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ARTICLE

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Quartz crystal microbalance investigation of the interaction of bacterial toxins with ganglioside containing solid supported membranes

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Abstract The binding of cholera toxin, tetanus toxin and pertussis toxin to ganglioside containing solid supported membranes has been investigated by quartz crystal microbalance measurements. The bilayers were prepared by fusion of phospholipid-vesicles on a hydrophobic monolayer of octanethiol chemisorbed on one gold electrode placed on the 5 MHz AT-cut quartz crystal. The ability of the gangliosides G_{M1}, G_{M3}, G_{D1a}, G_{D1b}, G_{T1b} and *asialo*-G_{M1} to act as suitable receptors for the different toxins was tested by measuring the changes of quartz resonance frequencies. To obtain the binding constants of each ligand-receptor-couple Langmuir-isotherms were successfully fitted to the experimental adsorption isotherms. Cholera toxin shows a high affinity for G_{M1} ($K_a = 1.8 \cdot 10^8 M^{-1}$), a lower one for *asialo*-G_{M1} ($K_a = 1.0 \cdot 10^7\ M^{-1}$) and no affinity for G_{M3}. The C-fragment of tetanus toxin binds to ganglioside G_{D1a}, G_{D1b} and G_{T1b} containing membranes with similar affinity ($K_a \sim 10^6\ M^{-1}$), while no binding was observed with G_{M3}. Pertussis toxin binds to membranes containing the ganglioside G_{D1a} with a binding constant of $K_a = 1.6 \cdot 10^6\ M^{-1}$, but only if large amounts (40 mol%) of G_{D1a} are present. The maximum frequency shift caused by the protein adsorption depends strongly on the molecular structure of the receptor. This is clearly demonstrated by an observed maximum frequency decrease of 99 Hz for the adsorption of the C-fragment of tetanus toxin to G_{D1b}. In contrast to this large frequency decrease, which was unexpectedly high with respect to Sauerbrey's equation, implying pure mass loading, a maximum shift of only 28 Hz was detected after adsorption of the C-fragment of tetanus toxin to G_{D1a}.

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Key words Quartz crystal microbalance (QCM) · Cholera toxin · Tetanus toxin · Pertussis toxin · Solid supported membranes · Gangliosides

Abbreviations Cer ceramid · CT cholera toxin · Gal galactose · GalNac N-acetylgalactosamine · Glc glucose · LUV large unilamellar vesicle · NANA N-acetyl-neuraminic acid · PNA peanut agglutinin · PT pertussis toxin · POPC 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine · QCM quartz crystal microbalance · TT tetanus toxin

1 Introduction

The interaction of bacterial exotoxins, such as cholera toxin, tetanus toxin or pertussis toxin, with cellular target membranes is usually initiated by a specific recognition process with well defined receptors on the membrane of the attacked cell. These toxins often possess at least two different domains, one is responsible for binding to the receptor, the other one penetrates the cell-membrane and induces the typical toxic effect attributed to the bacterium, mainly by disturbing the signal transduction of the eukaryotic target cell. In this study we have investigated the specific binding of three different bacterial toxins to receptor lipids.

Tetanus toxin (TT), the exotoxin of *Chlostridium tetani* is one of the most effective toxins from bacteria. It consists of two subunits, fragment B (M = 99 kDa) and fragment C (M = 52 kDa) (Weller et al. 1989). Fragment C binds to the membrane confined receptors, the gangliosides of the b-series (Winter et al. 1996), whereas the B-fragment carries the catalytic unit of the toxin. Here the binding of the C-fragment to the gangliosides G_{M3}, G_{D1a}, G_{D1b} and G_{T1b} has been investigated.

The second ligand-receptor couple under investigation was G_{M1} – cholera toxin (CT). CT is the enterotoxin of *Cholera vibrio*, an 87 kDa protein with the subunit composition AB_5 in which the five identical B subunits form a pentagonal ring surrounding the A subunit. The B sub-

unit possesses the binding site for the cell surface receptor, the ganglioside G_{M1} (Fishman 1990). Upon binding, the A subunit divides into two parts, the A1 subunit penetrates the cell membrane and catalyzes the ADP-ribosylation of stimulatory G-proteins, thus activating the adenylate cyclase and producing large amounts of cAMP. This results in the emission of huge quantities of digestive fluids into the lumen of the intestine (up to 20 l/day), the major symptom of cholera.

Pertussis toxin (PT) from *Bordetella pertussis*, the pathogen causing whooping cough, was the third toxin investigated in this study. PT, a 105 kDa protein, has a similar A–B structure to cholera toxin. It consists of an enzymatically active A subunit and a B oligomer, composed of five subunits (S2, S3, 2×S4 and S5), which is responsible for the binding of the toxin to the target cell (Hausman and Burns 1993; Saukkonen et al. 1992). Like CT, pertussis toxin exerts its effect on mammalian cells by catalyzing the transfer of the ADP-ribose moiety from NAD to certain inhibitory G-proteins, thus again leading to highly elevated intracellular levels of cAMP. PT starts its action, as do other bacterial toxins, by first binding to its cellular receptors, which may be a glycolipid or a glycoprotein. Hausman and Burns (1993) studied the interaction of PT with different glycolipid containing vesicles and found that the ganglioside G_{D1a} exhibits the highest affinity for PT. In contrast to this study, Brennan et al. (1988) found no evidence for PT binding to glycolipids in general. In order to determine the contribution of gangliosides as possible receptors for PT we investigated the binding of PT to different gangliosides.

The quantification of protein binding to a special membrane receptor is usually a demanding experimental procedure which often requires artificial lipid bilayers doped with the isolated receptor to determine the binding constant of the ligand-receptor couple. Several approaches have been made to obtain stable planar lipid bilayers. In the last decade solid supported lipid bilayers have played an important role in imitating biological membranes because of their long-term stability and the possibility of constituting them on electrically conductive supports (Kalb et al. 1992). An overview on current techniques to deposit bilayers on a solid support, especially on gold, is given by Steinem et al. (1996). Terrettaz et al. (1993) used gold-electrodes with a first chemisorbed monolayer of alkanethiols and a second phospholipid monolayer doped with the ganglioside G_{M1} to study the specific adsorption of cholera toxin by surface plasmon resonance spectroscopy. We have now used the quartz crystal microbalance (QCM) to quantify the amount of protein bound to receptor lipids embedded in a phospholipid matrix. Since Sauerbrey's pioneering work (Sauerbrey 1959), piezoelectric quartz crystals have been used widely in chemistry and physics as vacuum deposition monitors and gas-phase sensors. In the last decade the use of the QCM has been extended to the liquid phase, opening up a variety of new applications of in situ measurements of adsorption processes. In addition to its application in electrochemistry, the QCM offers an inexpensive and very sensitive method to detect the adsorption of small amounts of proteins (Muratsugu et al. 1993) and nucleic acids (Yamagushi and Schimomura 1993) from solution. For example, shear wave resonators have been used as immunosensors to quantify the binding of protein antigens, haptens and antibodies (Muramatsu et al. 1987; König and Grätzel 1994).

In this study we immobilized solid supported membranes on top of a quartz plate. The bilayer consisted of a self-assembled monolayer of octanethiol on gold and a second monolayer of ganglioside containing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) deposited by fusion of large unilamellar vesicles on the hydrophobic alkanethiol-monolayer. Impedance spectroscopy was used to characterize the integrity of the membrane. The specific interactions of the three different toxins with these bilayers were investigated by measuring the frequency shift of the QCM upon binding of the proteins to the surface-receptors.

2 Materials and methods

2.1 Materials

Cholera toxin was purchased from List Biological Laboratories (Campbell, CA, USA), the C-fragment of tetanus toxin was a kind gift from Prof. Habermann, University of Giessen, pertussis toxin was obtained as a generous gift from the Institute Pasteur Mérieux Connaught. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine was purchased from Avanti Polar Lipids (Pelham, AL, USA). All gangliosides were from Sigma (Deisenhofen, Germany). Octanethiol and pronase E were from Fluka (Neu Ulm, Germany). Water was first purified by a Millipore water purification system MilliQ RO 10 Plus and finally by the Millipore ultrapure water system MilliQ Plus 185 (18 MΩ/cm). The 5 MHz overtone polished AT-cut quartz crystals (plano-plano) were from KVG (Niederbischofsheim, Germany), the silver conductive adhesive was from the Epoxy-GmbH (Fürth/Odenwald, Germany) and the silicon glue (Elchsiegel) from Rhône Poulenc (Leverkusen, Germany). The gold (99.99% purity) used for the gold electrodes of the surface of the quartz plates was a generous gift from DEGUSSA (Hanau, Germany), the chromium was purchased from Bal-Tec (Balzers, Liechtenstein).

2.2 Impedance spectroscopy

AC impedance spectroscopy was performed as described previously (Janshoff et al. 1996b). The continuous wave impedance gain/phase analyzer SI 1260 was from Solartron instruments (Farnborough, Great Britain). The magnitude of the impedance $|Z(f)|$ and the phase angle $\Phi(f)$ were recorded in the frequency range of 0.1 to 10^6 Hz with an AC amplitude of 30 mV and 0 mV DC offset-potential. The electrochemical cell consists of the quartz crystal with one gold electrode serving as the working electrode and a

platinized platinum wire as the counter electrode. Quantitative analysis of the spectra was performed by fitting the parameters of the equivalent circuit shown in Fig. 2 to the data by a non-linear-least-squares-fit using the Levenberg-Marquardt algorithm (Bevington 1969). The equivalent circuit used for the fitting procedure simply consists of a resistance in series with a capacitance. The resistance represents the ohmic nature of the electrolyte and the capacitance the electrical behavior of the membrane. The resistances of the monolayer and the bilayer were not detectable in the applied frequency range and were therefore neglected in the analysis.

2.3 QCM-measurements

The experimental setup for the QCM-measurements used in the present study is shown schematically in Fig. 1. It basically consists of the quartz resonator, a flow system and the oscillator circuit (Janshoff et al. 1996 b). We used overtone polished plano-plano 5 MHz AT-cut quartz resonators with 14 mm in diameter, which were coated with gold electrodes with an area of 0.33 cm^2 on both sides as described elsewhere (Steinem et al. 1996). The gold electrodes were connected to the oscillator circuit via thin silver wires which were fixed on the gold electrodes by a conductive adhesive. The measuring chamber, formed by small glass tubes fixed to the quartz plates with a silicon glue was closed by a stopper equipped with an inlet and an outlet to connect the chamber with a peristaltic pump. The whole flow system includes a volume of 2 ml pumped with a flow rate of 500 µl/min through the quartz chamber. To keep the temperature constant, the crystal and the oscillator circuit were placed in a temperature controlled chamber which also served as a Faraday cage. All experiments were performed at 21 °C. The oscillator circuit consists of an integrated circuit SN74LS124N from Texas Instruments connected to a frequency counter from Hewlett Packard (HP 53181 A) and an oscilloscope in order to control the oscillation.

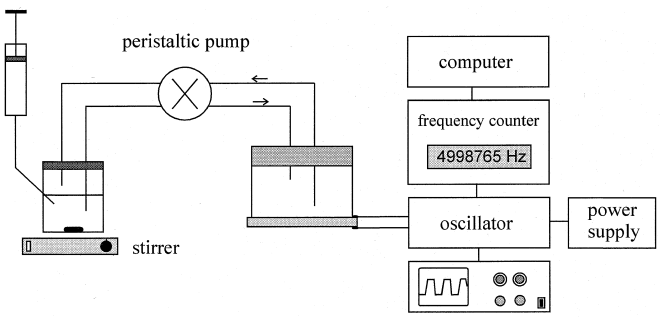

Fig. 1 Experimental setup of the quartz crystal microbalance

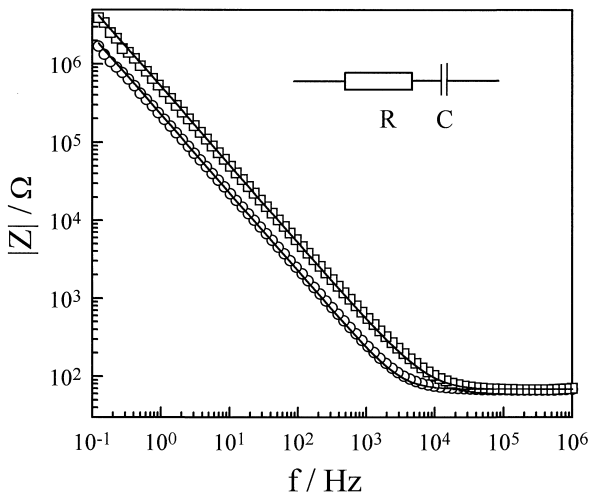

Fig. 2 Impedance spectra of a chemisorbed monolayer of octanethiol (OT) and a bilayer consisting of OT and a second physisorbed monolayer of POPC. The *solid lines* are the results of the fitting procedure according to the Levenberg-Marquardt-algorithm with the following parameters: $C_{OT} = 2.11\ \mu F/cm^2$, $C_{OT/POPC} = 0.93\ \mu F/cm^2$

The calibration of the QCM was performed by electrodeposition of copper as described elsewhere (Hillier & Ward 1992). Following the theory of Sauerbrey (Sauerbrey 1959) the observed decrease in frequency should be proportional to the change in mass of the quartz resonator:

$$\Delta f = \frac{-2 f_0^2 \Delta m}{A \sqrt{\rho_q \mu_q}} = -C_f \Delta m , \qquad (1)$$

where f_0 denotes the fundamental resonant frequency, A the electrode area, ρ_q the density of the quartz ($\rho_q = 2.648\ g/cm^3$) and μ_q the shear modulus of the quartz ($\mu_q = 2.947 \cdot 10^{11}$ dyne/cm^2). We determined an integral mass sensitivity of $C_f = 0.11$ Hz/ng, corresponding to 0.036 Hz cm^2/ng. Although this value is lower than the numerical value of 0.057 Hz cm^2/ng obtained from Sauerbrey's equation it is still reasonable considering the study of Hillier and Ward (1992) who determined the same value as we did for 5 MHz plano-plano AT-cut quartz resonators plated with copper.

2.3.1 Binding assay

As indicated in Fig. 1 the buffer solution consisting of 50 mM Tris, 200 mM NaCl, pH 7.4 was pumped through the measuring cell with a flow rate of 500 µl/min until a stable frequency was reached. The protein solution, dissolved in the same buffer, was added by a syringe to the small vessel outside the Faraday cage. The experiments were carried out at 21 °C in a temperature controlled chamber.

2.4 Preparation of bilayers on gold

The preparation of the solid supported membranes was performed as described elsewhere (Steinem et al. 1996; Janshoff et al. 1996 a). In the first step the gold electrode of the quartz crystal was immersed for 30 min in an ethanolic solution containing 1 mM octanethiol. Directly

before use, the electrode was extensively rinsed first with ethanol, and then at least five times with buffer to remove remaining thiols.

Vesicles (LUVs) of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) with different amounts of the appropriate ganglioside were prepared by the extrusion method as described elsewhere (Steinem et al. 1996). The bilayer was formed by adding the preformed vesicles to the hydrophobic monolayer of octanethiol in 50 mM Tris, 200 mM NaCl, pH 7.4 at 40 °C. With respect to impedance measurements the fusion process was finished after one hour. Remaining vesicles were removed by rinsing the electrode surface several times with buffer solution.

3 Results

3.1 Characterization of the bilayer formation on the quartz-crystal

Impedance spectroscopy is a sensitive method to determine several relevant physical parameters of thin organic films such as self-assembled monolayers or lipid bilayers on a solid support. Membrane thickness, dielectric constant, conductivity through pin holes and electrode coverage may be easily determined (Terrettaz et al. 1993; Stelzle et al. 1993; Janshoff et al. 1996 a). Here we made use of this method only to control the deposition process of the lipid bilayer to ensure that the electrode coverage is more than 95%. Figure 2 shows typical impedance spectra of a chemisorbed monolayer of octanethiol (OT) and the bilayer consisting of octanethiol with a second monolayer of the phospholipid POPC subsequently adsorbed on top of the hydrophobic OT-layer by vesicle fusion. The solid lines represent the fitting results according to the equivalent circuit which is also depicted in Fig. 2. As the conductivity of the solid supported membrane is very low the use of this simple model is justified in order to keep interpretable results. The mean capacitance of an OT-monolayer is about $C_{OT} = (2.2 \pm 0.1)\ \mu F/cm^2$, whereas the capacitance of the bilayer consisting of the OT-layer and a monolayer of the phospholipid POPC provides a mean value of $C_{OT/POPC} = (1.0 \pm 0.1)\ \mu F/cm^2$. Assuming a serial connection of C_{OT} and C_{POPC} the POPC-monolayer exhibits a mean capacitance of $C_{POPC} = (1.8 \pm 0.2)\ \mu F/cm^2$. The values for the capacitances and the standard deviations are obtained from 20 independent measurements. The incorporation of gangliosides up to 10 mol% did not significantly influence the impedance behavior of the solid supported membranes.

3.2 Binding of the C-fragment of tetanus toxin to different gangliosides

The specific binding of the C-fragment of tetanus toxin to different gangliosides was investigated using the QCM. The measurements were performed two times and the results were reproducible. The gangliosides G_{M3}, G_{D1a}, G_{D1b} and G_{T1b}, shown schematically in Fig. 3, were incorporated into the outer leaflet of a solid supported lipid bilayer by fusing POPC vesicles doped with 10 mol% of the corresponding ganglioside on a hydrophobic octanethiol-monolayer chemisorbed on the gold electrode of the quartz plate.

The protein was added to the QCM after a constant baseline was reached. For the determination of the binding constant of the C-fragment to various gangliosides the time resolved resonant frequency change was monitored as a function of the protein concentration in solution. Figure 4 shows the time course of the resonant frequency shift due to the adsorption of the C-fragment of tetanus toxin on the bilayer consisting of octanethiol and POPC doped with 10 mol% G_{D1b}. The arrows in Fig. 4 indicate the time of

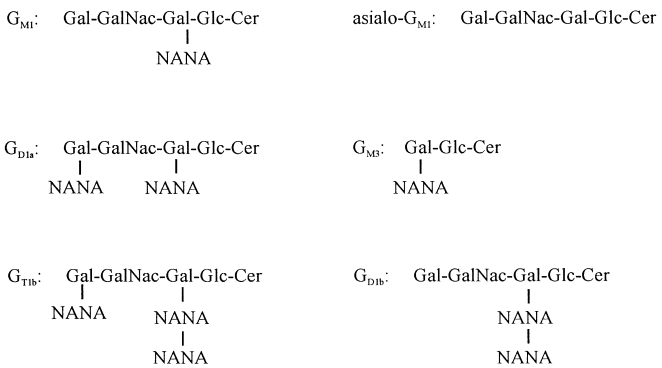

Fig. 3 Schematic representation of the gangliosides used in this study. *Cer*: ceramid, *Gal*: galactose, *GalNac*: *N*-acetylgalactoseamine, *Glc*: glucose, NANA: N-acetyl-neuraminic acid

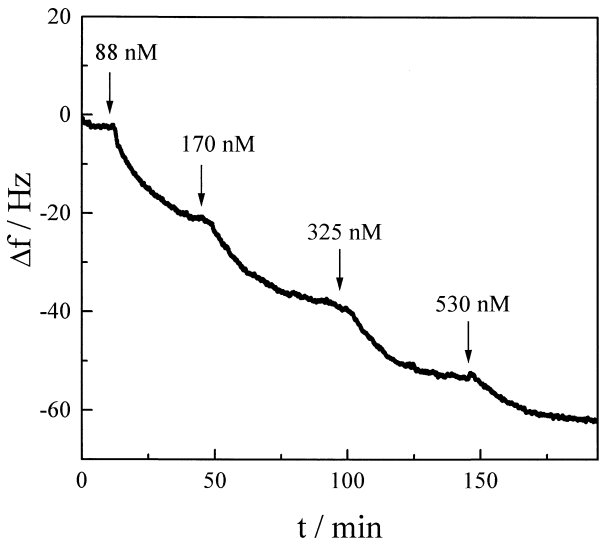

Fig. 4 Time course of the resonant frequency shift after adsorption of the C-fragment of tetanus toxin on a bilayer of OT and POPC doped with 10 mol% G_{D1b} in the outer leaflet. The *arrows* indicate the time of protein injection and the corresponding protein concentration in solution. Each quantity of toxin was allowed to reach an equilibrium value in order to extract the frequency shift for the determination of the adsorption isotherm

protein injection as well as the actual concentration of the C-fragment in the bulk phase. The resonant frequency was decreased by addition of the C-fragment with a typical exponential decay. Determination of the equilibrium frequency shifts after injection of the corresponding toxin concentration in solution resulted in the hyperbolic dependence shown in Fig. 5. In order to obtain the binding constant of the ligand receptor couple a Langmuir adsorption isotherm was used to fit the experimental data:

$$\Delta f = \Delta f_{max} \frac{K_a\, c_{toxin}}{1 + K_a\, c_{toxin}}. \quad (2)$$

Δf_{max} is the frequency shift upon saturation and K_a the binding constant of the toxin to the corresponding receptor lipid. This model implies a homogeneous distribution of the ganglioside in the membrane phase, no energetic differences between the adsorption sites and no interaction between the bound proteins. The solid lines in Fig. 5 are the results of the fitting procedures. Clearly, the experimental data are in good agreement with the calculated fits. The binding constant for the ganglioside G_{D1b} amounts to $K_a = (3.0 \pm 0.4) \cdot 10^6\ M^{-1}$ with a maximum frequency shift of $\Delta f_{max} = -(99 \pm 4)$ Hz. Since it is known that the gangliosides of the b-series exhibit the best binding constants, we also investigated the binding of the C-fragment of tetanus toxin on a G_{T1b} doped POPC-monolayer (Fig. 5). The maximum frequency shift of $\Delta f_{max} = -(66 \pm 7)$ Hz of the adsorption isotherm is significantly lower, whereas the binding constant $K_a = (1.7 \pm 0.4) \cdot 10^6\ M^{-1}$ is in the same range. The analysis of the binding properties of the toxin to a G_{D1a} doped POPC-monolayer yielded, in contrast to all expectations, a similar binding constant. As shown in Fig. 5 the binding constant of $K_a = (2.4 \pm 0.3) \cdot 10^6\ M^{-1}$ is in the same range as the binding constants of the two other gangliosides. The maximum frequency shift amounts only to $\Delta f_{max} = -(28 \pm 1)$ Hz. It is important to note that a frequency shift could not be detected by the addition of the C-fragment to a POPC-monolayer doped with 10 mol% of the ganglioside G_{M3} up to a final protein concentration of 1.2 μM in solution, which excludes a pure electrostatic interaction.

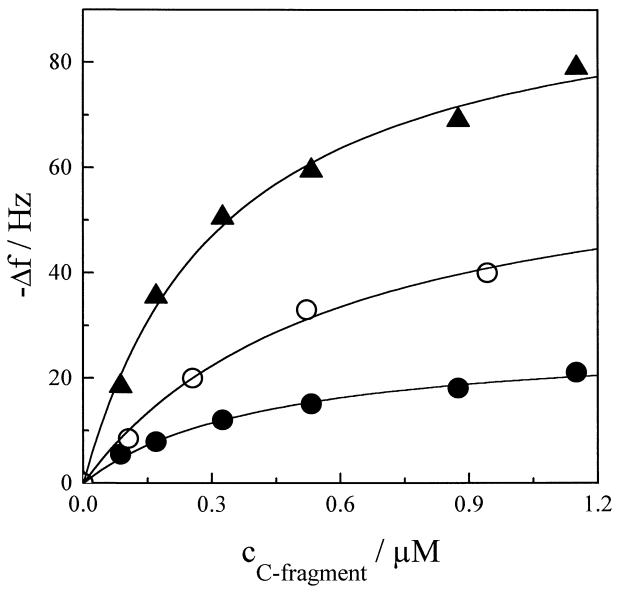

Fig. 5 Adsorption isotherm of the C-fragment of tetanus toxin on the ganglioside containing lipid layer: (●) 10 mol% G_{D1a}, (▲) G_{D1b} and (○) G_{T1b}, respectively. The *solid lines* represent the fitting results according to Eq. (2) with the following parameters: (●) $K_a = (2.4 \pm 0.4 \times 10^6)\ M^{-1}$, $\Delta f_{max} = -(28 \pm 1)$ Hz, (▲) $K_a = (3.0 \pm 0.4 \times 10^6)\ M^{-1}$, $\Delta f_{max} = -(99 \pm 4)$ Hz, (○) $K_a = (1.7 \pm 0.4 \times 10^6)\ M^{-1}$, $\Delta f_{max} = -(66 \pm 7)$ Hz

3.3 Digestion of the adsorbed C-fragment of tetanus toxin by pronase E

Although the previous section showed that there are only small differences between the binding constants K_a of the C-fragment of TT to the different gangliosides, there may be a different kind of interaction and orientation of the C-fragment adsorbed on the membrane. This hypothesis is supported by the remarkable differences in the maximum decreases of the resonant frequency (see Table 1). In order to test the accessibility of the bound protein in the presence of the different ganglioside-receptors, the unspecific protease, pronase E, was added at a final concentration of 0.2%. Figure 6 shows the proteolytic effect of pronase E on the C-fragment of tetanus toxin adsorbed on a bilayer of octanethiol and POPC doped with 10 mol% G_{D1b} and G_{D1a}, respectively. The resonant frequency of the shear wave resonator immediately increases, due to the digestion of the protein. In the case of G_{D1a} the maximal observed frequency increase of 20 Hz means that about 71% of the initial frequency decrease caused by the C-fragment adsorption was regained by the protease action. In the case of G_{D1b} only 38% of the initial frequency decrease was regained.

3.4 Binding of cholera toxin to G_{M1} and *asialo*-G_{M1}

The binding of cholera toxin to G_{M1} containing POPC-membranes was also investigated using the QCM and compared with the binding to G_{M3} containing membranes.

Table 1 Binding constants and maximum frequency shifts of the different toxin-ganglioside couples studied in this paper

Toxin	Ganglioside	K_a/M^{-1}	$-\Delta f_{max}$/Hz
Cholera toxin	G_{M3}/10 mol%	–	–
	G_{M1}/10 mol%	$1.8 \cdot 10^8$	111
	asialo-G_{M1}/10 mol%	$1.0 \cdot 10^7$	34
C-fragment	G_{M3}/10 mol%	–	–
	G_{D1a}/10 mol%	$2.4 \cdot 10^6$	28
	G_{D1b}/10 mol%	$3.0 \cdot 10^6$	99
	G_{T1b}/10 mol%	$1.7 \cdot 10^6$	66
Pertussis toxin	G_{D1a}/40 mol%	$1.6 \cdot 10^6$	34
	G_{D1a}/10 mol%	–	–
	ganglioside mix/40 mol%	–	–

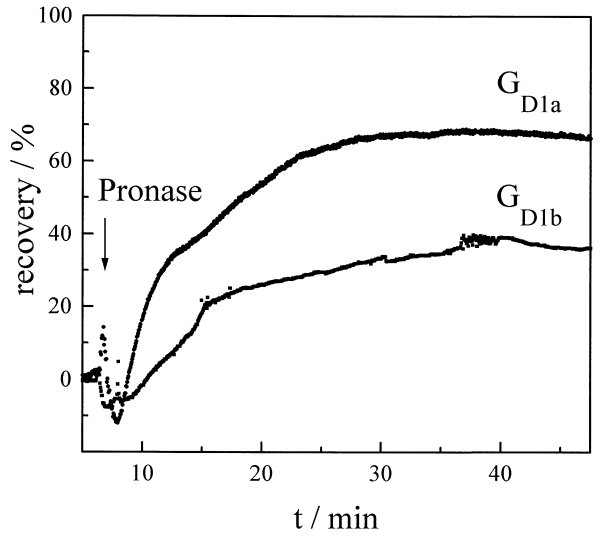

Fig. 6 Recovery in % of the initial frequency decrease, which was caused by the adsorption of a monolayer of the C-fragment of tetanus toxin on G_{D1a} and G_{D1b}, respectively, due to the addition of pronase E at a final concentration of 0.2% (w/w)

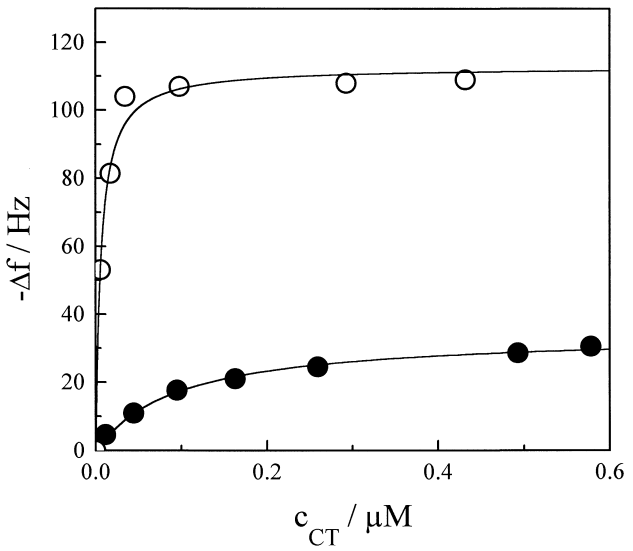

Fig. 8 Adsorption isotherm of cholera toxin on a POPC-monolayer containing (○) 10 mol% G_{M1} and (●) 10 mol% *asialo*-G_{M1}. The *solid lines* represent the results of the fitting procedure according to Eq. (3). Fitting results: (○) $K_a = (1.8 \pm 0.1) \cdot 10^8$ M^{-1}, $\Delta f_{max} = -(111 \pm 2)$ Hz, (●) $K_a = (1.0 \pm 0.1) \cdot 10^7$ M^{-1}, $\Delta f_{max} = -(34 \pm 2)$ Hz

Figure 7 shows the time course of the resonant frequency shift of two quartz-crystals, one with a solid supported bilayer containing 10 mol% G_{M3} in the outer POPC-monolayer (A.) and the other doped with 10 mol% G_{M1} (B.). Only the resonator functionalized with the ganglioside G_{M1} shows a response on the addition of the toxin. In Fig. 7 each protein injection is indicated by an arrow labeled with the actual concentration of the toxin in solution. The resulting adsorption isotherm of the binding of CT to a G_{M1} containing bilayer shown in Fig. 8 was obtained by plotting the equilibrium values of the frequency change as a function of the CT-concentration in the bulk phase. A binding constant of $K_a = (1.8 \pm 0.1) \cdot 10^8$ M^{-1} with a maximum frequency decrease of $\Delta f_{max} = -(111 \pm 2)$ Hz was obtained for G_{M1} by fitting the experimental adsorption isotherm with a Langmuir-isotherm (Eq. (2)).

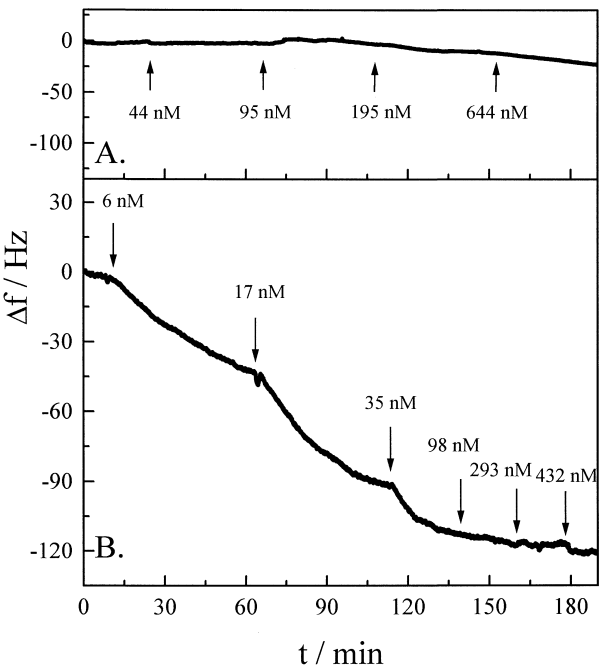

Fig. 7 Time course of the resonant frequency shift after the addition of cholera toxin to a lipid layer consisting of POPC and **A** 10 mol% G_{M3} and **B** 10 mol% G_{M1}. The *arrows* indicate the time of injection and the actual protein concentration in solution

To demonstrate the importance of the sialic-acid in the receptor-structure of G_{M1} we also used a bilayer consisting of OT and POPC doped in the outer leaflet with 10 mol% *asialo*-G_{M1} (Fig. 8). Both the maximum frequency decrease $\Delta f_{max} = -(34 \pm 2)$ Hz and the binding constant $K_a = (1.0 \pm 0.1) \cdot 10^7$ M^{-1} were significantly lower compared to G_{M1} containing membranes, which is the putative receptor for cholera toxin. All experiments presented in this section were performed three times with reproducible results.

3.5 Digestion of CT adsorbed on G_{M1} and *asialo*-G_{M1} by pronase E

The proteolytic effect of pronase E on the adsorbed cholera toxin was similar to the one observed during the digestion of the tetanus toxin C-fragment. The resonant frequency of a quartz crystal with G_{M1} bound cholera toxin increased only by 35 Hz compared to an initial frequency decrease of 110 Hz at $c_{CT} = 0.5$ μM. The digestion of CT adsorbed on an *asialo*-G_{M1} doped POPC-monolayer resulted in an increase of the resonant frequency by about 30 Hz, which is close to the initial frequency decrease of

32 Hz observed after the adsorption of CT from a 0.5 μM solution.

3.6 Binding of pertussis toxin to different gangliosides

Since it is not yet confirmed whether pertussis toxin binds to gangliosides, we investigated the binding of PT to a POPC-monolayer doped with different amounts of mixed brain gangliosides up to 40 mol%. As shown in Fig. 9 even at this high ganglioside content no binding of PT could be monitored up to a concentration of 7 μg/ml PT in solution. In order to prove that the gangliosides are accessible for the protein the lectin peanut agglutinin (PNA) was added to the reaction vessel at a concentration of 2 μM. PNA is known to bind specifically to the ganglioside G_{M1} and, as expected, the frequency of the quartz resonator decreases after the addition of the protein, corresponding to an adsorption of PNA to the surface.

We also studied the binding of PT to a monolayer of POPC doped with G_{D1a}, as a potential receptor (Hausman and Burns 1993). With a content of 10 mol% ganglioside in the POPC monolayer no frequency shift of the quartz resonator could be detected up to a concentration of 0.8 μM. The concentration of the toxin was not further increased in order to maintain physiological conditions. However, an increase of the G_{D1a}-content up to 40 mol% in the POPC monolayer finally resulted in binding of the toxin. The corresponding adsorption isotherm is shown in Fig. 10. Fitting the data according to Eq. (2) yields a binding constant of $K_a = (1.6 \pm 0.5) \cdot 10^6\ M^{-1}$ and a maximum frequency shift of $\Delta f_{max} = -(34 \pm 4)$ Hz.

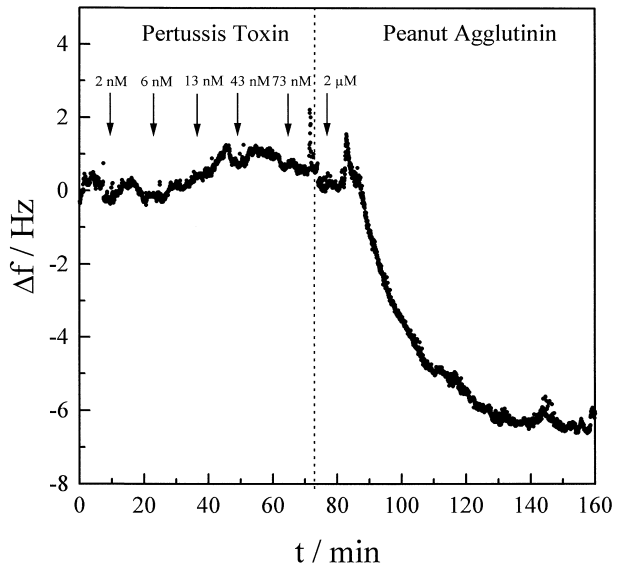

Fig. 9 Time course of the resonant frequency shift after the addition of pertussis toxin and peanut agglutinin to a POPC monolayer containing 40 mol% of mixed brain gangliosides. The *arrows* indicate the time of protein injection and the actual protein concentration in solution

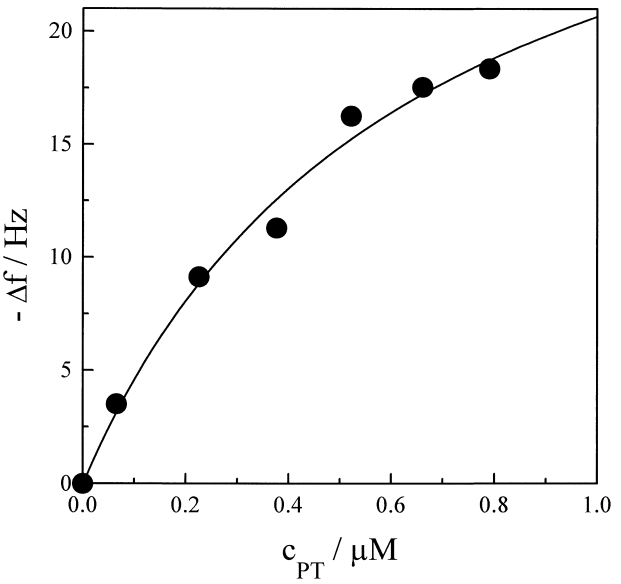

Fig. 10 Adsorption isotherm of pertussis toxin on a POPC-monolayer doped with 40 mol% G_{D1a}. The corresponding fit using Eq. (2) is given as a *solid line* with the following parameters: $K_a = 1.6 \cdot 10^6\ M^{-1}$, $\Delta f_{max} = -(34 \pm 4)$ Hz

4 Discussion

The present study demonstrates the applicability of the quartz crystal microbalance to quantify the specific adsorption of proteins on membrane confined receptors in real time. Solid supported lipid bilayers with incorporated receptor molecules are appropriate model membranes because of their long-term stability, the low level of non-specific binding of proteins to the phospholipid matrix and ease of preparation (Stelzle et al. 1993). Moreover the suitability of membrane preparations can be easily tested by impedance spectroscopy, guaranteeing a high degree of reproducibility.

4.1 Binding of the C-fragment of tetanus toxin to ganglioside containing membranes

The specific adsorption of tetanus toxin on the ganglioside containing solid supported lipid membrane follows a Langmuir-isotherm (Fig. 5), which means that no interaction between the ligands on the surface occurs and that the maximum amount of adsorbed protein is organized in a well defined protein-monolayer. This fact is in good agreement with the observations of Ebato et al. (1992), Okahata et al. (1995) and Janshoff et al. (1996 a).

We were able to show that the C-fragment of tetanus toxin binds to the gangliosides G_{D1a}, G_{D1b} and G_{T1b} with almost the same affinity. The binding constants for these ganglioside-receptors are close to a range from $1.7 \cdot 10^6$ to $3.0 \cdot 10^6\ M^{-1}$. However, no adsorption of the C-fragment of tetanus toxin on G_{M3} was observable. These findings are consistent with the study of Holmgren et al. (1980) who

pointed out that the number and positions of the sialic acid residues linked to the receptor are important for the binding affinity. The authors concluded from their experimental results that successful binding requires at least one sialosyl residue linked to the proximal galactose, while maximum binding occurs if a disialosyl group is linked to the proximal galactose (b-series). Additional sialic acid residues do not contribute to the recognition structure. Holmgren et al. also noticed that the oligosaccharide backbone is crucial for the binding affinity of tetanus toxin. No adsorption of the toxin was observed using G_{M3}, which lacks the terminal galactose and the N-acetylgalactoseamine, although a sialosyl group is present at the galactose.

The lower binding affinity predicted for the ganglioside G_{D1a} could not be confirmed. We found no significant difference between G_{D1a} and the gangliosides of the b-series (G_{D1b} and G_{T1b}). This could be explained by the observation of Habermann and Dreyer (1986) who found that tetanus toxin did not interact with G_{M1} and G_{D1a} if physiological phosphate buffer was used but considerable binding affinity was reported in a Tris-HCl buffered system.

4.2 Binding of cholera toxin to G_{M1} and *asialo*-G_{M1} containing membranes

This study confirms that the most effective lipid-receptor for the binding of cholera toxin to the cellular surface is the ganglioside G_{M1}. The important role of the sialic acid residue was demonstrated by the fact that the ligand receptor couple CT-*asialo*-G_{M1} exhibits a considerably lower binding constant of $K_a = 1.0 \cdot 10^7\ M^{-1}$ compared to the ligand receptor couple CT-G_{M1} with $K_a = 1.8 \cdot 10^8\ M^{-1}$. Thus we may conclude that the binding affinity also depends on the composition of the oligosaccharide backbone of the ganglioside with special respect to the sialic acid residue. Compared to the binding of the lectin PNA to G_{M1}, which exhibits a binding constant of $K_a = 8.3 \cdot 10^5\ M^{-1}$, also determined by QCM-measurements, the affinity of CT for the ganglioside is significantly higher (Janshoff et al. 1996 b).

4.3 Binding of pertussis toxin to ganglioside containing membranes

Until now it is not clear whether the natural receptor of pertussis toxin is a glycolipid or a glycoprotein. Brennan et al. (1988) have shown that the presence of pertussis toxin does not induce the clustered growth pattern in CHO cells, if the cells lack the terminal NeuAc-Gal-β-4-GlcNAc oligosaccharide sequence on the glycoproteins. Therefore, they concluded that the natural receptor in cell membranes is a glycoprotein. In contrast, Saukkonen et al. (1992) have demonstrated that the binding of pertussis toxin to mixed gangliosides was detectable by a TLC-assay when the chromatograms were submersed in a solution of saline containing 5 µg/ml PT. Hausman and Burns (1993) studied the binding of pertussis toxin to ganglioside doped vesicles and found that G_{D1a} was the receptor with the highest binding affinity although the signal of PT-binding to vesicles which do not contain glycolipids was 50% of that of the specific binding. In our experiments reported here we could not detect any significant binding of pertussis toxin to ganglioside containing lipid bilayers. We also found no evidence that pertussis toxin binds to bilayers doped with 10 mol% G_{D1a}. Adsorption of pertussis toxin on ganglioside containing membranes could only be detected if high amounts of G_{D1a}, up to 40 mol%, were used. The adsorption of the toxin to the surface in the presence of 40 mol% G_{D1a} may be an electrostatically induced process rather than a specific binding. Another possible explanation for the PT binding could be ganglioside clustering, induced by the high ganglioside content in the membrane, which is possibly neccessary for the binding process. So we cannot exclude the possibility that glycolipids in general are natural receptors of pertussis toxin, but we conclude that gangliosides do not serve as receptors in cell membranes.

4.4 Frequency shift of the resonator depending on the immobilized receptor

With respect to the results summarized in Table 1 a considerable difference exists between the maximum frequency decrease of the adsorption isotherm of cholera toxin and the C-fragment of tetanus toxin depending on the molecular structure of the ganglioside used. A possible explanation for the difference in the frequency response on the specific interaction of cholera toxin with G_{M1} and *asialo*-G_{M1} could be found in the excess charge of G_{M1}, due to the negative charge of the sialic acid at the proximal galactose. According to Tsionsky et al. (1996) the electrochemical double layer influences the frequency response of the shear resonator since the thin layer can be treated as an additional rigid mass. The treatment of the electrochemical double layer as a rigid layer is justified when one considers its thickness of about 1 nm in a 100 mM solution of 1 : 1 electrolytes, which is thin compared to the penetration depth of the shear wave of a 5 MHz-quartz in water, amounting to 250 nm. For this reason it is justified to assume that the double layer oscillates in phase with the surface of the resonator. According to a study of Wang et al. (1992) the electrochemical structure of the lipid-water-interface plays an important role. They observed that self-assembled monolayers of mercaptoundecanol strongly influence the frequency response of an AT-cut quartz upon pH changes. Ohlsson et al. (1993) found similar frequency shifts upon addition of cholera toxin to a 5 MHz quartz functionalized with a lipid bilayer containing 6 mol% G_{M1}. They observed frequency shifts larger than 100 Hz for the injection of 100 µg/ml of the B-subunit of CT to the quartz chamber. Unfortunately they did not determine the binding constant and the maximum frequency shift.

The different frequency responses to the absorption of CT to G_{M1} and *asialo*-G_{M1} was explainable by changes in the surface charges. For the binding of the C-fragment of

tetanus toxin to gangliosides the situation is more complicated. Specific adsorption of the C-fragment of tetanus toxin on G_{D1b}-containing lipid bilayers results in a maximum decrease of the resonant frequency of about 99 Hz, while binding to G_{D1a} exhibited a frequency decrease of only 28 Hz (see Table 1). The explanation for this difference in Δf_{max} cannot be found in the different charge of the receptor. Perhaps a different kind of coupling of the toxin to the receptor, which depends on the molecular structure of the ganglioside, influences the properties of the protein-layer and therefore the frequency response of the shear resonator. A crucial point in this context could be the distance of the toxin from the surface. This assumption is supported by the fact that the percentage of the initial frequency decrease which is regained by the digestion of the corresponding protein-monolayer is low for protein adsorption accompanied by a high initial frequency shift. This fact could be explained in terms of a reduced accessibility of the proteins which have a lower distance to the surface.

5 Conclusions

The investigation of the specific adsorption of proteins on receptor containing planar surfaces requires sensitive methods which are often based on optical techniques such as ellipsometry or surface plasmon resonance spectroscopy. The quartz crystal microbalance became popular since high gain amplifiers were developed to operate the microgravimetric device under liquid loading. In this study we show the applicability of the quartz crystal microbalance in biochemical research. The binding of cholera toxin, tetanus toxin and pertussis toxin to different gangliosides embedded in a phospholipid matrix immobilized via self-assembly on gold electrodes on top of the quartz crystal were investigated by frequency measurements. The specific adsorption of the toxins showed an ideal behavior with regard to a Langmuir isotherm, which means that the proteins form a defined monolayer on the solid supported membrane, in which no interaction between the adsorbed proteins occurs. The study confirms the preference of cholera toxin for the receptor G_{M1} over other gangliosides such as G_{M3}. A significantly lower binding constant was observed using *asialo*-G_{M1}, which emphazises the importance of the terminal sialic acid for the molecular recognition of the B-subunit. The binding domain (C-fragment) of tetanus toxin preferentially attaches to the gangliosides G_{D1b}, G_{D1a} and G_{T1b}, whereas no interaction with G_{M3} was observed. In contrast to these well known ligand-receptor couples the receptor of pertussis toxin is not exactly known. As far as our results are concerned we were only able to detect a low affinity of pertussis toxin for high amounts of G_{D1a}.

Beside the determination of the binding constants the maximum decrease of the resonant frequency is a specific parameter of the ligand-receptor couple. Sauerbrey's equation, which predicts that the frequency shift is only caused by pure mass loading could no longer hold with regard to protein adsorption from liquid media. Other effects such as viscoelasticity, the influence of the electrochemical double layer and changes in hydrophobicity have to be considered. It remains to be elucidated which one has the strongest influence on the frequency decreases.

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