Process development for functional membrane receptor production in mammalian cells

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Abstract

Two model G-protein coupled membrane receptors (GPCRs), a serotonin (5HT) and a metabotropic glutamate (mGlu) receptor, stably expressed in CHO cells were used to characterize culture conditions for maximum receptor expression and functional activity in membrane preparations. Expression levels of the 5HT receptor were affected by the growth phase of the cell culture. Maximum receptor density, as measured by ligand binding per mg membrane protein, was observed when cells were harvested in late exponential growth phase. Expression could be increased further by addition of 10 mM sodium butyrate and incubation at 31 °C for 24 hours prior to cell harvest. In contrast, functional activity as determined by agonist-stimulated GTP γ S binding was independent of the growth rate. For both receptors, butyrate treatment at decreased temperature negatively affected functional activity. The mGlu receptor membranes lost functional activity considerably when the cells were cultured in an agitated system either on microcarriers or as aggregates in suspension. Functional activity could be restored and further improved compared to a control grown in T-flasks when the cell culture was incubated at 31 °C for 48 hours following a complete medium exchange and omission of sodium butyrate.

Introduction

It is estimated that approximately 20% of all drug targets are G-protein coupled membrane receptors (GPCR), which illustrates their outstanding importance as target proteins within the drug discovery process. GPCRs represent an inhomogeneous superfamily of transmembrane proteins involved in signal transduction into the cell (Ji et al., 1998; Gether et al., 1998). Ligand binding assays are the typical assay format for high throughput screening of large chemical libraries for hit identification. However, this assay format is unable to predict the pharmacological profile of a novel ligand. Therefore, assay formats measuring protein function have gained increasing importance. In drug discovery, stable expression in mammalian cells is typically applied to the production of membrane receptors to be used in binding assays or

functional assays. Traditionally, labor-intensive static culture has been used for propagation of cells for assays or subsequent membrane preparation. Although this technique has many advantages, a drawback is the need for a highly sophisticated automation approach for large-scale production of cells required to meet the increasing demand for high throughput screening campaigns. Important determinants for the quality of the preparations are level of receptor expression as determined by ligand binding and maintenance of functional response of the membrane preparations as quantified in assays like the GTP γ S binding assay (Lazareno 1999).

Cultivation of mammalian cells in stirred tank bioreactors both in suspension and on microcarriers is an established method for industrial production of vaccines and recombinant therapeutic proteins. It of-

fers the advantage of supplying large lots of identical material produced under controlled conditions e.g. for screening campaigns. Moreover, process engineering and scale-up principles are well understood and documented in the literature (Hu et al., 1997). In this study we investigated how different culture conditions and formats affect the yield and quality of two model receptors. It is common practice to harvest cells for subsequent membrane preparation when the culture has reached 80% confluence. However, there is very little quantitative data available in the literature on how the time of harvest affects membrane preparation quality (Zaworski et al., 1995). Additionally, the degree of confluence is difficult to quantify especially when working in stirred tanks. Therefore, we investigated the effect of growth rate on receptor expression and functional response. In order to further optimize receptor expression we studied the effect of butyric acid in combination with decreased temperature. Butyric acid added to the culture medium in millimolar concentrations has been shown to improve expression of a series of different, mainly secretory recombinant proteins expressed in CHO or NS0 cells (Palermo et al., 1991; Oh et al., 1993; Ganne et al., 1991; Chevalot et al., 1995). Other authors could show the beneficial effect of a decreased culture temperature on protein expression (Chuppa et al., 1997; Junker et al., 1994; Reuveny et al., 1986; Rössler et al., 1996). Also a combination of both, i.e. butyrate addition at decreased temperature has recently been shown to increase recombinant protein expression (Santel et al., 1999; Hendrick et al., 2001; Rasmussen et al., 1998). Especially the concept of a separated growth and expression phase has been discussed in order to improve product yields (Fussenegger et al., 1997).

In our study we used a serotonin receptor and a metabotropic glutamate receptor as model GPCRs for process development and optimization of cell mass production for membrane preparations used in ligand binding and functional assays. The family of GPCRs that respond to serotonin (5HT) are major targets for psychoactive drugs and have been pursued as drug targets for many years (Martin et al., 1998). The 5HT receptor used in this study is stably expressed in a CHO cell-line lacking dihydrofolate reductase activity (Newman-Tancredi et al., 1992). The cells can be cultured in monolayer and suspension. Expression levels of 3 pmol mg⁻¹ protein were reported which is approx. 7 times that of brain tissue. Increasing methotrexate concentrations in the culture medium (5 nM – 80 µM) are commonly used to amplify the gene

of interest in DHFR negative CHO cell line (Kingston et al., 1997). During routine culture the selection pressure is sometimes maintained by methotrexate addition at levels not affecting cell growth even when working with a clonal cell line. A number of authors have investigated the effect of methotrexate on cell growth, gene copy number, productivity and stability (Pendse et al., 1992; Kim et al., 1998; Gu et al., 1996). However, in this study methotrexate addition at a very low concentration was only tested in regard to a maintained productivity.

Metabotropic glutamate receptors (mGluR) define a new family of GPCRs that do not share any sequence homology with previously known members of that gene superfamily (Conn et al., 1997). They may provide a novel target for development of therapeutic agents with significant impact in neuropharmacology. The recombinant CHO cell line stably expressing

Figure 1. Effect of culture system on functional activity of a mGluR (a) and 5HT (b) receptor expressed in CHO cells grown at 37 °C. Cells were grown in parallel in stationary culture systems (T-flask, Cell factoryTM) and in spinner flasks on solid collagen coated (Cytodex 3) microcarriers, on macroporous gelatin (CultisphereTM G) microcarriers and in suspension. The mGlu cells grew strictly adherently in the presence of an attachment substrate whereas the 5HT cells had a strong tendency to grow in suspension in stirred systems even when cultured in the presence of Calcium.

Figure 2. Suspension growth of 5HT cells in 500 ml (a), 250 ml (b) and 125 ml (c) spinner scale with (w/) and without (w/o) methotrexate (MTX). In experiment (a) cells were harvested on day 2 (late exponential growth phase), in experiment (b) cells were harvested in stationary phase on day 4 for further membrane preparation. In experiment (c) the cell culture was split 1:4 with fresh medium on day 2 and harvested two days later. Results obtained for ligand binding and functional activity determined in the GTP γ S assay are shown in fig 3 and 4 respectively.

Figure 3. Ligand binding of 5HT membrane receptor preparations determined for cells harvested in late exponential growth phase (a), stationary phase (b) and after 1:4 dilution (c) with (gray bars) and without (black bars) methotrexate (MTX). The growth characteristics of the cells in the different experiments is shown in Fig. 2.

a specific mGlu receptor applied in this study was developed in-house.

Materials and methods

Cell lines and culture medium

All cell lines were cultivated in amino acid enriched DMEM/F12 supplemented with 10% FCS. For suspension culture a Ca^{2+} free medium batch

was applied. The 5HT receptor was expressed in CHO DHFR⁻ negative cells (Newman-Tancredi et al., 1992) cultured in nucleoside free basal medium supplemented with dialysed serum. A clonal cell line derived from CHO K1 was developed in-house expressing a metabotropic glutamate receptor (mGluR) using the Geneticin selection system. This cell line was routinely maintained in 0.2 mg ml^{-1} G 418 supplemented medium.

Figure 4. Functional activity expressed in stimulation over background for 5HT membrane receptor preparations when harvested in late exponential growth phase (a), stationary phase (b) and after 1:4 dilution (c) with (gray bars) and without (black bars) methotrexate (MTX). Growth curves for the different conditions are shown in Fig. 2.

Culture conditions

Adherent culture was performed in T-flasks or Cell factories (NalgeNunc). Suspension culture and adherent culture on Cytodex 3 (Amersham Pharmacia Biotech) and CultiSphere G (Perstorp Biolytica) carriers were performed in spinner flasks kept in a humidified CO₂ incubator or in stirred tank bioreactors (B. Braun Biotech) equipped with a caged sparger (Fenge et al., 1993) for bubble free aeration. During cell line expansion cultivation temperature was kept at 37 °C, DOT at 50%, stirrer tip speed at values below 0.5 m s⁻¹ and pH 7.2. Carriers were prepared according to supplier's instructions. A carrier density of 2 g l⁻¹ and 3 g l⁻¹ respectively was applied for the 5HT and the mGluR experiments. Inoculum for carrier culture was grown in T-flasks.

Cell harvest and membrane preparation

Cell cultures were harvested in late exponential growth phase if not otherwise indicated. Adherent cells were detached enzymatically. All cells were harvested at 2500 × g and washed once with phosphate buffered saline (PBS). Membrane preparations were performed from frozen cell pellets. The pellets were thawed in buffer A (50 mM Tris/HCl; 2.5 mM EDTA, pH 7 at 4 °C, supplemented with 1 mM protease inhibitor PefablocTM (Roche) prior to use) resulting in a cell concentration of 1 × 10⁸ cells ml⁻¹. The cells

were disintegrated for 15 seconds at low speed (approx. 8000 rpm) using a Turrax (T-25 basic) and subsequently centrifuged at 2500 × g for 15 minutes. The supernatant was stored on ice and the pellet treated as before. The supernatants were pooled and centrifuged at 100000 × g for 45 minutes. The resulting pellet was carefully resuspended in buffer B (50 mM Tris/HCl, pH 7 at 4 °C, 20% sucrose) in a volume corresponding to an initial cell concentration of 1 × 10⁸ per ml. All steps were performed at 0 to 4 °C. Protein concentration was determined using the Bradford reagent (BioRad) according to the supplier's instructions. Membrane preparations were kept in aliquots at -80 °C.

Assays

Routine cell density determination was performed with the automatic cell counter CASY TTC. Nuclei count was determined after citric acid/Triton X 100 treatment in a hemacytometer (Lin et al., 1991). Ligand binding capacity is proportional to receptor expression and was determined according to a proprietary protocol for the serotonin receptor. Functional activity of the G-protein coupled receptors was determined in a GTPγS assay as described by Lazareno (Lazareno, 1999) and is expressed as stimulation over background. Typically, the standard deviation of the applied analysis methods was less than 10%.

Figure 5. Effect of Na-butyrate addition and temperature decrease prior to harvest on ligand binding of 5HT membrane preparations (a) and functional activity as measured with the GTP'S assay (b). Cells were cultured in Ca²⁺-containing medium in the presence of CultiSphereTM G microcarriers until late exponential growth phase when the cultures were diluted 1:1.7 with fresh medium. Subsequently, Na-butyrate was added (10 mM final concentration) and the culture temperature was decreased to 31 °C 20 h prior to harvest. As control a parallel spinner was maintained without Na-butyrate addition and kept at 37 °C. Experiments w/ (gray bars) and w/o MTX addition (black bars) were performed in parallel.

Results

Effect of growth format on receptor expression and functional activity

Conventionally, cells harvested from static cultures are used to prepare membranes for subsequent use in drug discovery assays. A loss of functional activity has been observed occasionally when suspension adapted cells were used to prepare membranes for assays in previous experiments in our laboratory. Therefore, the effect of culture format on specific binding and stimulation was investigated for the two model GPCR's (see Fig. 1). For the metabotropic glutamate receptor

expressed in CHO K1 cells it was observed that T-flask and Cell factoryTM derived membranes gave comparably high stimulation results. Significantly lower stimulation results were obtained from the suspension adapted cell culture. Although this cell line grows strictly adherently in Ca²⁺ containing medium, low functional activity was observed for both Cytodex 3 and CultiSphereTM G microcarriers. In contrast, this observation was not confirmed for the 5 HT expressing CHO DHFR⁻ cells. This cell line gave similar stimulation results in suspension and on CultiSphereTM G carriers. Interestingly, this cell line had a strong tendency to grow in suspension, which is reflected by the high percentage of non-attached cells in the carrier culture (> 75 %), which may explain the comparable stimulation results for the two culture systems.

Effect of growth phase on receptor expression and functional activity

The effect of growth phase on receptor expression and activity was investigated for the 5HT expressing cell line. Suspension cultures with and without 5 nM methotrexate were inoculated at 2×10^5 cells ml⁻¹ in 125 ml, 250 ml and 500 ml spinner culture after complete medium change. Cell density was monitored daily to characterize cell growth. Cells were harvested after 2 and 4 days in culture. Additionally, on day 2 the 125 ml spinners were split 1:4 and harvested two days later.

As seen from Fig. 2 cell growth did not depend on the chosen spinner scale and was not affected by the applied methotrexate concentration of 5 nM. The cells grew exponentially after inoculation with 0.2×10^6 cells ml⁻¹ until a cell density of approx. 0.8×10^6 cells ml⁻¹ was reached on day 2. A maximum cell density of 1×10^6 cells ml⁻¹ was determined on day 4. A 1:4 fold dilution resulted in slowed growth probably as a result of the low dilution factor.

From the binding results shown in Fig. 3 it can be seen that the growth rate affects receptor expression. Cells harvested in late exponential growth phase, at maximum growth rate, resulted in highest binding results. This observation has been confirmed for several other recombinant cells expressing GPCRs in our lab and was previously described in the literature for the 5-HT₂ serotonin receptor (Zaworski et al., 1995). Therefore, it was concluded that it is extremely important to keep the culture at maximum growth rate prior to harvest if high receptor expression levels are required. In contrast, receptor stimulation as de-

Figure 6. Functional activity of mGluR membrane preparations derived from cells cultured on Cultisphere™ G (gray bars) and Cytodex 3 carriers (black bars) as measured in the GTP γ S assay. Parallel cell cultures (500 ml) were initiated on Cultisphere™ G and Cytodex 3 microcarriers at an inoculum density of 0.1×10^6 cells ml $^{-1}$. After 5 days in culture a 100 ml aliquot was harvested from each spinner for membrane preparation (results shown in Fig. 1a). After medium exchange the residual carrier cultures were divided into aliquots and incubated w/ and w/o 10 mM Na-butyrate at 31 °C for 24 h and 48 h prior to harvest.

Figure 7. Functional activity of mGluR membrane preparations derived from cells grown in suspension as measured in the GTP γ S assay. Suspension adapted cells were inoculated at a density of 0.4×10^6 cells ml $^{-1}$ and cultured for three days at 37 °C. After medium exchange the culture was divided into aliquots, supplemented either with (w/) or without (w/o) 10 mM Na-butyrate (Nab) and incubated for 24 h or 48 h at 31 °C prior to harvest. As control stimulation results obtained from a stationary cell culture are given.

terminated by the GTP γ S assay did not seem to be significantly dependent on growth rate or cell density. Only a slightly decreased stimulation was observed for

the stationary culture as can be seen from Fig. 4. All conditions resulted in stimulation ≥ 2 except the stationary culture with methotrexate. However, there is

no evidence for a negative effect of methotrexate on stimulation from this data.

Effect of temperature decrease and butyrate addition on receptor expression and functional activity

The effect of Na-butyrate addition and culture temperature on receptor expression and functional activity was investigated for both model GPCRs. In case of 5HT expression a CultisphereTM carrier culture was initiated at a cell density of 0.7×10^6 cells ml⁻¹. 48 hours after inoculation when the cell density had reached approx. 1.3×10^6 ml⁻¹ the cell suspension was diluted 1:1.7 and an aliquot of a 1 M Na-butyrate stock solution was added to the culture medium resulting in a final concentration of 10 mM Na-butyrate. The culture temperature was decreased to 31 °C and the cells were harvested for membrane preparation after approx. 20 hours. As already previously noted it was found that the majority of cells did not adhere to the CultisphereTM carriers but grew in suspension although a culture medium for adherent cells was used.

Upon induction with sodium butyrate at decreased culture temperature an increased receptor expression in the membrane preparation could be detected (see Fig. 5a). This finding is in agreement with reports in the literature where an increased product formation has been reported for secretory proteins expressed in CHO cells after butyrate treatment or at decreased temperatures as stated in the introduction. The positive effect of these culture conditions on recombinant membrane receptor formation in CHO cells could be confirmed in our lab for a number of different GPCRs. Highest receptor expression given by a maximum ligand binding value B_{max} of 3.8 pmol mg⁻¹ crude membrane protein was observed in the methotrexate (MTX) containing cultures upon Na-butyrate induction at 31 °C. In general slightly higher receptor concentrations were observed in the cultures with maintained selection pressure. In contrast to the positive effect of butyrate induction on receptor expression, a negative effect on G-protein coupling was detected in the GTPγS assay as can be seen from the stimulation over background results under these conditions (see Fig. 5 b). This reciprocal relation of increased receptor expression and G-protein coupling upon sodium butyrate treatment was not expected. Best stimulation results were obtained for the MTX free cultures without butyrate induction. However, the major contribution to a decreased stimulation seems to be due to the Na-butyrate induction. Cell growth was compar-

able under all test conditions (data not shown). The negative effect of butyrate treatment on stimulation was confirmed with the other model GPCR, a mGlu receptor (see Fig. 6). In this experiment cell cultures were initiated either on CultisphereTM or Cytodex 3 carriers at an inoculum density of 0.1×10^6 cells ml⁻¹ and grown for 5 days until late stationary growth phase was reached. Prior to induction with sodium butyrate the culture medium was completely exchanged and the culture temperature lowered to 31 °C. Applying a longer incubation time of 48 h in the presence of butyrate decreased functional activity even further. A semi-quantitative estimation of receptor density using Western blotting revealed that the functional response of the mGlu receptor was not correlated to receptor density (data not shown), which is in agreement to the 5HT ligand binding results. This observation indicates that receptor density does not necessarily predict the degree of potential receptor stimulation. Rather, conditions that improve the efficiency of coupling of the receptor to the G-protein increased the measurable stimulation of a membrane preparation. Using the mGluR as a model GPCR it was observed that medium exchange and incubation at a lowered temperature of 31 °C for 48 h prior to cell harvest improved G-protein coupling significantly as concluded by a comparison of results shown in Fig. 1a and 6. All data shown in Fig. 6 were derived from the same carrier cultures used to produce the data of Fig. 1a but after medium exchange and temperature decrease. The best stimulation results were obtained for cells grown on Cytodex 3. However, from our data we cannot conclude if the differences in functional activity observed for the Cytodex 3 and CultisphereTM G are significant. The improvement in receptor stimulation upon media exchange and decreased culture temperature could be confirmed for the suspension-adapted cells (Fig. 7) which otherwise showed very poor functional activity (Fig. 1a). Medium exchange and incubation at lowered temperature for 48 h led to even higher functional activity than the reference membrane preparation from T-flasks. Generally, medium exchange combined with 48 h incubation at lower temperature resulted in ≥ 2 fold improvement in functional activity when compared to membrane preparations from cells harvested prior to this treatment. Indirectly, this experiment also confirms the observation made for 5HT receptor stimulation that growth phase upon cell harvest does not have a significant impact on receptor stimulation since the cells were harvested in late stationary phase. Rather, improved nutrient supply of

Figure 8. Suspension growth of a 5HT receptor producing CHO cell in a 50 l bioreactor in the presence of 5 nM methotrexate. Three consecutive batches were initiated at 0 h, 98 h and 218 h respectively. Temperature was lowered to 31 °C and 10 mM Na-butyrate added 24 h prior to each harvest performed at 96 h, 216 h and 336 h respectively. Ligand binding results given as Bmax values are indicated as diamonds.

Table 1. Comparison of 20 l carrier culture with 29 Cell factories

MGlur production system	Cell yield [10 ¹⁰]	Crude protein [mg]	Stimulation over background	Available surface [m ²]
20 l reactor, 3 g l ⁻¹ Cytodex 3	5.4	730	2.26	16.2
29 Cell Factories TM	6.7	720	0.78	18.3

the cultured cells in combination with a growth arrest obtained via a decreased culture temperature seemed to be important to obtain highly functionally active membrane preparations.

Production scale results

Based on the finding that 10 mM Na-butyrate together with a culture temperature decreased to 31 °C resulted in maximum receptor expression after 24 h, 3.3 g of crude 5HT membrane preparation was produced in 3 consecutive 50 l suspension culture batches (Fig. 8). The highest receptor density was obtained in the first batch due to optimum induction in late exponential growth phase. Difficulty in determining a reliable cell count of the aggregate forming cells may explain the poor ligand binding results for the subsequent two batches. This could be a consequence of induction at a sub-optimal growth rate. Optimization towards reliable growth monitoring is expected to result in improved binding results.

A 20 l bioreactor culture of the mGluR-CHO cells grown on 3 g l⁻¹ Cytodex 3 carriers was conducted using temperature decrease and medium exchange to improve the functional activity of the receptor. In par-

allel, cells were harvested from 29 Cell factoriesTM at 80% confluence and pooled for a subsequent membrane preparation. A comparison of both conditions revealed similar cell and protein yields. However, an increased functional response of the reactor-derived membranes may have resulted from the improved culture conditions (Table 1). The reactor production procedure demands fewer manual manipulations and is faster than the Cell factory-based production. A further advantage of reactor production is non-linear scale-up. However, the carrier-based process is still complex during inoculation and harvest when compared to a simple suspension culture.

Conclusion

We have shown that for 2 different membrane receptors expressed in CHO cells, conditions typically used to increase recombinant protein expression (i.e., Na-butyrate addition and decreased temperature) are not recommended when receptor preparations are intended for functional assays. Clearly, receptor expression and functional activity are affected by different conditions. Highly functionally active mGluR preparations

were obtained after incubation of the cells in fresh medium for 48 h at 31 °C. Moreover, functional activity is not generally marginal in suspension cultures but rather, is dependent on the cell line/receptor construct and culture conditions. Sodium butyrate treatment at decreased temperature is recommended when the membrane preparation is intended for ligand binding assays since membrane receptor expression is significantly improved upon this treatment. Interestingly, it was observed that receptor expression is affected by the growth phase of the culture which was not confirmed for functional activity. The optimum time point for harvesting the culture or inducing expression further with butyrate at low temperature for subsequent membrane preparation used in ligand binding assays is in late exponential growth phase.

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