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Site-Specific Incorporation of Unnatural Amino Acids into Receptors Expressed in Mammalian Cells

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Summary

We describe an approach to achieve unnatural amino acid incorporation into channels and receptors expressed in mammalian cells. We show that microelectroporation provides a general method to deliver DNA, mRNA, and tRNA simultaneously. In both CHO cells and cultured neurons, microelectroporation efficiently delivers an in vitro transcribed, serine amber suppressor tRNA, leading to nonsense suppression in a mutant EGFP gene. In CHO cells, both natural and unnatural amino acids chemically appended to a suppressor tRNA are site specifically incorporated into the nicotinic acetylcholine receptor (nAChR). Electrophysiology confirms the expected functional consequences of the unnatural residue. The microelectroporation strategy described here is more general, less tedious, and less damaging to mammalian neuronal and nonneuronal cells than previous approaches to nonsense suppression in small cells and provides the first example of unnatural amino acid incorporation in mammalian cells using chemically aminoacylated tRNA.

Introduction

Unnatural amino acid incorporation into proteins by nonsense suppression has proven to be a valuable tool for structure-function studies [1–4]. Using the in vivo nonsense suppression methodology [5], information on ligand binding and ion channel gating mechanisms has been obtained on a variety of ion channels including the nicotinic ACh receptor (nAChR) [6–11], 5-$HT3_A$ receptor [12], and the Shaker [13] and Kir2.1 [14] potassium channels. To date, such studies have been limited to the *Xenopus* oocyte heterologous expression system. There would be clear benefits to expanding the technology to a mammalian cell expression system. This would provide a more relevant environment for many proteins of mammalian origin and would allow for studies of cell-specific signal transduction pathways.

We describe here unnatural amino acid incorporation into channels and receptors expressed in mammalian cells. Presented is a general method to deliver mRNA or DNA that codes for a protein of interest, amber suppressor tRNA, and a reporter gene to mammalian cells. Initial studies, aimed at optimizing the protocol, involved coelectroporation of a human serine amber suppressor (HSAS) tRNA [15] with the DNA or mRNA corresponding to the protein of interest into adherent cells. This leads to highly efficient delivery of these components and efficient nonsense suppression. We demonstrate this for both enhanced green fluorescent protein (EGFP) and nAChR expression in CHO-K1 cells. We also show that the approach is successful in cultured hippocampal neurons.

We then employed the amber suppressor THG73, which has previously been successful in *Xenopus* oocytes [5]. In the present experiments, when chemically aminoacylated with natural or unnatural amino acids, THG73 delivers the amino acid site specifically into nAChR expressed in CHO cells. Electrophysiology clearly reveals the expected shift in dose-response relations, establishing that the desired unnatural amino acid has been incorporated. Other strategies, including lipofection, microinjection, and bioloistics, did not lead to successful transfection and nonsense suppression. Our findings establish that electroporation is an effective and general method to deliver not only mRNA and DNA, but also tRNA into a variety of adherent mammalian cell types, and that in vitro transcribed suppressor tRNA is fully functional in these cells. These results thus significantly expand the scope and applicability of the nonsense suppression methodology.

Results

Electroporation of tRNA into Adherent Mammalian Cells: EGFP Expression by Nonsense Suppression

Our initial assay involved suppression of EGFP with a human serine amber suppressor tRNA (HSAS) that was first described by RajBhandary and coworkers [15]. This tRNA is efficiently charged with serine by endogenous aminoacyl tRNA synthetases of the cell. The EGFP DNA gene was mutated to introduce a TAG (amber) stop codon at Ser29. This DNA was combined with in vitro transcribed HSAS to a volume of 5 μl. A microelectroporator [16, 17] was used to transfect adherent mammalian cells with tRNA and DNA. The electroporator was designed to transfect a small section (~1 cm^2) of cells in a 35 mm dish, and therefore requires only small volumes of the transfection solution. Transfection was achieved by applying this solution to adherent CHO-K1 cells (or HEK, data not shown) and applying four 120 V, 50 ms square wave pulses.

As shown in Figure 1, 2 hours after transfection there is high EGFP expression in cells transfected with either wild-type pEGFP-F or mutant S29TAG pEGFP-F DNA and HSAS. When only the S29TAG mutant DNA is transfected without the HSAS, no EGFP expression is observed (not shown). This demonstrates that both DNA and tRNA can be coelectroporated into cells with high efficiency, that in vitro transcribed HSAS is aminoacylated by the endogenous CHO-K1 synthetase, and that HSAS then functions as a suppressor tRNA in mammalian cells.

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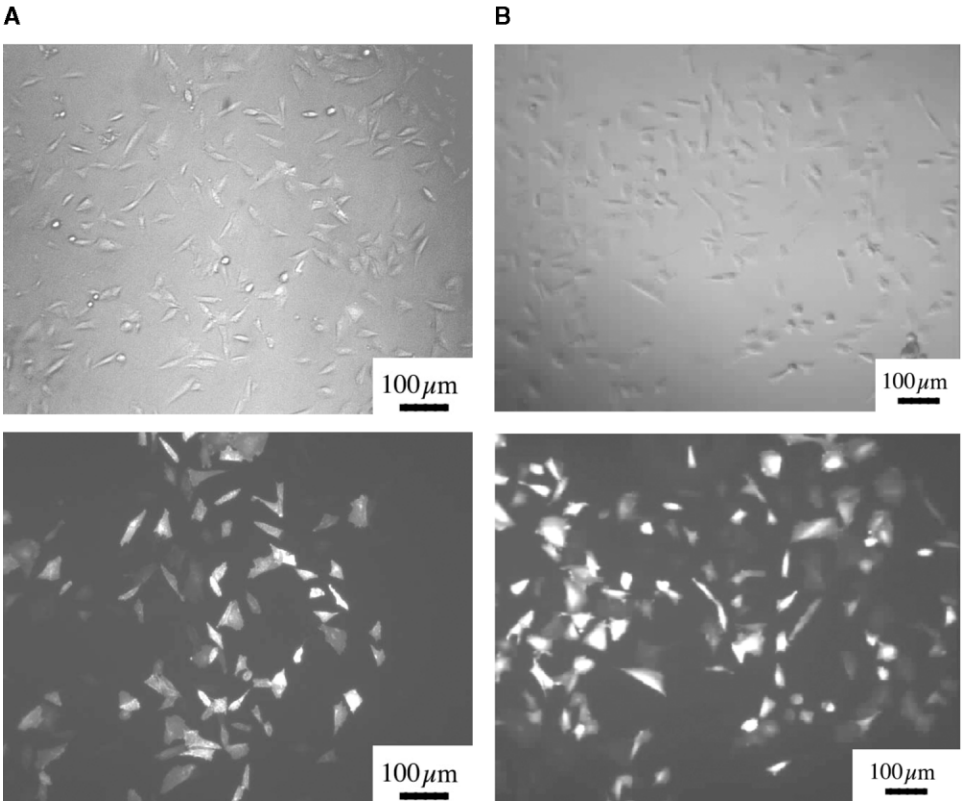


Figure 1. EGFP Expression in CHO Cells by Nonsense Suppression Using HSAS tRNA

(A) CHO-K1 cells were electroporated with a 5 μl solution of HSAS (4 μg/μl) and Ser29TAG EGFP-F DNA (2.5 μg/μl).
(B) CHO-K1 cells were electroporated with wt EGFP-F DNA (2.5 μg/μl). For both cases, four 120 V, 50 ms pulses were delivered to the cells. The bright-field (upper) and fluorescent (lower) images were taken 2 hr after transfection.

Nonsense Suppression in Hippocampal Neurons

In order to demonstrate the generality of this method, we tested the EGFP suppression assay in neurons. As with CHO cell transfection, HSAS tRNA and Ser29TAG EGFP DNA were coelectroporated into E18 rat hippocampal neurons (5 days in culture). As can be seen in Figure 2, 24 hr after transfection EGFP suppression by HSAS leads to comparable expression levels as electroporation of wild-type EGFP DNA. This demonstrates that electroporation also efficiently delivers tRNA and DNA to neurons and that the neurons also readily use in vitro transcribed tRNA for nonsense suppression. As shown in Figure 2C, only low levels of fluorescence were detected when no tRNA is added, indicating minimal readthrough of the Ser29TAG construct.

Expression of the Muscle Type nAChR by Nonsense Suppression

In initial experiments with the nAChR, we relied on the mutation of a Leu residue in the M2 pore lining region, termed Leu9′ [18], that is conserved in all known nAChR subunits. Earlier studies in *Xenopus* oocytes showed that the L9′S mutation of the β subunit leads to a ~40-fold decrease in the EC_{50} compared to wild-type channel [9, 19]. Therefore, suppression of the βL9′TAG by HSAS should lead to expression of channels that display a substantial shift in the dose-response relation, the characteristic βL9′S phenotype.

Transfection of CHO-K1 cells was achieved by electroporation of a 5 μl solution containing HSAS, mutant β subunit mRNA (L9′TAG), and mRNA for the remaining wild-type subunits (α, γ, and δ). Also included was a reporter EGFP plasmid. Expression of the nAChR was determined from whole-cell recordings of ACh-induced currents in EGFP-expressing CHO-K1 cells.

As shown in Figure 3, 24 hr after transfection the cells exhibit a strong ACh response that is not observed in nontransfected cells (data not shown). All GFP-expressing cells exhibit an ACh response. Both the receptors generated from HSAS-suppressed β9′TAG mutant mRNA and from the β9′Ser conventional mutant showed substantial decreases in their EC_{50} values, relative to wild-type. Interestingly, the shift seen in CHO cells (~10-fold) is smaller than that seen in *Xenopus* oocytes, perhaps due to differential processing in the two different cell types. For the present purposes, however, the key is that the shift seen is the same whether the L9′S mutant is made by conventional mutagenesis or nonsense suppression. This demonstrates that HSAS did indeed deliver serine during translation of the βL9′TAG subunit. Importantly, the magnitude of the ACh response for the HSAS-suppressed channels is comparable to those for the wild-type and βL9′S nAChR systems. This estab-

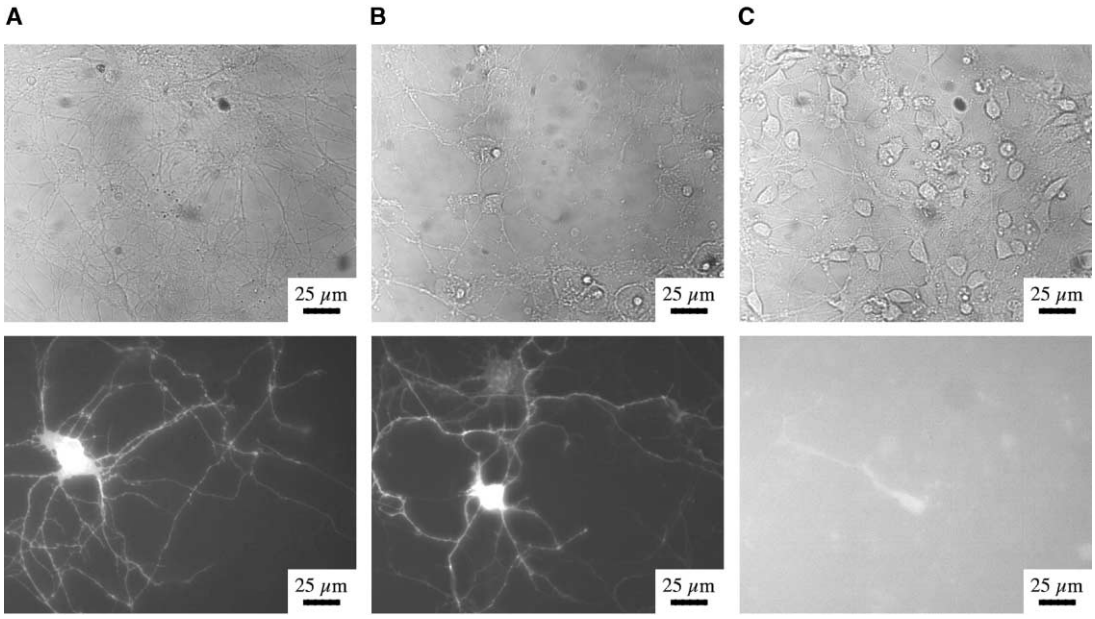


Figure 2. EGFP Expression in Hippocampal Neurons by Nonsense Suppression Using HSAS tRNA

E18 rat hippocampal neurons (5 days in culture) were electroporated with a 5 μl solution of (A) HSAS (4 μg/μl) and Ser29TAG EGFP-F DNA (2.5 μg/μl); (B) wt EGFP-F DNA (2.5 μg/μl); (C) Ser29TAG EGFP-F DNA in the absence of HSAS tRNA. In all cases, four 160 V, 25 ms pulses were delivered to the cells. The bright-field (upper) and fluorescent (lower) images were taken 24 hr after transfection.

lishes that ion channel expression is not tRNA limited, validating that electroporation leads to highly efficient delivery of tRNA to the cells.

Incorporation of an Unnatural Amino Acid

With the basic protocol being established using the HSAS suppressor, we turned our attention to unnatural amino acid incorporation. Again, we designed an experiment that would produce a distinct phenotype upon unnatural amino acid incorporation. Our studies of the agonist binding site of the nAChR established a critical role for Trp α149 in agonist binding [11]. The most telling evidence for this arises from substitution of fluorinated Trp derivatives, which produce systematic shifts in the EC_{50} for ACh activation. In these initial studies, we employed the THG73 amber suppressor that has proven to be effective for studies in *Xenopus* oocytes [20].

Using the electroporation protocol described above, we delivered mutant α subunit mRNA (TAG 149), mRNA for the remaining subunits (βL9′S mutation, γ, and δ), and a reporter EGFP plasmid. Also included was the tRNA THG73 that had been chemically aminoacylated with either Trp (wild-type) or 5,7-difluorotryptophan (THG-F_2Trp).

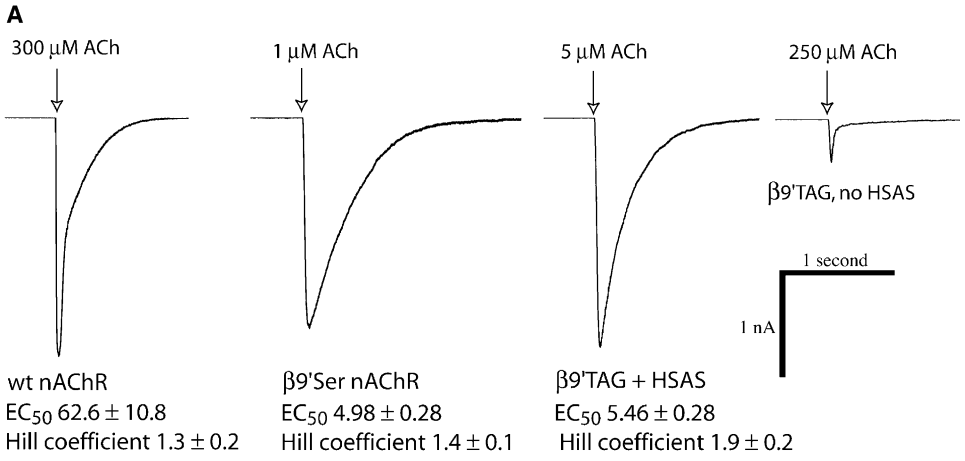
In measurements 24 hr after transfection, currents from 100 pA to 2 nA are seen in response to saturating ACh concentrations. As shown in Figure 4, when THG73 is used to deliver Trp, a wild-type channel is produced. Most importantly, THG73 aminoacylated with F_2Trp leads to a characteristic shift in the dose-response curve to higher EC_{50}. The results agree well with analogous experiments performed in *Xenopus* oocytes, and they convincingly demonstrate successful incorporation of an unnatural amino acid. Control experiments using THG73 that has not been aminoacylated gave no current.

Discussion

We present here a general microelectroporation method to transfect mammalian cells with amber suppressor tRNA and mRNA or DNA simultaneously. We demonstrate that CHO-K1 cells and hippocampal neurons readily aminoacylate in vitro transcribed human amber suppressor tRNA (HSAS), and that this tRNA is efficiently used by the translational machinery of these cells. This is shown for both an EGFP suppression assay and an nAChR suppression assay. We also show that microelectroporation can deliver chemically aminoacylated tRNA, allowing the first example of site-specific incorporation of an unnatural amino acid into a protein expressed in a mammalian cell using chemically aminoacylated tRNA.

Our results with the HSAS tRNA appear to contradict early reports by Deutscher and coworkers, who found that exogenous tRNA was not used by the translation machinery of mammalian cells [21]. They concluded that tRNA is "channeled" within the cell and that exogenous tRNA cannot enter the translational apparatus. Although their experimental design differed somewhat from ours (their tRNA was isolated from rabbit liver), the reason our results differ so significantly is unclear. What is clear from our results is that exogenous tRNA can easily enter into the protein synthesis pathway.

In addition to the electroporation approach described here, we investigated several other transfection techniques, including commercially available transfection reagents (Effectene and Polyfect [Qiagen], GeneJammer



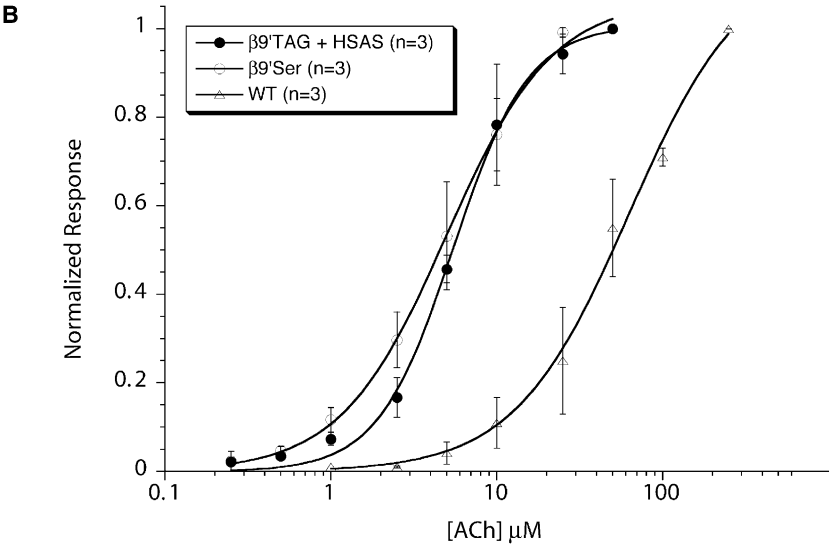


Figure 3. nAChR Expression in CHO Cells by Nonsense Suppression Using HSAS tRNA

(A) CHO-K1 cells were electroporated with a 5 μl solution containing α, β, γ, and δ, nAChR subunit mRNA, and reporter plasmid. Arrows indicated 25 ms pulses of ACh application. The first trace shows a typical response from cells transfected with the wild-type nAChR subunits (2 μg/μl α, 0.5 μg/μl each β, γ, and δ). The second trace is a response of cells transfected with the β Leu9′Ser mutant (0.15 μg/μl) and the remaining wild-type subunits (2 μg/μl α, 0.5 μg/μl each γ and δ). The third trace is a response from cells transfected with mutant β Leu9′TAG mRNA (0.15 μg/μl), the remaining wild-type subunits (1 μg/μl α, 0.5 μg/μl each γ and δ), and HSAS tRNA (2 μg/μl). The fourth trace shows a response from cells transfected with mutant β Leu9′TAG, in the absence of HSAS tRNA (read-through). The reporter plasmid EGFP-N1 (0.5 μg/μl) was included in all cases, and recordings were done from EGFP-expressing cells. The corresponding EC_{50} values are also shown.
(B) ACh dose-response curves for wt nAChR (triangle), β Leu9′Ser nAChR (L9′S, open circle), and HSAS-suppressed β Leu9′TAG nAChR (L9′TAG, closed circle).

[Stratagene], Lipofectamine [Invitrogen]), microinjection, and biolistics. All these approaches resulted in lower DNA delivery, with no convincing evidence of tRNA delivery (data not shown). In our hands, electroporation is the most efficient method for transfection of mammalian cells with multiple components, including tRNA. The microelectroporator used here typically results in less than 50% cell death and over 80% transfection efficiency [16, 17]. An advantage of the microelectroporator is the small electroporation volume (5 μl), which consumes minimal quantities of aminoacyl tRNA. Furthermore, protein expression can be seen as soon as 2 hr after transfection in the HSAS experiments, and cell health appears not to be compromised.

Recently, RajBhandary and coworkers [22] have shown the delivery of aminoacyl-tRNA obtained intact from *E. coli* to COS-1 cells using the transfection reagent Effectene (Qiagen). As noted above, we saw no success with this strategy. Most likely, the difference between the two studies is the nature of the assays employed. In their studies, Kohrer et al. harvested transfected COS-1 cells and then employed the highly sensitive biochemical CAT assay because protein expression was too low to be observed on a single-cell level. In the present

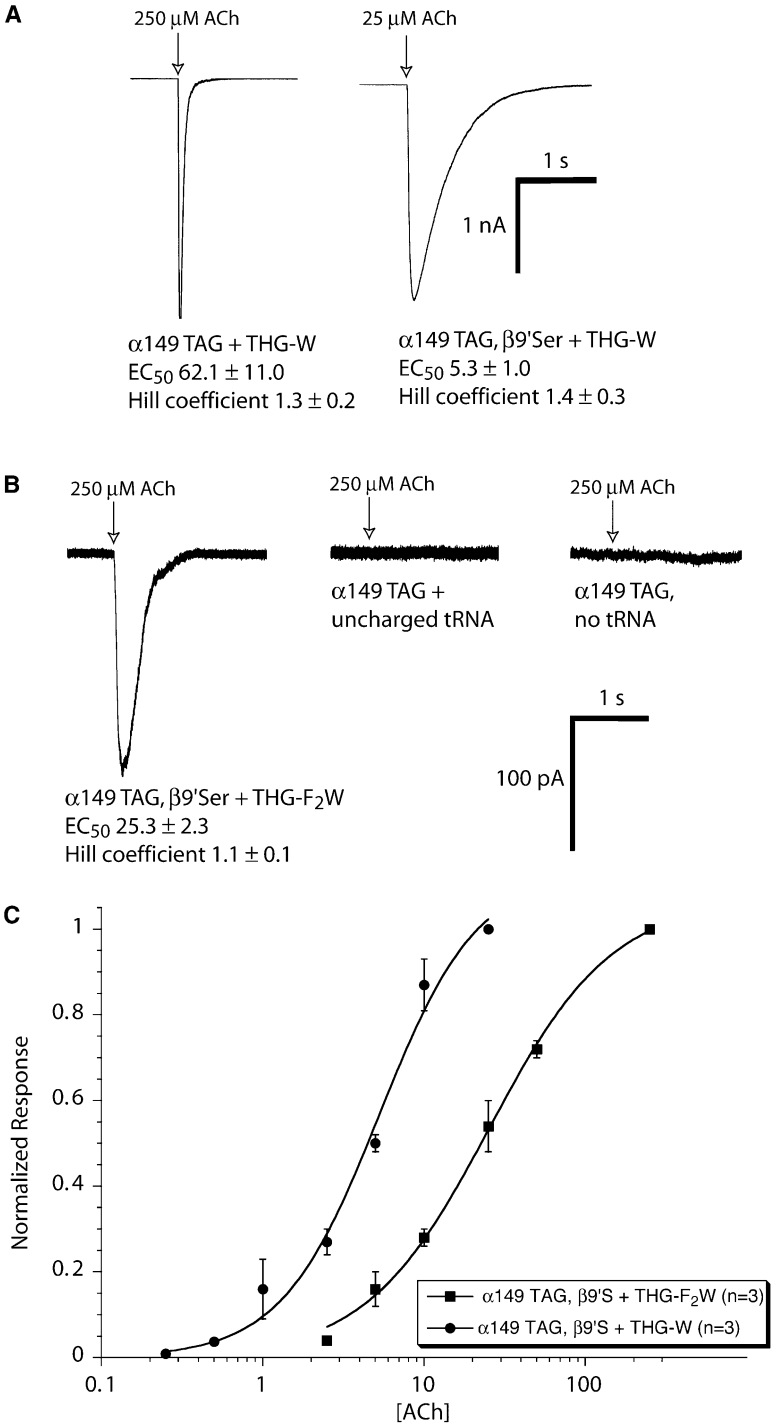


Figure 4. Incorporation of Natural and Unnatural Amino Acids into the nAChR Expressed in CHO Cells by Nonsense Suppression Using Chemically Aminoacylated THG73 tRNA

CHO cells were electroporated with a 5 μl solution containing α149TAG, β or β9′Ser, γ and δ nAChR subunit mRNA, THG-aa tRNA, and reporter plasmid. Arrows indicate 25 ms pulses of ACh application.

(A) The first trace shows wild-type recovery of the nAChR by suppression of α149TAG mRNA with THG73 tRNA aminoacylated with tryptophan (THG-W) (2 μg/μl α149TAG, 0.5 μg/μl each β, γ, and δ, 4 μg/μl THG-W). The second trace shows wild-type recovery of the β9′Ser mutant nAChR channel by suppression of α149TAG mRNA with THG-W (2 μg/μl α149TAG, 0.5 μg/μl each β9′Ser γ and δ, 4 μg/μl THG-W).

(B) The first trace shows ACh response from a cell expressing the unnatural amino acid 5,7-difluorotryptophan (THG-F_2W) at α149 of the nAChR (2 μg/μl α149TAG, 0.5 μg/μl each β9′Ser γ and δ, 3 μg/μl THG- F_2W). The second and third traces show that there is no ACh response from cells transfected with mRNA only, with or without uncharged tRNA.

(C) ACh dose response curves for THG-W suppressed α149TAG/β9′Ser nAChR (closed circle) and THG- F_2W suppressed α149TAG/β9′Ser nAChR (closed square).

work, we observe much higher levels of protein expression, and single cells can be assayed. While there are other important differences between the two studies, our work thus far indicates that for studies at the single-cell level, electroporation is a more promising transfection method.

A recent report from Yokoyama and coworkers showed site-specific incorporation of unnatural amino acids in proteins in mammalian cells [23]. They expressed in CHO-Y cells a mutant *E. coli* tyrosine synthetase that aminoacylates *B. stearothermophilus* amber suppressor tRNA with 3-iodo-L-tyrosine. This is significant work toward engineering cells with novel amino acids, but is complicated by the requirement that each new amino acid has a specific engineered synthetase and tRNA. For our purposes, chemical aminoacylation of tRNA has the distinct advantage of not being amino acid specific and no protein engineering is required, and therefore it is a more general technique.

During the preparation of this manuscript, Vogel and

coworkers independently demonstrated nonsense suppression of EGFP with aminoacyl-tRNA [24]. They microinjected CHO cells with in vitro transcribed *E. coli* amber suppressor tRNA that was chemically aminoacylated with wild-type leucine, along with the Leu64TAG mutant EGFP mRNA reporter gene, leading to the recovery of wild-type EGFP expression. This is promising work because like THG73, this tRNA was shown to be orthogonal, such that delivery of nonaminoacyl tRNA did not lead to EGFP expression. For many systems, microinjection may represent a viable approach. However, in our hands electroporation is far less tedious, since hundreds of cells can be transfected in a matter of seconds. The present method also appears to be more general because we were able to transfect different types of adherent cells with equal efficiency and with less cell mortality than single-cell gene transfer methods such as microinjection.

Significance

In the present work, we describe a general and efficient method to deliver tRNA, mRNA, and DNA to mammalian neuronal and nonneuronal cells by electroporation. We have also shown that exogenous in vitro transcribed amber suppressor tRNA is readily used by the protein synthesis machinery in these cells for nonsense suppression. This is shown by wild-type EGFP recovery using a serine amber suppressor tRNA in both CHO cells and cultured neurons. Furthermore, we demonstrate both natural and unnatural amino acids chemically appended to a suppressor tRNA are site specifically incorporated into the nicotinic acetylcholine receptor (nAChR) in CHO-K1 cells. Electrophysiology confirms the expected functional consequences of the unnatural residue.

The present methodology has many significant advantages. First, it is highly general since many types of adherent cells can be electroporated with equal efficiency and low cell mortality. Second, the method and instrumentation are very simple. The microelectroporator is easily built, is small and portable, and is easy to use [17]. Electroporation of adherent cells can be done on the benchtop or in a biological safety cabinet, and hundreds of cells can be transfected in a matter of seconds. Furthermore, protein expression is observable as soon as 2 hr after transfection.

In conclusion, we describe the first general method for unnatural amino acid incorporation in mammalian cells. By not being limited to the *Xenopus* oocytes or in vitro expression systems, this will greatly expand the use of unnatural amino acids to studying protein structure-function relationships in cell-specific signaling cascades. We therefore feel that this method will advance our own studies on neuronal ion channels, as well as making the use of unnatural amino acids more attainable to a broader cross-section of researchers.

Experimental Procedures

Materials

Synthetic DNA oligonucleotides were synthesized on a ABI 394 DNA Synthesizer on site. Restriction enzymes and T4 RNA Ligase were purchased from New England Biolabs (Beverly, MA). The mMessage mMachine and MegaShortScript in vitro trancription kits were purchased from Ambion (Austin, TX). Maxiprep kits used for plasmid isolation were purchased from Qiagen (Valencia, CA). Two membrane-localized GFP mammalian expression vectors were used, pCS2gapEGFP (Jack Horne, Caltech) and pEGFP-F (BD Biosciences Clontech, Palo Alto, CA). pEGFP-N1, a soluble GFP construct, was also purchased from BD Biosciences Clontech (Palo Alto, CA). Ham's F12 tissue culture media was purchased from Irvine Scientific (Santa Ana, CA), and CO_2 independent and Neurobasal Media were purchased from GIBCO Invitrogen Corporation (Carlsbad, CA). The microporator was built on site.

Mutagenesis, mRNA Synthesis, and tRNA Synthesis

The HSAS gene was constructed as follows: two complementary synthetic oligonucleotides encoding for the T7 promoter, the HSAS gene, and the Fok 1 restriction site were annealed and ligated into the EcoR 1 and BamH 1 restriction sites of pUC19 [5]. After linearization of the DNA with Fok 1, in vitro transcription with the MegaShortScript kit yields 74-mer tRNA (i.e., lacking the 3′ terminal CA nucleotides).

THG73 tRNA [5], THG73-W, and THG73-F_2W [11] have been described elsewhere. Briefly, linearization of pUC19 containing the THG73 gene with Fok 1 yields 74-mer tRNA upon in vitro transcription with the MegaShortScript kit. THG73 74-mer was then ligated to dCA-W or dCA-F_2W with T4 RNA ligase to generate THG73-aa.

EGFP mutants (pCS2gapEGFP and pEGFP-F Ser29TAG) and nAChR β subunit mutants (Leu9′Ser and Leu9′TAG) were made following the Quickchange mutagenesis protocol (Stratagene).

The mRNA that codes for the muscle type nAChR subunits (α, β, δ, and γ) was obtained by linearization of the expression vector (pAMV) with Not 1, followed by in vitro transcription using the mMessage mMachine kit.

Tissue Culture

CHO cells were grown at 37°C and 5% CO_2 in Ham's F12 media, enriched with glutamine, fetal bovine serum (FBS, 10%), penicillin, and streptomycin. 1 to 2 days prior to electroporation, the cells were passaged onto 35 mm tissue culture dishes such that confluency was typically 50% or less at the time of transfection.

Rat E18 hippocampal neurons were prepared as described previously [25]. Briefly, hippocampi were digested with 0.25% trypsin and then triturated. Cells plated in polylysine-coated 35 mm plastic dishes were maintained in Neurobasal medium supplemented with B27, 500 μM Glutamax, and 5% horse serum (Invitrogen). Transfections were done after 5 days in culture.

Electroporation

The DNA, mRNA, or tRNA to be electroporated into either CHO cells or neurons was precipitated alone or as coprecipitates in ethanol and ammonium acetate, and left at −20°C for at least 1 hr. For THG73-aa, the amino acids have a o-nitroveratryloxycarbonyl (NVOC) protecting group at the N terminus and were photo-deprotected immediately prior to electroporation. This consisted of irradiating a 15 μl solution of THG73-aa (1 μg/μl) in 1 mM NaOAc (pH 4.5) for 6 min, using a 1000 W Hg/Xe arc lamp (Oriel, Stratford, CT) operating at 400 W, equipped with WG355 and UG11 filters (Schott, Duryea, PA). Reporter EGFP DNA and nAChR subunit mRNA was then combined with this and precipitated with ammonium acetate and ethanol. This was then microcentrifuged at 15,000 rpm, 4°C for 15 min, vacuum dried for 5 min, and resuspended in CO_2 independent medium to the desired final concentration. Immediately prior to electroporation, the cell tissue culture media was swapped to CO_2 independent media (with no glutamine, FBS, or antibiotics). Approximately 5 μl of the electroporation solution was applied to the cells, followed by application of electrical pulses. For CHO cells, this was typically four 120 V pulses of 50 ms duration, and for neurons, four 150 V pulses of 25 ms duration. The CO_2 independent media was immediately replaced with fresh Ham's F12 for CHO cells, or the original neurobasal media for neurons, and the cells were placed back into the 37°C incubator. Imaging of EGFP was done as soon as 2 hr after transfection, and electrophysiogical recordings were done 24 hr after transfection.

CHO cells and neurons were typically transfected with EGFP DNA (2 μg/μl) with or without HSAS tRNA (4 μg/μl). CHO cells were transfected with nAChR mRNA for each of the subunits α (1 to 2 μg/μl), β, δ, and γ (0.5 μg/μl each), with or without HSAS tRNA (2 μg/μl) or THG73-aa (3 to 4 μg/μl) and wt pEGFP-N1 DNA (0.5 μg/μl).

Microscopy

CHO cells and neurons were visualized with an inverted microscope (Olympus IMT2), a 250 W Hg/Xe lamp operating at 150 W, a GFP filter set (Chroma, model 41017) with an excitation band pass of 450 to 490 nm and an emission band pass of 500 to 550 nm, 10×/0.25NA or 40×/1.3NA lens, and a Photometrix Quantix CCD camera running Axon imaging Workbench 4.0.

Electrophysiology

Whole-cell recordings were performed on EGFP-expressing cells. The cells were visualized using an inverted microscope as described above. Patch electrodes (borosilicate, 4–6 MΩ) were filled with a pipette solution containing 88 mM KH_2PO_4, 4.5 mM $MgCl_2$, 0.9 mM EGTA, 9 mM HEPES, 0.4 mM $CaCl_2$, 14 mM creatine phosphate, 4 mM Mg-ATP, 0.3 mM GTP (Tris salt), adjusted to pH 7.4 with KOH. The recording solution contained 150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, and 1 μM atropine, adjusted to pH 7.4 with NaOH. Standard whole-cell recordings were done using an Axopatch 1-D amplifier, low-pass filtered at 2–5 kKz and digitized online at 20 kHz (pClamp 8, Foster City, CA). The membrane potential was held at −60 mV.

Acetylcholine (ACh) was delivered using a two-barrel glass theta tube (outer diameter ~200 μm, pulled from 1.5 mm diameter theta borosilicate tubing) connected to a piezo-electric translator (Burleigh LSS-3100, Fisher, NY). Each barrel of the theta tube was fed from a 12-way manifold. This allowed up to 12 different solutions to be fed in either the control or agonist barrel. Agonists were applied for 25 ms, which was triggered by pClamp 8 software. The voltage input to the high-voltage amplifier (Burleigh PZ-150M, Fishers, NY) used to drive the piezo translator was filtered at 150 Hz by an 8-pole Bessel filter (Frequency Devices, Haverhill, MA), to reduce oscillations from rapid pipette movement. Solution exchange rates measured from open tip junction potential changes upon application with 10% recording solution were typically ~300 μs (10%–90% peak time).

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