Micromechanical measurement of AChBP binding for label-free drug discovery

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A potential binding assay based on binding-driven micromechanical motion is described. Acetylcholine binding protein (AChBP) was used to modify a microcantilever. The modified microcantilever was found to bend on application of the naturally occurring agonist (acetylcholine) or the antagonist (nicotine and d-tubocurarine). Control experiments show that microcantilevers modified without AChBP do not respond to acetylcholine, nicotine, and d-tubocurarine. Kd values obtained for acetylcholine, nicotine, and d-tubocurarine are similar to those obtained from radio-ligand binding assays. These results suggest that the microcantilever system has potential for use in label free, drug screening applications.

Introduction

Ligand gated ion channels (LGICs) are involved in a wide range of physiological processes including nerve conduction, regulation of blood flow, fluid balance and gastric motility. In the central nervous system, these important proteins mediate fast excitatory and inhibitory neurotransmission. The importance of ligand gated ion channels and their endogenous ligands is reflected in the substantial effort that has been applied toward understanding their pharmacology, physiology, and biochemistry. The LGIC superfamily includes 5-HT3, GABA, nicotinic acetylcholine, glycine receptor, and ion-gated and histamine channels (Zn2+).1–7 These are pentameric membrane bound proteins with an amino terminal domain containing the multiple binding sites and a transmembrane region that anchors the protein in the membrane and forms the gated ion channel. Even though LGIC receptors have different channel specificities, they appear to utilize similar mechanisms of ligand specificity, channel opening, ion selectivity and desensitization. Research in the LGIC structure has been greatly facilitated by the discovery of a related acetylcholine binding protein (AChBP).8–10 The AChBP is a naturally occurring, soluble homolog of the amino terminal region of the Nicotinic acetylcholine receptors (nAChR). This protein assembles in a pentameric form and displays structural and functional similarity to the amino terminal domains of the homomeric α7 nAChR and 5-HT3A R. The crystal structure of the AChBP has been determined and provides the framework for developing homology models of 5-HT3A R (as well as other ligand gated ion channels)11 although only a limited amount of information about the specific pharmacology of the AChBP itself has been reported. In its native state this protein can be used in high throughput screening and drug discovery for nAChR ligands.

Current high throughput analyses commonly utilize membrane bound receptor proteins or cell lines that express cloned receptor subtypes. These assays typically provide high throughput using either radioligand or fluorescent binding assays in combination with low to moderate throughput functional assays. Functional assays for LGIC receptors often use either automated patch clamp,12 automated Xenopus oocyte two electrode voltage clamp,13 or a system using a fluorescent calcium or voltage sensing dye.14 While these assays provide reasonably high throughput for evaluation of drug candidates, they have significant limitations. Chief among these are the need to prepare isolated membrane preparations and/or maintain cell cultures for each receptor type being evaluated. Receptor proteins are not particularly stable and cannot be easily stored for long periods of time. Cellular assays require continually maintained cell cultures. The assays themselves must be designed so that as many potential cross-reactions with other receptors are identified as possible. While these assays are reasonably high throughput, it is still time consuming to evaluate interactions on large numbers of receptors. In addition, commonly used assays do not identify all interactions with a receptor but only interactions that produce effects observable in the assay system used. As the technology for the rapid production of new chemical entities is extended, so the analytical characterization efforts must increase to support these programs. Such drug screening technologies will need to provide rapid evaluation of drug molecules, multiple receptor analysis, and must be cost efficient. One such technology involves the use of microcantilevers.

Advances in the field of micro-electro-mechanical systems (MEMS) and their uses offer unique opportunities in the design...
of small-size and cost-effective analytical methods. Microcantilevers are the simplest MEMS device that can be micromachined and mass-produced. The unique characteristic of microcantilevers is that they can be made to undergo bending due to molecular adsorption by confining the adsorption to one side of the microcantilever. Microcantilever sensors hold a position as a cost-effective and highly sensitive sensor platform for medical diagnostics, and environmental and high throughput analysis. Since the microcantilever's deflection is derived from molecular binding, no labeled compound is needed, and this technology could undoubtedly be used to study the interaction of small molecules with drug targets for drug screening. Microcantilevers can also be incorporated into multichannel microcantilever chips that offer improved dynamic response, greatly reduced size, high precision, increased reliability and integration of micromechanical components with on-chip electronic circuitry.

The low cost of the microcantilever technology would be more accessible to smaller laboratories than current high throughput systems, thus providing an added advantage over existing technology.

In our previous work, we have studied interactions of small molecules with the 5-HT3R receptor embedded membrane by using the microcantilever method. This membrane immobilized microcantilever and its binding with the naturally occurring agonist serotonin (5-hydroxytryptamine) and the antagonist MDL-72222 were determined. However, although hydrophobic chip surfaces have been created for coupling membrane receptors, these receptors produce much less stable and reliable surfaces than covalently linked proteins. On the other hand, receptor proteins, such as AChBP, could be produced in large quantities and distributed for use in a variety of assay systems, since they are soluble receptor proteins they are relatively easily attached to chip based biosensors and they are stable. In this work, microcantilever binding is used for an AChBP-analyses binding study, which has applications in drug screening and drug development.

Methods

The cDNA sequences for AChBP from Lymnaea stagnalis (GeneBank accession number AF364899.1) were used to synthesize a full length cDNA. A 6X polyHis tag was added to the 3’ end of the cDNA to provide efficient purification of the expressed protein. cDNA synthesis was conducted by GeneArt Inc. (Burlingame, CA). The synthetic AChBP cDNA was inserted into a p3XFLAG-CMV-9 expression vector (Sigma Aldrich). Incorporation of the AChBP sequence into this vector provides an amino terminal Flag tag in addition to the C-terminal 6X His tag. The vector construct was transfected into HEK-293 cells and maintained in 0.4 mg ml⁻¹ G418® (Sigma Aldrich) antibiotic for several weeks. Surviving HEK-293/AChBP cells stably expressed the AChBP protein. The protein was secreted into the extracellular medium and was purified using the Profinia® (Bio-rad) immobilized metal ion chromatography (IMAC) purification system. The purified protein was quantified by UV spectroscopy and further characterized using denaturing and native PAGE to confirm that the expressed protein was pure and present in its pentameric form. The protein was prepared in a 0.01 M phosphate buffer solution (PBS, pH = 7.0) that was used in all subsequent experiments.

Purity and assembly of AChBP were determined using native and denaturing PAGE as shown in Fig. 1. The left hand gel shows the results of PAGE (non-denaturating conditions) and the right hand gel shows the result of separation under denaturing conditions (SDS-PAGE). Gels were stained with Coomassie Blue stain (BioRad). The protein sample lane is indicated by S and the BioRad Kaleidoscope standard is indicated by Std. Under non-denaturing conditions, a distinct band is shown to migrate similar to the 150 kDa standard. Under denaturing conditions, the dominant band shifts to slightly less than 37 kDa. The molecular weight of the native protein of about 150 kDa is consistent with the predicted molecular weight of the pentameric AChBP. The 37 kDa single band in the denaturing gel is as expected for the monomeric protein. In the non-denatured protein, a smaller band is evident at around 37 kDa as well suggesting some unassembled monomers are present.

We used commercially available silicon microcantilevers (Veeco Instrument, CA, http://store.veeco.com) in these experiments. The dimensions of the V-shaped microcantilever are 200 μm in length, 20 μm in width, and 1 μm in thickness. One side of the cantilever had a thin film of chromium (3 nm) followed by a 20 nm layer of gold deposited by e-beam evaporation. The deflection experiments were performed in a flow-through glass cell (Veeco, CA) similar to those used in atomic force microscopy (AFM). For continuous flow-through experiments, initially, the electrolyte solution was driven through the cell using a syringe pump. A constant flow rate at 10 ml h⁻¹ was maintained during each experiment. The cantilever was immersed in the electrolyte solution until a baseline was obtained and the voltage of the position-sensitive detector was set as background corresponding to 0 nm.

Experimental solutions containing different concentrations of analytes were injected directly into the flowing fluid stream via a low-pressure injection port sample loop arrangement with a loop volume of 0.5 ml. This arrangement allows for continuous exposure of the cantilever to the desired solution without disturbing the flow cell or changing the flow rate. Since the volume of the glass cell, including the tubing, was only 0.3 ml, a relatively fast replacement of the liquid in contact with the cantilever was achieved. Deflection measurements were determined using the
The bending of the cantilever was measured by monitoring the position of a laser beam reflected from the gold-coated side of the cantilever onto a four-quadrant AFM photodiode. We define bending toward the gold side as "bending up"; "bending down" refers to bending toward the silicon side. In case the adsorption occurs on the gold surface, in general, the downward bending is caused by repulsion or expansion of molecules on the gold surface, which is the so-called compressive stress; *vice versa*, the upward bending is caused by attraction or contraction of molecules on the gold surface, which is called tensile surface stress. Three cantilevers were prepared for each of the individual experiments to allow statistical comparison of repeatability and efficiency between devices. To eliminate thermomechanical motion of the silicon cantilever caused by temperature fluctuations, we mounted the fluid cell on thermoelectric coolers so that the temperature of the fluid cell could be controlled to 20 ± 0.2 °C.

The $K_d$, Hill coefficient and $B_{\text{max}}$ were calculated by fitting the data to the following equation: $B/B_{\text{max}} = 1/(1 + (K_d/[L]^n))$, where $B$ is the microcantilever bending, $B_{\text{max}}$ is the maximum bending at equilibrium, $L$ is the free ligand (acetylcholine) concentration and $n$ is the Hill coefficient.

### Results and discussions

#### Conjugation chemistry

Significant effort has been focused on microcantilever fabrication and simulation of responses. While this is essential to developing inexpensive, practical microcantilever instrumentation, the production of specific biological sensor molecules and optimization of their attachment to the microcantilever surface are equally important. It is often assumed that surface conjugation chemistries utilized by other chip-based microsensors can be transferred to microcantilever devices; however, the mechanism of surface stress induced bending is substantially different from the mechanisms of other sensor platforms such as surface plasmon resonance. Optimization of a biosensor requires surface characteristics such as packing density, be appropriate to the sensor device, and the detection mechanism in use. Surface packing and orientation can be altered by using different conjugation chemistries. It has been observed that microcantilevers modified by different conjugation chemistry responded differently to the analytes. The approach using succinic anhydride did not work for microcantilever sensors. The approach using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) (Scheme 1) provided a relatively more reliable surface modification approach than the succinic anhydride approach. The chemistry of EDC/NHS has been studied in the past. In this work, we further investigated the effort of conjugation time on the performance optimization of our microcantilever sensors with improved response characteristics. Improved response characteristics will produce receptors with increased sensitivity.

In our experiments, clean microcantilevers were stored for 48 hours in a 1 mM ethanolic solution of 11-mercaptoundecanoic acid. These microcantilevers were then thoroughly rinsed with distilled water before being immersed in a solution of 0.5 M NHS and 0.2 M EDC in distilled water for a time period of 12–72 hours. This was followed by thorough rinsing of cantilevers in DI water to remove excess chemicals. The final step was the immobilization of the AChBP on the microcantilever. 5 μl of the AChBP was dissolved in 1 ml of phosphate buffer solution. The microcantilevers were incubated in this solution for a period of 12–48 hours. The microcantilevers were then rinsed to remove any excess AChBP. The chemically modified microcantilevers were stored in buffer solution before use.

Table 1 shows the effect of conjugation time of the EDC/NHS methods on the deflection of the AChBP-modified microcantilever surfaces. It shows that the conjugation time of EDC/NHS is more important than that of the following conjugation time of AChBP. It also showed that a protocol of EDC/NHS reaction time 48 hours and the following cross-linking for 48 hours provided the maximum deflection upon exposure to the same concentration of analytes and the standard error was within 7%. We used this protocol in our entire sensor test in this work.

#### Microcantilever deflection

AChBP coated microcantilever was initially exposed to a constant flow (4 ml h$^{-1}$) of 0.010 M phosphate buffer solution (pH = 7.0) and the cantilever equilibrated until a stable baseline was obtained (*i.e.*, 0 nm deflection). When a 10$^{-5}$ M solution of acetylcholine in buffer (pH = 7.0) was switched into the fluid cell to replace the buffer solution at the same flow rate, the microcantilever was deflected until it reached equilibrium (450 seconds). The maximum amplitude of the microcantilever

| Table 1 Effect of conjugation time on the bending of AChBP-modified microcantilevers upon exposure to 10$^{-5}$ M acetylcholine |
|---|---|---|
| EDC/NHS reaction time/h | AChBP conjugation time/h | Deflection amplitude/nm |
| 12 | 12 | 69.0 ± 20.4 |
| 12 | 24 | 75.2 ± 15.9 |
| 12 | 48 | 75.2 ± 17.7 |
| 24 | 12 | 81.4 ± 17.7 |
| 24 | 24 | 83.2 ± 16.8 |
| 24 | 48 | 83.2 ± 17.8 |
| 48 | 12 | 88.5 ± 9.73 |
| 48 | 24 | 86.7 ± 10.6 |
| 48 | 48 | 90.2 ± 6.20 |
| 72 | 12 | 77.0 ± 13.5 |
| 72 | 24 | 83.2 ± 17.7 |
| 72 | 48 | 85.8 ± 11.5 |
The bending amplitudes of the microcantilevers at equilibrium vs. the log of the concentration of acetylcholine are shown in Fig. 3. Each point shown on the curve represents the mean ± SE of three experiments. The standard deviation induced by variation in the cross-linked AChBP on different cantilevers and the position of the focused laser spot at the end of the cantilever was found to be within ±25%. The data in Fig. 3 were used to determine dissociate constants (K_d) of the AChBP to acetylcholine. A K_d value of 1.83 × 10^{-8} M was calculated from these data using non-linear curve fitting techniques and Graphpad Prism software, which is close to 4.3 × 10^{-6} M from Smit et al.\textsuperscript{28} for radioligand binding. A B_{max} value of 107 ± 1.7 nm was also determined.

Competitive antagonists were also tested to verify the specificity of microcantilever bending resulting from binding of ligands to the AChBP receptor and to identify any variation in binding kinetics for agonists versus antagonists. Nicotine and d-tubocurarine were chosen due to their availability and they were well studied for ready comparison. Similar to application of acetylcholine, 10^{-6} M nicotine or d-tubocurarine produced a deflection of the microcantilever coated with the AChBP (Fig. 4). The time to plateau is much faster for d-tubocurarine and nicotine compared to that for acetylcholine, indicating a high binding constant between AChBP with nicotine or d-tubocurarine. In the absence of AChBP no bending was observed on application of 10^{-6} M nicotine or d-tubocurarine indicating that nicotine or d-tubocurarine by itself does not produce a deflection of the microcantilever. The deflection amplitude at equilibrium of the microcantilever vs. the concentration of nicotine in the buffer solutions is shown in Fig. 5. K_d values of 1.92 × 10^{-8} M and 7.1 × 10^{-8} M\textsuperscript{-1} for nicotine and d-tubocurarine, respectively, were calculated from these data as described above. The B_{max} values are 13 ± 0.47 nm and 32 ± 0.67 nm for nicotine and d-tubocurarine, respectively.
probe.

and antagonists has been demonstrated by using a fluorescent conformational change of the AChBP upon binding to agonists. The microcantilever derived from the binding of the ligand to the AChBP. The mechanism responsible for producing deflections of the AChBP-modified microcantilever in response to ligand binding may be due to a slight conformational change of the AChBP on the microcantilever from the binding of the ligand to AChBP. The conformational change of the AChBP upon binding to agonists and antagonists has been demonstrated by using a fluorescent probe. The surface stress resulting from protein conformation change has been a recent focus of MCL research. Conformational changes are capable of altering immobilization of molecules on the surface, distances between molecules, relative orientations and surface interactions; thus it is reasonable that conformational changes will alter MCL bending. The $B_{\text{max}}$ for nicotine and $d$-tubocurarine is much less than that for acetylcholine, which reflects a difference in conformational change of the AChBP on the surface. Partial agonists and antagonists, such as nicotine and $d$-tubocurarine, would likely produce smaller conformational changes compared to a full agonist like acetylcholine.

Fig. 5 Maximum deflection of a silicon cantilever coated with AChBP as a function of the concentration of nicotine (left) and $d$-tubocurarine (right) in 0.01 M phosphate buffer at pH 7.0. The $K_d$ and $B_{\text{max}}$ were calculated as described in Fig. 2 for acetylcholine.

The data presented here demonstrate that the deflections we observed are the result of binding of acetylcholine, nicotine, and $d$-tubocurarine with AChBP. From a molecular point of view, binding can result in electrostatic repulsion, attraction, steric effects, intermolecular interactions or a combination of effects that alter the surface stresses on the cantilever. The mechanism responsible for producing deflections of the AChBP-modified microcantilever in response to ligand binding may be due to a slight conformational change of the AChBP on the microcantilever from the binding of the ligand to AChBP. The conformational change of the AChBP upon binding to agonists and antagonists has been demonstrated by using a fluorescent probe. The surface stress resulting from protein conformation change has been a recent focus of MCL research. Conformational changes are capable of altering immobilization of molecules on the surface, distances between molecules, relative orientations and surface interactions; thus it is reasonable that conformational changes will alter MCL bending. The $B_{\text{max}}$ for nicotine and $d$-tubocurarine is much less than that for acetylcholine, which reflects a difference in conformational change of the AChBP on the surface. Partial agonists and antagonists, such as nicotine and $d$-tubocurarine, would likely produce smaller conformational changes compared to a full agonist like acetylcholine.

Conclusion

In summary, we demonstrated that AChBP-modified microcantilevers can be used to study the interactions of small molecules with AChBP. The microcantilever technology provides new opportunities for drug identification in an increasing number of therapeutic areas. This approach has the advantage that it might be applicable to a large number of proteins. A state-of-the-art screening microchip capable of developing and running biochemical assays such as enzyme inhibition as well as binding assays could be established.

The bending approach based on adsorption-induced surface stress is of particular interest in the study of conformational change in proteins. Surface stress changes due to protein conformational change on interaction with analytes could act as transducers of chemical information. The AChBP is an example of a protein that undergoes conformational change on ligand binding. Microcantilevers responding to these stresses would be ideal for high sensitivity detection of the small dimensional changes expected. These microcantilevers could also be used to investigate conformational changes that do not involve analyte interactions.

While the use of the AChBP in high throughput applications may be limited, the similarity of this protein to the LGIC family makes it likely that other similar proteins might be developed with similar functionality on microcantilevers. Kostelidou et al. have reported the expression of multiple nicotinic subunits in a soluble form similar to the AChBP. Further advance in this direction could provide a wide array of human LGICs for use in microcantilever arrays. The development of microchip arrays makes it possible to evaluate a single drug candidate on a wide variety of receptors simultaneously. In addition to the development of large arrays containing multiple receptor subtypes, it would also be possible to develop arrays designed to identify the specific binding site on a receptor at which a drug candidate binds. This array would be composed of fully functional receptor proteins along with receptors mutated at different known binding sites on the protein. An inkjet printing technique can be used to modify the cantilever array, each cantilever modified by specific AChBP mutants or references.

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