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EFFECT OF FREE FATTY ACIDS ON $GABA_A$ RECEPTOR LIGAND BINDING

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Abstract—Phospholipase A_2 (PLA_2) treatment of synaptosomal membranes, which causes the release of fatty acids, particularly unsaturated fatty acids, inhibits the flux of chloride ions through the γ-aminobutyric acid (GABA) benzodiazepine receptor ion channel in response to activation by agonists. PLA_2 treatment has also been shown to affect ligand binding to the receptor. In the present study, we have investigated the effect of unsaturated free fatty acids, arachidonic acid and oleic acid and saturated free fatty acids, arachidic acid and stearic acid on various characteristics of $GABA_A$ receptor ligand binding. Only the unsaturated fatty acids showed any effect: arachidonic acid and oleic acid enhanced flunitrazepam binding and muscimol binding but inhibited *tert*-butylbicyclophosphorothionate (TBPS) binding in a dose-dependent manner. The effects on muscimol and TBPS binding were shown to be due to changes in receptor density by saturation analysis. Oleic acid and arachidonic acid also decreased the enhancement of flunitrazepam and muscimol binding by cartazolate and pentobarbital but did not affect GABA enhancement of flunitrazepam binding. These data indicate that unsaturated free fatty acids can mimic the effects of PLA_2 treatment and underline the importance of the lipid microenvironment on ligand binding to the $GABA_A$ receptor.

The γ-aminobutyric acid (GABA†) benzodiazpine receptor ($GABA_A$ receptor) is widely distributed throughout the mammalian central nervous system. Agonist activation of this protein oligomer leads to the opening of an integral anion channel which generally results in cellular hyperpolarization and a reduction in excitability. The receptor *in vivo* is subject to control by a number of distinct allosteric sites, the most notable of which are those for the benzodiazepines, the barbiturates and several metabolites of progesterone and deoxycorticosterone [1]. There are additional allosteric sites on this receptor the function of which is less clearly defined. *tert*-Butylbicyclophosphorothionate (TBPS) is thought to interact within the ion channel itself and has been used, in radioligand binding experiments, to report on occupancy of other recognition sites within the receptor oligomer [2, 3].

In addition, it is clear that phospholipase treatments of membrane preparations also affect the characteristics of the $GABA_A$ receptor complex. The action of phospholipase A_2 leads to the accumulation of free fatty acids, especially of unsaturated fatty acids such as arachidonic acid and docosahexenoic acids which are found in greatest concentrations in neural tissue [4]. The addition of free fatty acids to membrane binding assays can mimic all of the effects of phospholipase A_2 treatment ([5], this study.)

Free fatty acids are known to affect the function of many receptors including oestradiol [6] and muscarinic [7] receptors, potassium channels [8, 9] and the nicotinic acetylcholine receptor-cation channel [10]. The purpose of this study is to examine the effects of free fatty acids on flunitrazepam, muscimol (a GABA agonist) and TBPS binding to rat brain synaptic membranes.

MATERIALS AND METHODS

Materials. All chemicals were reagent grade and obtained from either Sigma or BDH with the following exceptions. Radiolabelled compounds were obtained from NEN [^{35}S]TBPS, [^{3}H]flunitrazepam, [^{3}H]muscimol). Unlabelled TBPS was from NEN, cartazolate was a gift from Squibb Inc. and clonazepam was a gift from Hoffman-La Roche.

Tissue preparation. Rats (male Sprague–Dawley, 200–250 g) were killed by stunning and decapitation and the brains removed. The brainstem was removed and the remaining forebrain and cerebellum, usually 40 g from 25 rats, was scissor minced and homogenized in 320 mL of ice-cold buffer A (0.32 M sucrose, 1 mM EDTA, 50 mg/L bacitracin, 50 mg/L ovomucoid trypsin inhibitor, 1 mM benzamidine, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) adjusted to pH 7.4 with Tris) at 170 rpm for 8–10 passes. The homogenate was centrifuged at 1000 *g* for 10 min, the supernatant kept aside on ice and the pellet resuspended in 160 mL buffer A, homogenized (four strokes) and centrifuged at 1000 *g* for 10 min. The pellet was discarded and the supernatant combined with that obtained above and centrifuged at 20,000 *g* for 35 min at 4°.

The resulting pellet was resuspended in 72 mL buffer A and carefully layered onto discontinuous sucrose gradients prepared as follows: 12 mL

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† Abbreviations: GABA, γ-aminobutyric acid; PLA_2, phospholipase A_2; PMSF, phenylmethylsulfonyl fluoride; TBPS, *tert*-butylbicyclophosphorothionate.

Table 1. Binding assay conditions

	[^{3}H]Flunitrazepam	[^{3}H]Muscimol	[^{35}S]TBPS
Buffer	Tris-citrate 100 mM	Buffer R*	Buffer R*
pH	7.1	7.4	7.4
Temperature (°C)	4	4	25
Time (min)	90	75	180
Non-specific ligand	Clonazepam	GABA	Picrotoxin
Concentration (μM)	6	100	10
Specific activity (Ci/mmol)	80–85	7 or 25	60–120†
Membrane protein concentration (mg/mL)	0.16–0.26	0.34–0.42	0.13–0.18

* Buffer R: 140 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes pH 7.4.
† Adjusted for half life = 87 days.

homogenate above 13 mL 0.8 M sucrose (including EDTA and protease inhibitors as in buffer A) above 13 mL 1.2 M sucrose (including inhibitors) and centrifuged at 100,000 *g* for 115 min in a swinging bucket rotor. The synaptosomal layer was collected from the 0.8 M/1.2 M interface, diluted 1:5 with 0.2 M sucrose (including inhibitors as above) and centrifuged at 20,000 *g* for 40 min. The pellet was resuspended in 120 mL buffer B (5 mM Tris (pH 7.1—citrate), 1 mM EDTA, 50 mg/L bacitracin, 50 mg/L ovomucoid trypsin inhibitor, 1 mM benzamidine, 0.1 mM PMSF) using a Polytron homogenizer at setting 5 for 10 sec. After the addition of a further 240 mL buffer B, the synaptosomal membranes were washed five times by centrifugation and resuspension in 360 mL of fresh buffer B. The final suspension was stored at −15° for up to 3 months. On the day of the binding assay, the synaptic membranes were thawed, then washed five times by centrifugation and resuspension in assay buffer.

Ligand binding assays. Binding assay conditions particular to each ligand are summarized in Table 1. The final incubation volume was 1 mL. Each point was measured in triplicate. The incubation was terminated by addition of ice-cold buffer (4 mL) and filtration through Whatman GF/B filters. Free fatty acids, oleic acid, arachidonic acid and stearic acid were dissolved in water by sonication and added to the assay in 100 μL, control tubes contained 100 μL water. Arachidonic acid was dissolved in ethanol then diluted in water. These fatty acids exhibit limited solubility in aqueous solution and while the stock solutions (1 mM) in water appeared as a clear solution, it is not possible to assess the "solution concentration" under the assay conditions. At concentrations above 100 μM both arachidonic acid and oleic acids had limited solubility under the assay conditions. Control tubes contained the appropriate concentration of solvent.

GABA (100 μM), cartazolate (1 μM) and pentobarbital (1 mM) enhancement of [^{3}H]flunitrazepam binding was determined in the presence of 150 mM NaCl. Scatchard analysis of muscimol binding was performed by using increasing concentrations of [^{3}H]muscimol from 0.4 nM to either 5 or 10 nM and then increasing the non-labelled muscimol concentration from 0 to 200 or 500 nM, typically 10–14 different concentrations were used in each experiment. Saturation analysis of TBPS binding was performed by displacement of radiolabelled TBPS (3 nM) with non-labelled TBPS using eight different concentrations over the range 0–450 nM.

Data analysis. The data were analysed by Scatchard transformation using linear regression analysis with the EBDA program of McPherson [11]. The resultant parameters were then used as initial parameter estimates for non-linear regression using the LIGAND program of Munson and Rodbard [12]. A more complex model was accepted only when there was a statistically significant (F test, $P = 0.05$) improvement in the fit.

Protein concentration was determined according to the method of Lowry *et al.* [13] using bovine serum albumin as a standard. Standard curves included appropriate amounts of either Hepes or Tris buffer as necessary.

RESULTS

[^{3}H]*Flunitrazepam binding*

The unsaturated free fatty acids oleic acid and arachidonic acid enhanced [^{3}H]flunitrazepam binding (Fig. 1) and decreased the modulatory effects of GABA, cartazolate and pentobarbital on [^{3}H]-flunitrazepam binding although the effect on GABA modulation was not significant (Fig. 2). The saturated free fatty acids stearic acid and arachidic acid had no effect at 100 μM (data not shown).

[^{3}H]*Muscimol binding.*

Similarly oleic acid and arachidonic acid enhanced muscimol binding (Fig. 1) and decreased the modulatory effects of cartazolate and pentobarbital (Fig. 3). Stearic acid and arachidic acid had no effect at 100 μM (data not shown). Scatchard analysis of the effect of 50 μM arachidonic acid showed that this enhancement was due to an increase in the maximal number of binding sites rather than a change in the affinity of muscimol. A typical experiment is shown in Fig. 4. The average binding affinity in the control experiment was 2.7 ± 0.4 nM ($N = 4$; $P = 0.05$) which is not significantly different from the affinity observed in the presence of arachidonic acid (K_d = 3.8 ± 1.0 nM). However, the maximal number of binding sites (B_{max}) was significantly different ($P = 0.05$): B_{max} (control) =

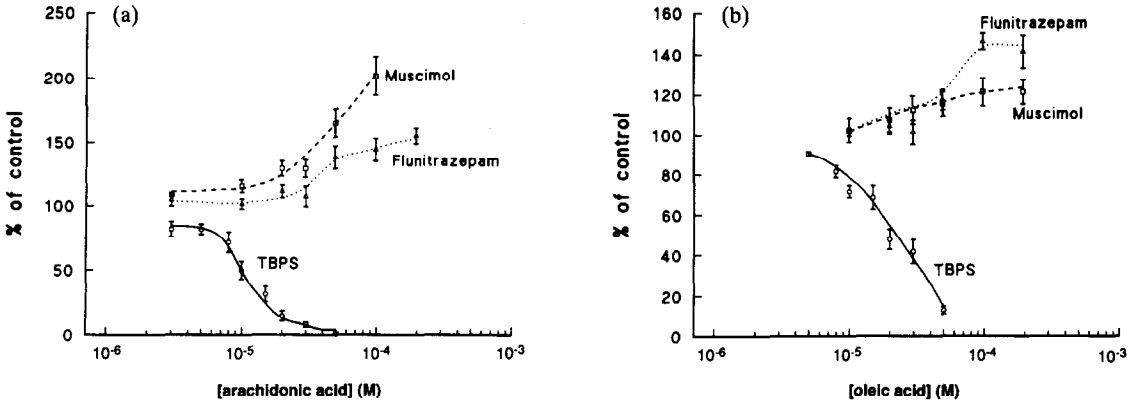

Fig. 1. Dose–response curve of the effect of fatty acids (a) arachidonic acid and (b) oleic acid on the binding of [^{3}H]muscimol (10 nM), [^{3}H]flunitrazepam (0.5 nM) and [^{35}S]TBPS (3 nM) to synaptic membranes. Data are expressed as a percentage of control values: error bars represent ± SEM (N = 5 experiments). The average specific binding in the control was 3400 dpm for 10 nM [^{3}H]muscimol, 5700 dpm for 0.5 nM [^{3}H]flunitrazepam and 6300 dpm for 3 nM [^{35}S]TBPS.

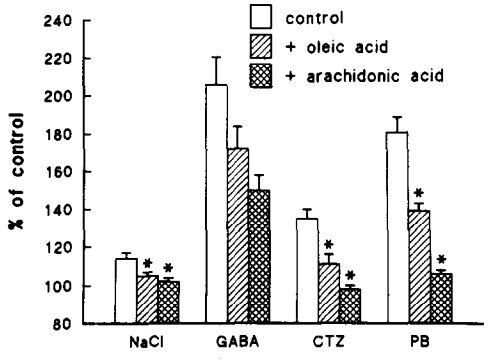

Fig. 2. Effect of oleic and arachidonic acids on the allosteric modulations of [^{3}H]flunitrazepam binding to synaptic membranes. Synaptic membranes were incubated with [^{3}H]flunitrazepam (0.5 nM) and various allosteric modulators, namely; NaCl (150 mM) alone and in combination with; GABA (100 μM), cartazolate (CTZ, 1 μM) or pentobarbital (PB, 1 mM). In addition tubes contained either water, oleic acid (100 μM in water) or arachidonic acid (100 μM in water). Data are expressed as a percentage of the control value: error bars represent ± SEM (N = 4 separate experiments). The average control specific binding of 0.5 nM [^{3}H]flunitrazepam was 6500 dpm. *Significantly different from the respective control (P = 0.05, ANOVA test).

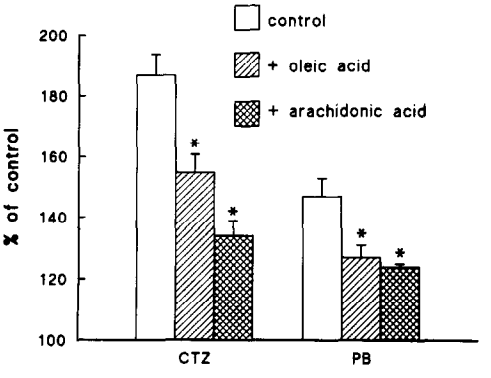

Fig. 3. Effect of oleic and arachidonic acids on the allosteric modulations of [^{3}H]muscimol binding to synaptic membranes. Synaptic membranes were incubated with [^{3}H]muscimol (10 nM) and either cartazolate (CTZ, 10 μM) or pentobarbital (PB, 1 mM) in the presence of either water, oleic acid (100 μM) or arachidonic acid (100 μM). Data are expressed as percentages of the control: error bars represent μ SEM (N = 5 separate experiments). The average control specific binding was 3400 dpm for 10 nM [^{3}H]muscimol. *Indicates a significant difference from the respective control (P = 0.05, ANOVA test).

0.99 ± 0.20 pmol/mg compared to B_{max} (+ arachidonic acid) 1.73 ± 0.33 pmol/mg.

[^{35}S]TBPS binding.

Oleic acid and arachidonic acid inhibited [^{35}S]-TBPS binding in a concentration-dependent manner (Fig. 1) but stearic acid and arachidic acid were ineffective at 100 μM (data not shown). The IC_{50} for oleic acid was 19 μM and for arachidonic acid was 9.7 μM. Scatchard analysis of the effect of 20 μM oleic acid showed that the inhibition of binding is due to a decrease in the maximal number of binding sites rather than a change in affinity (Fig. 5). The average control affinity was 29 ± 2 nM (N = 5) which was not significantly different to the average affinity observed in the presence of oleic acid (K_d = 26 ± 3 nM; N = 5). However, the control B_{max} was 2.4 ± 0.4 pmol/mg which is significantly different (P = 0.05) from the B_{max} in the presence of oleic acid (1.2 ± 0.2 pmol/mg). Figure 5 shows one of five such experiments.

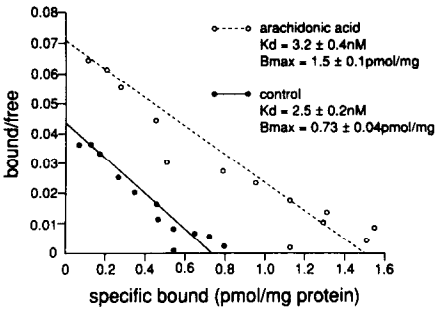

Fig. 4. Scatchard plot of [^{3}H]muscimol binding to synaptic membranes: effect of arachidonic acid (50 μM). Representative example of saturation analysis of [^{3}H]-muscimol binding to synaptic membranes. The replicate error was less than 5% (SD).

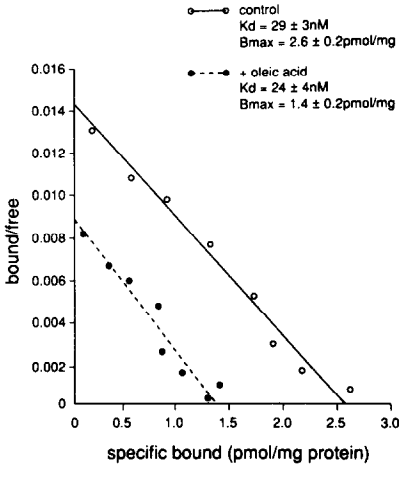

Fig. 5. Scatchard plot of [^{35}S]TBPS binding to synaptic membranes: effect of oleic acid (20 μM). Representative example of saturation analysis of [^{35}S]TBPS binding to synaptic membranes. The replicate error was less than 5% (SD).

DISCUSSIONS

Arachidonic and oleic acids enhance both [^{3}H]-flunitrazepam and [^{3}H]muscimol binding and inhibit [^{35}S]TBPS binding in a dose-dependent manner (Fig. 1). The mechanism of enhancement of benzodiazepine binding involves a change in affinity [5] while Figs 4 and 5 show that the mechanisms of muscimol enhancement and TBPS inhibition involve a change in receptor density. This pattern is similar to that of barbiturates [14–18], pyrazolopyridines [19, 20] and anaestheric steroids [21, 22]. These drugs also enhance benzodiazepine binding by a change in affinity and enhance GABA agonist binding and inhibit TBPS binding by a change in receptor density.

It is not clear whether the effects of the fatty acids, barbiturates, pyrazolopyridines and steroids are due to a direct interaction with the receptor or an indirect action at the lipid–protein boundary layer. The clear structure activity profile of steroids suggests a receptor effect [21] although certain anaestheric steroids are known to have a membrane disordering effect and therefore an interaction with the lipid environment cannot be ruled out [22]. It is interesting to note that a number of other anaesthetic compounds such as alcohols and chloroform which also cause increased membrane fluidity show dose-dependent inhibition of TBPS binding [23].

Free fatty acids also affect the allosteric modulations of flunitrazepam and muscimol binding by cartazolate and pentobarbital. The effect of unsaturated free fatty acids on the allosteric modulations of flunitrazepam and muscimol is to cause a decrease in the effects of cartazolate and pentobarbital but not GABA (Figs 2 and 3). These effects are similar to those of phospholipase A_2 treatment [24–26] which causes the release of free fatty acids.

The effects of free fatty acids on GABA/benzodiazepine binding characteristics also shows a similar pattern to the effects seen after washing synaptic membranes with Triton X-100 detergent. Yang and Olsen [15] noted that washing of synaptic membranes with this detergent causes reduced pentobarbital enhancement of muscimol binding and reduced TBPS binding. The explanation proposed was that Triton treatment causes a shift in binding of GABA agonists from low to high affinity states. Although, in this study, the low affinity muscimol binding sites could not be observed because a filtration assay was used, there was an increase in the receptor density of high affinity muscimol binding, a decrease in pentobarbital enhancement of muscimol and reduced TBPS binding which is compatible with the rationalization forwarded by Yang and Olsen [15] concerning a shift in receptor conformation from low affinity to high affinity agonist binding sites.

In contrast though, it has been suggested that GABA enhancement of flunitrazepam binding occurs through a low affinity GABA binding site [27]. There was some effect of fatty acids on the modulation of flunitrazepam by GABA but this effect did not reach statistical significance.

There is not yet much data regarding the effects of fatty acids on the function of the GABA/benzodiazepine receptor either by electrophysiological or biochemical methods. Schwartz *et al.* [28] have shown that PLA_2 treatment, which results in an increase in free fatty acids, causes a decrease in the activation of $^{36}Cl^-$ flux by muscimol and pentobarbital. This is consistent with the view that binding to low affinity sites results in opening of the chloride channel and the mechanism of fatty acids in reducing chloride flux is by a conversion of low affinity binding states to high affinity states.

Whatever the mechanism involved in the action of free fatty acids on the GABA/benzodiazepine receptor, their effects may explain, in part, why there is such large variation in the results of membrane binding assays. It is evident that different methodologies, even subtle variations of one method, may lead to different levels of fatty acids and this

will affect the apparent coupling between the allosteric sites and affect the apparent receptor density for each ligand and thus the ratio of ligand binding sites. For example, a preparation with higher levels of free fatty acids will appear to have a higher density of muscimol and flunitrazepam binding sites and a lower density of TBPS binding sites.

This study and others [5, 28] have shown that fatty acids have a complex effect on the binding characteristics of the GABA/benzodiazepine receptor. It remains to be seen whether such endogenous molecules have a role to play *in vivo*.

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