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NOISE ANALYSIS OF DRUG INDUCED VOLTAGE CLAMP CURRENTS IN DENERVATED FROG MUSCLE FIBRES

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SUMMARY

1. Voltage clamp currents were recorded during iontophoretic application of steady doses of acetylcholine (ACh), carbachol or suberyldicholine to hyperpersensitive extrasynaptic regions of chronically denervated frog muscle fibres. Autocorrelation functions of drug induced current fluctuations were calculated and estimates of conductance γ and average open time τ of the extrasynaptic ion channels were derived.

2. The average open time of an extrajunctional channel induced by acetylcholine is $\tau_{ACh} = 11 \pm 1{\cdot}6$ msec ($\pm$S.E.) at -80 mV and 8° C. Carbachol and suberyldicholine open channels of $\tau_{Carb} = 3{\cdot}9 \pm 0{\cdot}4$ msec and $\tau_{SubCh} = 19 \pm 2{\cdot}5$ msec ($\pm$S.E.) duration under the same conditions. The average open time of the extrasynaptic channel produced by each drug is three to five times longer than the value found for junctional channels in normal fibres.

3. The average open time of the extrajunctional channel is dependent on temperature and membrane potential. Lowering the temperature or increasing the membrane potential increases the average open time of channels induced by any one of the drugs.

4. The conductance of a single extrajunctional channel opened by the action of acetylcholine is estimated to be $\gamma_{extra} = 15 \pm 1{\cdot}8$ pmho ($\pm$S.E.) This is somewhat lower than the value of $\gamma_{ep} = 23 \pm 2$ pmho ($\pm$S.E.) found for the conductance of a single open channel in the junctional membrane of normal fibres. The extrasynaptic channels opened by the action of carbachol and suberyldicholine have similar conductances to those produced by ACh.

5. The autocorrelation function of drug-induced current fluctuations, recorded at the former end-plate region of chronically denervated fibres often shows both a fast and a slow time constant. They correspond in value to the time constant of the autocorrelation function obtained from

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end-plate currents in normal fibres and from extrasynaptic currents in denervated fibres respectively. This could indicate that two populations of channels exist at the former end-plate region of denervated muscle fibres.

INTRODUCTION

When skeletal muscle fibres are denervated chronically, the entire extrasynaptic muscle membrane becomes sensitive to acetylcholine (ACh). This is due to new ACh receptors which appear in the extrasynaptic membrane (Axelsson & Thesleff, 1959; Miledi, 1960). These 'extrajunctional' receptors resemble junctional receptors in many respects but have different pharmacological properties since they are less sensitive to curare (Jenkinson, 1960; Beranek & Vyskocil 1967). It has been reported that the ion channels opened by the binding of ACh to these extrajunctional receptors are different in ion selectivity from the channels in the junctional membrane (Feltz & Mallart, 1971; Mallart & Trautmann, 1973).

Junctional ion channels have recently been characterized in terms of their open channel conductance γ and average open time τ. These two properties have been derived from statistical analysis of the 'membrane noise' which is recorded at the end-plate in the presence of steady doses of cholinergic drugs (Katz & Miledi, 1970, 1971, 1972, 1973*a*; Anderson & Stevens, 1973; Colquhoun, Dionne, Steinbach & Stevens, 1975, Ben Haim, Dreyer & Peper, 1975). This type of analysis can also be used to characterize the ion channels induced by cholinergic drugs in the extrasynaptic membrane of chronically denervated muscle fibres.

It has already been noted by Katz & Miledi (1972) that the power spectrum of ACh-induced voltage noise recorded from former end-plate of chronically denervated muscles is shifted towards lower frequencies indicating a longer life span of the open channel in these fibres. The aim of the present experiments is to further elucidate possible differences in conductance and average lifetime between the ion channels in the junctional and extrajunctional membrane. We therefore determined both quantities from the autocorrelation function of current fluctuations induced by steady iontophoretic application of ACh and two other depolarizing drugs, carbachol (Carb) and suberyldicholine (SubCh) to voltage clamped extrasynaptic portions of chronically denervated muscle fibres. We compared the values of τ and γ of 'extrajunctional' channels with the values derived from end-plate currents of junctional channels in normal fibres. The results show that drug-induced extrasynaptic channels have a considerably longer open time and a somewhat smaller conductance than synaptic channels. Some of the results have appeared in abstract form (Neher & Sakmann, 1975*a*).

METHODS

Denervated and normal *cutaneous pectoris* muscle preparations from *Rana esculenta* and *Rana temporaria* were used. Denervation of muscles was performed under ether anaesthesia by cutting the n.cut. pectoris close to its entry to the muscle and removing a nerve segment of 2–10 mm length from the proximal nerve trunk. After denervation frogs were kept for 40–70 days in tanks under running tap water of 16–18° C. Muscles were dissected and maintained in Ringer solution (114 mM-NaCl, 2·5 mM-KCl; 2·5 mM-$CaCl_2$; 2·15 mM-NaH_2PO_4; 0·5 mM-NaH_2PO_4; 5 mM glucose; pH = 7·1) and were pinned out in a Plexiglas chamber with a glass bottom covered with translucent Sylgard resin. Tetrodotoxin at a concentration of 5×10^{-8} g/ml. was added to the bath routinely. Bath temperature was continuously monitored with a thermistor placed beside the preparation and was kept constant at either 8 or 18° C by means of two Peltier cooling elements mounted beneath the metal plate on which the chamber rested.

Experiments on denervated muscles were made under a Zeiss stereomicroscope (× 120 magnification). Since after denervation the layer of connective tissue on the muscle is increased considerably this layer as well as the upper muscle fibre layer was dissected away in the region of former end-plates. Most experiments were carried out on fibres near the medial edge of the muscle where, by stretching the muscle transversely, fibres can be separated from each other. Sometimes small bundles of three to five fibres had to be dissected because of movement artifacts caused by the contraction of adjacent unclamped fibres during drug application. This kind of preparation was necessary especially in the experiments with SubCh and in the experiments in which end-plate currents at the former end-plate were recorded.

The localization of the former end-plate was made by mapping the distribution of ACh sensitivity with brief iontophoretic pulses of ACh. The fibre was thereafter repenetrated at least 0·5 mm away from the suspected end-plate region. Location of the electrodes in the fibre was marked on a Polaroid photomicrograph of the muscle using landmarks such as myelin fragments and blood vessels. At the end of an experiment muscles were fixed briefly and stained for acetylcholinesterase (AChE) activity (Karnovsky, 1961) which delineated the former synaptic region. The distance between the locus of the micro-electrodes and the nearest part of AChE staining profile was then measured with an ocular micrometer.

Experiments on normal muscles were done either under the stereomicroscope or using a Zeiss STANDARD compound microscope fitted with a × 20 U.D. objective. End-plates were localized by the rise time of intracellularly recorded m.e.p.p.s and mapping the distribution of ACh sensitivity with brief pulses of ACh or by direct visual identification of nerve terminals with the compound microscope.

Muscle fibres were impaled with two glass micro-electrodes filled with 3 M-KCl or 0·5 M-K_2SO_4. Electrode resistances were in the range of 2–6 MΩ. Voltage recording and current injecting electrodes were either separated by 40–60 μm along the fibre length or both electrodes penetrated the fibre transversely opposite to each other. Micro-electrodes filled with KCl were used to record the membrane potential whereas the current injecting micro-electrode was filled with KCl or K_2SO_4. When using KCl-filled electrodes for current injection the holding current after drug application often did not return completely to its value before drug application. The amount by which the holding current was increased after drug application was dependent on the size of the drug-induced current and was often about 15–30% of the drug-induced current plateau. This effect is not seen when K_2SO_4 filled micropipettes are used for current injection and therefore these micro-electrodes were preferred (Adams, 1975*b*).

A conventional two micro-electrode clamp circuit was used (Takeuchi & Takeuchi, 1959). The input stage was a differential amplifier with FET inputs. The feed-back amplifier consisted of a combination of Tektronix AM501/AM502 amplifiers which has an output swing of ± 40 V. Amplification factors of 2000–6000 were used to clamp the fibre locally. The quality of potential control was checked routinely by applying hyperpolarizing steps of 40 mV amplitude. The gain of the feed-back loop was adjusted so that the new potential was established with 50 μsec after the beginning of the command pulse. Voltage clamp currents were measured by an operational amplifier (Burr-Brown, 3522) supplying a virtual ground to the bath via a feed-back resistor of 100 kΩ.

Drugs were released iontophoretically from pipettes located between 20 and 30 μm above the fibre. Drug pipettes were filled with either 3 M acetylcholine chloride, 3 M carbamylcholine chloride, both obtained from Sigma, or with 1 M suberyldicholine iodide synthesized by J. Heesemann. The drug pipettes had resistances of 40–80 MΩ and required bucking currents of 5–8 nA. For iontophoresis a floating constant current circuit with a separate bath electrode was used. Iontophoresis currents of 2–10 nA were required for ACh and Carb application whereas with SubCh it was often sufficient to reduce the holding current. Iontophoretic currents were adjusted manually to give drug-induced membrane current plateaus of 30–70 nA amplitude. Due to densensitization current plateaus of this amplitude could be maintained only for short periods of 10–25 sec and sometimes repeated drug applications with the same drug were made on the same fibre.

Voltage clamp currents were recorded simultaneously on a low gain d.c. channel and a high gain a.c. channel (bandpass between 0·1 Hz and 5 kHz) on a FM tape recorder (Hewlett Packard, 3960 instrumentation recorder, 15 in./sec, band width 5 kHz). Current fluctuations were analysed by means of a Saicor 42A, 100 point auto-cross-correlation device after they had been filtered by a bandpass (Krohn Hite, model 3700 filter, 24 db per octave attenuation factor, high pass 0·2–0·5 Hz; low pass 2·5 kHz). Before analysing the current record was inspected for electrical artifacts on a storage oscilloscope.

The autocorrelation functions of current fluctuations were calculated from current records both in the absence of drugs and during current plateaus produced by iontophoretic application of the drugs. They are based upon 8–128 thousand digitalized data points, being sampled at intervals between 0·05 and 2 msec depending on the time constant of the process studied. Therefore for each autocorrelation function total sample periods ranging from 4 to 32 sec had to be obtained which sometimes was achieved by repeated application of the drugs. Routinely the autocorrelation function obtained in the absence of the drug was subtracted from the autocorrelation function obtained in the presence of the drug in order to account for the contribution of extraneous noise, unrelated to drug-induced noise. The difference procedure is legitimate if it can be assumed that drug-induced noise is statistically independent from background noise (Anderson & Stevens, 1973). The difference between the two functions was plotted semilogarithmically. A straight line was fitted to the points using least squares regression. It yielded the parameters of the autocorrelation function. Values below 3–5% of the maximum value were not taken into account for fitting.

When autocorrelation functions had to be fitted by a fast and a slow time constant a voltage of the form $V(T) = V_1 \exp(-T/\tau_1) + V_2 \exp(-T/\tau_2)$ was generated electronically and displayed on an oscilloscope screen. The autocorrelation function was projected from a photomicrograph on to the oscilloscope screen and the parameters of the electronically generated function were adjusted until good visual fit was obtained. Essentially the same results were obtained by plotting the autocorrelation function in semilogarithmic co-ordinates and fitting by eye a straight

line to the slow component. Plotting the difference between the straight line and the initial values of the autocorrelation function yielded the parameters of the fast component (see Fig. 9).

In some experiments, after an initial fast decrease, the holding current returned only very slowly (3–5 min) or not at all back to its initial value when drug application was discontinued. This effect of a 'residual current' made it difficult to measure the mean value of the drug-induced current plateau. In these experiments the value of zero current level was estimated by extrapolating back the 'residual current' to the time of the current plateau and forming the average of the initial holding current and the residual current (see Fig. 1). This value was then used to measure the amplitude of the current plateau. When the residual current amounted to more than 20% of the drug-induced current plateau the experiment was rejected.

Relaxation of drug-induced currents following step changes in membrane potential were measured according to Neher & Sakmann (1975*b*). Means ± S.E. of the means are given.

RESULTS

Noise analysis of end-plate currents using the autocorrelation function

In a first series of experiments the value of τ and γ for junctional channels were derived from drug-induced end-plate currents (e.p.c.s) in normal fibres for comparison of the properties of extrajunctional and junctional ion channels. So far drug-induced end-plate potentials (e.p.p.s) (Katz & Miledi, 1972) and e.p.c.s (Anderson & Stevens, 1973; Colquhoun *et al.* 1975; Ben Haim *et al.* 1975) have been analysed by measuring the power spectrum of e.p.p. or e.p.c. fluctuations to derive the values of conductance and average lifetime of a single open channel. An alternative method yielding the same information is the calculation of the autocorrelation function. The two methods are merely different representations of the same information since according to the Wiener–Kintchin theorem the one is the Fourier-transform of the other. We chose the autocorrelation function for better comparison of the data obtained by noise analysis with those derived from relaxation experiments.

The same assumptions about the nature of the 'elementary event' giving rise to the current fluctuation are made for autocorrelation analysis as for power spectrum analysis (Katz & Miledi, 1972; Anderson & Stevens, 1973). (i) The extra current, induced by a drug, arises from statistically independent elementary current pulses which add linearly. (ii) The elementary current pulse has a fixed amplitude i but varies in duration. The durations are distributed in an exponential fashion with a mean length of τ.

With these assumptions the autocorrelation function $\phi(T)$ of fluctuations around a steady drug-induced current of the mean amplitude I is given by (Rice, 1944)

$$\phi(T) = \sigma^2 \exp(-T/\tau), \quad (1)$$

where σ^2 denotes the total variance of current fluctuations, T the time

interval and τ the time constant of the autocorrelation function. The mean duration τ of the elementary current pulse (or channel open time) is then given by the value of the time constant of the autocorrelation function. The amplitude i of the elementary current pulse is estimated from σ^2 and I using the relation:

$$i = \frac{\sigma^2}{I}, \tag{2}$$

σ^2 is obtained using $\phi(0) = \sigma^2$. The mean single channel conductance γ is estimated from:

$$\gamma = \frac{i}{(V - V_{eq})}, \tag{3}$$

where V_{eq} is the reversal potential and V the holding potential.

A typical experiment illustrating the derivation of the values of τ and i by measurement of the autocorrelation function of ACh-induced e.p.c. fluctuations at a normal end-plate is illustrated in Fig. 1. The characteristic temporal properties of e.p.c. fluctuations induced by ACh, Carb or SubCh under various experimental conditions derived with correlation analysis are summarized in Table 1.

The reversal potentials of drug-induced e.p.c.s were obtained from the analysis of instantaneous currents following hyperpolarizing step changes in membrane potential (from -80 to -120 mV) applied during e.p.c. plateaus (Neher & Sakmann, 1975*b*). This procedure yields two points on the instantaneous I–V relation of the 'activated' end-plate, when channels are open. Assuming ohmic behaviour of end-plate channels (Magleby & Stevens, 1972) the reversal potential can be obtained by extrapolation. For all the drugs tested the mean value of V_{eq} obtained by extrapolation was found to be close to 0 mV (within ± 5 mV). This is in accordance with Colquhoun *et al.* (1975) who directly measured a V_{eq} close to 0 mV for the e.p.c.s induced by various cholinergic drugs. Taking a reversal potential of 0 mV the grand average of γ derived from different experiments at either 8 or 18° C and at different membrane potentials are for ACh: $\gamma_{ACh} = 23 \pm 2$ pmho ($n = 31$); Carb: $\gamma_{Carb} = 19 \pm 2{\cdot}0$ pmho ($n = 10$); SubCh: $\gamma_{SubCh} = 20 \pm 2$ pmho ($n = 17$). The mean values of γ as well as the τ values of end-plate channels derived by the autocorrelation function are in good agreement with values reported by others using power spectrum analysis (Katz & Miledi, 1972; Colquhoun *et al.* 1975; Ben Haim *et al.* 1975).

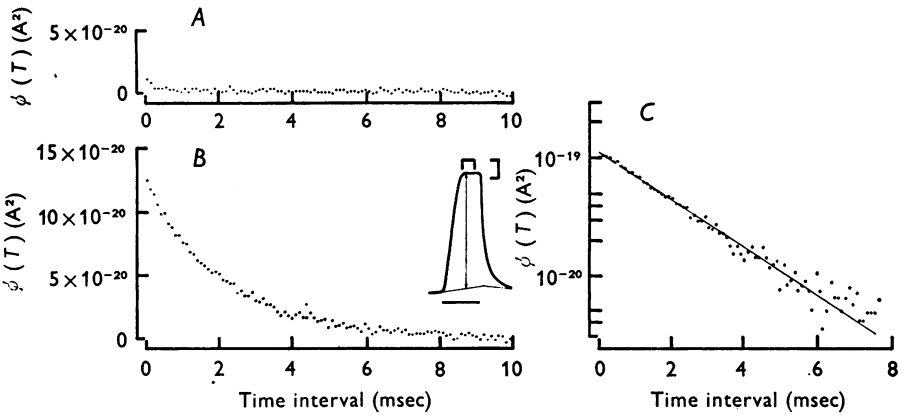

Fig. 1. Evaluation of amplitude and mean duration of the elementary current pulse from the autocorrelation function of ACh-induced end-plate current fluctuations. *A*, autocorrelation function of fluctuations of voltage clamp current before application of ACh. *B*, autocorrelation function of current fluctuations recorded during steady iontophoretic application of ACh to the end-plate. The current records are filtered with a bandpass (0·5 Hz–2·5 kHz) before the autocorrelation function was calculated. Sampling interval 100 μ sec. The inset shows a pen-writer record of the time course of voltage clamp current before, during and after ACh application. Bar below the record indicates the period of ACh application. Horizontal bar above the trace shows the 6·4 sec time interval during which the autocorrelation function of e.p.c. fluctuations was calculated. Vertical bar represents 10 nA inward current. The arrowed bar represents the mean ACh-induced current I = 64 nA. *C*, semilogarithmic plot of the autocorrelation function shown in *B* after subtraction of the autocorrelation function shown in *A*. A straight line was fitted to the points according to relation (1) using linear regression. The slope of this line yields an estimate of the time constant of the autocorrelation function which is τ = 2·1 msec in this experiment. The extrapolated intercept with the ordinate yields the variance $\sigma^2 = 11{\cdot}5 \times 10^{-20}$ A². A value of i = 1·79 pA for the amplitude of the elementary current pulse is estimated using relation (2). Holding potential: −80 mV; temperature 14° C.

TABLE 1. Temporal properties of current fluctuations at normal end-plates at −80 mV holding potential. τ-values (mean ± S.E.) of the autocorrelation function calculated from drug-induced e.p.c.s. Numbers in parentheses refer to numbers of experiments

Substance	Temp. (°C)	τ value (msec)
ACh	8	3·1 ± 0·16 (11)
	18	1·1 ± 0·1 (13)
Carbachol	8	1·0 ± 0·1 (7)
Suberyldicholine	8	5·3 ± 0·3 (7)

Properties of ACh-induced extrasynaptic currents

Denervation periods of 40–60 days were required for the development of high extrajunctional ACh sensitivities. Even after these long denervation times drug-induced current plateaus suitable for noise analysis could be obtained in most fibres only in the vicinity of the former end-plate region when standard doses of drugs were applied (see Methods). To avoid contributions from the end-plate only experiments in which the distance between the site of drug application and the former end-plate region was larger than 300 μm were regarded as extrasynaptic (see Methods). In three control experiments it was found that with standard iontophoretic doses of ACh no drug-induced current was recorded when the drug pipette was moved away from the fibre by more than 150 μm sideways or in the upward direction. This insures that the range of drug action is limited to the extrasynaptic parts of the fibre. No systematic change in the values of τ and i was observed when current records at the beginning and at the end of a drug-induced current plateau were analysed.

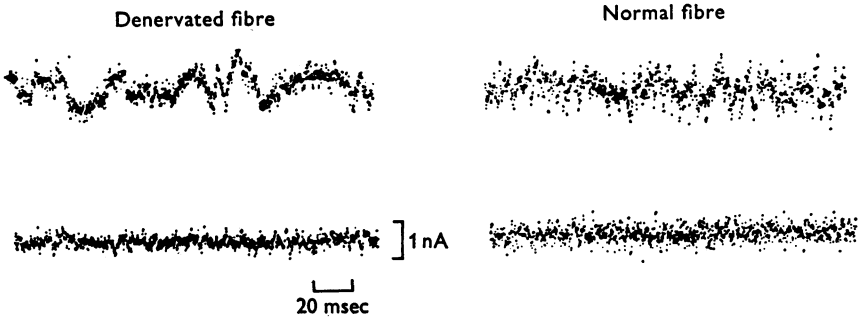

Fig. 2. Comparison of current fluctuations produced by ACh application to an extrajunctional area of a 61 day denervated fibre (left side) and the neuromuscular junction of a normal fibre (right side). The traces are digitalized records of filtered (0·5 Hz–2·5 kHz) voltage clamp currents recorded before (lower traces) and during iontophoretic application of ACh (upper traces). ACh was applied 620 μm away from the former end-plate region in the experiments with the denervated fibre. In both experiments the mean ACh-induced current was I = 40 nA. Temperature 18° C, holding potential: −80 mV.

Duration of the elementary ACh current pulse. Fig. 2 (left side) shows the effect of ACh on a chronically denervated muscle fibre under voltage clamp conditions when ACh is applied to a hypersensitive extrasynaptic area of the fibre. It is obvious that the ACh-induced current fluctuations in the denervated fibre are similar in amplitude but of a slower time course than those recorded under the same conditions from a voltage clamped

end-plate of a normal fibre (Fig. 2, right side). The autocorrelation function of the ACh-induced current fluctuations recorded from the denervated fibre is shown in Fig. 3. The value of τ derived from the semi-logarithmic plot of this function is $\tau_{extra} = 5{\cdot}6$ msec. This compares with a value $\tau_{ep} = 1{\cdot}3$ msec found for the autocorrelation function of the e.p.c. fluctuations of the normal fibre illustrated in Fig. 2. The average duration of the elementary ACh current pulse of extrasynaptic currents is thus much longer than that derived from e.p.c. records in normal fibres. For instance the mean value of τ for ACh-induced extrasynaptic currents of seven experiments at 18° C and -80 mV holding potential was $\tau_{extra} = 5{\cdot}2 \pm 0{\cdot}4$ msec. This compares with a mean value of $\tau_{ep} = 1{\cdot}1 \pm 0{\cdot}1$ msec found for the open time of end-plate channels under the same conditions in thirteen experiments, the ratio of the means τ_{extra}/τ_{ep} being 4·73 at this temperature.

The τ value of ACh-induced extrasynaptic channels is temperature dependent. Comparing seven experiments at 18° C with seven experiments at 8° C at a holding potential of -80 mV a Q_{10} of 2·1 is found (Table 2). This

TABLE 2. Temporal characteristics of extrasynaptic current fluctuations. τ values (mean ± S.E.) of the autocorrelation functions are given at two holding potentials. Numbers in parentheses refer to number of experiments

Substance	Temp. (°C)	τ value (msec) (-80 mV)	τ value (msec) (-120 mV)
ACh	8	11 ± 1·6 (7)	18 ± 3·5 (2)
	18	5·2 ± 0·4 (7)	8·6 ± 0·8 (5)
Carbachol	8	3·9 ± 0·4 (6)	6·5 ± 0·4 (3)
Suberyldicholine	8	19 ± 2·5 (7)	28 ± 6 (3)

temperature dependence is comparable to the one described for the τ values of end-plate channels (Katz & Miledi, 1972; Anderson & Stevens, 1973). It also confirms the observation of Katz & Miledi (1972) that the half power point of the voltage noise spectrum recorded at former end-plates of denervated fibres is shifted towards lower values at low temperatures.

At the end-plate it has been shown that increasing the membrane potential shifts the spectrum of ACh-induced e.p.c. fluctuations towards lower frequencies without changing its shape indicating a longer open time of the single end-plate channel at higher membrane potentials (Anderson & Stevens, 1973). We therefore examined the voltage dependence of τ of extrajunctional channels by measuring the autocorrelation functions of extrasynaptic current fluctuations at -80 and -120 mV holding potential. In Fig. 4 the autocorrelation functions of ACh-induced current fluctuations recorded from the same fibre at two different holding potentials are shown. Increasing the membrane potential by 40 mV lengthened the

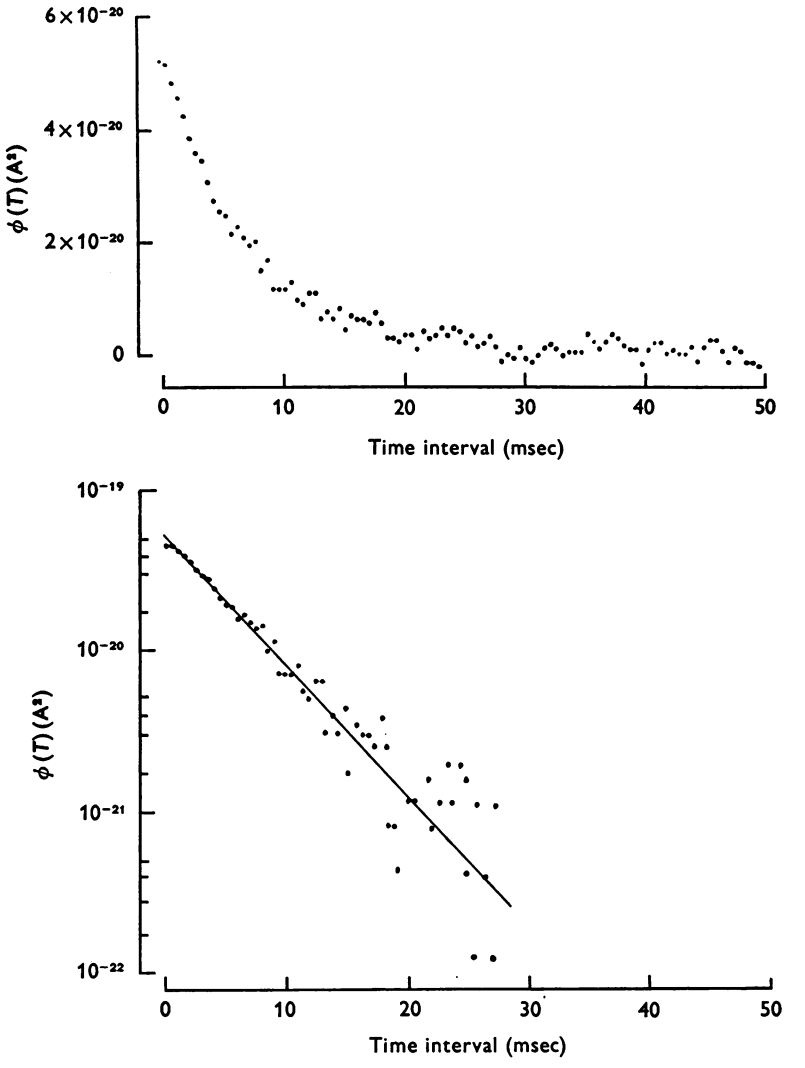

Fig. 3. Autocorrelation function calculated from fluctuations of extrasynaptic current induced by iontophoretic ACh application to a hypersensitive area of a 56 day denervated fibre. Distance of the locus of drug application to the old end-plate region was 620 μm. ACh application produced a mean current of 40 nA. Length of current record used to calculate the function was 8 sec. 0·5–2·5 kHz bandpass, sampling interval: 500 μsec. Upper graph: autocorrelation function in linear co-ordinates. Lower graph: in semilogarithmic co-ordinates. The time constant of the autocorrelation function is $\tau = 5{\cdot}6$ msec, the variance $\sigma^2 = 5{\cdot}3 \times 10^{-20}$ A^2. This yields an estimate of $i = 1{\cdot}33$ pA for the amplitude of the elementary current pulse.

time constant of the autocorrelation function from $\tau_{80} = 5{\cdot}4$ msec to $\tau_{120} = 8{\cdot}4$ msec in this experiment. The mean ratio τ_{120}/τ_{80} found in four paired experiments at 18° C was $1{\cdot}6 \pm 0{\cdot}1$. This is in the same range as the ratio found for the voltage dependence of the τ values of junctional channels (Anderson & Stevens, 1973; Neher & Sakmann, 1975*b*).

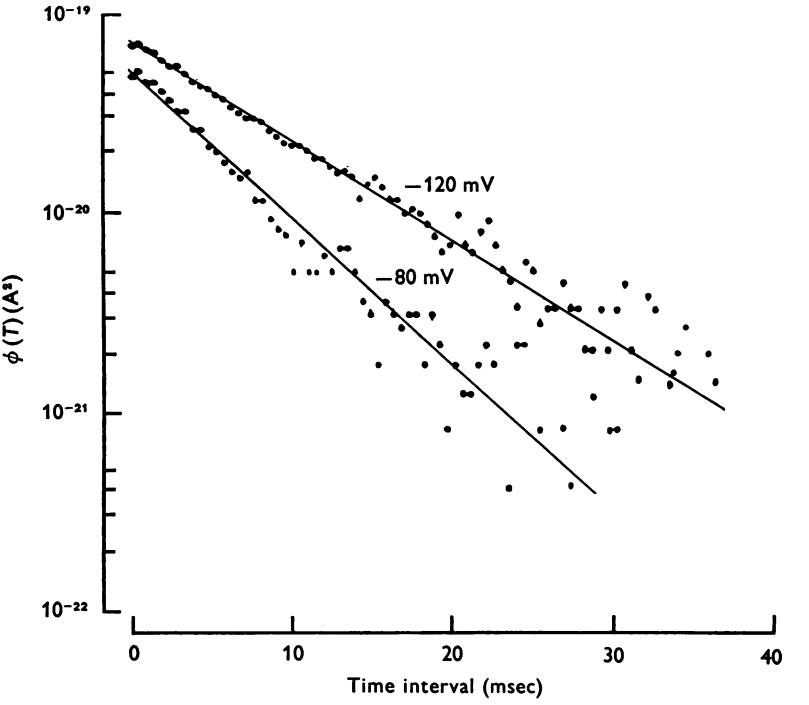

Fig. 4. Effect of membrane potential on the autocorrelation function of ACh-induced extrasynaptic current fluctuations at 18° C. Autocorrelation functions of current records obtained from the same fibre at −80 and −120 mV holding potential are plotted in semilogarithmic co-ordinates. The fibre had been denervated for 56 days. ACh was applied iontophoretically 370 μm away from the former end-plate region and induced a mean inward current of 38 nA at −80 mV and 37 nA at −120 mV membrane potential. At −80 mV the time constant of the autocorrelation function is $\tau_{80} = 5{\cdot}4$ msec, the variance $\sigma^2 = 5{\cdot}1 \times 10^{-20}$ A^2; at −120 mV the corresponding values were $\tau_{120} = 8{\cdot}4$ msec and $\sigma^2 = 7{\cdot}3 \times 10^{-20}$ A^2.

The voltage sensitivity of the open channel lifetime can be used to derive the values of τ at different membrane potentials from the relaxation of the mean drug-induced current I to a new equilibrium value, following step changes in membrane potential during drug-induced current plateaus (Adams, 1975*a*; Neher & Sakmann, 1975*b*). Fig. 5 shows the relaxation of the mean ACh-induced current following a 40 mV hyperpolarizing step

change in membrane potential during an ACh current plateau recorded from an extrasynaptic hypersensitive region of a denervated fibre. The mean values of the relaxation time constants at 18° C derived from four experiments were $\tau_{120} = 7{\cdot}8 \pm 0{\cdot}6$ msec for steps from -80 to -120 mV and $\tau_{80} = 5{\cdot}1 \pm 0{\cdot}6$ msec for steps from -120 to -80 mV. The corresponding mean values from three experiments at 8° C are $\tau_{120} = 15 \pm 2$ msec and $\tau_{80} = 9 \pm 1$ msec. The ratio τ_{120}/τ_{80} is $1{\cdot}53 \pm 0{\cdot}2$ at the higher, and $1{\cdot}67 \pm 0{\cdot}1$ at the lower temperature. The voltage dependence of the

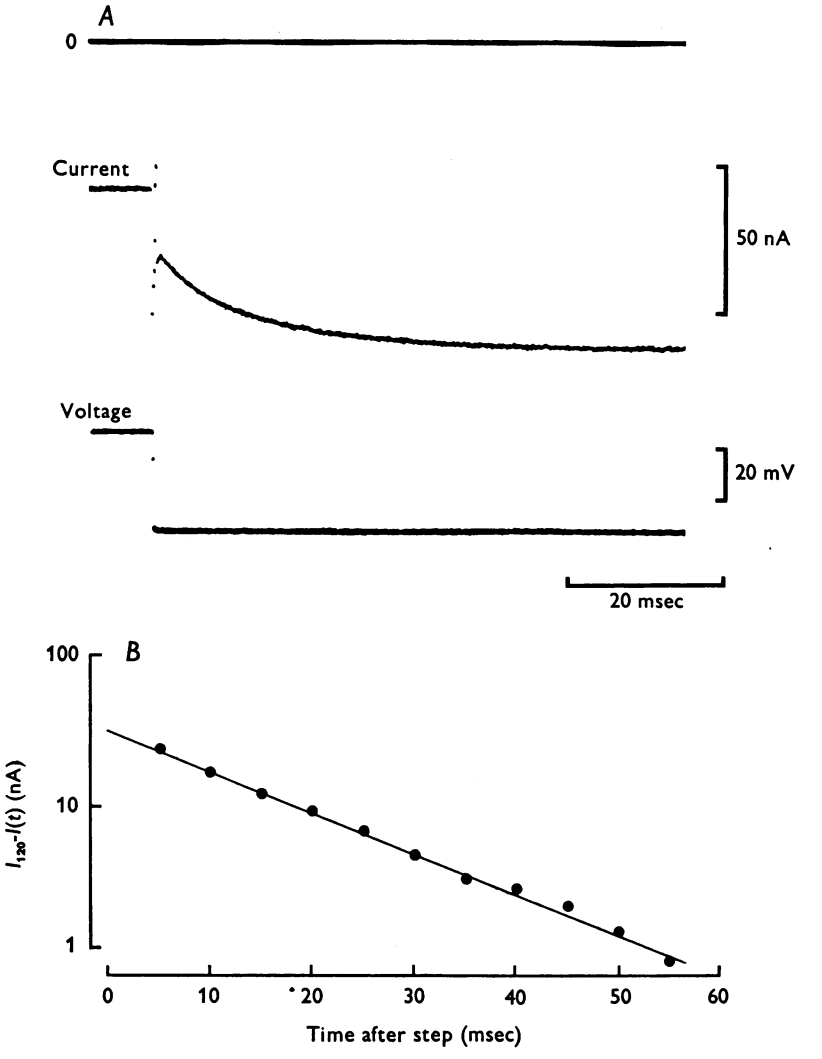

Fig. 5. For legend see facing page.

relaxation time constant of extrasynaptic currents, described by the ratio τ_{120}/τ_{80}, is therefore similar to the voltage dependence of τ derived from relaxation experiments of e.p.c.s in normal fibres (Neher & Sakmann, 1975*b*). With both kinds of analysis therefore a comparable voltage dependence of τ is found for junctional and extrajunctional channels although it seemed somewhat more pronounced in denervated fibres.

Amplitude of the elementary ACh-current pulse. The estimate of i derived from the autocorrelation function of ACh-induced extrasynaptic current fluctuations was consistently somewhat smaller than the value derived from e.p.c.s in normal fibres. For instance, at -80 mV holding potential and 18° C the mean value from seven experiments with denervated fibres was $i_{extra} = 1{\cdot}4 \pm 0{\cdot}18$ pA which compared to a value of $i_{ep} = 1{\cdot}9 \pm 0{\cdot}2$ pA derived from e.p.c. fluctuations in thirteen normal fibres, the ratio i_{extra}/i_{ep} being 0·74 under these conditions. The values of i tended to be higher at 18° C than at 8° C. In seven different experiments at 8° C a mean value of $i_{extra} = 1{\cdot}1 \pm 0{\cdot}15$ pA was found.

When the membrane potential was raised to -120 mV the amplitude of i increased by the amount predicted from the slope of the I–V relation of the open channel (next section). In four paired experiments at 18° C the values of i were determined in the same fibre at -80 and -120 mV

Fig. 5. Relaxation of ACh-induced extrasynaptic current following a hyperpolarizing step change in membrane potential. *A*, the trace marked 'current' shows the time course of relaxation of the ACh-induced current. This record is obtained by measuring the voltage clamp currents during the ACh-induced plateau when the membrane potential is stepped for 100 msec from -80 to -120 mV. Four relaxation responses were averaged. The voltage clamp current recorded during the same step change in membrane potential but in the absence of ACh was subtracted. Thereby the capacitative transients which partially obscure the time course of relaxation are eliminated. The subtraction procedure was carried out after digitalization of the current records (for details see Neher & Sakmann, 1975*b*). The zero level for the ACh-induced current is indicated by the line above current trace. ACh-application induced a mean current of $I_{80} = 49$ nA at -80 mV membrane potential. Following the step change in membrane potential an instantaneous current is seen followed by the relaxation of the ACh-induced current to its new equilibrium value $I_{120} = 103$ nA at -120 mV membrane potential. The trace marked 'voltage' shows the time course of step change in membrane potential. *B*, semilogarithmic plot of $I_{120} - I(t)$ *vs.* t, where $I(t)$ is the amplitude of the ACh-induced current at different times t after the step change in membrane potential. This plot yields the time constant of relaxation at -120 mV membrane potential which is $\tau_{120} = 16$ msec. The amplitude of the instantaneous current was 73 nA. It is obtained by extrapolating the relation current back to the time of the voltage step. 52 day denervated fibre at 8° C. ACh was applied iontophoretically 380 μm away from the former end-plate region.

membrane potential. The mean values found were $i_{80} = 1{\cdot}4 \pm 0{\cdot}21$ pA and $i_{120} = 2{\cdot}1 \pm 0{\cdot}25$ pA.

Instantaneous current–voltage relation of the extrasynaptic membrane. Drug-induced current fluctuations are caused, according to the assumptions made for the interpretation of their autocorrelation function, by the random opening and closing of individual ion channels. In order to compare the physical properties of the open channels in the synaptic and the extrasynaptic membrane conductances have to be derived from the current measurements. To do so the current–voltage relations of synaptic and

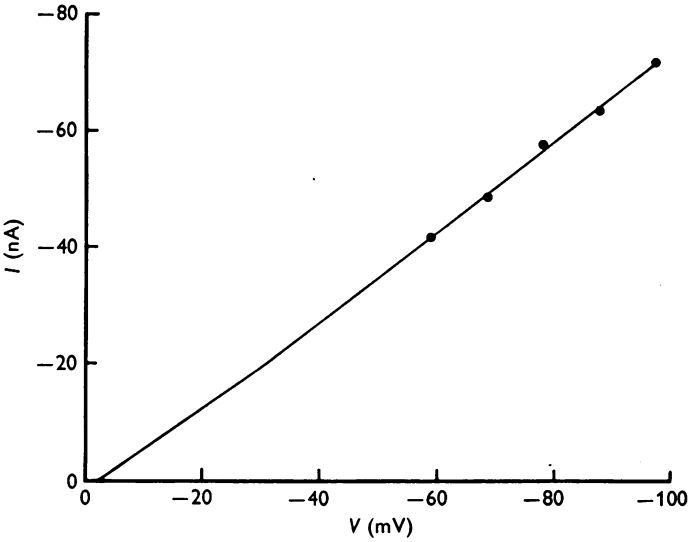

Fig. 6. Instantaneous current–voltage relation of the extrasynaptic membrane during an ACh current plateau. The amplitudes of instantaneous currents are plotted versus the amplitude of the membrane potential change. The steps in membrane potential were made from a holding potential of −60 mV during an ACh-induced current plateau of 42 nA. The straight line is fitted by linear regression, correlation coefficient: 0·95. An equilibrium potential of −4 mV was found in this experiment by extrapolation. The fibre had been denervated for 44 days. ACh was applied 400 μm away from the former end-plate region. Temperature 8° C.

extrasynaptic channels must be known. It is not yet possible to measure this relation directly for a single channel, but it can be determined for an ensemble of channels by measuring the instantaneous I–V relation of the activated membrane during drug-induced current flow where ion channels are open. Magleby & Stevens (1972) have measured the instantaneous I–V relation of the end-plate membrane by causing step changes in membrane potential during the peak of nerve-evoked e.p.c.s and found a linear I–V relation for end-plate channels. We have obtained the instantaneous

I–V relation of the extrasynaptic membrane during ACh-induced current flow in a similar way.

During a current plateau of 25–45 nA amplitude, induced by iontophoretic application of ACh, the membrane potential is stepped for 100 msec from a holding potential of -60 mV in the hyperpolarizing direction to various potentials ranging from -70 to -100 mV. The amplitudes of the instantaneous currents following a step are obtained by extrapolating the relaxing mean current back to the time of the voltage step as illustrated in Fig. 5 (for details see Neher & Sakmann, 1975*b*). In five experiments on denervated fibres the instantaneous I–V relation of the 'activated' extrasynaptic membrane was linear in the range measured (between -60 and -100 mV). The result of one experiment is shown graphically in Fig. 6. Assuming ohmic behaviour, extrapolation of the I–V curve yielded in all experiments a reversal potential V_{eq} for ACh-induced extrasynaptic currents which was close to 0 mV. The value of V_{eq} derived by linear extrapolation from the instantaneous I–V relations measured in five fibres was $V_{eq} = +2{\cdot}6 \pm 2{\cdot}7$ mV (mean ± S.E., range +10 to -6 mV).

Thus calculating the average value of the conductance of the single open extrasynaptic channel from measurements of I at different holding potentials and using an equilibrium potential of 0 mV a mean value of $\gamma_{extra} = 15 \pm 1{\cdot}8$ pmho (twenty-one experiments) is obtained. This is somewhat lower than the average value of $\gamma_{ep} = 23 \pm 2$ pmho obtained for the conductance of the single open end-plate channel using the same reversal potential of 0 mV, the ratio of the average values being $\gamma_{extra}/\gamma_{ep} = 0{\cdot}65$.

Properties of extrasynaptic currents induced by other agonists

In view of the pharmacological difference between junctional and extrajunctional ACh-receptors (Jenkinson, 1960; Beranek & Vyskocil, 1967) it was interesting to compare the properties of junctional and extrajunctional channels opened by two other cholinergic drugs, Carb and SubCh. At the end-plate these drugs are known to induce ion channels of very different duration (Katz & Miledi, 1973*a*). Fig. 7 shows the autocorrelation functions of current fluctuations induced by Carb and SubCh during iontophoretic application of these drugs to an extrasynaptic area of a 58 day denervated fibre. With both drugs autocorrelation functions are obtained which are characterized by a single time constant, their values being $\tau_{Carb} = 4{\cdot}1$ msec and $\tau_{SubCh} = 20$ msec in this particular experiment. The relation between the τ values characteristic for each drug is roughly the same for end-plate and extrasynaptic channels, i.e. Carb induces ion channels with a much shorter open time than SubCh. However,

the absolute values of τ are considerably larger for extrasynaptic channels. This is similar to the result obtained in the experiments with ACh. The mean values of τ measured at -80 mV at 8° C are listed in Table 2. Comparing the mean values of τ derived from e.p.c.s and from extrasynaptic currents at -80 mV and 8° C the ratio τ_{extra}/τ_{ep} is 3·9 for Carb and 3·6 for SubCh. Temperature and voltage dependence of τ was, for both drugs, similar to that found for ACh. The voltage dependence of τ was measured for both drugs with noise analysis as well as by relaxation experiments (see Fig. 8). At 8° C the ratio τ_{120}/τ_{80} of the mean τ values at

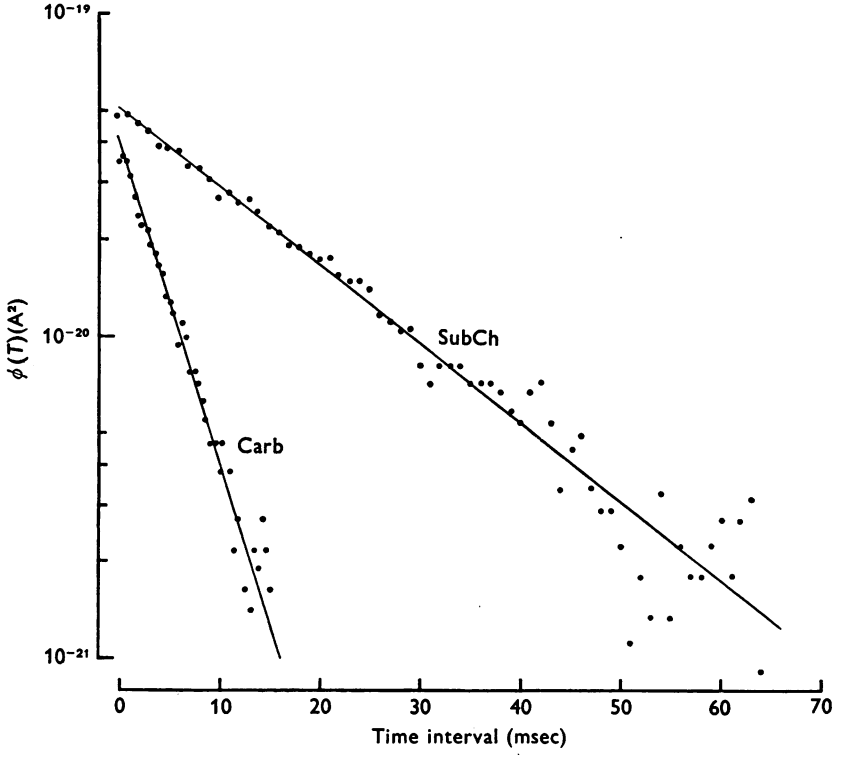

Fig. 7. Autocorrelation functions calculated from fluctuations of extrasynaptic currents induced by iontophoretic application of Carb and SubCh. Both functions were obtained from the same, 48 day denervated fibre. Drugs were applied 300 μm away from the former end-plate region. Bandpass 0·2 Hz–2·5 kHz. -80 mV holding potential, 8° C. The autocorrelation function marked Carb was calculated from a 4 sec current record. Mean current I = 36 nA. Sampling interval was 200 μsec. Only every other point of the autocorrelation function is shown. The values of τ = 4·1 msec and $\sigma^2 = 4{\cdot}2 \times 10^{-20}$ A² are obtained. The function marked SubCh was calculated from a 16 sec current record. Mean current I = 42 nA. Sampling interval 1 msec. It yields τ = 20 msec and $\sigma^2 = 5{\cdot}1 \times 10^{-20}$ A².

-80 and -120 mV obtained from noise analysis was 1·66 for Carb and 1·47 for SubCh (Table 2).

In three relaxation experiments with Carb and six with SubCh at 8° C, the mean value of the relaxation time constant at -120 mV was $\tau_{Carb} = 5{\cdot}9 \pm 0{\cdot}4$ msec and $\tau_{SubCh} = 35 \pm 4$ msec. At -80 mV the values were $\tau_{Carb} = 3{\cdot}5 \pm 0{\cdot}3$ msec and $\tau_{SubCh} = 21 \pm 3$ msec. The ratio of the means τ_{120}/τ_{80} is 1·68 for Carb and 1·66 for SubCh, which is similar to the ratio found in relaxation experiments with ACh.

The estimate of τ and σ^2 values derived from extrasynaptic currents at low temperature and -120 mV membrane potential is subject to a number of errors, especially in experiments with SubCh. This arises from the fact that long current records of 16–32 sec were required to get reliable estimates of the autocorrelation function. These records were analysed with a high pass filter having its 3 db point at 0·2 Hz. Slow changes in the value of I due to desensitization are inevitable during prolonged drug applications and show up as oblique base lines in the autocorrelation function. These have to be taken into account for exponential curve fitting. In several experiments repeated drug applications were made and current plateaus suitable for noise analysis were selected. Due to the fact that for relaxation analysis

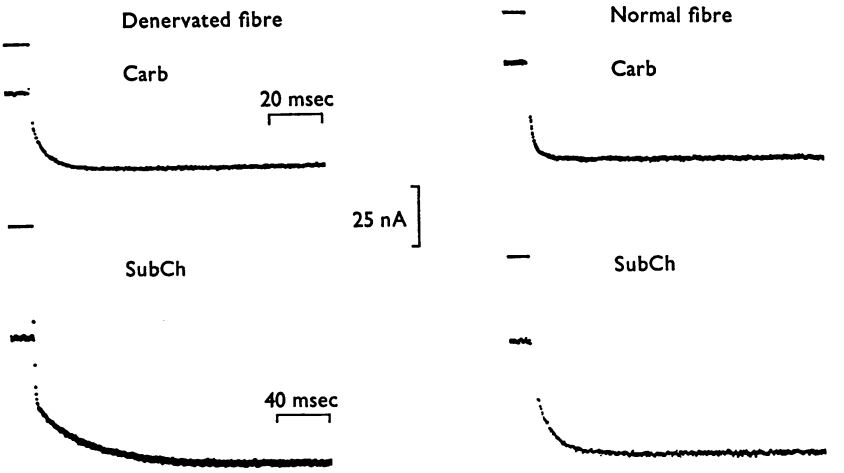

Fig. 8. Relaxation of drug-induced currents when, during a Carb- or SubCh-induced current plateau, the membrane potential is changed stepwise from -80 to -120 mV. Drugs were applied iontophoretically to an extrasynaptic area of a 58 day denervated fibre (left side). For comparison relaxations of e.p.c.s induced by the same drugs in a normal fibre are shown on the right side. Each trace is obtained by the procedure described in the legend of Fig. 5. The bar above each trace indicates zero level for drug-induced current. The time constants of relaxation at -120 mV are $\tau_{Carb} = 5{\cdot}5$ msec and $\tau_{SubCh} = 37$ msec in the experiment on the denervated fibre and $\tau_{Carb} = 1{\cdot}53$ msec and $\tau_{SubCh} = 11{\cdot}5$ msec on the normal fibre. Temperature 8° C. Note different time bases for experiments with SubCh and Carb.

current plateaus of much shorter duration (3–5 sec) are required the τ values derived from relaxation analysis were less variable than those obtained from noise analysis under these conditions.

The amplitudes of the elementary current pulses were, as it was found for ACh, somewhat lower than the corresponding values derived from e.p.c.s induced by these drugs under similar conditions. At -80 mV holding potential, averaging the values obtained at 8 and 18° C, the mean value found for Carb-induced extrasynaptic currents was $i_{Carb} = 1{\cdot}2 \pm 0{\cdot}16$ pA (nine experiments) and $i_{SubCh} = 1{\cdot}2 \pm 0{\cdot}25$ pA (ten experiments) for SubCh-induced currents.

The evaluation of instantaneous currents in relaxation experiments with Carb and SubCh yielded a reversal potential close to 0 mV for the extrasynaptic currents induced by these drugs. In these experiments the membrane potential was stepped from -80 to -120 mV during a drug-induced current plateau and the equilibrium potential was determined by extrapolation from two points of the instantaneous I–V relation assuming ohmic behaviour of the channel conductance. The mean value of V_{eq} obtained in this way was -2 ± 5 mV (six experiments) for Carb-induced currents and $\pm 2{\cdot}5 \pm 2$ mV (eight experiments) for SubCh. Taking a reversal potential of 0 mV and averaging over all experiments at different temperatures and membrane potentials the mean value of the conductance of a single open extrasynaptic channel is $\gamma_{Carb} = 14 \pm 1{\cdot}2$ pmho (thirteen experiments) and $\gamma_{SubCh} = 14 \pm 3$ pmho (fourteen experiments) for Carb and SubCh respectively. Comparing these conductances with the corresponding ones of end-plate channels the ratio of the means $\gamma_{extra}/\gamma_{ep}$ is 0·73 for Carb and 0·70 for SubCh.

Properties of end-plate currents in denervated muscle fibres

In fifteen experiments drug-induced currents at the former end-plate of 40–70 day denervated fibres were analysed. In only four of the experiments the time course of the autocorrelation function of e.p.c. fluctuations could be fitted by a single time constant. In one experiment with ACh and one with SubCh the time constant was $\tau_{ACh} = 3{\cdot}4$ msec and $\tau_{SubCh} = 6{\cdot}8$ msec and thus corresponded to the mean value for e.p.c.s in normal fibres. In two other fibres, one tested with Carb and one with ACh, the values of τ were similar to those found for extrasynaptic currents and were $\tau_{Ach} = 13$ msec and $\tau_{Carb} = 3{\cdot}2$ msec. In the remaining eleven experiments the autocorrelation function could be separated into a 'fast' and a 'slow' component. This observation of two components was made for currents induced by any one of the three drugs. In all these cases two time constants were fitted which differed by a factor of 3–5. The 'fast' component corresponded roughly to the mean values found for e.p.c.

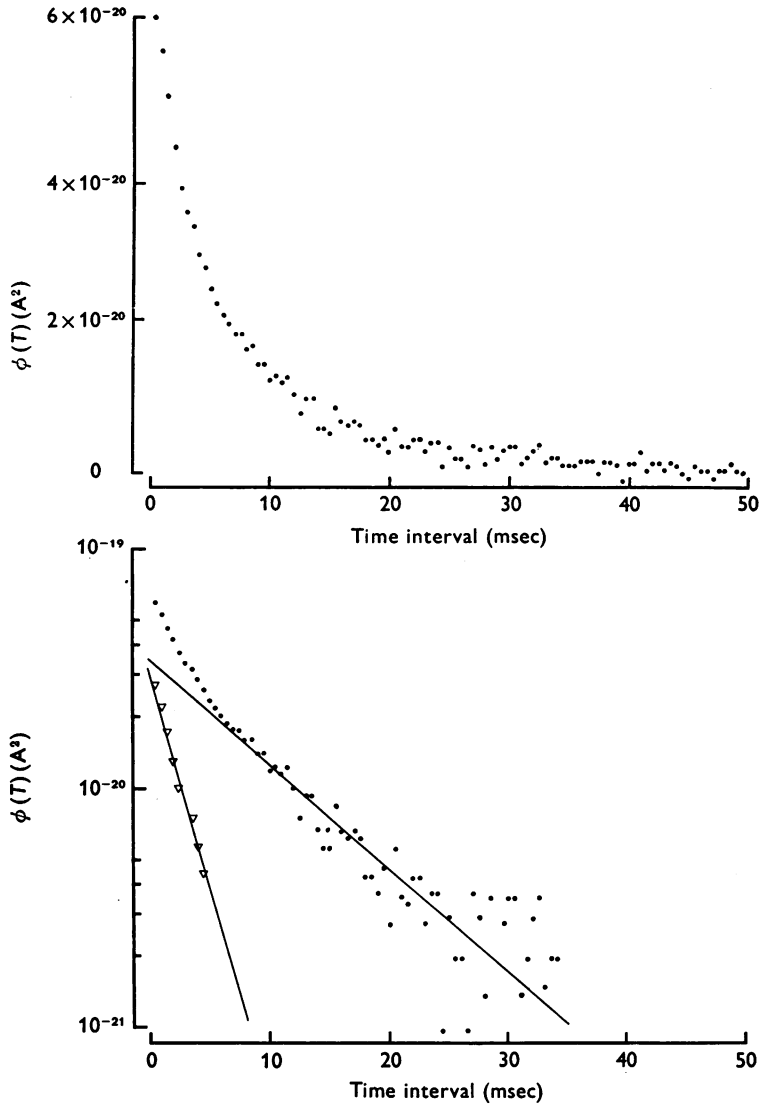

Fig. 9. Autocorrelation function of ACh-induced e.p.c. fluctuations recorded from a 62 day denervated fibre. Holding potential: −80 mV, temperature: 8° C. Duration of current record was 8 sec (sampling interval: 500 μsec), mean amplitude of current plateau: 60 nA. Upper graph: autocorrelation function in linear co-ordinates. Lower graph: in semilogarithmic co-ordinates. Straight lines were fitted by assuming that the autocorrelation function is the sum of two exponential components. Triangles represent difference between the straight line fitted to the slow component and the initial values of the autocorrelation function. Curve fitting yielded values of $\tau = 2{\cdot}6$ msec and $\sigma = 3{\cdot}2 \times 10^{-20}$ A² for the fast component and $\tau = 11$ msec and $\sigma = 3{\cdot}5 \times 10^{-20}$ A² for the slow component of the autocorrelation function.

fluctuations in normal fibres whereas the 'slow' component was similar to the value of τ derived from purely extrasynaptic currents. An example of an autocorrelation function of current fluctuations produced by ACh application to a former end-plate of a 62 day denervated fibre is shown in Fig. 9. The two time constants derived from this function are $\tau_1 = 2{\cdot}6$ msec and $\tau_2 = 11$ msec. The mean values of τ derived from the 'fast' and the 'slow' component of the autocorrelation functions obtained by application of different drugs are listed in Table 3. In two experiments with ACh in

TABLE 3. Temporal characteristics of current fluctuations recorded at the former end-plate of denervated fibres. Means ± S.E. of fast and slow τ values of the autocorrelation function are given. Numbers in parentheses refer to number of experiments. All experiments are at 8° C and −80 mV holding potential

Substance	τ_{fast} (msec)	τ_{slow} (msec)
ACh (4)	$2{\cdot}7 \pm 0{\cdot}2$	12 ± 1
Carbachol (4)	$0{\cdot}8 \pm 0{\cdot}13$	$4{\cdot}3 \pm 0{\cdot}4$
Suberyldicholine (3)	$4{\cdot}3 \pm 0{\cdot}3$	25 ± 4

which the autocorrelation function of the e.p.c. could be separated into two components, it was possible to repenetrate the fibre more than 300 μm away from the end-plate region and measure the autocorrelation function of the extrasynaptic current. In these experiments a single time constant was then obtained which was similar to the slow component found in the e.p.c. autocorrelation function.

DISCUSSION

In the present experiments values for conductance and mean open time of drug-induced extrasynaptic ion channels have been derived from the fluctuations of extrasynaptic membrane currents. For this derivation we made assumptions about the elementary event underlying these fluctuations as have been made for the analysis of membrane noise recorded at the end-plate (Anderson & Stevens, 1973). When discussing the results in terms of molecular reaction rates it will be implied therefore that the same two step reaction scheme describes the interaction of agonist and receptor in the synaptic as well as in the extrasynaptic membrane. According to this scheme (del Castillo & Katz, 1957; Katz & Miledi, 1972; Magleby & Stevens, 1972) an agonist molecule A reacts with an ACh-receptor R and forms an inactive AR complex. In a second step the inactive complex is reversibly transformed to the active conformation AR* which causes a channel to open as long as the complex is in the active conformation.

$$A + R \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} AR \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} AR^*. \qquad (4)$$

The second step is assumed to be rate limiting. The drug-induced membrane noise is due to the fluctuations of the complexed receptor between AR and AR*. For such a model relations (1) and (2) are valid under the condition that only a small percentage of all channels are in the open state (Stevens, 1972), i.e. only under the assumption $k_2 < k_{-2}$ can the mean duration and the size of the elementary current pulses be derived from the present data.

The condition of a low concentration limit has been documented for drug-induced e.p.p.s and e.p.c.s in normal fibres (Katz & Miledi, 1972; Anderson & Stevens, 1973). In our experiments on denervated fibres the amplitudes of the drug-induced current plateaus used for noise analysis varied between 30 and 70 nA (at -80 mV holding potential). Within this range plotting I *vs.* σ^2 can be fitted by a straight line passing through the origin, but the range of mean currents is probably to small to prove that the low concentration limit indeed applied to these experiments. In the more general case the time constant τ and amplitude σ^2 of the autocorrelation function are connected with the parameters of the model in a more complicated fashion

$$\tau^{-1} = k_{-2} + k_2 \frac{k_1 C}{k_1 C + k_{-1}}; \quad \frac{\sigma^2}{I} = i\left(1 - \frac{I}{I_{\max}}\right), \tag{5}$$

where C is the concentration of agonist and $I_{\max}$ is the current that would flow if all available channels were open simultaneously. According to eqn. (5) an experimental situation in denervated fibres where the low concentration limit no longer applies could well account for the smaller value of i found for extrasynaptic channels as compared to synaptic ones. Such a situation could arise under conditions of high receptor occupancy, i.e. at saturating concentrations of agonist. It could, however, not explain the larger values of τ obtained for extrasynaptic currents. Therefore a genuine difference in the rate constants of the kinetic scheme (3) must be present in the synaptic and the extrasynaptic membrane. The simplest explanation for the larger τ values which is also consistent with interpretations of noise experiments on normal end-plates (Katz & Miledi, 1972, 1973; Anderson & Stevens, 1973) is a reduced rate constant k_{-2} to about 25 % of the value at the end-plate membrane.

Unfortunately it is not yet possible to establish unequivocally that the low concentration limit applies to our experiments on denervated fibres; only a very rough estimate can be made. With ACh application the highest current plateaus recorded were about $I = 70$ nA at -80 mV holding potential. Taking a mean value of $i_{\mathrm{ACh}} = 1{\cdot}4$ pA for the amplitude of the elementary current pulse it follows that about 5×10^4 channels open during the current plateau. The muscle surface area contributing to this current is about $6 \times 10^3\ \mu^2$ (assuming that the upper half of a 30 μm thick

200 μm long portion of the fibre contributes to the mean current). Receptor density has not been measured for the extrasynaptic membrane of chronically denervated frog muscle fibres. In rat it has been shown by autoradiographic studies with radioactively labelled α-bungarotoxin that in denervated diaphragm fibres receptor density is between 140 and 635 receptors/μm^2 (Fambrough, 1974). Taking the lower limit of this figure for the extrasynaptic membrane for frog muscle fibres, one gets an estimate in the order of 10^6 receptors in the membrane portion under consideration of which about 5 % are open. This is necessarily a very rough estimate which does not take into account the spatial inhomogeneity of ACh concentration, ACh-receptor distribution and the effects of desensitization. But given these uncertainties it would indicate that the low concentration limit indeed applied to our experiments.

Apart from the problem of low concentration limit, other factors such as the exact shape of the I–V relationship complicate a comparison of the conductances of single synaptic and extrasynaptic channels. In our experiments the shape of the I–V relationship of the activated extrasynaptic membrane was linear over the limited range tested. By extrapolating the reversal potentials for drug-induced currents from the instantaneous I–V relation measured during the current flow, we could not detect a significant difference between the synaptic and the extrasynaptic membrane. In both cases V_{eq} was close to 0 mV. This conflicts with measurements of Feltz & Mallart (1971) who reported a reversal potential of -42 mV in denervated fibres obtained by extrapolation of iontophoretic ACh-responses at different membrane potentials. Under equilibrium conditions, the ACh-induced current amplitude is voltage dependent. Since with iontophoretic drug application presumably equilibrium conditions apply (Adams, 1976; Dionne & Stevens, 1975), the extrapolation procedure may have caused the more negative value of V_{eq} in their measurements. More direct evidence, however, is needed to solve this question. Another possible source of error in our experiments might be inadequate voltage control over the region of drug action in denervated fibres. This would result in an underestimate of i and yield too large τ values, i.e. produce changes in the observed direction. With the iontophoretic doses of drugs used in our experiments the region of drug action is restricted to about 150 μm in both directions from the voltage clamp electrodes. This fibre length is well under voltage control during a drug-induced plateau as measured with a third independent micro-electrode (Neher & Sakmann, 1975*b*). The fact that estimates of γ made at -80 and -120 mV holding potential are similar makes it unlikely that inadequate voltage clamping of denervated fibres produced the different i and τ values.

To summarize these points: the data presented suggest that there is a

genuine difference in the open–close kinetics of extrasynaptic and synaptic ion channels produced by cholinergic drugs, whereas the difference in the size of open channels is less well established and must be substantiated by more direct evidence. There is no obvious explanation for the slower kinetics of extrasynaptic channels and it is interesting that apart from the difference in the absolute values of τ, both are similarly dependent on changes of experimental conditions such as temperature, membrane potential and the type of agonist. Possibly after denervation a type of ACh-receptor different from the synaptic one is incorporated into the extrasynaptic membrane. So far no data are available on the biochemistry of frog muscle ACh-receptors, but for ACh-receptors isolated from normal and denervated rat muscle it has been concluded, on the basis of biochemical criteria, that junctional and extrajunctional receptors are 'very similar but not identical molecules' (Brockes & Hall, 1975).

Alternatively the kinetics of ion channels could, in part, be influenced by their lipid environment or by interaction between individual channels. Both factors might be different in the junctional and extrajunctional membrane. It has been shown that aliphatic alcohols slow the kinetics of channel gating at the end-plate (Gage, McBurney & Schneider, 1975). This effect has been attributed to a change in the physical environment of the ion channels and it will be interesting to compare the effect of these alcohols and other substances which affect the membrane lipid structure, on the noise properties of synaptic and extrasynaptic currents. Likewise it has been found that the open time of ion channels formed by gramicidin A in lipid bilayers depends on the type of lipid used for the formation of the bilayer (Neher & Eibl, 1976). But so far no difference in the lipid composition of junctional and extrajunctional membrane has been reported.

The observation that the autocorrelation function of drug-induced current fluctuations at end-plates of denervated fibres can be fitted by the sum of two single time constant functions suggests that there are two populations of channels in this region present which are activated simultaneously by the iontophoretically applied drugs. The fact that the faster time constant obtained in these experiments corresponds to the time constant found in normal end-plates makes it likely that it is due to the original end-plate channels which remain unchanged in the subsynaptic membrane after denervation. One can, however, not exclude the possibility that a single species of channels with altered gating properties is present in these end-plates. It has been shown recently that in rat diaphragm the turnover of end-plate receptors, measured by the loss of radioactivity after labelling the receptors with radioactive bungarotoxin is slow (Berg & Hall, 1975; Chang & Huang, 1975). This could be the case in

frog muscle fibres as well. The noise experiments would then indicate that in adult muscle fibres the open-close kinetics, a characteristic property of end-plate channels, is independent of the nervous influence for a long period of time. On the other hand, it willbe interesting to establish whether during synapse formation a change in this aspect of receptor function occurs.

Note added in proof

Since the preparation of this manuscript Dreyer, Walther & Peper (1975) have reported similar experiments on denervated muscle fibres.

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