Video Article

A Calcium Bioluminescence Assay for Functional Analysis of Mosquito (*Aedes aegypti*) and Tick (*Rhipicephalus microplus*) G Protein-coupled Receptors

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Abstract

Arthropod hormone receptors are potential targets for novel pesticides as they regulate many essential physiological and behavioral processes. The majority of them belong to the superfamily of G protein-coupled receptors (GPCRs). We have focused on characterizing arthropod kinin receptors from the tick and mosquito. Arthropod kinins are multifunctional neuropeptides with myotropic, diuretic, and neurotransmitter function. Here, a method for systematic analyses of structure-activity relationships of insect kinins on two heterologous kinin receptor-expressing systems is described. We provide important information relevant to the development of biostable kinin analogs with the potential to disrupt the diuretic, myotropic, and/or digestive processes in ticks and mosquitoes.

The kinin receptors from the southern cattle tick, *Boophilus microplus* (Canestrini), and the mosquito *Aedes aegypti* (Linnaeus), were stably expressed in the mammalian cell line CHO-K1. Functional analyses of these receptors were completed using a calcium bioluminescence plate assay that measures intracellular bioluminescence to determine cytoplasmic calcium levels upon peptide application to these recombinant cells. This method takes advantage of the aequorin protein, a photoprotein isolated from luminescent jellyfish. We transiently transfected the aequorin plasmid (mtAEQ/pcDNA1) in cell lines that stably expressed the kinin receptors. These cells were then treated with the cofactor coelenterazine, which complexes with intracellular aequorin. This bond breaks in the presence of calcium, emitting luminescence levels indicative of the calcium concentration. As the kinin receptor signals through the release of intracellular calcium, the intensity of the signal is related to the potency of the peptide.

This protocol is a synthesis of several previously described protocols with modifications; it presents step-by-step instructions for the stable expression of GPCRs in a mammalian cell line through functional plate assays (Staubly *et al*., 2002 and Stables *et al*., 1997). Using this methodology, we were able to establish stable cell lines expressing the mosquito and the tick kinin receptors, compare the potency of three mosquito kinins, identify critical amino acid positions for the ligand-receptor interaction, and perform semi-throughput screening of a peptide library. Because insect kinins are susceptible to fast enzymatic degradation by endogenous peptidases, they are severely limited in use as tools for pest control or endocrinological studies. Therefore, we also tested kinin analogs containing amino isobutyric acid (Aib) to enhance their potency and biostability. This peptidase-resistant analog represents an important lead in the development of biostable insect kinin analogs and may aid in the development of neuropeptide-based arthropod control strategies.

Video Link

The video component of this article can be found at http://www.jove.com/video/2732/

Protocol

1. Establishment of stable cell lines

1. Clone your GPCR of interest and insert it into an expression vector incorporating a 5' Kozak consensus sequence (GCCA/GCCATGG) around the start codon for optimal ribosomal binding in a mammalian system (Kozak, 1986). Here we use the plasmid pcDNA3.1/Bm-KR for the tick kinin receptor, and the plasmid pcDNA3.1/Aedae-KR for the mosquito kinin receptor (Holmes *et al*., 2003; Pietrantonio *et al*., 2005). The pcDNA3.1(-) vector encodes ampicillin resistance for selection in bacteria and neomycin (G418) resistance for selection in mammalian cells.

2. Grow CHO-K1 empty cells (without plasmids) (ATCC, Manassas, VA, USA) or other desired cell line in a T-25 flask (BD Falcon) at 37°C in a 5% CO_2 humidified incubator (Holmes *et al*., 2003). All further incubations should be done under these conditions unless specified otherwise. Maintain the empty cells in growth medium (F12K medium with 10% fetal bovine serum) with 1X Antibiotic-Antimycotic (Invitrogen,CA).
3. To split cells, warm up all solutions to 37 °C. Remove old medium in the T-25 flask and rinse with 5 ml PBS and then remove PBS. To trypsinize cells, add 2 ml PBS-Trypsin-EDTA (34 ml PBS, 2 ml 7.5% sodium bicarbonate, 4 ml 10X trypsin-EDTA) for 2 min. Add 3 ml medium and aspirate medium up and down to mix cells. Transfer medium with cells into a conical tube and centrifuge for 2 min at ~200-300 g (1,000 rpm). Discard supernatant and re-suspend cells in 5 ml medium. Dilute the resuspended cells to a concentration of 1:5 or 1:10 with fresh medium and transfer 5 ml in a new T-25 flask.
4. After the cells are growing healthily (around 2-3 days), seed the CHO-K1 cells into T-25 tissue culture flasks and grow them overnight in growth medium without antibiotics until they are about 30% confluent (approximately 18 hours). The degree of confluency can be determined by observing cells under inverted fluorescent microscopy.
5. Prepare the following mixtures for each sample:
    1. Combine 1-2 µg DNA (example: use 4µl of 265µg/µl pcDNA3.1/Aedae-KR) with 100 µl Opti-MEM I Reduced Serum Medium (Invitrogen).
    2. Mix 6 µl Lipofectin Reagent (InvitrogenTM) into 100 µl F12K serum free medium, making a 1:1 ratio of Lipofectin to DNA. Incubate at room temperature for 30-45 min.

    Gently mix the solutions from 1.5.1 with 1.5.2 in a drop-wise fashion (total volume = 200 µL). Let stand at room temperature for 10-15 min.
6. Remove the old growth medium from the cells and wash the cells with 5 ml F12K serum free medium and then remove the F12K serum free medium. In a 15 ml tube, gently mix the transfection mixture into 1.8 ml of fresh F12K medium in a drop-wise fashion. Then, washed the cells from step 1.5 with PBS and add this new transfection solution to the cells. Incubate for 18 hours.
7. Change the medium to F12K medium plus 10% fetal bovine serum without antibiotics and incubate overnight. Split the cells into two T-25 flasks with F12K medium plus 10% fetal bovine serum without antibiotics for another 18 hours (for splitting cells please see step 1.3).
8. Replace the medium with selective medium (F12K medium plus 10% fetal bovine serum with 800µg/ml GENETICIN, Invitrogen). Culture the cells using the selective medium for 3-4 weeks. Over time this will select for cells that have stably incorporated the plasmid into their genomic DNA. Continue maintaining the cells using maintenance medium (F12K medium plus 10% fetal bovine serum with 400µg/ml GENETICIN). Periodically freeze cell lines with 1:1 ratio of freezing media (selective medium with 20% DMSO) to prevent loses in case of unexpected contamination.
9. Selecting clonal cell lines: as a first step, trypsinize and centrifuge the cells from step 1.8 with maintenance medium as in step 1.3. Re-suspend cells in 5 ml maintenance medium, take 0.5 ml of the cell suspend and add 4.5 ml of fresh maintenance medium to make a 10x dilution. Transfer the 10x dilution into 12 wells of a 96 well plate, adding 100 µl to each well to select for single cells.
10. Continue to make 10x serial dilutions of these cells for a theoretical final suspension of one cell per 100 µl (normally the final dilution will be in the range of 10^{-11} to 10^{-19}; the total number of 10x serial dilutions is ~19). Immediately following each dilution, transfer 100 µl of the dilution into 12 wells of the 96 well plate. After 18 hours incubation, observe 96 well plates under an inverted light or fluorescence microscope and mark wells that appear to only contain one single cell or contain two cells that obviously divided from one single cell. Keep observing every day and change medium every three days.
11. When the wells are 80% confluent (about a week), rinse marked wells with 200 µl PBS and trypsinize them with 100 µl of PBS-trypsin-EDTA solution. Transfer cells from each marked well into one well of a 6-well plate with 1 ml maintenance medium. Grow those cells for 3 days then transfer cells into individual T25 flask. Test these cells using the calcium bioluminescence assay with agonist peptides (please see section 2).
12. Select 1 cell line from step 1.11 with the highest response in the calcium bioluminescence plate assay and perform for a second time the single cell selection following steps 1.9-1.11. Periodically freeze cell lines.
13. From the secondary selection described in step 1.12, choose 2-3 cell lines with the highest response in the calcium bioluminescence plate assay and maintain them in culture for further calcium bioluminescence plate assays. Keep track of passage numbers. From time to time, freeze cell lines from early passages so that you can always go back to them if the cell lines with more passages stop performing consistently.

2. The calcium bioluminescence plate assay

1. Ligate the reporter gene of interest into an expression vector. Here we used aequorin plasmid mtAEQ/pcDNA1 (a gift from Drs. C. J. P. Grimmelikhuijzen and Michael Williamson, University of Copenhagen, Denmark). Transform the plasmid in *E. coli* cells MC1061/PS (Invitrogen) and purify them using a QIAprep spin miniprep kit (Qiagen Inc.). In the final step elute the plasmid with Tris buffer without EDTA, not water.
2. Grow cell lines from step 1.13 expressing the desired receptor in maintenance medium. When the cells are 90% confluent, trypsinize, centrifuge and then re-suspend the cells in 5 ml maintenance medium as in step 1.3. Dilute cells (about 10x with maintenance medium) and count cell number with cell counter (Bright-Line Hemacytometer) under microscopy. Adjust the cell number to approximately 2 x 10^5 cells/ ml (average 20 cells in one of the 9 squares showed in the Hemacytometer). Seed 2 ml diluted cells in media into each well of a six well plate. Incubate for 24 hours (the cells should reach about 60% confluency after incubation).
3. Change media in the 6 wells plate to OPTI-MEM medium. For each well, mix 96 µl OPTI-MEM with 4 µl FuGENE 6 Transfection reagent (Roche Biochemicals) in a microfuge tube and let sit at room temperature for 5 min. Add 1 µg of aequorin/pcDNA 1 plasmid DNA into each tube and then gently shake the sample for 1 min, incubate at room temperature for 15-20 min. Add each mixture into each well in a dropwise fashion while gently shaking the well plate. Incubate the plates for 6 hours and change the medium to F12K medium containing 10% fetal bovine serum without antibiotic.
4. After incubating the cells in six well plate for 24 hours, trypsinize, centrifuge and re-suspend the cells as step 1.3. Count the cell number to 400,000 cells/ml as step 2.1 and transfer 100 µl (total 40,000 cells/100 µl) into each well of a 96-well white thin bottom microtitre plate (Costar 3610). Incubate for another 24 hours until about 80% cell confluency. This is the optimal concentration of cells for the bioluminescence assay.
5. Prepare 90µl/well of a calcium-free DMEM media (Invitrogen) containing 5 µM coelenterazine (Invitrogen) in the dark (coelenterazine is light sensitive). Take the plate from 2.4, remove old media and add this 90µl into each well. Incubate plates for 3 hours in the dark at 37°C and 5% CO_2, after which the cells in the plate are ready to be tested.

3. Instrument operation and data analysis

1. Each bioluminescence plate reader is different. We perform our assay using a NOVOstar (BMG Labtechnologies) plate reader in bioluminensence mode. If you use a different instrument, you must adapt the protocol.
2. Purge the plate reader pumps (or PRIME PUMPS) before use. Turn off the light in the room before putting the plate on the plate holder. Once the plate holder has closed, turn the lights on.
3. Solubilize peptides (in a 1.5 ml Eppendorf tube) in the calcium-free DMEM media. Set the "Aspirate Depth" and "Position Determination" of the peptide solution before use. Challenge the cells with 10 µl (10x) of varying concentrations of the peptides (FFFSWG-NH2, Aedes-K1-3, or other desired peptide) and immediately begin recording the light emission. We have set the instrument to record the light emission (465 nm) for each well every 2 seconds for a total time of 50 seconds.
4. Make sure to include a positive control such as an active analog (analog FFFSWGa has been used, Taneja-Bageshwar *et al*., 2009) and a negative control such as vector only transfected cells. The negative control will be necessary during data analysis to set the baseline threshold (see representative results). An unrelated, inactive peptide can also be added as a negative control.
5. After the run is complete, wash the instrument pump (or PRIME PUMPS) then place your next peptide sample. Save your data and wash the pumps again.
6. Data handling: Transfer the data of light emission from each well into a Microsoft Excel data sheet.
7. Paste the data from Excel to Prism software 4.0 from GraphPad Software Inc. (San Diego, CA, USA). The various peptide concentrations is the X-axis and bioluminescence units is the Y-axis. To normalize the data, plot a log-response curve. Select a nonlinear regression curve fit analysis (sigmoidal dose-response equation with variable slope) to obtain concentration-response curves for each peptide. The program plots the values in the end and gives the EC_{50}.
8. Each experiment should be repeated three times for data analysis.

4. Representative Results:

When expressed in CHO-K1 cells, the mosquito Aedes aegypti kinin receptor behaved as a multiligand receptor and functionally responded to concentrations as low as 1 nM of the three endogenours Aedes kinins, Aedae kinins 1-3, tested singly using the calcium bioluminescence plate assay. Figure 1 shows that the rank order of potency obtained was Aedae-K-3 > Aedae-K-2 > Aedae-K-1, based on the respective EC_{50} values of Aedae-K-3, 16.04 nM; Aedae-K-2, 26.6 nM and Aedae-K-1, 48.85 nM, which were statistically significantly different ($P < 0.05$) (Pietrantonio *et al*., 2005).

We also used this assay to determine which kinin residues are critical for the kinin peptide-receptor interaction. Insect kinin peptides share a C-terminal pentapeptide that represents the minimal sequence required for biological activity, also known as core. In Table 1, the kinin peptide core analogs were synthesized as an Alanine replacement series of the core kinin pentapeptide FFSWGa and tested by a calcium bioluminescence plate assay (Taneja-Bageshwar *et al*., 2006). We found that the amino acids Phe^1 and Trp^4 were essential for activity of the insect kinins for both receptors.

The assay also can be used to test peptides designed for enhanced biostability. Table 2 shows the designed kinin analogs containing amino isobutyric acid (Aib) tested on the tick recombinant kinin receptor and Figure 2 shows the activity comparison of six alpha-amino isobutyric acid analogs on the tick kinin receptor expressing CHO-K1 cell line by a calcium bioluminescence plate assay (Taneja-Bageshwar *et al*., 2009). The hexapeptide analog FFFSWGa is added for a positive control for receptor activity. The analog FF[Aib]WGa resulted more active than this hexapeptide control analog. The analog with two aminoisobutyric acid replacements, [Aib]FF[Aib]WGa, was the most potent of the double-replacement analogs tested (Table 2 and Figure 2).

For more examples of how this assay has been and can be applied see Nachman and Pietantonio (2010), Nachman *et al*. (2009), Taneja-Bageshwar *et al*. (2008a), and Taneja-Bageshwar *et al*. (2008b).

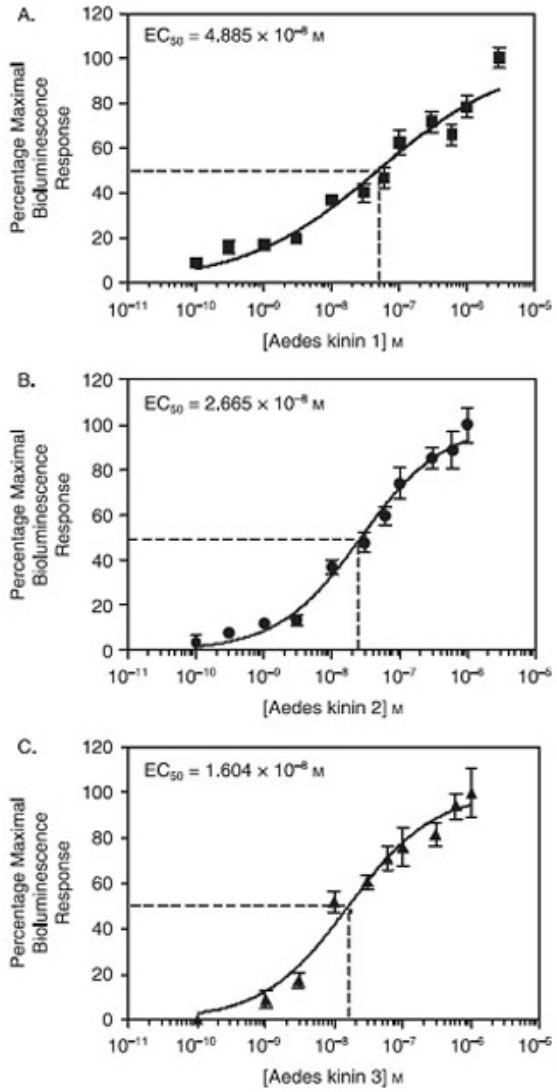

Figure 1. Estimation of *Aedes* kinins effective concentration (EC_{50}) on CHO-K1 E10 cells by a calcium-bioluminescence plate assay. The y-axis in the concentration-response curves was obtained from bioluminescence units expressed as a percentage of the maximal response observed for each peptide. Data points represent the average of six replicates obtained during three independent experiments. Bars represent the standard error. (A) Estimation of Aedae-K1 EC_{50} = 48 nM. (B) Estimation of Aedae-K2 EC_{50} = 26 nM. (C) Estimation of Aedae-K3 EC_{50} = 16 nM. EC_{50} Aedae-K3 < EC_{50} Aedae-K2 < EC_{50} Aedae-K1; $P < 0.05$. Statistical analysis and graphs were with the GraphPad Prism 4.0 software.

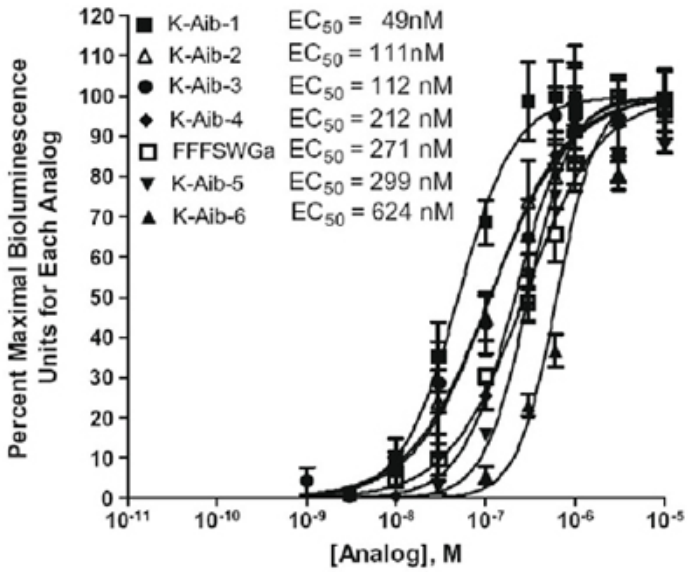

Figure 2. Activity comparison of six alpha-amino isobutyric acid analogs on the tick kinin receptor expressing CHO-K1 cell line by a calcium bioluminescence plate assay. The y-axis represents percent maximal bioluminescence units for each analog expressed as a percentage of bioluminescence observed at a concentration versus the maximal response observed among all concentrations tested for each analog. Statistical analysis and graphs were performed with GraphPad Prism 4.0 software.

	Tick receptor cell line		Mosquito receptor cell line	
Peptides	EC_{50} (nM)	Maximal bioluminescence response at 1 mM	EC_{50} (nM)	Maximal bioluminescence response at 1 mM
AFSWGa	I	I	I	I
FASWGa	586	5,600	N.D.	400
FFAWGa	64	12,800	621	3,050
FFSAGa	I	I	I	I
FFSWAa	417	10,600	2,800	1,830
FFSWGa	590	10,800	N.D.	525
FSWGa	I	I	I	I
FFSWa	I	I	I	I
FFSWG-OH	I	I	I	I
FFFSWGa	259	13,000	562	10,000
FF[Aib]WGa	29	12,700	445	9,300

Table 1. Estimated potencies (EC_{50}) and maximal bioluminescence response of all the peptides tested on tick and mosquito receptor transfected cell lines*.

*The EC_{50} estimates the concentration required to induce a half-maximal response. I: Inactive if bioluminescence response is less than 300 units (level of vector-only transfected cells). A: The position where the respective residue in the peptide FFSWGa has been replaced by alanine. N.D.: The analog was tested but was either not very active or was not active at lower molarities, thus an EC50 could not be determined.

K-Aib-1	[Aib]FF[Aib]WGa
K-Aib-2	[α MeF]FF[Aib]WGa
K-Aib-3	Ac-R[Aib]FF[Aib]WGa
K-Aib-4	Ac-R[β3F]FF[Aib]WGa
K-Aib-5	[Aib]RFF[Aib]WGa
K-Aib-6	[Aib-Aib-Aib-Aib]RFF[Aib]WGa

Table 2. Kinin analogs (K) containing amino isobutyric acid (Aib) tested on the tick recombinant kinin receptor. Ac: acetyl; α Me: α methyl-phenylalanine; β3F: β3 -phenylalanine; a: amide.

Discussion

We were able to perform the functional characterization of the first neuropeptide receptor discovered from the Arachnida (ticks, mites and spiders), the tick kinin receptor, using this protocol. This method has three primary applications. First, the technique can be applied for receptor deorphanization through ligand activity measurements. Second, the assay can resolve ligand-receptor structure-activity relationships (SAR). Third, the methods can be used in drug discovery. Furthermore, this protocol can be used to study the activity of agonists or antagonists on almost any GPCR. We are beginning to adapt this protocol for screening of small libraries. The cell line we utilized does not express the ubiquitous G protein G_{16}. We did not need it because arthropod kinin receptors signal through the Gq protein and the intracellular calcium cascade and they conserve this signaling properties in mammalian cells as shown here.

Disclosures

No conflicts of interest declared.

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