Stable expression in HEK-293 cells of the rat α3/β4 subtype of neuronal nicotinic acetylcholine receptor

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Abstract **The α3/β4 subtype of neuronal nicotinic acetylcholine receptor (nAChR) was stably expressed in human embryonic kidney (HEK) 293 cells that co-expressed a voltage-gated Ca^{2+} channel. α3/β4-nAChR-expressing clones were identified using the fura-2 Ca^{2+} imaging technique, and were further characterised by single-cell and whole-cell patch-clamp studies. Acetylcholine (ACh) induced fast activating currents which showed desensitisation and inward rectification. The conductance of the ACh-activated channel was 29 pS. The order of potency of the nicotinic agonists tested was cytisine ≅ nicotine > acetylcholine. The EC_{50} value for ACh was 145 μM; the Hill coefficient was close to 2. The currents elicited by ACh were effectively blocked by nicotinic antagonists, but not by the muscarinic antagonist atropine. These properties are comparable to the pharmacological and physiological profile of ganglionic nicotinic receptors and type III currents of cultured hippocampal neurons.**

Key words: Stable expression; HEK cell; α3/β4 nAChR; Ganglionic nAChR; Ca^{2+} imaging

1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChR) exist in multiple subtypes and display functional diversity [1,2]. As has been concluded from subunit expression studies in *Xenopus* oocytes and mammalian cell lines, functional neuronal nAChRs are hetero-pentamers of α and β subunits, or homo-pentamers of α subunits, [3–6]. To date, eight α subunits (α2–α9), which contain in their N-terminal extracellular domain vicinal cysteine residues characteristic of the ACh binding site, and three structural β subunits (β2–β4), which can modify the pharmacological and functional properties of the nAChRs, have been identified. Because native neurons and neuronal cell lines usually express several nAChR subtypes [7–10], the assignment of neuronal nicotinic responses to distinct nAChR subtypes is just at the beginning [10–14]. Ectopic expression systems for individual nAChR subtypes play an essential role in these studies. They are also important for drug screening and mechanistic studies, e.g. single-channel analysis. With a few notable exceptions [14,15], most ectopic expression systems for neuronal nicotinic receptors so far are based on transient expression. It is not clear, at present, why stable ectopic expression of nAChRs is more difficult to achieve than for other ionotropic neuroreceptors [16–18].

Here we report on the stable expression of the α3/β4 subtype of nAChR in human embryonic kidney (HEK) 293 cells. α3 and β4 subunits are expressed, among others, in the chicken superior cervical ganglion and the ciliary ganglion, and in PC12 cells [6,10,13,19,20], suggesting that the α3/β4 subtype of nAChR is a major ganglionic nicotinic receptor. Transient expression of the α3/β4 nAChR subtype in HEK-293 cells has previously been achieved by others [21].

For the selection of cell clones stably expressing the α3/β4 subtype of nAChR, we employed Ca^{2+} imaging with the Ca^{2+}-chelating agent fura-2 [22]. By using as ectopic expression system HEK-293 cells that stably express an L-type voltage-gated Ca^{2+} channel ($L\alpha_{1C-b}$ [23]), we achieved high assay sensitivity, due to subsequent activation of Ca^{2+} channels by nAChR-mediated depolarisation. The $L\alpha_{1C-b}$ Ca^{2+} channel facilitates Ca^{2+} currents after repeated depolarisation [24]. The assay permits sensitive detection of nAChR-expressing cell clones and quantitative assessment of the stability with time of culture of these clones. The results of functional studies obtained with HEK-293/α1 cells stably expressing the α3/β4 subtype of nAChR are similar to those of Wong et al. [21] obtained with HEK-293 cells transiently expressing the same nAChR subtype.

2. Materials and methods

2.1. Materials

The cDNA clones for the rat α3 [25] and β4 [26] nAChR subunits were kindly provided by Drs. Steve Heinemann and Jim Boulter of the Salk Institute, San Diego. HEK-293 cells stably expressing the $L\alpha_{1C-b}$ Ca^{2+} channel (HEK-293/α1 [23]) were a generous gift from Dr. Franz Hofmann (Institute of Pharmacology and Toxicology, Technical University Munich).

2.2. Eukaryotic expression vectors

The rat α3 (PCA48E) nicotinic acetylcholine receptor subunit coding sequence and the rat β4 (ZPC13) coding sequence were subcloned into the expression vector pcDNA 3 (Invitrogen, San Diego, CA), and the constructs were introduced into *E. coli* strain C600 [27,28]. Plasmid DNA was purified using a column based method (Qiagen) and stored in TE buffer (20 mM Tris; 1 mM EDTA, pH 8.0) at −20°C until transfection.

2.3. Cell culture

Human embryonic kidney cells (HEK-293) cells were obtained from the American Tissue Type Culture Collection (ATCC CRL 1573; Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 15% foetal calf serum (FCS) and maintained in a water-jacketed incubator at 37°C, 10% CO_2. To keep the cells in the exponential phase of growth, they were harvested every 3 days and plated at a density of 1.5×10^4 cells/cm^2. HEK-293 cells stably transfected with the $L\alpha_{1C-b}$ Ca^{2+} channel (HEK-293/α1 [23]) were cultured under the same conditions as HEK-293 cells, except

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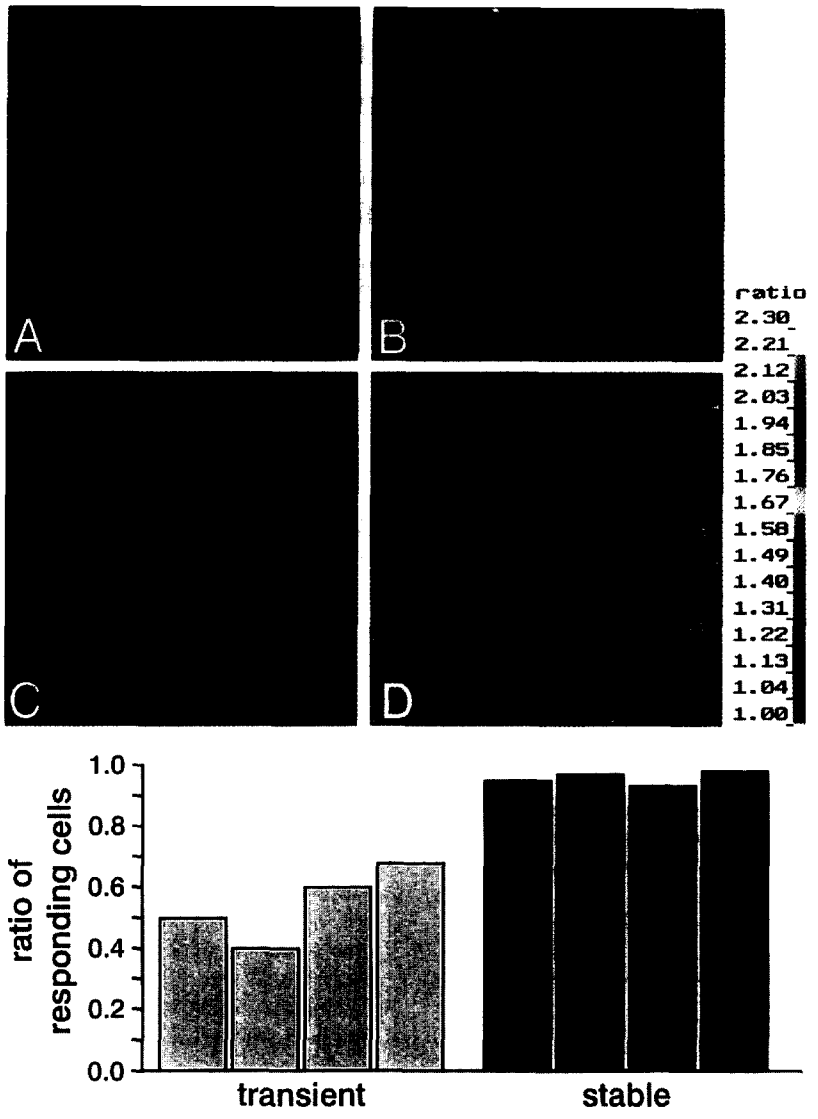

Fig. 1. Expression of rat α3/β4 nAChR in HEK-293/α1 cells, as monitored by Ca^{2+} imaging. The relative intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ was determined by fura-2 fluorescence emission at 510 nm, at the two excitation wavelengths 340 and 380 nm, and intensity ratios were calculated, as described in Section 2. The left micrographs (A,C) show the transfected cells (24 h after initiation of transfection) prior to the application of nicotine. In (A) HEK-293/α1 cells transiently transfected with α3/β4 nAChR plasmids are shown. They display only background levels of fura-2 fluorescence. In the experiment shown, the density of cells was kept low in order to permit single-cell resolution of the fluorescence measurements. Following stimulation with nicotine (100 μM), a portion of the cells responded with a strong increase in $[Ca^{2+}]_i$ (B). In (C,D) HEK-293/α1 cells stably transfected with α3/β4 nAChR plasmids are shown. $[Ca^{2+}]_i$ before (C) and after (D) stimulation with nicotine (100 μM). In the experiment shown, the density of cells was kept relatively high (approx. 150 cells in the section shown) in order to permit estimation of the high efficiency of transfection (close to 100%). (Bottom) The ratio of HEK-293/α1 cells showing robust Ca^{2+} influx (ratio value above 1.5) upon stimulation by nicotine, and the total number of cells is displayed for four experiments each of transient and stable transfection. In these experiments, the transfection efficiencies were 54 and 96%, respectively.

that the medium was supplemented with 0.75 μg/ml G418 (Gibco/BRL).

2.4. Transient transfection of HEK-293 and HEK-293/α1 cells

Cells of passages 4–15 were used for transfection experiments. 24 h prior to transfection, cells were plated on fibronectin-coated coverslips in 24-well culture dishes. Transfection was achieved by calcium phosphate-DNA precipitation [29], using 1 μg DNA/cover-slip, i.e. 0.5 μg DNA of each subunit. The DNA was dissolved in 90 μl sterile H_2O and 100 μl 2×BBS (50 mM BES (*N,N*-bis[2-hydroxyethyl]-2-ethanesulfonic acid, Sigma); 1.5 mM Na_2HPO_4; 280 mM NaCl, adjusted to pH 6.95; 22°C). After drop-wise addition of 10 μl 2.5 mM $CaCl_2$, the solution was gently mixed and incubated for 15 min at room temperature. The solution was then added drop-wise to the medium (1 ml) covering the cells, and the mixture was incubated for 24 h at 37°C, 10% CO_2. After replacing the medium, cells were cultured for another 18–24 h and were then subjected to the screening procedures described below.

2.5. Stable transfection of HEK-293/α1 cells

HEK-293/α1 cells were plated on 35 mm^2 cell culture dishes 24 h prior to transfection. A mixture of 1 μg each of the expression vectors for the nAChR subunits and puromycin (Gibco/BRL), in 600 μl transfection solution, and 2.4 ml medium were used to transfect the cells by means of calcium-DNA precipitation. The cells co-transfected with the three expression vectors were selected on the basis of puromycin/geniticin resistance.

Cells were maintained for 2 weeks in DMEM containing 15% FCS, 0.75 μg/ml G418 and 0.5 μg/ml puromycin. After massive cell death had occurred, a few double-resistant clones could be isolated by the use of cloning rings. Isolated clones were cultivated in selection medium for another 6 weeks. They were then screened by Ca^{2+} imaging for functional expression of nicotinic receptors. Positive clones were submitted to electrophysiological studies.

2.6. Fura-2 measurements

Ca^{2+} influx induced by stimulation with nicotine was measured

with single-cell resolution using the Hamamatsu Argus 50/CA imaging system [30]. Cells transfected with α3/β4 nAChR DNA were plated on fibronectin-coated cover-slips for 72 h, followed by incubation (for 1 h at 37°C) with a solution (2 μM) of membrane-permeable fura-2-acetoxy methyl ester (fura-2-AM, Molecular Probes), washing and incubation for 1 h at room temperature in Ringer's solution (130 mM NaCl; 5 mM KCl; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 2 mM KH_2PO_4; 5 mM glucose; 20 mM HEPES; pH 7.4). The latter incubation results in the conversion by cytoplasmic esterases of fura-2-AM to water-soluble, membrane-impermeable Ca^{2+}-chelating fura-2. Cover-slips were then placed into the recording chamber (1 ml volume) mounted on the stage of an Axiovert 135TV inverted microscope (Zeiss), and the fluorescence intensities at 510 nm were measured every 20 s at two excitation wavelengths, 340 and 380 nm, respectively. Fluorescence ratio data analysis [30] was performed with the software provided by Hamamatsu and yielded relative values of intracellular calcium ion concentration, $[Ca^{2+}]_i$.

Usually 30 fluorescence measurements were taken at intervals of 20 s. During measurements 2–6 and 17–21 the medium (Ringer, containing 2.5 mM $CaCl_2$) was exchanged for one containing nicotine (100 μM). In between and thereafter the cells were kept in medium without nicotine. Blocking experiments (with hexamethonium (100 μM, Fluka) and nitrendipine (1 μM, Research Biochemicals Int.), respectively) were performed by supplementing the medium of fluorescence measurements 7–21 with the blocking agent. All medium changes were performed manually using 5 ml of solution in a Gilson pipette, which sufficed to wash out and replace the previous solution in the 1 ml recording chamber.

2.7. Electrophysiological measurements

Whole-cell current recordings from transiently transfected cells were performed 12–24 h after transfection, those with stably transfected cells 12–48 h after plating, according to standard patch-clamp techniques [31] using an EPC-7 patch-clamp system (List Electronics, Darmstadt). The bathing solution was composed of (mM) NaCl 124; KCl 3.25; $MgCl_2$ 2; $CaCl_2$ 2; D-glucose 11; HEPES 10 (pH 7.4; 275 mOsm), and the internal pipette solution contained (mM) CsF 120; CsCl 10; Cs-EGTA 10; HEPES 10 (pH 7.3; 280 mOsm). The microelectrodes were pulled from borosilicate glass (external diameter 1.6 mm), and the resistance of the fire-polished pipettes was 5–7 MΩ using the external and internal solutions described above. After formation of a high-resistance seal with the cell under investigation, capacitance transients were minimised using the C-Fast facility of the system. No compensation was made for series resistance.

Single-channel recordings were made on outside-out patches using a double-barrelled glass theta tube for fast drug application, as described previously [10]. Whole-cell currents were induced by fast application of the test substances by means of a U-tube reversed-flow device [32], positioned near the cell. The cells were superfused with the bathing solution at a rate of 1.5–3.0 ml/min. In most whole-cell experiments, atropine (1 μM) was included in the bathing solutions in order to inhibit intrinsic muscarinic response. For voltage-jump experiments, the command potentials were applied and computer-controlled using the CLAMPEX application of pClamp. The cell membrane was held at −70 mV and pulsed to the test potentials (−30 to +10 mV) for 50 ms by a 1 s interpulse interval at the holding potential before application of the next pulse.

Signals were low-pass (Bessel) filtered at 3.15 kHz (whole-cell measurements) or 1 kHz (single-channel recordings), digitised at 1 kHz (whole-cell) or 10 kHz (single-channel), and analysed on a PC using the pClamp software package version 6.03 (Axon Instruments). The whole-cell responses were normalised to the response induced by the maximal agonist concentration used. Dose-response curves represent fits of the data to the Hill equation. All data are presented as mean ± standard error.

3. Results

3.1. Ca^{2+} imaging as a detection method for nAChR-expressing cells

Transient expression of the rat α3/β4 subtype of neuronal nAChR was initially achieved in normal HEK-293 cells, with the expression level of functional nAChR tested by whole-cell patch-clamp recordings in the presence and absence of nicotinic agonist. Transfection efficiencies of 40–60% were routinely achieved, with robust nicotinic responses detected between 12 and 48 h after initiating transfection. Transfection with the DNA of only one subunit did not render the cells responsive to nicotinic agonist, as has been observed previously by others for the expression in *Xenopus* oocytes and HEK-293 cells [21,25,26]. This finding also excludes the presence of endogenous nAChR subunits in HEK cells that couple to the introduced exogenous subunit.

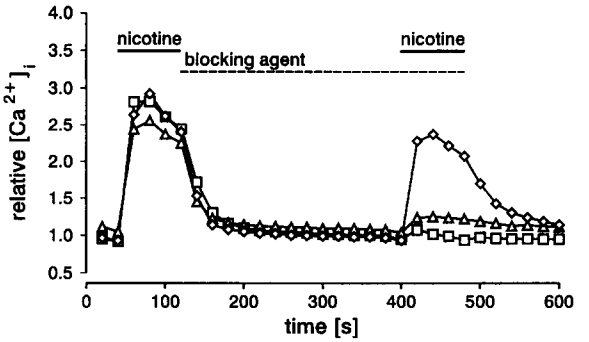

Fig. 2. Pharmacological specificity of Ca^{2+} influx into rat α3/β4 nAChR-expressing HEK-293/α1 cells. The relative intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ was determined at intervals of 20 s in the course of experiments in which nicotine (100 μM) was applied twice (for 100 s each), as indicated by the bars. In the control experiment (◇), normal medium was given before, in between and after the application of nicotine. In the other two experiments, hexamethonium (100 μM; □) and nitrendipine (1 μM; △), respectively, were given after the first application of nicotine. The nicotinic antagonist and the Ca^{2+}-channel blocker reduced the second nicotine-induced response to 8.8 and 19.1%, respectively, of the previous value.

Because whole-cell electrophysiological recordings are rather time-consuming and hence do not lend themselves to the assessment of nAChR expression levels of large numbers of cells, we used a screening assay that takes advantage of the functional coupling of ectopically expressed nicotinic receptors to voltage-gated Ca^{2+} channels and monitors agonist-induced Ca^{2+} influx into transfected cells. For this purpose, we employed as ectopic expression system HEK-293/α1 cells, which stably express a voltage-gated Ca^{2+} channel [23]. The presence of functional nAChR channels can then be monitored by the activity of Ca^{2+} channels, provided that sufficient local depolarisation is produced when the cells are exposed to nicotinic agonist. The functional coupling of nicotinic receptors with voltage-gated Ca^{2+} channels results in an amplification and an elongation in time of the agonist-induced ion influx into the cell. As shown in Fig. 1A,B, intracellular calcium ion levels $[Ca^{2+}]_i$ well above background (minimal ratio value 1.5) were observed in more than 50% of HEK-293/α1 cells co-transfected with rat nAChR α3 and β4 plasmids. The nicotine-induced stimulation of Ca^{2+} influx into cells transiently transfected with nAChR could be blocked by saturating concentrations of the nicotinic antagonist hexamethonium (100 μM) and (to a lesser extent) by nitrendipine (1 μM), a blocking agent of voltage-gated Ca^{2+} channels (not shown). These findings suggest that agonist-induced activation of nicotinic receptors results in Ca^{2+} influx through voltage-gated Ca^{2+} channels (which are activated by nAChR-mediated local depolarisation). In addition, some Ca^{2+} influx may occur through the nAChR channel itself. These results show that Ca^{2+} imaging is a suitable method for the identification of HEK-293/α1 cells transfected with α3/β4 nAChR.

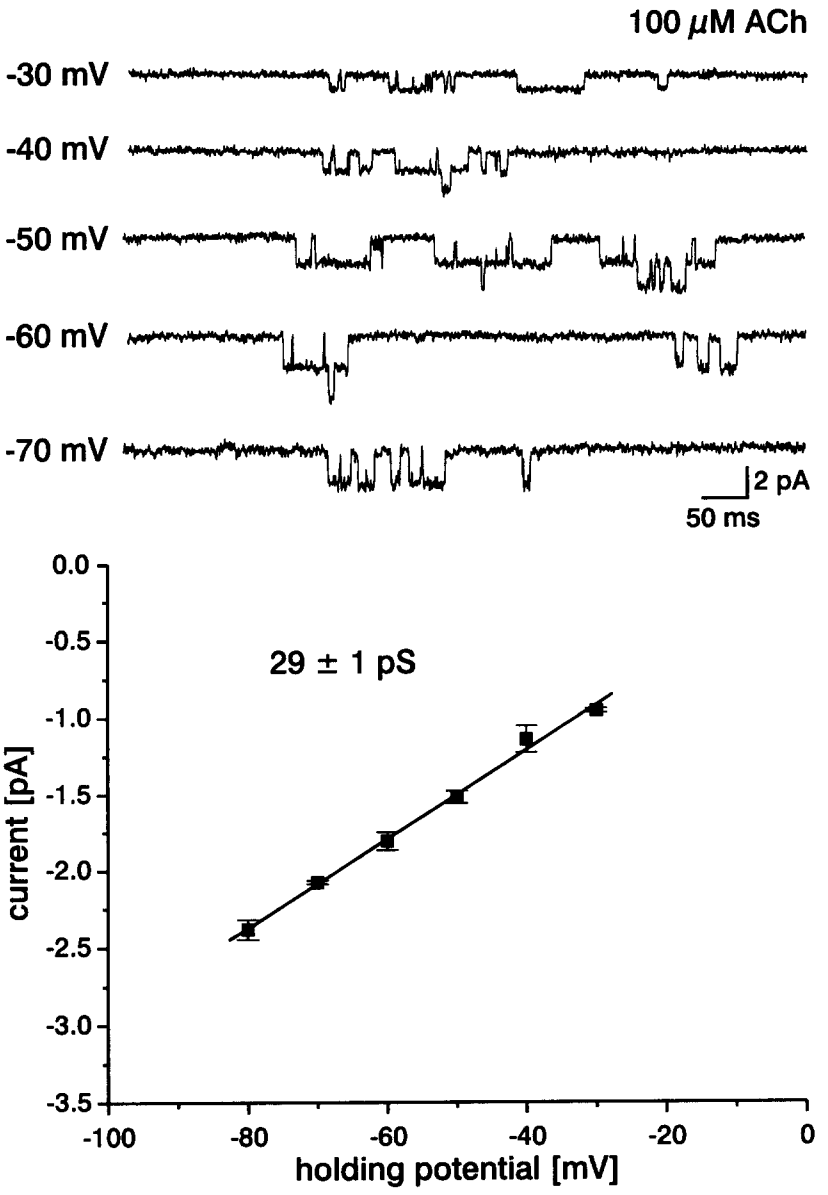

Fig. 3. Samples of recordings and current-voltage relationship of single-channel currents activated by acetylcholine (100 μM) in an outside-out patch excised from a HEK-293/α1 cells stably expressing the rat α3/β4 nAChR. Data shown were filtered at 1.0 kHz. Within the linear range of the *I-V* plot, the channel conductance was determined to 29 ± 1 pS ($n = 3$).

3.2. Stable expression of α3/β4 nAChR in HEK-293/α1 cells

In order to obtain stable clones of α3/β4 nAChR-expressing HEK-293/α1 cells, the transfected cells were cultured for 2 weeks in the presence of geneticin (0.75 μg/ml) and puromycin (0.5 μg/ml), as described in Section 2. 12 double-resistant clones were obtained, separated, and were grown for another 6 weeks under selection conditions. Using Ca^{2+} imaging as functional assay, three of the clones responded to the application of nicotine (100 μM) with a Ca^{2+} influx (150 cells per dish and 3 dishes per clone were tested). Approx. 96% of the cells of each dish/clone responded to nicotine with a strong Ca^{2+} influx (Fig. 1C,D). This value compares well with electrophysiological tests of 100 cells (see below), of which 98 cells produced sizeable whole-cell currents when exposed to nicotine. Agonist-induced Ca^{2+} influx was reversible upon washing with agonist-free solution, reproducible when agonist was applied again, and could be blocked with pharmacological specificity (Fig. 2). Meanwhile, the stable α3/β4 nAChR-expressing HEK-293/α1 cells have been kept in culture for several months, without significant loss of their nAChR activity.

3.3. Preliminary electrophysiological characterisation of α3/β4 nAChR-expressing HEK-293/α1 cells

In outside-out patches excised from HEK-293 cells stably expressing the α3/β4 nAChR, in addition to the $L\alpha_{1C-b}$ Ca^{2+} channel, ACh (100 μM) activated single-channel currents (Fig. 3). Only one channel conductance was observed, which from the linear range of the *I-V* plot, in four independent sets of experiments, was 29.4 ± 1.0 pS. This is in close agreement with the major conductance we observed previously in PC12 cells [10].

In whole-cell recordings the following were present: (i) an L-type Ca^{2+} current, which could be blocked by the dihydropyridine derivative nimodipine (Fig. 4); and (ii) an ACh-acti-

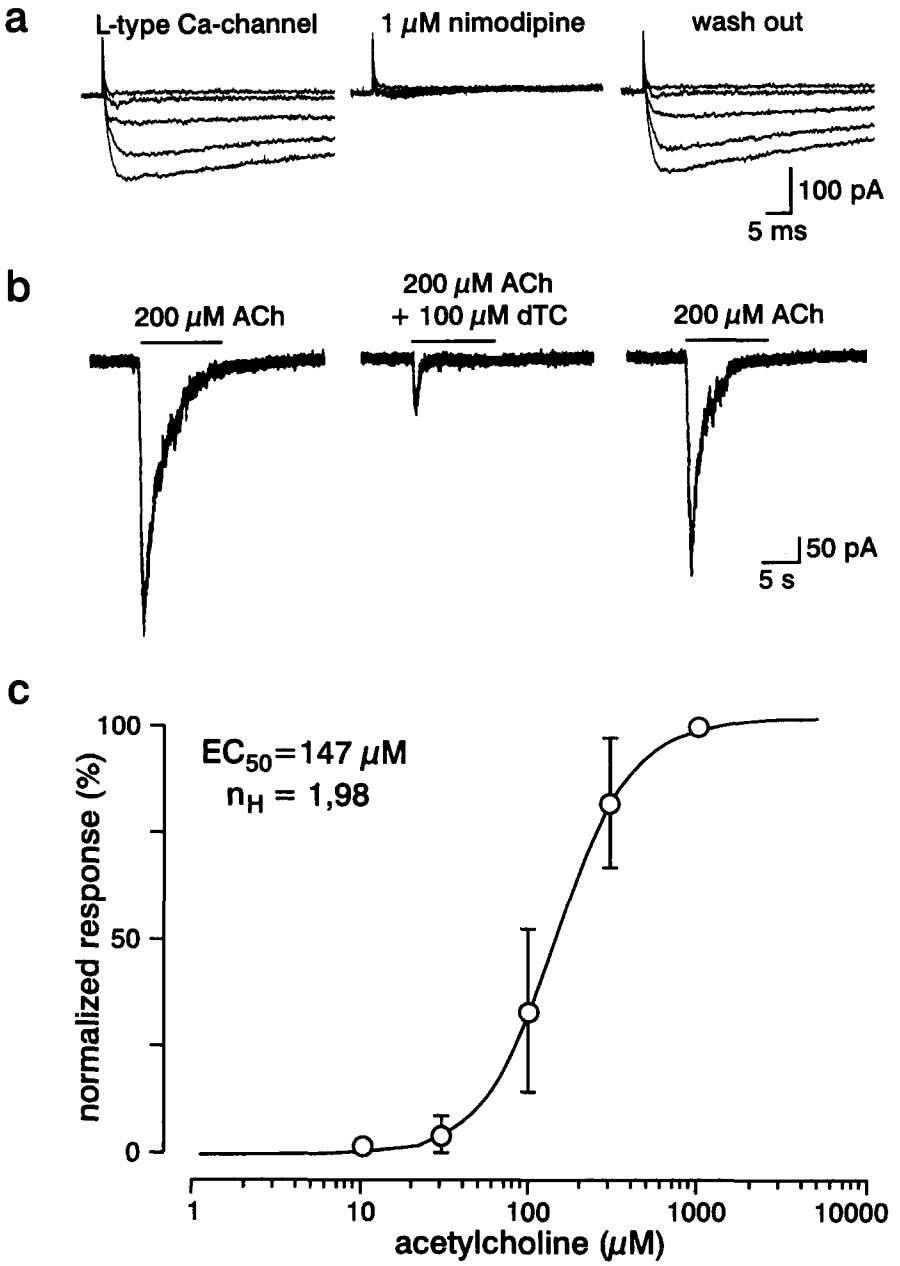

Fig. 4. Whole-cell responses of HEK-293/α1 cells stably expressing the rat α3/β4 nAChR and an L-type Ca^{2+} channel. (a) Activation by depolarisation of an L-type Ca^{2+} channel that is inhibited by preincubation (1 min) with nimodipine (1 μM). After wash-out for 3 min, the block by nimodipine was removed. (b) Sample recordings (representative of four series of experiments) of whole-cell currents activated by 2-s pulses of ACh (200 μM) in the absence (left) and presence (middle) of d-tubocurarine (100 μM), and after wash-out for 10 min (right). (c) Dose-response curve for acetylcholine. Each open circle denotes the mean response value obtained from three cells. The solid line represents the Hill equation fit which yielded an EC_{50} of 147 ± 4 mM and a Hill coefficient of 1.98.

vatable current, which could be blocked by (in the following order) mecamylamine, hexamethonium, decamethonium, dihydro-β-erythroidine (all not shown) and d-tubocurarine (Fig. 4). At more positive membrane potentials, inward rectification was observed. Interestingly, the ACh-induced currents recorded from different cells showed considerable variation in the rate and level of desensitisation. From dose-response curves for ACh, EC_{50} values of 145 ± 12 μM, and Hill coefficients between 1.6 and 1.98 were obtained (3 experiments, Fig. 4). Together with dose-response curves for the agonists cytisine and nicotine (not shown), an order of potency of cytisine ≅ nicotine > acetylcholine was deduced. The Hill coefficients for all agonists tested were significantly higher than unity, suggesting that two (or more) molecules of agonist are required to activate this nAChR subtype. The distribution of response amplitudes to 100 μM ACh of HEK-293 cells stably expressing the α3/β4 nAChR cultured for 6–8 weeks was quite broad, varying between 10 and 2000 pA, with an average value of 360 pA. It is unlikely that this was due to the presence of varying amounts of homopentameric and heteropentameric nAChRs because (i) α3 subunits apparently do not form functional homopentamers [21,25], and (ii) the Hill coefficients obtained with low responding cells were not significantly smaller than those obtained with high responding cells.

4. Discussion

Human embryonic kidney (HEK) cell lines are well established ectopic expression systems [16–18,21,33,34]. Although relatively high levels of transient expression of α3/β4 nicotinic receptors have been achieved in these cells ([21] and this paper), stable expression should provide several advantages, including more reproducible and longer lasting levels of nAChR expression, which are prerequisites for detailed single-channel studies and the practical application of these cells for drug screening. At present, we have achieved in HEK cells the expression of α3/β4 nAChR that persists for several months, but the level of receptor expression is still quite variable between different transfected cells. The functional assay employed in the selection of nAChR-expressing cells, however, may also be suitable for the selection of high responders from among transfected cells.

The functional assay employed takes advantage of the fact that activation by agonist of nicotinic receptors produces a local depolarisation that, if large enough, could trigger the activation of voltage-gated Ca^{2+} channels. By employing a HEK cell line that stably expresses an L-type Ca^{2+} channel, the desired functional coupling of the two types of ion channels was indeed achieved (Figs. 1 and 2). The assay can detect HEK-293/α1 cells that have a sufficiently high level of nAChR expression in order to produce, in the presence of agonist, membrane depolarisation that is above the threshold of Ca^{2+}-channel activation. Only properly assembled and functional nAChR are detected, which is an important advantage in comparison to binding assays. Binding assays are also more time-consuming, more prone to experimental artefacts, and less sensitive.

The electrophysiological data obtained so far are only preliminary, yet it appears that the α3/β4 subtype of nAChR, when stably expressed in HEK-293 cells, closely resembles the major nAChR of PC12 cells [10], and also type III responses recorded from cultured embryonic hippocampal neurons [11,12]. Based on patch-clamp studies with *Xenopus* oocytes ectopically expressing various combinations of nAChR subunits, it has been proposed that type III responses are subserved by a combination of α3 and β4 subunits [11]. Therefore, our results suggest that this subtype may indeed represent the major ganglionic nAChR. The HEK cell line stably expressing this receptor may help to characterise further its pharmacology and functional properties.

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