Comparison of Cell Expression Formats for the Characterization of $GABA_A$ Channels Using a Microfluidic Patch Clamp System

Qin Chen,[1] Peter D. Yim,[2] Nina Yuan,[3] Juliette Johnson,[1] James M. Cook,[3] Steve Smith,[1] Cristian Ionescu-Zanetti,[1] Zhi-Jian Wang,[3] Leggy A. Arnold,[3] and Charles W. Emala[2]

[1]Fluxion Biosciences, South San Francisco, California.
[2]The Department of Anesthesiology, Columbia University, New York, New York.
[3]The Department of Chemistry and Biochemistry, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin.

ABSTRACT

Ensemble recording and microfluidic perfusion are recently introduced techniques aimed at removing the laborious nature and low recording success rates of manual patch clamp. Here, we present assay characteristics for these features integrated into one automated electrophysiology platform as applied to the study of $GABA_A$ channels. A variety of cell types and methods of $GABA_A$ channel expression were successfully studied (defined as $I_{GABA} > 500$ pA), including stably transfected human embryonic kidney (HEK) cells expressing $\alpha_1\beta_3\gamma_2$ $GABA_A$ channels, frozen ready-to-assay (RTA) HEK cells expressing $\alpha_1\beta_3\gamma_2$ or $\alpha_3\beta_3\gamma_2$ $GABA_A$ channels, transiently transfected HEK293T cells expressing $\alpha_1\beta_3\gamma_2$ $GABA_A$ channels, and immortalized cultures of human airway smooth muscle cells endogenously expressing $GABA_A$ channels. Current measurements were successfully studied in multiple cell types with multiple modes of channel expression in response to several classic $GABA_A$ channel agonists, antagonists, and allosteric modulators. We obtained success rates above 95% for transiently or stably transfected HEK cells and frozen RTA HEK cells expressing $GABA_A$ channels. Tissue-derived immortalized cultures of airway smooth muscle cells exhibited a slightly lower recording success rate of 75% using automated patch, which was much higher than the 5% success rate using manual patch clamp technique by the same research group. Responses to agonists, antagonists, and allosteric modulators compared well to previously reported manual patch results. The data demonstrate that both the biophysics and pharmacologic characterization of $GABA_A$ channels in a wide variety of cell formats can be performed using this automated patch clamp system.

INTRODUCTION

γ-Amino butyric acid (GABA) is the primary inhibitory neurotransmitter in the central nervous system. $GABA_A$ receptors belong to a family of ligand-gated ion channels mediating fast synaptic transmission. Ionotropic $GABA_A$ receptors consist of 5 protein subunits arranged around a central pore that constitutes the actual ion channel. There are 16 different subunits comprising the $GABA_A$ receptor family, including α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, and θ. $GABA_A$ receptor subtype combinations, $\alpha_1\beta_{2/3}\gamma_2$, $\alpha_2\beta_3\gamma_2$, and $\alpha_3\beta_3\gamma_2$, are the most abundant, and are mainly present in the brain. These channels conduct a Cl^- current when exposed to agonists. The increased Cl^- conductance drives the membrane potential toward the reversal potential of the Cl^- ion (~75 mV in neurons) inhibiting the firing of new action potentials.[1,2] $GABA_A$ receptors are of major importance as pharmacological targets for anxiolytics, anti-schizophrenics, anesthetics, and sleep aids.

Patch clamp recording is an information-rich assay that has become the gold standard in the study of ion channels, as well as for the selection and development of ion channel drug candidates. Patch clamp recordings are real-time measurements of the ionic currents passing through the cellular membrane of a single cell, providing a real-time recording of ion channel activity. Patch clamp studies define a compound's effect on channel desensitization, kinetics, and gating, which reveals many aspects of drug and ion channel interaction. While cell-based fluorescence assays for screening ion channels has proven to be powerful for assessing a large number of compounds quickly in ion channel targeted drug discovery efforts,[3,4] hit confirmation and mechanism of action studies require the use of patch clamp recordings.

Automated patch clamp systems have been developed over the last decade to achieve higher experimental throughput as compared to conventional manual patch clamp, which is slow and laborious. Automated patch clamp systems integrate robotics for liquid handling, and enables multiple recordings in parallel.[5–8] It is expected that increasing capacities of such systems will allow for a greater capacity in the conduct of ion channel studies and ion channel targeted compound screening.

ABBREVIATIONS: DMSO, dimethyl sulfoxide; EC_{50}, compound concentration at which ion channel current is 50% of the maximum response; FBS, fetal bovine serum; GABA, γ-amino butyric acid; HEK293, human embryonic kidney; hTERT, human telomerase reverse transcriptase; IC_{50}, compound concentration at which ion channel current is 50% of the current in the absence of compound; MEM/EBSS, minimum essential medium with Earle's balanced salts; PBS, phosphate-buffered saline; RTA, ready-to-assay.

DOI: 10.1089/adt.2011.415

The overwhelming majority of automated electrophysiology experiments employ recombinant cell lines stably overly expressing a particular ion channel of interest. Such recombinant cell lines are expensive to establish or acquire from vendors and may lack the normal channel kinetics of endogenously expressed channels. Currently, only a small number of stable recombinant cell lines expressing different $GABA_A$ channels are commercially available, which limits essential investigations to determine ligand effects based on different $GABA_A$ receptor subunit compositions. Furthermore, the gradual loss of expression levels of stably transfected plasmids at higher passages can influence the integrity of results. Recently, a new assay format, frozen ready-to-assay (RTA) cells, has been introduced to the market.[9] These cryopreserved cells are thawed on demand and offer convenience and improved consistency by avoiding variations between different cell passages. Furthermore, using frozen RTA cells decouples the experimental schedule from cell culture, which leads to important time savings. For smaller scale studies, RTA cells are also more affordable as compared to acquisition of the immortalized stable cell lines.

If the study of different $GABA_A$ subunit compositions is desired, transiently transfected cells allow maximum flexibility. The transient transfection of a specific combination of $GABA_A$ subunits can be performed on demand as long as the cDNA is available, while only maintaining one cell type in culture. The limitations of this approach include the expression heterogeneity of transfected plasmids, uncertainty in the true subunit combination achieved for a given ion channel and damage to the cell membrane during the transfection, which can lead to low success rates using manual patch or single-cell configured automated patch clamp systems.

Despite recent advances in transfection technology, disparities persist between experiments using recombinant cell lines as compared to primary cells with endogenous levels of ion channel expression that are extracted from tissue samples. In nontransfected cells, the assay targets are expressed in a more physiologically relevant environment, and the complex interplay of the endogenously expressed ion channels, second messengers, and other cell signaling proteins can be better recapitulated than in recombinant cell lines. Therefore, there is a growing interest in studying endogenously expressed ion channels to achieve a higher level of physiological relevance. Limitations of transiently transfected cells and natively expressing cells are overcome by the use of the IonFlux™ instrument, which records the average current and resistance of 20 cell ensembles and utilizes continuous microfluidic perfusion.

Previous publications have described the principles of operation for the IonFlux system, and shown electrophysiology and pharmacology characterization for both ligand-gated and voltage-gated ion channel targets on the IonFlux platform, which applies whole cell voltage clamp to an ensemble of 20 cells in parallel.[7] The IonFlux is the first microfluidic patch clamp system featuring continuous recording coupled with fast solution exchange—an ideal combination for the study of ligand gated channels.[7] In the present study, we focused on determining the robustness of GABA assays in a number of different cell expression formats and report the success rate, throughput, and accuracy of pharmacology results as compared to available manual patch clamp references. The different assay types explored were as follows: recombinant human embryonic kidney (HEK293) cells stably expressing the $\alpha_1\beta_3\gamma_2$ $GABA_A$ channel; frozen RTA recombinant HEK293 cells expressing either the $\alpha_1\beta_3\gamma_2$ or $\alpha_3\beta_3\gamma_2$ $GABA_A$ channel; transiently transfected HEK293T cells expressing the $\alpha_1\beta_3\gamma_2$ $GABA_A$ channel; immortalized *ex vivo* human airway smooth muscle cells endogenously expressing $GABA_A$ channels composed of α_4, α_5, β_3, γ_1, γ_2, δ, or θ subunits.[10–12] The side by side comparison of different cell expression formats provides an overview for the selection of the appropriate protocol and expression systems, outlining tradeoffs between biological relevance, assay flexibility, and raw experimental capacity. Ensemble recording coupled with continuous fluid perfusion yields high success rates for the study of $GABA_A$ receptors in transfected HEK293 cells or endogenously expressed in smooth muscle cells.

MATERIALS AND METHODS

Reagents

GABA, bicuculline, picrotoxin, muscimol, and diazepam were purchased from Sigma-Aldrich, and used without further purification. HZ166 was provided by Dr. James Cook.

Cell Culture and Preparation of Recombinant $GABA_A$ Receptor-Expressing HEK293 Cells

Cells expressing $hGABA_A$ (Millipore PrecisION™ $hGABA_A$ $\alpha_1\beta_3\gamma_2$-HEK Recombinant Cell Line; Cat# CYL3053) were cultured in 175-cm^2 filter-top flasks containing DMEM/F12 glutamax (Invitrogen #10569-010), 10% fetal bovine serum (FBS) (Thermo #SH30070.02), 1% nonessential amino acids (Invitrogen #11140), 400 µg/mL geneticin (Invitrogen #10131), 100 µg/mL hygromycin B (Invitrogen #10687), and 0.625 µg/mL puromycin (Clontech #631306) at 37°C and 5% CO_2. The cells were kept below 80% confluency and split once a week. For the cell preparation, flasks were first washed with 2 mL of Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS) followed by the addition of 5 mL of Detachin™ solution for cell detachment. After release, the cell suspension was spun for 90 s (1,000 rpm) prior to being resuspended in extracellular solution (5×10^6 cell/mL).

Cell Culture and Preparation of Frozen RTA Recombinant $GABA_A$ Receptor-Expressing HEK293 Cells

Two RTA cell lines—one expressing $hGABA_A$ $\alpha_1\beta_3\gamma_2$ (Millipore #CYL3053RTA) and the other one expressing $hGABA_A$ $\alpha_3\beta_3\gamma_2$ (Millipore #CYL3068RTA)—were tested for this study. For storage, cells were kept in liquid nitrogen before use. For the assay, cells were thawed by immediately immersing the vial in a 37°C water bath. As soon as the ice was thawed, 1 mL of prewarmed HEK293 serum-free media (Invitrogen #11686) was added to the vial. The cell solution was transferred dropwise into a 15-mL conical tube holding 10 mL of prewarmed HEK 293 serum-free media. The cell suspension solution was centrifuged at 1,000 rpm for 90 s to separate cells from freezing media. The cell pellet was resuspended in 10 mL warm HEK 293 serum-free media, and the cells were incubated for 30 min at 37°C,

washed, and resuspended in extracellular solution at a density of 3–5×10^6 cells/mL before the electrophysiology experiment.

Cell Culture and Preparation of Transiently Transfected HEK293T Cells Expressing the $\alpha_1\beta_3\gamma_2$ $GABA_A$ Channel

HEK293T cells (ATCC) were cultured at 37°C and 5% CO_2 in 75 cm^2 flasks using growth media composed of MEM/EBSS without phenol red but with L-glutamine (2 mM) and glucose (1 mM) (Hyclone #SH30024FS), 1% nonessential amino acids (Hyclone #SH3023801), 1 mM sodium pyruvate (Hyclone SH3023901), penicillin (100 units/mL) and streptomycin (100 μg/mL) (Hyclone #SV30010), and 10% heat inactivated FBS (Hyclone #SH3008803HI). All flasks were treated for 10 min at 37°C with a 1% Matrigel (BD #CB40230A) solution in MEM/EBSS and split three times a week. The transfections were performed at 50%–70% confluency. Therefore, growth media were aspirated and replaced with assay media containing MEM/EBSS without phenol red but with L-glutamine (2 mM), glucose (1 mM) (Hyclone #SH30024FS), 1% nonessential amino acids (Hyclone #SH3023801), 1 mM sodium pyruvate (Hyclone SH3023901), penicillin (100 units/mL) and streptomycin (100 μg/mL) (Hyclone #SV30010), and 10% dialyzed FBS (Invitrogen #26400-036). A transfection mixture was then added to the flask. The mixture consisted of 1.5 mL of serum-free MEM/EBSS media, 5 μg of each of the $GABA_A$ receptor subunit DNA (α_1, β_3, and γ_2), Lipofectamine™ LTX (75 μL), and PLUSTM reagent (25 μL) (Invitrogen, #15338-100). After 24 h, without changing media, the cells were washed with 10 mL of Ca^{2+} and Mg^{2+}-free PBS, followed by the addition of 3 mL of Detachin solution, after which the cells were incubated for 2 to 5 min depending on their confluency. The cell suspension was added to 7 mL of growth media to inhibit Detachin, followed by centrifugation (2 min at 1,000 rpm) and resuspension in serum-free MEM/EBSS media (5 mL). The centrifugation and resuspension were repeated two more times. Cells were then resuspended in HEK293 serum-free media (Invitrogen #11686) (5 mL) and placed on a shaker for 30 min. The cells were centrifuged again and then resuspended in extracellular solution (5 mL). The centrifugation and resuspension in extracellular solution was repeated two more times, resulting in cell suspension of 5×10^6 cells/mL, which was then used for electrophysiology experiments.

Cell Culture and Preparation of Immortalized Human Airway Smooth Muscle Cells Endogenously Expressing $GABA_A$ Channels

Human bronchial smooth muscle cells were a kind gift from Dr. William Gerthoffer (University of South Alabama) and were immortalized by the stable expression of human telomerase reverse transcriptase (hTERT).[10] Expression of hTERT extends the life span of smooth muscle cells, but does not affect the endogenous level of expressed channels. Cells were originally isolated from human lung surgical specimens under approved institutional review board (IRB) protocols. Cells were grown to confluence in 15-cm-diameter collagen-coated (BD Biosciences [354236] rat tail collagen type 1, using 5 $\mu g/cm^2$) plastic dishes. Cells were grown in M199 media containing 10% fetal bovine serum, 0.25 ng/mL epidermal growth factor, 1 ng/mL fibroblast growth factor, ITS supplement (1 mg/mL insulin, 0.55 mg/mL transferrin, and 0.67 μg/mL sodium selenium), and antibiotics (100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B) in a humidified atmosphere of 5% CO_2 at 37°C. Media were aspirated from the dishes and collagenase type IV (Sigma C5138) (500 units/mL in 5 mL basal M199 media) was added, and the dishes were incubated 15 min at 37°C in a cell culture incubator. Cells were released from the dish by gentle trituration using a pipette and transferred to a 15-mL conical tube for centrifugation (300 *g*, 3 min). The cell pellet was resuspended in the desired external buffer solution and washed twice. Cell solution ($3–5 \times 10^6$ cells/mL) was then loaded to the inlet wells in a 96-well IonFlux microfluidic plate.

Automated Patch Clamp and Ensemble Recording

The IonFlux platform utilizes integrated microfluidics and the wells on the 96-well plate are interconnected through microfluidic channels. Buffers, compound solutions, cells, and waste are deposited and handled in a single plate within a standard 96-well-plate format. The solutions are moved by pneumatic pumps, eliminating automated pipetting and possible cross contamination. The IonFlux plate layout consists of units of 12 wells: two wells contain intracellular solutions, 1 contains extracellular solution plus cells, 8 contain compounds of interest, and 1 well is used for waste collection. *Table* 1 shows an example of an assay protocol with the 12-well plate layout, loading parameters and assay steps. A 96-well plate contains 8 patterns, and can provide 16 recordings (384-well plates provide 64 recording channels). Cells are captured from suspension by applying suction to microscopic channels in the ensemble of the recording array. Once the array is fully occupied by 20 individual cells, the applied suction breaks the cell membrane of captured cells, establishing whole cell voltage clamp. The calculated current over time is dictated by the compound affinity, ion channel kinetics, and solution exchange speed. IonFlux utilizes microfluidics with the fluid flow regulated by valves and regulators that control the duration and timing of compound application, which are synchronous across the plate. Compound solutions can be introduced or replaced within ~100 ms (0%–90% washout),[7] which can well accommodate $GABA_A$ channel recording.

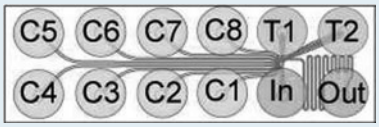
For compound applications, pressure was applied to the appropriate compound wells, introducing a compound dissolved in extracellular solution rapidly to the patch clamped cells. For recording of $GABA_A$ channel currents, cells were clamped at a holding potential of −80 mV. The extracellular solution was (in mM):138 NaCl, 4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, 5.6 glucose, pH 7.4 with NaOH. The intracellular solution was in mM: 60 KCl, 70 KF, 15 NaCl, 5 EGTA, 5 HEPES, pH 7.2 with KOH.

Each GABA application lasted for 3 s, followed by a 30 to 60 s of wash to allow the channels to recover in stably transfected cultured or RTA HEK293 cells. Bicuculline or picrotoxin at each concentration was preincubated for 3 min before GABA (10 μM) and bicuculline or picrotoxin co-application. Allosteric ligands diazepam or HZ166 were co-applied for stably transfected and transiently transfected cell with 1 or 0.3 μM GABA, respectively.

Table 1. Example of Assay Protocol

Step	Procedure	Value
1	Remove IonFlux™ plate from 4°C refrigerator, empty, and warm up to room temperature. Each 96- or 384-well plate allows 8 or 32 experimental paradigms, respectively.	
2	Dispense intracellular buffer into well T1 and T2 of the IonFlux plate.	250 µL/96-well plate 70 µL/384-well plate
3	Dispense desired compounds into wells C1–C8 of the IonFlux plate.	200 µL/96-well plate 50 µL/384-well plate
4	Dispense external buffer into well IN of the IonFlux plate.	250 µL/96-well plate 70 µL/384-well plate
5	Prime plate.	
6	Remove external buffer from IN and OUT wells.	
7	Dispense cell suspension into IN well of the customized IonFlux plate.	250 µL/96-well plate 70 µL/384-well plate
8	Run experiment.	

Nonsterile well arrangement:



Cells are resuspended in serum-free media and placed on a shaker for 30 min, followed by three times media exchange with extracellular solution at a density of 5×10^6 cells/mL prior to assay.

Data Analysis

Data analysis and graphical presentation were performed using a combination of IonFlux software, GraphPad Prism 5, and Microsoft Excel. Dose–response curves were analyzed using nonlinear regression (GraphPad Prism 5, variable slope, sigmoidal dose response). All data are shown with arithmetic mean ± standard error of mean (SEM).

RESULTS

The Current Response to GABA or Muscimol in Cultured Recombinant HEK293 Cells Stably Expressing the $\alpha_1\beta_3\gamma_2$ $GABA_A$ Channel Was Reliably Determined with the Automated Patch Clamp Instrument IonFlux

The inward Cl^- current ensemble of 20 HEK293 cells, stably expressing the $\alpha_1\beta_3\gamma_2$ $GABA_A$ channel in the presence of 10 µM GABA, is shown in *Figure 1a* as a screen capture of IonFlux software. The GABA-evoked Cl^- current increased to peak (10%–90%) within 400 ± 50 ms. GABA concentrations between 0.1 and 30 µM were applied to determine the dose response of HEK293 cells that stably express the $\alpha_1\beta_3\gamma_2$ $GABA_A$ channel (*Fig. 1b*). At higher GABA concentrations, the channel showed faster desensitization (*Fig. 1b*, *inset*). The wash out of GABA using extracellular solution took ~500 ms. The calculated EC_{50} value from dose response was 2.87 ± 0.24 µM, which is similar to the values reported in the literature, which ranged from 2.00 to 3.29 µM.[13–15] A second classic $GABA_A$ agonist, muscimol, was used to demonstrate correct pharmacology and desensitization kinetics using this microfluidic platform in these HEK293 cells stably transfected with the $\alpha_1\beta_3\gamma_2$ $GABA_A$ channel (*Fig. 1c*). Muscimol showed faster desensitization than GABA stimulation as expected. Muscimol (0.1–100 µM) induced a dose-dependent current with an EC_{50} of 1.43 ± 0.38 µM consistent with values published with this $GABA_A$ channel composition in HEK cells.[16] Additionally, the consistency of measurements from plate to plate was determined for eight plates comparing the GABA (10 µM)-induced Cl^- currents of 128 cell ensembles across 8 plates compared to a vehicle control (0.1% dimethyl sulfoxide [DMSO]) in the extracellular solution (*Fig. 1d*). Average current from 10 µM GABA was 14.95 ± 3.21 nA, whereas vehicle control (0.1% DMSO) yielded 0.16 ± 0.03 nA, which was about 2 orders of magnitude lower than the measured GABA current.

Pharmacological Responses of Cultured Recombinant HEK293 Cells Stably Expressing the $\alpha_1\beta_3\gamma_2$ $GABA_A$ Channel in the Presence of the Antagonists Bicuculline or Picrotoxin or the Allosteric Modulator Diazepam as Determined in the IonFlux Microfluidic Platform

Figure 2a depicts the dose–response inhibition of 10 µM GABA by the competitive $GABA_A$ antagonist bicuculline at the $\alpha_1\beta_3\gamma_2$ $GABA_A$ channel stably expressed in HEK293 cells. The calculated IC_{50} value of 13.9 ± 0.5 µM is higher than the reported IC_{50} value of 1.9 ± 0.3 µM.[17–19] Similarly, 10 µM GABA was dose dependently inhibited by the $GABA_A$ channel antagonist picrotoxin (0.3–100 µM) with an IC_{50} of 13.6 ± 0.3 µM in HEK293 cells stably expressing the $\alpha_1\beta_3\gamma_2$ $GABA_A$ channel (*Fig. 2b*).[13,20] $GABA_A$ receptors containing α_1, α_2, α_3, or α_5, any β subunit, and γ_2 subunits are sensitive to benzodiazepine modulation.[1,21] Benzodiazepines function as positive allosteric modulators of GABA at $GABA_A$ channels and have been the focus of intense research due to the extensive therapeutic use of benzodiazepines. The dose-dependent potentiation of diazepam (0.01–10 µM) in the presence of 1 µM GABA in HEK293 cells stably transfected with the $\alpha_1\beta_3\gamma_2$ $GABA_A$ channel is shown in *Figure 2c*. The calculated EC_{50} value for diazepam was 420 ± 10 nM with a current potentiation of ~300%, which is similar to values reported in the literature using manual patch clamp techniques.[22]

Frozen RTA HEK293 Cells, Stably Expressing $GABA_A$ Channels $\alpha_1\beta_3\gamma_2$ or $\alpha_3\beta_3\gamma_2$, Yielded Similar Results as Cultured HEK293 Cells Stably Expressing the Same Channels

Two RTA HEK293 cell lines stably expressing $\alpha_3\beta_3\gamma_2$ or $\alpha_1\beta_3\gamma_2$ $GABA_A$ channels were compared with cultured recombinant cells

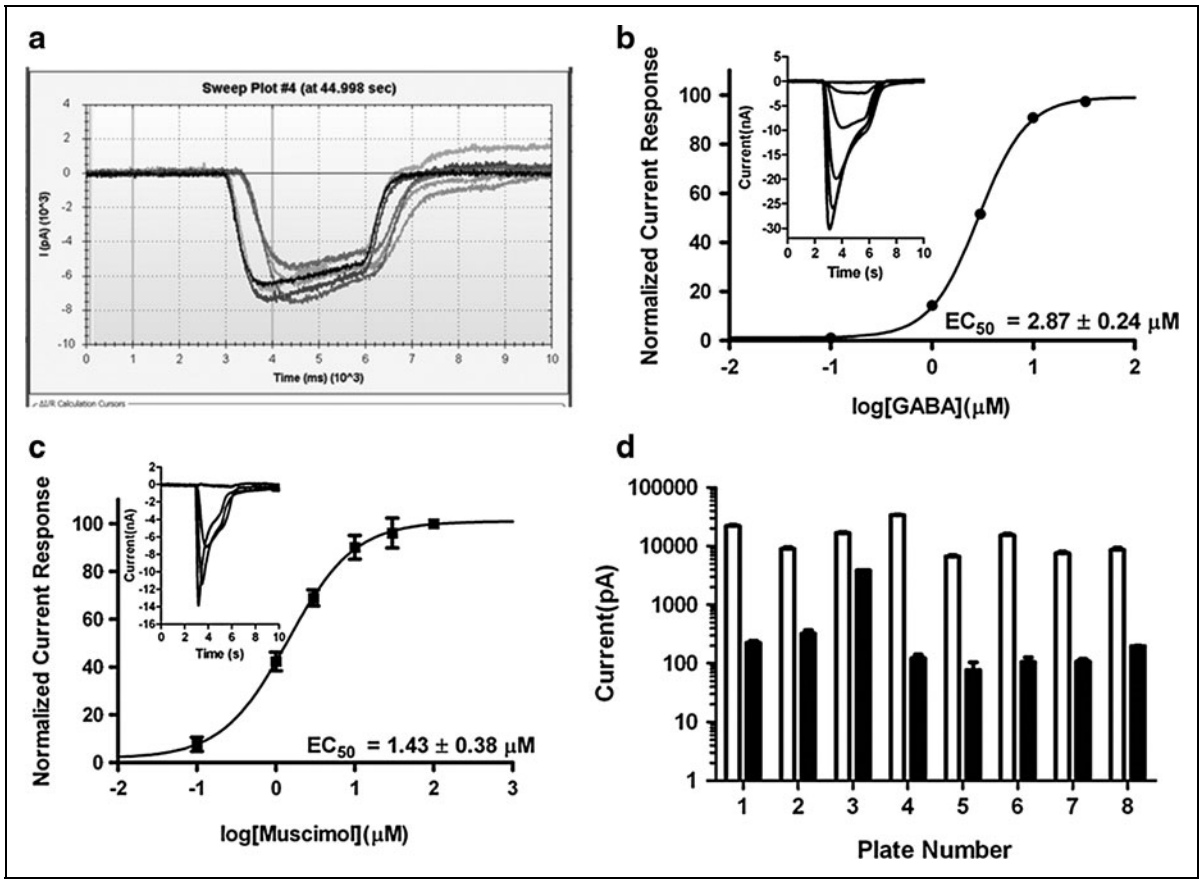


Fig. 1. **(a)** Representative current sweeps of GABA-induced current from HEK cells stably transfected with $\alpha_1\beta_3\gamma_2$ GABA$_A$ receptors. **(b)** GABA dose–response curve (0.1–30 μM) in recombinant HEK cells expressing the $\alpha_1\beta_3\gamma_2$ GABA$_A$ receptor ($n=40$). Current data of independent measurements in duplicate were normalized to highest response for each dose response used for fitting data to a four-parameter (variable slope) nonlinear regression equation. (*Inset*) Inward Cl^- currents are shown from one ensemble of cells exposed to GABA with increasing concentrations (0.1–30 μM). **(c)** Muscimol dose–response curve (0.1–100 μM) in stably transfected HEK cells expressing the $\alpha_1\beta_3\gamma_2$ GABA$_A$ receptor ($n=6$). Current data of independent measurements in duplicate were normalized to highest response for each dose response used for fitting data to a four-parameter (variable slope) nonlinear regression equation. (*Inset*) Inward Cl^- currents are shown from one ensemble of cells exposed to muscimol with increasing concentrations (0.1–100 μM). **(d)** Consistency of GABA-induced current responses in HEK cell stably expressing the $\alpha_1\beta_3\gamma_2$ GABA$_A$ receptor. Eight plates were tested, and the average current at 10 μM GABA was ~10 nA (shown as white bars), whereas the vehicle control (0.1% dimethyl sulfoxide) yielded an ~100 pA current, which was 2 orders of magnitude lower than GABA current, and shown as black bars ($P=0.003$). GABA, γ-amino butyric acid; HEK, human embryonic kidney.

expressing the $\alpha_1\beta_3\gamma_2$ GABA$_A$ channel using an IonFlux-HT plate, which can perform 64 recordings in parallel. *Figure 3* depicts representative dose–response sweeps of GABA dose responses (0.3–30 μM) in HEK293 RTA cells expressing the $\alpha_1\beta_3\gamma_2$ (*Fig. 3a*) or $\alpha_3\beta_3\gamma_2$ (*Fig. 3b*) GABA$_A$ channels. GABA was more potent at $\alpha_3\beta_3\gamma_2$ GABA$_A$ channel (EC_{50} value = 0.9 ± 0.04 μM) as compared to cells expressing $\alpha_1\beta_3\gamma_2$ GABA$_A$ channels (EC_{50} value = 1.8 ± 0.1 μM) (*Fig. 3c*). The normalized response at 10 μM GABA showed that the $\alpha_3\beta_3\gamma_2$ GABA$_A$ channel had a slower on time and desensitization as compared to the $\alpha_1\beta_3\gamma_2$ GABA$_A$ channel (*Fig. 3d*). Cultured recombinant HEK293 cells and RTA HEK293 cells with the same GABA$_A$ channel, $\alpha_1\beta_3\gamma_2$, showed very similar kinetics profiles. Previous studies substantiate that the EC_{50} value and affinity of GABA agonists are highly dependent on subunit composition of GABA$_A$ channels.[17,23–25] The EC_{50} values are in good agreement with previous manual patch clamp studies. The average seal resistance from cultured HEK cells on the same plate was ~700 MΩ, as compared to ~500 MΩ from RTA HEK cells.

Transiently Transfected HEK293T Cells Expressing GABA$_A$ Channel $\alpha_1\beta_3\gamma_2$ Yield Robust Currents in Response to GABA, Bicuculline, and the Benzodiazepine-Site Modulator HZ166

The GABA dose response (0.3–30 μM) for transiently transfected HEK293 cells expressing the $\alpha_1\beta_3\gamma_2$ GABA$_A$ channel is shown in *Figure 4a* along with an inset of current sweeps. The GABA response kinetic profile in these transiently transfected cells was similar to the kinetic profile in response to GABA seen in stably transfected

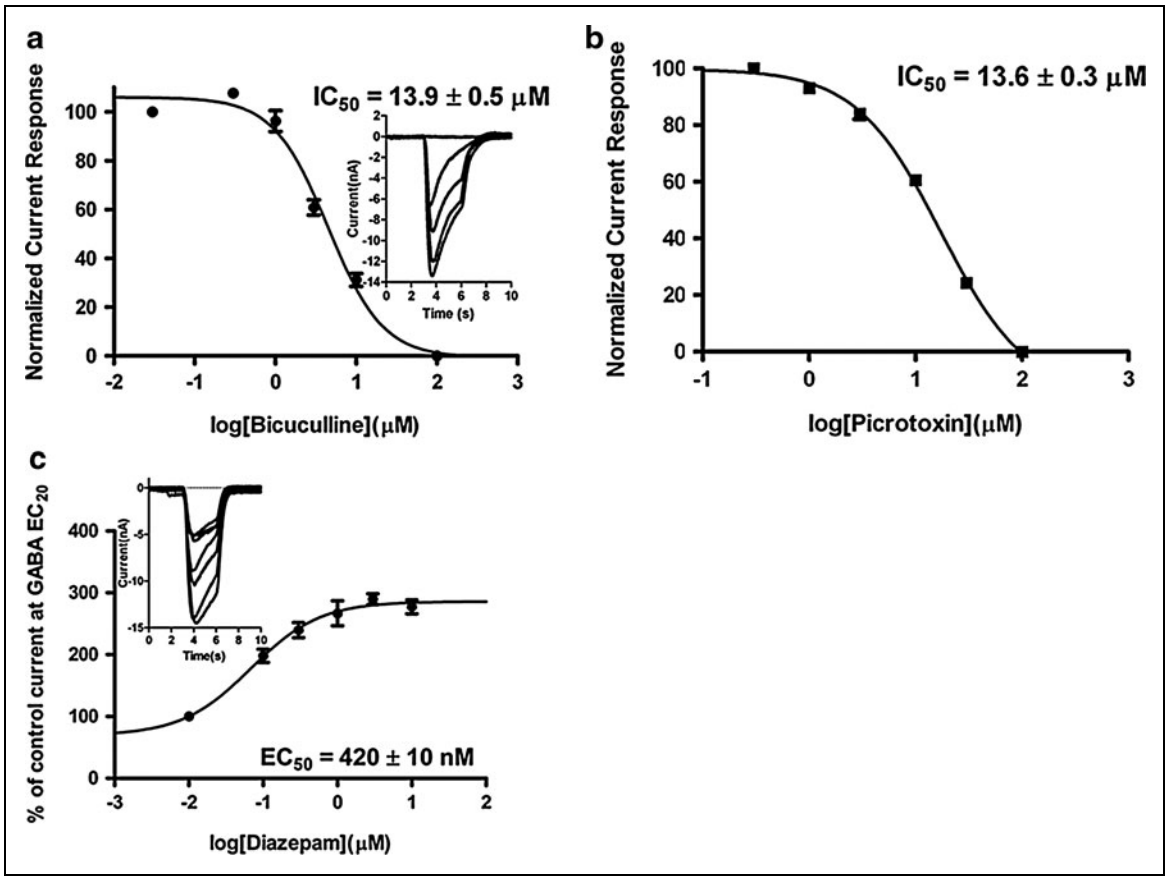


Fig. 2. **(a)** Dose-dependent inhibition of 10 μM GABA (an EC_{80}) by co-application of the $GABA_A$ receptor antagonist bicuculline (0.03–100 μM) in HEK cells stably expressing $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors. Current data of $n = 5$ independent measurements in duplicate were normalized to highest response for each dose response and used for fitting data to a four-parameter (variable slope) nonlinear regression equation. (*Inset*) Representative dose–response sweeps of bicuculline (0.03–100 μM) inhibiting 10 μM GABA-induced current from one ensemble of HEK cells stably transfected with $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors. **(b)** Dose-dependent inhibition of 10 μM GABA (an EC_{80}) by co-application of the $GABA_A$ receptor antagonist picrotoxin (0.5–100 μM) in HEK cells expressing $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors. Current data of $n = 6$ independent measurements in duplicate were normalized and used for fitting data to a four-parameter (variable slope) nonlinear regression equation. **(c)** Dose-dependent potentiation of 1 μM GABA (an EC_{20}) by co-application of the $GABA_A$ receptor allosteric enhancer diazepam (0.01–10 μM) in HEK cells expressing $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors. Current data of $n = 4$ independent measurements in duplicate were normalized and used for fitting data to a four-parameter (variable slope) nonlinear regression equation. (*Inset*) Inward Cl^- currents are shown from one ensemble of cells exposed to diazepam (0.01–10 μM) in the presence of 1 μM GABA (EC_{20}).

HEK293 cells expressing the same subtype ($\alpha_1\beta_3\gamma_2$) of $GABA_A$ channels (*Fig. 1b*). The calculated EC_{50} value was 1.3 ± 0.29 μM, which is well in the range with EC_{50} values of 1.8–2.9 μM from the cultured or RTA HEK293 cells expressing this same $GABA_A$ channel subtype (*Fig. 3c*). Dose-dependent inhibition of GABA-induced currents was demonstrated by bicuculline (0.03–100 μM). After a 3-min pretreatment with varying concentrations of bicuculline, a 4-s co-application of bicuculline with 1 μM GABA (EC_{80}) (*Fig. 4b*). The calculated IC_{50} value was 15.9 ± 5.45 μM, a value similar to the IC_{50} of bicuculline in HEK293 cells stably expressing the $\alpha_1\beta_3\gamma_2$ $GABA_A$ channel (*Fig. 2a*). HZ166 is benzodiazepine-based compound that exhibits antiseizure activities in mice and rats.[26] To determine its ability to modulate the $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptor, transiently transfected HEK293T cells were first exposed to 0.3 μM GABA (EC_{20}) followed by an application of HZ166 and a co-application of HZ166 and 0.3 μM GABA. At a concentration of 30 μM, HZ166 showed a current potentiation of ~200%. The modulation and the determined IC_{50} value of 670 ± 290 nM was similar to previously reported results using transfected oocytes.[26,27]

The Activity of Endogenous $GABA_A$ Channel in Human Airway Smooth Muscle Cells Can Be Determined with the IonFlux

The expression and function of endogenous ionotropic $GABA_A$ channels on airway smooth muscle cells was reported recently.[11,12,28–30] *Figure 5a* demonstrates a GABA dose response (1–1,000 μM) eliciting current in human immortalized airway smooth muscle cells using the IonFlux platform. The determined EC_{50} value

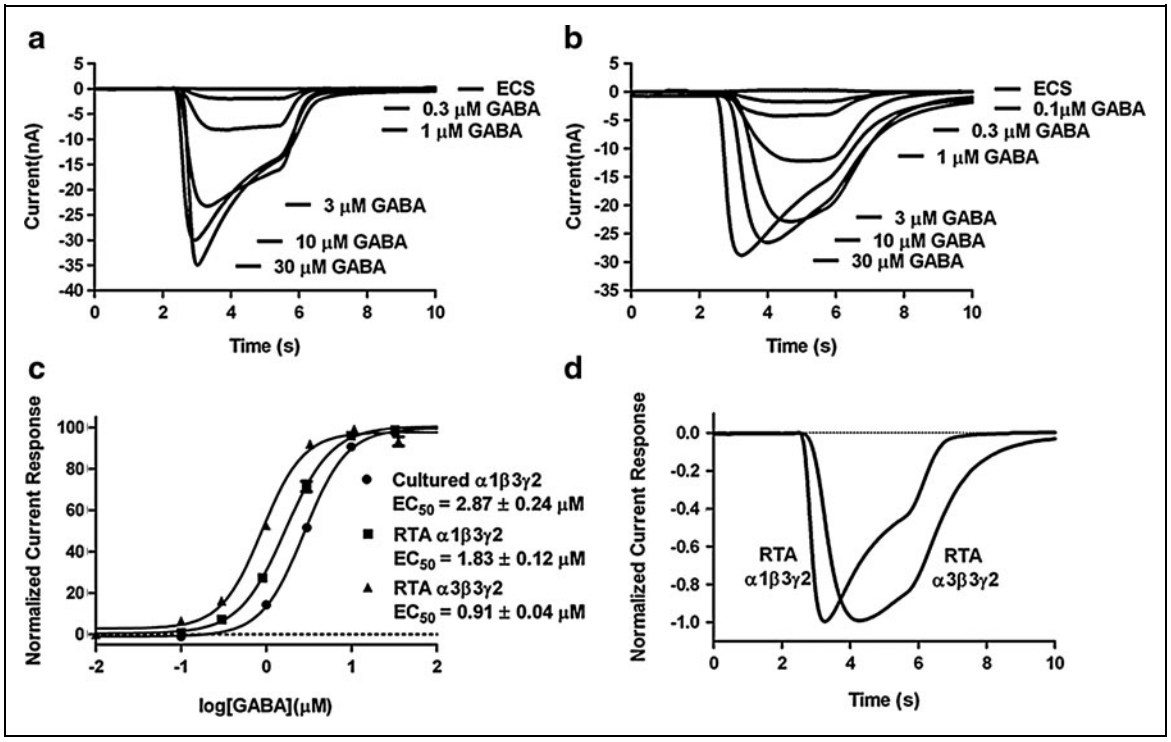


Fig. 3. **(a)** Representative dose–response sweeps of GABA (0.3–30 μM)-induced inward Cl^- current from one ensemble of ready-to-assay (RTA) HEK cells transfected with $\alpha_1\beta_3\gamma_2$ GABA$_A$ receptors. **(b)** Representative dose–response sweeps of GABA (0.3–30 μM)-induced inward Cl^- current from one ensemble of RTA HEK cells transfected with $\alpha_3\beta_3\gamma_2$ GABA$_A$ receptors. **(c)** Dose–response curves of GABA (0.1–30 μM)-induced currents in cultured HEK cells stably expressing the $\alpha_1\beta_3\gamma_2$ subtype, (●) ($n=16$), RTA HEK cells expressing the $\alpha_1\beta_3\gamma_2$ subtype (■) ($n=14$), and RTA HEK cells expressing the $\alpha_3\beta_3\gamma_2$ subtype (▲) ($n=8$). **(d)** Overlay of representative current sweeps with 10 μM GABA in RTA HEK cells expressing either the $\alpha_1\beta_3\gamma_2$ or $\alpha_3\beta_3\gamma_2$ GABA$_A$ receptor. ECS, extracellular solution.

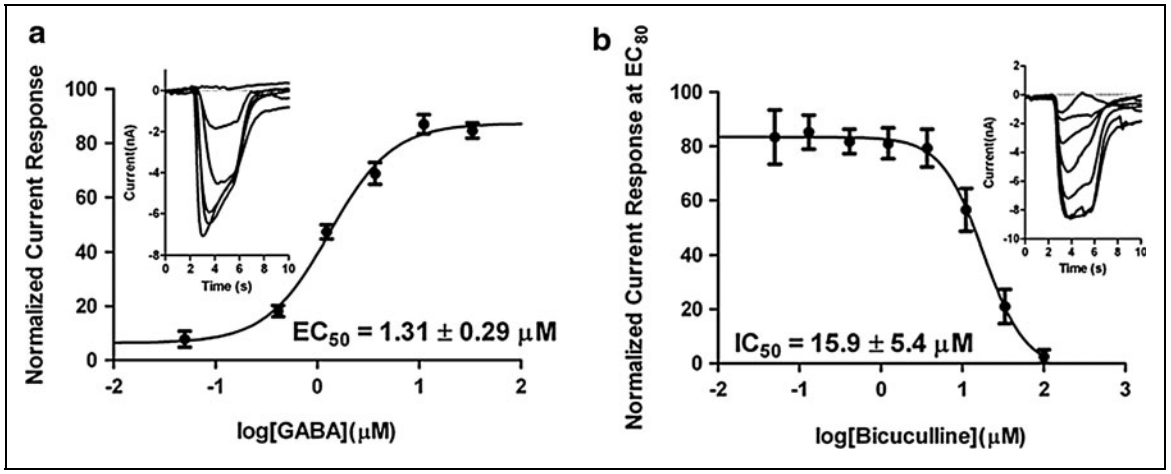


Fig. 4. **(a)** GABA dose–response curve (0.03–30 μM) using transiently transfected HEK293T cells expressing the $\alpha_1\beta_3\gamma_2$ channel. (*Inset*) Inward Cl^- currents are shown from one ensemble of cells exposed to GABA with increasing concentrations (0.03–30 μM). Current data of $n=26$ independent measurements in duplicate were normalized to highest response for each dose response used for fitting data to a four-parameter (variable slope) nonlinear regression equation. **(b)** Bicuculline dose–response curve (0.03–100 μM) using transiently transfected HEK293T cells expressing the $\alpha_1\beta_3\gamma_2$ channel. Current data of $n=8$ independent measurements in duplicate were normalized to highest response for each dose response and used for fitting data to a four-parameter (variable slope) nonlinear regression equation. (*Inset*) Inward Cl^- currents are shown from one ensemble of cells exposed to GABA (EC_{80} concentration = 10 μM) and increasing concentrations of bicuculline (0.03–100 μM). (*Continued*)

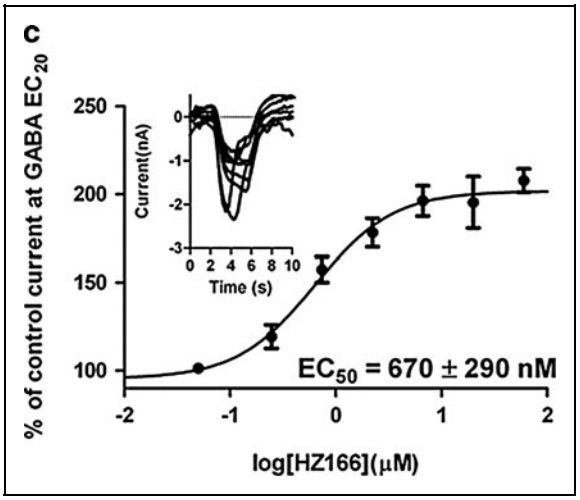


Fig. 4 (*continued*). **(c)** HZ166 dose–response curve (0.03–60 μM) using transiently transfected HEK293T cells expressing the $\alpha_1\beta_3\gamma_2$ channel. Current data of $n = 8$ independent measurements in duplicate were normalized to highest response for each dose response used for fitting data to a four-parameter (variable slope) nonlinear regression equation. The GABA concentration of 0.13 μM was potentiated 200% in the presence of HZ166. (*Inset*) Inward Cl^- currents are shown from one ensemble of cells exposed to GABA (EC_{20} concentration = 0.3 μM) with increasing concentrations of HZ166 (0.03–60 μM).

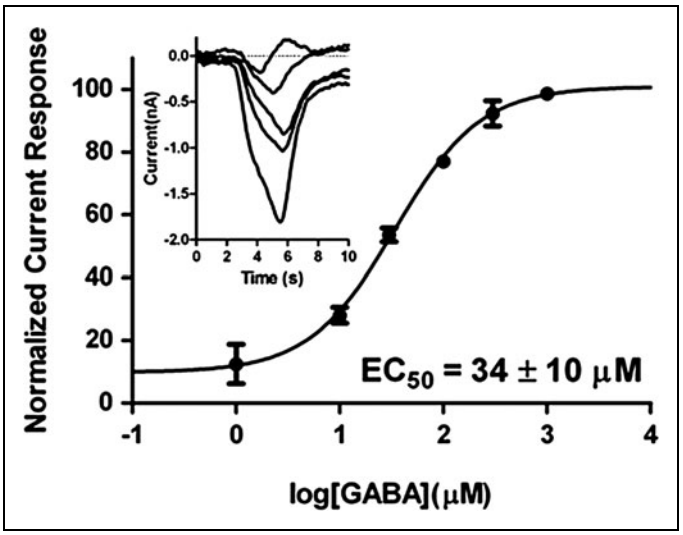


Fig. 5. GABA dose–response curve (1–1000 μM) in cultured human airway smooth muscle cells. Current data of $n = 6$ independent measurements in duplicate were normalized to highest response for each dose response used for fitting data to a four-parameter (variable slope) nonlinear regression equation. (*Inset*) Inward Cl^- currents are shown from one ensemble of cells exposed to GABA with increasing concentrations (1–1000 μM).

was 34 ± 10 μM. The average seal in these airway smooth muscle cells on IonFlux was ~100 MΩ. About 12/16 human airway smooth muscle cell ensembles demonstrated a detectable current (defined as current >500 pA). The success rate of measuring these GABA-induced currents in an ensemble of cells was ~75% as compared to manual whole cell patch clamping of single cells that had a success rate of 5% (data not shown).

High Success Rates for GABA Responses in Stably Transfected (in Active Culture or RTA), Transiently Transfected, and Native Cells with Endogenous $GABA_A$ Channel Expression Using the IonFlux Were Observed

A high average seal resistance for patch clamp assays is essential in order to measure changes in ion flux. Although seal resistances of higher than 1 GΩ were reported for manual patch clamp, similar values were observed for the automated patch clamp. Recombinant HEK293 cells, expressing $GABA_A$ channels, exhibited seal resistances between 500 and 700 MΩ in whole cell (*Fig. 6a*). Transient transfected cells and native airway smooth muscle cells showed seal resistances between 60 and100 MΩ. Nevertheless, high success rates were observed, defined as a measurable current of >500 pA (*Fig. 6b*). The difference in success rates of GABA-induced currents was most pronounced in native human airway smooth muscle cells endogenously expressing $GABA_A$ channels. This automated microfluidic platform method of measuring current from an ensemble of cells yielded detectable currents in 75% of experiments compared with only a 5% success rate using traditional single whole cell manual recordings, representing a significant advance and throughput for current studies in these cells.

DISCUSSION

Electrophysiology studies of $GABA_A$ channels were performed on the IonFlux automated patch clamp system, in various cell expression formats including recombinant cell lines stably expressing a specific $GABA_A$ channel (Millipore, $\alpha_1\beta_3\gamma_2$), frozen RTA recombinant cells expressing two different subtypes of $GABA_A$ channels (Millipore RTA, $\alpha_1\beta_3\gamma_2$ and $\alpha_3\beta_3\gamma_2$), transiently transfected HEK293T cells expressing the $\alpha_1\beta_3\gamma_2$ GABA channel, and immortalized human airway smooth muscle cells endogenously expressing $GABA_A$ channels. One $GABA_A$ channel ($\alpha_1\beta_3\gamma_2$) was tested in three different assay formats using cultured recombinant HEK293 cells, RTA-frozen recombinant stably transfected HEK293 cells, and transiently transfected HEK293T cells (*Figs. 3a, 4c*). The results for this particular subtype showed a high level of consistency for GABA-induced current kinetics profile with EC_{50} values of 2.9 ± 0.1 μM (stably transfected), 1.8 ± 0.1 μM (RTA), and 1.3 ± 0.29 μM (transiently transfected), respectively. Different $GABA_A$ channels showed distinctive current desensitization, and potency profiles in response to GABA stimulation as expected given the different biologic function of the receptor subtypes. Dose–response studies of the $GABA_A$ receptor antagonists bicuculline and picrotoxin and the allosteric modulators diazepam and HZ166 confirmed the correlation of responses between automated and manual patch clamp. Overall, the improved success rates observed for the IonFlux in comparison with manual patch clamp demonstrates that automation does not compromise quality.

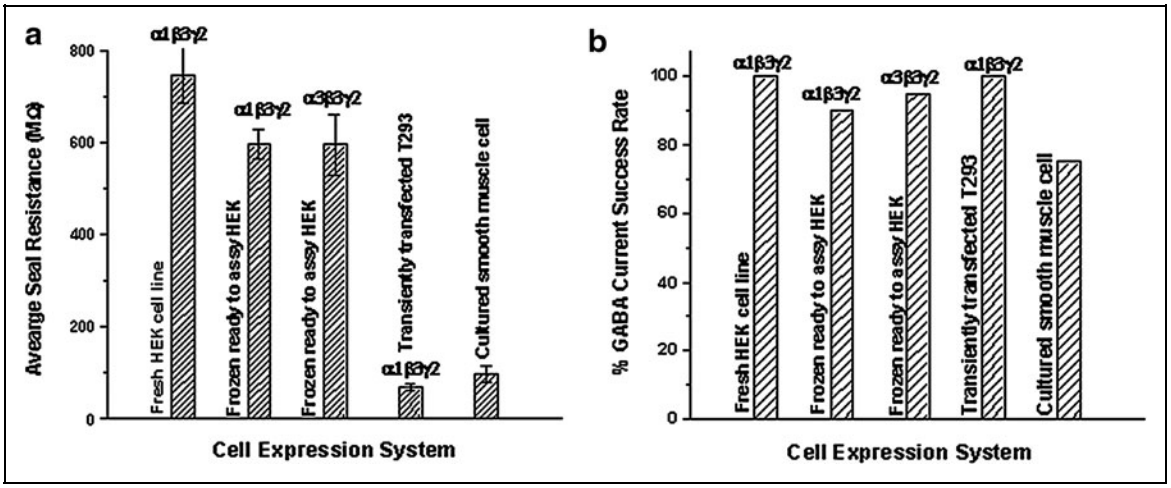


Fig. 6. **(a)** Average seal resistance (MΩ) for different cell types expressing GABA$_A$ receptors as measured on IonFlux™. **(b)** The success rate of recording GABA-induced currents (defined as measurable current >500 pA) on IonFlux™ from ensembles of 20 cells from different cell types expressing GABA$_A$ receptors.

HEK293 cells are useful for the performance for the automated patch clamp assay, because HEK293 cells can be easily prepared as a monodisperse suspension. Nevertheless, other cell lines such as primary cells have been successfully used. The IonFlux utilizes the ensemble recording with 20 cells patched per ensemble. Both current and seal resistance are averaged during the recording, significantly improving the success rate for assays where cells have heterogeneous expression levels, such as transiently transfected and primary cells.

Endogenous GABA$_A$ channels expressed by primary cells provide the most physiological relevant information, but also present the most challenging assay format due to the low expression level and heterogeneity of channel expression. The results from endogenous GABA$_A$ channels from immortalized human airway smooth muscle cells demonstrated that IonFlux system can measure GABA-dependent currents from endogenously expressed ion channels at a much higher success rate (75% for immortalized smooth muscle cells in a 20-cell ensemble, as compared to <5% success using manual patch clamp in single cells).

The EC$_{50}$ values for GABA are very different in transfected HEK cells overexpressing large numbers of pure populations of a given GABA$_A$ receptor subunit combination compared to the GABA$_A$ receptors endogenously expressed in native human airway smooth muscle cells. We believe that this difference is accounted for by the subunit composition of the GABA$_A$ channel in human airway smooth muscle cells whose native stoichiometry and composition are not yet fully elucidated.[11] We were the first to describe GABA$_A$ receptors on human airway smooth muscle cells and a complete mRNA survey of all known subunits demonstrated a limited repertoire of subunits including the α_4, α_5, β_3, γ_1, γ_2, γ_3, δ, π, and θ subunits.[11] However, the precise combinations of subunits that comprise the native GABA$_A$ receptor in native human airway smooth muscle cells remain unknown. It is possible that compositions that include the atypical subunits (*e.g.*, π and θ) may account for a decreased affinity for GABA in smooth muscle as has been demonstrated by the co-expression of the θ subunit with the $\alpha_2\beta_3\gamma_1$ subunits in HEK cells, which demonstrated an increased GABA EC$_{50}$ value of 62 μM.[31] Further support of this altered agonist affinity of GABA$_A$ receptors in airway smooth muscle is supported by a recent publication[12] using the same cells in this same microfluidic platform demonstrating an EC$_{50}$ value of 15 μM for THIP (gaboxadol), which is 10-fold higher than EC$_{50}$ values reported for classic heterologously expressed GABA$_A$ channels not containing an α_4 subunit and nearly 100-fold higher than EC$_{50}$'s for heterologously expressed GABA$_A$ channels containing α_4 subunits.

The GABA$_A$ receptors belong to an important family of ligand-gated ion channels, which are widely distributed in human central nervous system and serve as the predominant inhibitory channel. However, it is challenging to characterize the large number of subunit combinations expressed in GABA$_A$ channels with traditional recombinant cell lines because only a few types of stably transfected cell lines expressing different GABA$_A$ channels are available. Furthermore, it is very costly and time consuming to maintain these cells in culture. Commercially available frozen RTA cells are an excellent alternative, allowing researchers to determine ion channel activity without being equipped to maintain a large number of mammalian cells in culture, and provide larger, well-characterized production lots.

Transiently transfected cells, on the other hand, provide maximum flexibility in studying different ion channel subtypes, even those that are not available as stably transfected cells. The IonFlux system records from a 20-cell ensemble and features continuous microfluidics compound perfusion, which overcomes the challenges from expression heterogeneity, and achieves a better success rate. These features compensate for heterogeneous expression levels and reduce the total assay time as compared to other systems that require robotic pipetting for compound applications.

The average seals across the ensemble varied between 100 and 600 MΩ, depending on the cell type used. This is due to both the substrate used, and the fact that resistances are in parallel and the overall resistance will be dominated by the lowest resistance in the 20-cell ensemble.

In conclusion, we have evaluated the performance of the IonFlux-automated patch clamp systems for use with a variety of $GABA_A$ channel cell expression systems. Manual patch clamp is a recognized standard for obtaining electrophysiology data, but also a labor-intensive and low throughput approach, which usually requires years of user training to master; the presented approach increases throughput by over an order of magnitude and reduces the training period to weeks. For the cell lines tested, we also found the success rates to be increased considerably through the use of ensemble recording as compared to single cell recording.

We anticipate that automated patch clamp instruments will accelerate current electrophysiology research and provide us with the opportunity to better understand the distribution and function of ion channels. Additionally, these instruments will facilitate the screening of small molecules to identify new drug candidates. Finally, it makes ion channel research accessible to a broader spectrum of researchers, who may otherwise be discouraged by the challenges of traditional patch clamping techniques.

ACKNOWLEDGMENTS

The α_1, β_3, and γ_2 plasmids were kindly provided by Prof. Dr. Werner Sieghart (Department of Biochemistry and Molecular Biology of the Nervous System, Medical University of Vienna, Austria).

DISCLOSURE STATEMENT

J.M.C., N.Y., and L.A.A. are employees of the University of Wisconsin–Milwaukee. Their work was supported by the National Institutes of Health NIMH MIL100371. P.D.Y. was supported by NIH GM008464 and C.W.E. was supported by GM065281. Q.C., J.J., S.S., and C.I.Z. are all employees of Fluxion Biosciences.

REFERENCES

1. Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN, Langer SZ: International Union of Pharmacology. XV. Subtypes of gamma-aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. *Pharm Rev* 1998;50:291–313.
2. Mohler H: GABA(A) receptor diversity and pharmacology. *Cell Tissue Res* 2006;326:505–516.
3. Lubin ML, Reitz TL, Todd MJ, Flores CM, Qin N, Xin H: A nonadherent cell-based HTS assay for N-type calcium channel using calcium 3 dye. *Assay Drug Dev Technol* 2006;4:689–694.
4. Schroeder KS, Neagle BD: FLIPR: a new instrument for accurate, high throughput optical screening. *J Biomol Screen* 1996;1:75–80.
5. Finkel A, Wittel A, Yang N, Handran S, Hughes J, Costantin J: Population patch clamp improves data consistency and success rates in the measurement of ionic currents. *J Biomol Screen* 2006;11:488–496.
6. Dunlop J, Bowlby M, Peri R, Vasilyev D, Arias R: High-throughput electrophysiology: an emerging paradigm for ion-channel screening and physiology. *Nat Rev Drug Discov* 2008;7:358–368.
7. Golden AP, Li N, Chen Q, Lee T, Nevill T, Cao X, Johnson J, Erdemli G, Ionescu-Zanetti C, Urban L, Holmqvist M: IonFlux™: a microfluidic patch clamp system evaluated with human Ether-a-go-go related gene channel physiology and pharmacology. *Assay Drug Dev Technol* 2011;9:608–619.
8. Farre C, Haythornthwaite A, Haarmann C, Stoelzle S, Kreir M, George M, Bruggemann A, Fertig N: Port-a-patch and patchliner: high fidelity electrophysiology for secondary screening and safety pharmacology. *Comb Chem High Throughput Screen* 2009;12:24–37.
9. TerWee JA, Chin CL, Watrin S, Tello RF, Rieder NJ, Lowell JD, Latham-Timmons D: Increased consistency and efficiency in routine potency testing by bioassay with direct use of cryopreserved (ready-to-plate) cells. *J Immunol Meth* 2011;370:65–74.
10. Gosens R, Stelmack GL, Dueck G, McNeill KD, Yamasaki A, Gerthoffer WT, Unruh H, Gounni AS, Zaagsma J, Halayko AJ: Role of caveolin-1 in p42/p44 MAP kinase activation and proliferation of human airway smooth muscle. *Am J Physiol Lung* Cell Mol Physiol 2006;291:L523–L534.
11. Mizuta K, Xu D, Pan Y, Comas G, Sonett JR, Zhang Y, Panettieri RA, Jr., Yang J, Emala CW, Sr.: $GABA_A$ receptors are expressed and facilitate relaxation in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L1206–L1216.
12. Gallos G, Yim P, Chang S, Zhang Y, Xu D, Cook JM, Gerthoffer WT, Emala CW, Sr.: Targeting the restricted alpha subunit repertoire of airway smooth muscle $GABA_A$ receptors augments airway smooth muscle relaxation. *Am J Physiol Lung Cell Mol Physiol* 2012;302:L248–L256.
13. Hollands EC, Dale TJ, Baxter AW, Meadows HJ, Powell AJ, Clare JJ, Trezise DJ: Population patch-clamp electrophysiology analysis of recombinant $GABA_A$ alpha1beta3gamma2 channels expressed in HEK-293 cells. *J Biomol Screen* 2009;14:769–780.
14. Thompson SA, Arden SA, Marshall G, Wingrove PB, Whiting PJ, Wafford KA: Residues in transmembrane domains I and II determine gamma-aminobutyric acid type AA receptor subtype-selective antagonism by furosemide. *Mol Pharm* 1999;55:993–999.
15. Khom S, Baburin I, Timin EN, Hohaus A, Sieghart W, Hering S: Pharmacological properties of $GABA_A$ receptors containing gamma1 subunits. *Mol Pharm* 2006;69:640–649.
16. Mortensen M, Ebert B, Wafford K, Smart TG: Distinct activities of GABA agonists at synaptic- and extrasynaptic-type $GABA_A$ receptors. *J Physiol* 2010;588:1251–1268.
17. Krehan D, Storustovu SI, Liljefors T, Ebert B, Nielsen B, Krogsgaard-Larsen P, Frolund B: Potent 4-arylalkyl-substituted 3-isothiazolol GABA(A) competitive/noncompetitive antagonists: synthesis and pharmacology. *J Med Chem* 2006;49:1388–1396.
18. Huang SH, Duke RK, Chebib M, Sasaki K, Wada K, Johnston GA: Bilobalide, a sesquiterpene trilactone from Ginkgo biloba, is an antagonist at recombinant alpha1beta2gamma2L GABA(A) receptors. *Eur J Pharm* 2003;464:1–8.
19. Ueno S, Bracamontes J, Zorumski C, Weiss DS, Steinbach JH: Bicuculline and gabazine are allosteric inhibitors of channel opening of the $GABA_A$ receptor. *J Neuroscience* 1997;17:625–634.
20. Etter A, Cully DF, Liu KK, Reiss B, Vassilatis DK, Schaeffer JM, Arena JP: Picrotoxin blockade of invertebrate glutamate-gated chloride channels: subunit dependence and evidence for binding within the pore. *J Neurochem* 1999;72:318–326.
21. Korpi ER, Grunder G, Luddens H: Drug interactions at GABA(A) receptors. *Prog Neurobiol* 2002;67:113–159.
22. Baumann SW, Baur R, Sigel E: Forced subunit assembly in alpha1beta2gamma2 $GABA_A$ receptors. Insight into the absolute arrangement. *J Biol Chem* 2002;277:46020–46025.
23. Browne SH, Kang J, Akk G, Chiang LW, Schulman H, Huguenard JR, Prince DA: Kinetic and pharmacological properties of GABA(A) receptors in single thalamic neurons and GABA(A) subunit expression. *J Neurophysiol* 2001;86:2312–2322.
24. Wang P, Slaughter MM: Effects of GABA receptor antagonists on retinal glycine receptors and on homomeric glycine receptor alpha subunits. *J Neurophysiol* 2005;93:3120–3126.

25. Huang RQ, Dillon GH: Functional characterization of GABA(A) receptors in neonatal hypothalamic brain slice. *J Neurophysiol* 2002;88:1655–1663.
26. Rivas FM, Stables JP, Murphree L, Edwankar RV, Edwankar CR, Huang S, Jain HD, Zhou H, Majumder S, Sankar S, Roth BL, Ramerstorfer J, Furtmuller R, Sieghart W, Cook JM: Antiseizure activity of novel gamma-aminobutyric acid (A) receptor subtype-selective benzodiazepine analogues in mice and rat models. *J Med Chem* 2009;52:1795–1798.
27. McCartney MR, Deeb TZ, Henderson TN, Hales TG: Tonically active $GABA_A$ receptors in hippocampal pyramidal neurons exhibit constitutive GABA-independent gating. *Mol Pharm* 2007;71:539–548.
28. Gallos G, Gleason NR, Zhang Y, Pak SW, Sonett JR, Yang J, Emala CW: Activation of endogenous $GABA_A$ channels on airway smooth muscle potentiates isoproterenol mediated relaxation. *Am J Physiol Lung Cell Mol Physiol* 2008; 295:L1040–L1047.
29. Gallos G, Gleason NR, Virag L, Zhang Y, Mizuta K, Whittington RA, Emala CW: Endogenous gamma-aminobutyric acid modulates tonic guinea pig airway tone and propofol-induced airway smooth muscle relaxation. *Anesthesiology* 2009; 110:748–758.
30. Gleason NR, Gallos G, Zhang Y, Emala CW: The $GABA_A$ agonist muscimol attenuates induced airway constriction in guinea pigs *in vivo*. *J Appl Physiol* 2009;106:1257–1263.
31. Bonnert TP, McKernan RM, Farrar S, le BB, Heavens RP, Smith DW, Hewson L, Rigby MR, Sirinathsinghji DJ, Brown N, Wafford KA, Whiting PJ: theta, a novel gamma-aminobutyric acid type A receptor subunit. *Proc Natl Acad Sci USA* 1999;96:9891–9896.

Address correspondence to:
Charles W. Emala, MS, MD
Department of Anesthesiology
Columbia University
622 West 168th St.
P&S Box 46
New York, NY 10032

E-mail: cwe5@columbia.edu