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Artículo Original

Cholinergic currents in *Xenopus* oocytes transplanted with human brain membranes

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ABSTRACT

Cholinergic human brain currents were recorded in *Xenopus laevis* oocytes transplanted with human cerebral membranes from two different zones, the frontal cortex and the hippocampus. The recorded currents were supported by the nicotinic or the muscarinic acetylcholine receptor. We tested the effects of a number of several nicotinic agonists acetylcholine, nicotine and 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP), and the nicotinic receptor antagonists a-bungarotoxin and d-tubocurarine on the transplanted oocytes. We detected four kinds of nicotinic current kinetics. The differences in the amplitude and in the total electric charge of the currents elicited by various agonists at a range of holding potentials were not significant, except in the case of DMPP at a holding potential of -90 mV. Our results indicate that $\alpha4\beta2$, $\alpha3\beta4$ and $\alpha7$ are the main nicotinic receptors in human brain.

Key words: Human cortex.—Neuronal nicotinic currents.—*Xenopus* oocytes.—Membrane transplant.—Alzheimer.—Brain bank.

RESUMEN

Corrientes colinérgicas en oocitos de *Xenopus* trasplantados con membranas de cerebro humano

Corrientes colinérgicas de cerebro humano fueron registradas en oocitos de *Xenopus laevis* trasplantados con membranas de cerebro humano procedentes de

dos zonas diferentes, la corteza frontal y el hipocampo. Las corrientes registradas fueron activadas por el receptor nicotínico o por el receptor nicotínico o muscarínico de la acetilcolina. Se probaron los efectos de diferentes agonistas nicotínicos como acetilcolina, nicotina y yoduro de 1,1-dimetil-4-fenil-piperazinio (DMPP), y antagonistas del receptor nicotínico como a-bungarotoxina y d-tubocurarina en los oocitos transplantados. Detectamos cuatro clases de cinéticas de corrientes nicotínicas. Las diferencias en la amplitud y en la carga eléctrica total de las corrientes provocadas por varios agonistas en el rango de potencial mantenido no fueron significativas, excepto en el caso del DMPP a un potencial mantenido de -90 mV. Nuestros resultados indican que las formas $\alpha 4\beta 2$, $\alpha 3\beta 4$ y $\alpha 7$ son los principales receptores nicotínicos en el cerebro humano.

Palabras clave: Córtex humano.—Corrientes nicotínicas neuronales.—Oocitos de *Xenopus*.—Trasplante de membrana.—Alzheimer.—Banco de cerebros.

INTRODUCTION

The cholinergic pathways in the central nervous system play a significant role in cognitive and behavioral functions such as learning, memory, arousal, cerebral blood flow and metabolism. Neuronal nicotinic and muscarinic receptors are involved in these functions (1-3).

The neuronal nicotinic acetylcholine receptors are a family of acetylcholine-gated ion channels which has been characterized combining molecular cloning and pharmacology. Several subtypes with a specific pharmacology, physiology and anatomical distribution, have been found. A gene family encoding 11 of these receptors, has been identified, and most of them have been cloned and expressed in transfected cells (4). Two main subunits have been documented: a and b chains. To date 8 types of α and 3 types of β chains have been detected. Mutations in one of the subunit genes account for some pathological conditions such as a specific form of epilepsy or schizophrenia and in neurodegenerative illnesses, such as Alzheimer's and Parkinson's disease, high affinity neuronal nicotinic acetylcholine receptors sites are reduced (3).

Studies of recombinant neuronal nicotinic receptor expressed in *Xenopus* oocytes show that coexpression of the α and β subunits is needed to form an active receptor with a pentameric structure.

However, expression systems α7, α8 and α9 result in active homo-oligomeric receptors (3, 5). The expression of combinations of distinct mRNA subunits in *Xenopus* oocytes shows the individual pharmacological profiles of the subunit combinations and which makes it possible to identify the type of nicotinic receptor according to its profile (6). The specific combination of the types of subunits determines the pharmacology of these receptors (7).

The presence of distinct subunits in human brain has been established by *in situ* hybridization techniques, but we do not know the combination of the subunits in each native receptor. In addition functional activity, has been tested by expressing the mRNA encoding subunits in *Xenopus* oocytes. Here we assessed the physiology of the human neuronal nicotinic acetylcholine receptor by transplanting membranes obtained from human brain into *Xenopus* oocytes. This technique alowed us to study the pharmacology of native receptors in their original lipid and proteic environment.

MATERIALS AND METHODS

1. Subcellular fractionation protocol in human brain

Frozen brain tissue was obtained from the «Banc de Teixits Neurològics. Serveis Científico-Tècnics. Universitat de Barcelona. Hospital Clínic», at 7 h post-mortem from a 66-year-old male donor with no history of neurological disorder (reference BK360). The samples were from the frontal cortex and hippocampus.

Subcellular fractionation was performed following the method described by Blackstone *et al*. (8). All procedures were performed at 4° C. About 1 g of frozen brain was homogenized 10 times at 600 r.p.m. with a glass-teflon homogenizer (Potter S, B. Braun Biotech International) in 10 volumes with a solution 4 mM HEPES, 1 mM EDTA (pH 7.4) with 2 μg/ml leupeptin, 1mM phenyl-methylsulfonyl fluoride (PMSF), 20 μg/ml aprotinin, 2.5 μg/ml pepstatin. The homogenate was agitated for 30 min and centrifuged at 1000 *g* for 10 min. The supernatant was then centrifuged at 25000 *g* for 20 min. The resulting sediment was resuspended in the solution plus 0.25 M sucrose and layered onto a discontinuous sucrose gradient

containing 0.8 M/1 M/1.2 M sucrose and centrifuged for 2 h at 65000 *g* in a Beckman SW-28 rotor. A synaptic membrane fraction was yielded at the 1 M/1.2 M sucrose interface, myelin in the floating fraction and a mitochondrial fraction as the pellet. The synaptic plasma membrane fraction was diluted 1/3 in a solution HEPES 10 mM and centrifuged for 30 min at 50000 g. The resulting sediment was resuspended in a HEPES 10 mM, KCl 140 mM (pH 7.4) solution, frozen with liquid nitrogen and stored at –80 °C until use.

2. Animals and solutions

Mature females of *Xenopus laevis* were purchased from the «Centre d'Elevage des Xenopes» (Montpellier, France), and were anaesthetized by immersion in water containing 0.17 % tricaine. A few lobes were removed from an ovary through a small incision in the abdomen.

Solutions for *Xenopus* oocytes: Barth's solution contained: 88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.40 mM $NaHCO_3$, and 20 mM HEPES at pH 7.4, supplemented with 100 IU/ml penicillin and 0.1 mg/ml streptomycin. Recording solution: 115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, and 10 mM HEPES at pH 7.4. None of the *Xenopus* female donors used in this study exhibited muscarinic acetylcholine receptor currents in their oocytes.

3. Oocyte preparation, membrane transplantation and recording

Oocytes at stages V and VI (9) were removed out and kept at 15-16° C in sterile Barth's solution. Healthy oocytes were microinjected with volumes within 50-100 nl of thawed suspension of human brain membranes (3-10 mg/ml) following Marsal *et al*. (10), by means of an injector (WPI, model A203XVZ). Samples were sonicated prior to injection.

Before recording (3-4 h), oocytes were treated with collagenase type 1A (Sigma) (0.5 mg/ml) for 45-50 min at room temperature to remove the surrounding layers (11). They were then voltage-clamped

with a two-electrode system (Axoclamp-2A, Axon Instruments, USA) 20-48 h after the membrane transplant. Intracellular electrodes (1-3 MW resistance) were filled with 3 M KCl for voltage recording and current injection. The volume of the oocyte recording chamber was 200 µl. Membrane currents were sampled by Lab PC+ (National Instruments, USA) at twice the filter frequency, low-pass filtered at 10 Hz and recorded on a PC using the Whole Cell Analysis v. 2.1. program which was kindly provided by Prof. J. Dempster (Strathclyde University, Scotland, UK). Currents were elicited by challenges of 1-3 mM cholinergic agonist. The interval between consecutive responses was systematically set to 10-12 min (flow rate 6-8 ml. min^{-1}), as we had previously established that this was sufficient to ensure a complete recovery from receptor desensitization. Oocytes from *Xenopus* donors were tested for muscarinic acetylcholine receptors. To ensure the consistency of the response amplitude, three consecutive agonist challenges were applied before changing to other agonist or antagonist.

We tested the effects of the nicotinic agonists, nicotine, acetylcholine, 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP), at concentrations from 1 to 3 mM, on the *Xenopus* oocytes transplanted with the human brain membranes. The membranes from frontal cortex were obtained in two independent fractionations. The results were recorded in oocytes from 12 *Xenopus* donors.

4. Measurement and calculations

The amplitude and time constant of responses was measured through Whole Cell Analysis. Statistical analysis was done using SigmaStat 2.0 (SPSS Inc, Chicago, IL), using the one way Anova Test.

5. Drugs

(-)-Nicotine, acetylcholine chloride, 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP), α-bungarotoxin and d-tubocurarine chloride were obtained from Sigma, St Louis, MO, USA.

RESULTS

Transplant of human brain membranes, either from cortex or hippocampus, into *Xenopus* oocytes significantly decreased the oocyte resistance ($p < 0.05$); (0.3 ± 0.05 MΩ) (n = 16) vs (0.8 ± 0.2 MΩ) (n = 12) in oocytes injected with H_2O. Usually, after 20-48 h of membrane injection, we recorded an inward current in response to cholinergic agonists in 30-40% of the injected oocytes. When acetylcholine was used some of the responses (between 40-50% of the oocytes tested) were from the muscarinic receptor (Fig. 1a) because the response was of the oscillatory type and remained active for several minutes and was sensitive to atropine (Fig. 1b). In other oocytes we found a non-oscillatory response after perfusing with the nicotinic agonists acetylcholine, or nicotine or DMPP.

We found four kinds of nicotinic currents depending on the agonist applied, Figs. 1 C, D, E, F. In the first kind of response (Fig. 1 C) the current opened fast ($\tau = 6.9 \pm 2.2$ s) and was maintained while perfused with the agonist. This kind of current, which was not so usual (8/83 responses) was observed using acetylcholine (7/8 responses) and nicotine (1/8 responses) but not with DMPP. The second kind of current (Fig. 1 D), the most common (53/83 responses) opened slowly ($\tau = 19.7 \pm 4.7$ s) and increased until the agonist was washed out (in some cases, we exposed the oocyte to the agonist for 90 s and the inward current increased at a constant rate throughout exposure. A third kind of response (Fig. 1 E, 17/83 responses) exhibited dual kinetics: it started as a fast opening ($\tau = 8.12 \pm 1.4$) but then slowed down until the agonist was washed out ($\tau = 16.2 \pm 1.7$). Finally, we identified a fourth kind of current (Fig. 1 F) that opened with a $\tau = 6.4 \pm 0.8$ s and closed spontaneously before the agonist was washed out. This kind of current was not usual (5/83 responses) and was observed only using DMPP (4/5) and nicotine (1/5).

The total recording time in one oocyte varied from 40 to 90 s. Table 1 summarizes the amplitude of responses obtained at a range of holding potentials, –80, –90, –100 mV. In each holding potential group, the amplitude of the currents was independent of the agonist used, except in the case of DMPP when was measured at –90 mV.

When the membranes were obtained from oocytes injected with hippocampus membranes, we found the same results as described in cerebral cortex, except that no differences between the agonist were detected.

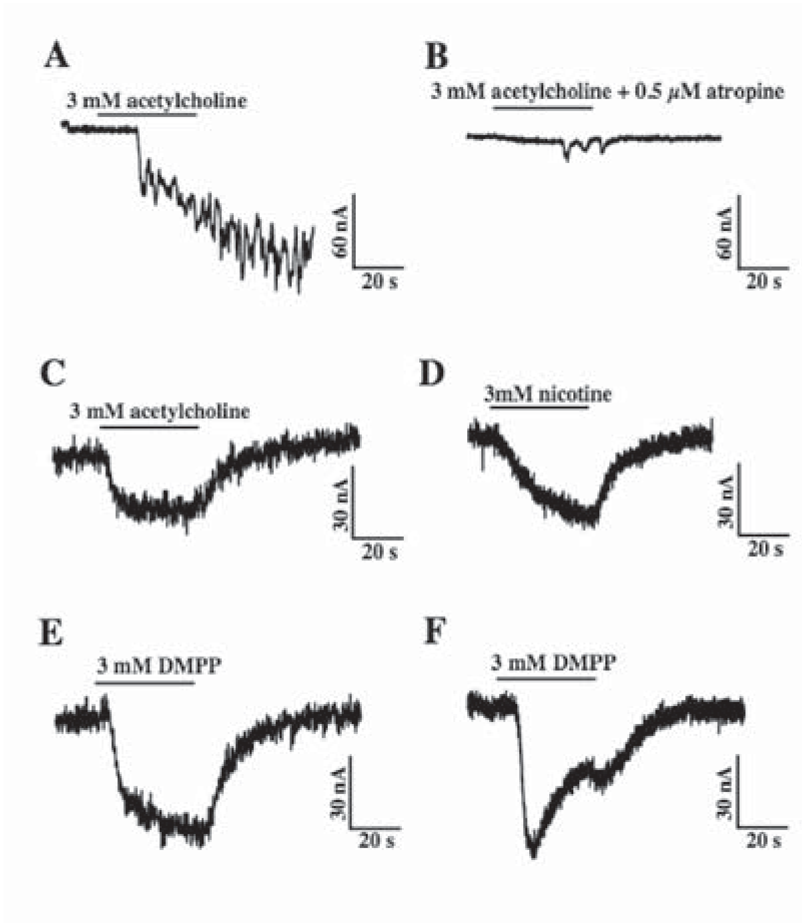

FIGURE 1. *Effects of nicotinic agonists on the size of the currents at a holding potential of –90 mV with an exposure of 40 s. In this case the membranes were from frontal cortex. (A) Muscarinic current induced by 3mM acetylcholine. (B) Muscarinic current inhibited by 0.5 mM atropine. (Current obtained in the same oocyte as point A). (C) Nicotinic current induced by 3 mM acetylcholine. (D) Nicotinic current induced by 3 mM nicotine. (E) Nicotinic current induced by 3 mM DMPP. (F) Nicotinic current induced by 3 mM DMPP, note that in this case the channel closed before the end of exposure to the agonist.*

TABLE 1. *Effects of a range of concentrations of agonist at distinct membrane potentials on the size of the induced currents*

	Amplitude (*nA*)	**Charge (*nQ*)**	***n***
[agonist] (time exposure) at –100mV			
1 mM acetylcholine (40 s) FC	29.5 ± 4.5	440 ± 223	8
1 mM nicotine (40 s) FC	33.8 ± 4.7	736 ± 106	6
1 mM DMPP (40 s) FC	33 ± 4	812 ± 215	5
[agonist] (time exposure) at –90mV			
3mM acetylcholine (40 s) FC	18 ± 2.5 [a]	418 ± 83 [a]	5
3mM nicotine (40 s) FC	25 ± 5 [a]	584 ± 138 [a]	6
3mM DMPP (40 s) FC	47 ± 10[a,b]	1094 ± 206[a,b]	6
[agonist] (time exposure) at –80mV			
3mM acetylcholine (30 s) FC	8 ± 5	113.5 ± 78.5	2
3mM nicotine (30 s) FC	15 ± 2	271 ± 79	4
3mM DMPP (30 s) FC	15 ± 4[b]	295 ± 142[,b]	4
[agonist] (time exposure) at –90mV			
3mM acetylcholine (30 s) H	24 ± 10	207 ± 28	3
3mM nicotine (30 s) H	23 ± 2	522 ± 79	3
3mM DMPP (30 s) H	22 ± 6	253 ± 103	2

FC = frontal cortex, H=hippocampus.

The values are in amplitude nA and in nQ area, total surface under the profile of the current. Results are presented as mean ± S.E.M, differences between values[a] and between values[b] were significant $p < 0.05$.

The nicotinic antagonists, α-bungarotoxin (10 nM) and d-tubocurarine chloride (10 μM), had an inhibitory effect on the currents reducing their size (Fig. 2 and Table 2). Both α-bungarotoxin and d-tubocurarine antagonize the effect of acetylcholine, nicotine and DMPP.

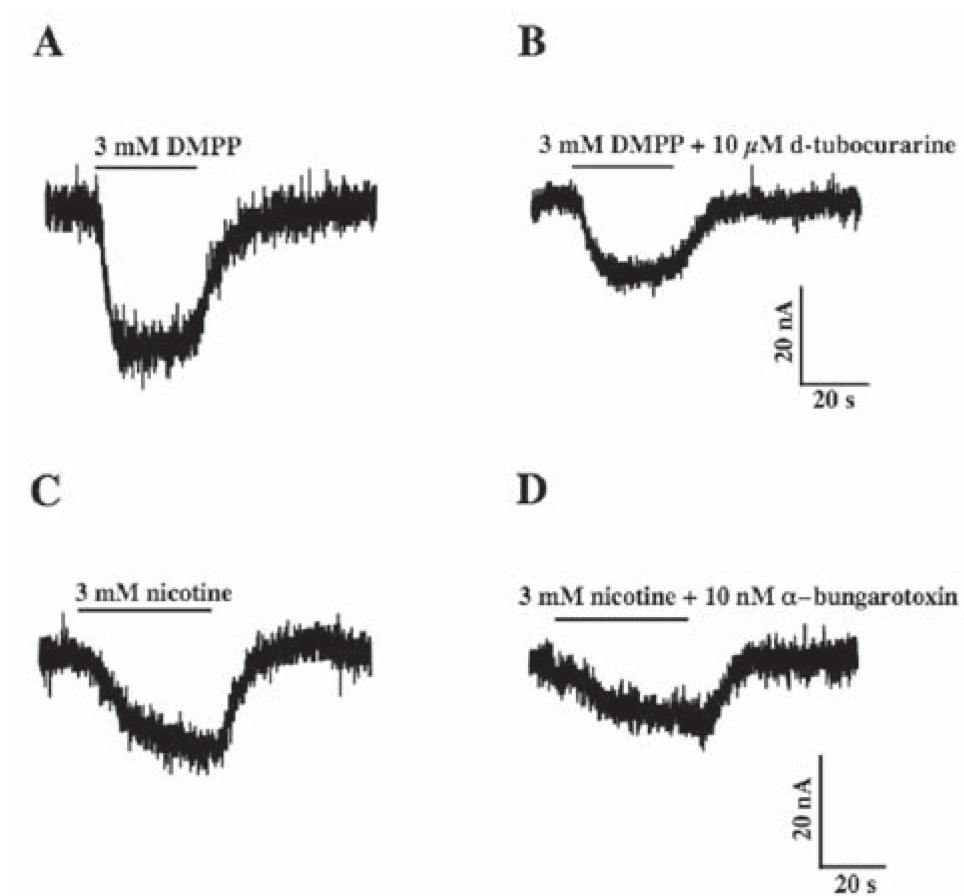

FIGURE 2. *Effects of nicotinic antagonists on the currents induced by nicotinic agonists. (A) Current induced by 3 mM DMPP. (B) Current induced by 3 mM DMPP with the presence of d-tubocurarine (10 μM) 1 min before the exposure to DMPP and during this exposure. (C) Current induced by 3 mM nicotine. (D) Current induced by 3 mM nicotine with the presence of α-bungarotoxin (10 nM) 1 min before the exposure to nicotine and during exposure.*

TABLE 2. *Effects of the aplication of antagonists in the presence of agonists. Results are in % of inhibition*

Agonist / agonist+antagonist	Amplitude (% inhib.)
FRONTAL CORTEX	
nicotine/nicotine+d-tubocurarine	42 ± 4 n = 8
DMPP/DMPP+a-bungarotoxin	42 ± 4 n = 3
DMPP/DMPP+d-tubocurarine	43 ± 1 n = 3
HIPPOCAMPUS	
acetylcholine/acetylcholine+d-tubocurarine	29 n = 1
Nicotine/nicotine+d-tubocurarine	26 ± 2 n = 2

d-tubocurarine was at a concentration of 10 μM and α-bungarotoxin at a concentration of 10 nM

DISCUSSION

In the human brain most high affinity neuronal nicotinic acetylcholine receptors seem to comprise an arrangement of α4β2 subunits. Combinations with α3 subunits and homomeric α7 constitute the other major part. However various combinations of α2, α3, α5, α6 and β2 and β4 subunits are also coexpressed to form heteromeric receptors (3, 12-14).

In previous studies that have involved injecting human mRNA to *Xenopus* oocytes, recombinant human neuronal nicotinic acetylcholine receptors displayed differential sensitivity to nicotinic agonists and antagonists and distinct kinetics, depending on the subunit combinations and on the concentration of agonist used. The sensitivity to the antagonists also differed depending on combination of the subunits present in the receptor (3, 6).

We must point out that native human nicotinic acetylcholine receptors are still active 7 hours after death, the brain extraction and even after freezing and thawing. Here we recorded small currents by perfusing acetylcholine over oocytes transplanted with human brain membranes. The small amplitude of the currents may be due to: a small number of receptors incorporated into the *Xenopus* oocyte or to the poorly represented nicotinic receptors in the zones

of human brain where membranes were obtained (3). In some cases, using acetylcholine, we recorded currents from human muscarinic receptors with the characteristic oscillatory profile for several minutes because this profile was caused by fluctuations in intracellular IP4 which led the opening of calcium activated chloride channels (15).

The oocytes in which were stimulated by all the agonists tested were taken as the most representative, with respect to sensitivity to the agonists. Generally, we recorded differences in the effect of these agonists in the same oocyte but did not find any significant difference between the means. However, with DMPP at a holding potential of –90 mV, the difference was significant and may be due to a greater incorporation of α7 or α3β4 receptors, in which DMPP is the most potent agonist (6).

The distinct kinetics of the currents detected can be explained by the predominant presence of some subunit combinations and to the concentration of the agonist dose used. Currents elicited in α3β4 and α3β2 decay substantially faster than those recorded in α2β4 or α4β4, and the currents elicited by the α7 receptor are faster that any other heteromeric combination (6). The currents that we recorded could be the addition of the distinct kinds of receptor currents, in which, in some cases, the prevalence of one of the receptors determines the profile or the kinetics of the current. We found that sensitivity to a-bungarotoxin was about 40%, a result which indicated the presence of the human neuronal nicotinic acetylcholine receptor α7 in the sample, the only neuronal subunit with sensitivity to a-bungarotoxin. Sensitivity to d-tubocurarine was variable, with mean inhibition between 26% and 43% of the current induced by the agonist, depending on the brain zone from where membranes were obtained. These results indicate the major presence of α4β2 and α7 subunits in the frontal cortex sample, because these receptors are sensitive to d-tubocurarine of 50% (6). In the currents obtained from hippocampus this inhibition was lower.

In conclusion, active nicotinic receptors survive for at least several hours after death. The profiles of the currents of transplanted human brain nicotinic receptors are very similar to those recorded from oocytes expressing human *mRNA*, indicating that the latter model is

similar to the actual currents in human brain, and not merely a chimeric combination of different receptor subunits. Our results, therefore reinforce the usefulness of *Xenopus* oocytes expressing human nicotinic mRNA as a tool for pharmacological research to design drugs to ameliorate the symptoms in Alzheimer patients. The pharmacology of the nicotinic current recorded reveals that the main native combination of subunits corresponds to $\alpha4\beta2$, $\alpha3\beta4$ and $\alpha7$ receptors which is consistent with the major presence of nicotinic receptors in human brain (12-14).

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