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ORIGINAL PAPER

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# Recombinant aequorin as a reporter for receptor-mediated changes of intracellular $Ca^{2+}$-levels in *Drosophila* S2 cells

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**Abstract** The bioluminescent $Ca^{2+}$-sensitive reporter protein, aequorin, was employed to develop an insect cell-based functional assay system for monitoring receptor-mediated changes of intracellular $Ca^{2+}$-concentrations. *Drosophila* Schneider 2 (S2) cells were genetically engineered to stably express both apoaequorin and the insect tachykinin-related peptide receptor, STKR. *Lom*-TK III, an STKR agonist, was shown to elicit concentration-dependent bioluminescent responses in these S2-STKR-Aeq cells. The $EC_{50}$ value for the calcium effect detected by means of aequorin appeared to be nearly identical to the one that was measured by means of Fura-2, a fluorescent $Ca^{2+}$-indicator. In addition, this aequorin-based method was also utilised to study receptor antagonists. Experimental analysis of the effects exerted by spantide I, II and III, three potent substance P antagonists, on *Lom*-TK III-stimulated S2-STKR-Aeq cells showed that these compounds antagonise STKR-mediated responses in a concentration-dependent manner. The rank order of inhibitory potencies was spantide III > spantide II > spantide I.

**Keywords** Agonist · Antagonist · Assay · Calcium · Coelenterazine

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## Introduction

The photoprotein aequorin derived from the coelenterate jellyfish, *Aequorea victoria*, is a bioluminescent complex of apoaequorin with the luminophore cofactor, coelenterazine. Shimomura and co-workers (1962) discovered that aequorin-derived luminescence is triggered by $Ca^{2+}$-ions. Apoaequorin (21 kDa), a protein of 189 amino acids, contains three calcium binding sites consisting of EF-hand structures (Inouye et al. 1985). In the presence of calcium ions, coelenterazine is oxidised to coelenteramide with a concomitant release of carbon dioxide and emission of light [$\lambda_{max} = 469$ nm (blue light)] which can be detected by conventional luminometry.

Aequorin has been used for many years as a reporter of changes in intracellular calcium concentration in mammalian cells or *Xenopus* oocytes. In the initial experiments, loading of cells with aequorin involved microinjection of the purified protein (Ashley and Cambell 1979). Later, the cloning of the apoaequorin cDNA (Inouye et al. 1985) has allowed recombinant expression of this protein, both transiently and stably, in a broad range of cell types and this has greatly expanded the utility of aequorin as a calcium indicator (Prasher et al. 1985). From then on, several groups have successfully produced recombinant apoaequorin-producing cells (Tanahashi et al. 1990; Button and Brownstein 1993; Ungrin et al. 1999; Stables et al. 2000). In these cells, reconstitution of aequorin can be obtained by simple addition of coelenterazine to the medium. Coelenterazine is highly hydrophobic and has been shown to easily permeate cell membranes of various cell types, ranging from the slime mould, *Dictyostelium discoideum*, to mammalian and plant cells (Inouye et al. 1986; Knight et al. 1991a, b; Nakajima-Shimada et al. 1991).

Agonist activity at G protein-coupled receptors (GPCRs) that couple to G protein $\alpha$ subunits of the $G\alpha_{q/11}$ class of heterotrimeric G proteins usually results in the activation of phosphoinositidases of the phospholipase C$\beta$ family to generate the second messenger

metabolites diacylglycerol and inositol 1,4,5-trisphosphate ($IP_3$). In many cell types, this leads to a temporal increase of the intracellular calcium concentration as a consequence of $IP_3$-activated release of calcium ions from intracellular stores. Traditionally, the measurement of these agonist-induced calcium responses was based on the detection of an increase in fluorescence of cells preloaded with a calcium-sensitive fluorescent dye. This was the conventional way to study these receptor-mediated responses and the majority of studies are still following this fundamental principle. Since the first expression studies of apoaequorin cDNA in several cell types, the number of reports in which aequorin is employed to detect agonist-dependent activation of GPCRs steadily increased (MacNeil et al. 1997; Kolakowski et al. 1998; Ungrin et al. 1999; Detheux et al. 2000; Stables et al. 2000). Mitochondrially targeted aequorin was shown to provide the most robust bioluminescent GPCR responses (Stables et al. 1997, 2000). Moreover, injector luminometers which detect aequorin-based luminescence in 96- or 384-well plates, can allow for high-throughput screening of receptor ligands (Ungrin et al. 1999; Detheux et al. 2000; Stables et al. 2000).

In this paper, we have explored the possibility of employing aequorin as a reporter system for detection of intracellular calcium changes in insect cells. Therefore, Schneider 2 (S2) cells, derived from *Drosophila melanogaster* embryos (Schneider 1972), were genetically engineered to co-express apoaequorin and STKR, an insect tachykinin-related peptide receptor which was previously shown to mediate agonist-dependent $IP_3$/$Ca^{2+}$ responses (Torfs et al. 2000).

## Materials and methods

### Expression and selection vector constructs

The apoaequorin expression vector pERZaeq (obtained from Euroscreen, Belgium) was digested with EcoR I. The resulting apoaequorin-encoding cDNA fragment was placed under control of a constitutive insect actin promoter in pAc5.1V5His (Invitrogen). The resulting vector construct (pAc5.1aeq; Fig. 1) was checked by restriction enzyme digestion and by DNA sequencing (Applied Biosystems; ABI Prism 310). A second vector, pFJ*iegprom* (Torfs et al. 2000), contained the STKR-encoding region controlled by another constitutive insect promoter, *Bm*NPVieg (Vulsteke et al. 1993; Vanden Broeck et al. 1995). The antibiotic selection construct, pUC*hshyg*, was kindly provided by Dr. J. Carlson (Colorado State University, Fort Collins, Colo., USA).

### Transfection and selection of cell cultures for stable expression studies

S2 cells were transfected with three plasmids, the expression vector (pFJ*iegprom*) containing the cDNA coding for STKR, the apoaequorin expression vector (pAc5.1aeq) and the selection plasmid (pUC*hsHyg*) encoding the hygromycin phosphotransferase gene, by employing the liposomal transfection agent Cellfectin (Gibco-BRL, Life Technologies). The transfected cells were grown in Schneider's medium (Serva) supplemented with $CaCl_2$ (0.6 g/l), $NaHCO_3$ (0.4 g/l), 10% heat-inactivated fetal calf serum (Gibco-BRL, Life Technologies) and antibiotics (25 U/ml penicillin G and 25 μg/ml streptomycin; Sigma). The pH of the prepared medium was adjusted to 6.45 by adding NaOH. Cells were grown in monolayers at 23°C. For selection, 400 μg/ml hygromycin B (Duchefa) was added to the culture medium. After 4 weeks of continuous selection, the resulting hygromycin-resistant cell population was diluted and subdivided in six-well plates to obtain clonal cell lines. Clones were examined by northern analysis (see below) and by means of the aequorin-based luminescence assay by adding 10 nM and 1 μM of the insectatachykinin receptor agonist *Lom*-TK III. The best-responding cell clone (S2-STKR-Aeq) was selected for further experiments. We also selected an apoaequorin-expressing S2-Aeq cell line for negative control experiments.

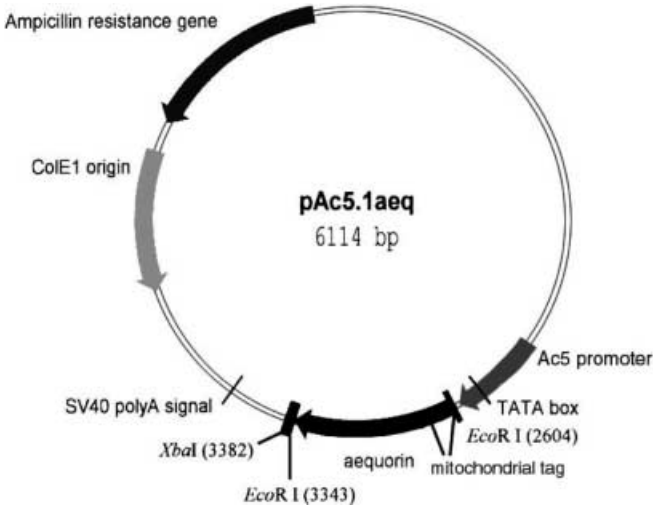


**Fig. 1** Schematic representation of the apoaequorin expression vector construct pAc5.1aeq (Vector NTI software, InforMax). The apoaequorin-encoding DNA sequence, fused to a mitochondrial matrix targeting sequence, (ca 740 bp) was inserted into the EcoR I restriction site of pAc5.1 (Invitrogen). As a result, apoaequorin gene expression is placed under control of a constitutive insect actin gene promoter

### Northern blot analysis of the clones

Poly(A)$^+$ RNA was prepared from $10^7$ cells of each transfected S2 clone under RNase-free conditions using the QuickPrep Micro mRNA purification kit (Amersham-Pharmacia-Biotech). Two micrograms of each sample were separated by denaturing electrophoresis in 1.5% agarose/formaldehyde gels (Sambrook et al. 1989). Northern blotting and hybridisation analyses were performed as described by Janssen et al. (2001).

### Peptide ligands

*Lom*-TK III, employed as an STKR agonist, was synthesised by means of Fmoc polyamide chemistry (kindly provided by Dr. H.B. Oonk and Dr. R. Meloen, Department of Molecular Recognition, ID-DLO, Lelystad, The Netherlands). The neurokinin receptor antagonists, spantide I, II and III, were purchased from Sigma.

### Aequorin charging protocol

S2-STKR-Aeq cells, expressing apoaequorin and STKR, were examined for viability and counted by Trypan Blue exclusion. Cells were spun down (5–8 min at 900 rpm) and resuspended in basal Schneider's medium [Serva; pH 6.45; supplemented with $CaCl_2$ (0.6 g/l), $NaHCO_3$ (0.4 g/l)], at a density of $1\times10^7$ cells/ml. Coel-

enterazine h (Molecular Probes) was added to a concentration of 5 μM and the cells were incubated in the dark at room temperature for 2–4 h. These coelenterazine-loaded cells were diluted 5 times in basal Schneider's medium immediately before starting the experiments.

### Aequorin luminescence assay protocol

Dilution series (100 μl) ranging from $10^{-10}$ to $10^{-5}$ M *Lom*-TK III (final concentrations) were prepared in 96-well plates. In addition, each row of the 96-well plate contained one positive (0.1% Triton X-100) and one negative (medium only) control sample. Plates were analysed in a Microlumat plus, LB96V microplate luminometer (Berthold, Perkin Elmer Life Sciences). The instrument was programmed by means of Winglow software (Perkin Elmer). A suspension containing $2\times10^5$ S2-STKR-Aeq cells (100 μl) was injected into each well. Light detection started at the moment of injection and was recorded for 30 s.

### Statistical processing of the results

Luminescence data (peak integration) were analysed by means of Winglow software which is linked to the Microsoft Excel program. The resulting data were then transferred to and processed by SigmaPlot 4.0 (SPSS) software. This program was employed to analyse the results by means of a three-parameter sigmoidal curve-fitting algorithm, to plot dose-response curves and to calculate the $EC_{50}$ value.

### Calcium measurements

The procedure for determination of intracellular calcium ion concentrations by means of Fura-2 fluorescence measurements was described previously (Torfs et al. 2000). Data were analysed by the SigmaPlot 4.0 program.

## Results and discussion

### Aequorin-based functional assay for STKR-mediated calcium responses

To analyse the utility of an insect cell-based aequorin assay for studying receptor-mediated calcium responses, S2 cells were transfected with apoaequorin and STKR expression constructs and with a hygromycin B resistance construct. After several weeks of antibiotic selection, different cell clones were obtained. Northern blot analysis revealed the presence of both STKR and apoaequorin mRNA in most of the 12 clones that were analysed: 11 proved to contain detectable levels of the STKR transcript, whereas all 12 contained the apoaequorin mRNA. In addition, these cell clones were also tested in luminescence assays and the clone that displayed the highest aequorin signal (S2-STKR-Aeq) was utilised in all further experiments. This is the first report on the development of an aequorin-based functional assay system in a stably transfected insect cell line.

STKR is a G protein-coupled receptor that, upon activation by an insectatachykinin-like agonist, induces a transient increase in the concentration of intracellular calcium ions in S2-STKR cells (Torfs et al. 2000, 2001). This agonist-dependent calcium rise can now also be visualised by measuring light emission from S2-STKR-Aeq cells. This response was not obtained in negative control cells (S2-Aeq). Figure 2 shows that the bioluminescent response to the STKR agonist, *Lom*-TK III, was concentration dependent. The $EC_{50}$ values, as determined for this agonist by means of the aequorin-based and the Fura-2-based calcium assay methods, were not significantly different. Nevertheless, a comparison of the dose-response curves obtained for each method suggests that the luminescent assay is more sensitive than the fluorescent one. At low agonist concentrations ($10^{-9}$ to $10^{-8}$ M *Lom*-TK III) aequorin still displays agonist-dependent activity, while such responses could not be obtained with Fura-2 in similar conditions. This higher sensitivity is probably due to the high signal-to-noise ratio of the aequorin assay and/or

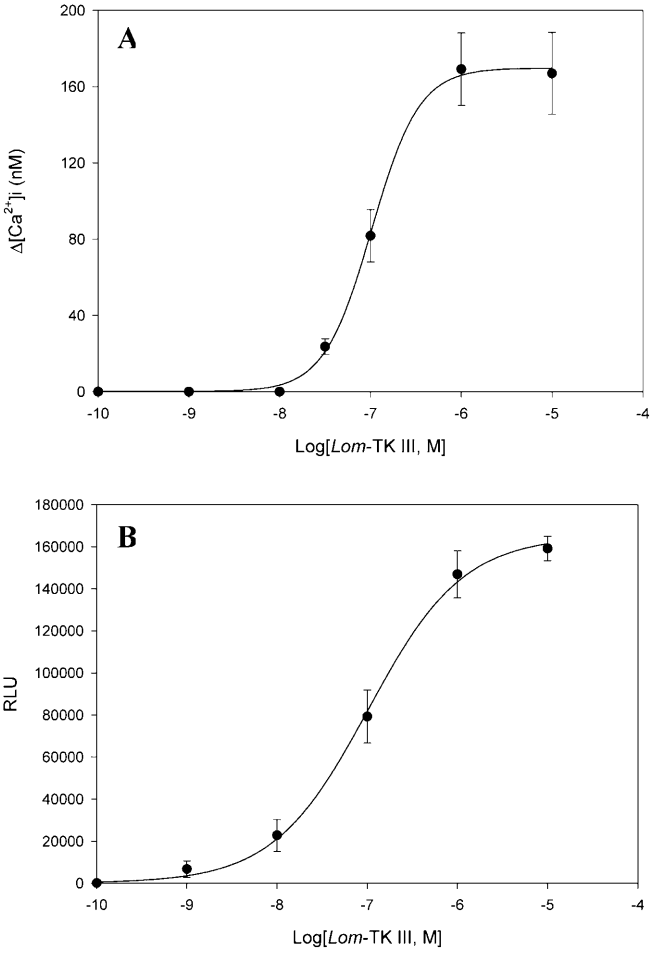


**Fig. 2** Dose-response curves for the calcium rise induced by *Lom*-TK III in S2-STKR-Aeq cells were measured by means of Fura-2 fluorescence ($EC_{50}$ ca $1.0\times10^{-7}$; **A**) and aequorin luminescence ($EC_{50}$ ca $1.0\times10^{-7}$; **B**). **A** Fura-2 fluorescence. Fluorescence data (shown in nM $Ca^{2+}$) are the average ± standard deviation (SD) of $n$ measurements ($n=8$ for each data point, obtained from four experiments done in duplicate). **B** Aequorin luminescence. Bioluminescence data are the average ± SD of $n$ measurements ($n=8$ for each data point, obtained from four experiments done in duplicate). Relative light units (*RLU*) are displayed on the vertical axis

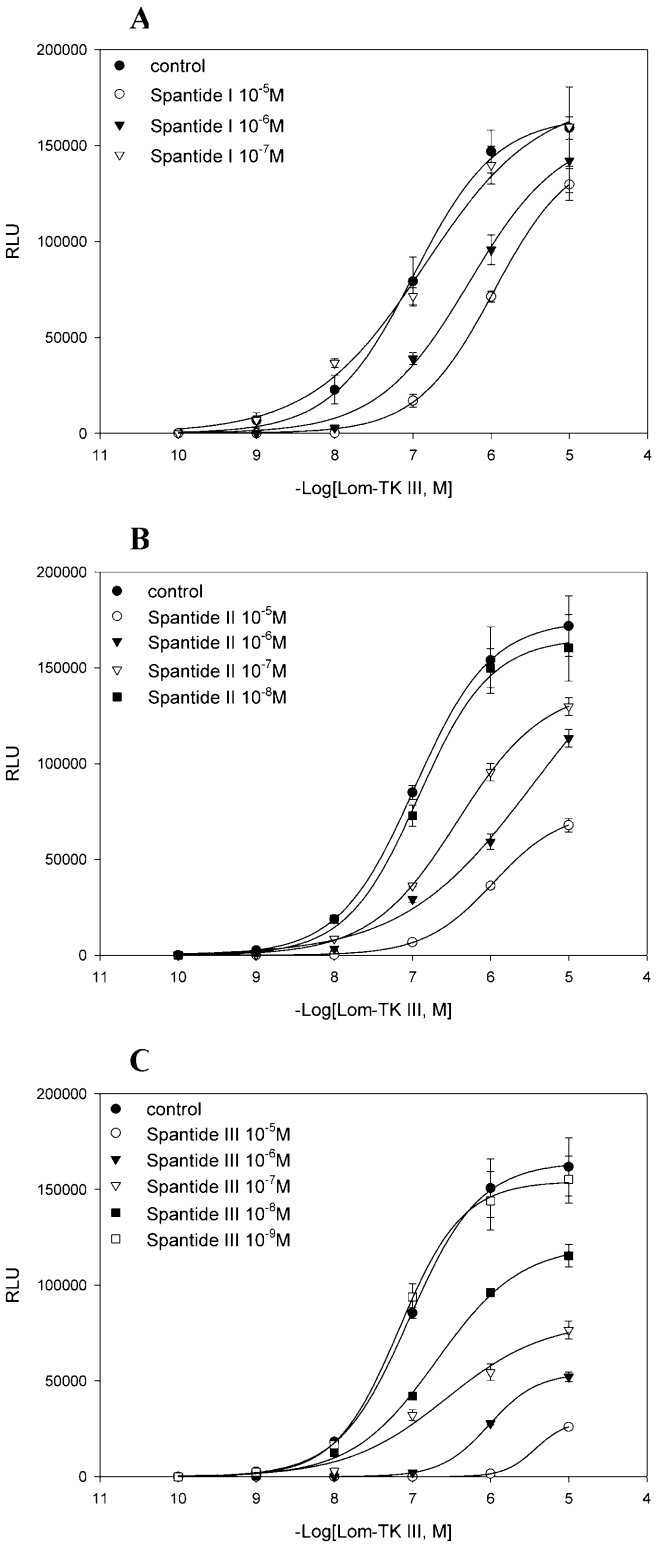


**Fig. 3A–C** Concentration-dependent effects of spantides I–III on the dose-response curve for *Lom*-TK III-induced bioluminescence in S2-STKR-Aeq cells. Data are the average ± SD of *n* measurements ($n = 9$ for each point, obtained from three experiments done in triplicate). Relative light units (*RLU*) are displayed on the vertical axis

to its lower $Ca^{2+}$-buffering capacity. In addition to its high sensitivity and wide dynamic range, the stability of coelenterazine-loaded insect cells is an important practical advantage of this detection method. Moreover, since the aequorin-based functional assay can be performed in multiwell plates using a microplate luminometer, it can be easily adapted to high throughput analysis of experimental samples.

## Effects of spantides I–III on agonist-evoked responses in S2-STKR-Aeq cells

Stimulatory effects of the insect tachykinin-related peptides, *Lom*-TK II (Monnier et al. 1992) and *Lem*TRP I (Winther et al. 1998), on cockroach hindgut contractility can be blocked by the substance P antagonist, spantide I (*D-Arg*-Pro-Lys-Pro-Gln-Gln-*D-Trp*-Phe-*D-Trp*-Leu-Leu-$NH_2$). Recently, we have also shown that another spantide I-related neurokinin receptor antagonist, spantide II (*N-nicotinoyl-D-Lys*-Pro-*3-Pyridyl-Ala*-Pro-*D-*$Cl_2$*Phe*-Asn-*D-Trp*-Phe-*D-Trp*-Leu-Nle-$NH_2$), reduces the effects of *Lom*-TK III in S2-STKR cells as well as on cockroach hindgut (Torfs et al. 2000, 2001). In this paper, the potential of all three members of the spantide family of neurokinin antagonists (all known as potent substance P antagonists), spantide I, spantide II and spantide III (*N-nicotinoyl-D-Lys*-Pro-*3-Pyridyl-Ala*-Pro-*D-*$Cl_2$*Phe*-Asn-*D-Trp*-Phe-*3-Pyridyl-Ala*-Leu-Nle-$NH_2$) to inhibit *Lom*-TK III-induced bioluminescent responses of S2-STKR-Aeq cells was analysed and compared. The experimental results shown in Fig. 3 clearly demonstrate that all three compounds are STKR antagonists inhibiting the response generated by *Lom*-TK III. The potency, with which the STKR-mediated calcium responses are antagonised, appears to depend on the type of compound. The concentrations needed to obtain a significant inhibitory effect (compared with the control condition without antagonist) were $10^{-6}$ M for spantide I, $10^{-7}$ M for spantide II and $10^{-8}$ M for spantide III. Therefore, the main conclusion of these experiments is that spantides are antagonists for the STKR-mediated calcium responses and that the rank order of potencies is spantide III > spantide II > spantide I. Recently, these three spantides have also been tested in a mammalian assay system by Zubrzycka and co-workers (2000). The effects of these antagonists were recorded on evoked tongue jerks (ETJ) in rats. The conclusion was that spantide III was also more potent than spantide II in antagonising the dose-dependent substance P-evoked increase in ETJ amplitude. Spantide I had an additional effect on this system (complete respiratory arrest) and, as a result, its specific effect on ETJ could not be evaluated. The present publication is the first one in which the activities of all three spantides are compared in a single study on a well-defined receptor preparation. Other reports of the effects of spantide antagonists on tachykinin-induced effects and on neu-

rokinin receptors partially demonstrated the relative selectivity and potency of these antagonists (Regoli et al. 1985; Hakanson et al. 1990; Maggi et al. 1991; Folkers et al. 1993).

## General conclusion

The present study shows that agonists as well as antagonists can be studied by analysing bioluminescent responses generated in an insect cell line expressing both apoaequorin and STKR, an insect tachykinin-related GPCR. The major practical advantages of the aequorin system are its high signal-to-noise ratio, its relatively low cost and its high throughput of experimental samples. Since measurements can be easily performed in a microplate luminometer, this functional, insect cell-based, receptor assay system can be utilised as a powerful tool for screening natural or synthetic compound libraries resulting in the discovery of new receptor ligands.

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