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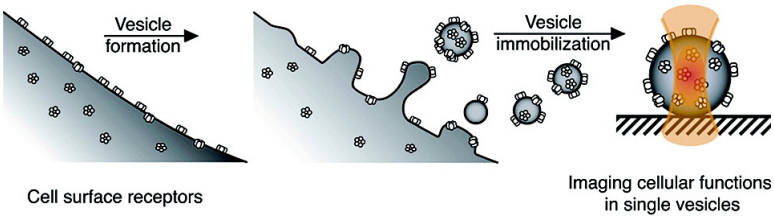

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Investigating Cellular Signaling Reactions in Single Attoliter Vesicles

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Abstract: Understanding cellular signaling mediated by cell surface receptors is key to modern biomedical research and drug development. The discovery of a growing number of potential molecular targets and therapeutic compounds requires downscaling and accelerated functional screening. Receptor-mediated cellular responses are typically investigated on single cells or cell populations. Here, we show how to monitor cellular signaling reactions at a yet unreached miniaturization level. On the basis of our observations, cytochalasin induces mammalian cells to extrude from their plasma membrane submicrometer-sized native vesicles. They comprise functional cell surface receptors correctly exposing their extracellular ligand binding sites on the outer vesicle surface and retaining cytosolic proteins in the vesicle interior. As a prototypical example, ligand binding to the ionotropic 5-HT_3 receptor and subsequent transmembrane Ca^{2+} signaling were monitored in single attoliter vesicles. Thus, native vesicles are the smallest autonomous containers capable of performing cellular signaling reactions under physiological conditions. Because a single cell delivers about 50 native vesicles, which can be isolated and addressed as individuals, our concept allows multiple functional analyses of individual cells having a limited availability and opens new vistas for miniaturized bioanalytics.

Introduction

The ability of organisms or individual cells to sense and react to different external signals (light, hormones, odorants, taste) is crucial for their survival. External signals interact with cell surface receptors, which transmit the signals across the membrane and activate a variety of intracellular processes leading to a cellular response. It is therefore not surprising that many disease processes are initiated at the level of cell surface receptors influencing ligand−receptor interactions and leading to activation or modulation of receptor-dependent signaling pathways.[1,2] Cellular responses upon activation of cell surface receptors, such as intracellular calcium ion signals or transmembrane ion fluxes, are typically investigated measuring optical or electrical properties of either single cells or cell populations in microliter volume.[3−5] Recent advances in different genome projects have deciphered the genetic code fundamental to many organisms, including humans, animals, microbes, and plants, however, leaving the function and the interplay of most proteins unresolved.[6] In addition, medical and biological research are going to provide large numbers of mutant proteins with modified functions, either from patients (with single or multiple nucleotide polymorphism[2]) or from combinatorial genetic engineering.[7−9] Combinatorial chemistry, on the other side, produces huge libraries of compounds with potential therapeutic value.[10,11] In this context, there is a continuous trend to develop miniaturized efficient analytical techniques for unraveling molecular details of cell function, understanding diseases on a genetic basis, and discovering new medicines.[12−23]

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Here, we describe the utility of mammalian cell-derived native vesicles as a novel bioanalytical reagent allowing the miniaturization of receptor-based assays under physiological conditions. The present study was motivated by our recent finding to perform controlled chemical reactions in single micrometer-sized vesicles and to immobilize them in multiarrays with 100 nm precision.[24,25] Cultured mammalian cells are suitable for recombinant expression especially of human-derived membrane receptors because they provide post-translational modifications essential for receptor function as well as molecular determinants of endogenous signaling cascades.[26,27] To give an example for the versatility of our approach, we describe a comprehensive study on the ionotropic 5-HT_3 receptor[28] and monitor ligand binding to recombinant receptors and subsequent receptor-mediated transmembrane signaling in single submicrometer-sized vesicles derived from mammalian cells.

Although reconstituted lipid vesicles or cell-membrane-derived vesicles have been used in suspension for many biochemical and biophysical applications especially for investigating membrane transport processes,[29,30] the potential of these systems for miniaturizing bioanalytics has been realized only after extracting single vesicles from the ensemble and addressing them as individuals either by micromanipulation or by directed assembly on micropatterned surfaces.[24,31−35] This ultimate miniaturization opens novel routes in functional proteomics, such as multiplexing single-cell bioanalytics or investigating receptor-mediated signaling in multiarray format.

We describe the one-step production of native vesicles and demonstrate that in these vesicles cell surface receptors and cytoplasmic proteins retain their original cellular location, orientation, and function. Ligand binding to ionotropic 5-HT_3 receptors and subsequent transmembrane calcium ion flux, monitored by fluorescence confocal microscopy, are shown to be identical in live cells and single vesicles derived from those cells.

Because the native vesicles can be stored for weeks without losing their functional integrity, they may be used as a universal, efficient, and inexpensive bioanalytical reagent for investigating cellular signaling reactions in a highly parallel format, replacing live cells in many applications.

Materials and Methods

Chemicals. Cytochalasin B, serotonin, and Dulbecco's PBS containing 1 mM $CaCl_2$ (Sigma); [^{3}H]-GR65630 (61−63 Ci mmol^{-1}) (NEN-Dupont); quipazine; and mCPBG (Tocris-Cookson); PBG (Aldrich); granisetron, ondansetron, and GR−H (Institute of Molecular Biology, Glaxo Geneva, Switzerland). GR−H was chemically modified with rhodamine 6G-isothiocyanate (Fluka) yielding GR−Rho.[36] All other products were of highest quality available.

5-HT_3R Constructs. 5-HT_3 receptor sequences are based on the short splicing version of the murine 5-HT_{3A}R, corresponding to SwissProt entry p23979 lacking residues 360−365 (mature sequence numbering). Insertion of a 5-HT_3 receptor containing a C-terminal hexahistidine extension into the pCEP4 expression vector (Invitrogen) allowed either transient or stable expression in HEK293 EBNA cells. The 5-HT_3 receptor sequence was modified by insertion of the EGFP gene (Clontech) into the large cytoplasmic loop between the third and fourth predicted membrane-spanning domains of the 5-HT_3R coding sequence. The resulting 5-HT_3R−EGFP fusion protein DNA sequence was subcloned into the pEAK8 expression vector (Edge BioSystems), allowing puromycin selection for stable receptor expression in mammalian cells. Electrophysiological channel measurements and radioligand binding studies confirmed the receptor fusion protein to be fully functional active (Ilegems, unpublished).

Cell Production. Adherent human embryonic kidney HEK293 cells were grown in DMEM/F12 supplemented with either 2.2 or 10% fetal calf serum in plastic flasks. Cultures were kept at 37 °C in humidified, 5% CO_2 atmosphere. Exponentially growing cells were seeded (10^5 cells/ml) into 6-well plates and transfected after 16−20 h using Effectene (Qiagen). After 4 h transfection, solution was replaced by fresh medium.

Vesicle Formation. Native vesicles were produced from cells expressing either the 5-HT_3R or the 5-HT_3R−EGFP by a 10−20 min incubation at 37 °C with cytochalasin B (10 μg/mL) in serum-free DMEM/F12 medium. Cells were first separated from vesicles by centrifugation at 500 rpm for 5 min in an Eppendorf centrifuge. Vesicles were then collected by centrifugation at 2000 rpm for 20 min, resuspended in Dulbecco's PBS, and either used directly or stored in 10% DMSO at −20 °C for several weeks without loss of ligand binding activity.

Calcium Signaling. Cells were loaded at 37 °C for 30 min with 10 μM Fura-Red-AM (Molecular Probes) in serum-free DMEM/F12 medium and were then washed with PBS and incubated in DMEM/F12 medium containing 10% FCS for 30 min to allow complete hydrolysis of intracellular Fura-Red-AM. Subsequently, cells were treated with cytochalasin B as described above.

Vesicles containing Fura-Red were transferred to 8-well chambered coverglass slides precoated with 0.1 mg/L poly-L-lysine to ensure electrostatic binding of the vesicles to the glass surface and were investigated by confocal fluorescence microscopy (Zeiss LSM 510) using a water immersion objective (Zeiss Achroplan 63 NA 1.2). Excitation was at 488 nm (Ar+ laser); 505−530 nm band-pass or 650 nm long pass emission filters were used to image EGFP or Fura-Red, respectively. Vesicles comprising 5-HT_3R−EGFP, but not loaded with Fura-Red, served as a control to ensure that no crosstalk did occur from the green to the red measuring channel. Individual responses to the 5-HT_3R-specific agonist mCPBG were recorded for 100 s.

Binding Fluorescent Ligands to Native Vesicles. A quantity of 400 μL of freshly prepared native vesicles or 400 μL of frozen native vesicles, which were collected by centrifugation in an Eppendorf centrifuge at 2000 rpm for 20 min and resuspended in 400 μL of PBS buffer, were added per well to a 6-well plate in which a coverslide had been placed at the bottom. After vesicle immobilization, the coverslides were transferred to a microscope chamber and covered with 200−300

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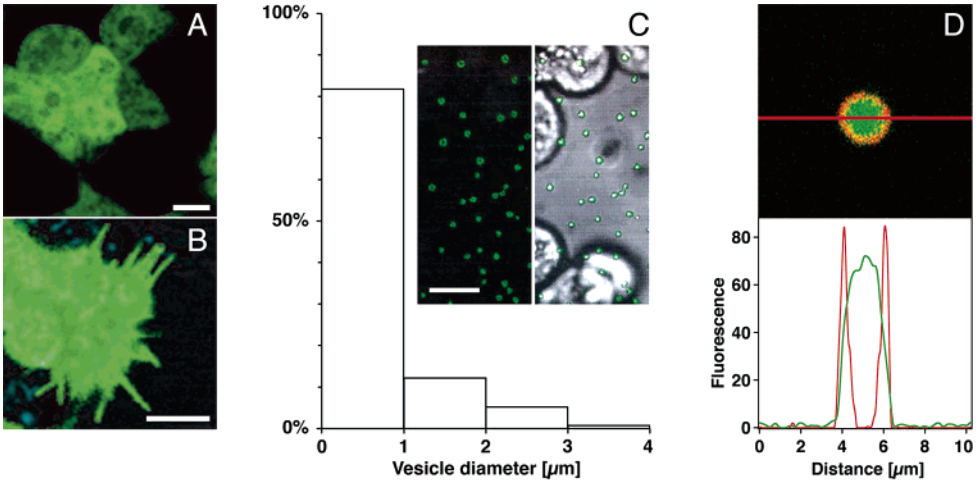

Figure 1. Formation and characterization of native vesicles from HEK293 cells. (A) Adherent HEK cells transiently coexpressing cytosolic GFP (green) and the 5-HT_3R were observed by fluorescence confocal microscopy. (B) Addition of cytochalasin B resulted, within a few minutes, in the formation of tubular protrusions, which contained the cytosolic GFP. (C) Agitation of cytochalasin-treated cells led to the release of about 50 native vesicles/cell, 80% of which have a diameter of 0.5−1 μm. The insert shows a typical vesicle population containing 5-HT_3R labeled with the fluorescent antagonist GR-flu[36] (left) transmission micrograph together with cells from which they are produced (right) fluorescence confocal micrograph. Scale bar represents 5 μm. (D) Native vesicles readily adsorb on the surface of a glass coverslip, retaining within their lumen cytosolic GFP (green). Incubation with the 5-HT_3R-specific ligand, GR−Rho[36] (5 nM), resulted in a bright staining (red) of the periphery of the vesicle (top). The fluorescence intensity profile at the indicated line across this vesicle shows clearly the GFP inside the lumen of the vesicle (green) and the GR−Rho ligand on the enveloping membrane (red). For experimental details, see Materials and Methods section and previous studies (ref 36).

μL of PBS buffer. The affinity of a fluorescent ligand for a population of immobilized native vesicles was determined by examining a series of coverslips, each incubated for 2 h with a solution containing the vesicles and various concentrations of fluorescent ligand. Nonspecific binding of the fluorescent ligand was determined in the presence of excess nonfluorescent competitor quipazine. The affinity of a fluorescent ligand for single immobilized native vesicles was determined by adding increasing concentrations of fluorescent ligand to a preparation of immobilized vesicles. Fluorescence intensities were measured by confocal microscopy.

Results and Discussion

Native Vesicles Comprise Cell Membranes and Cellular Cytosolic Components. The formation of native vesicles was monitored and characterized using HEK293 cells, co-expressing cytosolic green fluorescent protein (GFP), and the serotonin-gated mouse 5-hydroxytryptamine type-3 receptor (5-HT_3R) as a prototypic cell membrane receptor (Figure 1). Treatment of the cells with cytochalasin B leads to the appearance of tubular extensions (Figure 1B), which upon agitation, bud off as (sub)micrometer-sized vesicles (Figure 1C). Cytosolic components are enclosed in these vesicles as demonstrated by the presence of the GFP reporter protein (Figure 1D). Functional active cell surface receptors are present in the vesicle membrane as shown by specific binding of a fluorescent ligand to the 5-HT_3R (Figure 1D). Obviously, vesicles are formed from live cells by blistering off the plasma membrane without reorientation, thus enclosing parts of the cellular cytoplasm in the vesicle lumen. Native vesicles, therefore, represent miniaturized replicas of a living cell.

Receptor Activity and Orientation is Maintained throughout Native Vesicle Production. Native vesicle production was quantified by measuring binding of radioactive ligands to the 5-HT_3R. HEK293 cells transiently expressing 5-HT_3Rs at an average level of $(1.2 \pm 0.2) \times 10^6$ receptors on the surface of a live cell were used as starting material. Live cells were incubated with cytochalasin B, and vesicles were released from the cells by agitation. Low-speed centrifugation yielded a homogeneous preparation of 0.5−1 μm diameter vesicles (Figure 1C). Typically, a single live cell delivered 50 ± 10 vesicles. From radioactive ligand binding experiments, 15 ± 4% of the original average cellular receptor activity was found in vesicles, which corresponds to an average number of about 3500 receptors per vesicle. Comparing the average surface area of the native vesicles (2.0 ± 0.6 μm^2, histogram in Figure 1C) with the average surface area of a live cell (580 ± 20 μm^2, as measured elsewhere[37]) yields 17 ± 6% of the cell membrane transformed to native vesicles, in agreement with the number calculated above by ligand binding experiments.

That the topology of the plasma membrane of the parent cell is maintained in native vesicles was demonstrated by the following observations. (i) The 5-HT_3R fluorescent antagonist, GR−Rho, binds specifically to native vesicles when added from the bulk aqueous phase (Figure 1D), indicating that the ligand binding sites of the 5-HT_3R are accessible on the outer vesicle surface. (ii) Moreover, the binding of radioligands to the 5-HT_3R in native vesicle preparations was not increased in the presence of detergent, indicating that the 5-HT_3R binding sites are exposed on the outer surface of native vesicles. These findings are important because only native vesicles which retain both the original membrane topology and cytosolic components are of interest for bioanalytical applications.

The pharmacological properties of the receptor were investigated to show the power of native vesicles as bioanalytical reagents. Competitive radioligand binding assays using 5-HT_3R-specific agonists and antagonists performed on receptors in cell membranes and in native vesicles or purified, detergent-solubilized 5-HT_3R yielded virtually identical dissociation constants (Table 1). Also, the fluorescent 5-HT_3R ligand, GR−Rho, displayed identical affinities for the different 5-HT_3R constructs.

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Table 1. Binding of Ligands to the 5-HT_3R in Cell Membrane Fragments, Solubilized and Purified in Detergent and in Native Vesicles

	cell membranes pK_i[a]	detergent pK_i[a]	native vesicles pK_i[a]
	Antagonist		
GR−H	10.2 ± 0.1	9.9 ± 0.4	9.7 ± 0.1
granisetron	9.2 ± 0.1	9.4 ± 0.1	9.5 ± 0.1
ondansetron	8.5 ± 0.1	8.8 ± 0.1	8.6 ± 0.1
	Agonist		
quipazine	9.3 ± 0.1	9.3 ± 0.1	9.4 ± 0.2
mCPBG	8.2 ± 0.1	8.3 ± 0.1	8.3 ± 0.1
5-HT	7.5 ± 0.1	7.3 ± 0.1	7.7 ± 0.1
PBG	6.5 ± 0.1	6.9 ± 0.1	6.6 ± 0.1
	Fluorescent Ligand		
GR−Rho	9.0 ± 0.1	9.1 ± 0.1	8.8 ±0.2
	Radioligand[b]		
[^{3}H]-GR65630	9.5 ± 0.2	9.6 ± 0.2	9.4 ± 0.1

[a] HEK293 cells stably expressing the 5-HT_3R, membrane fragments, and vesicles produced thereof were used to determine dissociation constants (K_i) of various 5-HT_3R ligands by competitive displacement of the radioactive ligand, 3-(5-methyl-1*H*-imidazol-4-yl)-1-(1-[^{3}H]-methyl-1*H*-indol-3-yl)propanone ([^{3}H]-GR65630). Mean (± deviation) values of pK_i (negative logarithm of K_i) from two independent experiments are presented. [b] Saturation radioligand binding experiments delivered dissociation constants (K_d) of [^{3}H]-GR65630. Experiments were performed as described elsewhere.[36]

Other cell types, such as CHO or HEK293 cells transiently expressing 5-HT_3Rs and neuroblastoma N1E-115 cells expressing 5-HT_3Rs, naturally delivered comparable native vesicles with similar properties, indicating that the native vesicle approach is generic for a variety of cellular expression systems.

Ligand Binding and Receptor-Mediated Transmembrane Signaling can be Observed in Individual Immobilized Vesicles. Having characterized the topological and pharmacological properties of the 5-HT_3R in vesicle ensembles, we probed the following ligand binding and transmembrane signaling in individual vesicles as an ultimate step in downscaling cellular bioanalytics. For that reason, native vesicles comprising 5-HT_3R−EGFP were immobilized on glass slides. Ligand binding to individual vesicles was monitored by increasing the concentration of the fluorescent ligand, GR−Rho, in the bulk aqueous phase. Contributions of nonspecifically bound fluorescent ligands were determined to be less than 10% and were subtracted from the values of total binding. Figure 2 depicts typical results of such ligand binding experiments for three different vesicles. The variation of the intensities measured for the different vesicles originates from variations of receptor concentrations between particular vesicles. In all cases, data could be fitted by a Langmuir isotherm, delivering dissociation constants ranging from 4.4 to 5.9 nM. These values are in agreement with K_i values determined on ensembles of native vesicles (Table 1).

In the next step, we investigated whether receptor-mediated transmembrane signaling is preserved in native vesicles. It is known that agonist-induced activation of recombinant 5-HT_3R in HEK293 cells increases intracellular calcium ion concentration resulting from calcium influx across the cell membrane.[38] This intracellular calcium ion response is transient because calcium pumps restore the initial calcium ion concentration. On the basis of this finding, we measured agonist-activated calcium responses in individual native vesicles prepared from mammalian HEK293 cells stably expressing the 5-HT_3R−EGFP. Changes of calcium ion concentration in individual cells and native vesicles were monitored using the indicator Fura-Red, which upon binding Ca^{2+}, decreases its fluorescence emission at 650 nm. Transient calcium signaling was observed after the addition of the 5-HT_3R-specific agonist 1-(*m*-chlorophenyl)-biguanide (mCPBG). Addition of 500 nM mCPBG to either live cells or native vesicles derived from these cells induced an immediate increase of intracellular calcium ion concentration, followed by a slower restoration (15−20 s) of resting concentration (Figure 3). The preservation of the kinetics of the transient cellular calcium response in native vesicles, in turn, proves an unaltered function not only of the 5-HT_3Rs but also of the ion pumps, which restore the initial transmembrane calcium ion gradients.

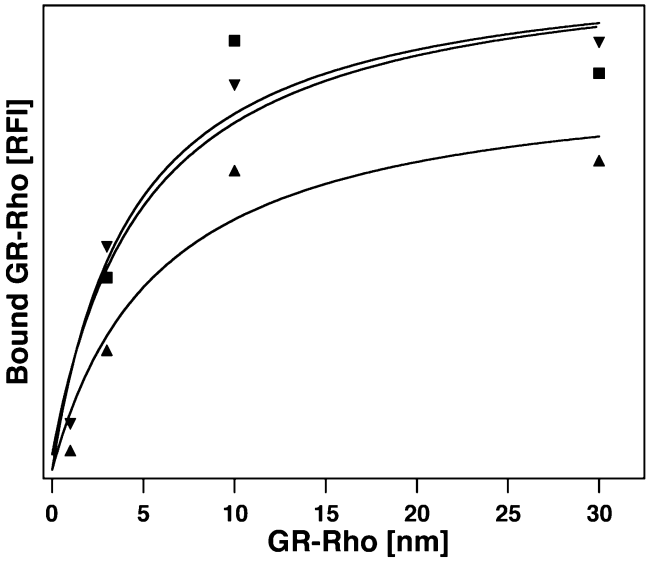

Figure 2. Binding of fluorescent ligands to single native vesicles comprising 5-HT_3R−EGFP. The vesicles, immobilized on the glass slide, were consecutively incubated for 20 min with the indicated concentrations of the fluorescent ligand, GR−Rho; binding was measured using confocal microscopy. Nonspecific binding, determined in the presence of 5 μM quipazine for independent vesicles, was found to be smaller than 10% at 30 nM GR−Rho. The specific binding was fitted to the Langmuir isotherm and yielded dissociation constants (K_d) for GR−Rho to the 5-HT_3R−EGFP receptor of 4.9 ± 3.6, 5.9 ± 3.1, and 4.4 ± 1.5 nM, and a maximal fluorescence intensity signal of 3970 ± 930, 3060 ± 530, and 3930 ± 410 for the vesicles indicated by the symbols ▼, ▲, and ■, respectively. Experiments were performed as described elsewhere.[36]

Conclusion

We have developed an efficient protocol to produce native cell membrane vesicles using cytochalasin B, which is known as an active agent for destabilizing cytoskeleton−membrane interactions.[39] Vesicle sizes were controlled from about 0.1 to a few micrometers in diameter depending on the shear forces applied during vesicle production. Mechanical shear forces released vesicles from the cell surface, whereas the remaining cell bodies were removed by differential centrifugation or filtration. In contrast to previous work,[40] the controlled vesiculation process induced by cytochalasin B did not involve any

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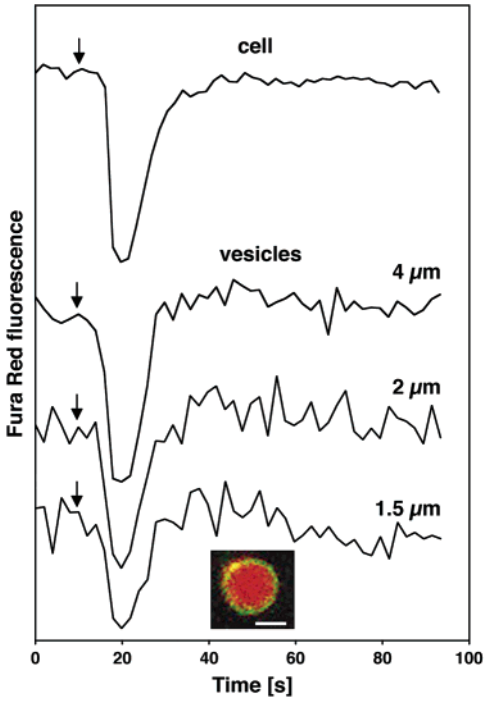

Figure 3. Transient Ca^{2+} signaling in mammalian cells expressing 5-HT_3R−EGFP (top trace) and in single native vesicles produced from these cells (lower trace). Changes of the Ca^{2+} concentration inside cells and vesicles in response to receptor activation by adding (arrow) the 5-HT_3R-specific agonist, mCPBG (500 nM), were recorded with confocal microscopy using Fura-Red (Ex 488/Em 650) as a fluorescent Ca^{2+} indicator. Decrease of the fluorescence signal corresponds to increase of the Ca^{2+} concentration. Inset shows a typical native vesicle loaded with the calcium indicator dye, Fura-Red. The vesicle membrane contains EGFP-tagged 5-HT_3 receptors (green). Size bar is 1 μm. For experimental details, see the Materials and Methods section.

cell homogenizing steps, which would release cytoplasmic proteins and change membrane protein orientation.

Orientation and functional activity of cell surface receptors and ion pumps as well as cytosolic proteins are preserved in native vesicles. These vesicles can be immobilized as functionally active containers on solid supports and, thereby, offer a number of interesting possibilities for investigating the function of receptor-mediated cellular signaling by surface-sensitive techniques. For instance, submicrometer-sized native vesicles immobilized on optical waveguides or surface plasmon sensors fit perfectly to and thus can be probed most efficiently by evanescent waves on these sensor surfaces,[12,41,42]

For the first time, ligand binding isotherms of 5-HT_3Rs on individual vesicles were measured using fluorescence confocal microscopy. Until now, ligand−receptor interactions in a natural plasma membrane environment were quantified on large numbers of individual living cells[36,43] or suspensions of membrane fragments and vesicles of variable size and composition.[40,44]

We also provided evidence that native vesicles are the smallest functional units capable of performing transmembrane signaling at fractions of cellular dimensions. Activation of 5-HT_3Rs expressed in HEK293 was shown to evoke increase in intracellular Ca^{2+} concentration resulting from calcium influx across the cellular membrane.[38] Increased cytosolic calcium ion concentrations as a response of agonist binding to the 5-HT_3R in cells were also observed in native vesicles. This finding demonstrates that cellular signaling reactions can be monitored in individual native vesicles.

Production of native vesicles is not limited to cultured mammalian cells. All eukaryotic cell types are amenable to cytochalasin B treatment since they possess an actin cytoskeleton.[45] Of importance would be the use of native vesicles from primary cells for functional screening, thus providing central biological information at an early stage in drug discovery. So far, such strategies were hampered by the limited availability of primary cells. Native vesicles allow the performance of multiple bioanalytical experiments starting with single live cells (e.g., cancer cells, stem cells, and neuronal cells obtained from living organisms) in multiplexing functional single-cell bioanalytics.

Because native vesicles can be prepared in large quantities, which can be frozen and stored for many weeks without losing biological activity, they serve as miniaturized artificial cells of constant quality for functional assays, replacing time-consuming, expensive analytical procedures based on cell cultures of highly variable quality.

Manipulating and addressing individual native vesicles either on surfaces or free in solution presents challenging opportunities for bioanalytics in the micro- and nanometer range. For example, proper tags at the vesicle surface (biotin, polyhistidine, oligonucleotides, lectins, antigens, etc.) provided by labeled lipids[33,46] or proteins[47−49] enable the formation of microarrays of vesicles[24] by self-assembly on complementarily micropatterned sensor surfaces.[20,50] Micromanipulating individual native vesicles by optical tweezers[51] and fibers, interconnecting them by lipid nanotubes,[52] or producing and sorting vesicles within microfluidic devices[21,53] are further interesting possibilities. Downscaling functional assays from large numbers and volumes to single native vesicles opens the door to new dimensions of miniaturized bioanalytics.

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