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Nicotinic acetylcholine receptors in cultured neurons from the hippocampus and brain stem of the rat characterized by single channel recording

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Single channel recording techniques have been applied to neurons cultured from the hippocampus and the respiratory area of the brain stem of fetal rats in order to search for nicotinic acetylcholine receptors (nAChR) in the central nervous system. In addition to acetylcholine (ACh), the potent and specific agonist (+)-anatoxin-a was also used to characterize nicotinic channels. nAChRs were concentrated on the somal surface near the base of the apical dendrite, and in some patches their density was sufficient to record 2 or more channel openings simultaneously. Although a multiplicity of conductance states was also evident, the predominant population showed a single channel conductance of 20 pS at 10°C. Thus, these neuronal nAChRs resembled the embryonic or denervated-type nAChRs in muscle. However, channel opening and closing kinetics were faster than reported for similar conductance channels in muscle. Therefore the nicotinic channels described here are similar but not identical to those of the well-characterized muscle nAChR, in agreement with biochemical, pharmacological, and molecular genetic studies on brain AChR.

Anatoxin; Acetylcholine; Patch clamp; Central nervous system; Nicotinic acetylcholine receptor; Ion channel kinetics

1. INTRODUCTION

Nicotinic acetylcholine receptor (nAChR) ion channels are present in many species at various levels of their nervous systems. While these receptors respond to the neurotransmitter acetylcholine (ACh), they are apparently heterogeneous with respect to their pharmacological characteristics. The nAChRs found at the neuromuscular junction and in the electric organ of the *Torpedo* are amongst the most well-characterized membrane macromolecules. They share similar binding properties of their agonist recognition sites and other site(s) for noncompetitive ligands. The physiology and the pharmacology of these nAChRs have been greatly detailed in the past few years by voltage clamp and single channel recordings [1–4]. Although there are some similarities between the nAChR located at the neuromuscular junction and nAChRs found in autonomic ganglia, pharmacological differences led to the subclassification of these receptors [5]. In the central nervous system (CNS) the disclosure of functional nAChRs has been a difficult endeavour. While peripherally potent nAChR ligands bind to brain tissues, it is controversial whether or not the CNS sites labelled by α-

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bungarotoxin (α-BGT) have any functional significance because, regardless of its binding, this toxin has often failed to antagonize nicotinic responses (review [6]). As a result the α-BGT-binding site was at one time purported to be a non-cholinergic receptor. A second population of putative nAChR is labelled by (−)-[^{3}H]nicotine and [^{3}H]ACh [7–10].

Furthermore, these two populations of binding sites are distributed differently in brain regions [6]. Whereas the α-BGT binding site has not yet been defined, a general consensus is that in the mammalian CNS, the agonist site labelled by [^{3}H]ACh and (−)-[^{3}H]nicotine [8,11] is responsible for some nicotinic cholinergic responses (see [9]). Recently, the peripherally selective and stereospecific nicotinic agonist (+)-anatoxin-a (Antx) [12,13] has also been shown to be a stereospecific competitor of the high affinity (−)-nicotine binding site in mammalian brain [10,14]. Additionally, recombinant DNA techniques have revealed that a separate gene family codes for two or more nAChR-like proteins in the CNS [15].

However, ligand binding and molecular biology studies do not shed any light on the functional capabilities of these nAChR sites. Biochemical studies of the action of (−)-nicotine at nerve terminals provided evidence that nAChRs facilitate the release of neurotransmitter [16]. The pharmacology of this mechanism corresponds to that of the (−)-[^{3}H]nicotine binding site and is insensitive to α-BGT. Furthermore, perhydrohistrionicotoxin, a well described ion channel probe for the peripheral nAChRs [4,17] is able to block nicotine-induced transmitter release with a similar K_i to that reported for *Torpedo* electric organ and frog skeletal muscles [18]. This is evidence that the ion channel of the neuronal nAChR is related to that of the muscle nAChR. To probe this relationship further requires the application of sophisticated electrophysiological techniques.

Thus, the purpose of this investigation was to unveil the presence of functional central nAChRs using single channel recording techniques. The hippocampus [19] and the brain stem reticular formation [20] have been reported to carry nicotinic cholinergic pathways and the excitatory responses to iontophoretically applied nicotine could be blocked by dihydro-β-erythroidine. This is, however, a controversial point [21]. Therefore in our studies single channel currents were recorded from cells isolated from the hippocampus and from the medullary region of brain stem of rats. ACh and (+)Antx were used as agonists. (+)Antx has several important advantages over the putative nicotinic neurotransmitter ACh: (+)Antx is not inactivated by acetylcholinesterase or other esterases; the toxin is a semirigid molecule with high nicotinic agonist potency and selectivity, which is nearly devoid of muscarinic activity; and (+)Antx also lacks the noncompetitive blocking effects of (−)-nicotine [22]. Thus, (+)Antx can most simply unveil the kinetics of the nAChR activation process. Here we describe the abilities of this powerful toxin and ACh to disclose the presence in brain neurons of nAChR channels which differ subtly from their counterparts at the neuromuscular junction.

2. MATERIALS AND METHODS

2.1. *Tissue culture*

The method of culturing hippocampal neurons and brain stem medullary neurons was similar to that described by Banker and Cowan [23]. Briefly, female Sprague-Dawley rats (16–18 days gestation) were sacrificed by CO_2 narcosis and cervical dislocation. Forebrains and brain stems from embryos were removed and maintained in cold physiological solution of the following composition (mM): 140 NaCl, 5.4 KCl, 0.32 Na_2HPO_4, 0.22 KH_2PO_4, 25 glucose and 20 Hepes. This solution had a pH of 7.3 and its osmolarity was adjusted to 325 mosM with sucrose.

Hippocampi or portions of medulla lying rostral to the obex were dissected free, minced with iridectomy scissors and incubated with trypsin (0.25%, Gibco) for 15 min at 35.5°C. The enzymatic activity was terminated by pipetting the tissue sections into 6–7 ml of modified Eagle's medium (MEM, Gibco) containing 10% fetal calf serum and 10% horse serum (MEM 10/10). The neurons were dissociated by trituration and suspended in MEM 10/10 to yield about 700000 cells per 2 ml plating volume per culture dish. The dissociated cells were co-cultured with mouse astrocytes derived from the cerebral hemispheres of DUB:(ICR) randombred mice. The method of obtaining confluents of astrocytes was according to the procedure described by Booher and Sensenbrenner [24] and

modified by Brookes and Yarowsky [25]. The hippocampal cell cultures were incubated for 3–4 h and the medium changed to growth medium containing MEM plus 10% horse serum (MEM 10). The cell cultures were incubated at 35.5–36.5°C in an atmosphere of 10% CO_2/90% air and the media were replaced with fresh MEM 10 every 3 days. Five days after plating, the cultures were exposed to 5′-fluoro-2′-deoxyuridine (53 μM final concentration) for 3 days to reduce the proliferation of background cells.

Hippocampal cultures contained mostly pyramidal cells, because granule cells are not yet present at the pre-natal stage of these animals [23]. Cultures derived from the medulla rostral to the obex include neurons from the ventral and dorsal respiratory groups. These include the nucleus ambiguus and the nucleus tractus solitarius. The neurons are related to respiratory (inspiratory and expiratory phase) functioning [26]. In addition to the above neuronal groups, neurons comprising cranial motor nuclei (vagal, hypoglossal, facial and trigeminal), which are responsible for motor innervation to accessory respiratory musculature, are also included in the region dissected [27]. In the rat, in particular, many neurons in the reticular formation have also been shown to respond to the iontophoretic application of ACh [20]. It is likely that neurons from the lateral reticular nucleus were also included in the region of the medulla selected for enzymatic dissociation and subsequent tissue culture. The cells in the brain stem culture were either pyramidal, fusiform or spherical, as described in a recent morphological and electrophysiological study of guinea pig brain stem neurons [28].

One to four-week-old hippocampal cultures and brain stem cultures as young as 3–4 days were used for single channel recordings. The membrane potentials of these neurons were between −50 and −65 mV. In the absence of tetrodotoxin, spontaneous synaptic potentials could be recorded from all the cells tested.

2.2. *Single channel recording techniques*

The patch clamp technique [29] was used to record single channel currents from the somal surface membrane close to the apical dendrite of cultured neurons. For recording, the neurons were maintained in a physiological buffer containing (mM): 116 NaCl, 5.4 KCl, 3.0 $CaCl_2$, 1.3 $MgCl_2$, 26.0 $NaHCO_3$, 1.0 NaH_2PO_4, 11.0 dextrose at 315 mosM. After bubbling with a 95/5% O_2-CO_2 mixture the pH was 7.4. Tetrodotoxin (0.1 μM) was included in the solution to prevent spontaneous activity. The patch-clamp microelectrodes were made from borosilicate capillary glass (A & M Systems) and their resistances ranged between 3 and 5 MΩ when filled with recording solution. Single channel currents were recorded from cell-attached patches with micropipettes filled with the same solution and the desired concentrations of the agonist being tested. An LM-EPC-7 patch clamp system (List Electronic, FRG) was used to record single channel currents at various holding potentials.

The data were stored on FM magnetic tapes (Racal 4DS) for later computer analysis. The data were filtered at 3 kHz (−3 dB) with an 8-pole Bessel filter, digitized at 12.5 kHz and analyzed using IBM XT and AT microcomputers. Automated programs (M. Sloderbeck and C.J. Lingle, Florida State University) were used for data acquisition, detection and analysis of single channel currents. The average amplitude of a single channel current was determined as the difference between the current peak and the baseline peak in histograms generated from all digitized points. Open time was defined as the duration of an open event that was terminated by a closing transition, recognized by a decrease in current to below 50% of the unitary channel amplitude. A burst was the sum of open and short closed events terminated by a closure lasting more than 1.6 ms. For kinetic analyses, histograms of event durations were fitted with single exponential decay functions to determine the time constants, τ.

3. RESULTS

3.1. *Identification of nAChR on central nervous system neurons*

Neurons cultured from the hippocampus and brain stem regions of fetal rats were used to study the agonist properties of ACh and (+)Antx. In contrast to glutamate (personal observations) and GABA activated-channels [30] which seem to be homogeneously distributed in a rather high density in the somal surface membrane, we found that nAChR activity was likely to occur close to the

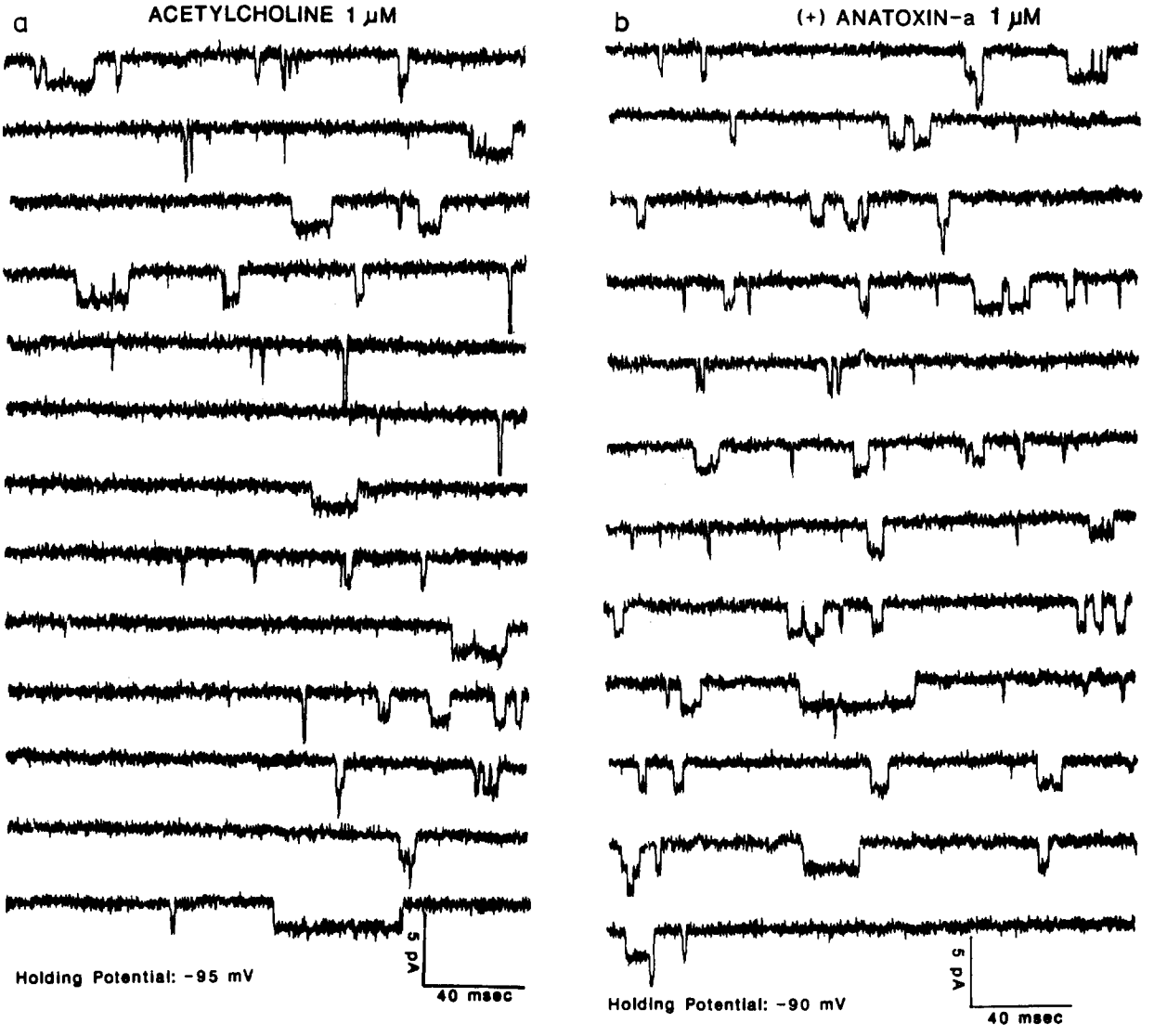

Fig.1. Single channels currents activated by (a) ACh and (b) (+)Antx (1 μM each) were located at the region of the apical dendrite of a brain stem medullary neuron. Typical channel activity is shown for each agonist.

apical dendrite. Thus, most of our recordings were obtained from this region. In some patches, the frequency of openings was high enough that currents resulting from the simultaneous openings of two or more channels with similar or different conductance states could be recorded (fig.1).

3.2. *Conductance states of the central nAChR*

A concentration of (+)Antx 10-fold higher (0.2–1 μM) than that necessary to activate muscle nAChRs [13] was used to induce openings of channels in cultured neurons from both hippocampus and brain stem areas (fig.1). In some patches, a multiplicity of conductance states was evident, whereas in most a single conductance state was observed. The predominant population showed a single channel conductance of about 20 pS at 10°C as determined from the slope of the current-voltage relationship (fig.2). At room temperature (20–22°C), the current amplitude was increased by

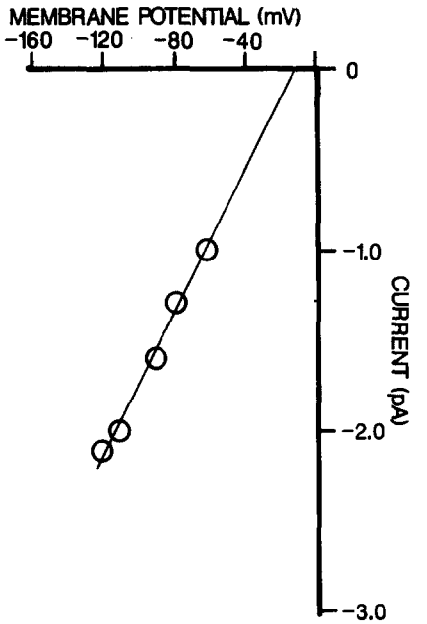

Fig.2. Slope conductance was determined as the slope of the current-voltage relationship for single channels.

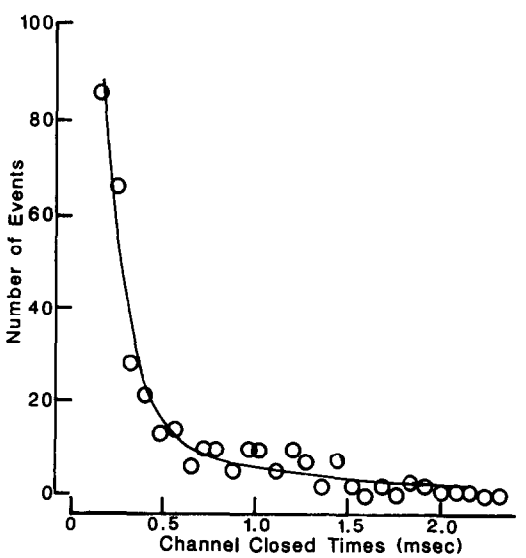

Fig.3. Stochastic analysis of single channel current open and closed durations was performed to determine the kinetic properties of the predominant 20 pS channel observed on the central neurons. The examples of the analysis of 1.6 pA channels, recorded at 10°C, are shown here and in figs 4 and 5. The histogram of durations of closed events was clearly distributed according to a double exponential. The decay constant of the shorter closed events was determined to have a τ of 0.2 ms. The minimum interburst interval was therefore defined as 1.6 ms.

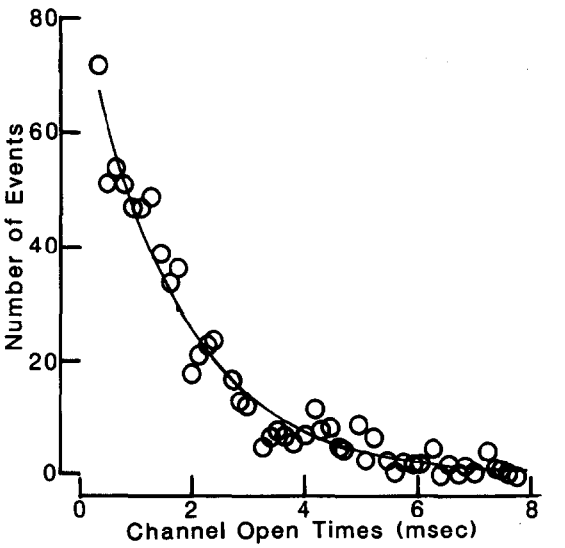

Fig.4. The distribution of durations of open events. All open events, whether occurring singly or in bursts were grouped to determine the mean open time. The open times were distributed according to a single exponential with a τ of 1.7 ms.

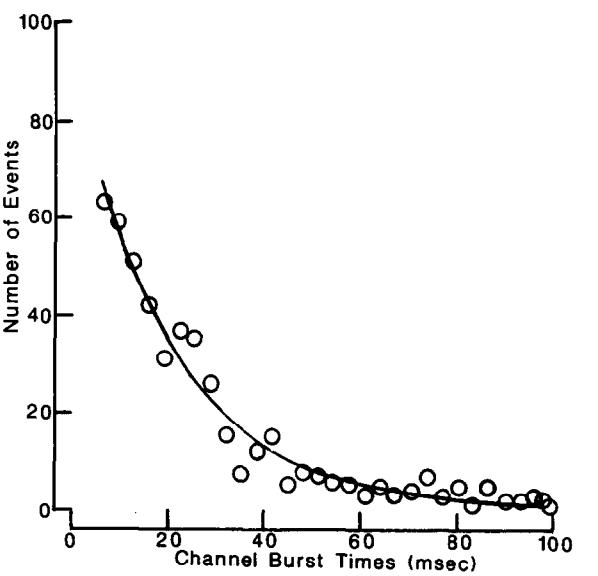

Fig.5. The distribution of durations of burst events. The durations of bursts, from the first opening to the beginning of a closure lasting 1.6 ms or more, were distributed according to a single exponential. The τ observed for burst durations was 2.7 ms.

a factor of 1.3–1.5 in agreement with the Q_{10} value reported for muscle nAChRs [31].

The presence of various conductance states was seen with both ACh and (+)Antx openings (fig.1a,b). In the case of ACh, a high conductance opening was noted in a few of the recordings (fig.1a). Due to the paucity of such events, we did not analyze them in this initial study. Smaller opening events which appeared at a lower frequency in some patches had about one-third of the amplitude of the prevalent current. This lowest conductance population of channels had apparently slower closure kinetics. The low frequency of appearance of this population, however, precluded a reliable quantitative analysis of its kinetics.

3.3. *Kinetic analysis of ionic channel activation*

The temporal analysis of the 20 pS-channels showed that (+)Antx-activated currents contained short interruptions, thus generating a bi-exponential distribution of closed times. The fast component corresponding to the intraburst fast gaps could be fitted to an exponential function with a τ of 0.2 ms (fig.3). Open times corresponding to either an isolated single opening or each of a number of openings within a burst had a single exponential distribution with a τ of 1.7 ms for channel currents with amplitude of 1.6 pA at 10°C (fig.4). The open times did not show a steep voltage-dependence through the limited voltage range thus far examined. Burst times, similar to the open times, had a single exponential distribution with a mean of 2.7 ms for currents of 1.6 pA. The mean number of events per burst ranged from 1.2 to 1.6 among different patches. This value did not show a clear correlation with the voltage or concentration of the agonist.

4. DISCUSSION

The present investigation conclusively demonstrates that nicotinic agonists such as (+)Antx and the neurotransmitter ACh are able to activate channel openings in neonatal cultures of hippocampus and brain stem cells. The most reactive site for the agonists was usually located at the region of the apical dendrite. Compared to peripheral nAChRs, 5–10-times greater concentrations of both (+)Antx and ACh were necessary to activate channels on the hippocampal and brain stem neurons. The clearly desensitizing, clustering pattern of channel activity, which has been observed in muscle at concentrations of either ACh higher than 1 μM [32] or 0.8 μM (+)Antx or greater [13], were not observed in the central neurons. Instead, in this concentration range, randomly occurring single events or sometimes stepwise multiple openings were recorded from these neurons. Indeed both (+)Antx and ACh were able to induce, in suitable concentrations, double and triple openings. The potency ratio of (+)Antx to ACh in this study was similar to that observed in the periphery; (+)Antx activated single channel currents at 5- to 10-fold lower concentrations than the neurotransmitter.

Regarding channel conductance in the cultured CNS neurons, the main population that was recorded had a slope-conductance of 20 pS, at 10°C. This finding compared closely with the observations in embryonic myoballs [31] and in chronically denervated skeletal muscles [33,34]. However, the high-conductance channels (about 32 pS, at 10°C), which are the predominant population in adult, innervated frog muscles [34] and which also appear at a lower frequency (5–10% of the total events recorded) in cultured rat myoballs [31], were not frequently observed in the CNS cultured neurons. Thus, it seemed that the central neurons in culture carried the embryonic or denervated-type nAChRs. This may reflect the immaturity of the preparation and/or its receptors; we previously demonstrated [30] that GABA-activated channels on cultured hippocampal neurons differed from those of adult neurons. Comparison of ACh- and GABA-activated channels assumes a new significance in the light of recent molecular biological evidence for their structural homology [35]. Alternatively, the ACh- and (+)Antx-activated channels may have an extrasynaptic localization on these neurons as proposed for α-BGT-binding sites on sympathetic ganglia [36].

Similarly to the muscle nAChR [13], the pattern of channels activated by (+)Antx in central neurons showed bursts with an increased number of short closures in contrast to the isolated openings induced by ACh. Because the individual (+)Antx-induced openings were also shortened, the resulting bursts remained shorter than those activated by ACh. The channel bursts induced in the

CNS neurons by (+)Antx at micromolar concentration did not resemble those elicited by desensitizing concentrations of agonists [13,32,37,38], by open channel blockers such as QX222 [39] or anticholinesterase agents such as neostigmine and edrophonium [38]. Rather, as reported for muscles [13,40], these fast closures observed with (+)Antx probably resulted from relatively more rapid transitions from the closed, doubly-agonist-bound state to the opened state as compared to the rates of dissociation of either of two agonist molecules. The τ_{closed} for the CNS receptor (0.2 ms) was shorter than that of muscle (0.4 ms; [13]), which implies that the rate of opening was greater; however, because the number of openings per burst remained similar to that in muscle, the dissociation of (+)Antx from the receptor was apparently also increased. This may account for the higher agonist concentrations necessary to elicit responses and the absence of desensitizing bursts at micromolar concentrations.

For the 20-pS channels, the nAChR in the cultured central neurons had a rapid rate of channel closure. For denervated muscles and cultured myoballs, at 10°C and at holding potentials between −80 and −140 mV, the mean channel open times for ACh-activated channels were reported to range from 10 to 45 ms, the rate of channel closure being faster at more positive potentials. However, different nicotinic agonists activate channels with different kinetics; for the 32-pS channel of innervated muscle, the duration of the open state induced by (+)Antx is approximately one-half of that induced by ACh [13]. While the 20-pS conductance channels activated by (+)Antx in denervated muscles had a burst pattern of multiple openings similar to the 32 pS channel typical of innervated muscles, the rate of channel closing was slower in the lower conductance channel (Albuquerque and Aracava, unpublished). For the predominant neuronal nAChRs reported here, the closing rate constant was faster than that observed for extrajunctional nAChRs in the skeletal muscles, thus the mean open and burst times of the channels activated by (+)Antx were only 1.6 and 2.7 ms (at −120 mV holding potential).

In conclusion, we have demonstrated using the patch clamp technique that the agonists ACh and (+)Antx activate channels in cultured hippocampal and brain stem neurons with a conductance similar to that reported for the embryonic and denervated muscle nAChRs. Although qualitatively the events opened by these two agonists were distinguishable, both ACh- and (+)Antx-activated channels had channel opening and closing kinetics faster than those reported for similar conductance nAChRs in muscle. These differences could be a functional consequence of the structural changes between neuronal and muscle nAChRs implicit in the molecular biological evidence for a separate gene family governing this macromolecule in the brain [15]. Application of the patch clamp technique to nicotinic channels on central neurons, successfully demonstrated here for the first time, will permit us to assess the functional status of nAChRs in the CNS. The apparent similarity of the ionic channels of the central and peripheral nAChRs [18] suggests that the use of Antx and its analogues, including the newly synthesized Antx derivatives with non-competitive antagonist properties [41], may help to clarify the relationship between the receptor subtypes.

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