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Effect of capsaicin on ligand binding activity of the hippocampal serotonin$_{1A}$ receptor

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Abstract The serotonin$_{1A}$ receptor is an important member of the G-protein coupled receptor family, and is involved in the generation and modulation of a variety of cognitive, behavioral, and developmental functions. In order to examine the role of membrane material properties in ligand binding activity of the hippocampal serotonin$_{1A}$ receptor, we monitored the function of the receptor in presence of capsaicin. Capsaicin has been previously shown to increase the elasticity of membrane bilayers. Our results show that the ligand binding activity of the hippocampal serotonin$_{1A}$ receptor is reduced in the presence of capsaicin in a linear concentration-dependent manner. This is accompanied by no appreciable change in G-protein coupling of the receptor and overall membrane order. We conclude that material properties of membrane bilayers could play an important role in the function of the serotonin$_{1A}$ receptor in particular, and membrane proteins in general.

Keywords Capsaicin · Hippocampal serotonin$_{1A}$ receptor · Fluorescence anisotropy · Membrane material property · Ligand binding activity

Abbreviations

5-HT$_{1A}$ receptor	5-hydroxytryptamine-1A receptor
DMPC	dimyristoyl-*sn*-glycero-3-phosphocholine
DMSO	dimethyl sulfoxide
DPH	1,6-diphenyl-1,3,5-hexatriene
GABA	γ-aminobutyric acid
GTP-γ-S	guanosine-5'-*O*-(3-thiotriphosphate)
GPCR	G-protein coupled receptor
PMSF	phenylmethylsulfonyl fluoride

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Introduction

The seven transmembrane domain G-protein coupled receptor (GPCR) superfamily is one of the largest and most diverse protein families in mammals and constitute ~1–2% of the human genome [1]. The primary function of GPCRs is to transduce signals across membranes [2, 3]. GPCRs represent major targets for the development of novel drug candidates in all clinical areas [4]. The serotonin$_{1A}$ (5-HT$_{1A}$) receptor is an important GPCR and is the most extensively studied among the serotonin receptors. The serotonin$_{1A}$ receptor is involved in the generation and modulation of a variety of cognitive, behavioral, and developmental functions (for a recent review, see [5]). The serotonin$_{1A}$ receptor agonists and antagonists represent major classes of molecules with potential therapeutic effects in anxiety- or stress-related disorders. Interestingly, mutant (knockout) mice lacking the serotonin$_{1A}$ receptor exhibit enhanced anxiety-related behavior [6] and represent an important animal model for the analysis of complex traits such as anxiety disorders and aggression in higher animals [7, 8].

Since a significant portion of integral membrane proteins remains in contact with the membrane [9], the structure and function of membrane proteins often depend on the membrane lipid composition [10, 11]. The function of membrane proteins can be modulated either by specific (local) lipid–protein interactions, or material (physical)

properties of the membrane, or both. We have previously shown that membrane cholesterol is necessary in maintaining ligand binding activity of the hippocampal serotonin$_{1A}$ receptor [12, 13]. The effect of cholesterol on the structure and function of integral membrane proteins and receptors has been a subject of intense investigation [14, 15]. It has been proposed that cholesterol can modulate the function of membrane proteins in two ways: (1) through a specific (local) interaction with the membrane protein, which could induce a conformational change in the protein [16, 17], or (2) through an indirect way by altering the membrane material properties in which the protein is embedded [11, 18–21] or due to a combination of both. Interestingly, membrane cholesterol has previously been shown to alter the material properties of the membrane [22, 23].

Capsaicin [*N*-(4-hydroxy-3-methoxybenzyl)-8-methylnon-6-eneamide] is a pungent plant alkaloid (see Fig. 1) from the hot pepper, *Capsicum*, and is used in the study of pain and analgesia [24]. It is known that capsaicin, which is an amphiphile, changes the physical properties of membrane bilayers [25–28]. In particular, it has been shown that capsaicin can increase the elasticity of membrane bilayers [21, 25]. In this paper, we have used capsaicin in order to monitor the effect of membrane bilayer elasticity on the ligand binding activity of the hippocampal serotonin$_{1A}$ receptor.

Materials and methods

Materials Capsaicin, DMSO, DMPC, Na_2HPO_4, DPH, EDTA, EGTA, $MgCl_2$, $MnCl_2$, iodoacetamide, PMSF, serotonin, sucrose, polyethylenimine, sodium azide, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO, USA). BCA reagent for protein estimation was from Pierce (Rockford, IL, USA). [^{3}H]8-OH-DPAT (specific activity 106 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA, USA). GTP-γ-S was purchased from Roche Applied Science (Mannheim, Germany). GF/B glass microfiber filters were from Whatman International (Kent, UK). Stock solution of capsaicin was prepared in DMSO. All other chemicals used were of the highest purity available. Water was purified through a Millipore (Bedford, MA, USA) 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.

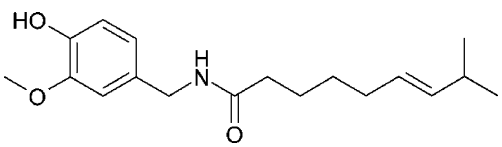

Fig. 1 Chemical structure of capsaicin

Preparation of native hippocampal membranes Native hippocampal membranes were prepared as described previously [12]. Bovine hippocampal tissue (~50 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×*g* for 10 min at 4°C. The resultant supernatant was filtered through four layers of cheesecloth and centrifuged at 50,000×*g* for 20 min at 4°C. The pellet obtained was suspended in 10 vol. 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×*g* for 20 min at 4°C. This procedure was repeated until the supernatant was clear. The final pellet (native membranes) was suspended in a minimum volume of buffer C (50 mM Tris, pH 7.4), homogenized using a hand-held Dounce homogenizer, flash frozen in liquid nitrogen and stored at −70°C. Protein concentration was assayed using BCA reagent [29].

Radioligand binding assays Receptor binding assays were carried out as described earlier [12] with some modifications. Tubes in duplicate with 1 mg protein in a total volume of 1 ml of buffer D (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM $MnCl_2$, pH 7.4) were incubated with the radiolabeled agonist [^{3}H]8-OH-DPAT (final concentration in assay tube being 0.29 nM) and increasing concentrations of capsaicin for 1 h at 25°C. DMSO content was 1% (*v*/*v*) in all assay tubes including control samples. Nonspecific binding was determined by performing the assay in the presence of 10 μM serotonin. The binding reaction was terminated by rapid filtration under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B 2.5 cm diameter glass microfiber filters (1.0 μm pore size), which were presoaked in 0.15% (*w*/*v*) polyethylenimine for 1 h [30]. Filters were then washed three times with 3 ml of cold water (4°C), dried and the retained radioactivity was measured in a Packard Tri-Carb 1500 liquid scintillation counter using 5 ml of scintillation fluid.

Estimation of inorganic phosphate Concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid [31] using Na_2HPO_4 as standard. DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings.

Fluorescence anisotropy measurements Stock solution of DPH was prepared in methanol. Hippocampal membranes

were treated with increasing concentrations of capsaicin for 1 h at 25°C, and 50 nmol of total phospholipid was suspended in 1.5 ml of buffer C and used for fluorescence anisotropy experiments. The amount of probe added was such that the final probe concentration was 1 mol% with respect to the total phospholipid content. This ensures optimal fluorescence intensity with negligible membrane perturbation. Membranes were vortexed for 1 min after addition of the probe and kept in the dark for 1 h before measurements. Background samples were prepared the same way except that the probe was omitted. The final probe concentration was 0.33 μM in all cases and the methanol content was low (~0.3%, *v*/*v*). Steady state fluorescence was measured in a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes at room temperature (~23°C). Excitation and emission wavelengths were set at 358 and 430 nm. Excitation and emission slits with nominal band passes of 1.5 and 20 nm were used. The excitation slit was kept low to avoid any photoisomerization of DPH. In addition, fluorescence was measured with a 30 s interval between successive openings of the excitation shutter (when the sample was in the dark in the fluorometer) to reverse any photoisomerization of DPH [32]. The optical density of the samples measured at 358 nm was 0.13±0.01. The anisotropy values remained identical even after dilution of membrane samples, indicating the absence of any scattering artifact [33]. Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory. Anisotropy values were calculated from the equation [34]:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

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 anisotropy are shown in Fig. 3.

GTP-γ-S sensitivity assay In experiments with GTP-γ-S, agonist binding assays were carried out (as described above), with increasing concentrations of GTP-γ-S in the presence and absence of 0.25 mM capsaicin. The concentrations of GTP-γ-S leading to 50% inhibition of specific agonist binding (IC_{50}) were calculated by nonlinear regression fit of the data to a four parameter logistic function [35]:

$$B = [a/(1 + (x/I)^s)] + b \quad (2)$$

where B is the specific binding of the agonist normalized to agonist binding at the lowest concentration 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.

Statistical analysis Significance levels were estimated using student's two-tailed *t*-test using Graphpad Prism software version 4.00 (San Diego, CA, USA).

Results and discussion

Capsaicin has previously been reported to modulate function of membrane proteins [36]. Figure 2 shows the effect of increasing concentrations of capsaicin on specific [^{3}H]8-OH-DPAT binding to hippocampal serotonin$_{1A}$ receptors. The figure shows that agonist binding is reduced in a concentration-dependent manner with ~50% reduction in specific binding activity when 0.25 mM capsaicin was used. The agonist binding is reduced to ~5% of its original value when 0.5 mM capsaicin was used. We interpret the reduction in agonist binding activity in presence of capsaicin due to its ability to increase the elasticity of the membrane bilayer. It therefore appears that the effect of capsaicin on ligand binding activity is nonspecific, possibly

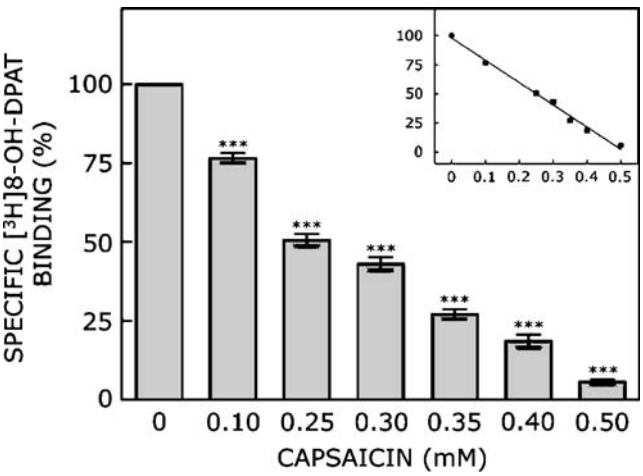

Fig. 2 Effect of capsaicin on specific binding of the agonist [^{3}H]8-OH-DPAT to the hippocampal serotonin$_{1A}$ receptor. Radioligand binding to the hippocampal serotonin$_{1A}$ receptor was monitored with increasing concentrations of capsaicin. A linear relationship of specific binding of [^{3}H]8-OH-DPAT to the hippocampal serotonin$_{1A}$ receptor as a function of increasing concentrations of capsaicin is observed (see *inset*). A linear regression analysis gave an excellent fit (R^2 ~ 0.99). Values are expressed as percentages of specific [^{3}H]8-OH-DPAT binding obtained in control membranes. Data shown are means± standard error from at least eight independent experiments in duplicate [*** correspond to *p*-value<0.0001, the differences in specific [^{3}H]8-OH-DPAT binding were tested against the corresponding value obtained in the absence of capsaicin (control value)]. See "Materials and methods" for other details

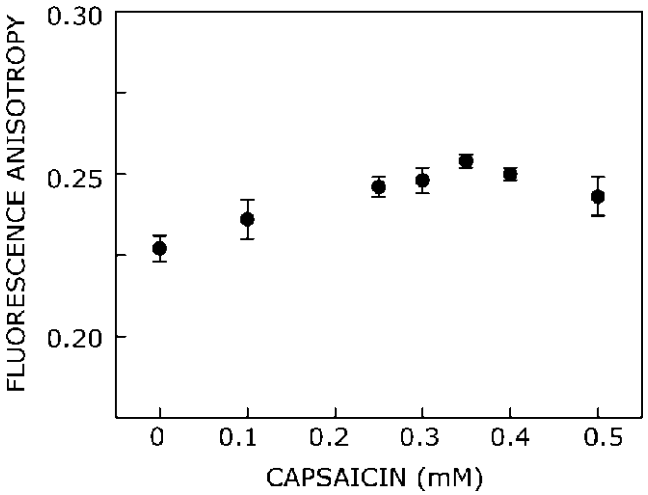

Fig. 3 Fluorescence anisotropy of the membrane probe DPH in hippocampal membranes monitored at varying concentrations of capsaicin. Fluorescence anisotropy measurements were carried out with membranes containing 50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (~23°C). Data represent means±standard error from at least three independent experiments in duplicate. See "Materials and methods" for other details

due to change in elasticity of the membrane bilayer induced by capsaicin. This is supported by the linear relationship between the agonist binding activity and capsaicin concentration (R^2~0.99, see inset of Fig. 2).

In order to monitor the effect of capsaicin on membrane order, we carried out fluorescence anisotropy measurements with the membrane probe, DPH. Fluorescence anisotropy measured using probes such as DPH is correlated to its rotational diffusion [34], which is sensitive to the packing of lipid chains. This is due to the fact that fluorescence anisotropy depends on the degree to which the probe is able to reorient after excitation, and probe reorientation is a function of local lipid packing. DPH, which is a rod-like hydrophobic molecule, partitions into the interior (fatty acyl chain region) of the bilayer. Figure 3 shows that the fluorescence anisotropy of DPH exhibits a modest (up to ~12%) increase with increasing concentrations of capsaicin. Similar change in fluorescence anisotropy of DPH [26] and other fluorescent probes [27] in presence of capsaicin have previously been reported.

As mentioned above, the reduction in agonist binding in presence of capsaicin could be attributed to its ability to increase the elasticity of the membrane bilayer rather than a direct effect. In such a scenario, it would be interesting to explore the effect of capsaicin on downstream signaling. To examine this, we monitored whether the G-protein coupling of the hippocampal serotonin$_{1A}$ receptor is altered upon capsaicin treatment. Most of the seven transmembrane domain receptors are coupled to G-proteins [37], and guanine nucleotides are known to regulate agonist binding. The serotonin$_{1A}$ receptor is negatively coupled to adenylate cyclase system through G-proteins [38]. Sensitivity of agonist binding to guanine nucleotides can be monitored by performing the agonist binding assay in the presence of GTP-γ-S, a non-hydrolyzable analogue of GTP. We previously showed that the specific binding of the agonist [^{3}H]8-OH-DPAT to hippocampal serotonin$_{1A}$ receptors is sensitive to guanine nucleotides and is inhibited with increasing concentrations of GTP-γ-S. Figure 4 shows the inhibition of specific [^{3}H]8-OH-DPAT binding to the serotonin$_{1A}$ receptor in hippocampal membranes by GTP-γ-S in a characteristic concentration-dependent manner, with an estimated half maximal inhibition concentration (IC_{50}) of 77 nM. This shows that the hippocampal serotonin$_{1A}$ 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, as reported previously [39]. Interestingly, the inhibition curve in case of membranes treated with capsaicin (0.25 mM) does not display significant shift, as apparent from an estimated IC_{50} value of 70 nM. This shows that capsaicin treatment does not alter G-protein coupling of the serotonin$_{1A}$ receptor.

Our results show that the ligand binding activity of the hippocampal serotonin$_{1A}$ receptor is reduced in the presence of capsaicin, possibly due to increase in membrane elasticity (*i.e.*, decrease in membrane stiffness). Such alteration in ligand binding by membrane elasticity has previously been observed for the $GABA_A$ receptor [21]. In addition, the function of membrane ion channels such as gramicidin [22], and the sodium channel [20] have been reported to be

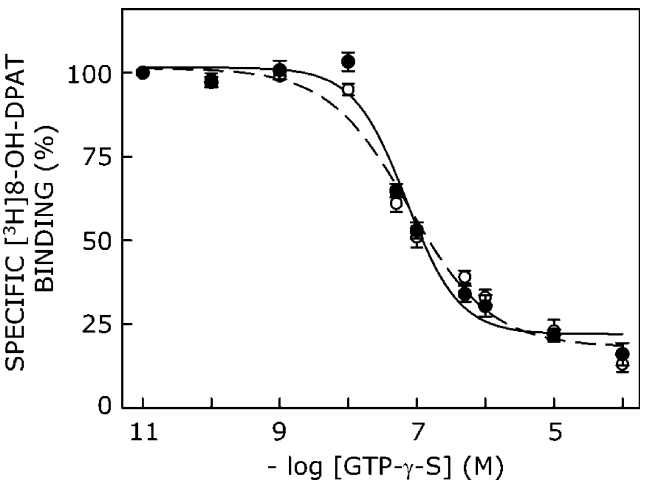

Fig. 4 Effect of increasing concentration of GTP-γ-S on the specific binding of [^{3}H]8-OH-DPAT to hippocampal serotonin$_{1A}$ receptors in the presence (*filled circle* in *solid line*) or absence (*empty circle* in *dashed line*) of 0.25 mM capsaicin. Values are expressed as percentages of the specific binding obtained at the lowest concentration of GTP-γ-S. The curves are non-linear regression fit to the experimental data using Eq. 2. The data points represent means± standard error from at least five independent experiments in duplicate. See "Materials and methods" for other details

dependent on membrane elasticity. To the best of our knowledge, this constitutes the first report describing the role of membrane mechanical properties in ligand binding of this important neurotransmitter receptor (serotonin$_{1A}$ receptor).

We have previously shown that depletion of membrane cholesterol results in reduction of the ligand binding activity of the hippocampal serotonin$_{1A}$ receptor [12]. Interestingly, cholesterol depletion also increases bilayer elasticity (reduces stiffness) [22, 23]. Whether the loss in activity of the hippocampal serotonin$_{1A}$ receptor, earlier reported by us [12], is due to a change in membrane elasticity induced by cholesterol depletion represents an interesting question. This could also be important in cases where the role of other types of membrane lipids (such as glycosphingolipids) are considered in relation to membrane protein function. We conclude that material properties of the bilayer are important in addressing the role of membrane lipids in the function of membrane proteins.

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