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Modulation of [^{3}H] TBOB binding to the rodent $GABA_A$ receptor by simple disaccharides

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Abstract

We have recently reported evidence that a simple β-linked alkylated mannose reversibly increased the magnitude of $GABA_A$ receptor currents evoked in cultured rat pyramidal neurons whilst concomitantly reducing the incidence of spontaneous synaptic activity. In this present study, the effects of the simple β-linked disaccharide, lactose was investigated using a [^{3}H] TBOB (*t*-[^{3}H] butylbicycloorthobenzoate) binding assay in adult rat forebrain and cerebellum membranes. Lactose elicited a significant potentiation of [^{3}H] TBOB binding to well-washed forebrain and cerebellar membranes (mean E_{max} values = 367 and 287%; mean EC_{50} values = 1.5 and 30 μM, respectively, N = 4). The α-linked disaccharides, maltose and sucrose also potentiated [^{3}H] TBOB binding, but with 100–600-fold higher EC_{50} values than lactose. The lactose-mediated potentiation of [^{3}H] TBOB in the forebrain and cerebellum was completely abolished in the presence of 0.3 μM GABA. Over the concentration range in which significant potentiation of [^{3}H] TBOB binding was detected, lactose elicited no significant effect upon [^{3}H] flunitrazepam binding. This study demonstrated that lactose can modulate the $GABA_A$ receptor channel, allosterically coupled to the agonist site, but independent of the benzodiazepine site. Furthermore, lactose displayed differential effects upon forebrain and cerebellar $GABA_A$ receptors indicating that it may be a novel subtype selective agent.

Keywords: $GABA_A$ receptor; Lactose; [^{3}H] TBOB; Forebrain; Cerebellum; β-Linked sugars

1. Introduction

Gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the vertebrate brain, mediates fast neuronal inhibition by binding to the $GABA_A$ receptor and opening an integral chloride channel. This receptor is of great interest therapeutically because it comprises several of the binding sites for pharmaceutically important drugs which interact allosterically with the GABA agonist site or the channel, including the anxiolytic benzodiazepines the anticonvulsant barbiturates, the anxiogenic beta-carbolines and the convulsant picrotoxin agents [1].

Deacetylated-caloporoside, a derivative of the natural fungal extract caloporoside, is a low-affinity $GABA_A$ receptor antagonist [2]. Based on this template, we have recently reported that a simple amphiphilic β-linked alkylated mannose, octyl-*O*-β-D-mannopyranoside significantly and reversibly increased the magnitude of $GABA_A$ receptor currents evoked in cultured rat pyramidal neurons, and exerted similar effects to those of commonly used depressant and hypnotic drugs [3]. These compounds share an apparent disaccharide moiety with a β-D-mannoside linkage, which we hypothesised underlies their interaction with the $GABA_A$ receptor. Based on this hypothesis we suggested that a simple disaccharide may display significant $GABA_A$ receptor modulatory properties. [^{3}H] TBOB is a well used channel-site radioligand which is a sensitive measurement of antagonists (e.g. picrotoxinin) and enhancers (e.g. benzodiazepine agonists) of $GABA_A$ receptor current (e.g. [4]). We report evidence to support the hypothesis that the β-linked disaccharide, lactose, modulates specific [^{3}H] TBOB binding to the central $GABA_A$ receptor channel.

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2. Materials and methods

2.1. Materials

[^{3}H] TBOB (*t*-[^{3}H] butylbicycloorthobenzoate) and [^{3}H] flunitrazepam were obtained from Amersham Biotech (Amersham) with specific activities of 30 and 70 Ci/mmol, respectively. Picrotoxinin and disaccharide sugars were obtained from Sigma Pharmaceuticals (Poole).

2.2. Methods

A series of dose–response competition binding experiments were performed using specific [^{3}H] TBOB binding defined with 10^{-4} M picrotoxinin to adult rat forebrain or cerebellar P_2 membranes [5].

2.2.1. Tissue preparation

Adult male rats (200–300 g), Wistar strain were maintained under a 12-hr light, 12-hr dark cycle at temperature of 23° and 65% humidity, with water and standard laboratory food *ad lib*. The animals were killed humanely, the brains rapidly removed and forebrain and cerebellum dissected out and snap-frozen. P_2 membranes were prepared as previously described [6]. ''Well-washed'' P_2 membranes were prepared using a 5-step freeze-thaw protocol, and aliquots frozen at −20° prior to use [5].

2.2.2. Radioligand binding assays

2.2.2.1. [^{3}H] TBOB binding assay. On the day of the assay the membranes were thawed and incubated with [^{3}H] TBOB (5 nM) for 90 min at 25° in 50 mM Tris–HCl, pH 7.4 containing 0.5 M NaCl. Nonspecific binding was defined in the presence of 100 μM picrotoxinin.

2.2.2.2. [^{3}H] Flunitrazepam binding assay. On the day of the assay, the membranes were thawed and incubated with [^{3}H] flunitrazepam (0.5–1 nM) for 1 hr on ice in 50 mM Tris, pH 7.4. Nonspecific binding was defined in the presence of 100 μM diazepam.

Bound radioactivity was captured for both radioligand binding assays by rapid filtration through a Glass-fibre filter (GF/B; Whatman). The filters were subsequently washed with 3 × 3 mL ice-cold buffer and then soaked in 1 mL scintilation fluid. Filter-bound radioactivity was determined by liquid scintilation spectroscopy.

2.2.3. Data analysis

Results from the radioligand binding data were analysed using nonlinear least squares regression analysis using GraphPad Prism (GraphPad Software). Biphasic curves were fitted as in [7]. The EC_{50} and IC_{50} values are the concentrations for half-maximal enhancement and displacement, respectively. Data were compared by ANOVA followed by a *post hoc* test. A P value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Effect of lactose upon [^{3}H] TBOB binding to rat forebrain membranes

The effect of the simple β-linked disaccharide, lactose was investigated using two different membrane preparations from rat forebrain: well-washed membranes (nominally devoid of endogenous GABA) and unwashed membranes (rich in GABA). The use of such preparations was suggested by the observation that the modulation of [^{3}H] TBPS and EBOB (related to TBOB) binding by different ligands on the $GABA_A$ receptor complex is strictly dependent on the absence or presence of GABA (e.g. [8,9]).

The addition of lactose to well-washed rat forebrain membranes elicited a marked increase in [^{3}H] TBOB binding in a concentration-dependent manner (E_{max} = 367 ± 66% and EC_{50} = 1.45 ± 0.96 μM, mean ± SD for at least three independent experiments) (Fig. 1). In contrast, addition of lactose to unwashed rat forebrain membranes elicited no significant effect upon [^{3}H] TBOB binding (−36 ± 45% at 1 mM lactose, mean ± SD for N = 4 independent experiments performed in triplicate) (Fig. 1). The lack of inhibitory action at high concentrations of lactose is a property shared by diazepam, but not loreclezole or propofol. This property has been previously attributed to the lack of ability of diazepam to activate the $GABA_A$ receptor channel in the absence of GABA [8]. However, unlike diazepam, lactose does not inhibit [^{3}H] TBOB binding in unwashed membranes. This suggests that lactose may thus be a neutral antagonist *in vivo*.

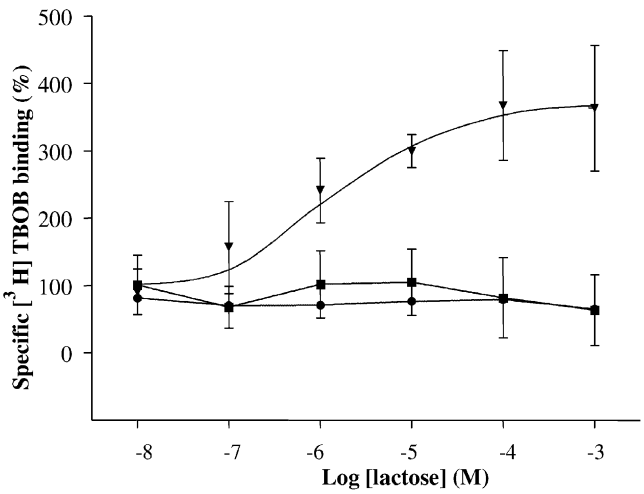

Fig. 1. Effect of lactose upon [^{3}H] TBOB binding to adult rat forebrain membranes: influence of GABA. Effects of lactose on specific [^{3}H] TBOB binding to unwashed (■), well-washed (▼) and well-washed plus 0.3 μM GABA (●) adult rat forebrain membranes. Results are expressed as percentages (mean ± SD for three or four independent experiments) of control specific [^{3}H] TBOB binding in the absence of lactose.

3.2. Effect of GABA upon the lactose modulation of [^{3}H] TBOB binding

In order to confirm that the stimulatory response was GABA-dependent, 0.3 μM GABA was applied to the well-washed membranes. The presence of GABA abolished the stimulation elicited by 100 μM lactose (no GABA = +308 ± 117%; in the presence of 0.3 μM GABA = –20 ± 30%, mean ± SD for N = 4 independent experiments). This effect was consistent across the 0.1–100 μM concentration range of lactose (Fig. 1).

Therefore, the lactose-induced increase in [^{3}H] TBOB binding is sensitive to GABA, indicating that the modulatory action of lactose is related to the conformational state of the chloride channel [10]. GABA-sensitivity is shared by a number of $GABA_A$ receptor modulators, including loreclezole, propofol and diazepam. However, lactose did not display concentration-dependent inhibition of [^{3}H] TBOB binding in the presence of GABA, a property distinct from these other $GABA_A$ receptor modulators (compare to [8]). This property suggests that lactose may not potentiate $GABA_A$ receptor chloride currents *in vivo* and may indeed behave as a neutral antagonist at the sugar binding site. Further possibilities are that GABA directly competes for the lactose binding site itself or that lactose is binding to a distinct site which is occluded when GABA is bound to its site.

3.3. Significance of β-glycosidic linkage

In order to investigate whether the nature of the sugar linkage is important, we compared the effect of lactose (β-glycosidic-linked) to maltose and sucrose (both α-glycosidic-linked sugars). All three common sugars (at 100 μM) significantly potentiated [^{3}H] TBOB binding to forebrain $GABA_A$ receptors, but to similar degrees (2.5 ± 0.5-fold, 1.9 ± 0.5-fold and 3.2 ± 0.6-fold for sucrose, maltose and lactose, respectively; mean ± SD for at least three separate experiments) (Fig. 2). Interestingly, although both maltose and sucrose stimulated [^{3}H] TBOB binding, significantly higher effective concentrations of these sugars were required (EC_{50} values of approximately 900 and 200 μM, respectively), in comparison to lactose (β-glycosidic-linked sugar) (EC_{50} = 1.5 μM) (Fig. 2). This indicates that the β-glycosidic linkage is important for high-affinity interaction with the $GABA_A$ receptor.

3.4. Effect of lactose upon [^{3}H] TBOB binding to cerebellar $GABA_A$ receptors

Many allosteric modulators have been shown to display differential effects upon $GABA_A$ receptor subtypes [11]. The cerebellum possesses a unique population of $GABA_A$ receptors which display a distinct pharmacology [1,12]. In contrast to the forebrain membrane preparation, lactose elicited a biphasic action upon [^{3}H] TBOB binding in well-washed whole cerebellar membranes. Over the 10^{-10} to 10^{-8} M concentration range, lactose elicited a modest yet significant inhibition of [^{3}H] TBOB binding (I_{max} = 54 ± 23%, $P < 0.05$). In contrast, over the 10^{-7} to 10^{-4} M concentration range, lactose potentiated [^{3}H] TBOB binding (E_{max} = 287 ± 132% and EC_{50} of *circa* 30 μM; mean ± SD for at least three separate experiments) (Fig. 3). It is noteworthy that little or no specific [^{3}H] TBOB binding was detected in unwashed membranes which reflects the higher affinity for GABA displayed by cerebellar $GABA_A$ receptors (reviewed in [9]). Therefore, lactose was not tested on unwashed cerebellar membranes. These data with well-washed membranes suggest a distinct mode of action of lactose towards cortical and cerebellar $GABA_A$ receptors, which provide evidence that lactose may distinguish between distinct $GABA_A$ receptor subtypes in the mammalian brain. The E_{max} values for the two tissues are similar indicating a common mode of action of lactose upon $GABA_A$ receptor channels, but the partial inhibitory action and the 20-fold higher EC_{50} value for the stimulatory component displayed by the cerebellar preparation may relate to the influence of cerebellar-specific $GABA_A$ receptor

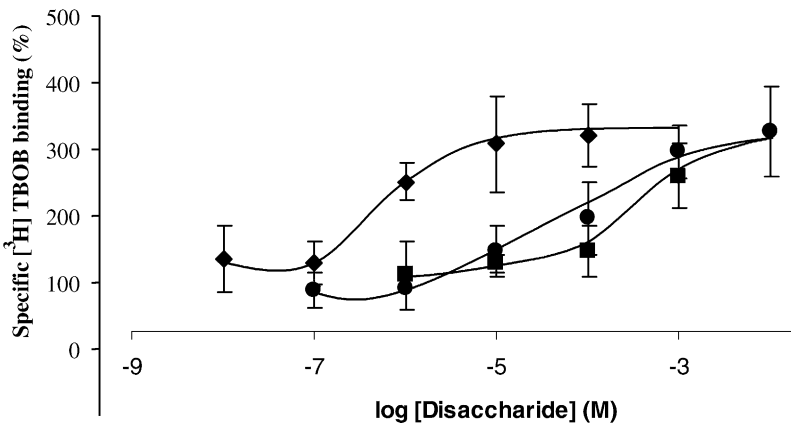

Fig. 2. Comparison of α- and β-glycosidic-linked disaccharides. Effects of lactose (◆), maltose (●) and sucrose (■) on specific [^{3}H] TBOB binding to well-washed adult rat forebrain membranes. Results are expressed as percentages (mean ± SD for at least four independent experiments) of control specific [^{3}H] TBOB binding in the absence of lactose.

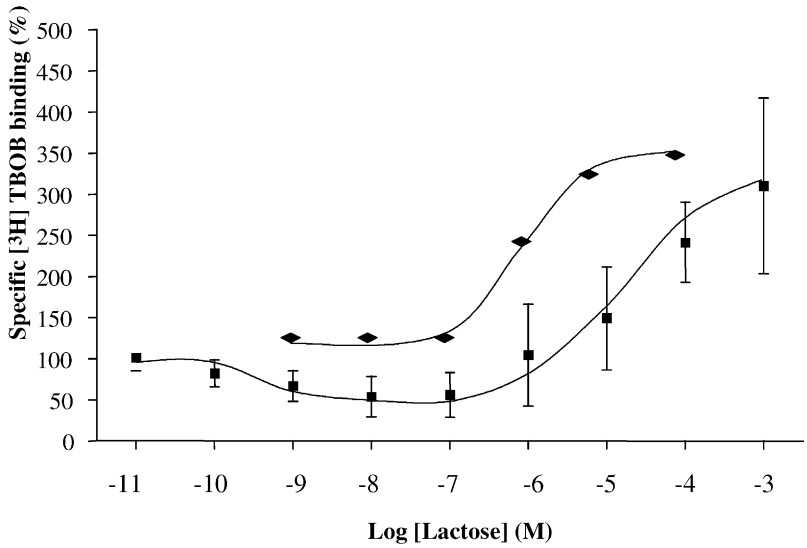

Fig. 3. Effect of lactose upon [^{3}H] TBOB binding to adult rat cerebellar membranes. Effects of lactose on specific [^{3}H] TBOB binding to well-washed adult rat whole cerebellar membranes (■). Results are expressed as percentages (mean ± SD for three or four independent experiments) of control specific [^{3}H] TBOB binding in the absence of lactose. Mean values for well-washed adult rat forebrain membranes are displayed for comparison (◆).

subtypes (e.g. α6-containing). The difference in K_d values for TBOB and structurally-related ligands between forebrain and cerebellum is small (up to 2-fold), and would not account for the differences in lactose effects in the two tissues (reviewed in [9]). Such complex binding profiles have been reported for many different $GABA_A$ receptor allosteric modulators, some of which are GABA-dependent and subtype-selective, including benzodiazepines and loreclozole (e.g. [8]). It is noteworthy that both effects (stimulatory and inhibitory) in the cerebellum were abolished in the presence of 0.3 μM GABA (not shown).

3.5. *Effect of lactose upon [^{3}H] flunitrazepam binding to the $GABA_A$ receptor*

Based on some shared properties of lactose and diazepam in modulating [^{3}H] TBOB binding, we also directly investigated the effect of lactose on the benzodiazepine binding site of the $GABA_A$ receptor, labelled by [^{3}H] flunitrazepam, using the well-washed forebrain or cerebellar membrane preparations. Lactose was without significant effect (positive or negative) upon either forebrain or cerebellar [^{3}H] flunitrazepam binding, even at 100 μM ($P > 0.05$ for $N = 3$ independent experiments), which suggested a lack of allosteric linkage of lactose binding and the benzodiazepine site (Fig. 4). This property is in marked contrast to other allosteric modulators, such as loreclezole and propofol which potentiate [^{3}H] flunitrazepam binding under these conditions [8,10]. These data suggest lactose binds to a novel site on the $GABA_A$ receptor.

In conclusion, this work has confirmed the initial hypothesis which proposed that a simple dissacharide

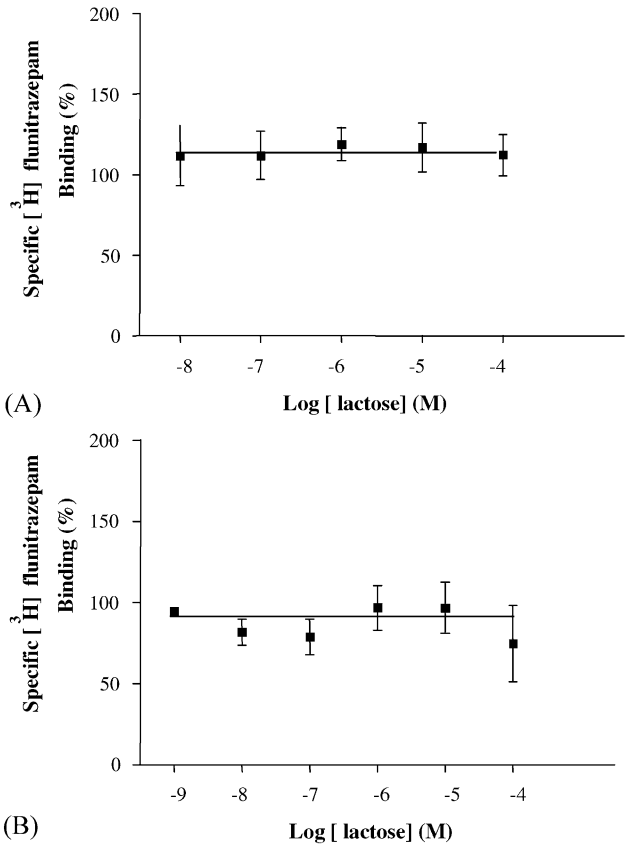

Fig. 4. Effects of lactose upon [^{3}H] flunitrazepam binding to adult rat forebrain and cerebellar membranes. Effects of lactose on specific [^{3}H] flunitrazepam binding to well-washed adult rat (A) forebrain and (B) cerebellar membranes. Results are expressed as percentages (means ± SD for three independent experiments) of control specific [^{3}H] flunitrazepam binding in the absence of lactose.

can modulate binding of a channel site ligand to the $GABA_A$ receptor. Furthermore, the work provides evidence, firstly, that lactose binding is independent of the benzodiazepine site, secondly, that the sugar linkage is an important determinant of affinity for the $GABA_A$ receptor and, finally that lactose may represent a novel subtype selective structural drug template.

The normal physiological concentration of lactose in the brain is unknown, but it is conceivable that in pathophysiological circumstances (e.g. hyperlactosaemia), a high μM concentration of lactose could be reached.

We intend to extend these findings to examine whether lactose can modulate native $GABA_A$ receptor-mediated functional responses in primary cortical and cerebellar cultures [3].

Acknowledgments

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References

[1] Mohler H, Crestani F, Rudolf U. $GABA_A$ receptor subtypes: a new pharmacology. Curr Opin Pharmacol 2001;1:22–5.

[2] Tatsuka K, Yasuda S. Synthesis and biological evaluation of caloporoside analogues. J Antibiot 1991;49:713–5.

[3] Lees G, Chazot PL, Vankayalapati H, Singh G. A simple polar deacetylated caloporoside derivative is a positive modulator of the $GABA_A$ chloride channel complex in cortical mammalian neurones. Bioorg Med Chem Lett 2000;10:1759–61.

[4] Van Rijn CM, Dirksen R, Willems-van Bree E, Maksay G. Diazepam biphasically modulates [^{3}H] TBOB binding to the convulsant site of the $GABA_A$ receptor complex. J Recept Signal Transduct Res 1995; 15:787–800.

[5] Thomas P, Sundaram H, Krishek BJ, Chazot PL, Xie X, Bevan P, Brocchini SJ, Latham CJ, Charlton P, Moore M, Lewis SJ, Thornton DM, Stephenson FA, Smart TG. Regulation of neuronal and recombinant $GABA_A$ receptor ion channels by xenovulene A, a natural product isolated from *Acremonium strictum*. J Pharm Ex Ther 1998;282:513–20.

[6] Chazot PL, Fotherby A, Stephenson FA. Evidence for the involvement of a carboxyl group in the vicinity of the MK801 and magnesium ion sites of the NMDA receptor. Biochem Pharmacol 1993;45:605–10.

[7] Maksay G, Korpi EA, Oukari MU. [^{3}H] EBOB binding to cerebellar and cortical $GABA_A$ receptors. Neurochem Int 1998;33:353–8.

[8] Ghiani CA, Tuligi G, Maciocco E, Serra M, Sanna E, Biggio G. Biochemical evaluations of the effects of loreclezole and propofol on the $GABA_A$ receptor in rat brain. Biochem Pharmacol 1996;51: 1527–34.

[9] Korpi ER, Grunder G, Luddens H. Drug interactions at $GABA_A$ receptors. Prog Neurobiol 2002;67:113–59.

[10] Xue BG, Friend JM, Gee KW. Loreclezole modulates [^{35}S]*t*-butylbicyclophosphorothionate and [^{3}H] flunitrazepam binding via a distinct site on the $GABA_A$ receptor complex. Eur J Pharmacol 1996;300:125–30.

[11] Sieghart W. Unravelling the function of $GABA_A$ receptor subtypes. Trends Pharmacol Sci 2000;21:411–3.

[12] Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G. $GABA_A$ receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 2000;101:815–50.