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Three Conductance Classes of Nicotinic Acetylcholine Receptors Are Expressed in Developing Amphibian Skeletal Muscle

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Two previously described classes of nicotinic AChRs in vertebrate skeletal muscle have conductances of 40 and 60 pS. In addition, a third conductance class of AChR channels is present in developing *Xenopus* muscle. This class appears to represent an independent channel type, rather than a subconductance state of the larger conductance channels. The channel has a slope conductance of 25 pS and a reversal potential of about 0 mV membrane potential. Its kinetic properties resemble those of the 40 pS channels present in early embryonic myotomal muscle. The channel has a mean open time of about 6 msec (at 40 mV applied potential). The open time is dependent on membrane potential and increases *e*-fold for every 60 mV of hyperpolarization. Consecutive openings were often separated by brief closures of about 0.4 msec in duration. The identity of the channel as a nicotinic AChR was established by blocking the channel openings with α-BTX and by demonstrating bursting and desensitization in the presence of high agonist concentrations. In some muscles (e.g., extraocular), this channel may be a predominant form at early developmental stages and could therefore be important to the function of developing synapses in those muscles.

Patch-clamp studies of amphibian and mammalian skeletal muscle have revealed the existence of 2 classes of AChRs having conductances of 40 and 60 pS (reviewed by Schuetze and Role, 1987). AChR channels have also been shown to have subconductance states with reduced current amplitude (Hamill and Sakmann, 1981; Auerbach and Sachs, 1983, 1984; Takeda and Trautmann, 1984; Colquhoun and Sakmann, 1985; Sigworth, 1985; Morris and Montpetit, 1986). In addition to these 2 classes of AChR channels and their related subconductance states, smaller, independent channel openings have been observed while recording AChR activity in muscle membrane (Brehm et al., 1984a; Auerbach and Lingle, 1986; Leonard et al., 1988). The electrophysiological properties of the smaller channels have not been characterized in detail nor has their identity as AChR channels been established. These channels have tended to be overlooked because their openings were less frequently observed than those of the main conductance classes. However, while studying the development of AChR channels in *Xenopus* muscle *in vivo*, we noticed that they contributed the majority of events in some patches and therefore might play a significant role in neurotransmission or synapse development. The purpose of the present study was to describe the activity of these smaller channels and confirm their identity as AChR channels.

Materials and Methods

Single-channel recordings were obtained *in vivo* from developing myotomal, interhyoideus, and superior oblique muscle of *Xenopus laevis* embryos and tadpoles. Animals were reared as described previously (Owens and Kullberg, 1989), and were staged according to the criteria of Nieuwkoop and Faber (1967). Myotomal muscle is the first muscle to develop in the embryo and the first myotomes can be detected as early as stage 17. The superior oblique is an extraocular muscle that can first be visualized by dissection at about stage 39. The interhyoideus, a flat muscle of the lower jaw, begins to develop at about the same time. In other studies, we have shown that these muscles differ in their development of endplate currents and probably have 3 distinct patterns of AChR development (Kullberg et al., 1985; Kullberg and Owens, 1986).

Preparation of muscles for recording. Dissection and recording were carried out in a saline solution consisting of 110 mM NaCl, 1 mM KCl, 1.8 mM $CaCl_2$, 8 mM HEPES buffer adjusted to pH 7.4 with NaOH, and tetrodotoxin (1 μg/ml). Tadpoles were pithed prior to dissection. The skin overlying the muscles was removed and the preparation was secured to the bottom of a Sylgard (Dow-Corning) covered petri dish with stainless steel pins or clips. A chamber with a coverslip on top was placed over the preparation to improve visualization of the electrode tip. All experiments were done at room temperature (22–24°C).

The amount of connective tissue overlying the muscle cells increases with age, and collagenase treatment is usually necessary to obtain seals in older animals. However, we wished to restrict our use of enzyme treatment in order to avoid any possible effects of enzymes on receptor function. Our study was therefore limited to early developmental stages. All data reported in this study were obtained without enzyme treatment, with the exception of recordings from myotomal muscle older than stage 40.

Recording techniques and data analysis. All recordings were obtained from cell-attached patches, using standard single-channel recording techniques (Hamill et al., 1981). Electrodes were made from borosilicate glass and had outer tip diameters of about 1 μm. The tips were not fire-polished but were occasionally coated with Sigmacoat to reduce noise. Electrodes were filled with saline having the same composition as the bath and contained 500 nM ACh for most recordings or else 20–50 μM ACh for eliciting bursts of openings. Details of the performance characteristics of our recording system and the methods of data analysis have been described previously (Owens and Kullberg, 1989). Briefly, recordings of single-channel events were obtained at several different applied potentials (0–100 mV), Bessel-filtered at 3 kHz, digitized at 10 kHz, and stored on disk. The open and closed durations and amplitudes of all events were measured and compiled into histograms. Open and closed duration histograms were fitted by single- and double-exponential functions, by the method of maximum likelihood (Colquhoun and Sigworth, 1983). All data are expressed as means ± SD, and the sample sizes are given in parentheses and refer to the number of different recording sites.

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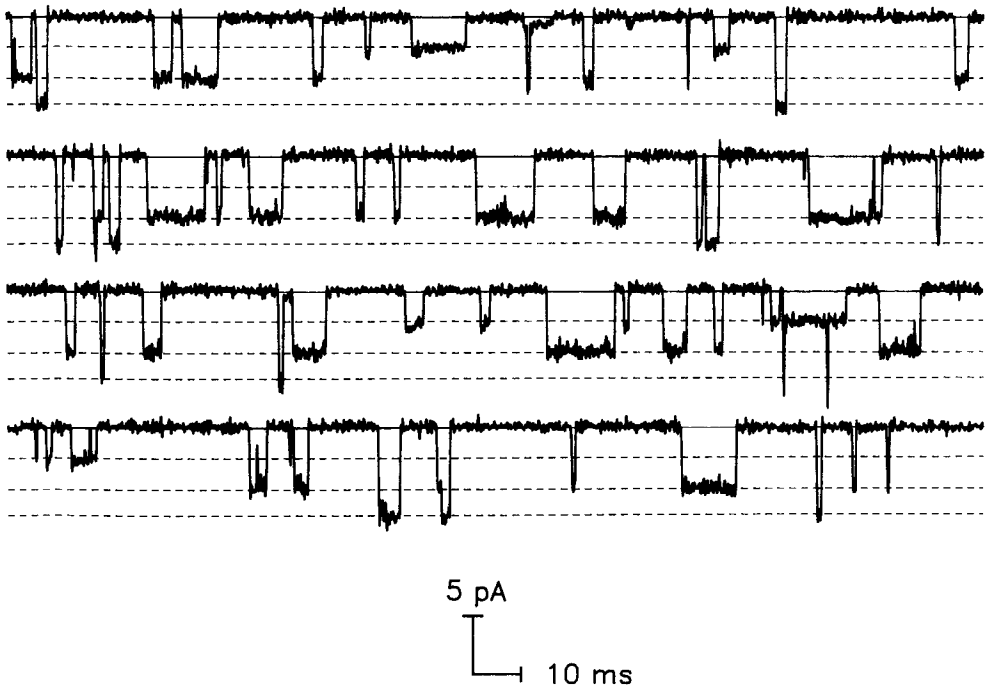

Figure 1. Single-channel events recorded from a patch of nonjunctional myotomal muscle membrane (stage 37). These records exhibit 3 discrete current levels, as indicated by *dashed lines.* Records are nonsequential and were selected to demonstrate the different amplitude classes. Applied pipette potential was 60 mV and the pipette contained 500 nM ACh.

We routinely tested patches for the presence of stretch-activated channels (Brehm et al., 1984b) by applying suction to the recording pipette during the recording. To avoid confusing AChRs and stretch-activated channels, records containing channel activity elicited by suction were not analyzed.

Results

Channel conductance

Single-channel recordings of nonjunctional AChR activity were obtained *in vivo* from myotomal, superior oblique, and interhyoideus muscles. Records of single-channel currents revealed up to 4 discrete current levels in each of these muscles. Examples of single-channel records with 3 current levels are shown in Figure 1. Three discrete classes of events were also evident in some amplitude histograms, such as shown in Figure 2, where the 2 larger-amplitude peaks correspond to the 40 and 60 pS channels previously described in myotomal muscle (Brehm et al., 1984a, b; Leonard et al., 1988; Owens and Kullberg, 1989). A number of such histograms revealed a class of events that opened to a current level which was about ⅔ that of the 40 pS channel. Plotting the amplitudes of the smaller-channel currents against applied pipette potential gave a slope conductance of about 25 pS with little rectification in the hyperpolarizing range (Figs. 3, 4). As noted previously (Brehm et al., 1984a, b; Owens and Kullberg, 1989), some rectification was evident in the 2 higher conductance channels. We will refer to the 3 conductance classes of channels as "60 pS," "40 pS," and "25 pS" channels.

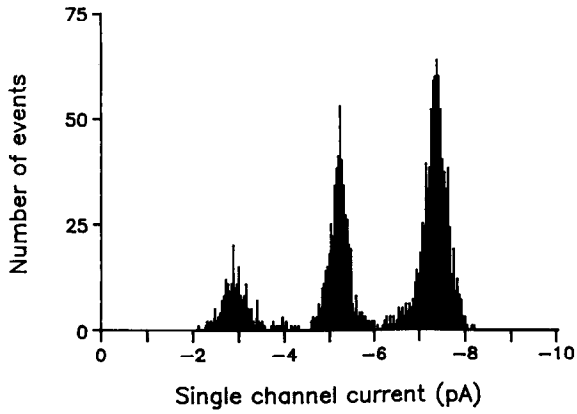

Figure 2. Amplitude histograms of single-channel events. Data were obtained from nonjunctional myotomal muscle membrane, at stage 37. The applied pipette potential was 60 mV. The peaks correspond to the 25, 40, and 60 pS conductance classes.

In addition to the 3 current levels associated with these channel classes, we observed even smaller currents in some records. These events had slope conductances of 10–15 pS. Because of their low amplitude, they were not analyzed in detail in the present study.

Reversal potential

Linear extrapolation of current–voltage plots of the 25 pS channels gave a predicted reversal potential of -80 ± 25 mV ($n = 47$) mV in terms of applied pipette potential. Assuming resting potentials of about 80 mV (Kullberg et al., 1985), this corresponds to a reversal potential of about 0 mV. This value is comparable to that found for the 40 and 60 pS AChR channels (Brehm et al., 1984a, b; Owens and Kullberg, 1989). There was little variability in either conductance or reversal potential among the 3 different muscles studied.

Relative frequency of 25 pS events

The relative frequency of the 25 pS events was variable and appeared to depend on the age and type of muscle. In myotomal muscle, 29 out of 174 patches (or 17%) exhibited 25 pS channel activity between stages 19 and 50, and the percentage did not change significantly with development during that age span (Table 1). However in patches where 25 pS channel activity was detected, openings of those channels contributed the majority of events (nearly 100%) at early stages (19–30), while at later

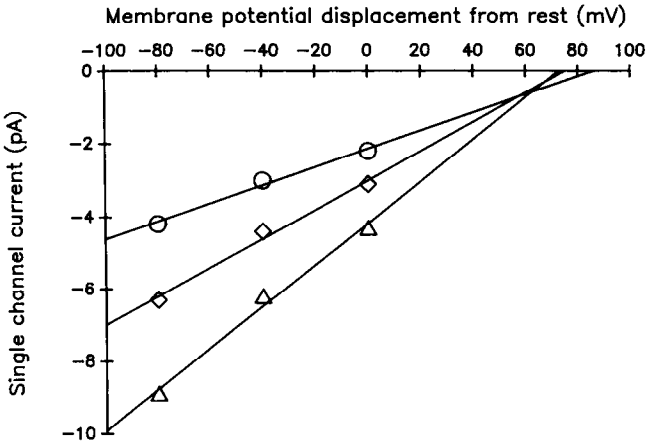

Figure 3. Single-channel current versus membrane potential displacement from a patch of myotomal muscle membrane with 3 channel types present (stage 35/36). Current amplitudes have been fitted by linear regression between 0 and 80 mV. The slope conductances were 24.9 pS (*circles*), 40.0 pS (*triangles*), and 57.4 pS (*diamonds*). The respective extrapolated reversal potentials were 85.9, 75.0, and 72.7 mV, in terms of membrane potential displacement from rest. Rectification of the 40 and 60 pS channels would result in an underestimate of the true reversal potentials.

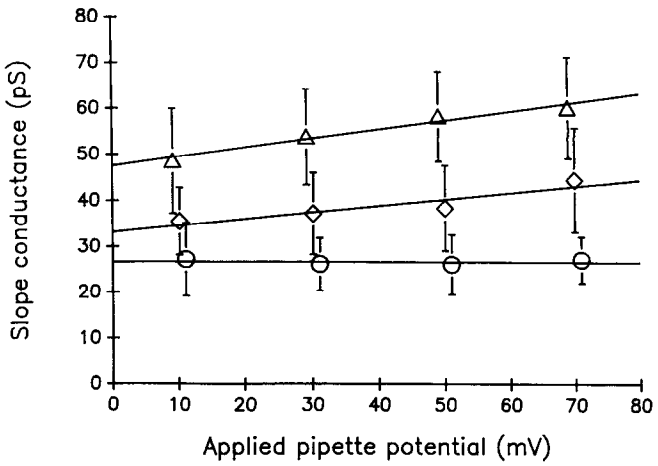

Figure 4. Slope conductance versus applied pipette potential. The data in this figure were pooled from myotomal muscle (stages 19–40), interhyoideus (stages 40–44), and superior oblique (stages 40–43). Recordings were taken at 0, 20, 40, 60, and 80 mV applied potentials. The slope conductances between adjacent 20 mV applied potentials were calculated at each recording site and then averaged to obtain the mean values plotted in this figure. *Bars* indicate SDs, and the mean values are fitted by linear regressions. The 40 pS channels (*diamonds*) and 60 pS channels (*triangles*) exhibit a small, but significant increase in conductance with increasing hyperpolarization (correlation coefficients = 0.93 and 0.98, respectively). By contrast, the slope conductance of the 25 pS channels (*circles*) shows little change with membrane potential (correlation coefficient = 0.06).

stages (41–50) their relative frequency was about 9-fold less (Table 1). The decline in relative frequency of 25 pS events was due to increased activity of the 40 and 60 pS channels.

The 25 pS channels were prevalent in newly developed superior oblique and interhyoideus muscle (stages 40–41). A total of 20 patches were examined in the superior oblique and 13 patches in the interhyoideus at these stages. In each of the muscles, the 25 pS channels were present at 70% of recording sites and constituted 85% of openings at those sites. We do not yet have data on developmental changes in frequency of 25 pS channel openings in these muscles.

Gating kinetics

The open times of the 25 pS channels were estimated by compiling open duration histograms from 25 pS events and fitting them with single- and double-exponential functions (Fig. 5). All data were obtained with 500 nM ACh in the recording pipette. The study of gating kinetics was limited to early stages of development (stages 19–30 in myotomal muscle, and stages 40–41 in superior oblique and interhyoideus). About half of the open duration histograms were fitted well by single-component exponential functions, with a mean time constant of 5.89 ± 1.32 msec (n = 7 sites, at 40 mV applied potential). This time constant is comparable to that estimated for 40 pS channels in early stage (19–30) myotomal muscle which was 6.2 msec at 40 mV applied potential (Owens and Kullberg, 1989). The remaining histograms appeared to have distinct fast and slow components and were fitted better by double-exponential functions, with mean time constants of 0.62 ± 0.38 and 4.23 ± 1.86 msec (n = 9 sites, at 40 mV applied potential). In this respect, the 25 pS channels also resemble the 40 pS channels, which frequently have double-exponential open duration histograms (Brehm et al., 1984a, b; Auerbach and Lingle, 1987; Igusa and Kidokoro, 1987; Leonard et al., 1988; Owens and Kullberg, 1989). In our measurements of channel open times, data were pooled from the 3 different muscles, since we did not notice any major differences between them. However, because of our limited sample size, small differences would not have been detected. We do not presently know whether there are developmental changes in the open time of the 25 pS channel, as found for the

Table 1. Relative frequency of 25pS events in myotomal muscle

	Stages		
	19–30	33–40	41–50
(A) Number of sites examined	92	52	30
(B) Percentage of sites with 25 pS events	16	21	13
(C) Relative percentage of 25 pS events	98	54	13
(Range, %)	(84–100)	(2–98)	(8–17)

This table documents changes in the relative frequencies of 25 pS channel openings during development of myotomal muscle. Data were pooled from 3 ranges of development, corresponding to early, intermediate, and late stages. Row (A) lists the total number of patches from which recordings were obtained at each range of stages. Row (B) lists the percentages of patches in which we detected activity of 25 pS channels. Row (C) indicates the mean percentages of observed 25 pS channel openings, relative to those of the 40 and 60 pS channels, in those patches that had identified 25 pS channel activity.

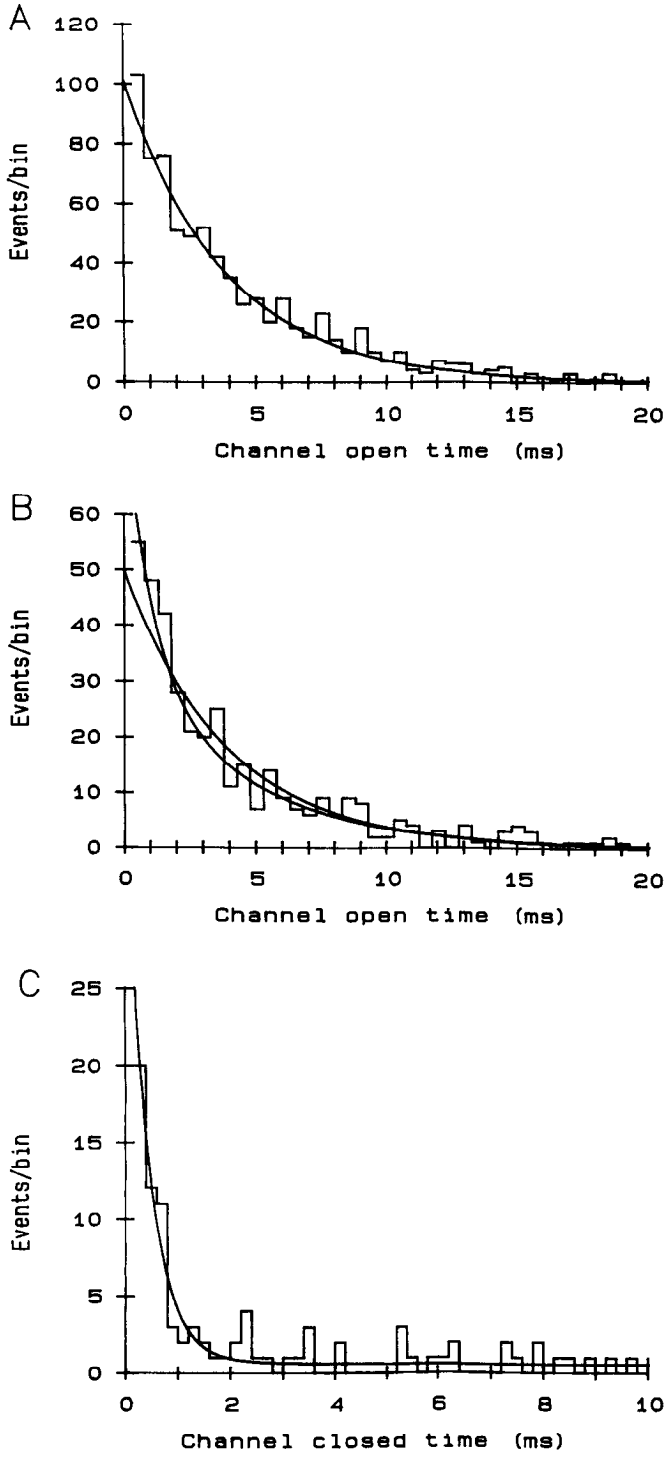

Figure 5. Open and closed duration histograms of the 25 pS channel. *A,* Open duration histogram fitted by a single-exponential function, with a time constant of 4.01 msec. Data were obtained from superior oblique, stage 40 muscle. *B,* Open duration duration histogram from stage 23 myotomal muscle. Both double- and single-exponential fitted curves are displayed. The histogram deviates obviously from a single-exponential distribution and is fitted better by a 2-component function. The time constants are 1.00 and 4.56 msec, with brief openings comprising about 22% of the total estimated number of openings (including unresolved openings less than 0.3 msec). *C,* Closed duration histogram from stage 25 myotomal muscle, showing 2 well-resolved kinetic components. The histogram is fitted by a double-exponential function with time constants of 0.44 and 59.5 msec, with brief closures comprising 30% of the total estimated number of closures, including unresolved events.

Table 2. Slope conductances of AChR channels in the presence of 20–50 μM ACh

Channel class	Slope conductance (pS)
60 pS	66.0 ± 22.1 (9)
40 pS	46.2 ± 10.4 (11)
25 pS	28.0 ± 5.6 (19)

Slope conductances were estimated at individual sites from linear regressions to the current–voltage plots of single-channel currents. Estimates were averaged for each conductance class to yield the mean slope conductances given. The values in parentheses refer to the number of sites at which data were obtained for each class.

40 pS channel (Leonard et al., 1984, 1988; Owens and Kullberg, 1989).

Based on pooled data from single-exponential histograms, the mean open time increased *e*-fold for every 62.3 ± 7.1 mV (n = 7 sites). This voltage dependency of open time is similar to that seen for the 40 pS channels at stages 19–30 in myotomal muscle and also to that of the 60 pS channels appearing later in myotomal muscle (Brehm et al., 1984b; Owens and Kullberg, 1989).

Closed duration histograms revealed 2 kinetic components (Fig. 5), as observed for the 40 pS channels in myotomal muscle (Auerbach and Lingle, 1987; Igusa and Kidokoro, 1987; Owens and Kullberg, 1989). The fast component had a mean time constant of 0.39 ± 0.12 msec (11) and constituted about 27% of the events (average of all 3 muscles, at 40 mV applied potential). The channel thus tends to open in bursts, as described for other AChRs. The average burst contained about 1.4 openings, which is similar to the value calculated for the embryonic 40 pS channels in myotomal muscle (Owens and Kullberg, 1989). The slow components, which are presumably due to periods of agonist dissociation and reflect receptor density, were quite variable and had an average value of 49.7 ± 19.5 msec (11) in the 3 muscles.

Recordings with high [ACh]

When the recording pipette contained high [ACh] (20 or 50 μM) channel openings occurred in bursts of many events, separated by long periods of silence, typical of desensitized AChRs (Sakmann et al., 1980; Auerbach and Lingle, 1987; Igusa and Kidokoro, 1987). Bursts of all 3 conductance classes were observed, sometimes within the same record as shown in Figure 6. The slope conductances of bursting channels (summarized in Table 2) were comparable to those obtained at low agonist doses.

α-BTX control experiments

The effect of α-BTX on 25 pS channel activity was tested in 4 myotomal muscles at stages 22, 23, 25, and 35/36. These muscles were selected for control experiments because of their relatively high abundance of 25 pS channel openings. Out of 13 patches in these muscles (2–4 patches/muscle), 11 exhibited 25 pS channel activity before α-BTX treatment. The muscles were then bathed for 30 min in 100 μg/ml α-BTX, and 26 patches (6 or 7 patches/muscle) were tested for AChR channel activity. No channel openings resembling those of the 3 conductance classes were seen following exposure to α-BTX during a total observation time of 64 min, with applied potentials of 60–80 mV.

In a different set of control experiments, the recording pipette

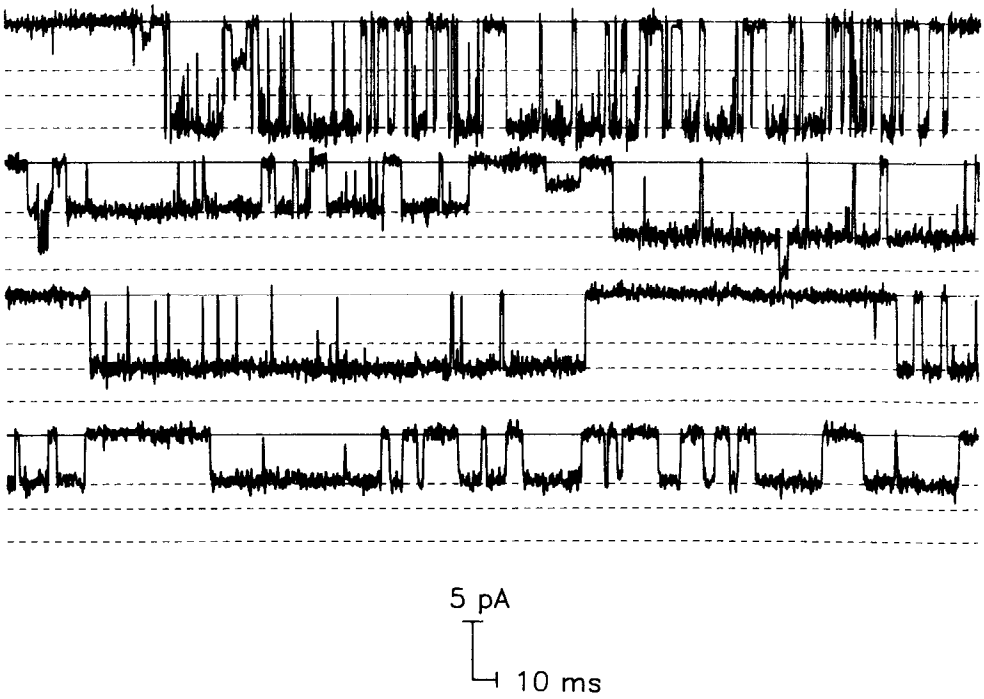

Figure 6. Single-channel events recorded from a patch of myotomal muscle membrane at stage 37 with 20 μM ACh in the patch pipette. These records show examples of bursts of openings belonging to 3 different amplitude classes. The applied pipette potential was 60 mV. Records are nonsequential and were selected to demonstrate the bursting behavior of the 3 classes of channels. An example of a possible 15 pS event is present in the second trace.

contained 1 μM ACh plus α-BTX (10–4 gm/ml). In these recordings, we observed the disappearance of single-channel events with time. Those channel openings that disappeared within a few minutes of the start of recording were considered to be blocked by toxin. In 39 patches we were able to determine the identity of the channels according to slope conductance before their disappearance. In 6 of these patches, 25 pS channel activity was present. In all 6 cases the 25 pS channel openings ceased within 4 minutes or less. In 5 of these 6 sites, there was also 40 or 60 pS channel activity, which disappeared within a similar period of time.

Discussion

The single-channel data reported here indicate that 3 conductance classes of AChRs are expressed by developing muscle in *X. laevis*. These classes have slope conductances of about 25, 40, and 60 pS at hyperpolarized membrane potentials. Previous studies in myotomal muscle have focused on the properties and developmental changes of the 2 higher conductance classes of channels, because of their relatively greater abundance in myotomal muscle. However, in other developing muscles, such as the interhyoideus and superior oblique, 25 pS channels may be a major class of channels, at least in early stages of development.

Several lines of evidence argue that the 25 pS channels are nicotinic AChR channels. They are blocked by α-BTX, and they exhibit desensitization and bursting in high concentrations of ACh. These are definitive properties of nicotinic AChRs. The 25 pS channels bear similarities to the other 2 classes of AChRs in terms of reversal potentials and voltage sensitivity of open time. Their gating properties, as exhibited by open and closed duration histograms, are similar to those of the 40 pS channels.

The 25 pS events do not appear to represent a subconductance state of either the 40 or 60 pS channels. Events that could be interpreted as possible transitions from either the 40 or 60 pS current level to the 25 pS level were extremely rare. In most cases, when more than one channel type was present, the 25 pS channel openings occurred in isolation or randomly overlapped the other events, and in several patches only openings of the 25 pS class were observed.

It seems unlikely that this channel is of artifactual origin. All of our recordings were made *in vivo* from healthy embryos and tadpoles, and all recordings were obtained without enzyme treatment, with the exception of older myotomal muscle. In early studies using the patch-clamp technique, "rim channels" were reported which had small and variable amplitudes (Neher et al., 1978). They were postulated to arise from channels that were partially occluded from the pipette interior by the glass–membrane seal. The occurrence of such channels was observed with seal resistances less than 1 GΩ. In the present study, seal resistances were always greater than 1 GΩ, making it unlikely that rim channels would have been observed. Rim channels, if present, should produce a wide distribution of amplitudes, depending on the access resistance between the channel and the pipette interior, rather than the discrete distributions which we observed. Furthermore, the relative frequency of the 25 pS channel activity declined with development in myotomal muscle, whereas the seal resistances were not noticeably greater in older stages.

The functional significance of the 25 pS class of channels is not yet clear. We do not know if these channels are present at endplates because all of our recordings were obtained from nonjunctional regions of membrane. However, other evidence suggests that the AChRs at nonjunctional regions are similar to those at junctional regions (Kullberg et al., 1981; Brehm et al., 1982; Kullberg and Kasprzak, 1985). If so, it is possible that this class of channel is the predominant form at endplates in some developing muscles.

Implications for AChR structure

Expression studies in oocytes using messenger RNAs derived from bovine muscle have shown differences in the subunit composition of 40 and 60 pS channels (Mishina et al., 1986). The 40 pS receptors are composed of $\alpha_2\beta\gamma\delta$ and the 60 pS channels

of $\alpha_2\beta\delta\epsilon$. Expression studies also suggest that functional receptors can be produced by other subunit combinations, for instance by deleting γ or δ subunits (Mishina et al., 1984). Although the natural occurrence of such altered receptors types has not been demonstrated, these studies raise the possibility that more than 2 stoichiometric classes of AChR may be expressed on muscle membrane and could account for the 3 conductance classes observed in the present study. Alternatively, the 25 pS channels might result from posttranslational modifications of either the 40 or 60 pS channels or possibly from as yet undescribed subunit types. In connection with the latter possibility, Baldwin et al. (1988), report that *Xenopus* δ subunit cDNA hybridizes to 2 transcripts of differing length in myotomal muscle. The smaller transcript (1.7 kb) is thought to encode the δ subunit, whereas the protein encoded by the larger (2.5 kb) has not been identified. In addition, a putative second form of α subunit cDNA has recently been isolated from *Xenopus* (Hartman and Claudio, 1988). Now that the subunit genes for *Xenopus* AChRs are being cloned, it should soon be possible to use expression systems to test hypotheses about the 25 pS channel subunit structure.

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