Time-resolved quantitative analysis of CCK1 receptor-induced intracellular calcium increase

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Abstract

Cholecystokinin (CCK) is a gastrointestinal hormone, which regulates many physiological functions such as satiety by binding to the CCK receptor (CCKR). Molecules, which recognize this receptor can mimic or block CCK signaling and thereby influence CCKR-mediated processes. We have set up a quantitative heterologous assay with CHO cells over-expressing the rat CCK1 receptor to screen for such candidate molecules. Receptor activation, induced by agonist binding, is followed by an intracellular calcium increase, which was monitored using a fluorescent sensor dye. For quantification of the calcium increase, a population average technique using a fluorescence plate reader was optimized and subsequently compared with a single-cell approach using confocal microscopy. With both strategies, dose-response curves were generated for the natural agonist CCK-8S, the partial agonist JMV-180 as well as the antagonist lorglumide. Significant differences were found between the ligands and a strong correspondence was observed between both methods in terms of maximum response and median effect concentrations. Both highly sensitive methods proved complementary: whereas the plate reader assay allowed faster, high throughput screening, the confocal microscopy identified single-cell variations and revealed factors that reduce specificity and sensitivity.

keywords: cholecystokinin 1 receptor, CCK, JMV-180, lorglumide, live cell imaging, cell-based bioassay, Fluo-4AM, fluorescence plate reader

1. Introduction

Cholecystokinin (CCK) is a hormone and neuropeptide, which induces satiety and which regulates many physiological processes like gall bladder contraction, gastrointestinal motility, pancreas secretion, gastric acid secretion, panic, and anxiety [12]. CCK exerts its effects via binding to members of the CCK receptor (CCKR) family. Among the major subtypes are CCK1 receptor (CCK1R), mainly expressed in the gastrointestinal channel, and CCK2 receptor (CCK2R), mainly expressed in the brain [7]. Due to their multifaceted role, CCK receptors form an important target in the treatment of several diseases. Both agonistic and antagonistic ligands for the CCKR are considered for therapeutic strategies [3,16].

CCKR are G protein-coupled receptors, which upon activation, elicit an inositol trisphosphate (IP_3)-induced calcium release from the endoplasmic reticulum [7,13]. This intracellular Ca^{2+}-flux is a measure for the activation of the receptor and can be visualized with fluorescent sensor dyes [12]. Different cell systems functionally expressing one of the CCKR subtypes exist and can be used to screen for ligands with agonistic or antagonistic CCKR binding activity [4,8,14,15]. However, the use of diverse cell types, fluorescent dyes and measuring techniques makes it difficult to compare the results from different studies on the effectiveness of several ligands. Here, we established a fully controlled, standardized and sensitive cell-based bioassay in 96-well plates to screen and characterize components with CCK1R activity. For validation, the changes in fluorescence intensity observed with a population average technique using a fluorescence plate reader were compared with a single-cell approach using confocal microscopy. Cross-validation of both measuring techniques resulted in a sensitive and specific assay, which can be used for high throughput screening of molecules and protein hydrolysates that interact with the CCK1R. The discovery of these products may underlie the development of novel therapeutic formulations or new functional

foods and nutraceuticals, which induce satiety or have an effect on other CCK1R-mediated pathologic conditions.

2. Materials and Methods

2.1. Cell lines and chemicals

CHO (Chinese Hamster Ovary) cells functionally expressing the rat CCK1R (CHO-CCK1R) were established by Prof. Peter Willems [15] and native CHO-K1 cells were obtained from Prof. Georges Leclercq (Ghent University Hospital, Department of Clinic Biology, Microbiology and Immunology, Ghent Belgium). Advanced Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) (DMEM-F12), fetal bovine serum (FBS), geneticin (G-418 antibiotic), Fluo-4AM, Pluronic F-127 and Hank's buffered salt solution (HBSS) were purchased from Invitrogen (Paisley, UK), probenecid, lorglumide ((±)-4-[(3,4-dichlorobenzoyl)amino]-5-(dipentylamino)-5-oxopentanoic acid sodium salt; CR-1409), bovine serum albumin (BSA) and HEPES from Sigma-Aldrich (St.-Louis, MO), sulfated cholecystokinin octapeptide (CCK-8S) and thrombin receptor activating protein (TRAP-7) from Bachem (Weil am Rhein, Germany), and JMV-180 (Boc-Tyr(SO_3H)-Nle-Gly-Trp-Nle-Asp-2-phenylethylester) from Research Inc. (Barnegat, NJ). Clear bottom black 96-well plates were purchased from Greiner (Frickenhausen, Germany).

2.2. Cell culture

CHO-CCK1R and CHO-K1 cells were grown at 37°C and 5% CO_2 in advanced DMEM-F12 supplemented with 1% streptomycin and penicillin, 1% L-glutamine and 10% FBS. The medium of CHO-CCK1R cells was supplemented with 10 µl/ml geneticin to maintain a stably transfected culture.

2.3. Cell-based bioassay to screen for CCK1R activity

The determination of the intracellular free Ca^{2+} concentration was adapted from a method previously reported by Foltz et al. [1]. The fluorescent probe that was used in this assay is the hydrophobic Fluo-4AM, a cell-permeant acetoxymethyl (AM) ester, which is hydrolyzed by cellular esterases and becomes fluorescent upon Ca^{2+}-binding. One half of a 96-well plate is seeded with CHO-CCK1R cells and the other half with CHO-K1 cells, in both cases at 40,000 cells per well. Cells were incubated at 37°C and 5% CO_2 for 20-24 h to allow attachment. Next, the medium was removed and 50 µl of DMEM-F12 supplemented with 4 µM Fluo-4AM, 0.02% (w/v) of the surfactant pluronic, 4.55 mg/ml BSA, and 1.6 mM of the anion transport inhibitor probenecid was added to the wells for 1 h at 19°C, as was determined as the ideal dye loading temperature in preliminary experiments. Subsequently, the wells were washed twice with 150 µl of HBSS supplemented with 20 mM HEPES, 2.5 mM probenecid and 10 mg/ml BSA and finally 100 µl of modified HBSS was added to the wells. Lorglumide was added at a final concentration of 0–40 µM, 20 min before the start of the experiment. Receptor activation leads to a rapid increase in intracellular calcium concentration (within 0–30 s). Hence, fluorescence intensity was measured kinetically. Two setups were used: a fluorescence plate reader and a confocal microscope. In the first setup, an Infinite pro 200 (Tecan, Männedorf, Switzerland) multimode plate reader with automated injection system was handled using i-control™ software. Excitation and emission wavelengths were set to 480 nm and 520 nm respectively, using Quad4 monochromators™ technology. In the second setup, a Nikon A1r confocal laser scanning microscopy system (Nikon Instruments Inc., Melville, NY) was used, mounted on a Nikon Ti-E inverted epifluorescence microscope and equipped with a microscope incubator, Perfect Focus System and resonant scanner. Multiwell

dishes were screened, with a Plan Fluor 40 x/0.75 dry objective at full field of view (636 µm x 636 µm), resulting in a pixel size of 1.24 µm x 1.24 µm. Fluo-4AM was excited using a 488 nm multi-line Ar laser and fluorescence was detected through a 525/50 nm bandpass filter.

On both platforms a similar measurement protocol was applied. Measurement started 30 min after washing to allow complete intracellular cleavage of the Fluo-4AM ester. After washing, the plates were immediately placed in the plate reader or microscope incubator to equilibrate at the measurement temperature of 31°C, as determined in preliminary experiments. Each well was measured separately. Fluorescence was acquired at 2.5 fps with the plate reader and 3 fps with the confocal microscope. Ligands were added *in fluxo*, i.e. during acquisition: the basal fluorescence of a well was measured for 6 s after which 100 µl of the sample (diluted in modified HBSS) was added instantaneously while the measurement continued for another 34 s. In the plate reader, sample addition was performed automatically, while in the confocal microscopy the sample was added using an electronic repeating pipette (Handystep, BrandTech Scientific Inc, Essex, CT). Each sample concentration was measured in 5 wells (technical replicates) for both cell types and every experiment was repeated 3-4 times (biological replicates).

2.4. Image analysis

Confocal images were analyzed with ImageJ freeware (National Institute of Health, Bethesda, MD). For population average measurements, the average fluorescence intensity (per pixel) over the entire image was measured in unprocessed recordings. For single-cell analysis, the following workflow was designed: first, images were aligned by means of a rigid registration (translation and rotation) to remove superimposed motion *e.g.* due to thermal drift. Next, an average projection was made of the complete time stack to provide a well-contrasted image of

all the cells by averaging out the noise. This approach of temporal averaging works efficiently even in the absence of an overt calcium flux (*e.g.* in CHO-K1 cells, cfr. Supplementary Fig. 1). An extra smoothing step, by means of Gaussian filtering (sigma=1) removed the remaining noise and allowed for segmenting the cells in a subsequent thresholding step (Huang autothreshold). Touching cells were separated by local maxima finding and conditional region growing, resulting in a complete set of regions of interest (ROI), corresponding to the cell boundaries. All ROI were inspected and manually corrected, where required. Finally, a particle analysis was performed to measure the average pixel intensity per cell ROI (*i.e.* integrated intensity in a cellular ROI, divided by the ROI area in pixels) through time. This metric was selected to avoid cell-size dependent variations in signal intensity. Single cell results were processed and summarized using Matlab 7.10.0 (R2010a, The Mathworks Inc., Natick, MA).

2.5. Data analysis and statistics

For calculation of dose-response curves the following procedure was applied. Per time point i, fluorescence measurements (F_i) were normalized to the average fluorescence before sample addition, which corresponds to the average fluorescence in the first 6 s of the recording (F_0), thereby correcting for differences in the amount of basal fluorescence, due to variations in cell density, dye concentration and/or free calcium concentration. Average normalized fluorescence values of 5 technical replicates were corrected for non-specific responses and background fluorescence by subtracting the average normalized fluorescence values obtained for CHO-K1 cells per time point and per condition. Hence, the relative fluorescence (RF) was calculated using the following formula: $RF_i = \frac{F_i(CHO-CCK1R)}{F_0(CHO-CCK1R)} - \frac{F_i(CHO-K1)}{F_0(CHO-K1)}$ and

plotted as a function of time. Subsequently, a single metric was derived from the RF curves: the net response was calculated as the sum of relative fluorescence values, from the moment of sample addition until the end of the acquisition (net response = $\sum_{i=6s}^{40s} RF_i$). All net responses were expressed as a percentage of the maximal net response of CCK-8S (induced by a concentration of 1 nM). The inhibiting effect of the antagonist was calculated as 100% minus the net response. From these results, sigmoid dose-response curves for the percentage of the maximum response/percentage of inhibition versus sample concentration were derived with Prism v4 software (GraphPad Prism, La Jolla, CA) and median response concentrations, *i.e.* effective EC_{50} and/or inhibitory IC_{50} values, were calculated for the agonists and antagonists, respectively [19]. The EC_{50} value represents the agonist concentration at which 50% of its maximum response is reached. The IC_{50} value is the antagonist concentration, which inhibits 50% of the maximum response of CCK-8S (1 nM). The median response concentrations of agonists and antagonists are the mean of at least three independently repeated dose-response curves (biological replicates), which are based on 5 repeated measurements (technical replicates) for each concentration.

Median effect concentrations were statistically compared in S-plus (TIBCO Software Inc., Palo Alto, CA) by means of a One-way ANOVA analysis and the significance of individual differences were calculated using Tukey post hoc tests.

3. RESULTS

3.1. Optimization

First, we optimized and benchmarked our assay in terms of sensitivity, stability and specificity. When using fluorescent dyes, there is a risk of active dye uptake (*e.g.* by

pinocytosis) leading to compartmentalization of the dye in vesicles [11]. Indeed, using epifluorescence microscopy, we found that the fluorescent dye showed significant compartmentalization when the dye was loaded at 37°C. By lowering the loading temperature to 19°C, we observed a homogeneous cellular distribution of the dye indicating passive diffusion into the cell.

Given that a complete experiment, corresponding to one 96-well plate, takes about one hour to screen, it is imperative that the cellular response in different wells remains stable through time. When performing pilot experiments using the plate reader, we observed at 37°C a gradual decrease in cell response to a single concentration of CCK-8S (0.1 and 1 nM) from well to well. Conversely, at lower temperatures (28°C) a gradual increase in response was observed. The most stable overall response through time was obtained at a measurement temperature of 31°C (Supplementary Fig. 2).

To ensure ligand specificity, native CHO-K1 cells were included in the assay as negative controls. When stimulated with CCK-8S, CHO-K1 cells did not show a significantly different response from cells treated with buffer. However, both CHO-K1 as CHO-CCK1R showed a similar response when stimulated with TRAP-7, an agonist for the thrombin receptor (Supplementary Fig. 3). This confirmed that CHO-K1 cells could be used to correct for non-specific responses.

3.2. The plate reader assay allows for sensitive and accurate measurement of agonist and antagonist effects on the cell population level

First, we measured the cellular response to the natural ligand, CCK-8S. The change in fluorescence was monitored in time for increasing concentrations of CCK-8S (0.001 nM–1 nM) (Fig. 1). Typically, RF curves (Fig. 1a) showed strong dose-dependent kinetics in terms

of the time point and height of the maximum RF, but also in terms of peak persistence. The integrated area below the curve incorporates these different parameters and was therefore used in the calculations. A significant increase in signal could be detected down to a concentration of 0.01 nM CCK-8S. Dose-response curves were established from calculating the net response per condition (Fig. 1b) and were used to derive the EC_{50} values (Table 1).

Next, the effect of a partial agonist, JMV-180, was determined. The EC_{50} of JMV-180 was 1,000-fold higher with respect to the natural ligand, pointing at a much lower affinity towards the receptor (Table 1). Moreover, the maximum response that could be evoked by JMV-180 compared to 1 nM CCK-8S was more than halved (mean ± SEM; 38 ± 8%), illustrating the lower potency of this partial agonist to activate the CCK1R (Supplementary Fig. 4a).

In addition to the aforementioned agonists, the full antagonist lorglumide was tested for its potential to inhibit a CCK-8S-induced response. Increasing concentrations of lorglumide clearly demonstrated a dose-dependent inhibition of the response to 1 nM CCK-8S. The IC_{50} value was 3 ± 1 µM (Supplementary Fig. 4c, Table 1). Full inhibition of 1 nM CCK-8S was obtained at 40 µM lorglumide.

3.3. Confocal microscopy provides both population as well as single-cell information

Using confocal microscopy, the same kinetic experiments were performed as with the plate reader. First, the population-average response was determined on whole images. Per well, one single region was acquired at full field of view (636 µm x 636 µm), corresponding to 100-150 cells, and the average pixel intensity was measured over the entire image through time (Fig. 2a, Supplementary Movies 1 and 2). This assay was performed for CCK-8S with and without lorglumide and for JMV-180 and resulted in highly similar RF curves, dose-response curves

and median effective concentration values (EC_{50} and IC_{50}) as those obtained for the plate reader (Fig. 2b,c; Table 1; Supplementary Fig. 4b,d).

To determine the actual single-cell response, we measured fluorescence kinetics in individual cells by means of automated image analysis (Fig. 3a, Supplementary Fig. 1). The individual fluorescence kinetics in time varied from cell to cell, not only in magnitude and time point of the maximum fluorescence intensity but also in fluorescence fluctuation behavior (Supplementary Fig. 5). The time lag between the moment of sample addition and the moment of maximum fluorescence intensity became progressively shorter with increasing agonist concentration. In parallel, the synchrony with which the fluorescence of individual cells changed increased as well. This is reflected in the magnitude of the standard deviation of the time point of maximum fluorescence (Supplementary Fig. 6). Especially at low doses a cellular behavior became evident that was not observed in population averages: calcium fluxes show several less intense oscillations after the first primary fluorescence peak (Supplementary Fig. 5g). These fluctuations were no longer observed from a dose of 0.1 nM CCK or higher. At high concentrations, the oscillatory mode changed into one single increase after which no oscillations are seen anymore. In brief, fluorescence kinetics In CCK-8S stimulated cells became more uniform with increasing concentrations, which is in accordance with earlier observations [20]. In comparison, fluorescence oscillations were more pronounced in cells treated with JMV-180 and also persisted at higher doses (Supplementary Fig. 7).

Despite the inherent variability, all doses were significantly different from each other (Fig 3a). The median effective concentrations (EC_{50} and/or IC_{50}) based on single-cell analysis were not significantly different from those based on the average image intensity nor from those derived from the plate reader (Table 1).

To determine the robustness of the single-cell analysis, we calculated the number of cells required to discriminate dose-dependent responses using an *in silico* approach. To this end, image data sets containing all individual cell responses were progressively eroded by omitting one single, randomly selected cell at a time (on permutated data sets), down to one single cell, and by calculating the average response with each step. This was repeated 100 times to obtain a distribution that represents the variability within the data set (Fig. 3b). The covariance (std/mean) plots of these distributions demonstrated that as little as 10 cells are sufficient to determine the population mean with 95% accuracy, or 25 cells are sufficient to determine the population mean with 97.5% accuracy (Fig. 3c). *In silico* erosion was performed for all experiments and confirmed that 10-20 cells are sufficient to determine the population mean with 95% accuracy (Supplementary Fig. 8).

4. DISCUSSION

We have established a robust cell-based bioassay to screen for compounds which activate CCK1R or inhibit its binding to the natural ligand, and which works on two different platforms. This technology was first developed by Foltz et al. [9] for a fluorescence plate reader. We have now optimized this technology by making it more sensitive, specific and more robust by cross-validating this assay on a different platform, namely the confocal microscope. During optimization, we discovered that lowering the loading and measurement temperature had an important influence on the sensitivity and temporal stability of the cell response. It is conceivable that the degree of cell compartmentalization and the decay of the fluorescence through time, which were observed at higher temperatures, are due to a higher cell metabolism. At higher temperatures, transport mechanisms like endocytosis/pinocytosis (leading to compartmentalization) and exocytosis (leading to loss of signal) become more

prominent [11] and active breakdown of the fluorescent dye could be promoted [6].

The EC_{50} measured for CCK-8S in this project with the fluorescence plate reader and the confocal microscope were not significantly different, although purely based on the SEM one could say a higher accuracy was obtained with the plate reader. This could be due to a different demand for manual interaction between both approaches: the measurements with the fluorescence plate reader were almost fully automated, whereas the measurements with the confocal microscope were carried out manually, thereby introducing pipetting inaccuracies and causing complete experiments to last longer and have more fluorescent probe decay. Also, sample mixing in the medium may have occurred faster in the plate reader causing cells to react more synchronously. On the other hand, a bigger SEM in confocal measurements may be the reflection of the high heterogeneity between individual cells, as such providing more accurate information of the true cellular response. In addition, the visualization of cells allows immediate verification of cellular health and density. Confocal microscopy also allows discrimination of off-target effects like bad solubility and background fluorescence of the sample (*e.g.* when protein hydrolysates are tested). With the fluorescence plate reader, autofluorescence of the sample causes a major concern as the fluorescent signal of the entire well is measured, which may obscure more subtle fluorescence increases at the level of the cells. In contrast, the confocal microscope only detects fluorescence in a selected focal section on the bottom of the well, which is predominantly from within the cells. Hence, due to its optical sectioning capability, confocal microscopy allows measuring a more specific response and testing higher sample concentrations when working with autofluorescent samples.

Experiments carried out with cells in suspension under continuous stirring revealed 10 to 50-fold higher EC_{50} values (around 0.19-1.08 nM) for CCK-8S compared to our experiments [4,8,14]. However, the EC_{50} of the partial agonist JMV-180 measured with cells

in suspension (25 nM) was comparable to the EC_{50} measured in this project [14]. These variations could not be ascribed to large differences in medium composition (*e.g.* BSA concentration) or probenecid concentration, but may be due to differences in sample composition and stability as well as the experimental setup. Receptor activation can also be measured by inositol trisphosphate (IP_3) production. Cawston et al. [5] reported an EC_{50} value for IP_3 production of JMV-180 of 18 nM, which is similar to the EC_{50} value of JMV-180 we found for Ca^{2+}-increase (Table 1). In addition, the maximum concentration only induced 9% of the IP_3 production compared to that of CCK-8S. The lower potency of JMV-180 compared to the natural ligand can be explained by the differential positioning of the C-terminal end within the binding site of CCK1R [1]. A partial agonist only exerts a subset of the biological functions of the natural ligand and therefore has a reduced tendency to evoke side effects and cause receptor intolerance. This makes a partial agonist more suitable as a therapeutic than the natural ligand [5]. In this context, it is also noteworthy that JMV-180 elicited highly different fluorescence kinetics compared to CCK-8S. In accordance with previous observations [15,17], more pronounced and more frequent oscillations were observed, even at high doses, indicating an altered cellular response. These differences could be due to differential coupling of low- and high affinity CCK1R binding sites to second-messenger systems and Ca^{2+}-signal transduction pathways, *e.g.* differences in activation of protein kinase C and IP_3 production [18,21]. Lorglumide is a full antagonist of the CCK1R and it is 2,300 times more selective for CCK1R than for CCK2R. It is a potent inhibitor in comparison to other glutaramic acid analogues [2]; an IC_{50} value of 0.13 µM was reported by Makovec et al. [10] for this antagonist. The latter IC_{50} value is 15 times lower compared to the IC_{50} measured in this project, which is probably due to the use of a different experimental design. Hence it can be concluded that comparison between the median effect concentrations (EC_{50} and IC_{50}) of

different CCK receptor agonists and antagonists between studies should be made with caution, as different cell types, measuring techniques and controls are used. Therefore, we propose this standardized procedure to enable comparison of the CCK1R activity of different ligands. Moreover, with little adaptations, this assay could be converted into a screening system for ligands for the CCK2R, the sulfakinin receptor and other Ca^{2+}-influencing GPCRs.

In conclusion, the proposed cell-based bioassay can be used to screen for protein hydrolysates and molecules with CCK1R activity in a standardized manner. Measurement can be accomplished on two platforms. The fluorescence plate reader is more suitable to perform a primary screen, but the confocal microscope can be used to validate the activity of the components on the single-cell level, especially in a context of strong autofluorescence background. The selected components might be an aid in the battle against obesity or other CCK1R-involved diseases like diabetes type II.

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Figure legends

Fig. 1. Dose-dependent CCK1R-mediated calcium fluxes in cell populations monitored with a plate reader. (a) Kinetics of relative fluorescence (RF) of Fluo-4AM labeled CHO-CCK1R cells to increasing concentrations of CCK-8S (0.001–1 nM). The curves represent the mean of 5 technical replicates (wells). (b) Representative dose-response curve for CCK based on 4 experiments (biological replicates) in which the measurements for each concentration were repeated 5 times, expressed as a percentage of the maximum net response, *i.e.* the net response induced by 1 nM CCK.

Fig. 2. Dose-dependent CCK1R-mediated calcium fluxes in cell populations monitored with a resonant scanning confocal microscope. (a) Montage from *in fluxo* confocal microscopy, combined from equally cropped regions selected from raw time-lapse series, acquired as described in the Materials and Methods section. (b) Kinetics of relative fluorescence (RF) of Fluo-4AM labeled CHO-CCK1R cells to increasing concentrations of CCK-8S (0.001–1 nM). The curves represent the mean of 5 technical replicates (wells). (c) Representative dose-response curve for CCK based on 3 experiments (biological replicates) in which the measurements for each concentration were repeated 5 times, expressed as a percentage of the maximum net response, *i.e.* the net response induced by 1 nM CCK.

Fig. 3. Dose-dependent CCK1R-mediated calcium fluxes in individual cells monitored with a resonant scanning confocal microscope (a) Boxplot representing the single-cell response of CHO-CCK1R cells to increasing concentrations of CCK-8S (0.001–1 nM), measured as the average intensity of individual cells and expressed as a percentage of the maximum response, *i.e.* the average response induced by 1 nM CCK. (b) *In silico* erosion. Per condition, the

response was calculated for all cells within one representative image per CCK-8S concentration. Next, cells were progressively removed from permutated data sets, one by one, down to the single cell and per step the average net response was calculated. This was repeated 100 times per condition. Every line represents one complete erosion cycle. (c) From the eroded data sets the covariance (CoV) was calculated and plotted as a function of the number of cells and represented on a logarithmic scale for visualization purposes. Ten cells reduce the CoV to 5% and 25 cells reduce the CoV to 2.5% (dotted black lines).

Supplementary Material

Supplementary Fig. 1. Illustration of temporal averaging for image segmentation. (a) original raw image of one single time point of a CHO-K1 cell labeled with Fluo-4AM acquired for 40 s at 3fps. (b) average intensity projection of the complete time stack (40 s, 150 frames) (c) result of segmentation, displayed as white outlines superimposed on the average projection image. Scale bar represents 100 µm.

Supplementary Fig. 2. Temperature and concentration dependence of cell response from well to well. Fluo-4AM CHO-CCK1R cells in 96-well plates were exposed to 0.1 nM (a) or 1 nM CCK-8S (b) at different measurement temperatures. Every well, represented by a single data point in the plot, was treated (and measured) sequentially. Trend lines from a linear regression were plotted for demonstrational purposes. The response has been expressed relative to that of the first well.

Supplementary Fig. 3. Kinetics of normalized fluorescence (F_i/F_0) ± SD in Fluo-4AM labeled CHO-CCK1R and CHO-K1 to 250 µM of TRAP-7. Experiments were carried out in the presence of 50 µM lorglumide to inhibit effects via the CCK1R (n=6).

Supplementary Fig. 4. Representative dose-response curves for JMV-180 (a,b) and lorglumide (c,d) based on 3 experiments in which the measurements for each concentration are repeated 5 times, expressed as a percentage of the maximum response and the percentage of inhibition from the maximum response, respectively. Graphs on the left are the results measured with the fluorescence plate reader (a,c) and on the right with the confocal microscope (b,d).

Supplementary Fig. 5. Inter-individual and temporal variation in normalized fluorescence kinetics (F_i/F_0) in response to CCK-8S, monitored in Fluo-4AM labeled CHO-CCK1R cells with a resonant scanning confocal microscope, decreases with increasing concentration: (a) 1E-03 nM, (b) 1E-02 nM, (c) 2.5E-02 nM, (d) 1E-01 nM, (e) 2.5E-01 nM, and (f) 1n M. (g) 10 randomly selected curves from cells treated with 2.5E-02 nM CCK-8S demonstrate the fluorescence fluctuations in individual cells at low concentrations.

Supplementary Fig. 6. Histogram comparing the average peak time (maximum fluorescence) of individual cells of all experiments, for increasing concentrations of CCK-8S, JMV-180 and lorglumide. Error bars represent standard deviations.

Supplementary Fig. 7. Inter-individual and temporal variation in normalized fluorescence kinetics (F_i/F_0) in response to JMV-180, monitored in Fluo-4AM labeled CHO-CCK1R cells with a resonant scanning confocal microscope, is more pronounced than after treatment with CCK-8S and persists with increasing concentration: (a) 5.0 nM, (b) 12.5 nM, (c) 50 nM, (d) 125 nM, and (e) 500 nM. For comparison, (f) shows the profiles of Fluo-4AM labeled CHO-K1 cells treated with 500 nM JMV-180. (g) 10 randomly selected curves from cells treated with 500 nM JMV-180 illustrate the persistence of fluorescence fluctuations in individual cells at high concentrations.

Supplementary Fig. 8. Histogram comparing for all experiments the number of cells required to obtain a coefficient of variation of the cell response equal to or smaller than 5%, as calculated from *in silico* erosion data sets. Error bars represent standard deviations.

Supplementary Movie 1. Time-lapse recording (subregion of full field of view) of Fluo-4AM labeled CHO-CCK1R cells acquired with resonant scanning confocal microscopy, stimulated *in fluxo* with 0.01 nM CCK-8S at 6 s of acquisition. A Green Hot lookup table was applied for accentuating intensity fluctuations.

Supplementary Movie 2. Time-lapse recording (subregion of full field of view) of Fluo-4AM labeled CHO-CCK1R cells acquired with resonant scanning confocal microscopy, stimulated *in fluxo* with 1 nM CCK-8S at 6 s of acquisition. A Green Hot lookup table was applied for accentuating intensity fluctuations.

Table 1

Median effect concentrations (EC_{50} and IC_{50}) for the natural ligand CCK-8S, the partial agonist JMV-180 and the antagonist lorglumide, measured with the fluorescence plate reader (population average) and the confocal microscope (population average and single-cell). Median effect concentrations for a ligand do not significantly differ between measuring techniques, calculated with ANOVA followed by a post hoc Tukey test (df = 2; CCK-8S: F = 3.05, p = 0.11; JMV-180: F = 0.48 p = 0.64; lorglumide: F = 0.72, p = 0.52).

Ligand	**Median effect concentration (EC_{50} and/or IC_{50} ± SE)**		
	Fluorescence plate reader	**Confocal microscope**	
	Population average	**Population average**	**Single-cell average**
CCK-8S	24 ± 4 pM	50 ± 13 pM	47 ± 13 pM
JMV-180	20 ± 4 nM	31 ± 12 nM	31 ± 12 nM
Lorglumide	3 ± 1 µM	4 ± 1 µM	5 ± 2 µM