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Biochemical Pharmacology, Vol. 31, No. 9, pp. 1787–1790, 1982.
Printed in Great Britain.

0006-2952/82/091787-04 $03.00/0

Displacement of Ca^{2+} bound to synaptosomal membrane sialoglycoconjugates by serotonin and serotonergic drugs and the effect on endogenous sialidase (neuraminidase) activity

(*Received* 13 *July* 1981; *accepted* 4 *November* 1981)

Gangliosides are a class of complex glycosphingolipids which contain one or more residues of the negatively charged sugar, sialic acid (*N*-acetylneuraminic acid) (for review, see Ref. 1). Gangliosides are abundant in nervous tissue and are thought to enrich neuronal synaptic membranes [2, 3]. Recent evidence suggests that membrane gangliosides modify various neuronal enzyme activities [4, 5], drug interactions [6, 7] and receptor properties for certain neurotoxins [8] and peptide hormones [9, 10]. The possibility of a membrane ganglioside being the receptor for serotonin was first suggested by Woolley and Gommi [11, 12], and in recent years this concept has again received much attention [13–15].

Synaptic membranes also contain an intrinsic sialidase (*N*-acetyl neuraminosyl glycohydrolase, EC 3.2.1.18), which resides in close association with its preferred substrate, membrane gangliosides [16]. By enzymatic removal of sialic acid from gangliosides in synaptic membranes, oligosialosyl gangliosides are reduced to the monosialosyl ganglioside G_{M1} (for a complete description of ganglioside nomenclature, see Ref. 17). One of the many consequences of such an intrinsic enzyme–substrate system functioning to modify the synaptic membrane glycocalyx may be an alteration of net surface negative charge density [18]. Calcium ions have been shown to interfere with this system by inhibiting endogenous sialidase activity [19]. Others have demonstrated that gangliosides bind Ca^{2+} with high affinity [20–22], and it has been suggested that Ca^{2+} binding to sialic acid residues of gangliosides in synaptic membranes is a critical step in synaptic transmission [22, 24]. The present experiments were designed to examine more closely the interactions of serotonin and serotonergic drugs with Ca^{2+} bound to synaptosomal sialoglycoconjugates and the effect of these interactions on the endogenous membrane sialidase activity.

Synaptosomes were prepared from bovine brain frontal cortical gray matter by techniques previously described [25]. After hypo-osmotic lysis of the synaptosomes at pH 8.4 [26], the plasma membranes were collected by centrifugation at 100,000 *g* for 60 min. Synaptosomal plasma membranes (2 mg protein) were suspended in 2 ml of 1 mM HEPES* buffer, pH 7.5, containing 10 mM [^{45}Ca]Cl_2 (New England Nuclear Corp., Boston, MA, 0.8 mCi/mmole). After incubation at room temperature for 10 min, membrane samples were collected by centrifugation and were washed twice with buffer. Samples were then suspended in 2 ml of the same buffer containing one of the following compounds at various concentrations: serotonin, quipazine [2-(1-piperazinyl)quinoline maleate], fluoxetine [Lilly 110140, 3-(*p*-trifluoromethylphenoxy)-*N*-methyl-3-phenylpropylamine], acetylcholine, ruthenium red and EGTA. Membranes were collected by centrifugation after 10 min at room temperature, and radioactivity in the supernatant fraction was determined by liquid scintillation spectrometry in Aquasol (New England Nuclear Corp.). After suspension in 20 mM acetate buffer, pH 3.9, endogenous sialidase activity (i.e. activity of the intrinsic membrane enzyme toward native membrane sialoglycoconjugates) was determined as described elsewhere [27].

The displacement of bound [$^{45}Ca^{2+}$] from synaptosomal membranes by various agents, and the effect of this displacement on the endogenous sialidase activity are shown in Fig. 1. Exposure of $^{45}Ca^{2+}$-labeled synaptosomal membranes to 10 mM $^{40}Ca^{2+}$ (non-radioactive) resulted in a 40% inhibition of the endogenous sialidase activity, whereas less than 5% of the radioactive [$^{45}Ca^{2+}$] was displaced. This observation suggests that bound [$^{45}Ca^{2+}$] does not readily exchange with subsequently added Ca^{2+}. The degree of inhibition of sialidase activity is similar to that reported previously [19].

Acetylcholine (ACh) at 2 and 10 mM displaced approximately 50 and 63% of bound [$^{45}Ca^{2+}$] respectively; sialidase activity was increased 10 and 20% above the level seen in the presence of 10 mM Ca^{2+}. Serotonin (5-HT) displaced 90% of the bound [$^{45}Ca^{2+}$] at each concentration studied. Endogenous sialidase activity increased 15 and 22% above the Ca^{2+} level at 2 and 10 mM serotonin. The finding that serotonin was more effective than acetylcholine in displacing Ca^{2+} bound to synaptosomal membranes parallels that made by others when examining displacement of bound Ca^{2+} from purified gangliosides by the same agents [28].

* Abbreviations: HEPES 4-(2-hydroxyethyl-1-piperazine-ethanesulfonic acid; and EGTA, ethyleneglycolbis(amino-ethylene)tetra-acetate.

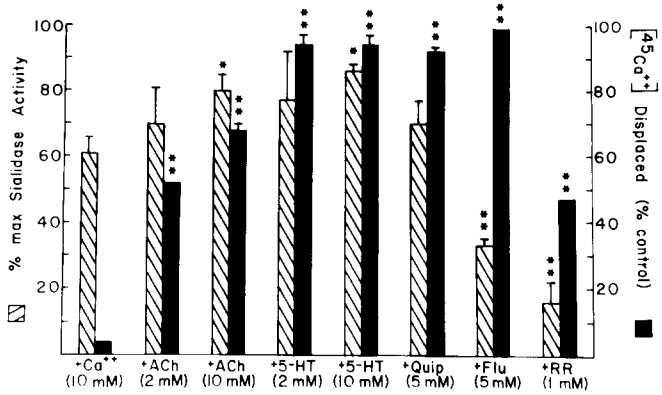

Fig. 1. Displacement of bound [$^{45}Ca^{2+}$] from synaptosomal membranes and changes in endogenous sialidase activity caused by various neuroactive substances. Synaptosomal membrane samples were exposed to 10 mM [^{45}Ca]Cl_2, washed twice by centrifugation, and exposed to the compounds presented at the bottom of the histogram. Displacement of radioactivity into a supernatant fraction caused by 50 mM EGTA was taken to represent the control total bound Ca^{2+}. Percentages of this total are represented by the black bars. The membranes were resuspended in 20 mM acetate buffer, pH 3.9, and incubated for 90 min at 37°. After centrifugation, percent total endogenous sialidase activity was determined [27], which is represented by the hatched bars (100% value = 11 nmoles NeuNAc per mg protein per 90 min). The average of triplicate determinations ± S.E.M. is reported. Replicate experiments gave similar results. Abbreviations: Ach, acetylcholine; 5-HT, serotonin; Quip, quipazine; Flu, fluoxetine; and RR, ruthenium red. Key: (*) $P < 0.05$ compared to $+Ca^{2+}$; and (**) $P < 0.001$ compared to $+Ca^{2+}$ value.

The displacement of synaptic membrane-bound [^{45}Ca] by several different concentrations of serotonin is shown in Fig. 2. Over 90% of the bound ion was displaced by concentrations of serotonin at or above 1 mM, indicating that the data shown in Fig. 1 represents a maximum effect. Below 1 mM the percentage of displaced [$^{45}Ca^{2+}$] dropped sharply, but a significant 26% displacement was still observed at the lowest concentration examined, 10 nM.

Fluoxetine (Flu) is believed to operate exclusively at presynaptic serotonin reuptake sites by binding to reuptake receptors [29, 30]. Quipazine (Quip) is a serotonergic agent, binding to postsynaptic receptors and blocking the 5-HT sensitive adenylate cyclase activity [31–33]. Both drugs were seen to displace over 90% of the membrane-bound [$^{45}Ca^{2+}$]. A distinct difference was observed between the two in terms of overcoming the Ca^{2+}-induced inhibition of endogenous sialidase activity. Quipazine raised activity slightly above that seen in the presence of Ca^{2+}. Sialidase activity, in contrast, was reduced after treatment with fluoxetine to below 35% of the maximum activity.

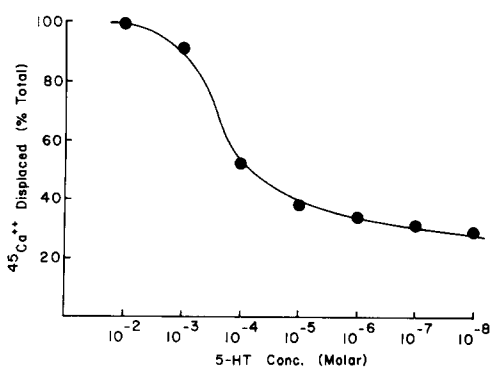

Fig. 2. Displacement of bound [$^{45}Ca^{2+}$] from synaptosomal membranes by various concentrations of serotonin (5-hydroxytryptamine, 5-HT). Methods are detailed in the legend to Fig. 1. The average of triplicate determinations is reported. Replicate experiments gave similar results.

Ruthenium red (RR) is widely used as a histochemical dye for Ca^{2+} binding sites, presumably being bound to the negative charge of sialic acid residues [34, 35]. In this study, the dye was found to displace only 43% of the bound [$^{45}Ca^{2+}$], but it caused the strongest inhibition of the endogenous sialidase activity, to a level lower than 20% of the maximum activity. These results may be partially explained by the presence of multiple binding sites, with different affinities for the dye, and possible subsequent membrane conformation changes [36]. Ruthenium red inhibits the Ca^{2+}-stimulated release of GABA from synaptosomes [37] and blocks transmission in cholinergic and noncholinergic synapses [38]. It seems possible that, by masking the negative charge of synaptic membrane-bound sialic acid and making it inaccessible to the intrinsic sialidase, ruthenium red may cause the same observed functional impairment as that seen when these residues are removed from the membrane by an added sialidase.

Experiments were next designed to determine whether serotonin, and drugs known to act at serotonergic sites, displace [$^{45}Ca^{2+}$] preferentially from membrane sialoglycoprotein or sialoglycolipid (ganglioside). Lipid-extracted glycoprotein was prepared from 1 mg synaptosomal membrane protein [39] and treated with [^{45}Ca]Cl_2, followed by various agents as described above for experiments with the intact synaptosomal membrane. The results of these experiments are summarized in Table 1. Both serotonin

Table 1. Displacement of bound [$^{45}Ca^{2+}$] from synaptosomal glycoprotein by serotonin and serotonergic drugs*

Compound	Concn (mM)	% Total
Serotonin	0.5	92.6 ± 8.0
Quipazine	2.5	91.0 ± 8.0
Fluoxetine	2.5	70.2 ± 7.1

* Radioactivity displaced into a supernatant fraction by 50 mM EGTA was taken to represent total bound Ca^{2+}. Percentages of this total for triplicate experiments were determined after subtracting cpm washed into the supernatant fraction by buffer alone (± S.E.M.).

and the postsynaptically-active drug, quipazine, displaced over 90% of the labeled Ca^{2+} bound to glycoprotein, whereas fluoxetine, the reuptake blocker, was 30% less effective.

These same compounds were tested for displacement of [$^{45}Ca^{2+}$] from gangliosides purified from bovine brain gray matter. Two groups of gangliosides were employed: a disialosyl–trisialosyl mixture (G_{D1a}:G_{D1b}:G_{T1}, 0.8:1.0:1.0), and a monosialosyl mixture (G_{M1}:G_{M2}:G_{M3}, 1.0:0.2:0.1). Ganglioside molar ratios were determined by spectrodensitometry after thin-layer chromatography [40]. Experimental details were identical to those used for the above membrane and glycoprotein studies, except that ganglioside concentrations were kept above critical micelle concentration and centrifugation was at 160,000 *g* for 6 hr. As shown in Table 2, serotonin displaced almost 90% of bound [$^{45}Ca^{2+}$], fluoxetine displaced a slightly higher percentage, and quipazine was least effective. The binding of [$^{45}Ca^{2+}$] was almost ten times higher in the oligosialosyl gangliosides than in the monosialosyl gangliosides (4.85×10^4 cpm/mmole compared to 4.92×10^3 cpm/mmole), demonstrating the importance of the sialic acid moeity in Ca^{2+} binding to gangliosides. Binding of [$^{45}Ca^{2+}$] by separated ganglioside and glycoprotein together accounted for over 85% of total binding observed with intact membranes.

Table 2. Displacement of bound [$^{45}Ca^{2+}$] from a disialosyl–trisialosyl mixture of gangliosides by serotonin and serotonergic drugs*

Compound	Concn (mM)	% Total
Serotonin	0.5	87.1 ± 8.0
Quipazine	2.5	71.0 ± 7.7
Fluoxetine	2.5	94.8 ± 5.0

* One milliliter of 1 mM ganglioside in 1 mM HEPES buffer, pH 7.5, was used (G_{D1a}:G_{D1b}:G_{T1}, 0.8:1.0:1.0). Total bound radioactivity was determined by counting a control sample after washing twice, and percentages of this total displaced into a supernatant fraction in the presence of the above compounds were determined. Values are of triplicate experiments ± S.E.M.

In summary, serotonin was capable of displacing Ca^{2+} bound to synaptosomal membranes and partially restored membrane sialidase activity inhibited by Ca^{2+}. Two serotonergic drugs, quipazine, which act postsynaptically, and fluoxetine, which acts on presynaptic reuptake sites, also displaced over 90% of the synaptic membrane-bound Ca^{2+}. Quipazine raised the endogenous sialidase activity above the Ca^{2+}-inhibited levels in a manner similar to that seen with serotonin. Fluoxetine, however, lowered sialidase activity to less than 35% maximum activity. A closer analysis revealed that quipazine was more effective in displacing Ca^{2+} from membrane glycoprotein than was fluoxetine, whereas the reverse was true when displacement from purified gangliosides was examined. Serotonin was much more effective in displacement of Ca^{2+} from isolated membrane glycoprotein and glycolipid than would be predicted from the studies with the intact membrane, a result which suggests partial masking of Ca^{2+} binding sites *in situ*. These results should help delineate the complex molecular interactions between serotonin and serotonergic drugs and the negatively charged amino sugar derivative, sialic acid, found in abundance in the synaptic region bound to both glycolipid and glycoprotein.

Acknowledgements—This work was supported by NIH Grant NS08258 from the NINCDS. Quipazine and fluoxetine were gifts from K. D. Yoder (Miles Laboratories, Inc.) and Dr. R. W. Fuller (Lilly Research Laboratories) respectively.

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Biochemical Pharmacology, Vol. 31, No. 9, pp. 1790–1792, 1982.
Printed in Great Britain.

0006-2952/82/091790-03 $03.00/0
Pergamon Press Ltd.

Ethanol and synaptosomal calcium binding

(*Received* 15 *August* 1981; *accepted* 4 *November* 1981)

Alcohols and barbiturates produce diverse alterations in calcium metabolism, and it has been suggested that these effects may mediate some of the pharmacological actions of these drugs [1–3]. Recent studies indicate that ethanol and pentobarbital alter the binding of calcium to biological membranes. *In vitro* addition of relatively low ethanol concentrations (10–100 mM) increases the binding of calcium to a cardiac membrane lipoprotein [4] and to a crude preparation of brain membranes [5]. In a study by Low *et al.* [6], the effects of anesthetic drugs on the binding of calcium to the cytoplasmic surface of erythrocyte membranes were explored. In that study, cells were made permeable to ^{45}Ca by incubation with the calcium ionophore A23187, which allows calcium binding to intracellular sites, and an NaCl–EGTA* wash solution was then used to remove (by displacement and chelation) the ^{45}Ca bound to the external surface. These investigators found that calcium binding to the cytoplasmic surface of erythrocyte membranes is increased by 400 mM ethanol and decreased by higher ethanol concentrations. In contrast to ethanol, pentobarbital (1–7 mM) merely decreases calcium binding [6]. Synaptic mechanisms are important in the actions of alcohols and barbiturates; therefore, our experiments were designed to answer these questions: (1) does *in vitro* addition of ethanol or pentobarbital alter calcium binding to either the cytoplasmic or external surface of brain synaptosomes? and (2) does chronic *in vivo* ethanol consumption alter synaptosomal calcium binding or the effects of *in vitro* addition of ethanol?.

Male BALB/C mice (20–28 g) (Harlan Laboratories, Indianapolis, IN) were decapitated and synaptosomes were isolated from whole brain homogenates by Ficoll gradient centrifugation, as described in previous reports [7, 8]. To study chronic ethanol ingestion, mice were fed a liquid diet conatining 7% (v/v) ethanol or were pair-fed an isocaloric sucrose diet [7]. Binding of ^{45}Ca to the external membrane surface was studied by suspending synaptosomes (0.3 mg protein/ml) in sucrose (300 mM)–Tris (20 mM) buffer (pH 7.6). A 1.7-ml aliquot was placed in a plastic minivial (RPI, Mt. Prospect, IL) that previously had been washed with 1 mM EGTA and rinsed three times with deionized H_2O. After 5 min at 30°, 0.2 ml $^{45}CaCl_2$ solution and 0.1 ml ethanol, pentobarbital sodium (all diluted in H_2O), or H_2O (control) were added. The final concentration of calcium, determined by atomic absorption spectroscopy, was 10^{-5} M. After 10 min at 30°, the vials were supported by 00 rubber stoppers in a Sorvall SS24 rotor and centrifuged at 10,000 rpm for 3 min without refrigeration. The supernatant fraction was immediately decanted, and the vial and pellet surface were rinsed with 1 ml H_2O. Pellets were digested overnight with 0.5 ml of 0.1% SDS; radioactivity was determined by liquid scintillation spectrometry. To estimate the volume of extrasynaptosomal fluid trapped in the vial, some samples in each experiment were incubated with [^{14}C]sorbitol rather than ^{45}Ca [9]. The extrasynaptosomal volume was used to calculate the amount of free ^{45}Ca in each vial, and this value was subtracted from the total ^{45}Ca to give the amount of ^{45}Ca bound.

Calcium binding to intrasynaptosomal sites was determined by the method of Low *et al.* [6]. Synaptosomes were suspended in sucrose–Tris buffer, as described above, and 1.7-ml aliquots were incubated for 20 min at 30° with 10 μM A23187 (Calbiochem-Behring Corp., La Jolla, CA) (dissolved in 2 μl DMSO), 0.1 ml ethanol, pentobarbital, or H_2O (control) and 0.2 ml ^{45}Ca–EGTA solution. The free concentration of calcium was calculated as 7 μM [10]. This value, however, did not take into account the binding of calcium to A23187 or to the membranes. Although A23187 may release calcium from synaptosomal storage sites (mitochondrial and non-mitochondrial), changes in free calcium concentration would be minimized by the EGTA buffer. The suspension was then diluted with 8 ml of 140 mM NaCl, 20 mM Tris, and 5 mM EGTA (pH 7.5) and rapidly filtered through Whatman GF/C discs which were washed with another 8-ml aliquot. Liquid scintillation spectrometry was used to determine radioactivity on the discs, the phenol method [11] to determine protein, and Student's *t*-test for paired observations to statistically evaluate drug effects.

In vitro addition of ethanol enhanced calcium binding to intrasynaptosomal sites (Fig. 1). Ethanol concentrations of 12–50 mM increased binding by about 30%; higher con-

* Abbreviations: EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate; SDS, sodium dodecylsulfate; and DMSO, dimethylsulfoxide.