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Synthesis of functional $GABA_A$ receptors in stable insect cell lines

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We have synthesised the β1-subunit of the bovine $GABA_A$ receptor in stable, continuous insect (*Spodoptera frugiperda*) cell lines. A cDNA was integrated randomly into the insect cell genome under control of a baculovirus immediate early (IE-1) gene promoter. Transformed cells were obtained by co-transfection of the insect cells with pIEK1.GRβ1, encoding the β1 subunit cDNA, and pIEK1.neo, encoding the neomycin resistance gene. G-418-resistant clones were selected and expanded into continuous cell lines synthesising functional, GABA-gated, homo-oligomeric chloride channels. These cell lines had significant advantages over the transient baculovirus expression system for the characterisation of receptors using electrophysiological recording techniques.

Insect cells (Sf9); Stable expression; $GABA_A$ receptor; β-1 subunit; Electrophysiology

1. INTRODUCTION

The use of the baculovirus expression vector system to produce neurotransmitter receptors in the membranes of insect cells, and their subsequent analysis using biochemical or physiological techniques has been well documented [1–3]. One drawback with transient expression under control of the baculovirus polyhedrin gene promoter is that the expression levels obtained for complex, membrane targeted proteins are significantly lower than those for proteins destined for the nucleus or cytoplasm [4–6]. This may be explained by the fact that the polyhedrin gene promoter is active in the very-late phase of gene expression, when it is likely that the host cells' secretory pathway is compromised [4,6]. In addition, virus infection affects the integrity of the plasma membrane, which may reduce the relative efficiency of electrophysiological analyses on the expressed receptors [7]. It may, therefore, be advantageous to use a stable cell line expression system to study the electrophysiological properties of ion channels in vitro.

The approach used in this study has been to use baculovirus promoters to express the β-1 subunit of the $GABA_A$ receptor in stably transformed insect cell lines, which then retain the physiological properties associated with non-infected cells.

2. EXPERIMENTAL

2.1. *Construction of the pIEK1 expression plasmids*

All DNA manipulations were carried out using standard techniques [8]. A plasmid containing the *Hin*dIII 'G' fragment of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome, pAcHG (Dr. R.D. Possee, Oxford), was digested with *Xba*I and *Cla*I to excise the IE-1 gene. Following a Klenow 'fill-in' reaction, this DNA fragment was inserted into pT7T3-*Sma*I, to produce pIE1. pT7T3-*Sma*I was produced by digestion of pT7T3 (Pharmacia) with *Eco*RI and *Hin*dIII to remove the polylinker and religation in the presence of a *Sma*I linker. The IE-1 gene coding region was removed by partial digestion with *Sph*I, followed by digestion to completion with *Hin*cII, producing a plasmid containing an intact IE-1 promoter but deleting part of the 5′ leader sequence. A 50 mer synthetic oligonucleotide encoding the missing IE-1 5′ leader sequences and a *Bam*HI cloning site, with compatible *Sph*I and *Hin*cII ends, was synthesised and ligated into the plasmid. This resulted in the formation of the expression vector, pIEK1, with an intact IE-1 promoter and 5′ leader sequences (Fig. 1). The plasmids pIEK1.neo and pIEK1.GRβ1 were prepared by insertion of the neomycin resistance gene (Pharmacia) or the $GABA_A$ receptor β1 subunit cDNA (from pbGRβsense [9]) respectively, at the *Bam*HI site of pIEK1 (Fig. 1).

2.2. *Production, selection and maintenance of insect cell lines expressing the $GABA_A$ receptor β1-subunit cDNA.*

The plasmids pIEK1.neo and pIEK1.GRβ1 (0.5 μg each) were mixed with an equal volume of Lipofectin (Gibco-Life Technologies) that had been previously diluted two parts to one with sterile water. Following gentle agitation and 15 min incubation at room temperature, the DNA mixture was added to 1×10^6 Sf9 cells in 1 ml of serum-free TC100 medium (J.R. Scientific). After incubation overnight at 28°C, 1 ml of TC100 medium supplemented with 10% foetal calf serum (growth medium) was added, and the incubation continued for 24 h. The cells were harvested by gentle scraping, the volume of growth medium was increased to 10 ml and G-418 (Gibco Life Technologies) was added to a final concentration of 1 mg/ml. The cells were then transferred in 100 μl aliquots to the wells of a 96-well plate. The 96-well plate was incubated for seven days at 28°C and then the media was changed to growth medium containing 0.6 mg/ml G418. After a further week, the medium was changed to G418-free growth medium and the incubation continued for seven days.

At this point, wells containing individual clones of Sf9 cells were harvested and transferred to 24-well plates. After 5–7 days, the cells were passaged and the excess cells were lysed with 200 μl of 0.2 M NaOH for analysis by dot-blotting. The cell lysates were applied to nylon filters (Amersham) and hybridised under stringent conditions with a $GABA_A$ receptor β1 subunit cDNA probe prepared by random

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primer labelling. Positive cell lines (Sf9.GRbeta1) were amplified and stocks frozen in liquid nitrogen, after the addition of DMSO to 10% [4]. The cell lines were passaged at weekly intervals for periods of up to six months, and during this time were periodically tested for the presence of functional $GABA_A$ receptors.

2.3. *Electrophysiology*

Electrophysiological recordings were performed using the whole-cell recording configuration of the patch clamp technique [10]. Sf9.GRβ1 cells (0.5×10^6) were placed in 60 mm Petri dishes and equilibrated in a bathing saline (120 mM NaCl, 4 mM KCl, 10 mM $CaCl_2$, 11 mM $MgCl_2$, 10 mM HEPES, pH 7.2). Patch electrodes (3–5 μM) were filled with 110 mM KCl, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES, pH 7.2. GABA (10^{-5} M; 1–2 s) was applied to the cells by pressure ejection (Picospritzer, General Valve Corporation) from 1 μM diameter micropipettes positioned 30–50 μm from the whole-cell clamped cells. In order to determine the pharmacology of the GABA-mediated currents, GABAergic substances were allowed to diffuse from the tips of broken pressure micropipettes positioned 50–60 μm from the whole-cell clamped cells.

3. RESULTS AND DISCUSSION

3.1. *Production of stable insect cell lines synthesising the bovine $GABA_A$ receptor β1-subunit*

A plasmid, pIEK1, was constructed containing the AcNPV IE-1 gene promoter and 5′ leader region, transcription termination sequences, and a *Bam*HI site to facilitate the insertion of foreign gene coding sequences (Fig. 1). Two recombinant plasmids were produced, pIEK1.GRβ1 containing the $GABA_A$ receptor β1 subunit cDNA, and pIEK1.neo containing the neomycin resistance gene (Fig. 1). The neomycin resistance gene was chosen as a selectable marker as Jarvis et al. [11] had previously shown that this gene confers resistance to G418 in insect cells. Sf9 cells, maintained in antibiotic-free TC100 growth medium, were co-transfected with pIEK1.GRβ1 and pIEK1.neo using lipofection [12], and G418-resistant clones were selected as described in section 2. Small clones of G418-resistant Sf9 cells were observed from days six to seven post-transfection, and after two weeks amplification in the presence of G418, the selective pressure was discontinued. DNA dot-blotting techniques demonstrated that 16 out of the 24 G418-resistant clones that were tested had incorporated the $GABA_A$ receptor β1 subunit sequences (data not shown).

After preliminary tests, one of the cell lines (Sf9.GRβ1), was amplified further and has been passaged continuously, without continued selective pressure, for more than six months (approximately 30 passages). The cell line expressed functional, homo-oligomeric, GABA-gated chloride ion channels when tested at periodic intervals (as described below). In addition, the cell line has been successfully frozen in, and retrieved from, liquid nitrogen using the same procedures adopted for Sf9 cells [4].

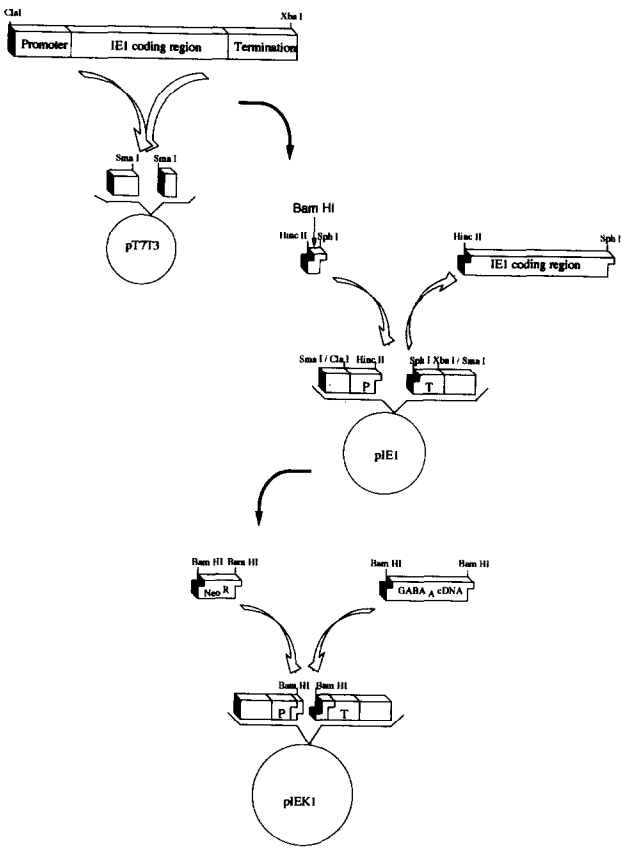

Fig. 1. Production of the expression vectors pIEK1.GRβ1 and pIEK1.neo. The IE-1 gene from the AcNPV genome was isolated on an *Xba*I–*Cla*I DNA fragment (from pAcHG) and inserted into pT7T3-*Sma*I, in which the polylinker cloning site had previously been removed, as described in section 2. The IE-1 gene coding region and part of the 5′ leader sequence was removed by partial *Sph*I digestion, followed by digestion to completion with *Hinc*II. A 50-mer synthetic oligonucleotide with compatible cohesive ends, containing a replacement for the deleted 5′ leader sequence and a *Bam*HI cloning site, was ligated into this plasmid to produce the expression vector pIEK1. The $GABA_A$ receptor β1 subunit cDNA and the neomycin resistance gene (neo^R) were both isolated as *Bam*HI DNA fragments and inserted, separately, into pIEK1 to produce pIEK1.GRβ1 and pIEK1.neo, respectively. P, IE1 promoter; T, IE1 terminator sequences.

3.2. *Electrophysiological analysis of the Sf9.GRβ1 cell line*

Recombinant cells were harvested from either suspension or monolayer cultures in mid log phase of growth and analysed for the presence of GABA-gated chloride ion channels using the path clamp technique, as previously described [2]. GABA (10^{-5} M) was applied by pressure ejection from micropipettes onto the surface of cells under whole-cell voltage-clamp. The results are illustrated in Fig. 2. Over 80% of the cells tested ($n > 20$), on each occasion, responded to GABA with an inward current which increased in amplitude with membrane hyperpolarisation. The GABA-evoked current reversed at around −10 mV, which is the Nernst equilibrium potential for chloride ions predicted for our exper-

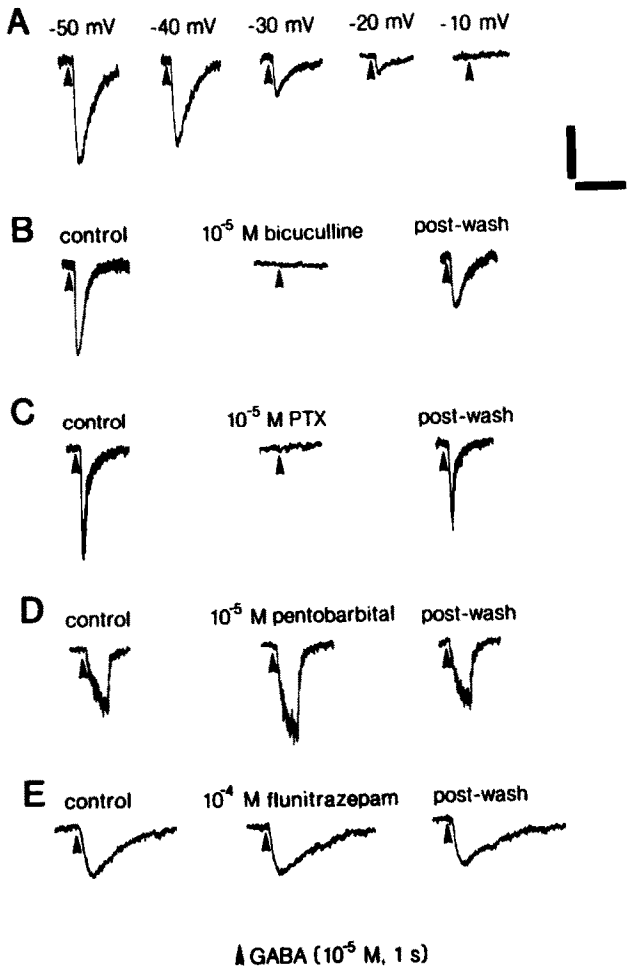

Fig. 2. GABA-evoked responses in Sf9.GRβ1 cells. GABA (10^{-5} M) was pressure-applied onto the surface of Sf9.GRβ1 cells. (A) In whole-cell voltage-clamped cells, GABA induced inward currents which reversed at around −10 mV. Calibration: 20 pA, 20 s. (B) The GABA-mediated currents were completely abolished by 10^{-5} M bicuculline. Calibration: 25 pA, 20 s. (C) Application of 10^{-5} M picrotoxin (PTX), blocked the GABA responses. This effect was reversible after prolonged wash-out (20 min or more). Calibration: 20 pA, 25 s. (D) The $GABA_A$ receptor potentiator pentobarbital increased the currents elicited by GABA in all cells tested. Calibration: 15 pA, 10 s. (E) The positive modulator flunitrazepam had no effect on the GABA-mediated currents even at concentrations as high as 10^{-5} M. Calibration: 10 pA, 10 S.

imental conditions. The GABA-gated response was blocked reversibly by bicuculline, the competitive antagonist of the $GABA_A$ receptor [13,14]. Picrotoxin, a blocker of the chloride ion channel of the $GABA_A$ receptor [13,14], completely abolished the response to GABA at 10^{-5} M, and application of the barbiturate sodium pentobarbital [13] enhanced the signals obtained. However, application of the benzodiazepine flunitrazepam [13,15–17] had no effect on the GABA responses. These results are in accord with previous studies showing that the β1-subunit of the $GABA_A$ receptor can form a GABA-gated chloride channel, which is insensitive to flunitrazepam but sensitive to bicuculline, picrotoxin and barbiturates [2,3,14– 17]. No GABA-gated chloride channels were detected in control Sf9 cells (data not shown). The recombinant Sf9 cell line demonstrated similar physiological characteristics as control Sf9 cells with regard to their amenability in performing the patch clamp analyses (Table I). One of the problems that had been encountered in analysing functional expression of neurotransmitter receptors in recombinant-baculovirus infected cells was the reduced efficiency of forming seals between the patch pipette and the plasma membrane of the insect cell (Table I). This was particularly pronounced when using the Sf21 cell line (Table I), and indicated that the Sf9 cell line was more amenable to electrophysiological analysis than the Sf21 cell line. Previous studies [7] using electron microscopy techniques have shown that the surface of the Sf9 cells is much smoother than that of the Sf21 cells, and this may explain the relative ease of forming seals with the Sf9 cells.

One other disadvantage of the baculovirus system for ion channel-receptor expression is that the window for experimental studies is restricted from about 24 to 36 hpi for Sf21 cells, and from about 24 to 72 hpi for Sf9 cells; in contrast the recombinant cell line could be used at any time. The currents obtained from the whole-cell recordings in the recombinant cell line were, however, slightly smaller than that previously obtained with recombinant baculovirus-infected cells [2]. This suggests that in common with the report by Jarvis et al. [11] on the secretion of tissue plasminogen activator, the levels of receptor synthesised are lower in the Sf9.GRβ1 cell line. In a recent study, Kleymann et al. [18] have also reported similar advantages, to those described above, when using stable insect cell lines for the analysis of receptors that are linked to second messenger systems. In this report the human β2-adrenergic receptor was functionally coupled via endogenous GTP-binding proteins to adenylyl cyclase. When the same receptor had been expressed in recombinant baculovirus-infected cells, functional coupling to adenyl cyclase had not been detected. Another advantage of the stable insect cell line expression system is the absence of endogenous receptors in the Sf9 cells, the common presence of which in many mammalian cell lines can often complicate the analysis of the introduced receptor.

Table I

Comparison of the successful seal formation between the electrophysiological patch pipette and membranes of Sf9 and SF21 cells

Cell type	Successful GΩ seal formations
Sf9 cell line	90%
AcGRβ1 virus-infected Sf9	60%
Sf9.GRβ1 cell line	90%
Sf21 cell line	20%
AcGRβ1 virus-infected Sf21	5%

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REFERENCES

[1] King, L.A., Atkinson, A.E., Obosi, L.A., Bermudez, I. and Beadle, D.J. (1992) in: Neurotox '91 (Duce, I.R., Ed.), pp. 191–202, Elsevier, Amsterdam.
[2] Atkinson, A.E., Bermudez, I., Darlison, M.G., Barnard, E.A., Earley, F.G.P., Possee, R.D., Beadle, D.J. and King, L.A. (1992) Neuroreport 3, 597–600.
[3] Carter, D.B., Thomsen, D.R., Im, W.B., Lennon, D.J., Ngo, D.M., Gale, W., Im, H.K., Seeburg, P.H. and Smith, M.W. (1992) Biotechnology 10, 679–681.
[4] King, L.A. and Possee, R.D. (1992) The Baculovirus Expression System, A Laboratory Guide, Chapman and Hall, London.
[5] Atkinson, A.E., Weitzman, M.D., Obosi, L., Beadle, D.J. and King, L.A. (1989) Pestic. Sci. 28, 215–224.
[6] Jarvis, D.L. and Summers, M.D. (1989) Mol. Cell. Biol. 9, 214–223.
[7] King, L.A., Mann, S.G., Marlow, S.A., Bermudez, I., Lawrie, A.M., Obosi, L.A. and Atkinson, A.E. (1992) in: Baculovirus and recombinant protein production processes (Vlak, J.M., Schlaeger, E.-J. and Bernard, A.R., Eds.) pp. 157–165, Editiones Roche, Basel.
[8] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
[9] Pritchett, D.B., Sontheimer, H., Gorman, C.M., Kettenmann, H., Seeburg, P.H. and Schofield, P.R. (1988) Science 242, 1306–1308.
[10] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, E.J. (1981) Pflugers. Arch. 391, 85–100.
[11] Jarvis, D.L., Fleming, J.G.W., Kovacs, G.R., Summers, M.D. and Guarino, L.A. (1990) Biotechnology 8, 950–955.
[12] Groebe, D.R., Chung, A.E. and Ho, C. (1990) Nucleic Acid. Res. 18, 4033.
[13] Stephenson, F.A. (1988) Biochem. J. 249, 21–32.
[14] Olsen, R.W. and Venter, R.C. (1986) in: Receptor Biochemistry and Methodology, Vol. 5. (Olsen, R.W. and Venter, R.C., Eds.) Liss, New York.
[15] Pritchett, D.B., Luddens, H. and Seeburg, P.H. (1989) Science 245, 1389–1392.
[16] Pritchett, D.B., Sontheimer, H., Shivers, B.D., Ymer, S., Kettenmann, H. and Seeburg, P.H. (1989) Nature 338, 582–585.
[17] Luddens, H. and Wisden, W. (1991) Trends Pharmacol. Sci. 12, 49–51.
[18] Kleymann, G., Boege, F., Hahn, M., Hampe, W., Vasudevan, S. and Reilander, H. (1993) Eur. J. Biochem. 213, 797–804.