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ACETYLCHOLINE-ACTIVATED ION CHANNELS IN EMBRYONIC COCKROACH NEURONES GROWING IN CULTURE

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Summary

Application of acetylcholine and carbamylcholine to cultured cockroach neurones held under whole-cell voltage-clamp conditions evoked an inward current that was accompanied by an increase in current noise. Fluctuation analysis of the noise revealed the existence of two Lorentzian components in acetylcholine, of corner frequencies $10 \pm 0{\cdot}6$ Hz and 116 ± 9 Hz, and one Lorentzian component in carbamylcholine, of corner frequency 35 ± 13 Hz. Single-channel analysis of the unitary currents evoked by acetylcholine or carbamylcholine in neurones held in the cell-attached mode of the patch-clamp technique revealed the presence of two categories of channel events. The large events had mean currents of 4·77 pA with acetylcholine and 5·09 pA with carbamylcholine, and the small events 1·92 pA (acetylcholine) and 1·72 pA (carbamylcholine) for a hyperpolarization of 60 mV. The reversal potentials for these currents relative to the resting potential were estimated to be −70 mV for acetylcholine and −68 mV for carbamylcholine, and the conductance values calculated from the I/V curves were 37 pS (large) and 19 pS (small) for acetylcholine and 52 pS (large) and 15 pS (small) for carbamylcholine. It is concluded that embryonic cockroach neurones growing *in vitro* possess two populations of acetylcholine-activated ion channels, and the possibility that one of these represents an embryonic receptor and the other an adult receptor is discussed.

Introduction

Neuronal cultures from embryonic cockroaches, *Periplaneta americana*, have been shown to possess binding sites for the snake toxin, α-bungarotoxin (α-BTX) and to be depolarized by pressure-applied acetylcholine (Lees *et al.* 1983; Beadle *et al.* 1984), suggesting that they possess nicotinic acetylcholine receptors and are therefore suitable for a detailed physiological analysis of insect central acetylchol-

Key words: cultured insect neurones, acetylcholine receptors, noise analysis, single-channel analysis.

ine receptors. Acetylcholine is a major neurotransmitter in the central nervous system of both vertebrates (Krnjevic, 1974) and insects (Callec & Boistel, 1967; Kerkut *et al.* 1969; Gerschenfeld, 1973; Sattelle, 1980; Sattelle & Breer, 1987) and is the neurotransmitter at the vertebrate muscle endplate (Katz & Miledi, 1972; Anderson & Stevens, 1973). Acetylcholine receptors with a pharmacological profile characteristic of nicotinic receptors that are antagonized by α-BTX have been identified on giant interneurones GI2 and GI3 of the sixth abdominal ganglion of the cockroach (Harrow *et al.* 1980, 1982; Sattelle *et al.* 1980) and on the slow (D_s) and fast (D_f) coxal depressor motoneurones of *P. americana* (Carr & Fourtner, 1980; David & Sattelle, 1984), and receptors with a nicotinic profile that are insensitive to α-BTX have been identified on dorsal unpaired midline (DUM) neurones of the grasshopper, *Schistocerca nitens* (Goodman & Spitzer, 1980). A detailed kinetic analysis of these receptors is made difficult, however, by the location of these neurones within the central nervous system which hinders the use of high-resolution patch-clamp techniques (Hamill *et al.* 1981).

Preliminary experiments with cultured cockroach neurones have resulted in the successful recording of unitary currents evoked by acetylcholine (Beadle *et al.* 1985, 1986) and similar recordings have been obtained from cultured *Drosophila* neurones (Wu *et al.* 1983) and freshly dissociated adult cockroach neurones (Sattelle *et al.* 1986). In addition, nicotinic acetylcholine receptors purified from the central nervous system of the locust have been reconstituted in planar lipid bilayer membranes and acetylcholine-evoked unitary currents detected (Hanke & Breer, 1986).

We present here the results of a detailed analysis of the acetylcholine-activated ion channels of cultured cockroach neurones using the patch-clamp technique in both the cell-attached and the whole-cell voltage-clamp mode. The analysis was carried out on neurones that had been growing *in vitro* for at least 14 days, at which stage the cells have become fully differentiated and express acetylcholine receptors (Beadle *et al.* 1982; Lees *et al.* 1983, 1985).

Materials and methods

Cell culture technique

Neuronal cultures were prepared from the brains of 21- to 23-day-old embryos of *P. americana*, as described elsewhere (Dewhurst & Beadle, 1985). The cultures were initiated in a medium consisting of five parts of Schneider's revised *Drosophila* medium and four parts of Eagle's basal medium containing streptomycin and penicillin. After 7 days growth they were transferred to a medium containing equal parts of Leibovitz's L-15 and Yunker's modified Grace's medium containing streptomycin and penicillin. The cells were grown at 29°C in air in 50 mm Falcon Petri dishes using a modification of the hanging column method. Fig. 1 shows embryonic cockroach neurones after 14 days growth *in vitro*.

Electrophysiology

For electrophysiological experiments the growth medium was removed and cells

were allowed to equilibrate in a saline solution modified from that of Pitman (1979) to meet the osmotic requirements of the cultured cells. The solution contained 210 mmol l^{-1} NaCl, 10 mmol l^{-1} $CaCl_2$, 3·1 mmol l^{-1} KCl and 10 mmol l^{-1} Hepes buffer at pH 7·2. The experiments were performed at room temperature (22–24 °C). For experiments using the cell-attached configuration the electrodes were filled with this saline containing low concentrations of either acetylcholine (ACh) or carbamylcholine (CCh). For voltage-clamp experiments the whole-cell clamp configuration of the patch-clamp technique was used (Hamill *et al.* 1981). The electrodes were filled with a solution containing 114 mmol l^{-1} KCl, 5 mmol l^{-1} EGTA, 1·6 mmol l^{-1} $MgCl_2$, 0·2 mmol l^{-1} $CaCl_2$ and buffered at pH 7·2 with 10 mmol l^{-1} Hepes. Electrodes were pulled in two stages from 1·5 mm haematocrit tubing on a modified Kopf 150C vertical puller and were polished and coated with Sylgard. The resistance ranged from 2 to 7 MΩ. The electrode was advanced towards the cell until gentle contact was made. A small amount of suction applied to the electrode usually resulted in a high-resistance seal of several gigaohms. When needed, rupture of the cell membrane was obtained by further suction and the resting potential of the cell was recorded.

The patch electrodes were connected to either a List (L/M-EPC5) or Dagan (8900) patch-clamp amplifier. The results were displayed on either a Tektronix or

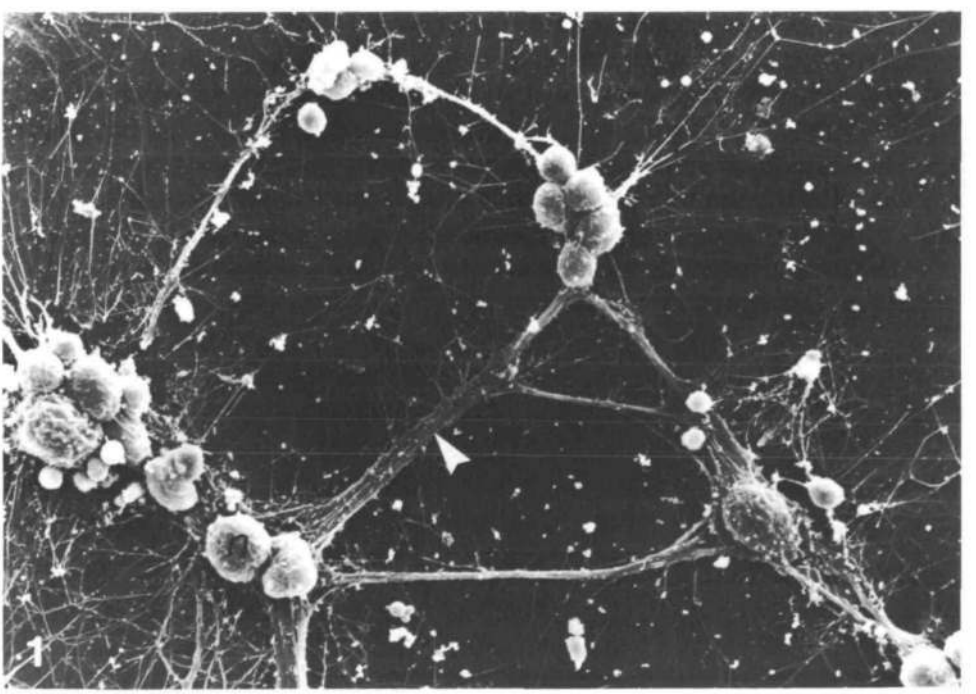

Fig. 1. Embryonic cockroach neurones after 14 days growth *in vitro*. The spherical neuronal somata (*n*) are connected by fascicles of neurites (arrowhead). Magnification, ×3500.

a Gould oscilloscope and stored either on analogue tape (Ampex PR 500) or on video cassettes using a modified Betamax video recorder and pulse code modulator at full amplifier bandwidth (Lamb, 1985), and were reproduced photographically from Gould 220 Brush chart records. Current fluctuations were analysed off-line using a spectrum analyser (HP3582A) connected to a desk computer (HP9825T) and a digital plotter (HP9872A). The fluctuations were analysed for bandwidths between 0 and 500 Hz using the method described by Shimahara *et al.* (1987). For analysis of single-channel activity, long stretches of stable recording are needed and the results from three cells were selected for detailed statistical analysis. The data were digitized on an IBM PC clone using a Data Translation (DT2801A) A/D converter. The original data were filtered at 5 kHz using a Tehebicheff filter and acquired at a 10 kHz sampling frequency. The sample used for analysis consisted of about 100 000 12-bit strings stored on the hard disc. The data were analysed using a computer program called IPROC-2 originally developed by Sachs *et al.* (1982) and donated by C. J. Lingle. The output of the program consists of a series of files containing information on the amplitude and duration of each valid event and on-time, off-time and duration histograms. A curve-fitting program linked to a statistical library was subsequently used to analyse the histograms except for on-time histograms of very short events (see next paragraph).

Limitations of the analysis

In cultured cockroach neurones, as in other preparations such as the frog muscle endplate (Colquhoun & Sakmann, 1981, 1985) and snake muscle fibre (Dionne & Leibovitz, 1982), the apparent single openings of the ion channel associated with the cholinergic receptor are interrupted by brief closed periods (see Fig. 4). These closed periods are usually too short to be resolved under our experimental conditions [by analogy with the results of Colquhoun & Sakmann (1985) using frog muscle endplate, the time constant of the briefest gap component for both ACh and CCh may be of the order of 10–20 μs]. This results in an overestimation of the open time of the channels and an underestimation of the single-channel amplitude (Sachs, 1983). These errors were minimized in the analysis by setting the parameters of the minimum closed time in a burst to its minimum value (100 μs). Another limitation was the short duration of many of the openings, especially with ACh, and the existence of 'triangular' openings. Here again, the minimum duration for an event to be accepted as an opening was set to the minimum value (100 μs). Under these conditions, the amplitude of the short events was underestimated and the amplitude histograms distorted and shifted towards smaller values (see Figs 5, 6). Furthermore, the first two bins of the on-time histograms (0·1 and 0·2 ms) were underestimated. This resulted in a substantial error in the fit of the on-time histograms. To overcome this difficulty, a different program was used in which the slow exponential components were calculated first from a semilogarithmic plot of the data and then subtracted from the fast component.

Results

Whole-cell voltage-clamp data

When 10–50 μmol l^{-1} ACh or CCh was applied from a pressure pipette onto the soma of cultured embryonic cockroach neurones held under whole-cell voltage-clamp conditions, an inward current was evoked that reached a peak value of 200–250 pA with the higher concentrations. The inward current was typically accompanied by an increase of current noise (Fig. 2). Spectral analysis of this noise revealed the existence of one (CCh) or two (ACh) Lorentzian functions. At rest, the mean corner frequency of the Lorentzian component in CCh was 35 ± 13 Hz, corresponding to a mean open time of 4·6 ms; the mean corner frequencies of the two Lorentzian functions in ACh were $10 \pm 0{\cdot}6$ Hz and $116{\cdot}8 \pm 9$ Hz, corresponding to mean open times of 15·9 ms and 1·36 ms, respectively (Fig. 3). Membrane hyperpolarization resulted in a shift of the spectrum towards lower frequencies, suggesting an increase of the mean open time of the single-channel events (the mean corner frequency with CCh decreased from 35 ± 13 Hz at rest to $16{\cdot}5 \pm 2$ Hz for an 80 mV hyperpolarization, corresponding to a lengthening of the mean open time from 4·6 ms to 9·7 ms).

Single-channel analysis

When added to the patch pipette at micromolar concentrations, both ACh and CCh induced small inward unitary currents. At the resting potential (RP), the mean amplitude and mean duration of these channels were, respectively, $1{\cdot}57 \pm 0{\cdot}5$ pA and $0{\cdot}342 \pm 0{\cdot}28$ ms for ACh and $1{\cdot}53 \pm 0{\cdot}64$ pA and $0{\cdot}415 \pm 0{\cdot}53$ ms for CCh. Membrane hyperpolarization revealed the existence of two categories of unitary currents (Fig. 4A,B): large events with a mean current of 4·77 pA with ACh and 5·09 pA with CCh and small events with a mean current of 1·92 pA with ACh and 1·72 pA with CCh (for a 60 mV hyperpolarization). These events could be classified into three categories from their time course: long bursts of openings (several tens of milliseconds), short openings (a few milliseconds) with occasional closings and substates and very short triangular openings (less than 0·5 ms). The bursting behaviour and triangular events were more frequent with ACh than with

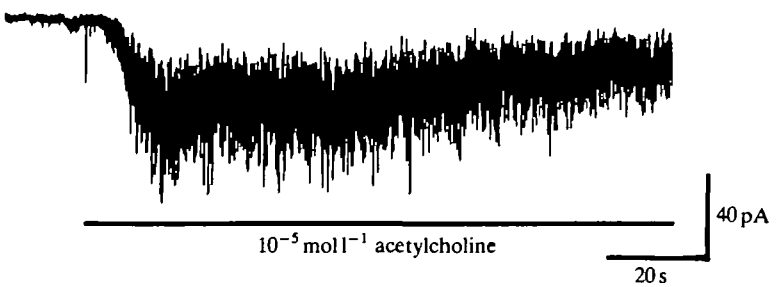

Fig. 2. Inward current evoked by prolonged application of 10^{-5} mol l^{-1} acetylcholine onto a neurone held under whole-cell voltage-clamp. The evoked current is accompanied by an increase in noise.

CCh. The mean open time of the short channels was longer with CCh than with ACh.

Amplitude histograms were constructed for the two agonists for membrane potentials between +100 mV and −40 mV relative to the resting potential (i.e. H100 to D40). In all experiments, the histograms could be reasonably well fitted with two Gaussian functions reflecting the existence of the two populations of events. Individual values of the amplitude and standard deviations of these Gaussian functions in two patches are presented in Table 1. It can be seen that the standard deviations are larger for the ACh-induced events than for those induced by CCh, reflecting the existence in ACh of a larger proportion of very short, partly unresolved triangular openings. Examples of such fits are given for the two agonists at four potential levels in Figs 5 and 6. It can be observed that the short duration of the events distorts the distribution and shifts the fitted curve to the left (i.e. towards lower conductance values).

Fig. 7 shows the effects of membrane hyperpolarization on the activity of channels induced by 5 μmol l^{-1} CCh: both amplitudes and durations are increased but this change is not associated with a significant increase of the noise during the openings (substates or flicker). For membrane potential values between 100 mV (H100) and −30 mV (D30) the current–voltage relationships were linear for the two agonists (Figs 8, 9). For ACh, the reversal potentials calculated from the

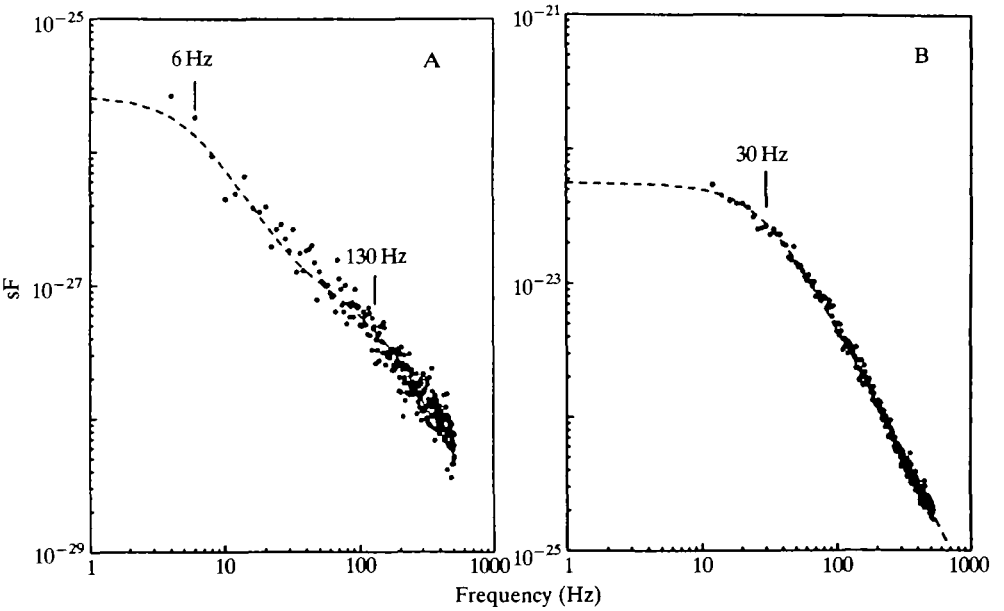

Fig. 3. Power spectra of the noise induced by pressure application of (A) 10 μmol l^{-1} acetylcholine (ACh) and (B) 50 μmol l^{-1} carbamylcholine (CCh) onto cockroach neurones growing *in vitro*. The spectrum for ACh was best fitted with two Lorentzian components with corner frequencies of 6 Hz and 130 Hz and the spectrum for CCh was fitted with a single Lorentzian component with a corner frequency of 30 Hz.

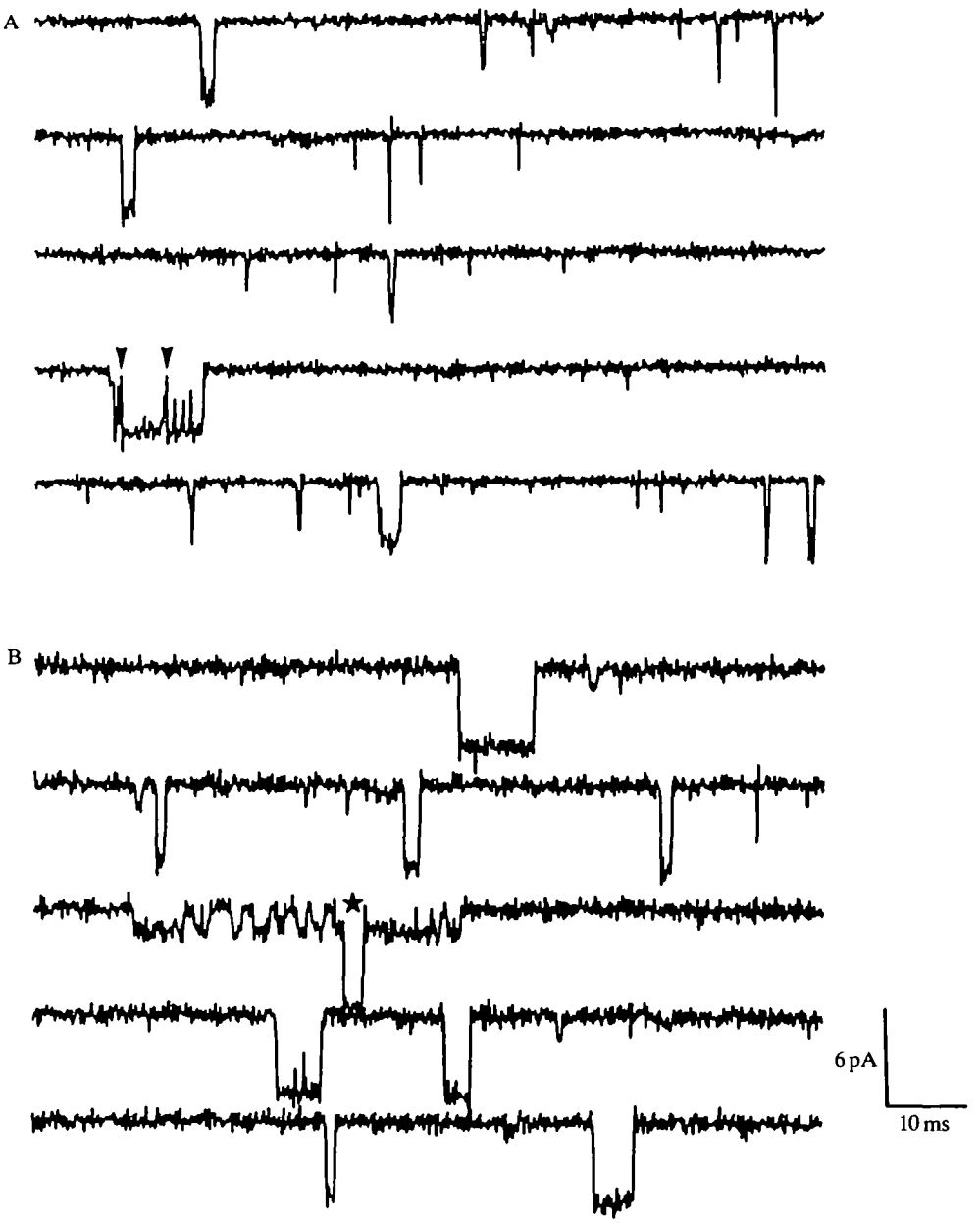

Fig. 4. Representative (nonconsecutive) traces of acetylcholine-sensitive (A) and carbamylcholine-sensitive (B) channels in cultured embryonic cockroach neurones. Note the existence of two distinct amplitudes of events. The membrane was hyperpolarized by 60 mV. Arrowheads in A indicate brief closures which reach the baseline. Star in B indicates the superimposition of a large event on top of a burst of small events. All records were low-pass filtered at 3 kHz. Agonist concentrations: A, 10 μmol l^{-1} ACh; B, 5 μmol l^{-1} CCh. Cell-attached configuration.

Table 1. *Effects of membrane potential on single-channel events induced by acetylcholine and carbamylcholine for two cell-attached membrane patches*

Agonist	Membrane potential (mV)	Number of events	First Gaussian function Peak (pA)	First Gaussian function S.D. (pA)	Second Gaussian function* Peak (pA)	Second Gaussian function* S.D. (pA)
Acetylcholine	−40	48			1·32	0·2
(10 μmol l^{-1})	−35	85			1·33	0·35
	−30	144			1·42	0·31
	−25	103			1·48	0·5
	−20	51			1·7	0·5
	−15	70			1·8	0·55
	−10	70			1·93	0·5
	−5	121			2·5	0·76
	0(RP)	78			3·35	0·4
	+10	334	1·84	0·4	3·61	0·71
	+20	321	1·67	0·37	3·0	1·2
	+30	350	1·8	0·35	3·5	1·1
	+40	287	2·1	0·49	3·9	0·84
	+50	127	2·17	0·4	4·0	1·2
	+60	123	2·35	0·3	4·5	1·3
	+70	266	3·0	0·6	5·8	0·9
	+80	79	3·0	0·5	6·0	1·3
	+90	120	3·23	0·99	5·89	1·1
	+100	186	3·1	0·88	6·2	1·1
Carbamylcholine	−60	367			1·6	0·15
(5 μmol l^{-1})	−40	305			1·59	0·15
	−30	711			1·61	0·25
	−20	990			1·91	0·55
	0(RP)	318	1·5	0·3	3·19	0·52
	+30	4089	1·8	0·55	5·0	0·8
	+60	4650	2·1	0·43	6·81	0·6
	+90	1316	2·88	0·58	8·6	0·67

The values of peak and standard deviations (S.D.) were calculated from the experimental data using the curve-fitting procedure.

* When the membrane was depolarized (negative values of membrane potential), the signal to noise ratio of the single events deteriorated and only the large events (i.e. the events corresponding to the second Gaussian function) could be detected from the background noise.

RP, resting potential.

linear portion of the curves were $-69{\cdot}7 \pm 2{\cdot}5$ mV ($N = 19$) for the large channels, $-73{\cdot}4 \pm 6{\cdot}2$ mV ($N = 9$) for the small channels and $-67{\cdot}9 \pm 1{\cdot}84$ mV ($N = 15$) for the mean amplitude of all the channels before curve fitting. For CCh, the corresponding values were $-68{\cdot}2 \pm 4{\cdot}15$ mV ($N = 8$) for the large channels, $-93{\cdot}9 \pm 10{\cdot}5$ mV ($N = 4$) for the small ones and $-78{\cdot}6 \pm 3{\cdot}7$ mV ($N = 8$)

for the mean amplitude. Single-channel conductance values obtained from the same curves were $37{\cdot}4 \pm 1{\cdot}90$ pS ($N = 19$) for the large channels in ACh, and $19{\cdot}0 \pm 2{\cdot}0$ pS ($N = 9$) for the small channels in ACh, with a mean of $28{\cdot}8 \pm 1{\cdot}4$ pS ($N = 15$). For CCh, the single-channel conductance values were $51{\cdot}8 \pm 4{\cdot}4$ pS ($N = 8$) for the large channels, and $14{\cdot}8 \pm 2{\cdot}8$ pS ($N = 4$) for the small channels with a mean of $33{\cdot}6 \pm 4{\cdot}8$ pS ($N = 8$). For a given ACh or CCh concentration, changes in membrane potential were found to alter the duration of the single-channel events: hyperpolarizations increasing the duration and depolarizations decreasing it. The effect of hyperpolarization is particularly striking in Fig. 7 between resting potential (RP) and 30H (30 mV hyperpolarization).

On-time histograms were constructed for the two agonists and tentatively fitted with one, two and three exponential functions. Within the limitations of the analysis, it was found that the best fit was obtained with two exponential functions. In ACh, the duration of the single channels was usually so short that it was not possible to obtain a reasonable estimate of the time constant of the fast exponential component but, as illustrated in Fig. 10, the data could not be fitted with a single exponential. The situation was slightly better with CCh and the

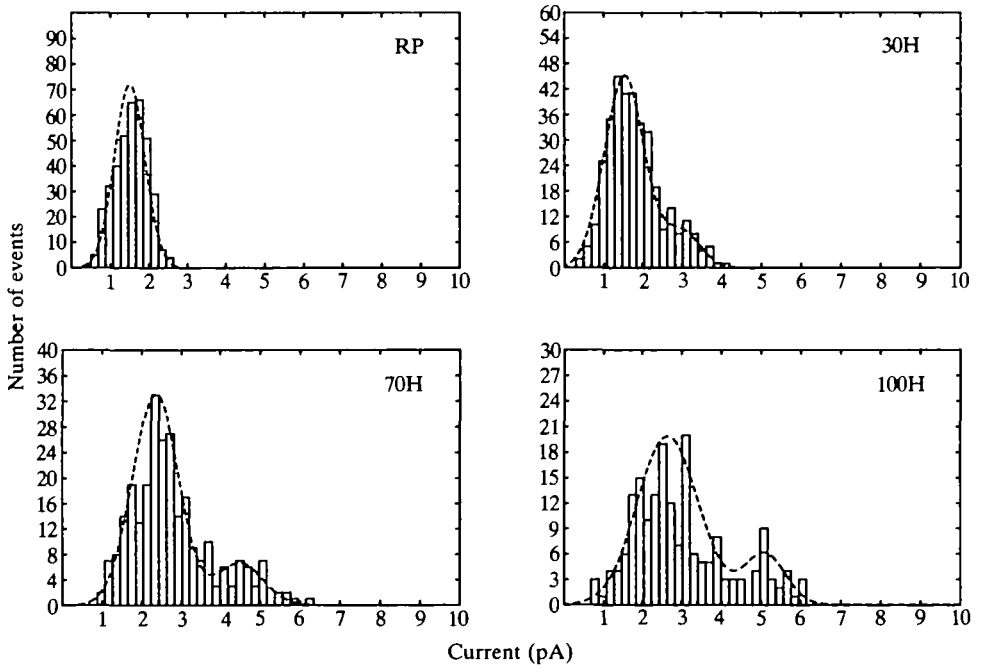

Fig. 5. Amplitude histograms of the single-channel events induced by 10 μmol l^{-1} acetylcholine in cultured embryonic cockroach neurones at four potential levels. The histograms were tentatively fitted with one (RP) or two Gaussian functions (interrupted lines). The mean values and standard deviations used for the fit were as follows: RP, $1{\cdot}54 \pm 0{\cdot}41$ pA; 30H, $1{\cdot}54 \pm 0{\cdot}53$ pA and $3{\cdot}04 \pm 0{\cdot}48$ pA; 70H, $2{\cdot}40 \pm 0{\cdot}6$ pA and $4{\cdot}6 \pm 0{\cdot}6$ pA; 100H, $2{\cdot}7 \pm 0{\cdot}8$ pA and $5{\cdot}2 \pm 0{\cdot}53$ pA. RP, resting potential; 30H, 30 mV hyperpolarized; 70H, 70 mV hyperpolarized; 100H, 100 mV hyperpolarized.

histograms illustrated in Fig. 11 were fitted with two exponential components. No systematic study of the off-time histograms was performed. Preliminary analyses with CCh at 60H suggested, however, the existence of three exponential components.

Examination of the cross-correlation histograms in both ACh and CCh revealed that events of both sizes contributed to the two time constants although separate analysis of the channels of the two sizes revealed on one occasion that the large events were somewhat faster than the small ones.

The voltage-dependency of the open time was studied for membrane potential values between H100 and D40. Fig. 12 illustrates the voltage-dependency of the mean open time for two patches in ACh (Fig. 12A) and one patch in CCh (Fig. 12B). In the three cases, the voltage-dependency was found to increase considerably when the membrane was depolarized. The data were thus fitted with two exponentials, a fast one for depolarized potentials and a slow one for potential values more positive than −20 mV. For the three illustrated patches, e-fold changes in duration for the fast component were observed for potential changes of 36·2 mV (open triangles in Fig. 12A), 24·1 mV (filled triangles in Fig. 12A) and 25·5 mV (open triangles in Fig. 12B). For the same three patches, e-fold changes

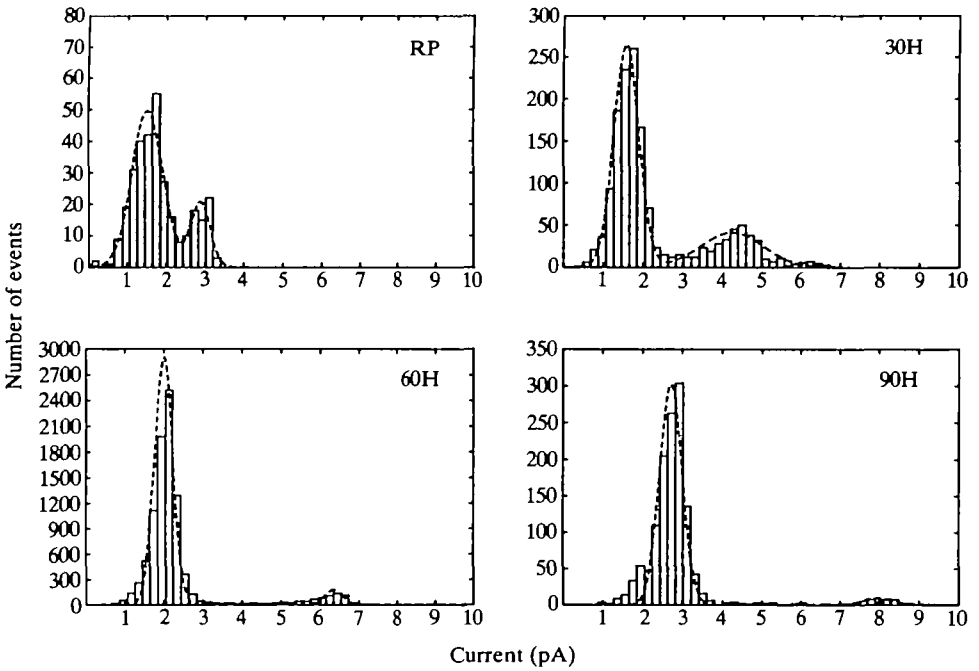

Fig. 6. Amplitude histograms of the single-channel events induced by 5 μmol l^{-1} carbamylcholine in cultured embryonic cockroach neurones at four potential levels. The histograms were tentatively fitted with two Gaussian functions (interrupted lines). The mean values and standard deviations used for the fit were as follows: RP, 1·52 ± 0·41 and 2·91 ± 0·26 pA; 30H, 1·58 ± 0·32 and 4·3 ± 0·8 pA; 60H, 2·03 ± 0·32 and 6·49 ± 0·23 pA; 90H, 2·76 ± 0·29 and 8·12 ± 0·4 pA. For further details, see Fig. 5.

in duration for the slow component were observed for potential changes of 603 mV (open triangles in Fig. 12A), 258 mV (filled triangles in Fig. 12A) and 1420 mV (open triangles in Fig. 12B).

When measurable, both fast and slow components of the on-time histograms were voltage-dependent. Thus, for the patch illustrated in Fig. 12B, e-fold changes in the time constant of the fast component of the on-time histogram were observed for potential changes of 7·89 mV (depolarizations) and 355 mV (hyperpolarizations), whereas an e-fold change in the slow component of the on-time histogram was observed for a 103 mV change in potential (not illustrated).

The mean frequency of the events induced by micromolar concentrations of the two agonists was low (around 10–15 s^{-1} for ACh and 30 s^{-1} for CCh at the resting potential level), corresponding to a mean open-time probability of 0·003 for ACh and 0·01 for CCh. The actual values are probably lower since there is evidence, that will be discussed below, that at least two channels were probably present under the patch pipette. The effects of membrane potential on the relative frequency of opening and the relative open-time probability were determined for membrane potential values between 100 mV (H100) and −40 mV (D40). In all cases, membrane hyperpolarization was associated with a statistically significant increase in the number of openings per unit time. For the three experiments illustrated in Fig. 13, an e-fold change in the frequency was obtained for 168·7 mV, 50·5 mV (Fig. 13A) and 80·66 mV (Fig. 13B). Since both open times and frequencies of opening increased with membrane hyperpolarization, the (apparent) open-time probability was also strongly voltage-dependent. Thus, in the exper-

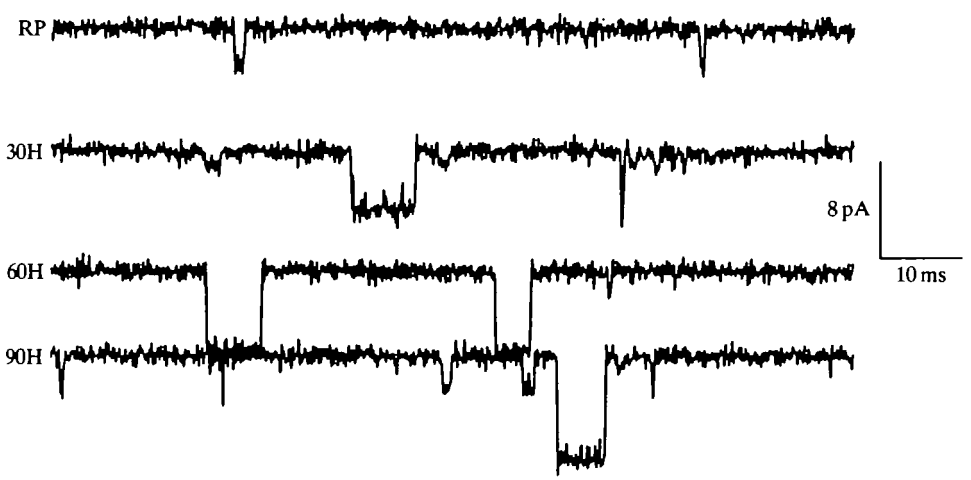

Fig. 7. Representative recordings of the effects of membrane potential on single-channel activity induced by 5 μmol l^{-1} carbamylcholine in cultured embryonic cockroach neurones. Note the increase in size and duration of the unitary currents. RP, resting potential; 30H, 30 mV hyperpolarization; 60H, 60 mV hyperpolarization; 90H, 90 mV hyperpolarization.

iment illustrated in Fig. 14, e-fold changes in the relative open-time probabilities were obtained for 115·1 mV and 46·4 mV (Fig. 14A) and 55 mV (Fig. 14B).

Discussion

The results presented here indicate that the somata of neurones from the brains of embryonic cockroaches growing in culture possess ion channels that are activated by acetylcholine and carbamylcholine. This confirms the results of previous microelectrode studies that have demonstrated that the somata of many insect central neurones are cholinoceptive despite their lack of synapses (Kerkut *et al.* 1969; Pitman & Kerkut, 1970; David & Pitman, 1979; Lees *et al.* 1983; Suter & Usherwood, 1985). Recent work involving the mapping of acetylcholine receptors on insect neurones using the radiolabelled ligand [^{125}I]α-BTX has also demonstrated their presence on neuronal cell bodies (Sattelle, 1980; Lees *et al.* 1983). The functional significance of these extrajunctional receptors has yet to be determined.

For both agonists, there is an apparent discrepancy between single-channel data and the results of spectrum analysis of the current fluctuations recorded in the whole-cell configuration. Amongst other possibilities, this difference could indi-

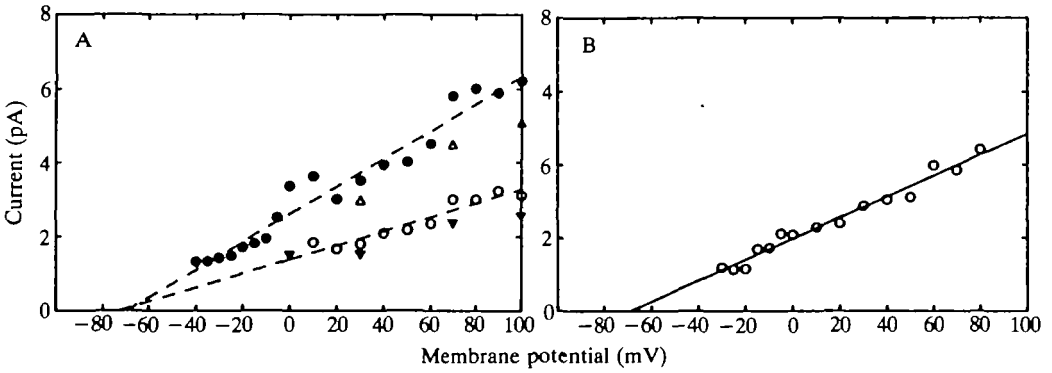

Fig. 8. Current–voltage relationships for acetylcholine-activated channels. In A, the data were obtained by curve-fitting the data with Gaussian functions, as illustrated in Fig. 5, the filled circles corresponding to the high conductance level and the open circles to the low conductance level. Triangles refer to the same experiments but were obtained by a different fitting procedure (the open triangles correspond to the large channels, the closed triangles to the small ones). In B, the open circles correspond to the mean current computed by the IPROC-2. The three sets of data can be fitted with straight lines: the high-conductance channel with a conductance of 37 pS, a reversal potential of −70 mV and a correlation coefficient of 0·978 ($N = 19$); the low-conductance channel with a conductance of 19 pS, a reversal potential of −73 mV and a correlation coefficient of 0·952 ($N = 9$); the mean current with a mean conductance of 29 pS, a reversal potential of −68 mV and a correlation coefficient of 0·986 ($N = 15$). In this figure, as in Figs 9, 12 and 13, the abscissa indicates the pipette potential (i.e. the relative membrane potential of the cell with respect to resting potential which is taken as 0).

cate that the ACh channels are not homogeneously distributed over the cell surface. Because of the specific limitations of these two methods of analysis (limited bandwidth, unequal representation of the different spectral components, importance of the background noise), randomly selected single-channel data were re-examined and it was found that the two categories of data could be reconciled to some extent. For example, in ACh (Fig. 4A) the mean duration of the short channels was 1·17 ± 0·87 ms, corresponding to a corner frequency of 136 Hz, and the duration of the burst was 12·5 ms, corresponding to a corner frequency of 12·7 Hz. Similarly, with carbamylcholine (Fig. 4B), the mean duration of the large events was 2·17 ± 1·8 ms, corresponding to a mean corner frequency of 73·4 Hz, and that of the small ones was 4·07 ± 0·9 ms, corresponding to a mean corner frequency of 39·1 Hz.

The conductance values calculated from the I/V curves were 37 pS (large) and 19 pS (small) for ACh and 52 pS (large) and 15 pS (small) for CCh. These are in reasonable agreement with our previously published values for CCh of 48 pS (large) and 18 pS (small) (Beadle *et al.* 1985). The larger value of conductance in CCh than in ACh is at variance with most reports on other preparations (see, for example, Colquhoun & Sakmann, 1985; Gardener *et al.* 1984) where the conductance was found to be independent of the nature of the agonist. As mentioned in Materials and methods, such a difference may not be genuine but may result from an underestimation of the current amplitude of the single channel in ACh because of the short open time (triangular events were not eliminated) or bursting behaviour. To test this hypothesis, single channels with durations longer than 1 ms

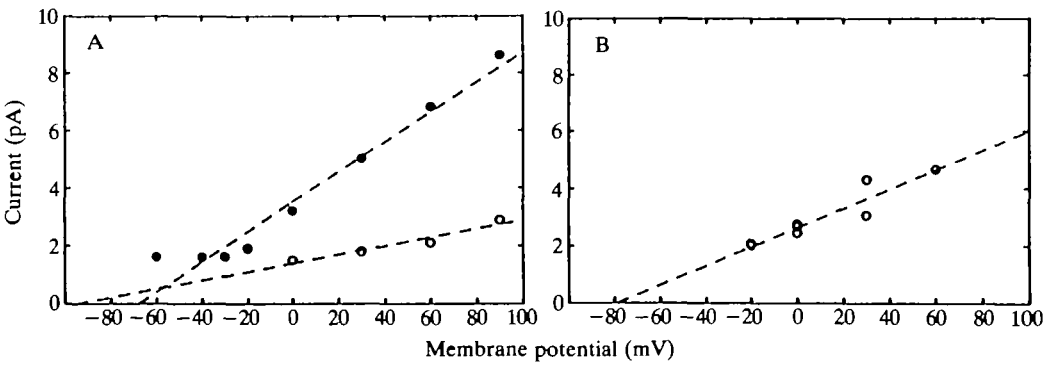

Fig. 9. Current–voltage relationships for carbamylcholine-activated channels. In A, the data were obtained by curve-fitting the data with Gaussian functions as illustrated in Fig. 6, the filled circles corresponding to the high conductance level and the open circles to the low conductance level. In B, the open circles correspond to the mean current computed by the IPROC-2. The three sets of data can be fitted with straight lines: the high-conductance channel with a conductance of 52 pS, a reversal potential of −68 mV and a correlation coefficient of 0·976 ($N = 8$); the low-conductance channel with a conductance of 15 pS, a reversal potential of −94 mV and a correlation coefficient of 0·962 ($N = 4$); the mean current with a mean conductance of 34 pS, a reversal potential of −79 mV and a correlation coefficient of 0·937 ($N = 8$).

and no visible burst or substate were selected for further analysis. Under these conditions, the mean single-channel conductance for the large events in ACh was found to approximate 50 pS (i.e. not significantly different from that induced by CCh). The larger conductance value is similar to the value of 40 pS for ACh channels in freshly dissociated adult cockroach neurones (Sattelle *et al.* 1986) but is considerably lower than the value of 75 pS reported by Breer (1986) for locust ACh channels reconstituted in planar lipid bilayers. The smaller conductance value is similar to that of 9–25 pS for ACh channels in cultured larval *Drosophila* neurones (Wu *et al.* 1983). Acetylcholine-activated ion channels with conductances of 38·42 pS have been reported in chick ciliary ganglion neurones grown in culture (Ogden *et al.* 1984) and of 34 pS in embryonic rat muscle in culture and 30 pS in adult frog muscle endplate (Gardner *et al.* 1984).

Do the two conductance states reported here correspond to two distinct populations of channels or two substates of the same channel? Substates have been reported for ACh-activated ion channel at the frog endplate (Colquhoun & Sakmann, 1985). In this case precise kinetic studies associated with modelling of channel activity clearly indicate that they are substates. Substates can also be seen in recordings of unitary ACh currents from dissociated adult cockroach neurones

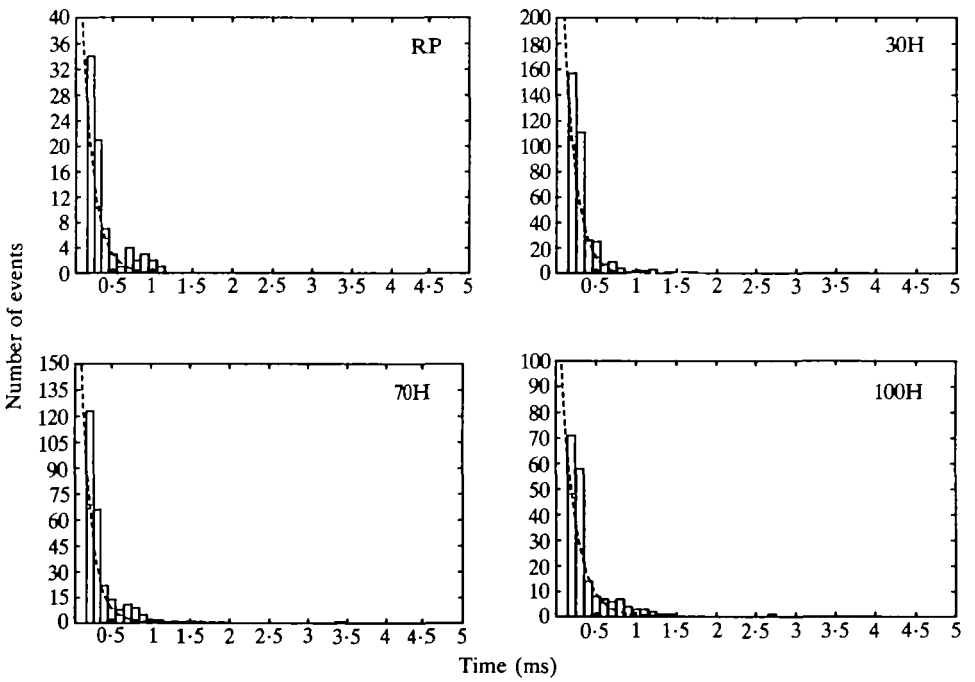

Fig. 10. Open-time histograms of the single-channel events induced by 10 μmol l^{-1} acetylcholine in cultured embryonic cockroach neurones at four potential levels. The histograms were tentatively fitted with one exponential function (interrupted lines). The time constants used for the fit were as follows: RP, 0·16 ms; 30H, 0·15 ms; 70H, 0·15 ms; 100H, 0·18 ms. For further details, see Fig. 5.

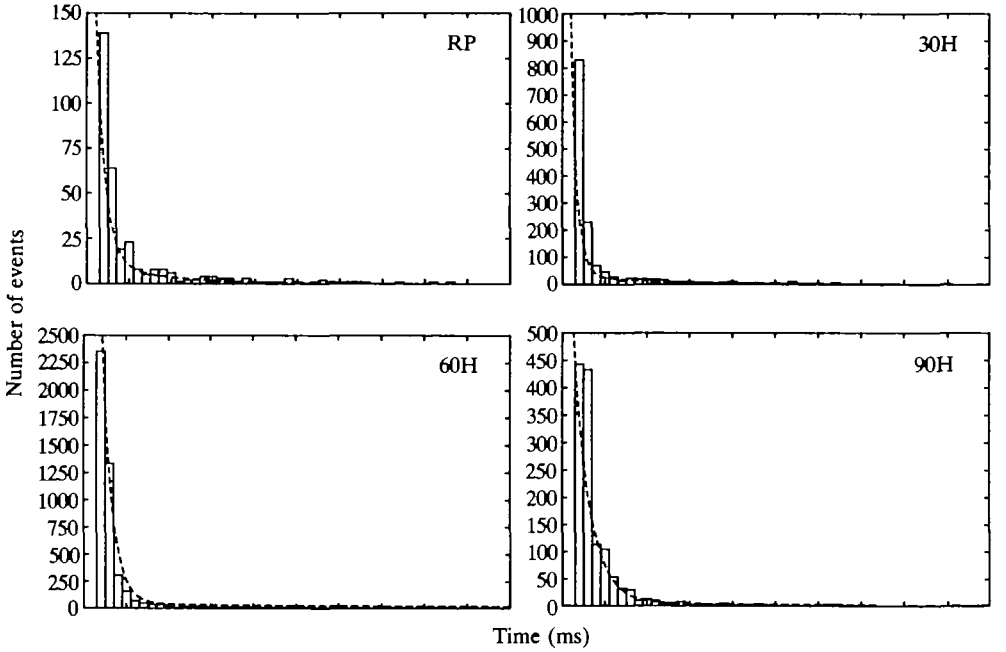

Fig. 11. Open-time histograms of the single-channel events induced by 5 μmol l^{-1} carbamylcholine in cultured embryonic cockroach neurones at four potential levels. The histograms were tentatively fitted with two exponential functions (interrupted lines). The time constants used for the fit were as follows: RP, 0·11 and 0·91 ms; 30H, 0·07 and 1·21 ms; 60H, 0·12 and 2·97 ms; 90H, 0·19 and 1·67 ms. See Fig. 5 for further details.

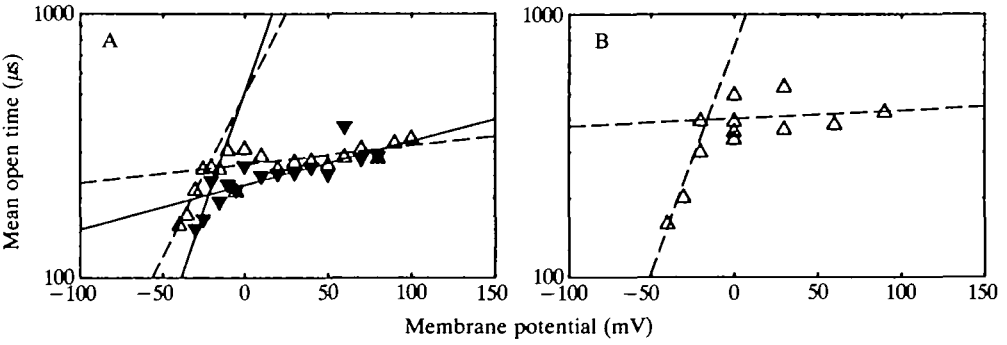

Fig. 12. Voltage-dependency of the mean open time for two patches in 10 μmol l^{-1} acetylcholine (A) and one patch in 5 μmol l^{-1} carbamylcholine (B). In all cases, the mean open time increased with membrane hyperpolarization, the voltage-dependency being more pronounced for membrane potentials more negative than – 20 mV than for more positive potentials. The experimental data were tentatively fitted with two exponentials (see text).

(Sattelle *et al.* 1986), although only one conductance value is given. The existence of substates is based on the frequent occurrence of conductance changes from one level to the other. Analysis of large numbers of channels in cultured cockroach neurones with both ACh and CCh failed to reveal such frequent conductance changes, with transitions from the small to the large conductance state occurring only very rarely. From this we conclude that embryonic cockroach neurones growing in culture possess two populations of ACh-activated ion channels. For embryonic frog muscle, two conductance states of 25 pS and 35 pS as well as a substate of 10 pS have been reported (Hamill & Sakmann, 1981), and cultured rat myocytes express an embryonic ACh-activated ion channel with a conductance state of 33 pS and an adult one of 58 pS (Jaramillo & Schuetze, 1988). Rat skeletal

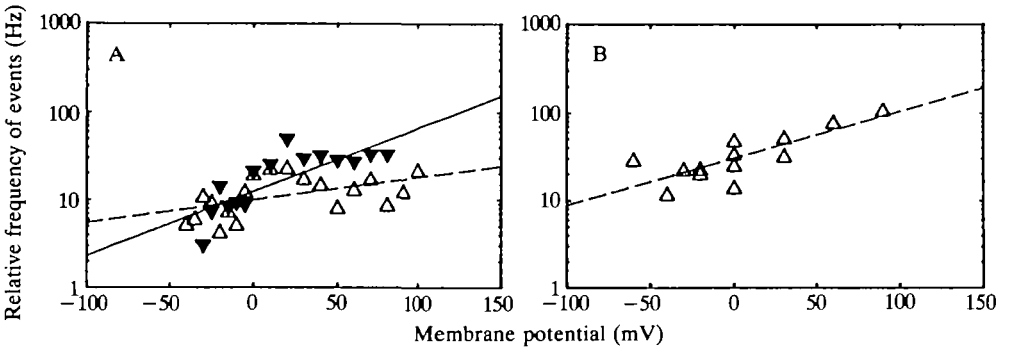

Fig. 13. Voltage-dependency of the relative frequency of opening for two patches in 10 μmol l^{-1} acetylcholine (A) and one patch in 5 μmol l^{-1} carbamylcholine (B). In all cases, the frequency increased with membrane hyperpolarization. The experimental data were tentatively fitted with exponential functions. In two cases out of three (filled triangles in A and open triangles in B) the slope was found to be statistically different from zero ($P < 0{\cdot}001$, Student's t-test), in the other case, the difference was not statistically significant ($0{\cdot}1 > P > 0{\cdot}05$).

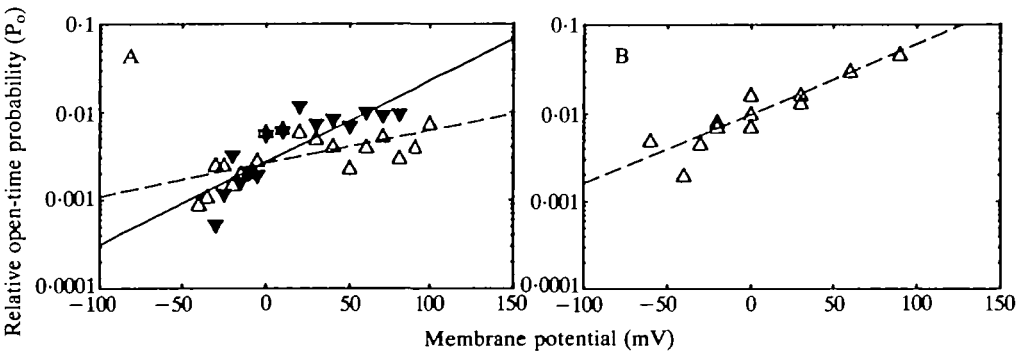

Fig. 14. Voltage-dependency of the relative open-time probability (P_o) for two patches in 10 μmol l^{-1} acetylcholine (A) and one patch in 5 μmol l^{-1} carbamylcholine (B). In all cases, P_o increased with membrane hyperpolarization. The experimental data were tentatively fitted with exponential functions. In all cases, the slope was statistically different from zero ($P < 0{\cdot}001$ in two cases, $P < 0{\cdot}01$ in one case, Student's t-test).

muscle fibres express at least two different types of ACh receptor differing in their conductance state and their gating properties (Hamill & Sakmann, 1981; Siegelbaum *et al.* 1984; Jamarillo & Schuetze, 1988). Recent results indicate that these two types differ in their subunit composition (Mishima *et al.* 1986), the gamma subunit of the low-conductance channel in the embryo (alpha2-beta-gamma-delta) being replaced by an epsilon subunit in the adult. It may be that one of the conductance states reported here represents an embryonic receptor and the other an adult receptor. Developmental studies using this culture system may resolve this question.

Bursting behaviour of the type illustrated in Fig. 4 was frequently observed, being more common with ACh, and this probably accounts for the apparently smaller conductance value for ACh channels than for CCh channels. More precise studies are needed to understand the bursting behaviour of these channels, although it appears to be very similar to that described by Colquhoun & Sakmann (1985) at the frog muscle endplate. Unitary events never appear in clusters with the range of concentrations (1–50 μmol l^{-1}) used in our experiments, suggesting that the receptor does not desensitize. The open-time probability was very low with both agonists, less than 1%, in contrast with that of adult dissociated cockroach neurones where the recordings show a high probability of channel openings (Sattelle *et al.* 1986). This could represent a difference between cultured neurones and those occurring *in situ* or between embryonic and adult neurones. The voltage-sensitivity of the mean channel open time is a classical feature of the N-ACh receptors in vertebrate preparations (see Jamarillo & Schuetze, 1988, for references). The observed increase in the open-time probability (which results from this effect and from the voltage-dependent increase in the frequency of events per unit time) should be reflected in the I/V relationship for a given agonist concentration, as in the fast coxal depressor motoneurones (D_f) in the metathoracic ganglion of the cockroach (Sattelle *et al.* 1986).

In conclusion, ACh receptors in cockroach neurones after 2 weeks in culture resemble in some aspects the nicotinic receptors at the vertebrate endplate. The stability of this preparation and its ease of use suggest that it may act as a useful model for the study of the basic properties and development of the neuronal nicotinic receptor. It would be very well suited for a study of the effects of various factors and other cell types such as glia on the development and characteristics of these receptors.

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