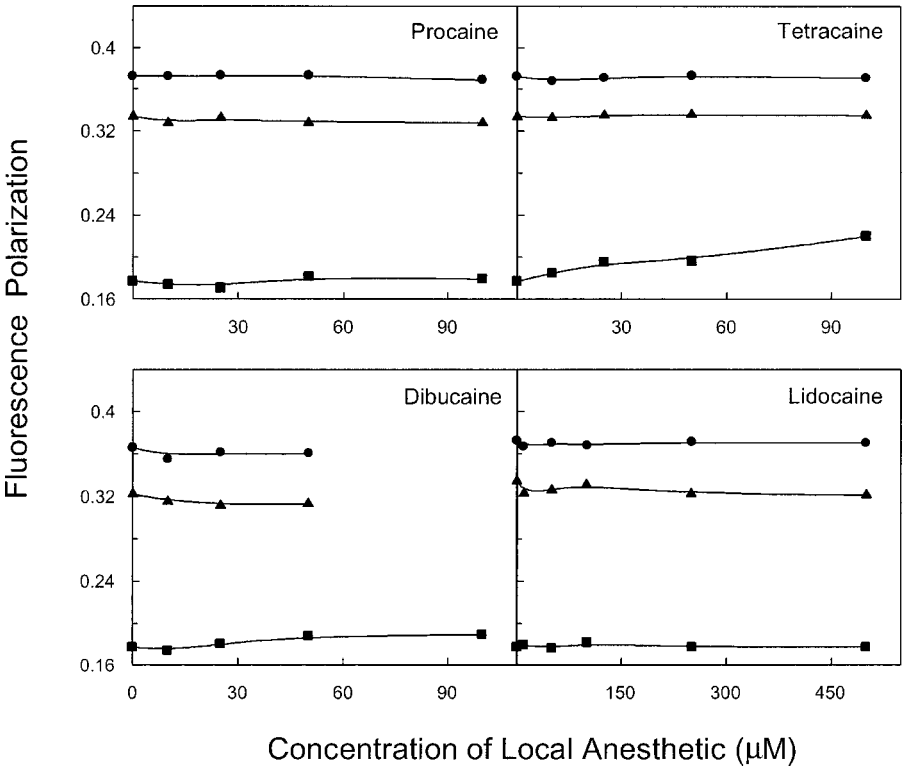

Fig. 7. Effect of increasing concentrations of local anesthetics on the fluorescence polarization of DPH (▲), TMA-DPH (●), and NBD-PE (■) in bovine hippocampal membranes. The data points represent means of duplicate determinations from three independent experiments. The SE in most cases were <3% of the mean values. In experiments where DPH or TMA-DPH is used as the probe with samples containing dibucaine, we used excitation/emission wavelengths of 365/428 nm, and avoided higher concentrations of dibucaine to avoid any interference from the anesthetic itself. The concentration of the probes was 1 μM in all cases and the polarization measurements were done at 23°C. See Materials and Methods section for other details.

anesthetics, the change in lipid dynamics (at least partially) could be a component of their mechanism of action although the effect is smaller in case of dibucaine.

Effect of Cholesterol Depletion

Cholesterol, an essential component of eukaryotic cell membranes, has previously been shown to be important for anesthetic action (Rehberg *et al.*, 1995). Cholesterol depletion from bovine hippocampal membranes has also been found to alter ligand-binding properties of the 5-HT$_{1A}$ receptor (Pucadyil, T. J., and Chattopadhyay, A., unpublished observations). To examine the effect of cholesterol depletion on the local anesthetic-induced inhibition of agonist binding, experiments were designed in which hippocampal membranes were treated with methyl-β-cyclodextrin (MβCD) which selectively extracts cholesterol by including it in a central nonpolar cavity.

The inhibition in specific agonist binding of the 5-HT$_{1A}$ receptor in native as well as cholesterol-depleted hippocampal membranes induced by specific concentrations of the local anesthetics is shown in Table IV. When hippocampal membranes were treated with 40 mM MβCD (which results in 85% cholesterol depletion as assessed by the cholesterol assay described in Materials and Methods section; data not shown) followed by treatment with the local anesthetics, no significant change was observed in the pattern of inhibition in the agonist binding. In other words, the local anesthetics show inhibition of agonist binding to the 5-HT$_{1A}$ receptor in membranes depleted of cholesterol more or less to the same extent as that of control membranes in all cases.

Table IV. Effect of Cholesterol Depletion on the Anesthetic-Induced Inhibition in Specific Binding Activity of [^{3}H]8-OH-DPAT to 5-HT$_{1A}$ Receptors From Bovine Hippocampal Membranes[a]

	Specific binding (%)	
Local anesthetic	Control membrane[b]	Depleted membrane[c]
Procaine	39.17 ± 0.20	44.24 ± 2.56
Tetracaine	57.10 ± 3.12	49.84 ± 1.60
Dibucaine	62.70 ± 6.98	59.46 ± 6.62
Lidocaine	64.10 ± 4.80	63.43 ± 6.30

[a] Data shown represents the means ± SE of duplicate points from three independent experiments. The local anesthetics were used at a concentration close to their respective IC$_{50}$ values.

[b] For preparing control samples, native hippocampal membranes were made to go through all steps of cholesterol depletion except that MβCD was not added to them. Values are expressed in all cases as a percentage of the specific binding obtained with native hippocampal membranes in the absence of any local anesthetics. See Materials and Methods section for other details.

[c] Cholesterol depletion was carried out as described in Materials and Methods section using 40 mM MCβD. Values are expressed in all cases as a percentage of the specific binding obtained with cholesterol-depleted hippocampal membranes in the absence of any local anesthetics.

DISCUSSION

G-protein-coupled receptors constitute a superfamily of proteins whose function is to transmit information across cell membranes from the extracellular environment to the interior of the cell thereby providing a mechanism of communication between the exterior and the interior of the cell. G-protein-coupled receptors are the single largest family of cell surface receptors involved in signal transduction and represent major targets for the development of novel drug candidates in all clinical areas (Bikker *et al.*, 1998). It is estimated that 30% of clinically prescribed drugs function as either agonists or antagonists of G-protein-coupled receptors which emphasizes their immense therapeutic potential (Kallal and Benovic, 2000; Stadel *et al.*, 1997).

We have explored the effects of tertiary amine local anesthetics on the 5-HT_{1A} receptor, an important member of the G-protein-coupled receptor superfamily. Our results show that local anesthetics inhibit specific agonist and antagonist binding to the 5-HT_{1A} receptor from a native source in a concentration-dependent manner. This is accompanied by a concomitant reduction in the binding affinity of the 5-HT_{1A} receptor to the agonist. More importantly, the extent of G-protein coupling is reduced in the presence of the local anesthetics. It is worth mentioning here that the concentrations of local anesthetics used by us are well within the range of clinical concentrations necessary for nerve block (Xiong *et al.*, 1999). In general, local anesthetics are believed to mediate their actions through membranes (Seeman, 1972). Mutagenesis (Ho *et al.*, 1992) and molecular modeling studies (Kuipers *et al.*, 1997) have shown that the ligand-binding site in serotonin receptors in general, and in the 5-HT_{1A} receptor in particular, is located in a transmembrane domain. It is therefore important to determine the actual concentration of the anesthetics partitioned into the membrane while considering their effects on the ligand-binding properties of the 5-HT_{1A} receptor. The concentrations of the local anesthetics in membranes can be determined from their membrane/buffer partition coefficients (De Paula and Schreier, 1995) using a formalism previously developed by us (Harikumar and Chattopadhyay, 1998b) to determine partitioning of alcohols into membranes. Calculations by this approach using literature values of the membrane/buffer partition coefficients of tertiary amine local anesthetics (De Paula and Schreier, 1995) showed that most (>99%) of the added anesthetics got partitioned into the membrane due to their high partition coefficient values in the range of concentrations used by us. It was therefore not necessary to calculate the membrane concentrations of the local anesthetics and the concentrations of the local anesthetics plotted in the figures represent the added concentrations of the anesthetics.

G-protein-mediated modulation of K^+ and Ca^{2+} currents has been shown to be inhibited in the presence of local anesthetics (Xiong *et al.*, 1999). For example, local anesthetics have been proposed to interact with $G\alpha_q$ thereby inhibiting signaling of G-protein-coupled receptors (Hollmann *et al.*, 2001). It is less likely that a similar mode of action of the anesthetics could occur in our system for the following reason. All the four local anesthetics tested show inhibition in the binding of the receptor to the agonist, 8-OH-DPAT as well as to the antagonist, *p*-MPPF. We have earlier shown that the antagonist binding shows no significant reduction over a large range

of GTP-γ-S concentration and is thus insensitive to guanine nucleotides (Harikumar and Chattopadhyay, 1999). If the inhibition in binding of the receptor to its ligands were solely due to the action of the local anesthetics on G-proteins, one would expect the antagonist binding to remain unaffected in the presence of the anesthetics. This suggests that the inhibition in ligand binding by the anesthetics is probably through a mechanism other than a direct interaction with G-proteins.

Results from experiments in which membrane dynamics was monitored in presence of the local anesthetics by measuring fluorescence polarization of added probes provide further information on molecular mechanism of the action of local anesthetics. Our results show that procaine and lidocaine do not show any significant change in membrane fluidity as indicated by the fluorescence polarization of all the three probes used which report local membrane dynamics from different regions of the membrane. Interestingly, procaine is found to inhibit agonist as well as antagonist binding of the 5-HT$_{1A}$ receptor with the lowest IC$_{50}$ of all the four anesthetics studied. Tetracaine and dibucaine were found to alter fluidity of the membrane as indicated from the fluorescence polarization values of NBD-PE reporting the headgroup region of the membrane with increasing concentrations of anesthetics. This indicates a decrease in the rotational mobility of the fluorescent probe in the headgroup region of the membrane. Earlier reports have described membrane perturbing effects of the local anesthetics in relation to their anesthetic action (Sweet and Schroeder, 1986; Yun *et al.*, 2002). However, while all the four local anesthetics affect the ligand binding and G-protein coupling of the serotonin$_{1A}$ receptor, only tetracaine and dibucaine are found to alter the fluidity of the membrane, indicating that one cannot exclude a role for the membrane environment in the action of local anesthetics.

Previous studies have highlighted the role of lipid composition in general (Harris and Groh, 1985; Sweet and Schroeder, 1986), and cholesterol in particular (Rehberg *et al.*, 1995), in the action of anesthetics. Location of the anesthetic in the bilayer and perturbations caused by anesthetics in membranes were found to be dependent on the presence of cholesterol in membranes (Mondal *et al.*, 2001). Also, cholesterol was found to modulate anesthetic actions on sodium channels from human brain cortex incorporated into planar bilayers (Rehberg *et al.*, 1995). Against this background, we addressed the role of cholesterol in the effects of local anesthetics by depleting cholesterol from bovine hippocampal membranes containing the 5-HT$_{1A}$ receptor. The local anesthetics showed inhibition of agonist binding to the 5-HT$_{1A}$ receptor in membranes depleted of cholesterol more or less to the same extent as that of control membranes in all cases. This suggests that the inhibition in ligand binding to the 5-HT$_{1A}$ receptor brought about by local anesthetics is independent of the membrane cholesterol content. Keeping in mind the well-known rigidifying effect of cholesterol in fluid membranes (Yeagle, 1985), this result also implies that fluidity changes are not major determinants in the action of local anesthetics. These results are difficult to interpret in view of the significance of lipid–protein interactions in the functioning of membrane proteins (Lee, 2003) and the sensitivity of the ligand-binding properties of the bovine hippocampal 5-HT$_{1A}$ receptor to membrane cholesterol content itself (Pucadyil, T. J., and Chattopadhyay, A., unpublished observations). Nonetheless, the present results suggest that membrane cholesterol does not have a major role in the effects of the anesthetics on the 5-HT$_{1A}$ receptor.

In summary, we show here that tertiary amine local anesthetics affect the ligand-binding properties and G-protein coupling of the hippocampal 5-HT_{1A} receptor. Further, we have addressed whether these effects on the 5-HT_{1A} receptor by local anesthetics are accompanied by changes in fluidity of the membrane or are dependent on cholesterol content of the membrane. Our results on the effects of the local anesthetics on the 5-HT_{1A} receptor support the possibility that G-protein-coupled receptors could be involved in the action of local anesthetics. Our results, however, do not rule out changes in the lipid dynamics as one of the probable components of the mechanism of action of local anesthetics. It is possible that action of local anesthetics involves a combination of direct interaction of the receptor with the anesthetics and modulation of properties of the lipid environment.

ACKNOWLEDGMENTS

This work was supported by the Council of Scientific and Industrial Research, Government of India. S. K. thanks the Council of Scientific and Industrial Research for the grant of a Senior Research Fellowship. We gratefully acknowledge R. Rukmini for help with fluorescence polarization measurements and S. Bala Tripura Sundari for help with the cholesterol estimation assay. We thank K. G. Harikumar, Thomas Pucadyil, Satinder Rawat, and H. Raghuraman for helpful discussions and members of our laboratory for critical reading of the manuscript.

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