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The effect of detergents on trimeric G-protein activity in isolated plasma membranes from rat brain cortex: Correlation with studies of DPH and Laurdan fluorescence

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ABSTRACT

The effect of non-ionic detergents on baclofen ($GABA_B$-R agonist)-stimulated G-protein activity was measured as a [^{35}S]GTPγS binding assay in the plasma membranes (PM) isolated from the brain tissue. The effect was clearly *biphasic* — a decrease in the activity was followed by an activation maximum and finally, at high concentrations, drastic inhibition of the G-protein activity was noticed. Contrarily, specific radioligand binding to $GABA_B$-receptor was inhibited in the whole range of detergent concentrations step by step, i.e. it was strictly *monophasic*. The magnitude of both detergent effects was decreased in the same order of potency: Brij58>Triton X-100>Digitonin. The identical order was found when comparing detergents ability to alter fluorescence anisotropy of the membrane probe 1,6-diphenyl-1,3,5-hexatriene (r_{DPH}) incorporated into the hydrophobic PM interior. Decrease of r_{DPH}, in the order of Brij58>Triton X-100>Digitonin, was reflected as decrease of the S-order parameter and rotation correlation time ϕ paralleled by an increase of diffusion wobbling constant D_w (analysis by time-resolved fluorescence according to "*wobble-in-cone*" model). The influence of the detergents on the membrane organization at the polar headgroup region was characterized by Laurdan generalized polarization (GP). As before, the effect of detergents on GP parameters proceeded in the order: Brij58>Triton X-100>Digitonin.

1. Introduction

Participation of the hydrophobic membrane interior and the influence of temperature and membrane phase transitions on the ligand binding to G-protein coupled receptors (GPCR) have been studied since the early days of G-protein oriented research. Coupling of receptors to guanine-nucleotide binding regulatory proteins was shown to induce a considerable increase of the affinity of the receptors for the agonists [1]. Analysis of the temperature dependence of the agonist binding to the two affinity states of A1 adenosine and α2-adrenergic receptors indicated marked thermodynamic differences between these two affinity states [2,3]. Agonist binding to the low-affinity state was enthalpy driven in a manner similar to that of antagonist binding whereas agonist binding to the high affinity state was entropy driven and thus clearly different from the former one. Measurements of the fluorescence of 1-anilin-8-naphtalenesulphonate (ANS) showed thermotropic phase transitions of platelet membranes at 17 °C [3]. The addition of 10 mM octanoic acid shifted the transition temperature by 12 °C; and additionally also shifted the break — points of van't Hoff plot of the antagonist and low affinity agonist binding. A high affinity agonist binding, however, remained unchanged.

The participation of the hydrophobic membrane interior in the GPCR-mediated signaling has been also reported for β-adrenergic receptors (β-AR). Beta-adrenergic blocking agents/partial agonists such as propranol and alprenolol were shown to decrease myocardial conduction velocity, to inhibit synaptosomal noradrenaline uptake, platelet aggregation and to exert the local anesthetic action. All these physiologically significant phenomena were found to be directly proportional to the hydrophobicity of a given β-adrenergic receptor ligand expressed as an n-octanol/water partition coefficient [4–7]. Partitioning of the hydrophobic β-adrenergic antagonist propanolol was shown to inhibit the fluoride-stimulated adenylyl cyclase activity in brain microsomes [8].

More recently, hydrophobic membrane constituents such as cholesterol were found to affect the ligand binding to GPCR and to

Abbreviations: GABA, γ-aminobutyric acid, $GABA_B$-R, metabotropic receptor for GABA, GPCR, G-protein-coupled receptor; G-proteins, heterotrimeric guanine nucleotide-binding regulatory proteins; Gsα, G protein stimulating adenylyl cyclase activity; Gi/Goα, G-proteins inhibiting adenylyl cyclase activity in pertussis-toxin sensitive manner; Gq/G11α, G proteins stimulating phoshoplipase C in pertussis-toxin independent manner; [35S]GTPγS, guanosine-5′-[γ-35S] triphosphate; PBS, phosphate-buffered saline; PM, plasma membrane, PMSF, phenylmethylsulfonyl fluoride; PT, pertussis toxin; TBS, Tris-buffered saline; w.w., wet weight

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modulate the functional coupling between GPCR and G protein(s) [9–14]. It has also been shown that the hydrohobicity of residue 351 of G-protein $G_i1\alpha$ determines the extent of the activation by α2A-adrenoceptor [15]. The same result was noticed for the functional coupling between the δ-opioid receptor (DOR) and $G_i1\alpha$ [16]. Direct correlation between the ligand-receptor binding and the organization of the plasma membrane (PM) has been observed on the oxytocin receptor and cholecystokinin receptor [17,18]. A significant decrease of the steady state anisotropy provoked by the cholesterol depletion was accompanied by the decrease in the ligand binding. While the cholecystokinin receptor showed monotonous decline of the ligand binding with the cholesterol depletion, the oxytocin receptor showed a sigmoidal dependence with a sharp decline at the critical cholesterol content. This finding indicates non-trivial and highly specific effects of the membrane organization on the GPCR activity.

As the examples above illustrate, there is a general awareness of the connection between the activity of G-proteins and the physical state of the membrane. To throw light upon the potential relationship of the membrane structure and the GPCR activity we decided to alter the state of the natural membranes such as brain cortex plasma membranes via detergent addition and follow the activation of the G-proteins by $GABA_B$-receptor agonist baclofen. At the identical detergent concentrations, the steady-state and time-resolved anisotropy of rod-like DPH fluorophore embedded in the plasma membrane was measured conveying the information on the organization of the hydrophobic membrane interior. In addition, the changes of the organization in the head-group region of the membrane were monitored by means of Laurdan generalized polarization.

2. Materials and methods

2.1. Isolation of plasma membrane fraction

Tissue pieces of rat brain cortex were minced with razor blade on pre-cooled plate, diluted in 250 mM sucrose, 20 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.6, fresh 1 mM PMSF plus complete protease inhibitors cocktail (STE medium), homogenized mildly in loosely-fitting, teflon-glass homogeniser for 5 min (1 g w. w. per 10 ml) and centrifuged for 5 min at 1500 rpm. Resulting post-nuclear supernatant (PNS) was filtered through a Nylon nets of decreasing size (330, 110 and 75 mesh, Nitex) and applied on top of 30% (v/v) PercollR in Beckman Ti60 tubes (3 ml of PNS plus 30 ml of PercollR in STE medium). Centrifugation for 30 min at 30,000 rpm (65,000 ×*g*) resulted in separation of the two clearly visible layers. The upper layer represented plasma membrane fraction; the lower layer contained mitochondria. The upper layer was removed, diluted 1:4 in distilled water and centrifuged in Beckman Ti60 rotor at 50,000 rpm (170,000 ×*g*) for 2 h. The membrane sediment was removed from the compact, gel-like sediment of Percoll®, re-homogenized by hand in small volume of STE medium, snap frozen in liquid nitrogen and stored at −80 °C at 10–15 mg/ml.

2.2. Steady-state DPH anisotropy measurements

Brain cortex membranes were diluted to 0.4 mg/ml and mixed at 0–4 °C with increasing concentrations of Triton X-100 or Brij58. The final concentration of detergents in detergent/membrane mix was 0.0005, 0.001, 0.002, 0.0039, 0.0078, 0.0156, 0.0313, 0.0625, 0.125, 0.25, 0.5 and 1%. Digitonin (12%) dissolved in DMSO was added to the same final concentrations. DPH incorporation was performed by the fast addition of 1 mM DPH in freshly distilled acetone to 1 μM final concentration (under mixing). After 30 min at 25 °C, which were allowed to ensure the optimum incorporation of the probe into the membrane interior, the anisotropy of DPH fluorescence was measured at Ex 365 nm/Em 425 nm wavelengths. Under these conditions, the fluorescence intensity of the membrane-bound DPH was ≈1000× higher than that of the free probe in aqueous medium alone; light scattering problems could be omitted. Steady-state fluorescence anisotropy r_{DPH} was calculated as described before by Shinitzky and Barenholz [19] and Svobodova and Svoboda [20].

2.3. Time-resolved fluorescence measurements

Fluorescence lifetime and polarization experiments were performed with a time correlated single photon counting (TCSPC) IBH 5000 U instrument equipped with a cooled Hamamatsu R3809U-50 microchannel plate photomultiplier detector. The sample was excited at 373 nm with a diode laser (IBH NanoLED-375 L, FWHM 80 ps, 1 MHz repetition rate). The emission monochromator was set to 450 nm. The anisotropy free decay $I(t)$ was obtained as follows:

$$I(t) = I_{vv}(t) + 2GI_{vh}(t) \tag{1}$$

where I_{vv} is the fluorescence decay measured with both excitation and emission polarized vertically, and I_{vh} with the vertically polarized excitation and horizontally polarized emission. The G-factor (G) was determined by measuring a standard solution of POPOP and calculated as:

$$G = \frac{\langle I_{hv}(t)\rangle_t}{\langle I_{hh}(t)\rangle_t} \tag{2}$$

where I_{hv} corresponds to the signal measured with the horizontally polarized excitation and vertically polarized emission, and I_{hh} to excitation and emission both polarized horizontally. In order to obtain fluorescence lifetimes, the $I(t)$ was fitted with two-exponential decay:

$$I(t) = B_1 \exp(-t/\tau_1) + B_2 \exp(-t/\tau_2), \tag{3}$$

yielding lifetimes, τ_1 and τ_2, and corresponding amplitudes B_1 and B_2.

The decay of the anisotropy $r(t)$ was determined as follows:

$$r(t) = \frac{I_{vv}(t) - GI_{vh}(t)}{I_{vv}(t) + 2GI_{vh}(t)} \tag{4}$$

and fitted with the formula:

$$r(t) = (r(0) - r(\infty)).\exp(-t/\phi) + r(\infty), \tag{5}$$

where $r(0)$, and $r(\infty)$ stands for the limiting and residual anisotropy, respectively. ϕ is the rotational correlation time. The anisotropy decays were fitted by the non-linear least squares method including the impulse reconvolution with the instrumental response function (fwhm ~100 ps). $\chi 2$ generated by the IBH software package served as goodness of fit criterion. The anisotropy data were then treated according to the "wobble in cone" model introduced by Kawato et al. [42]. The analysis is based on the interpretation of two parameters. Firstly, the S-order parameter S is defined as:

$$S = \left(\frac{r(\infty)}{r(0)}\right)^{1/2}, \tag{6}$$

and secondly, the wobbling diffusion constant D_w was calculated as:

$$D_w = \frac{\sigma_s}{\phi}, \tag{7}$$

where ϕ is the rotational correlation time and σ_s is the relaxation time which is a function of the S-order parameter and has been determined according to Kinosita, et al. [21].

2.4. Agonist-stimulated [^{35}S]GTPγS binding

Baclofen, an agonist of $GABA_B$-receptors ($GABA_B$-R), was used for determination of the agonist-stimulated [^{35}S]GTPγS binding in the

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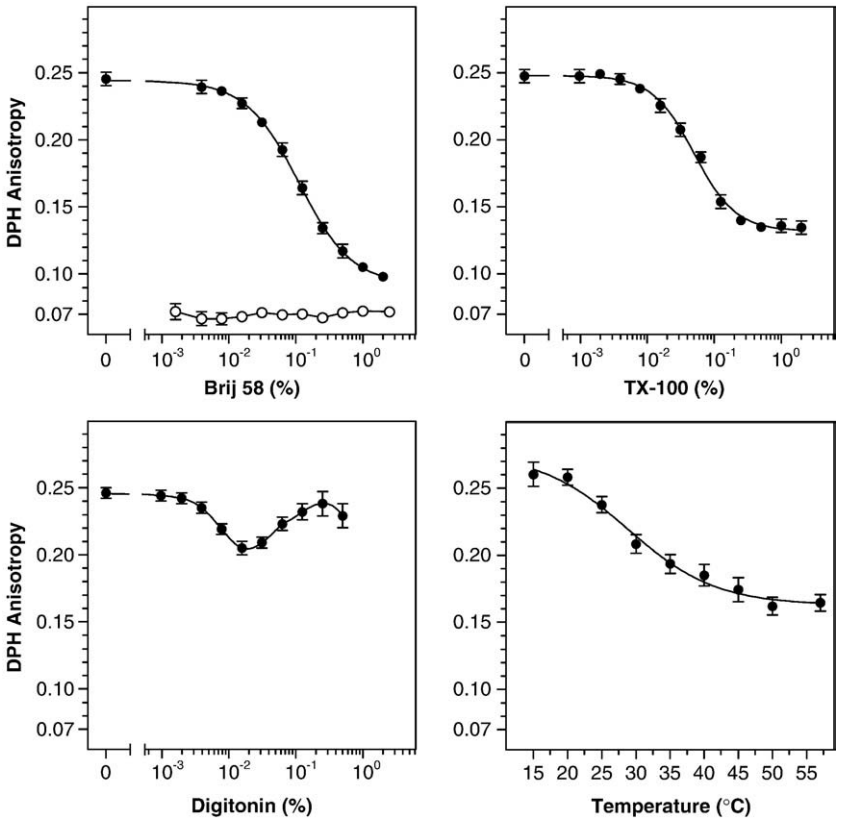

Fig. 1. The effect of Brij58 (upper left panel), Triton X-100 (upper right panel), Digitonin (bottom left panel), and temperature (bottom right panel) on the steady-state anisotropy of diphenylhexatriene (DPH). PM fraction was mixed 1:1 with the increasing concentrations of detergents at 0–4 °C; after 60 min on ice, 1 mM DPH in acetone (1 μM final concentration) was added quickly under stirring and incubated for 30 min at laboratory temperature, 25 °C. In the upper right panel, open symbols (○) correspond to Brij58 alone. The temperature dependence of DPH fluorescence was determined in the control, detergent-untreated membrane. The data represent the average of three experiments.

plasma membrane fraction isolated from the rat brain cortex. The binding assay was performed as previously described by Fong and Milligan [22,23]. Constant volume aliquots (20 μl) of control (detergent-untreated) or detergent-treated PM were incubated with (total) or without (basal) 100 μM baclofen in final volume of 100 μl of reaction mix containing 20 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl, 2 μM GDP, 0.2 mM ascorbate and [^{35}S]GTPγS (about 100,000 dpm per assay) for 30 min at 30 °C. The binding reaction was discontinued by dilution with 3 ml of ice-cold 20 mM HEPES, pH 7.4, 3 mM $MgCl_2$ and immediate filtration through Whatman GF/C filters on Brandel cell harvestor. Radioactivity remaining on the filters was determined by liquid scintillation using Rotiszint EcoPlus cocktail. Non-specific [^{35}S]GTPγS binding was determined by parallel assays containing 10 μM GTPγS.

2.5. $GABA_B$ receptor content

$GABA_B$-receptor content in PM isolated from the brain tissue was measured by binding of specific antagonist [^{3}H]CGP54626 as described before by Hejnova et al. [24]. Membranes (50 μg protein) were incubated with 2.5 nM [^{3}H]CGP54626 (total concentration) in 0.5 ml of 75 mM Tris–HCl, pH 7.4, 12.5 mM $MgCl_2$, 1 mM EDTA for 60 min at 25 °C. The bound and free radioactive antagonists were separated by rapid filtration through Whatman GF/B filters in Brandel cell harvestor. Filters were washed 3× with 3 ml of ice-cold incubation buffer and placed in 4 ml of scintillation cocktail (CytoScint, ICN). Radioactivity remaining on filters was determined after 16 h at laboratory temperature by liquid scintillation. The non-specific binding was defined as that remaining in the presence of 1 mM non-radioactive GABA.

2.6. Laurdan generalized polarization

The incorporation of the Laurdan to plasma membranes was performed in the following way: a small portion of Laurdan (Invitrogen, USA) dissolved in methanol was added to the plasma membrane solution (0.4 mM) to reach the 10 μM concentration. The sample was heated at 50 °C for 30 min to enable the proper incorporation of the probe. The detergent treatment was then performed at the identical conditions and temperature as described in Section 2.2. The emission and excitation spectra were recorded on Fluorolog 3 instrument (Horiba, Jobin-Yvon, USA). The emission scans were performed with the excitation wavelengths set to 340 nm and 410 nm, the excitation spectra were carried out at the emission wavelengths set to 440 nm and 490 nm. The generalized polarization (GP) spectra were calculated from the fluorescence intensities of Laurdan embedded in the membrane according to Parasassi et al. [25]. In the case of excitation GP the following equation was used:

$$GP^{\lambda}_{exc} = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \quad (8)$$

where I_{490} and I_{440} stands for the emission intensity detected at 490 nm and 440 nm, respectively, excited by the excitation wavelength λ. Similarly, the emission GP spectra were calculated via:

$$GP^{\lambda}_{em} = \frac{I_{410} - I_{340}}{I_{410} + I_{340}} \quad (9)$$

where I_{410} and I_{340} stand for the Laurdan excitation intensity at the wavelengths 410 nm and 340 nm, respectively, detected at the

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emission wavelength λ. Additionally, the excitation and emission GP spectrum slopes [26] were calculated by means of the equations:

$$GP_{exc}S = \frac{GP_{exc}^{410} - GP_{exc}^{340}}{410-340}, \quad (10)$$

$$GP_{em}S = \frac{GP_{em}^{490} - GP_{em}^{440}}{490-440} \quad (11)$$

where the GP_{exc}^{410} and GP_{exc}^{340} corresponds to the excitation GP values when exciting at 410 nm and 340 nm, respectively. GP_{em}^{490} and GP_{em}^{440} stand for the emission GP values determined for the emission wavelength of 490 nm and 440 nm, respectively.

3. Results and discussion

3.1. The effect of detergents on hydrophobic plasma membrane interior as revealed by steady-state fluorescence anisotropy of diphenylhexatriene (DPH)

Brij58 exhibited a strong "fluidization" effect of the hydrophobic membrane interior in the plasma membrane (PM) fraction isolated from the brain cortex. This is demonstrated in the left upper panel of Fig. 1 by a measurement of steady-state anisotropy of the hydrophobic membrane probe diphenylhexatriene (DPH). When increasing Brij58 above critical micelle concentration (CMC=0.0086% w/v), the highly polarized signal of DPH in the intact, detergent-untreated PM (r_{DPH}=0.245) was gradually decreased to highly depolarized signal (r_{DPH}=0.092), i.e. close to the anisotropy of Brij58 alone in aqueous medium measured at 25 °C (r_{DPH}=0.072). A magnitude of this effect was relatively large, (Δ r_{DPH}=0.153); when compared to the temperature-induced shift. Specifically, heating up the sample from 15 °C to 55 °C lead to the change in the anisotropy Δ r_{DPH}=0.100 (right bottom panel of Fig. 1D). Similar data as in the case of Brij58 were obtained when analyzing Triton X-100 (right upper panel of Fig. 1). The maximum change of DPH anisotropy caused by Triton X-100 (Δ r_{DPH}=0.116) was, however, less significant than in the case of Brij58 (Δr_{DPH}=0.153). The difference of CMC values between Brij58 (0.0086% w/v) and Triton X-100 (0.0155% w/v) might explain a higher penetration ability of Brij58 into the PM [27].

Digitonin was unable to induce a dramatic decrease of r_{DPH} as the former two water soluble detergents (left bottom panel of Fig. 1). Membranes exposed to the high concentrations of this detergent (0.1–0.5% w/v) exhibited the same r_{DPH} as the control, detergent-untreated membranes. A drastic decrease of r_{DPH} to values close to highly depolarized fluorescence signal (i.e. less than 0.01) was not observed. The magnitude of Digitonin effect was small and restricted to the "transient range" of concentrations between 0.005% and 0.1%; this transient range was characterized by the small decrease of r_{DPH} values followed by the increase back to the control values. Thus, r_{DPH}=0.230 measured at 0.5% Digitonin was close to the steady-state anisotropy of DPH fluorescence measured in detergent-untreated PM (r_{DPH}=0.245).

3.2. The time-resolved measurements of DPH fluorescence

The results of the time-resolved DPH fluorescence analysis by the wobble in cone model copies the trends already observed for the steady-state anisotropy measurements, i.e. the presence of detergents leads to the higher depolarization of the excitation light, in fact to a higher motional freedom of the dye. Nevertheless, the time resolved data provided more detailed insight into the organization of the membrane interior. Herein applied "wobble in cone" model retrieved the information on the membrane dynamics (the rate of dye rotational motion), which is characterized by the wobbling diffusion constant (D_w). In addition, this model enabled us to gain the static information about the degree of the orientational constrains due to the interaction of the dye with the aliphatic chains of fatty acids, which is characterized by the S-order parameter (S).

Table 1
The parameters obtained by the fitting and further analysis of the time resolved anisotropy decays

	r_0	r_∞	ϕ (ns)	S	D_w (ns^{-1})
Control, detergent-untreated PM	0.289± 0.018	0.165± 0.022	6.1± 0.7	0.68± 0.05	0.019± 0.001
PM+Digitonin	0.282± 0.021	0.111± 0.006	3.9± 0.4	0.56± 0.04	0.044± 0.008
PM+Triton X-100	0.286± 0.019	0.026± 0.003	3.2± 0.2	0.27± 0.02	0.072± 0.002
PM+Brij58	0.276± 0.020	0.074± 0.008	2.5± 0.1	0.46± 0.05	0.078± 0.006
SUVs	0.271± 0.018	0.115± 0.007	2.8± 0.2	0.65± 0.09	0.031± 0.006
SUVs+Digitonin (0.5% w/v)	0.140± 0.023	0.032± 0.006	11.3± 0.9	0.46± 0.07	0.017± 0.007
SUVs+Triton X-100 (1% w/v)	0.223± 0.018	0.002± 0.004	1.5± 0.2	0.10± 0.06	0.171± 0.030
SUVs+Brij58 (1% w/v)	0.201± 0.019	0.001± 0.007	1.4± 0.2	0.07± 0.06	0.173± 0.027

r_0 stands for the limiting anisotropy, r_∞ for the residual anisotropy, ϕ for the rotational correlation time, S for the S-order parameter, and D_w for the wobbling diffusion constant. In order to roughly mimic the composition of PM, the composition of small unilamellar vesicles (SUVs) was as follows: 50% of egg phosphatidyl-choline, 20% egg phosphatidyl ethanolamine, and 30% of cholesterol. The detergents were added in the ratio of 1 lipid molecule to 4 detergent molecules to make the micellization process as probable as possible.

As shown in Table 1 and Fig. 2, both of these parameters were changed by treatment of PM with detergents. In general, the S-order parameter was decreased upon the addition of the detergents while the wobbling diffusion constant D_w was raised significantly. It means that the disorganized, i.e. more chaotically organized aliphatic chains of fatty acids, provided more space for the movement of the DPH dye and simultaneously, the rate of the rotation of the DPH was increased by the presence of the detergents. The smallest effect was observed after Digitonin addition, yet, a significant drop of the S-order parameter and a noticeable decrease in the wobbling diffusion constant was observed. The water soluble detergents, Triton X-100 and Brij58, showed a stronger impact on the membrane organization — the wobbling diffusion coefficients were increased three times when compared with the native (detergent-untreated) membranes.

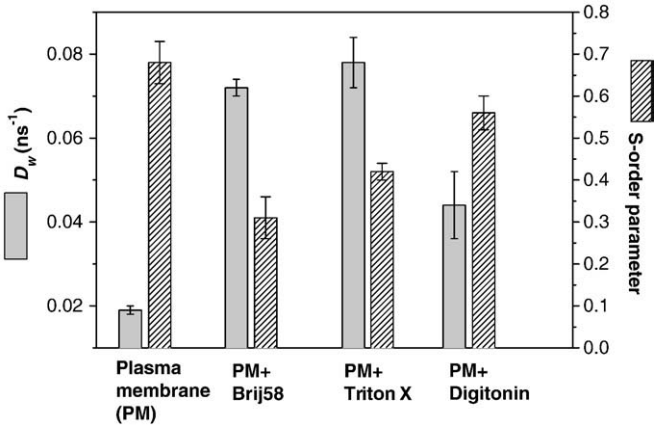

Fig. 2. The effect of detergents on the diffusion wobbling constant D_w (grey bars) and S-order parameter (hatched bars). Diffusion wobbling constant D_w and S-order parameter S were calculated from the time-resolved anisotropy decays of DPH fluorescence in the control, detergent-untreated PM (control) and PM exposed to 0.031% Digitonin, 0.05% Triton X-100 or 0.1% Brij58, respectively.

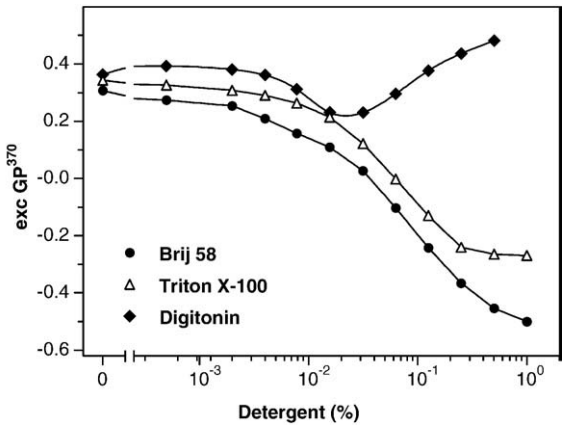

Fig. 3. The effect of detergents on the excitation generalized polarization GP_{exc} of Laurdan embedded in PM. Dependence of GP_{exc} of Laurdan on the concentration of Brij58 (●), Triton X-100 (△), and Digitonin (♦) was measured at the excitation wavelength of 370 nm in PM and GP_{exc} values were calculated as described in Materials and methods.

The effect of Brij58 on S-order parameter was stronger implying lower constrains of the motion of the DPH rod when compared to Triton X-100.

Table 1 also contains results for the small unilamellar vesicles (SUVs) composed of 50% of phosphatidyl-choline, 20% of phosphatidyl-ethanolamine and 30% of cholesterol. Such lipid composition might mimic the composition of lipids present in the PM to some extent. Obviously, the values of the S-order parameter ($S \cong 0.7$) lies in the same range. The wobbling constant, on the other hand, is significantly lower in the case of PM, indicating a lower motional freedom of the PM interior. The formation of micelles caused by the addition of high content of Brij58 and Triton X-100 to SUVs is accompanied by a significant change in all the parameters, i.e. faster rotational correlation time, a lower order parameter and a higher wobbling diffusion constant are observed (Table 1). This fact indicates that formation of micelles does not dominate even at the high concentrations of Brij58 (0.1%) and Triton X-100 (0.05%) when added to PM. Digitonin has a different effect on the model SUVs system and the formation of the slowly rotating aggregates is detected.

3.3. Laurdan generalized polarization measurements

Generalized polarization monitored by a fluorescent probe Laurdan is a tool for the investigation of lateral lipid organization in the headgroup region of phospholipid bilayers [25,28]. Specifically, we have shown that Laurdan is able to report on the hydration and mobility changes at the level of the sn-1 carbonyl group in phospolipid model membranes [34]. The solvatochromic properties of this dye lead to the significant red-shift of its emission spectra in the hydrated liquid-crystalline phase compared to the rigid gel phase. The value of the generalized polarization (GP) calculated from the fluorescence intensities at either the excitation or the emission wavelengths [25,28], yields the information on the phase state of the lipid microenvironment of Laurdan. Typically, GP reaches the values of approximately 0.6 and −0.2 in the gel and liquid crystalline phase, respectively. Nevertheless, the natural membrane contains noticeable amount of sphingolipids, whose content can reach up to 20% in the isolated cortex membranes [29,30]. It was demonstrated that the presence of the sphingolipids leads to the phase coexistence in the model membranes, which naturally makes the interpretation of the bulk GP experiment rather complex [31,32]. Moreover, the chemical structure of the sphingolipids is highly diverse and the effect on the lateral packing and water content in the membrane is strongly dependent on the type of sphingolipid [33]. For instance, model bilayers containing cholesterol and sphingomyelin coexist in the liquid ordered and liquid disordered phase, while model membranes containing ceramide and cholesterol show only gel–liquid crystalline phase coexistence [32]. Even though we believe that Laurdan GP can still provide us with the qualitative information on the average change in the hydration and/or mobility for complex membranes provoked by the detergents.

The effect of the detergents on the excitation GP is illustrated in Fig. 3. Obviously, the dependencies of the GP on the amount of Brij 58 and Triton X-100 follow a similar pattern. The GP decreases sigmoidally with increasing detergent concentration. It is reasonable to assume that this result indicates the increase in the hydration and mobility of the Laurdan microenvironment. Unlike the former two water soluble detergents, Digitonin takes a completely different action. After the initial decrease, the GP starts to rise when the 0.05% detergent concentration is reached. After that GP reaches even higher values in comparison to the initial non-treated plasma membranes.

To gain more detailed understanding of the detergent interaction with brain PM, the slope of the GP spectra (GPS) has been analyzed (Fig. 4). As demonstrated before [26,35], a thermotropic profile of the GPS has the potential to reveal the physical state of the membrane. For instance, it allows the detection of the main phase transition and serves as a good tool for the determination of the phase coexistence.

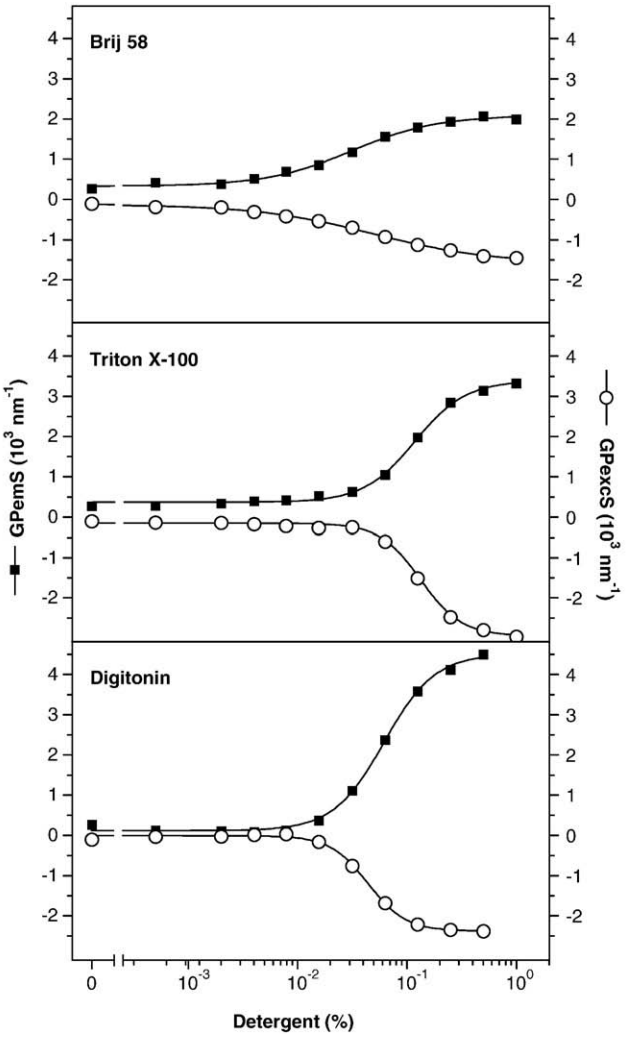

Fig. 4. The effect of detergents on the slope of the emission (GPemS) and excitation (GPexcS) generalized polarization of Laurdan embedded in PM. Dependence of GPemS (■) and GPexcS (○) on the detergent concentration was determined for the Brij 58 (upper panel), Triton X-100 (middle panel), and Digitonin (bottom panel). The values of GPemS and GPexcS were calculated as described in Materials and methods.

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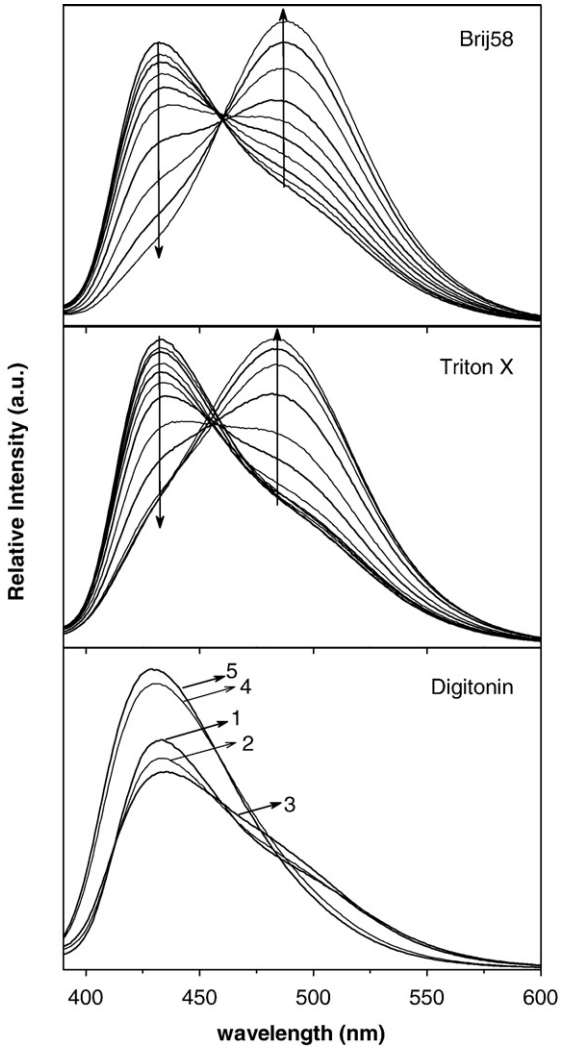

Fig. 5. The evolution of the steady state emission spectra of Laurdan embedded in PM upon the addition of Brij 58 (upper panel), Triton X-100 (middle panel) and Digitonin (bottom panel). In the upper and middle panel, the arrows indicate the change in the emission intensity caused by various concentration of Brij58 and Triton X-100. The emission spectra were recorded for the following Brij58 and Triton X-100 contents: 0.001, 0.002, 0.004, 0.0078, 0.0156, 0.0315, 0.063, 0.125, 0.25, 0.5 and 1.0%. With the increasing concentration of both Brij58 and Triton X-100 the intensity at the emission wavelength 435 nm was decreasing continuously. The opposite trend was detected for the emission intensity at 485 nm. In bottom panel, not all spectra recorded for Digitonin are shown for the purpose of the higher clarity. Numbers 1, 2, 3, 4, and 5 correspond to the emission spectra recorded at the following Digitonin concentrations: 0.001, 0.0078, 0.0315, 0.125, and 0.5%, respectively. Obviously, there is a blue shift of the spectra observed for the high Digitonin concentrations.

We decided to adopt this approach and followed the GPS evolution upon the addition of the detergents (Fig. 4).

At low detergent concentrations, the excitation and emission GP slopes run parallel and close to each other indicating phase coexistence. Some *nanoscale domains* are likely to be present as the composition of brain plasma membranes is largely heterogeneous and the lateral organization of such membrane has to be rather complex. For this reason, the interpretation of the GP slopes evolution is not as straightforward as for the pure model lipid bilayers and we were unable to bring more unambiguous description of the overall brain membrane phase. Nevertheless, since the GP values at low detergent concentrations are relatively low, the rigid gel-like phase may dominate. After the 0.01–0.05% detergent concentration is reached, the excitation and emission GPS traces diverge in a sigmoidal manner reflecting a significant change in the bilayer organization (Fig. 4).

When inspecting the evolution of the steady state spectra upon addition of Brij 58 and Triton X-100 (upper and middle panel of Fig. 5), the brain plasma membrane undergoes transition from the rigid-like membrane to the state similar to liquid crystalline phase since the isobestic point is detected and the "red edge" maximum of the emission spectra is steadily located at the wavelength of 485 nm corresponding to the liquid crystalline phase [36]. Marked micellisation of the membrane is unlikely for it is accompanied by an additional red-shift of the Laurdan emission spectrum up to 500 nm as soon as the first micelles are formed [36]. The formation of the hexagonal phase neither appears probable for the emission intensity would have to drop markedly just below the bilayer/hexagonal phase transition [37] and we have not observed this type of change.

In the case of Digitonin (Fig. 5, bottom panel), the evolution of the steady state spectra looks differently. Only the slight increase on the red edge of the spectra is observed at the low detergent concentrations. After the content of Digitonin reaches 0.01%, the increase of the intensity on the red side of the spectra decreases and the overall blue shift is observed. As already indicated by the DPH measurements Digitonin is fluidizing the membrane in a qualitatively similar manner as Brij 58 and Triton X-100 until its content reaches 0.01%. Apparently up to that concentration individual Digitonin molecules are incorporating into the membrane and by that –as the other investigated detergents– fluidizing the membranes. However, at higher Digitonin concentration the DPH anisotropy is again increasing and the Laurdan fluorescence gets again blue-shifted. Both experimental results indicate that the used dyes are probing a more rigid and/or less polar environment at higher Digitonin concentrations. We speculate that in that concentration range Digitonin is no more incorporating randomly into the membrane, but tightly packed aggregates or rigid domains with high Digitonin concentrations are formed. In this context it might be interesting to note, that similar blue-shifted spectra were observed when Laurdan was mixed with the Digitonin above the CMC.

3.4. The effect of detergents on trimeric G-protein activity

The effect of detergents on trimeric G-protein activity in brain cortex PM was strongly concentration dependent (Fig. 6). At very low concentrations (<0.003), the basal and baclofen-stimulated [^{35}S]GTPγS binding was not different from that in detergent-untreated PM. The increase of baclofen-stimulated binding was noticed at 0.006 and 0.013% Brij58 (Fig. 6, upper left panel) or at 0.013 and 0.025% Triton X-100 (Fig. 6, upper middle panel). At these concentrations, the net increment of baclofen stimulation, i.e. the difference between the agonist-stimulated and the basal level of binding for Brij58 and Triton X-100 was 1.8× and 1.3× higher than in detergent-untreated PM, respectively. Digitonin effect on G-protein activity was substantially different from the two former detergents. First of all, neither the basal nor baclofen-stimulated [^{35}S]GTPγS binding was significantly increased by this detergent. Secondly, the net-increment of baclofen-stimulation remained unchanged (Fig. 6, upper right panel). In order to underline the stimulatory effect of the detergents there is also depicted the difference between the baclofen stimulated and basal [^{35}S]GTPγS binding in the bottom panels of Fig. 6.

High concentrations of *all* three detergents were inhibitory for the baclofen-stimulated as well as basal level of [^{35}S]GTPγS binding: when increasing detergent concentration above "stimulatory range" of detergent concentrations, baclofen-stimulated as well as basal level of binding was diminished to zero level. The difference between the two water-soluble detergents Brij58 and Triton X-100 and Digitonin is clearly documented in Table 2 by comparison of the concentrations inducing an increase of the net-increment of baclofen-stimulation and those inducing the inhibition of [^{35}S]GTPγS binding.

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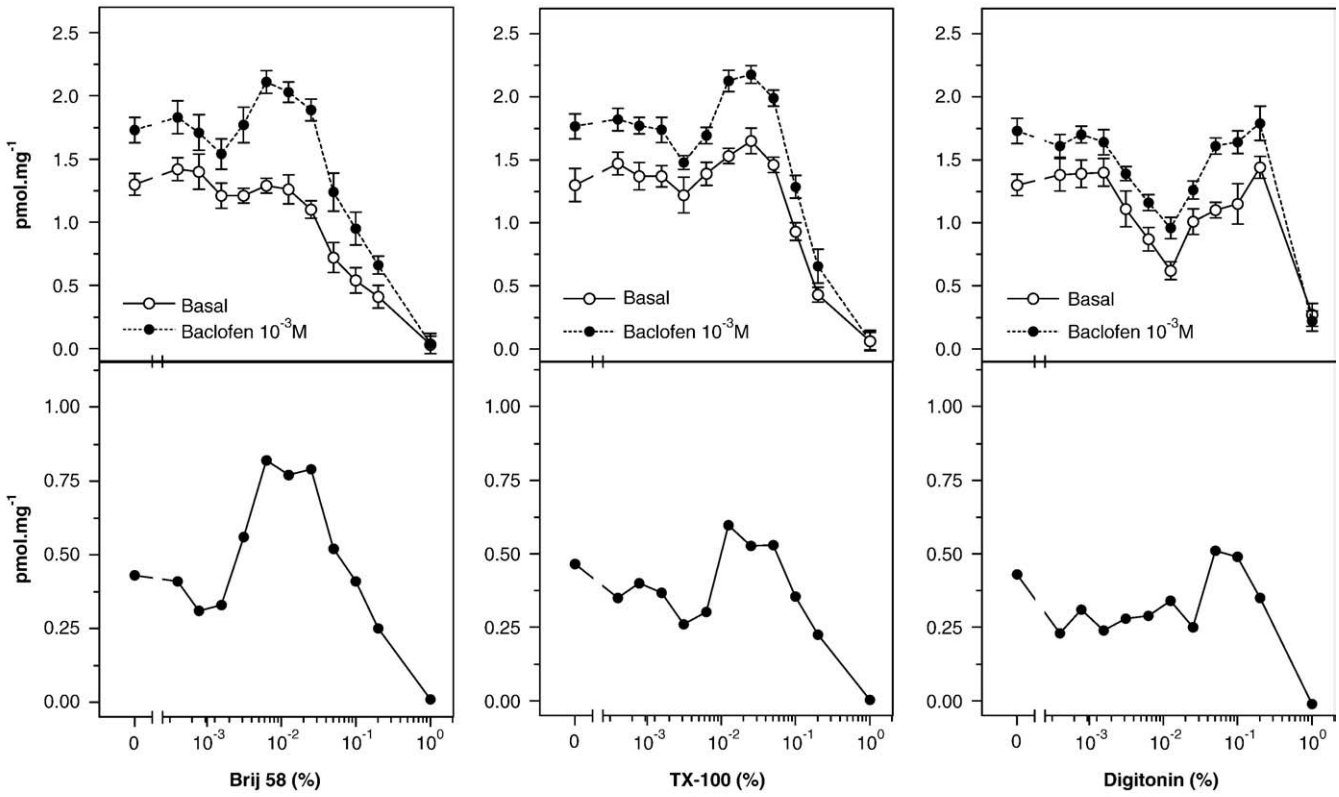

Fig. 6. Detergent effect on the baclofen-stimulated [^{35}S]GTPγS binding in brain cortex PM. The upper panels depict the activity of the basal (○) and baclofen-stimulated (●) [^{35}S]GTPγS binding. The bottom panels show the difference between the baclofen-stimulated and basal [^{35}S]GTPγS binding. PM fraction was mixed at 0–4 °C with increasing concentrations of Brij58 (right panel) or Triton X-100 (middle panel) to achieve the final concentration of 0.0004, 0.0008, 0.0016, 0.0031, 0.0063, 0.0125, 0.025, 0.05, 0.1, 0.2 and 1.0%; subsequently, an aliquot of membrane-detergent mix was transferred to [^{35}S]GTPγS binding assay medium and incubated at 25 °C for 30 min with [^{35}S]GTPγS as described in Materials and methods. Concentrated solution of Digitonin in DMSO (12%) was added to PM to achieve the same final concentration as in the case of the two water-soluble detergents (0.0004, 0.0008, 0.0016, 0.0031, 0.0063, 0.0125, 0.025, 0.05, 0.1, 0.2 and 1.0%), aliquots of membrane-detergent mix were transferred to [^{35}S]GTPγS binding assay medium and incubated at 25 °C for 30 min as in the case of Brij58 and Digitonin. Results represent the average of 3 experiments ± SEM.

3.5. The effect of detergents on radioligands binding to $GABA_B$-receptors

Detergent-induced changes of G-protein activity were also compared with detergent effect on specific radioligand binding to $GABA_B$-receptors measured as an antagonist [^{3}H]CGP54562 binding assay. Data presented in Fig. 7 indicated clearly the monotonous decrease of binding as a function of increasing detergent concentrations. From this point of view there was no difference between Brij58, Triton X-100 and Digitonin. The difference among the three detergents was manifested when comparing the quantitative parameters of this inhibition, i.e. concentrations inducing half-maximum decrease of binding. The potency of these detergents decreased with order of Brij58>Triton X-100>Digitonin. Thus, as shown previously in studies of DPH and Laurdan fluorescence and analysis of concentration dependence of detergent effect on the baclofen-stimulated [^{35}S]GTPγS binding, the same order of potency was detected.

When comparing the results of receptor binding and [^{35}S]GTPγS binding assays, a marked difference had to be noticed: inhibition of receptor binding capacity proceeded as monotonous decrease while concentration dependence of detergent effect on G-protein activity was clearly biphasic (decrease followed by maximum and sudden drop to very low or zero level of G-protein activity). This difference indicates that activation of G-protein activity by Brij58 cannot be explained simply as an increase in receptor binding capacity. This detergent, in a relatively narrow range of detergent concentrations and at a specific detergent/protein ratio is able to increase the intrinsic efficacy of receptor, i.e. ability of G-protein to respond to the conformational change induced by activated receptor. The thorough biophysical characterization of membrane state by fluorescence spectroscopy indicating the highest potency of Brij58 to alter structural as well as dynamic parameters of PM organization supports this view.

3.6. Summary of the DPH anisotropy, Laurdan generalized polarization, G-protein activity and $GABA_B$-receptor analysis

When summarizing our data, the direct effect of the detergents on G-protein activity in the isolated brain cortex PM was biphasic: i) *transient range of low* detergent concentrations was characterized by the decrease of baclofen ($GABA_B$-receptor)-stimulated [^{35}S]GTPγS binding followed by the increase back to the control or even higher levels of binding. The maximum of baclofen-stimulation observed at 0.006–0.013% Brij58 was 2× higher than in the control detergent/untreated PM. ii) The further increase of detergent concentrations

Table 2
The influence of Brij58, Triton X-100 and Digitonin on the G-protein activity

A		
Stimulatory range of detergent concentrations (% w/v)		
Brij58	0.006	0.013
Triton X-100	0.013	0.025
Digitonin	0.05	0.1
B		
Inhibitory range of detergent concentrations (% w/v)		
Brij58	>0.02	
Triton X-100	>0.05	
Digitonin	>0.2	

G-protein activity was measured as [^{35}S]GTPγS binding assay performed in the presence or absence of 1 mM baclofen ($GABA_B$-receptor agonist) as described in Materials and methods.

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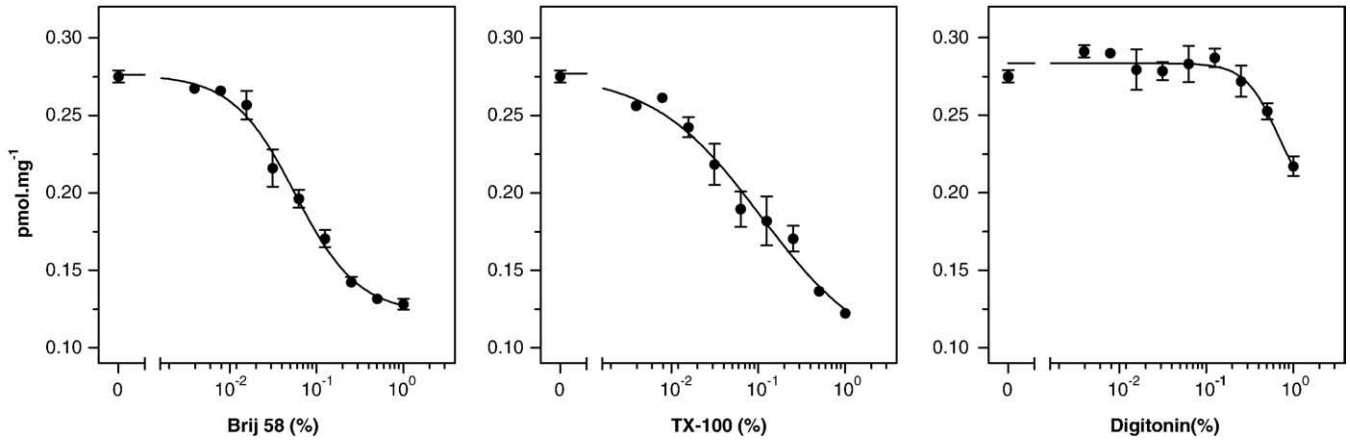

Fig. 7. Detergent-effect on the radioligand binding to $GABA_B$-receptors. Binding of specific antagonist [^{3}H]CGP54626 to $GABA_B$-receptors present in brain cortex PM was monitored at different concentrations of Brij58 (left panel), Triton X-100 (middle panel), and Digitonin (right panel) and was measured as described in Materials and methods. Membranes (50 μg protein) were incubated with 2.5 nM [^{3}H]CGP54626 (total concentration) in 0.5 ml of 75 mM Tris–HCl, pH 7.4, 12.5 mM $MgCl_2$, 1 mM EDTA for 60 min at 25 °C. The bound and free radioactivity was separated by rapid filtration through Whatman GF/B filters in the Brandel cell harvestor. Filters were washed 3× with 3 ml of ice-cold incubation buffer and placed in 4 ml of scintillation cocktail (CytoScint, ICN). Radioactivity remaining on filters was determined after 16 h at laboratory temperature by liquid scintillation. The non-specific binding was defined as that remaining in the presence of 1 mM non-radioactive GABA.

caused a drastic decrease/diminution of [^{35}S]GTPγS binding to zero level (Fig. 6). In contrast to the G-protein activity, the inhibitory effect of detergents on $GABA_B$-receptor was strictly monophasic, i.e. no stimulation/increase of specific radioligands binding was found (Fig. 7).

The ability of different detergents to induce the rise of baclofen-stimulated [^{35}S]GTPγS binding at low concentrations and a drastic decrease to the zero level at high concentrations grew in the order of potency Brij58>Triton X-100>Digitonin (Table 2). This order of potency was identical with the effect of detergents on the organization of the hydrophobic membrane interior characterized by the steady-state fluorescence anisotropy of DPH as well as on the lateral organization of the polar head-group region monitored by Laurdan generalized polarization. Additionally, Laurdan GP data indicate that Brij58 and Triton X-100 interact with the membrane in a completely different way than Digitonin. This can be explained by the fact that Triton X-100 and Brij58 preferentially incorporate into the bilayer present in the fluid, liquid crystalline phase and do not penetrate so effectively into the membranes possessing the gel or ordered phase states enriched in cholesterol [38,39]. In contrary, Digitonin is known to cause the clustering of the membrane cholesterol which results in the formation of the cholesterol enriched domains within the membrane [40,41]. Surprisingly, the GPCR activity was found to be affected to a greater extent by the fluidizing detergents, Triton X-100 and by Brij 58, than by Digitonin.

The time-resolved fluorescence measurements were performed with the aim to understand the effect of detergents on the hydrophobic membrane interior more clearly and to distinguish between the structural (S-order parameter) and dynamic (rotation correlation time) contributions to an overall steady-state anisotropy of DPH (r_{DPH}). Data presented in Fig. 2 indicated a dramatic decrease of the S-order parameter paralleled by an increase in the freedom of motion of DPH upon the addition of detergents. The analysis according to a model of restrained motions of Kawato et al. [42] and Kinoshita et al. [21] indicated a decrease of rotation correlation time accompanied by an increase of the diffusion wobbling constant D_w. The order of ability of different detergents to influence the time-resolved anisotropy parameters was identical as in the case of steady-state anisotropy and GP measurements: Brij58>Triton X-100>Digitonin.

In conclusion, the results of all experimental lines followed in this work (fluorescence, G-protein activity and receptor binding analysis) indicated the same order of potency of three different non-ionic detergents when modulating GPCR-G protein interaction or effecting the organization of the brain plasma membrane.

4. Conclusion

It is reasonable to assume that a significant increase of the net-increment of baclofen ($GABA_B$-R) stimulation of G-protein activity which was observed in the narrow range of *low* detergent concentrations reflects the *specific* alternation of plasma membrane structure while an over-all decrease of both total and basal activity of trimeric G proteins parallels the total degradation of membrane structure (at high detergent concentrations). Both these effects proceed in the order of efficiency: Brij58>Triton X-100>Digitonin.

Our data, thus, indicate that an optimum perturbation of the native PM structure is advantageous for the functional coupling between the receptor and its cognate G-protein in the narrow range of low detergent concentrations. Both hydrophobic membrane phase and the water-membrane interface participate in this process.

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