Development of Automated Patch Clamp Assay for Evaluation of α7 Nicotinic Acetylcholine Receptor Agonists in Automated QPatch-16

Yuchen Hao,[1] Jingshu Tang,[1] and KeWei Wang[1–3]

[1]*Department of Molecular and Cellular Pharmacology, State Key Laboratory of Natural and Biomimetic Drugs, Peking University School of Pharmaceutical Sciences, Beijing, China.*
[2]*PKU-IDG/McGovern Institute for Brain Research, Peking University, Beijing, China.*
[3]*Department of Pharmacology, Qingdao University School of Pharmacy, Qingdao, China.*

ABSTRACT

The α7 nicotinic acetylcholine receptor (α7 nAChR) is an important and challenging target for drug discovery in the area of neuropsychiatric disorders. The current screening for chemicals targeting α7 nAChRs is primarily achieved by the use of low-throughput assay two-electrode voltage clamp (TEVC) in non-mammalian Xenopus oocytes. Automated patch clamp system has emerged as an attractive approach compared to conventional electrophysiology. To develop a mammalian cell-based functional assay in an automated electrophysiology system, we in this study generated a stable expression of α7 nAChRs in GH3 cells that originated from a rat pituitary tumor cell line and utilized automated QPatch-16 to test a set of tool compounds and chemicals identified as α7 agonists by TEVC. For the improvement of evaluating weak or partial α7 nAChRs agonists, we achieved enhancement of the signal-to-noise ratio by the addition of a positive allosteric modulator PNU-120596, which only activates α7 current in the presence of agonist. This improved assay was further validated by using known α7 partial agonists, such as RG3487, EVP-6124, and A-P90. Using this validated assay, we were able to identify a novel agonist 140507C that partially activates α7 nAChRs. Taken together, our results validate the use of QPatch-16 for evaluation α7 partial agonists, demonstrating its utility as an effective tool for α7 ion channel drug discovery.

INTRODUCTION

The nicotinic acetylcholine receptors (nAChRs) are non-selective ligand-gated ion channels widely expressed in the nervous system, mediating synaptic transmission, regulating neuronal excitability, and participating in many cellular and physiological processes and functions.[1–4] As a subtype of pentameric nAChRs, the α7 nicotinic acetylcholine receptor (α7 nAChR) is characterized by high calcium permeability, rapid activation, and desensitization by agonists. Functional deficits of α7 nAChR are implicated in neuropsychiatric disorders, such as Alzheimer's disease and schizophrenia.[5–9] Thus, the activation of α7 nAChRs by selective agonists likely offers potential of novel therapies for the treatment of such disorders.[6,10]

The α7 nAChR remains important and an attractive target for the central nervous system drug discovery. Currently, the functional screening for chemicals targeting α7 nAChR is primarily accomplished by conventional two-electrode voltage clamp (TEVC) in nonmammalian *Xenopus* oocytes, presenting a major bottleneck and low throughput with only a small number of late-stage compound testing in drug discovery.[11–13] This dilemma primarily resulted from two reasons. First, obtaining stable expression of α7 nAChR in mammalian cells is extremely challenging compared with other α or β nAChR subunits.[1,14–18] To date, expression of α7 nAChR has only been successful in neuron-derived cells, such as GH4C1, SHSY5Y, PC12 and SH-EP1, which contain endogenous chaperone protein Ric-3, or by cotransfecting both α7 nAChR and exogenous Ric-3 cDNAs in nonneurogenous cells, such as CHO-k1 and HEK293 cells.[14–16,19–24] However, attempts for stably expressing α7 nAChR in mammalian cells, irrespective as to whether of neuronal origin, have proven successful for only a limited number of laboratories. Second, the effort to utilize automated patch clamp technologies has only demonstrated a limited success as an efficient approach in ligand-gated ion channels, suffering from the low ratio of signal over noise and low rate of successful experiments.[15,19]

QPatch-16 is an early version of automated whole-cell patch clamp system that is capable of simultaneous and independent recording of up to 16 cells.[25] QPatch has been used for functional validations and pharmacological characterizations of ion channels that include voltage-gated sodium channels, voltage- or calcium-gated potassium channels, calcium release-activated calcium channels, and TRP and nAChR

DOI: 10.1089/adt.2014.622

channels.[12,19,25–35] Although great efforts have been made in improving and validating the system, the most challenging issues come from cell lines that often encounter issues of unstable and low expression.[33] Nevertheless, automated patch clamp systems have emerged as an attractive and efficient approach with advantages of being higher throughput, user friendly, and cost effective per data point, which is beneficial to all phases of ion channel drug discovery.

To identify α7 nAChR agonists or positive allosteric modulators (PAMs), we generated a GH3 cell line stably expressing α7 nAChR and utilized automated QPatch-16 to extend the use of the system. Here, we report the development and validation of an automated QPatch-16 assay in GH3 cell line for evaluation of α7 agonists or PAMs.

MATERIALS AND METHODS

Chemicals

Acetylcholine (ACh) and PNU-120596 were purchased from Sigma-Aldrich. G418 was purchased from Amresco. Methyllycaconitine citrate (MLA) was purchased from Tocris Bioscience. α7 Agonists EVP-6124, RG3487, AP-90, and 140507C were all synthesized internally. ACh and G418 were dissolved in water as 1 M and 100 mM stocks, respectively, and kept at −20°C. All agonists, MLA, and PNU-120596 were dissolved in 100% dimethyl sulfoxide (DMSO) and kept as 100 mM stocks at −20°C. All working concentrations of compounds were made from the stock by serial dilutions in extracellular solution. The final concentrations of DMSO were no more than 0.1%.

Generation of α7 Stable Cell Line and Cell Culture

Rat pituitary origin GH3 cells were selected and transfected with human α7 cDNA (GeneID 1139) subcloned in pIRES2-EGFP vector using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. After 48 h of transfection, 0.6 mg/mL G418 was added into a culture medium for selection of positive clones. The culture medium with G418 was changed every 2 days to maintain screening pressure and dead cells were removed. After 10 days of screening, cells were dispensed by serial dilutions in 96-well culture plates at a density of one cell clone per well to obtain single cell clones. Selected single clones were monitored for enhanced green fluorescent protein (EGFP) expressions, and functional evaluations by manual patch clamp recordings were carried out on GFP-positive cells. After screenings, an ideal single cell clone was finally selected and amplified in T75 flasks for obtaining stable α7 nAChR cell line, named as GH3-hα7 in this study.

For the maintenance of stable expression, GH3-hα7 cells were cultured in the Ham F-12K (Kaighn's) medium (Gibco) supplemented with 15% inactivated horse serum, 2.5% fetal bovine serum, and 0.2 mg/mL G418 and maintained in 5% CO_2 at 37°C. Cells were passaged every 3–4 days at 70%–80% confluency.

Reverse Transcription–Polymerase Chain Reaction and Quantitative Real-Time Polymerase Chain Reaction

Total RNAs were isolated from GH3 or GH3-hα7 cells grown to about 80% confluency in a 60-mm culture dish using 2 mL TRIzol reagent. Briefly, cDNA was synthesized from 3 μg total RNAs using Promega GoScript reverse transcription kit according to the manufacturer's instructions. The primers used in polymerase chain reaction (PCR) amplification contain the following sequences: α7 sense, 5′-gttctatgagtgctgcaaagagcc-3′; α7 antisense, 5′-ctccacactggccaggctgcag-3′ (product size 497 bp); β-actin sense, 5′-cctgaaccctaaggccaaccg-3′; β-actin antisense, 5′-gctcatagctcttctccaggg-3′ (product size 391 bp). PCR amplification was performed in the Mx3000P QPCR system using Promega GoTaq kit with reaction conditions as follows: 95°C for 2 min; 95°C for 15 s, 60°C for 1 min, ×40 cycles.

Fluorescent Ca^{2+} Assay Using FlexStation3

GH3-hα7 or GH3 cells were seeded and incubated in a 96-well, optical-bottom black-walled plate (Thermo Scientific) with a density of about 50,000 cells per well for 5 h in 5% CO_2 at 37°C before experiments. Cells were incubated with 60 μL culture medium and 40 μL fluorescent dyes, including 4 μL Cal-520™ (AAT Bioquest) and 36 μL saline buffer (137 NaCl, 5.4 KCl, 0.4 KH_2PO_4, 0.1 Na_2HPO_4, 1.3 $CaCl_2$, 0.8 $MgSO_4$, 5.5 glucose, 4 $NaHCO_3$, 20 HEPES, mM, pH 7.4), for 1 h in 5% CO_2 at 37°C. The plate was placed in the FlexStation3 system (Molecular Devices) for reading. After 17 s of baseline reading, 3 μM PNU-120596 was added into the plate and cells were incubated for 300 s when ACh or saline was added for an additional 17 s. Fluorescent calcium signals were measured for 500 s at 525 nm.

TEVC Recordings in Xenopus Oocytes

Briefly, human α7 cDNAs were transcribed to hα7 cRNAs *in vitro* using T3 mMESSAGEmMACHINE kit (Ambion) following linearization of hα7-KSM (pBluescript) plasmids, as previously reported.[36] Isolated oocytes (stages V–VI) were injected with 46 nL of hα7 cRNAs and then kept at 17°C in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4 with NaOH) and used 2–5 days after injection. During recordings, oocytes were bathed in the Ringer solution (115 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.0005 mM atropine) and impaled with two microelectrode filled with 3 M KCl. Currents were recorded using the Axon GeneClamp 500B amplifier at the holding potential of −90 mV at room temperature.

Conventional Manual Patch Clamp Recordings

Cells were plated on poly-D-lysine-coated glass coverslips 1 day before experiments. Briefly, all experiments were performed at the holding potential of −60 mV and currents were recorded using the HEKA EPC10 amplifier with HEKA PatchMaster software.[16,20] Patch pipettes were pulled and polished to a resistance of 3–5 MΩ using DMZ universal. Extracellular saline was continuously applied to a cell through a gravity perfusion system (ALA Scientific Instruments, Inc.), while ACh was added to the cell surface through a focal pressurized perfusion system (ALA Scientific Instruments, Inc.). The extracellular solution contained (in mM) 145 NaCl, 3 KCl, 2 $MgCl_2$, 1 $CaCl_2$, and 25 HEPES (pH 7.4 with NaOH, 310 mOsm). The intracellular solution contained (in mM) 140 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, and 25 HEPES (pH 7.4 with KOH, 300 mOsm).

Table 1. Whole-Cell Protocol in QPatch-16

Cell positioning	
Wait time	5 s
Positioning pressure	−70 mbar
Resistance increase for success	750%
Positioning timeout	60 s
Gigaseal method	
Start pressure	−70 mbar
Wait time before Vhold change	8 s
Obtain gigaseal Vhold	−80 mV
Allowed pressure (minimum–maximum)	−20 mbar
Gigaseal formation timeout	180 s
Whole-cell method	
Use suction pulses	*Use suction ramp*
Order of use: 1	Order of use: 2
Number of pulses: 2	Ramp amplitude: −350 mbar
First pulse amplitude: −250 mbar	Ramp slope: 100 ms/mbar
Increment between pulses: −50 mbar	
Pulse duration: 1,000 ms	
Pulse period: 10,000 ms	
Whole-cell requirements	
Minimum seal resistance	0.1 GΩ
Holding potential	−90 mV
Holding pressure	−20 mbar

Table 2. Voltage Protocol for Data Acquisition

General	
Holding potential	−80 mV
Minimum seal resistance	0.1 GΩ
Liquid dispense delay	2,000 ms
Data acquisition time	10,000 ms
Amplifier range	Automatic
Filtering	
Sampling frequency	2,000 Hz
Filter type	Bessel
Filter order	4
Cutoff frequency	1,000 Hz
R_{series}	
Feedback factor	85.0%
Feedback cutoff frequency	800 Hz

Cell Preparations for the QPatch-16 Assay

GH3-hα7 cells grown in T175 flasks to 70%–80% confluency were mildly rinsed once with 5 mL Dulbecco's phosphate-buffered saline (Gibco), followed by the addition of 3 mL trypsin. Cells were incubated at 37°C for 1–2 min until a few cells floated, and then, 7 mL serum containing medium was added to stop digestion. Cells were dissociated from

Table 3. Day-to-Day Variability of Application of ACh and Co-Application of ACh Plus PNU-120596 in GH3-hα7 Cells Using QPatch

	ACh			ACh+PNU-120596		
	M[a]	N[b]	EC_{50} (μM)	M	N	EC_{50} (μM)
Day 1	1	3	170.4±29.6	1	6	5.0±0.5
Day 2	2	6	161.9±15.5	1	5	5.6±0.1
Day 3	1	4	223.8±65.5	1	5	3.9±0.1
	EC_{50} (GM[c]) = 183.5 (119.1–282.7) μM			EC_{50} (GM) = 4.8 (3.0–7.6) μM		

[a]M means the number of used QPlates.
[b]N means the amount of usable cells.
[c]GM means geometric mean with 95% confidence internal.
ACh, acetylcholine.

the flask aided by repeated trituration, transferred into a 15-mL conical tube, and centrifuged at 800 rpm for 1.5 min at room temperature. After removing the supernatant, cells were resuspended with the serum-free F12K medium supplemented with 10 mM HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were then transferred to the QPatch station (QStirrer) to maintain a single-cell suspension and allowed to recover for at least 30 min before the start of the experiment.

Automated QPatch-16 Recordings

Currents were recorded in the whole-cell patch clamp configurations on QPatch-16 using single-hole QPlate (orifice resistance 2.0 ± 0.4 MΩ, chip capacitance 55 ± 5 pF). QPatch-16 operation contained the following steps: cell positioning, gigaseal, whole-cell configuration, and final execution of current recording. Whole-cell protocols executed before data acquisition promote the formation of whole-cell configurations (*Table 1* for details). After obtaining whole-cell configuration, compounds were added in sequence and data were recorded with voltage protocols (*Table 2*) and application protocols (*Table 4*). The extracellular solution contained (in mM): 145 NaCl, 3 KCl, 2 $MgCl_2$, 1 $CaCl_2$, and 25 HEPES (pH 7.4 with NaOH, 310 mOsm). The intracellular solution contained (in mM): 140 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, and 25 HEPES (pH 7.4 with KOH, 300 mOsm).

Data Analysis

The real-time PCR data were measured using Mxpro QPCR software and exported to Microsoft Excel and GraphPad Prism 5 for analysis. FlexStation3 data were acquired using Softmax Pro software and exported to Microsoft Excel and Origin 8.0 for analysis. QPatch data were obtained using Sophion QPatch assay software and exported to Microsoft Excel and Origin 8.0 for analysis. All concentration–response data were fitted to Hill equation as follows: $I(c) = I_{fe}*C^n/(XC50^n + C^n)$, where C is X-input concentration, n is the Hill slope, and I_{fe} is full effect response. Normalized currents are presented as mean ± SEM, and data were fitted

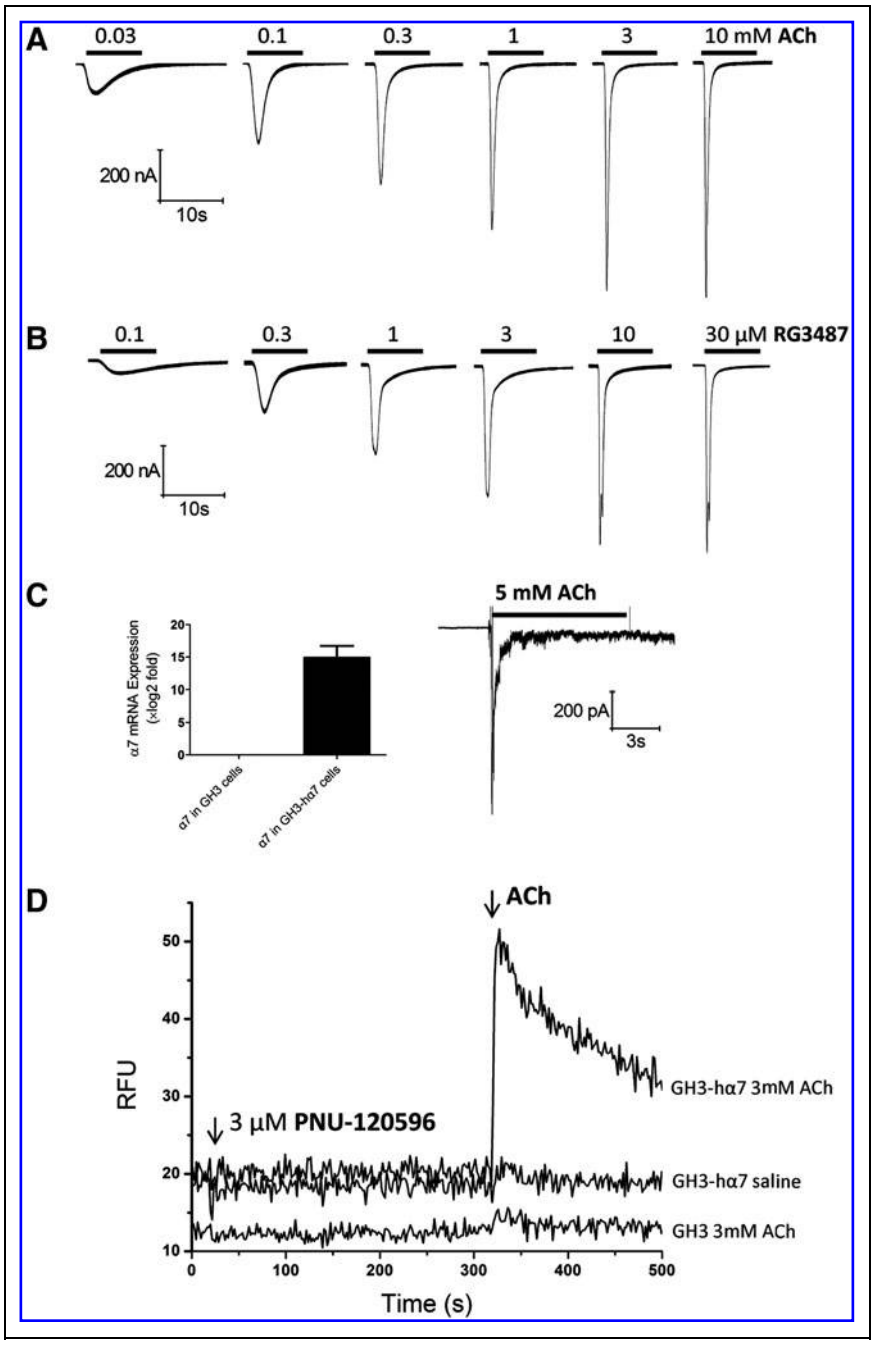

Fig. 1. Functional expression of human α7 channels in *Xenopus* oocytes and mammalian GH3-hα7 cells. **(A)** Representative traces of α7 currents recorded in *Xenopus* oocytes by two-electrode voltage clamp (TEVC) in response to increasing concentrations of full agonist acetylcholine (ACh) (0.03–10 mM). **(B)** Representative traces of α7 currents recorded in *Xenopus* oocytes by TEVC in response to increasing concentrations of partial agonist RG3487 (0.1–30 μM). **(C)** *Left panel*, comparison of mRNA expressions in GH3 cells and GH3 cells transfected with human α7 plasmids. Expression level is presented as fold in log 2 scale. Data are presented as mean ± SEM ($n = 3$). *Middle panel*, representative traces of α7 currents from stable GH3-hα7 cells recorded by manual whole-cell patch clamp in response to ACh (5 mM). **(D)** Fluorescent calcium signals in GH3-hα7 or GH3 cells were measured by the FlexStation3 assay in response to 3 mM ACh. PNU-120596 (3 μM) was added at the time point of 17th second, and testing compounds (ACh or saline) were added at 317th second.

by the Hill equation. Averaged EC_{50s} obtained from different days are expressed as geometrical means with 95% confidence interval.

RESULTS

Functional Expression of Human α7 in Xenopus Oocytes

The human α7 nAChR cDNA was subcloned into pBluescript KSM vector for functional expression. To confirm the function of hα7 cDNA plasmid, *in vitro* transcription of hα7 cRNA was made and microinjection into *Xenopus* oocytes was carried out for functional expression. α7 Currents were recorded by TEVC. As shown in *Figure 1A* and *B*, both α7 full agonist ACh ranging from 0.03 to 10 mM and partial agonist RG3487 ranging from 0.1 to 30 μM elicited α7 currents in a concentration-dependent manner. The results confirmed that our hα7 cDNA construct was functional and therefore was used for generation of α7 stable mammalian cell lines.

Generation of a GH3 Stable Cell Line Expressing Human α7 nAChR

To develop an automated QPatch assay for α7 nAChR, we started subcloning human α7 nAChR cDNA into mammalian pIRES2-EGFP vector. Stable transfection of α7 nAChR-pIRES2-EGFP plasmid resulted in expression of α7 nAChR in GH3-hα7 cells that were detected by real-time PCR (*Fig. 1C*, left panel). Functional evaluation of GH3-hα7 cells was carried out to confirm the activation of ACh-induced inward currents recorded by manual patch clamp, displaying rapid activation and desensitization (*Fig. 1C*, right panel). The stable GH3-hα7 cells were further validated by measurement of intracellular calcium signals using the FlexStation3 assay. As shown in *Figure 1D*, the application of either PAM PNU-120596 or saline caused no increased calcium, and the addition of 3 mM ACh elicited a robust elevation of intracellular calcium signal in GH3-hα7 cells, compared with saline control or untransfected GH3 cells that showed no change of calcium signals. These results suggested that human α7 nAChR was functionally expressed in mammalian GH3-hα7 cells.

Assay Development for GH3-hα7 Cells in QPatch-16

To develop automated QPatch assay in GH3-hα7 cells, we optimized the application protocol for the measurement of ACh responses in which saline was added as a negative control before sequential additions of increasing concentrations of agonist and saline wash after each administration. Using this protocol, we recorded α7 currents in the presence of ACh, a full agonist of α7 nAChR commonly used as positive control for inducing the maximum effect. As shown in *Figure 2B*, the application of ACh ranging from 10 μM to 3 mM elicited α7 currents in a concentration-dependent manner with the EC_{50} at 162 μM ($n=6$) (*Table 3*), similar to the EC_{50} value (177 μM) obtained from TEVC recordings in *Xenopus* oocytes.[37] The concentration–response of ACh in GH3 cells was also more potent than stably expressing α7 nAChR in GH4-C1 cells (> 1 mM for EC_{50}) in the QPatch system reported by Friis *et al.*[19] This result indicated that GH3-hα7 cells were functionally expressing α7 channels and suitable for functional evaluation of α7 agonists.

α7 PAMs are classified as type I and II. Type I PAMs enhance α7 current with characteristic rapid desensitization, whereas type II PAMs exhibit both augmented current and extended time of desensitization.[38] To validate the QPatch assay suitable for evaluating PAMs, we determined voltage-dependent activation of α7 currents by PNU-120596 (1.0 μM), a known type II PAM of α7 nAChR, in the presence of a fixed

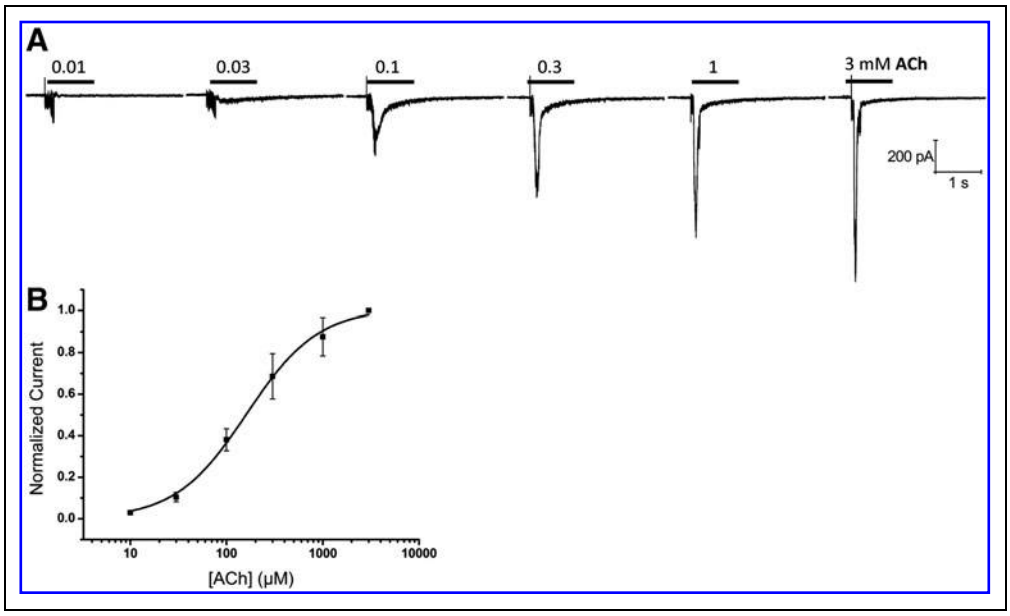

Fig. 2. Dose-dependent activation of α7 currents by ACh in stable GH3-hα7 cells using QPatch-16. **(A)** Representative traces of α7 currents in response to increasing concentrations of ACh (10 μM–3 mM) in GH3-hα7 cells. **(B)** Peak α7 currents were normalized as a function of ACh concentrations and fitted by the Hill equation with EC_{50} at 161.9 ± 15.5 μM ($n=6$). Data are presented as mean ± SEM.

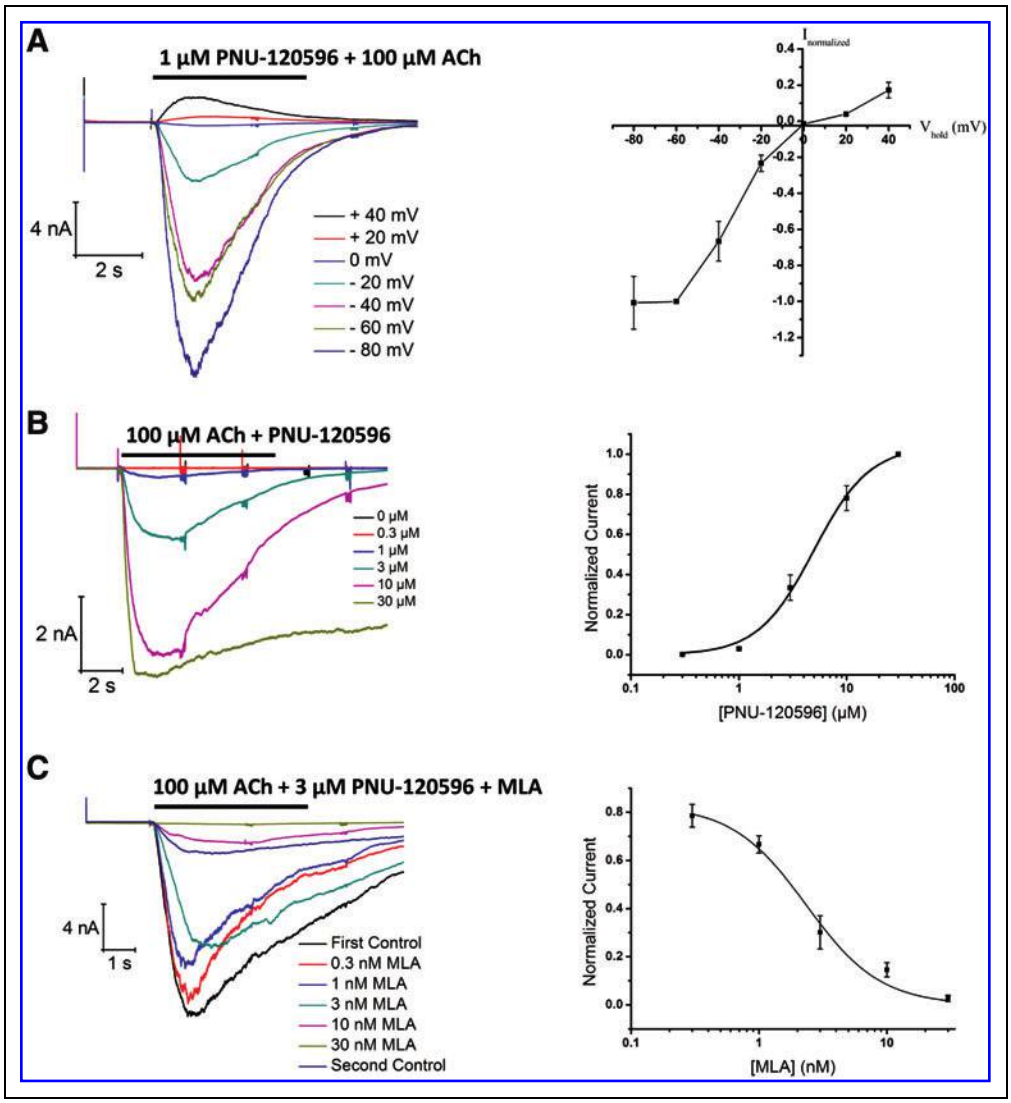

Fig. 3. Voltage- and concentration-dependent activation of α7 currents by positive allosteric modulator (PAM) PNU-120596 in the presence of ACh in GH3-hα7 cells using Qpatch-16. **(A)** Representative traces of voltage-dependent activation of α7 currents by 1 μM PNU-120596 in the presence of 100 μM ACh (*left panel*) and their I–V curve normalized at −60 mV ($n=4$, *right panel*). Cells were held at −80 mV and pulsed to +40 mV with an increment of 20 mV. **(B)** Representative traces of concentration-dependent activation of α7 currents by PNU-120596 (0.3–30 μM) in the presence of 100 μM ACh (*left panel*) and their concentration–response curve (*right panel*) that is fitted by the Hill equation with the EC_{50} at 4.95 ± 0.55 μM ($n=6$). Data are presented as mean ± SEM. **(C)** Representative traces of concentration-dependent inhibition of α7 currents by blocker methyllycaconitine citrate (MLA) (0.3–30 nM) in the presence of 100 μM ACh and 3 μM PNU-120596 (*left panel*) and their concentration–response curve (*right panel*) that is fitted by the Hill equation with the IC_{50} at 2.34 ± 0.36 nM ($n=7$). Data presented as mean ± SEM. Color images available online at www.liebertpub.com/adt

concentration of ACh (100 μM). As shown in *Figure 3A*, a family of pulses elicited voltage-dependent activation of α7 currents by PNU-120596 (1.0 μM) in the presence of ACh (100 μM) (*Fig. 3A*, left panel), and its I–V curve was normalized by −60 mV (*Fig. 3A*, right panel). Because holding potential at −40 mV resulted in more than 50% of the current, we used this holding potential for the rest of testing either for PAMs or partial agonists. In the presence of fixed ACh (100 μM), co-applications of ACh with increasing concentrations (0.3–30 μM) of PNU-120596 resulted in a concentration-dependent activation of α7 currents with the EC_{50} at 4.95 ± 0.55 μM ($n=6$) (*Fig. 3B; Table 3*). These results demonstrated that the assay can be used for evaluating α7 full agonists or PAMs.

To further confirm α7 current activated by ACh and PNU-120596 in GH3-hα7 cells, we used α7 selective inhibitor MLA and tested its inhibitory effects on α7. The addition of 100 μM ACh and 3 μM PNU-120596 resulted in robust activation of α7 current that was concentration dependently inhibited by increasing concentrations of MLA (0.3–30 nM) with an IC_{50} at 2.34 ± 0.36 nM ($n=7$) (*Fig. 3C*). This result indicated that ACh and PNU-120596 specifically activated α7 channels expressed in GH3-hα7 cells.

Evaluation of α7 Partial Agonists by QPatch-16

As partial agonists of α7 nAChR, compounds like nicotine, RG3487, and EVP-6124 can directly activate α7 nAChR without reaching maximal effect compared with full agonist ACh. To utilize the assay for evaluating partial α7 agonists, we tested whether partial agonists can induce significant activation of α7 current in stable GH3-hα7 cells. As shown in *Figure 4A*, ACh (300 μM) as a positive control gave rise to robust activation of α7 current. Repeated applications of increasing concentrations (0.1–10 μM) of partial agonist RG3487 resulted in current desensitization, in particular at high concentrations (*Fig. 4A*). The desensitization was further confirmed by repetitive application

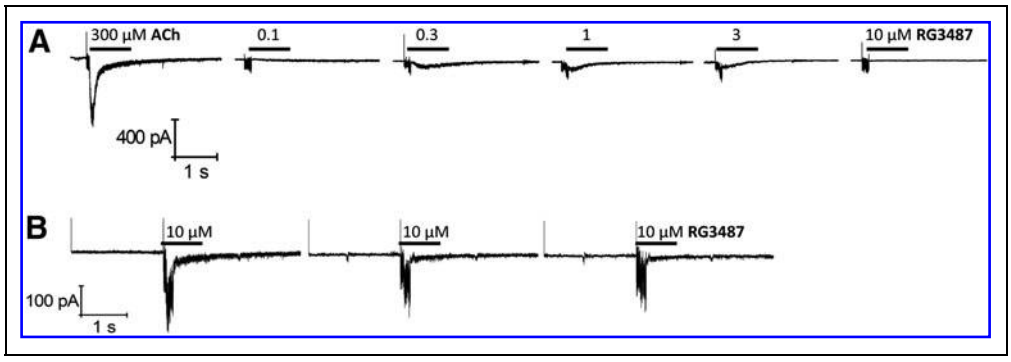

Fig. 4. Activation of α7 currents by partial agonist RG3487 in the QPatch-16 assay. **(A)** Representative traces of α7 currents in response to 300 μM ACh and subsequent increasing concentrations of RG3487 (0.1–10 μM) in GH3-hα7 cells. **(B)** α7 Current desensitization induced by repeated applications of RG3487 (10 μM) with wash interval of 2 min between each addition.

of high concentration (10 μM) of RG3487. First application of partial agonist RG3487 at high concentration (10 μM) resulted in time-dependent small α7 currents, and subsequent applications of RG3487 (10 μM) gave rise to smaller α7 currents, compared with the first application, due to desensitization (*Fig. 4B*), suggesting that current signals from partial agonists at low concentrations might be undetectable.

To overcome this issue, we generated a QPatch protocol that was designed to specifically identify partial agonists that induce smaller currents and render rapid desensitization. This protocol is composed of individual testing for partial agonist (such as 100 nM RG3487) and PAM alone (such as 3 μM PNU-120596) before their co-application of partial agonist (RG3487) and PAM (PNU-120596) (*Table 4* and *5*). For the groups of saline blank and PNU-120596 controls, there were no inducible currents (*Fig. 4B*). In contrast, the co-application of RG3487 (100 nM) with PNU-120596 (3 μM) resulted in a magnificent activation of α7 currents (*Fig. 5A*), resemblance of co-application of full agonist ACh and PAM (*Fig. 3B*). This result indicated that any weak or partial agonists can be identified upon co-application with type II PAM using this assay, which is an improvement for identifying partial or weak agonists. To further validate this assay protocol, we tested another three partial agonists EVP-6124 and A-P90 and an internally synthesized new chemical 140507C. As shown in *Figure 5B–D*, each of the three compounds (100 nM or 1 μM) in the presence of PAM PNU-120596 (3 μM) induced significant activation of peak currents with similar desensitization kinetics. The results showed that this assay can be used for evaluation of weak or partial agonists upon co-application of type II PAM using the QPatch system.

To further test whether this QPatch assay can be used for testing effects of a wide concentration range of partial agonists, we measured the effects of different concentrations of partial agonist RG3487 in the presence of PNU-120596 at fixed concentration (3 μM). As shown in *Figure 6*, RG3487 ranging from (0.1–10 μM) resulted in robust activation of α7 currents with the EC_{50} at 94 ± 4.0 nM ($n = 4$), similar to the range for the EC_{50} value (0.8 μM) with RG3487 alone obtained from TEVC recordings in *Xenopus* oocytes,[39] indicating that the QPatch assay is sensitive and capable of concentration–response evaluation over a wide concentration range of weak or partial α7 agonists.

Table 4. Application Protocol for Improved Evaluation of α7 Partial Agonists

Step	Liquid[a]	Volume (μL)	Wash or not[b]
1	Partial agonist concentration 1	10	N
2	Saline	10	Y
3	PNU-120596[c]	10	Y
4	PNU-120596 + partial agonist concentration 1	10	Y
5	PNU-120596 + partial agonist concentration 2	10	Y
6	PNU-120596 + partial agonist concentration 3	10	Y
7	Saline	10	N

[a]Holding potential was −40 mV.
[b]See *Table 5* for wash protocol.
[c]PNU-120596 concentrations in this table are all kept at 3 μM.

DISCUSSION

The goal of this study was to develop and validate the automated QPatch-16 assay for evaluation of α7 agonists. To

Table 5. Application Protocol for Each Wash

Step	Liquid	Volume (μL)	Waiting time (s)
1	Saline	10	3
2	Saline	10	3
3	PNU-120596	10	5

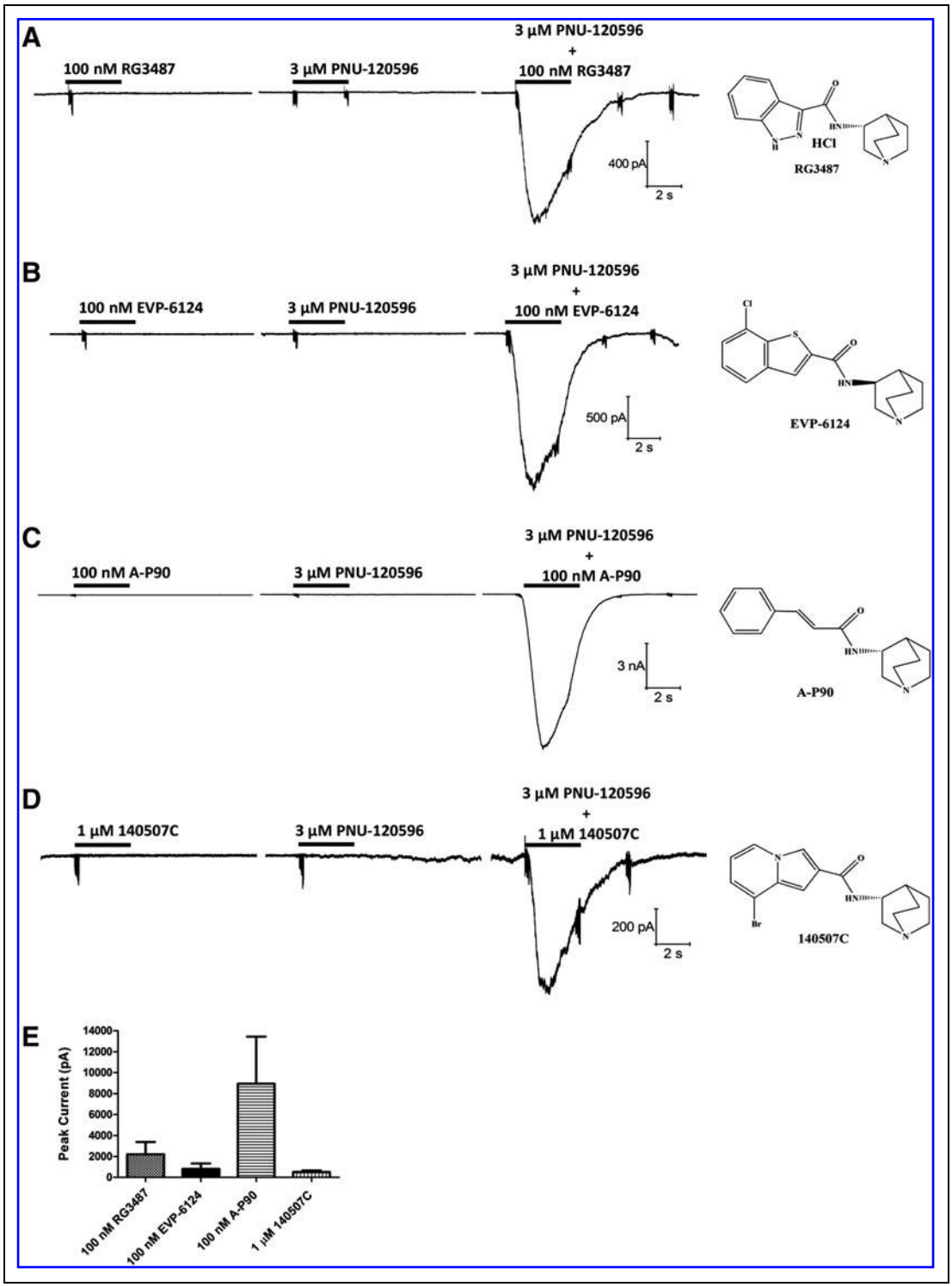

Fig. 5. Activation of α7 currents by different partial agonists in the presence of PAM PNU-120596 in the QPatch-16 assay. **(A)** α7 Currents induced by 100 nM RG3487 and 3 μM PNU-120596 and co-application of 100 nM RG3487 and 3 μM PNU-120596. PNU-120596 was preincubated for 2 min before co-addition of RG3487 and PNU-120596. **(B–D)** Similar to RG3487, α7 currents induced by EVP-6124 (100 nM), A-P90 (100 nM), 140507C (1 μM), and PNU-120596 (3 μM); co-application of each partial agonist and 3 μM PNU-120596. **(E)** Peak current of co-application of each partial agonist plus 3 μM PNU-120596 with means of 2,210, 807, 8,944, and 506 pA, respectively ($n = 3$). Data are presented as mean ± SEM.

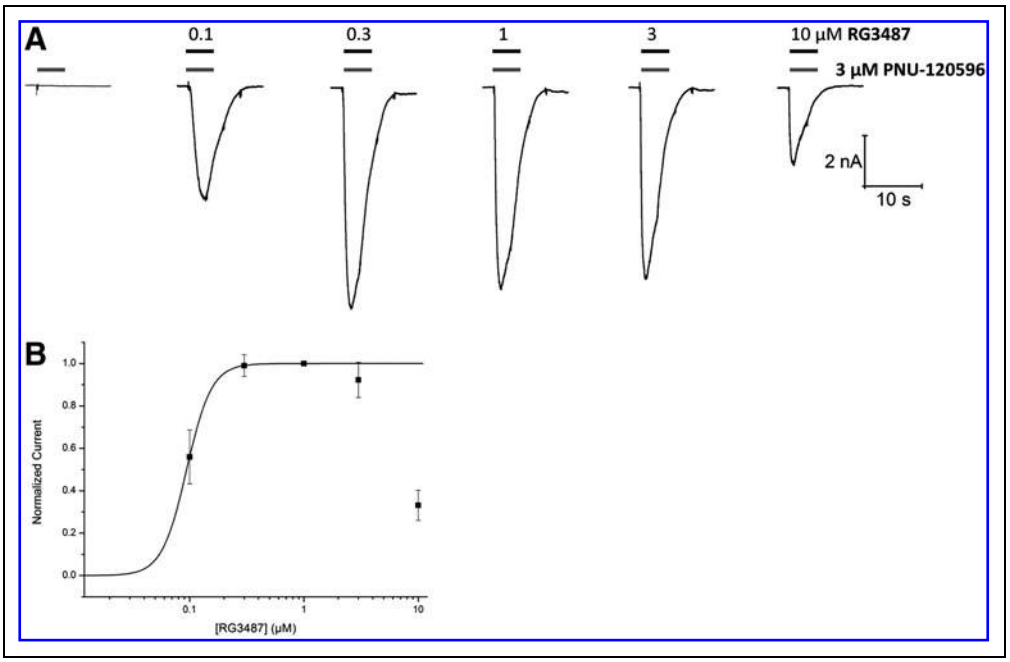

Fig. 6. Activation of α7 currents by partial agonist RG3487 in the presence of PAM PNU-120596 in GH3-hα7 cells using QPatch-16. **(A)** Representative traces of α7 currents in response to the co-application of increasing concentrations of RG3487 (0.1–10 μM) and fixed concentration of PNU-120596 (3 μM). The first trace from the left was recorded with 3 μM PNU-120596 alone. **(B)** Normalized peak α7 currents induced by the co-application of RG3487 and 3 μM PNU-120596 as a function of different concentrations of RG3487. EC_{50} (94 ± 4.4 nM, $n = 4$) was obtained by fitting the Hill equation. Data are presented as mean ± SEM.

achieve this goal, we started by generating a stable expression of human α7 nAChR GH3 cell line. Stable expression of α7 receptor has been challenging and a bottleneck for α7 drug discovery. To meet this challenge, we selected rat pituitary origin GH3 cells that have been shown to contain endogenous chaperones or factors such as Ric-3 likely critical for robust expression of α7.[16–18,24] Transfecting human α7 cDNA and subsequent screening of GH3 cell clones gave rise to stable and functional expression of α7 currents that were confirmed by real-time PCR and whole-cell patch clamp recordings. Using this stable GH3-hα7 cell line, we here present the development and validation of the automated QPatch-16 assay suitable for functional evaluation of full or partial α7 agonists or PAMs.

Compared to more commonly used CHO and HEK293 cells or GH4-C1 rat pituitary tumor cells expressing α7 nAChR,[15,16,19,22] our stable GH3-hα7 cell line worked very well, showing robust α7 current signal in response to ACh, which is sufficient for evaluation of full agonists or PAMs. In our QPatch experiments, more than 70% of the cells achieved gigaseal formation and 50%–60% of the total remained as tight whole-cell recordings and lasted for 30–40 min. We finally obtained about 30% of the total cells tested for usable recording data. The reduction in successful recordings was largely due to little or no functional expression of α7 nAChR in stable cell line, despite existence of endogenous chaperone Ric-3 in GH3 cells.

To overcome this issue of low expression that commonly faces α7 evaluation in automated patch-clamp system, we utilized PNU-120596, a type II PAM, to boost α7 signals in the QPatch assay. In the presence of PNU-120596, we tested several α7 partial agonists, including RG3487 and A-P90, and especially EVP-6124 (Encenicline) that is currently under evaluatzion in a clinical phase III trial.[40,41] The α7 currents induced by these agonists in the presence of PAM are significant and robust even when low concentrations of agonists are applied, demonstrating that this assay is suitable for evaluation of partial agonists over a wide range of concentrations. Using this assay, we successfully identified a novel α7 agonist 140507C that was confirmed by manual TEVC,

Table 6. Comparison of a Series of Success Rates During Experiments with GH3-hα7 Cells in QPatch-16 Between the Addition of ACh Alone and Addition of Agonists with PAMs

Compounds	ACh	Agonists+PNU-120596
Total cells in tests (*n*)	72	64
Primed (%)	100	100
Cell attached (%)	99	98
Seal[a] (%)	92	80
Whole-cell (%)	76	76
Success rate of QPlate[b] (%)	50	55
Usable current[c] (%)	31	52

[a]More than 200 MΩ.
[b]Remaining gigaseal for at least 20 min.
[c]More than 50 pA under a steady baseline.
PAM, positive allosteric modulator.

and the success rate of obtaining useful data was increased from 30% to about 50% (*Table 6*).

In comparison to conventional manual patch clamp, QPatch-16 still faces limitations of being difficult to repetitively apply agonist for α7 current recording without desensitization. This is due to the inability to continuously perfuse the QPlate with buffer, risking prolonged exposure to agonists and subsequent channel desensitization. With the co-application of PAM, we were able to get repetitive recordings of partial agonist-induced α7 currents, but still had problems of desensitization at high concentrations of test compounds and a large cell-to-cell variability (*Fig. 5E*). Nevertheless, this assay still offers an effective solution for evaluation of weak α7 partial agonists.

In conclusion, we developed and validated the automated QPatch-16 assay that is capable of evaluating partial or full α7 agonists as well as type II PAMs, thus providing an efficient functional platform for α7 target drug discovery.

ACKNOWLEDGMENTS

The authors thank Dr. Weifeng Yu and Dr. Binbin Qian (Sophion Bioscience, Inc.) for technical assistance in QPatch-16. We also thank laboratory members Bochuan Teng, Meng Li, Jingheng Zhou, and Yan Song for technical assistance in manual patch clamp and molecular subcloning. This work was supported by research grants to K.W.W. from the Ministry of Science and Technology of China (2013CB531302 and 2014ZX09507003-006-004) and the National Science Foundation of China (31370741 and 81221002).

DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

1. Albuquerque EX, Pereira EF, Alkondon M, Rogers SW: Mammalian nicotinic acetylcholine receptors: from structure to function. *Physiol Rev* 2009;89:73–120.
2. Dajas-Bailador F, Wonnacott S: Nicotinic acetylcholine receptors and the regulation of neuronal signalling. *Trends Pharmacol Sci* 2004;25:317–324.
3. Lendvai B, Vizi ES: Nonsynaptic chemical transmission through nicotinic acetylcholine receptors. *Physiol Rev* 2008;88:333–349.
4. Wang H, Yu M, Ochani M, *et al.*: Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. *Nature* 2003;421:384–388.
5. Deutsch SI, Schwartz BL, Schooler NR, Rosse RB, Mastropaolo J, Gaskins B: First administration of cytidine diphosphocholine and galantamine in schizophrenia: a sustained alpha7 nicotinic agonist strategy. *Clin Neuropharmacol* 2008;31:34–39.
6. Freedman R: Alpha7-nicotinic acetylcholine receptor agonists for cognitive enhancement in schizophrenia. *Annu Rev Med* 2014;65:245–261.
7. Freedman R, Adams CE, Leonard S: The alpha7-nicotinic acetylcholine receptor and the pathology of hippocampal interneurons in schizophrenia. *J Chem Neuroanat* 2000;20:299–306.
8. Lilja AM, Porras O, Storelli E, Nordberg A, Marutle A: Functional interactions of fibrillar and oligomeric amyloid-beta with alpha7 nicotinic receptors in Alzheimer's disease. *J Alzheimers Dis* 2011;23:335–347.
9. Wang HY, Lee DH, D'Andrea MR, Peterson PA, Shank RP, Reitz AB: Beta-amyloid(1–42) binds to alpha7 nicotinic acetylcholine receptor with high affinity. Implications for Alzheimer's disease pathology. *J Biol Chem* 2000;275:5626–5632.
10. Kawamata J, Suzuki S, Shimohama S: Alpha7 nicotinic acetylcholine receptor mediated neuroprotection in Parkinson's disease. *Curr Drug Targets* 2012;13:623–630.
11. Malysz J, Gronlien JH, Timmermann DB, *et al.*: Evaluation of alpha7 nicotinic acetylcholine receptor agonists and positive allosteric modulators using the parallel oocyte electrophysiology test station. *Assay Drug Dev Technol* 2009;7:374–390.
12. Dunlop J, Roncarati R, Jow B, *et al.*: In vitro screening strategies for nicotinic receptor ligands. *Biochem Pharmacol* 2007;74:1172–1181.
13. Papke RL, Thinschmidt JS: The correction of alpha7 nicotinic acetylcholine receptor concentration-response relationships in *Xenopus* oocytes. *Neurosci Lett* 1998;256:163–166.
14. Valles AS, Roccamo AM, Barrantes FJ: Ric-3 chaperone-mediated stable cell-surface expression of the neuronal alpha7 nicotinic acetylcholine receptor in mammalian cells. *Acta Pharmacol Sin* 2009;30:818–827.
15. Roncarati R, Seredenina T, Jow B, *et al.*: Functional properties of alpha7 nicotinic acetylcholine receptors co-expressed with RIC-3 in a stable recombinant CHO-K1 cell line. *Assay Drug Dev Technol* 2008;6:181–193.
16. Williams ME, Burton B, Urrutia A, *et al.*: Ric-3 promotes functional expression of the nicotinic acetylcholine receptor alpha7 subunit in mammalian cells. *J Biol Chem* 2005;280:1257–1263.
17. Lansdell SJ, Gee VJ, Harkness PC, *et al.*: RIC-3 enhances functional expression of multiple nicotinic acetylcholine receptor subtypes in mammalian cells. *Mol Pharmacol* 2005;68:1431–1438.
18. Aztiria EM, Sogayar MC, Barrantes FJ: Expression of a neuronal nicotinic acetylcholine receptor in insect and mammalian host cell systems. *Neurochem Res* 2000;25:171–180.
19. Friis S, Mathes C, Sunesen M, Bowlby MR, Dunlop J: Characterization of compounds on nicotinic acetylcholine receptor alpha7 channels using higher throughput electrophysiology. *J Neurosci Methods* 2009;177:142–148.
20. Zhao L, Kuo YP, George AA, *et al.*: Functional properties of homomeric, human alpha 7-nicotinic acetylcholine receptors heterologously expressed in the SH-EP1 human epithelial cell line. *J Pharmacol Exp Ther* 2003;305:1132–1141.
21. Blumenthal EM, Conroy WG, Romano SJ, Kassner PD, Berg DK: Detection of functional nicotinic receptors blocked by alpha-bungarotoxin on PC12 cells and dependence of their expression on post-translational events. *J Neurosci* 1997;17:6094–6104.
22. Gopalakrishnan M, Buisson B, Touma E, *et al.*: Stable expression and pharmacological properties of the human alpha 7 nicotinic acetylcholine receptor. *Eur J Pharmacol* 1995;290:237–246.
23. Puchacz E, Buisson B, Bertrand D, Lukas RJ: Functional expression of nicotinic acetylcholine receptors containing rat alpha 7 subunits in human SH-SY5Y neuroblastoma cells. *FEBS Lett* 1994;354:155–159.
24. Feuerbach D, Lingenhohl K, Dobbins P, *et al.*: Coupling of human nicotinic acetylcholine receptors alpha 7 to calcium channels in GH3 cells. *Neuropharmacology* 2005;48:215–227.
25. Mathes C: QPatch: the past, present and future of automated patch clamp. *Expert Opin Ther Targets* 2006;10:319–327.
26. Liu Y: Electrophysiological studies of voltage-gated sodium channels using QPatch HT, an automated patch-clamp system. *Curr Protoc Pharmacol* 2014;65:11.14.1–11.14.45.
27. Danker T, Moller C: Early identification of hERG liability in drug discovery programs by automated patch clamp. *Front Pharmacol* 2014;5:203.
28. McPate M, Bhalay G, Beckett M, *et al.*: The development of automated patch clamp assays for canonical transient receptor potential channels TRPC3, 6, and 7. *Assay Drug Dev Technol* 2014;12:282–292.

29. Jenkins DP, Yu W, Brown BM, Lojkner LD, Wulff H: Development of a QPatch automated electrophysiology assay for identifying KCa3.1 inhibitors and activators. *Assay Drug Dev Technol* 2013;11:551–560.
30. Zhang XF, Zhang D, Surowy CS, *et al.*: Development and validation of a medium-throughput electrophysiological assay for KCNQ2/3 channel openers using QPatch HT. *Assay Drug Dev Technol* 2013;11:17–24.
31. Beck EJ, Hutchinson TL, Qin N, Flores CM, Liu Y: Development and validation of a secondary screening assay for TRPM8 antagonists using QPatch HT. *Assay Drug Dev Technol* 2010;8:63–72.
32. Jones KA, Garbati N, Zhang H, Large CH: Automated patch clamping using the QPatch. *Methods Mol Biol* 2009;565:209–223.
33. Mathes C, Friis S, Finley M, Liu Y: QPatch: the missing link between HTS and ion channel drug discovery. *Comb Chem High Throughput Screen* 2009;12:78–95.
34. Korsgaard MP, Strobaek D, Christophersen P: Automated planar electrode electrophysiology in drug discovery: examples of the use of QPatch in basic characterization and high content screening on Na(v), K(Ca)2.3, and K(v)11.1 channels. *Comb Chem High Throughput Screen* 2009;12:51–63.
35. Kutchinsky J, Friis S, Asmild M, *et al.*: Characterization of potassium channel modulators with QPatch automated patch-clamp technology: system characteristics and performance. *Assay Drug Dev Technol* 2003;1:685–693.
36. Liang P, Wang H, Chen H, *et al.*: Structural insights into KChIP4a modulation of Kv4.3 inactivation. *J Biol Chem* 2009;284:4960–4967.
37. Briggs CA, McKenna DG: Activation and inhibition of the human alpha7 nicotinic acetylcholine receptor by agonists. *Neuropharmacology* 1998;37:1095–1102.
38. Gronlien JH, Hakerud M, Ween H, *et al.*: Distinct profiles of alpha7 nAChR positive allosteric modulation revealed by structurally diverse chemotypes. *Mol Pharmacol* 2007;72:715–724.
39. Wallace TL, Callahan PM, Tehim A, *et al.*: RG3487, a novel nicotinic alpha7 receptor partial agonist, improves cognition and sensorimotor gating in rodents. *J Pharmacol Exp Ther* 2011;336:242–253.
40. Study of EVP-6124 (Alpha-7 nAChR) as an Adjunctive Pro-Cognitive Treatment in Schizophrenia Subjects on Chronic Stable Atypical Antipsychotic Therapy. Available at: www.clinicaltrials.gov/ct2/show/NCT01714661?term=EVP-6124&rank=8 (Last accessed November 10, 2014).
41. Study of the Safety and Clinical Effects of 2 Doses of EVP-6124 in Subjects with Alzheimer's Disease Who Complete Study EVP-6124-024 or EVP-6124-025. Available at: www.clinicaltrials.gov/ct2/show/NCT02004392?term=EVP-6124&rank=11 (Last accessed November 10, 2014).

Address correspondence to:
KeWei Wang, MD, PhD
Department of Molecular and Cellular Pharmacology
State Key Laboratory of Natural and Biomimetic Drugs
Peking University School of Pharmaceutical Sciences
Beijing 100191
China

E-mail: wangkw@bjmu.edu.cn

Abbreviations Used

ACh = acetylcholine
DMSO = dimethyl sulfoxide
EGFP = enhanced green fluorescent protein
MLA = methyllycaconitine citrate
nAChR = nicotinic acetylcholine receptor
PAM = positive allosteric modulator
TEVC = two-electrode voltage clamp