

Calnexin co-expression and the use of weaker promoters increase the expression of correctly assembled Shaker potassium channel in insect cells

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Abstract

Voltage-gated potassium channels control the membrane potential of excitable cells. To understand their function, knowledge of their structure is essential. However, these channels are scarce in natural sources, and overexpression is necessary to generate material for structural studies. We have compared functional expression of the *Drosophila* Shaker H4 potassium channel in stable insect cell lines and in baculovirus-infected insect cells, using three different baculovirus promoters. Stable insect cell lines expressed correctly assembled channel, which was glycosylated and found predominantly at, or close to, the cell surface. In comparison, the majority of baculovirus-overexpressed Shaker was intracellular and incorrectly assembled. The proportion of functional Shaker increased, however, if the weaker basic protein promoter was used rather than the stronger p10 or polyhedrin promoters. In addition, co-expression of the molecular chaperone, calnexin, increased the quantity of correctly assembled channel protein, suggesting that calnexin can be used to increase the efficiency of channel expression in insect cells.

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1. Introduction

Voltage-gated potassium channels play a key role in the function of excitable cells. They respond to membrane depolarisation by opening and allowing potassium ions to flow across the plasma membrane. Their opening is involved in termination of the action potential [1]. Related channels include the voltage-gated sodium channels that initiate the action potential and the cyclic nucleotide-gated channels of the retinal and olfactory systems [2]. Defects in voltage-gated potassium channels cause loss of the careful regulation of our nervous systems, leading to neonatal human epilepsy [3], episodic ataxia [4] and long QT syndrome [5]. In addition, mutations in the KCNQ4 voltage-gated potassium channel gene are associated with deaf-

ness [6] and deletion or inhibition of Kv1.1 in mice leads to epilepsy [7] or impairment of associative memory [8].

Despite the importance of voltage-gated potassium channels, our insights into their structure are limited to a recent 25 Å resolution model of the Shaker potassium channel, determined by electron microscopy and image reconstruction [9] and to crystal structures of the tetramerization domain of Shaker [10] and of a bacterial potassium channel pore [11]. As yet, there is no structure for the voltage-sensing apparatus of the channel. To more fully understand its function, we need to determine the structure of a voltage-gated potassium channel at atomic resolution.

Voltage-gated potassium channels have been purified from rat brain [12] but appear heterogeneous due to the abundant variety of channel subunits expressed in the mammalian brain [13]. As structural analysis requires a reliable supply of pure, homogeneous protein, a heterologous expression system is required. Indeed, the mammalian Kv1.1 and Kv1.2 channels have been successfully expressed using a Semliki Forest virus expression system [14]. The surface expression levels of Kv channels can be increased by co-expression of their respective Kvβ subunits [14–17]. Co-expression of Kvβ2.1, with either Kv1.1 or Kv1.2, in the Semliki Forest virus system increased the expression levels

Abbreviations: CTX, charybdotoxin; DNA, deoxyribonucleic acid; HRP, horseradish peroxidase; K_d , dissociation constant; NTA, nitrilotriacetic acid; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Sf9, *Spodoptera frugiperda* 9

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of channel, allowing the purification of 80–150 μg of a complex consisting of four α and four β subunits.

The *Drosophila* Shaker voltage-gated potassium channels have been expressed using many systems, including mammalian cells [18], *Xenopus* oocytes [19], an in vitro expression system [20] and the baculovirus–insect cell system [21]. The baculovirus system has the considerable advantage of being readily scaled up, due to the ease of maintaining large volumes of *Spodoptera frugiperda* 9 (Sf9) cells in suspension culture. Previous studies of Shaker expression using the baculovirus system showed the presence of 10 μg of functional channel per litre of cell culture [21]. However, much of the expressed material did not reach the cell surface [22] and total expression levels of 5 mg of channel protein [23] per litre have been reported. A comparison of these reported expression levels suggested that much of the material expressed in the baculovirus system remains incorrectly assembled. In view of the ease with which large volumes of insect cells can be generated, we chose to attempt to increase the quantity of correctly assembled protein produced by this expression system. While the Shaker channels are able to form complexes with Kv β subunits, their co-expression does not lead to an increase in channel expression, suggesting that the beta subunit does not act as a chaperone for the Shaker protein [24]. Therefore, we investigated the expression of Shaker under the control of different baculoviral promoters and in the presence of molecular chaperones, in efforts to improve the functional expression of the potassium channel in insect cells.

2. Materials and methods

2.1. Construction of plasmid DNA and recombinant baculoviruses

Polymerase chain reaction (PCR) was used to amplify the *Drosophila melanogaster* Shaker H4 gene [25]. PCR primers were designed to contain restriction sites compatible for subcloning into baculovirus transfer vectors, using the polyhedrin promoter of pBacPAK8 (Clontech), the basic protein promoter of pAcMP3 (Pharmingen) and the p10 promoter of pBac4X (Novagen) while adding a His₁₀ tag to the C terminus of the protein. The forward (5'-GGA-ATT-CCA-TGG-CCG-CCG-TTG-CCG-GCC-TCT-AT-3') and backward (5'-CCG-GAA-TTC-CGG-TCA-(GTG)₁₀-ACC-GTC-GGT-CTC-GAT-ACT-AAC-GGC-3') primers were used to amplify the full-length Shaker gene. The PCR products were ligated into vectors using standard subcloning procedures [26] and the resulting plasmids were checked by deoxyribonucleic acid (DNA) sequencing. Baculoviruses were made using BaculoGold (Pharmingen), as described by the manufacturer. Recombinant viruses were isolated after one round of plaque purification and amplified using standard techniques [27,28].

2.2. Site-directed mutagenesis

The Quikchange mutagenesis method (Stratagene) was used to make the F425G mutant of Shaker H4. The forward (5'-GGA-AGC-GAA-AAT-TCC-GGC-TTC-AAG-TCC-ATA-CCC-G-3') and reverse (5'-CGG-GTA-TGG-ACT-TGA-AGC-CGG-AAT-TTT-CGC-TTC-C-3') primers were used. Plasmids were checked by DNA sequencing and recombinant baculoviruses were made as above.

2.3. Cell culture and protein expression

Sf9 cells were propagated in complete TNM-FH medium [TNM-FH medium (Sigma) with addition of 10% fetal calf serum (PAA) and lipid concentrate (Gibco)]. They were grown in 250 ml Erlenmeyer flasks (Corning) at 27 °C with shaking at 75 rpm and diluted 4-fold into fresh medium every 48 h. To analyse protein expression, log growth-phase cells (24 h after dilution) were infected in monolayer culture. 5×10^6 cells were added to each T 75 cm² dish (Corning). When the cells were adherent, the medium was replaced with fresh medium containing 10 virus particles per cell. After 60 min, the virus was replaced with 10 ml of complete TNM-FH and flasks were incubated at 27 °C for 48 h before analysis of the expressed protein. When used, 1 $\mu\text{g}/\text{ml}$ tunicamycin (Sigma) was added to the medium immediately after viral infection.

2.4. Generation of an insect cell line

The InsectSelect system (Invitrogen) was used to generate a stable insect cell line. The Shaker H4 F425G gene was amplified by PCR, using the primers described above, and was ligated into the pIZT vector (Invitrogen). Sf9 cells were transfected with this construct as described by the manufacturers. The 'dilution method', described in the InsectSelect handbook (Invitrogen), was used to isolate cell lines which were resistant to Zeocin and charybdotoxin (CTX) binding was used to determine which cell line generated the highest levels of folded Shaker protein.

2.5. SDS-PAGE and Western blotting

Gel electrophoresis of whole cells was used to analyse protein expression. Harvested cells were washed twice with phosphate-buffered saline (PBS), resuspended in reducing sample buffer [with a final concentration of 1.5% sodium dodecyl sulfate (SDS), 0.02% (v/v) β -mercaptoethanol, 10% glycerol and 0.005% bromophenol blue], and heated at 95 °C for 5 min. Protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% gels with 5×10^4 cells added to each well, except where otherwise indicated, and was detected using GELCODE Blue stain (Pierce). For Western blotting, protein was transferred onto nitrocellulose membrane (Schleicher and Schuell) using a MilliBlot-Graphite electroblotter (Millipore). The mem-

brane was incubated for 60 min in blocking buffer [5% (w/v) milk in PBS]. To detect His-tagged protein, a Ni^{2+} –nitrilotriacetic acid (NTA) horseradish peroxidase (HRP) conjugate (Qiagen) was diluted in PSB/0.5% Tween and added for 60 min. After three washes for 15 min each, in PBS/0.5% Tween, the blot was developed using the enhanced chemiluminescent system (ECL, Amersham Pharmacia Biotech) and developed using a KODAK RP X-OMAT processor.

2.6. Immunocytochemistry

To determine the intracellular location of expressed protein, log growth-phase cells were placed onto polylysine-coated coverslips. After they had adhered, they were infected as described above, and grown at 27 °C. Two days after infection, cells were fixed in 4% (w/v) paraformaldehyde for 30 min at 4 °C. They were permeabilised, and the remaining surface of the coverslips were blocked, at room temperature, by incubation for 30 min in PBS with 10% goat serum (Sigma) and 0.1% Triton X-100 (Fisons). All subsequent incubations were at room temperature in PBS which contained 1% goat serum and 0.1% Triton X-100. An anti-His₅ antibody (Qiagen) was added to the fixed, permeabilised cells at a dilution of 1/500 for 30 min and they were washed three times for 5 min using buffer without antibody. A Texas red-conjugated anti-mouse secondary antibody was diluted to 1/1000 and added for 30 min. After a further three washes using buffer without antibody, the cells were mounted by inverting the coverslip onto a droplet of 2.5% 1,4-diazabicyclo[2,2,2]octane (Sigma) in polyvinyl alcohol with an average molecular weight of 30 000–70 000 (Sigma). The mountant was allowed to set and the coverslips were stored in the dark until studied on an MRC 1024 confocal microscope (Bio-Rad).

2.7. CTX-binding assays

CTX binding was measured in permeabilised cells, using [¹²⁵I]-CTX (Dupont NEN) and a protocol similar to that of Ref. [29]. Infected Sf9 cells were washed in PBS and resuspended to 2×10^6 per millilitre in PBS that contained 0.1% saponin and 0.1% bovine serum albumin (Sigma). [¹²⁵I]-CTX was added to aliquots containing 1×10^5 cells to a final concentration of 2.5 nM. Different concentrations of unlabelled toxin were added to each aliquot and three aliquots were generated for each condition. After incubation at room temperature for 60 min, samples were filtered through GF/C microfibre filters (Whatmann) that had been presoaked for 30 min in 0.5% polyethylenimine. Unbound CTX was removed by washing these filters with three 1-ml aliquots of ice-cold PBS and the amount of bound [¹²⁵I]-CTX was determined using a 1261 Multigamma gamma counter (Wallac).

To determine a dissociation constant (K_d) for toxin binding, three aliquots were prepared for each of a series

of concentrations of unlabelled toxin (CTX). Measurements were taken in triplicate for each concentration of CTX and a mean was determined. GraFit (Erithacus software) was used to plot the CTX concentration against the mean number of counts generated from each sample and to determine the concentration at which the CTX displaced 50% of the bound [¹²⁵I]-CTX (the IC_{50}). Subtraction of the concentration of [¹²⁵I]-CTX from the IC_{50} yielded the K_d .

To determine the number of toxin-binding sites present under each condition, incubations containing 2.5 nM [¹²⁵I]-CTX, with and without the addition of 0.5 μM CTX, were generated. Measurements were taken in triplicate and a mean was determined. Error bars show the maximum spread of the data points. Each figure shows the result of one experiment. However, the results could be readily reproduced using different batches of infected cells.

3. Results and discussion

3.1. Expression of correctly assembled potassium channel using the baculovirus system

The binding of radiolabelled CTX has been used to investigate the quantity of voltage-gated potassium channels present in rat brain [29] and human T lymphocytes [30] and to quantify the amount of correctly assembled Shaker channel expressed heterologously in mammalian cells [18]. The toxin binds to correctly folded and assembled channel with one toxin molecule binding at the pore region and interacting with residues provided by all four subunits [31]. It also binds to detergent-solubilised channel [18], allowing purified protein to be analysed [9]. It is therefore a valuable tool that can be used to assess the quantity of correctly assembled channel generated by an expression system. Indeed, the approximate 50-fold differences in expression levels between individual cells infected together with a baculovirus that drives the expression of Shaker [21] makes it difficult to use electrophysiology as a means of determining average expression levels in a population of cells. In contrast, analysis of toxin binding allows an average expression level of toxin-binding sites per cell to be determined readily. The F425G mutation of the Shaker H4 variant is reported to increase the affinity of the channel for CTX by 1900-fold [32]. Therefore, recombinant baculovirus constructs were made that express both the Shaker H4 and the F425G mutant of Shaker H4 potassium channels.

The gene for the full-length Shaker H4 splice variant was amplified by PCR to include a His-tag at the C terminus and was inserted in the pBacPAK8 transfer vector, under the control of the polyhedrin promoter. Mutagenesis of this construct generated the F425G mutant of Shaker H4 in the same vector. Each of these constructs was co-transfected, together with linearised baculovirus DNA, into adherent Sf9 cells. The resulting viruses were plaque puri-

fied, amplified and used to infect small batches of cells. Forty-eight hours after infection, cells were studied for protein expression by SDS-PAGE. A band at 75 kDa was observed only in cells that had been infected with a virus that expressed a gene for the Shaker channel, and not in uninfected, or wild-type virus-infected cells (Fig. 1A). A band at a comparable molecular weight was observed on a Western blot that had been stained using a Ni^{2+} -NTA conjugate to detect His-tagged protein, only after infection with a virus that contained the His-tagged Shaker H4 gene (Fig. 1A). Comparison of the density of this band on a Coomassie-stained gel with a marker band of a known mass suggested that approximately 10 mg of channel protein was expressed from each litre of cell culture.

To assay for the presence of correctly folded channel, we studied CTX binding using a filter-binding assay. Sf9 cells were infected and incubated for 48 h at 27 °C. Infection with either wild-type baculovirus, or a virus that expressed the wild-type Shaker H4 gene did not cause the cells to produce protein that bound specifically to CTX (Fig. 1B). In contrast, cells infected with the F425G mutant of Shaker H4 bound toxin with a K_d of 1.7 ± 0.4 nM.

The dissociation constants for CTX binding to the Shaker H4 channel and its F425G mutant were determined by electrophysiology to be 152 and 0.08 nM, respectively [32]. In our studies, we detect no binding to the wild-type Shaker H4 channel. While this could be due to a lack of correctly folded channel produced by the infected Sf9 cells, we suggest instead that it is due to a difference in the method used to investigate binding. While electrophysiology is an equilibrium method, our filter binding assays involve a wash step. Therefore, a complex with a high off-rate will be lost during the wash and will not be detected. The F425G mutation is proposed to decrease the off-rate of CTX from Shaker H4 by 1800-fold [32]. We suggest that this decrease in off-rate allows us to detect the CTX–Shaker H4 F425G complex in a filter-binding assay while the complex between CTX and wild-type Shaker H4 is lost during the washes. We also suggest that the difference in methodology accounts for the difference in affinity determined for the F425G mutant of Shaker H4 by these different methods.

The specific interaction between CTX and the F425G mutant of Shaker H4 shows that the CTX binding site

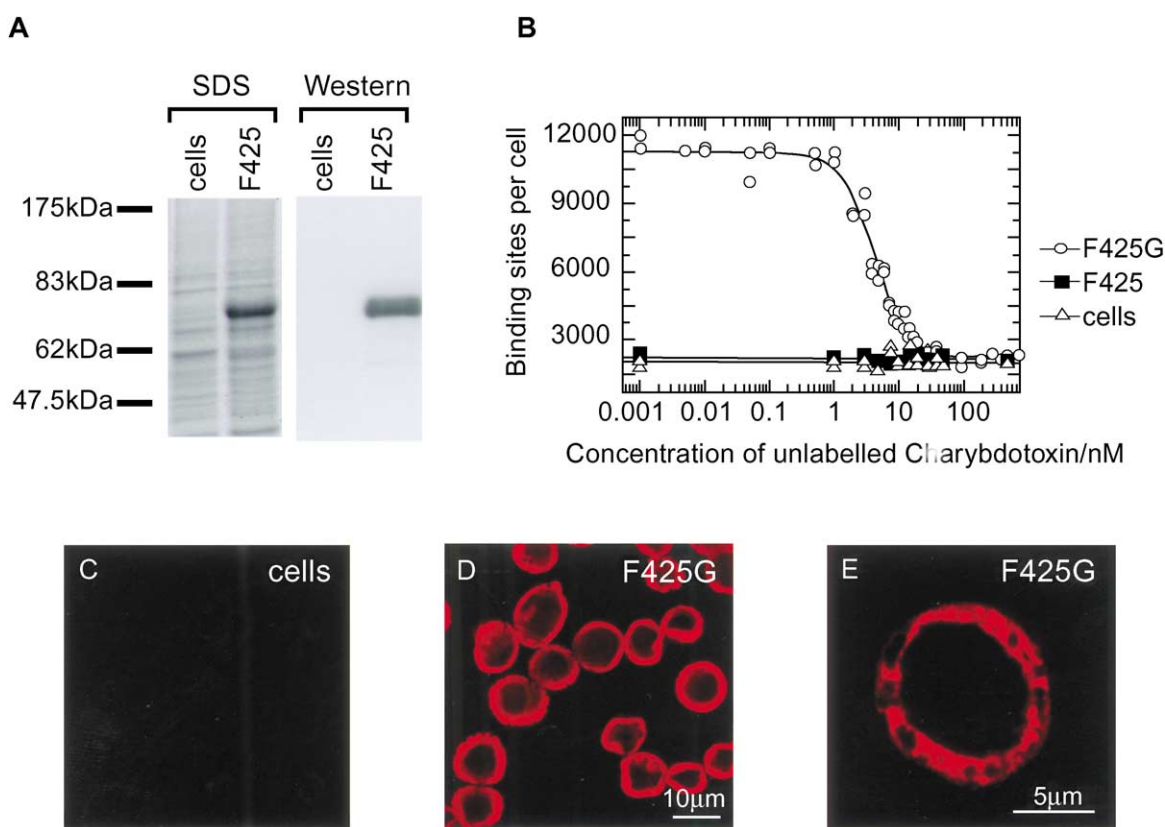


Fig. 1. Expression of Shaker using the baculovirus system. (A) SDS-PAGE with Coomassie staining (SDS) and Western blotting using a Ni^{2+} -NTA conjugate (Western) reveal the expression of a protein with an apparent molecular weight of 75 000. We analysed the protein present in uninfected cells (cells) and cells infected with a recombinant baculovirus that induced expression of wild-type Shaker H4 under the control of the polyhedrin promoter (F425). (B) CTX competition binding assays were used to study uninfected cells (cells), cells infected with a virus that contains the wild-type Shaker gene under the control of the polyhedrin promoter (F425) or a similar virus containing the F425G mutation of Shaker H4 (F425G). (C–E) Cells were fixed and stained with an antibody against the His-tag and a Texas red-conjugated secondary antibody. C shows uninfected Sf9 cells while D and E show cells expressing the F425G mutant.

adopts the correct conformation in the baculovirus-expressed channel protein. As the binding site for the toxin lies at the centre of the pore and its presence requires assembly of the four subunits, we assume that the number of correctly folded channels is equivalent to the number of toxin-binding sites observed.

To investigate the intracellular location of the expressed Shaker protein, we fixed cells 48 h after infection and stained them with an antibody targeted against the His-tag and a Texas red-conjugated secondary antibody. While wild-type virus-infected cells were not stained (Fig. 1C), cells expressing the F425G mutant of His-tagged Shaker H4 showed strong red fluorescence (Fig. 1D and E). This fluorescence was distributed throughout the region between the swollen nucleus of the cell and the plasma membrane. This stain distribution is similar to that seen when ion channels such as the acetylcholine receptor and the 5HT₃ receptor are expressed in baculovirus-infected insect cells [33,34].

To determine the maximum expression levels available from this system, we used CTX binding to study the effect of viral titre and the time after infection on the amount of expressed protein. As 0.5 μ M unlabelled CTX was sufficient to compete off all of the labelled toxin (Fig. 1B), we determined the total specific binding in each case by comparing the amount of bound radiolabelled toxin in the presence and absence of 0.5 μ M unlabelled toxin. We determined that 5 plaque forming units (pfu) of virus per cell generated maximal expression levels and that the amount of folded protein peaked at 48 h post-infection (data not shown). This gave maximum expression levels of approximately 11 000 toxin-binding sites per cell, or 10 μ g of the correctly assembled F425G mutant of Shaker H4 per litre of infected cell culture. This compared with 10 mg of total Shaker protein produced per litre, as determined by Coomassie staining, indicates that only 0.1% of the expressed Shaker protein is functional.

3.2. Using different baculovirus promoters to increase expression levels

Although the levels of mRNA produced from the polyhedrin promoter are greater than that from any other promoter in the wild-type baculovirus (reviewed in Ref. [35]), other baculovirus promoters have been used for the overexpression of many proteins. In some cases, the p10 promoter [36,37], or the basic protein promoter [38,39], have yielded larger quantities of correctly folded, expressed protein than the polyhedrin promoter. This is especially the case when the expressed protein requires considerable enzymatic processing during folding and assembly [37,39]. We therefore ligated the His-tagged F425G mutant of the Shaker H4 gene into pBac4X, under the control of the p10 promoter, and into pAcMP3, under the control of the basic protein promoter. Plasmids were checked by sequencing and viruses were generated as described above. Viruses were

used to infect Sf9 cells and, 48 h after infection, membrane proteins were separated by SDS-PAGE and analysed by Western blotting with a Ni²⁺-NTA conjugate. The largest amount of polypeptide was generated by the polyhedrin promoter and the least by the basic protein promoter (Fig. 2A). In addition, differences were seen in the state of glycosylation of the protein. Protein expressed under the control of the basic protein and p10 promoters formed two bands of mass 75 and 78 kDa on a Western blot. Only the lower molecular weight band could be seen clearly in protein expressed under the control of polyhedrin promoter. When the N-linked glycosylation inhibitor, tunicamycin, was added to the culture medium during infection, only the lower band was produced under the control of all three promoters, suggesting that the upper band is the result of N-glycosylation.

To determine which of the three promoters generated the largest quantity of correctly assembled channel, we used the CTX-binding assay described above. For each promoter, we set up a series of infections with different numbers of virus particles added for each cell to determine the maximum expression level available for each of the promoters. A comparison of these maximal levels of expression (Fig. 2B) revealed that the polyhedrin promoter generated the smallest quantity of correctly assembled protein, while the

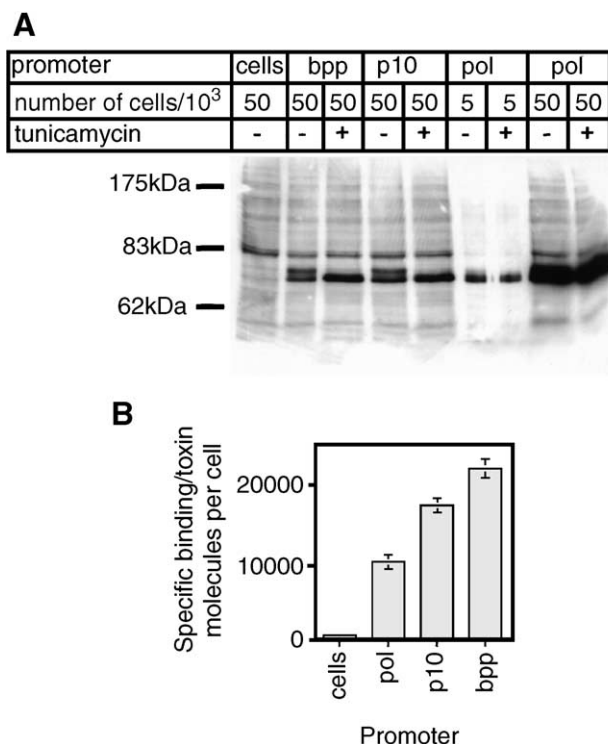


Fig. 2. The use of different baculovirus promoters in Shaker expression. Expression of Shaker under the control of different baculovirus promoters was analysed by Western blotting using a Ni²⁺-NTA conjugate (A) and CTX-binding assays (B). In each case, uninfected cells (cells) and cells infected with baculovirus constructs with the F425G mutant of Shaker H4 under the control of the polyhedrin (pol), p10 (p10) or basic protein (bpp) promoters were analysed.

p10 promoter produced 60% more toxin-binding sites per cell. The use of the basic protein promoter led to the expression of the largest quantity of correctly assembled channel, producing 2-fold more than under the control of the polyhedrin promoter. Therefore, despite the lower expression levels of Shaker polypeptide produced under the control of the earlier, ‘weaker’, basic protein promoter, this promoter generated the largest quantity of correctly assembled channel.

3.3. Co-expression of calnexin increased the expression of correctly assembled protein

The assembly and folding of ion channels is a complex process, involving membrane insertion and alternating multimer assembly and subunit folding [40]. Molecular chaperones are proteins that interact transiently with incorrectly folded proteins, preventing them from adopting incorrect interactions, and facilitating their folding. Calnexin is a chaperone in the endoplasmic reticulum and has been implicated in aiding the correct expression of membrane proteins. Co-expression of calnexin increases the expression both of the serotonin transporter [41,42] and the acetylcholine receptor [43]. In addition, calnexin has been shown to interact transiently with Shaker [44], but it has been suggested not to play a role in subunit folding. However, the effect of calnexin co-expression on the expression levels of Shaker had not been investigated. Therefore, we investigated the effect of four different molecular chaperones, including calnexin on the expression of Shaker.

Sf9 cells were co-infected with a virus containing the His-tagged F425G mutant of the Shaker potassium channel under the control of the polyhedrin promoter, together with calnexin, calreticulin, BiP, ERp57 or a control virus with no cDNA at the polyhedrin locus (pVL). Forty-eight hours after infection, cells were harvested and the total number of specific CTX binding sites was determined as above. Co-expression with calreticulin, BiP or ERp57 led to no increase in the expression of Shaker. However, although calnexin did not specifically bind to CTX (Fig. 3B), co-expression of calnexin increased the number of specific toxin-binding sites by 2-fold (Fig. 3A). Similar effects were seen when Shaker, expressed under the control of the polyhedrin, basic protein or p10 promoters, was co-expressed with calnexin under the control of the basic protein promoter (Fig. 3B). Therefore, calnexin increased the levels of correctly assembled Shaker protein by up to 2-fold, suggesting that, either directly or indirectly, it plays a role in Shaker potassium channel folding.

Calnexin binds to mono-glucosylated N-linked oligosaccharides and prevents the progress of the mono-glucosylated protein from the endoplasmic reticulum. Correct folding of the protein is accompanied by deglycosylation and the loss of the interaction with calnexin, leading to export from the ER only of correctly folded protein (reviewed in Ref. [45]). An alternative suggestion for the function of calnexin is that

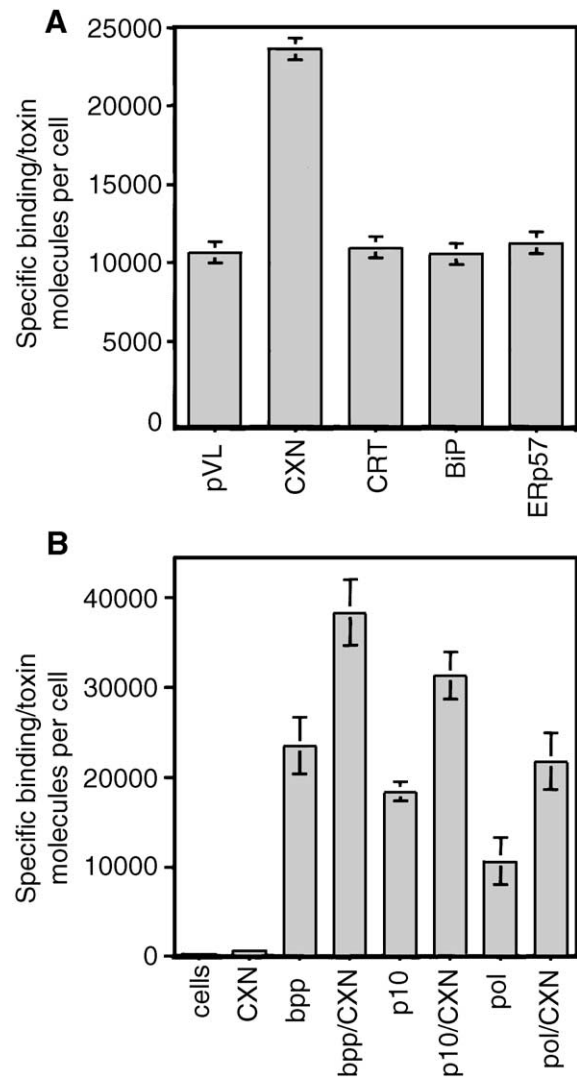


Fig. 3. The effect of molecular chaperones on Shaker expression. The effect of molecular chaperone co-expression was analysed by CTX binding. In A, cells were infected with a virus containing the F425G mutant of Shaker H4 under the control of the polyhedrin promoter, together with a recombinant virus with no gene at the polyhedrin locus (pVL), calnexin (CXN), calreticulin (CRT), immunoglobulin heavy chain binding protein (BiP) or ERp57. In B, the effect of calnexin (CXN) co-expression on expression of the F425G mutant of Shaker H4 expressed under the control of the polyhedrin (pol), p10 (p10) or basic protein (bpp) promoters was analysed.

it acts as a classical molecular chaperone, aiding the folding of glycosylated and unglycosylated proteins [46]. The Shaker potassium channel is N-glycosylated at two sites in the linker between the first and second transmembrane spans [22] and N-glycosylated Shaker co-precipitates with calnexin [44]. However, N-glycosylation is not required for correct assembly and targeting of expressed channel [44]. In addition, we have shown that calnexin has an effect on the expression levels both of the apparently unglycosylated protein expressed using the polyhedrin promoter and the partly glycosylated channel produced using the basic protein and p10 promoters. To assess whether calnexin acts directly to increase the expression of the unglycosylated channel

protein, or whether it acts indirectly by increasing the expression levels of a protein that aids in the folding of Shaker, requires further study. However, co-expression of calnexin does increase the expression levels of the Shaker channel in insect cells by approximately 2-fold.

3.4. Expression of Shaker in stable transfected insect cell lines

Insect cell lines have been used to express ligand-gated ion channels, such as the GABA_A receptor [47]. To investigate the expression of Shaker, we used the InsectSelect system (Invitrogen) and inserted the His-tagged F425G mutant of the Shaker H4 potassium channel into the pIZT vector, under the control of the OpIE2 promoter. This plasmid was checked by DNA sequencing and transfected into Sf9 cells. The pIZT plasmid containing the gene of interest also contains a constitutively expressed gene for the Zeocin resistance protein, linked to green fluorescent-protein. Hence, transfected cells display green fluorescence. Forty-eight hours after transfection, less than 5% of the cells were green and Shaker expression was not detected, either by Western blotting or by CTX binding (data not shown). However, immunocytochemistry showed that green cells also displayed red surface fluorescence when labelled with

an antibody against the His-tag and a Texas red-conjugated secondary antibody (Fig. 4A). This fluorescence appeared to be located predominantly at the cell surface (Fig. 4B), and was not observed in untransfected cells (Fig. 4C).

Zeocin resistance was used to select a stable cell line from this small proportion of transfected cells. When grown in the presence of Zeocin, the majority of cells died, leaving a small number of growing colonies. Single cell lines, each derived from one colony of growing cells, were amplified. They were analysed by SDS-PAGE and Western blotting, revealing the presence of His-tagged Shaker protein (Fig. 4D). Of the two bands observed after infection with a baculovirus that uses the basic protein promoter to express Shaker, only the upper band is observed in the cell line, suggesting that all of the expressed protein in the cell line is glycosylated. Also observed on a Western blot of protein from the insect cell line are bands of higher molecular weight. These may represent channels in which the carbohydrate additions have been further processed.

CTX binding showed the presence of approximately 4000 correctly assembled channel protein (Fig. 4E) in the cell line. However, the baculovirus system using the basic protein promoter generated 5-fold more correctly assembled channels. Therefore, stable insect cell lines provide a less effective means of generating the F425G mutant of Shaker

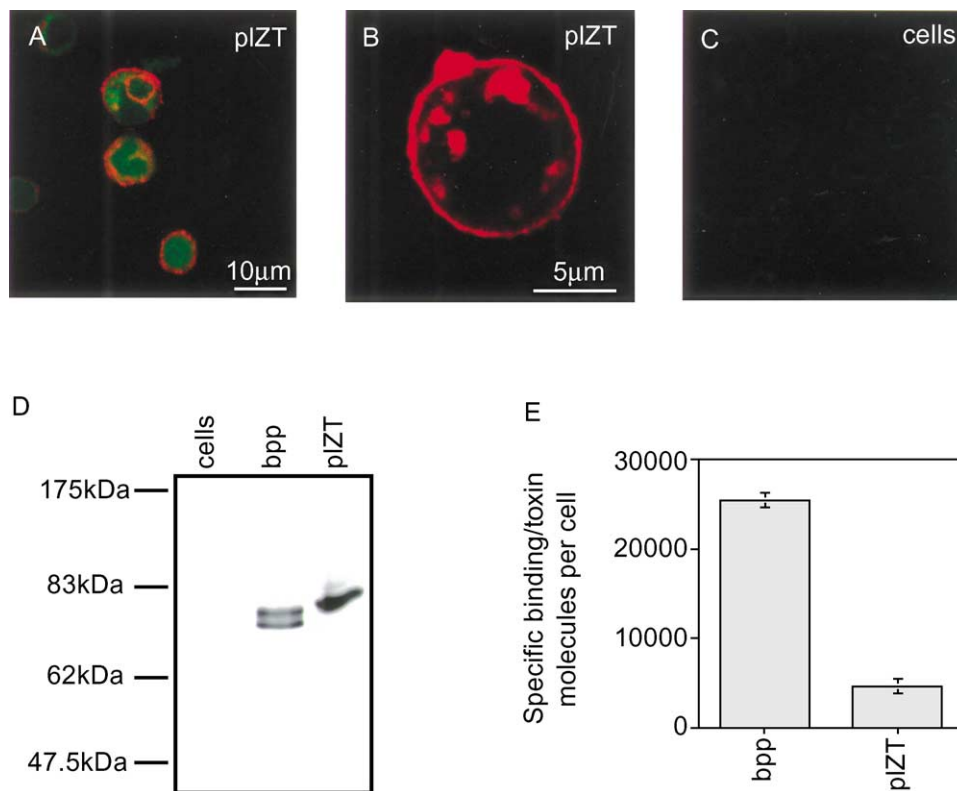


Fig. 4. Expression of Shaker in an insect cell line. Expression of the F425G mutant of Shaker H4 in an insect cell line (pIZT) was analysed by fixing and staining the cells using an antibody against the His-tag and a Texas red-conjugated secondary antibody (A–C). A and B are cells from the insect cell line, whereas C is an uninfected Sf9 cell (cells). In A and C, red fluorescence is due to the His-tagged material, and green fluorescence is a transfection marker. In B, only the red fluorescence is shown. Western blotting using a Ni²⁺-NTA conjugate (D) and CTX binding (E) were used to study uninfected cells (cells), the insect cell line (pIZT) or cells infected with a virus that contains the F425G mutant of Shaker H4 under the control of the basic protein promoter (bvp).

H4 in a functional form than the transient baculovirus system. However, the cell lines do produce correctly folded, processed and targeted protein. In addition, cell lines are more readily studied by electrophysiology than baculovirus-infected cells [47]. They may therefore provide a valuable alternative to oocytes or transient expression systems for analysis of channel function, folding and targeting.

4. Conclusions

We have investigated the production of the Shaker potassium channel in insect cell systems. Expression of the F425G mutant of Shaker H4 in the baculovirus system, under the control of the polyhedrin promoter, generates correctly assembled channel protein that binds to CTX. However, the expressed protein is predominantly intracellular and unglycosylated. While the overall levels of Shaker polypeptide are not increased, the expression of correctly assembled protein in the baculovirus system can be increased either by using the 'weaker' basic protein promoter, or by co-expression with the molecular chaperone, calnexin. In contrast, the use of stable insect cell lines generated 5-fold smaller quantities of protein. However, the expressed material is predominantly glycosylated and located at the cell surface. In conclusion, expression of Shaker H4 using the baculovirus system with the basic protein promoter and calnexin co-expression, allowed us to produce a total of 30 µg of correctly folded protein in each litre of insect cell culture.

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