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LIFE TIME AND ELEMENTARY CONDUCTANCE OF THE CHANNELS MEDIATING THE EXCITATORY EFFECTS OF ACETYLCHOLINE IN *APLYSIA* NEURONES

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SUMMARY

1. The excitatory effects of acetylcholine (ACh) on an identified group of *Aplysia* neurones have been studied under voltage clamp in an attempt to measure the average life time, τ, of the channels opened by ACh and the elementary current, i_{el}, flowing through these channels. The value of τ was determined both from spectral noise analysis and from current relaxations after voltage steps. Both methods lead to similar values. i_{el} was calculated from the ratio of the variance of the ACh induced noise to the mean ACh induced current.

2. τ is increased by hyperpolarization, or by lowering the temperature. At 12 °C, $\tau = 27$ msec at -80 mV, $\tau = 17$ msec at -40 mV. τ is about 5 times smaller at 21 °C than at 12 °C.

3. i_{el} increases linearly with hyperpolarization. At -80 mV, in Tris-buffered sea water, the mean value of i_{el} was $0{\cdot}8 \times 10^{-12}$ A at 12 °C. At 21 °C, this value was multiplied by 1·8.

4. The estimate of the ACh reversal potential E_{rev} obtained by extrapolation of the relation between i_{el} and the membrane potential V was $+30$ mV. The estimate obtained from the analysis of the instantaneous current changes produced by voltage steps was $+15$ mV. The difference between the two values appears to be due to the development of a K current activated by the entry of Ca into the cell during the ACh response. This current introduces an error in opposite directions into the two estimates of E_{rev}, which can therefore be assumed to be intermediate between $+15$ and $+30$ mV. An assumed value of $+20$ mV yields an elementary conductance of $8 \times 10^{-12}\ \Omega^{-1}$ at 12 °C in Tris-buffered sea water.

5. The total ACh induced current measured in steady-state conditions increases more with hyperpolarization than does i_{el}. The difference can be entirely accounted for by the fact that hyperpolarization increases τ.

6. When carbachol or tetramethylammonium is applied instead of ACh, the value of i_{el} is identical to that found with ACh, but τ is slightly shorter (about 75 %).

7. Inward ACh induced currents can still be observed in solutions where all Na has been replaced by Cs, Mg, or Ca.

8. i_{el} increases when Na is replaced by Cs; it decreases when Na is replaced by Mg or Ca. In all Na-free solutions, τ is larger than in Na sea water: the lengthening of τ is largest for Ca sea water, smallest for Cs sea water. An interpretation of these changes

of τ is proposed. This interpretation may also account for the voltage sensitivity of τ in normal sea water.

9. Partial replacement of NaCl by TrisCl strikingly reduces the ACh induced current. τ is not modified by Tris substitution, and the reduction of the total current is entirely accounted for by a steep decrease of i_{el}. Tris does not seem to affect the pore opening and closing processes, but to block the ACh controlled channel.

INTRODUCTION

The effects of acetylcholine (ACh) on molluscan neurones have been shown to involve three separate systems, one excitatory, two inhibitory, each corresponding to a specific permeability change (Kehoe, 1972*a*, *b*). We thought that it would be interesting to apply to these systems two methods (noise analysis and voltage jumps) which have recently shed new light on the properties of the ACh controlled channels in other preparations (Katz & Miledi, 1972; Anderson & Stevens, 1973; Neher & Sakmann, 1975; Adams, 1975; Sheridan & Lester, 1975). These methods allow the calculation of the average life time of the ACh controlled channels; noise analysis also provides an estimate of the current flowing through a single channel. Furthermore, the two methods provide a powerful tool for the study of the action of cholinomimetics and of ACh antagonists (Katz & Miledi, 1973; see Steinbach & Stevens, 1976).

In this and the following articles (Ascher, Marty & Neild, 1978; Marty, 1978) we have applied noise and relaxation methods to the excitatory effects produced by ACh on an identified group of *Aplysia* neurones. The first article is mainly concerned with an attempt to characterize the life time and the conductance of the ACh controlled channels in various solutions and at various membrane potentials. The results allow a comparison between *Aplysia* neurones and other systems, and also provide a basis for the pharmacological analysis reported in the following papers. In addition, they clarify the ionic mechanism of the ACh excitatory effects.

These ionic mechanisms had been the subject of contradictory observations, some suggesting that the excitatory effects of ACh were due to a specific increase in Na permeability, while others suggested that other cations than Na could carry the ACh induced current (Sato, Austin, Yai & Maruhashi, 1968; Blankenship, Wachtel & Kandel, 1971; Levitan & Tauc, 1972). Previously the reversal potential of the ACh response had only been measured by extrapolation of the total current, a dangerous method if the average life time of the channels varies with membrane potential (Dionne & Stevens, 1975). By analysing the elementary current at various membrane potentials, and by taking into account the voltage dependence of the channel life time, we have been able to obtain a better evaluation of the reversal potential. Furthermore, by analysing the 'elementary event' in solutions of various ionic compositions, we have obtained some new information on the passage of cations through the ACh induced channels. Our results suggest in particular that these channels are permeable to other cations than Na, and that their life time depends on the nature of the permeant ion.

A preliminary communication of this work has been presented (Marty, Neild & Ascher, 1976).

METHODS

We used in this study a family of small neurones of the mollusc *Aplysia californica*. These neurones, situated at the posterior pole of the right pleural ganglion (just at the level of attachment of the pleural pedal connective) are easily recognized because of their absence of pigmentation. They were chosen among the numerous *Aplysia* neurones excited by ACh because of four characteristics: their small size (about 60 μm in diameter) which allowed a reasonably fast and homogeneous voltage clamp; the stability of the membrane potential (the neurones have no spontaneous action potentials although they will fire when depolarized; they receive very little spontaneous synaptic input); their high sensitivity to ACh; and finally, the pharmacological 'purity' of the ACh response.

This last property deserves a special mention. Kehoe (1972*c*) has shown that in many *Aplysia* neurones excitatory responses to ACh are often the sum of simultaneous excitatory and inhibitory components, and can involve the activation of two and even three groups of ACh receptors. The best pharmacological test of such a mixed activation is the application of hexamethonium at high concentration (10^{-3} M). This compound specifically eliminates the 'excitatory' effect (Tauc & Gerschenfeld, 1962; Kehoe, 1972*b*) and reveals the inhibitory components when they are present. In the cells studied, application of hexamethonium even at very high concentration did not reveal any sign of an inhibitory component; we thus assume that we were dealing with the 'pure' excitatory effects of ACh.

The pleural ganglion was pinned on the bottom of a chamber covered with a silicone resin (Elastomere, Rhône-Poulenc), and the neurones were exposed by removal of the connective sheath. The composition of the artificial sea water and of the various solutions is given in Table 1.

TABLE 1. Composition of the solutions. Values are in mM. These compositions were calculated to obtain equal osmolarities. The pH was adjusted to 7·8 at room temperature. In HCO_3 buffered sea water, 5 mM-$NaHCO_3$ replaced 10 mM-TrisCl

	NaCl	KCl	$CaCl_2$	$MgCl_2$	TrisCl	CsCl
Na sea water	480	10	10	50	10	—
Cs sea water	—	10	10	50	10	480
Mg sea water	—	10	10	370	10	—
Ca sea water	—	10	403	—	10	—
Tris sea water	—	10	10	50	586	—

ACh was applied iontophoretically in most experiments, in the absence or in presence of neostigmine (5×10^{-5} M). In most cases the ACh pipette was positioned at some distance from the cell soma (about 50 μm) to avoid the local build up of high ACh concentrations, and we used low doses of ACh which caused currents of less than 10 nA at -40 mV. This is certainly much below the maximal value, since currents of more than 50 nA at -40 mV could easily be obtained by increasing the iontophoretic current. This precaution was taken in order to keep the ACh concentration within the 'low concentration limit', a critical condition for noise analysis (Katz & Miledi, 1972; Neher & Sakmann, 1976), and also because at high concentrations of ACh a series of new phenomena appears: the response to a steady ACh application shows a marked decline with time, and the relaxation patterns following voltage steps become complex.

Voltage clamp. The voltage clamp system was usually set at a gain of 500. The current injected into the cell was measured from the drop of potential across a 1 MΩ resistance inserted in series with the electrode. The voltage amplifier was a low noise Burr Brown 4320 K; all amplifiers were powered by batteries.

Frequency response. In the absence of agonists, the input resistance of the cell was high (up to 50 MΩ) and the major source of noise was the thermal noise due to the resistance of the voltage recording electrode, R_v. Theoretical calculations modelling the cell membrane as a simple RC network show that this noise should have an amplitude depending on frequency as

$$S(f) = 4R_v kT\left(\frac{1}{R^2} + 4\pi^2 f^2 C^2\right),$$

where k is the Boltzmann constant, T the temperature, R the resistance and C the capacity of the cell membrane. Assuming a cell radius of 30 μm and an apparent capacity per unit area of 12 μF/

cm² (see Gorman & Mirolli, 1972) C was estimated at a value of about 10^{-9}F. This was in satisfactory agreement with the observed spectra. To keep the background noise as low as possible, we used a low resistance (about 5 MΩ) voltage recording electrode, made with a de Fonbrune microforge, and filled with 3 M-KCl.

In the presence of agonists, the increase in conductance gave rise to an extra current I. Supposing that the cell membrane can be represented by a resistance and a capacitance in parallel, and supposing a small sinusoidal additional membrane conductance with a frequency f, the current would be of the form

$$I \propto \frac{1}{1+f^2/f_I^2} \quad \text{with} \quad f_I = \frac{G}{2\pi R_I C},$$

where C is the capacitance of the cell, G the gain of the clamp and R_I the resistance of the current passing electrode. This equation (see also Sachs & Lecar, 1977) shows the advantage of working with comparatively small cells (which have a low capacitance) and of lowering the resistance of the current electrode. We used low resistance (about 10 MΩ) de Fonbrune microelectrodes, which were filled with 0·5 M-K_2SO_4 to avoid excessive loading of the cell with Cl ions. With G = 500 and $C = 10^{-9}$F, the voltage clamp should have been accurate up to

$$f_I = \frac{500}{6 \times 1{\cdot}5 \times 10^7 \times 10^{-9}} \simeq 6000 \text{ Hz}.$$

This value was obviously not obtained, mainly due to capacitive coupling (between the electrodes, and between the electrodes and the bath) which we did not try to reduce. However the clamp appeared satisfactory in the frequency range analysed, i.e. up to 500 Hz. When the gain was lowered from 500 to 100, we could not detect any change in the clamp current or in the relaxation time course.

Noise analysis. The current was first filtered through a high pass first order filter and a low pass Butterworth active filter (Burr-Brown: 48 db/octave) before being stored on tape (Schlumberger MP 5519). The data were then either digitized and fed into the spectrum analyser, or into a variance to DC converter. The spectral analysis was done with a variety of computers, but mostly with a Hewlett Packard Fourier Analyser and later with a Hewlett Packard 9825 A.

In our initial experiments we used the AC input of a Tektronix 502 oscilloscope (which had a cut-off frequency of 1·5 Hz) as high pass filter. The low pass filter was set at 500 Hz. It later became clear, however, that the high pass filter was cutting off a significant fraction of the signal, especially at low temperatures and high membrane potentials. Later experiments were done using a first order 0·1 Hz high pass filter. Comparison of these results with those previously obtained confirmed the need for a correction, which partially accounts for quantitative discrepancies between the results presented here and those presented in our first communication (Marty *et al.* 1976): previously we had slightly underestimated the value of i_{el}, particularly at very negative membrane potentials, so that the value of the (extrapolated) ACh reversal potential was overestimated.

The spectral analysis was usually done in the range 1–100 Hz (using an additional low-pass 48 db/octave filtering at a cut off frequency of 100 Hz). The standard procedure for calculation of a 1–100 Hz spectrum was to take 12 independent records of 2·5 sec duration and to calculate the average of the corresponding spectra. A Hanning was performed after digitization of each sample (512 data points) to avoid 'leakage' effects. Here again, early experiments done with 1·5 Hz AC filtering had to be corrected in the low frequency range; the corrected results were in agreement with those obtained with 0·1 Hz AC filtering.

THEORY

Since in *Aplysia* little is known of the sequence of reactions involved in the ACh-receptor interaction we have tried to avoid as much as possible a specific reaction scheme. We have therefore preferred the use of probability densities to that of kinetic constants. Following other authors (cf. Neher & Stevens, 1977) we have made two sets of hypotheses.

1. The total ACh induced current, I, is the result of the random opening of a

homogeneous population of channels, each opening leading to an elementary current i_{el}. If the number of open channels is n,

$$I = n\, i_{el}.$$

This implies that

$$\text{var}\,(I) = (i_{el})^2\, \text{var}\,(n) = (i_{el})^2\, [\sigma(n)]^2, \tag{1}$$

where var (n) and $\sigma(n)$ are the variance and the standard deviation of n respectively.

2. Each individual channel has a given probability density p of opening if it is closed, and a given probability density q of closing if it is open. Each channel behaves independently of the others. If N is the total number of channels,

(a) the mean ACh induced current at equilibrium (q and p constant) will be

$$\langle I \rangle = \langle n \rangle\, i_{el} = N \frac{p}{p+q} i_{el}; \tag{2}$$

(b) if p (or q) are suddenly changed, the number of open channels $n(t)$ will relax exponentially to a new equilibrium with a time constant

$$\tau_{rel} = \frac{1}{p+q};$$

(c) the autocorrelation function for lag u will be

$$\psi(u) = e^{-(p+q)u}.$$

Since ψ is exponential, and the power spectrum $S(f)$ is its Fourier transform, $S(f)$ is lorentzian:

$$S(f) = \frac{S(0)}{1 + f^2/f_c^2} \quad \text{with} \quad f_c = \frac{p+q}{2\pi}$$

and the cut off frequency f_c of S gives a τ value:

$$\tau_{noise} = \frac{1}{p+q} = \frac{1}{2\pi f_c}.$$

Thus, relaxation and noise power spectra should both yield the same value of τ:

$$\tau_{noise} = \tau_{rel} = \frac{1}{p+q}. \tag{3}$$

This common value is, in general, different from the average life time of the channel τ_{ch} which is equal to $1/q$. It is only in the limiting case where p is negligible in comparison to q that one can equate the three values:

$$\tau_{noise} = \tau_{rel} \approx \tau_{ch} = \frac{1}{q}.$$

Still under the condition $p \ll q$, since we supposed the channels to be independent of each other, one can show that:

$$\sigma(n) = \sqrt{\langle n \rangle}. \tag{4}$$

Comparison of eqn. (1) with eqn. (2) and (4) gives

$$i_{el} = \text{var}\,(I)/\langle I \rangle. \tag{5}$$

RESULTS

Noise variance. Voltage dependence of the elementary current, i_{el}

The variance of the membrane current was very small in the absence of ACh: often less than 10^{-21} A^2 in the 1–100 Hz range, which corresponds to a r.m.s. noise of less than 30 pA. This background noise increased at very low and at high frequencies, probably due to mechanical noise in the first case (Katz & Miledi, 1975) and to electrode noise in the second (Anderson & Stevens, 1973). At 12 °C no spontaneous synaptic

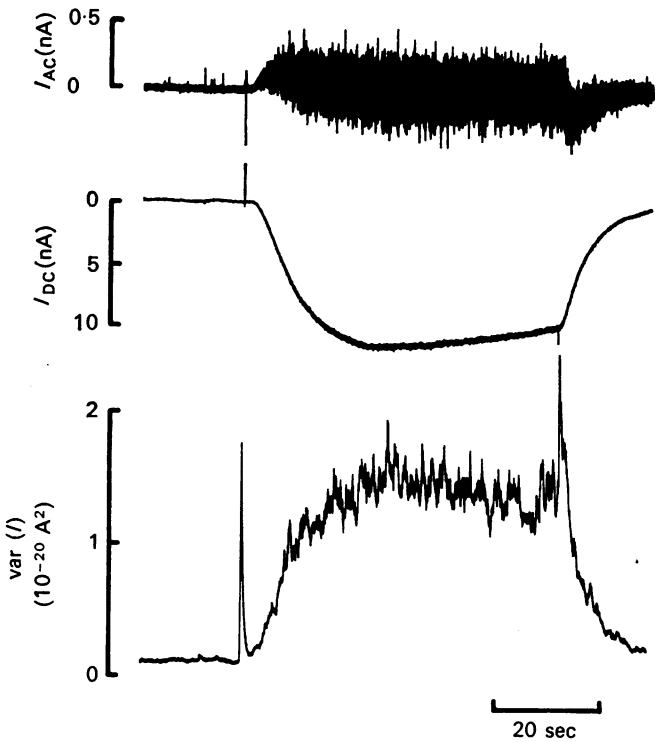

Fig. 1. Variance of the ACh noise. Upper trace: high gain AC record of the increase in current noise produced by an application of ACh of 60 sec. The recording system filtered at 1·5 and 500 Hz, but additional low pass filtering is introduced by the pen recorder (Brush 280). Middle trace: low gain DC record of the same current. Lower trace: output of a variance to DC converter fed with the current illustrated in the upper trace. Notice that, except for the transients appearing at the onset and at the offset of the iontophoretic current, the variance is roughly proportional to the DC current. The ratio of the variance over the DC, ACh induced current, i_{el}, was $1{\cdot}2 \times 10^{-12}$ A. Temperature 21° C. Membrane potential −60 mV. The cut off frequency of the noise power spectrum in this experiment was $f_c = 39$ Hz.

currents were observed; at 22 °C there were occasional synaptic currents, but never frequent enough to require systematic editing of the records before further analysis.

During the application of ACh the noise variance increased noticeably (Fig. 1). The additional variance was proportional to the total ACh induced current; they both varied in parallel during a given ACh application and when the ACh dose was modified

by varying the iontophoretic current. This proportionality allowed, at a given membrane potential, the calculation of a value of the elementary current, i_{el}, as the ratio of the variance to the mean ACh induced current (see eqn. (5)).

In Fig. 2. average values of i_{el} at 12 °C are compared in 'normal' sea water (containing 10 mM-Tris) and in HCO_3 sea water (see Table 1). It appears that i_{el} is slightly larger in HCO_3 sea water, and that the difference tends to increase for negative membrane potentials.

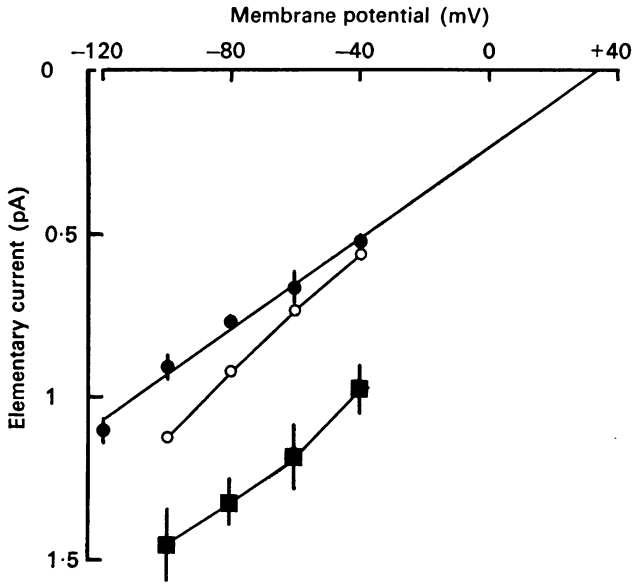

Fig. 2. Voltage dependence of the elementary current. ●, 12 °C, Tris buffered sea water; ○, 12 °C, HCO_3 buffered sea water; ■, 21 °C, Tris buffered sea water. Each point is the average of 8–23 (●), 7 (○) and four to eight (■) experiments. Error bars: ± S.E. of mean. The extrapolated value of the reversal potential for the points at 12 °C, Tris, is 33 mV.

The data points obtained in Tris sea water at 12 °C may be fitted by a straight line. This suggests that the variation of i_{el} with voltage is simply a reflexion of the change in driving force. Assuming this to be true, an estimate of the reversal potential E_{rev} may be obtained by extrapolation of the $i_{el}(V)$ relationship and calculation of its intersect with the voltage axis. The validity of this procedure will be further considered later, and the value obtained (+33 mV) should be considered as tentative at this point because the range of potentials where i_{el} can be determined is comparatively narrow, and moderate errors on i_{el} may result in large errors on the apparent value of E_{rev}. Fig. 2 also illustrates that at 21 °C, in Tris sea water, i_{el} is 1·6–1·9 times larger than at 12°C.

The noise power spectrum. Voltage dependence of τ_{noise}

In principle the power spectrum of the ACh induced current noise is obtained by subtracting the spectra of the currents obtained with and without ACh. As illustrated in Fig. 3*A*, however, the amplitude of the control noise was usually so small that in practice the spectral analysis was directly done on the current recorded in the presence of ACh.

Within the low range of ACh concentration (see Methods) a single lorentzian component could satisfactorily account for the shape of the spectrum (Fig. 3*B*), and allowed the determination of a half power frequency, f_c, from which τ_{noise} could be deduced by

$$\tau_{noise} = \frac{1}{2\pi f_c}.$$

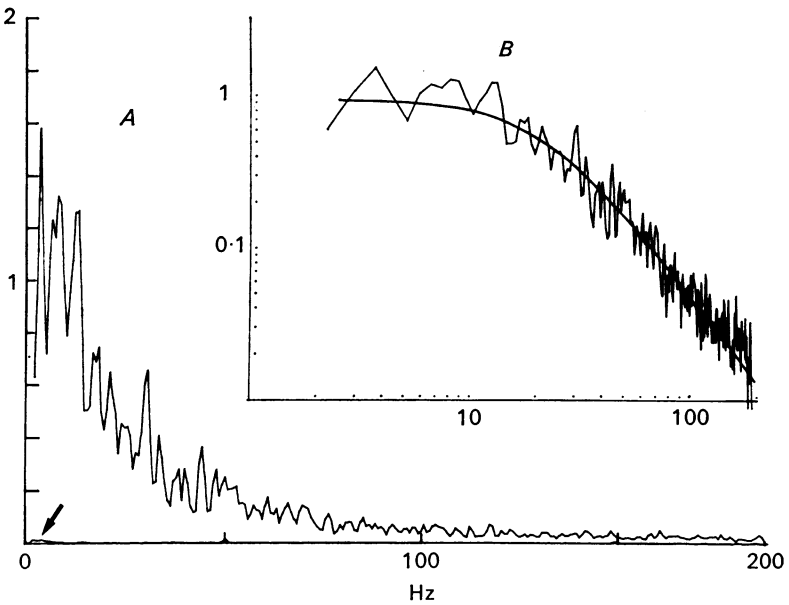

Fig. 3. A noise power spectrum. Linear co-ordinates in *A*, double logarithmic co-ordinates in *B*. Each spectrum is the average of twelve separate spectra. *A*, spectra obtained both in the absence of ACh (arrow) and in the presence of ACh. *B*, the second spectrum may be fitted with a Lorentzian curve having a cut off frequency $f_c = 27$ Hz. Vertical calibration: arbitrary units. Membrane potential: -80 mV. Steady-state ACh induced current, $I_{ss} = 7{\cdot}3 \times 10^{-9}$ A. Elementary current $i_{el} = 1{\cdot}16 \times 10^{-12}$ A. Temp. 21 °C.

No dependence of τ_{noise} on iontophoretic dose could be detected in the conditions of our experiments. The increase of τ_{noise} with hyperpolarization is illustrated in Fig. 4 from results obtained at 12 and 21 °C. Results obtained with Tris and bicarbonate buffered sea waters are identical. In our initial experiments the increase of τ measured at 12 °C seemed to level off around -100 mV (Marty *et al.* 1976); this trend was a consequence of an underestimate of the plateau level due to low frequency filtering (see Methods) and was not observed at 12 °C after modifying the filter. However, the trend persisted at 21 °C.

Voltage jumps

Adams (1975) and Neher & Sakmann (1975) have shown that, at the frog neuromuscular junction, the application of sudden voltage steps in the presence of ACh induces an instantaneous current change followed by an exponentially relaxing current. The voltage dependence of the instantaneous current allows an estimation of the ACh reversal potential which agrees with that obtained from direct measurement. The time constant of the relaxation is identical to that obtained from spectrum

analysis. The study of the ACh induced current during voltage jumps thus appears complementary to noise analysis. As we shall see, it presents certain advantages in the case of *Aplysia* neurones.

In most of our experiments the voltage was changed, for a few hundred milliseconds, from a holding value of -40 mV to a more hyperpolarized level, varying between -50 and -120 mV. This was done first in the absence of ACh, then during the plateau of an ACh response. In the experimental conditions used (see Methods) the response

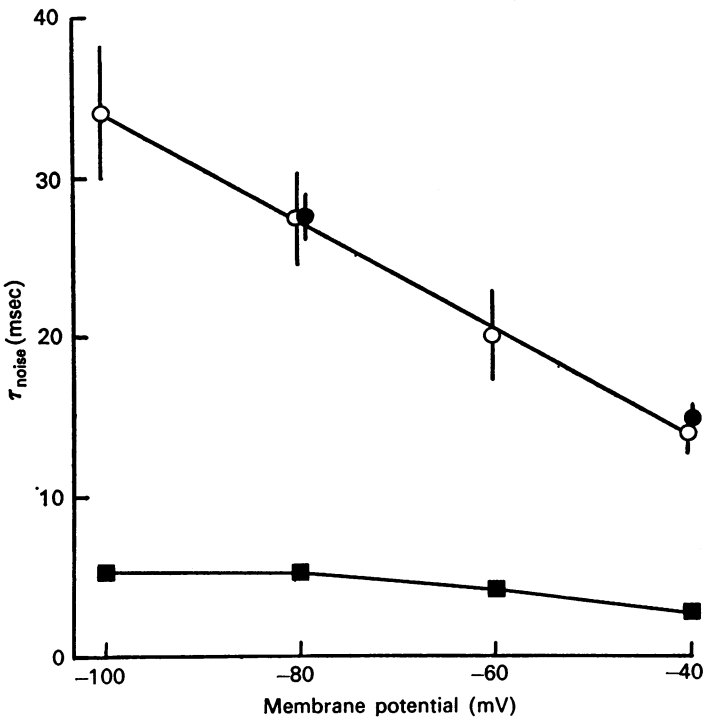

Fig. 4. Voltage dependence of τ_{noise}. τ_{noise} was calculated from the cut off frequency of the power spectra. ●, Tris sea water, 12 °C (twelve experiments); ○, HCO_3 sea water, 12 °C (eight experiments); ■, Tris sea water, 21 °C (three or four experiments). Error bars: ± S.E. of mean. Notice the identity of τ_{noise} in Tris and HCO_3 buffered sea water at 12 °C.

did not decline with time at -40 mV. The ACh induced current was calculated as the difference between the currents measured in these two situations. In a few experiments depolarizing pulses were applied from a more hyperpolarized holding potential.

A typical record is shown in Fig. 5*A*. The evolution of the ACh induced current after the hyperpolarizing step consists of an instantaneous component followed by a progressive increase towards a final steady value. A semilog plot of I *vs.* time indicates that the relaxation is described by a single exponential, with a time constant $\tau_{rel}(-80) = 33$ msec.

A similar relaxation is observed when the potential is stepped back to the holding value, and its analysis yields a shorter time constant, $\tau_{rel}(-40) = 17$ msec. The time constants in this experiment appear to be close to the values of τ_{noise} shown in Fig. 4.

Figs. 6 and 7 show the variation of τ_{rel} with membrane potential. Again, the results resemble those of Fig. 4 obtained from noise analysis. The equality between τ_{noise} and τ_{rel} was more directly tested in a series of eight measurements of the two values on four different cells. The ratio τ_{rel}/τ_{noise} had a mean value of 0·94 (S.D. ± 0·15). A similar agreement between noise and relaxation measurements of τ was reported by Neher & Sakman (1975) at the frog neuromuscular junction.

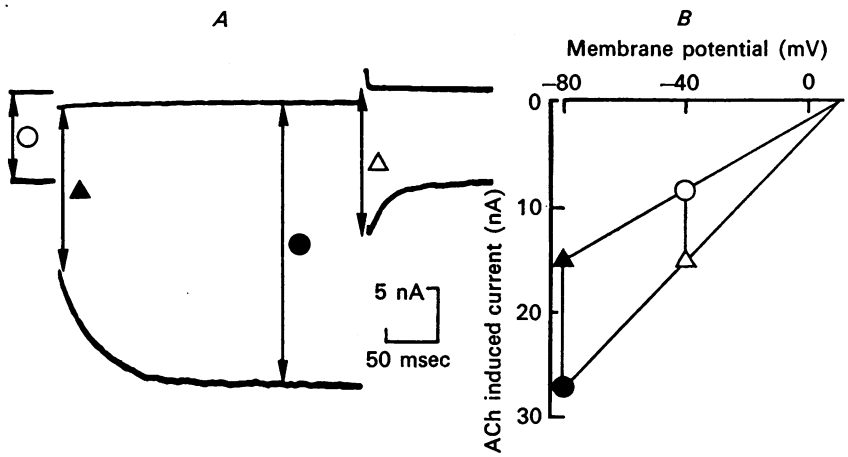

Fig. 5. A relaxation experiment. *A*, current traces recorded during a 300 msec hyperpolarizing step (from −40 to −80 mV) first in the absence (upper trace) then in the presence (lower trace) of ACh. Inward current is downward, as in all following figures. I_{ACh} is measured by the interval between the upper and the lower trace. 100 Hz low pass filter. Temp. 12 °C. Calibration: 50 msec; 5 nA. The arrows indicate the points at which were measured the values of the stationary currents (circles) and the instantaneous currents (triangles) which were calculated by back extrapolation of the exponential. *B*, the stationary currents I_{ss} (circles) and the instantaneous currents I_{in} (triangles) measured in Fig. 5*A* have been plotted at −40 and −80 mV. The lines joining I_{in}, after a step, to I_{ss}, before a step, cross the abscissa at about +12 mV, which gives an evaluation of the reversal potential, E_{rev}, of the ACh response.

In a few experiments two sets of records were taken corresponding to ACh currents differing by a factor of about 10. The time constants obtained from the two sets were identical (see Fig. 6*C* at −120 mV). Thus τ_{rel} does not depend on the ACh concentration in the range used in these experiments.

Instantaneous and steady-state currents

In experiments like that of Fig. 5*B*, it is possible to measure at −80 and −40 mV both the value of the ACh currents flowing in the steady state (which we will note I_{ss}) and, by extrapolation of the relaxation to its origin, the instantaneous currents (I_{in}). These values can be used for two different purposes.

First, as illustrated in Fig. 5*B*, they permit the calculation of the reversal potential of the ACh effects. Assuming that the sudden variation from I_{ss} (before the voltage step) to I_{in} (just after the step) reflects only the change in driving force, one should have

$$\frac{I_{in}(-80)}{I_{ss}(-40)} = \frac{I_{ss}(-80)}{I_{in}(-40)}$$

and it should be possible to determine E_{rev} by extrapolating the line joining $I_{in}(-80)$ to $I_{ss}(-40)$, or $I_{in}(-40)$ to $I_{ss}(-80)$. The values obtained by this method in Fig. 5*B* (+13 mV for the step to −80 mV, +10 mV for the step to −40 mV) are in close agreement. They are different, however, from the value obtained by extrapolating the relation between i_{el} and V (+33 mV, Fig. 2). This discrepancy, which we attribute to the development of a K current triggered by Ca entry, will be discussed later (p. 195).

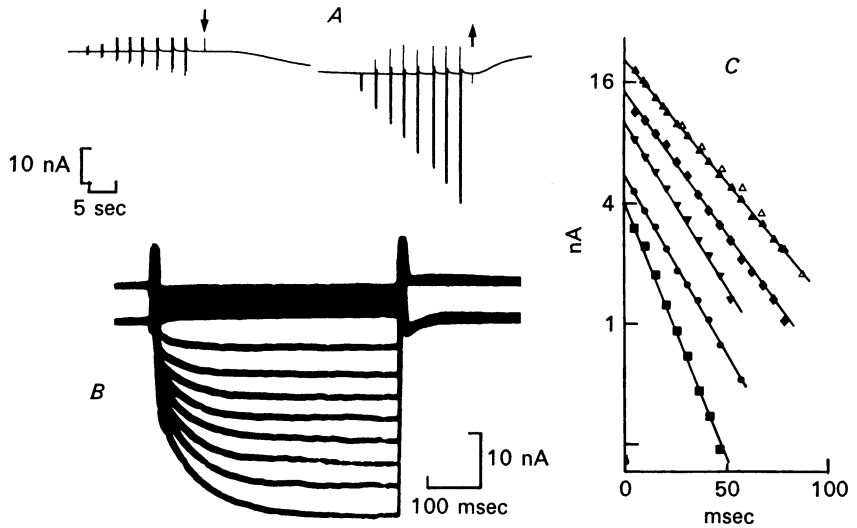

Fig. 6. Voltage dependence of τ_{rel}. *A* and *B* are records of the same experiment. *A* is a pen recorder trace, taken at low speed. The left part of the record shows the inward currents triggered by a series of eight hyperpolarizing steps bringing the membrane potential from a holding potential −40 mV to successively more hyperpolarized values (from −50 to −120 mV in 10 mV steps). The downward arrow indicates the beginning of the ACh iontophoresis, which induces an inward current. During the break in the record (12 sec), the current reached a steady-state value. Then the same series of hyperpolarizing voltage steps was applied again. At the upward arrow, the application of ACh was stopped. Calibration: 10 nA; 5 sec. *B*, the currents triggered by the hyperpolarizing pulses before (upper traces) and during (lower traces) the application of ACh have been superimposed. Calibration: 100 msec; 10 nA. *C*, semilogarithmic plot of six relaxations. Four were taken from Fig. 6*B* (● from −40 to −60 mV; ▼ from −40 to −80 mV; ◆ from −40 to −100 mV; ▲ from −40 to −120 mV). The two others were obtained on the same cell, but the records are not shown: △ from −40 to −120 mV, i.e. as ▲, but with a lower dose of ACh (a scaling factor of 8 has been introduced to show the agreement between the two time constants); ■ from −80 to −40 mV. The voltage records are not shown. During the pulse to −120 mV, and for the 'high' dose of ACh (*A* and *B*) the application of ACh only changed the test potential by 2 mV. Temp. 12 °C. 100 Hz second order low pass filter.

Measurement of the instantaneous and steady state currents can be used for a second purpose, namely an evaluation of the voltage sensitivity of p, the probability of transition from closed to open channels (Neher & Sakmann, 1975). In the model described in the Methods section, if $p \ll q$

$$\frac{I_{ss}(-80)}{I_{in}(-80)} = \frac{p(-80)}{p(-40)} \frac{q(-40)}{q(-80)} = \frac{p(-80)}{p(-40)} \frac{\tau(-80)}{\tau(-40)}. \quad (6)$$

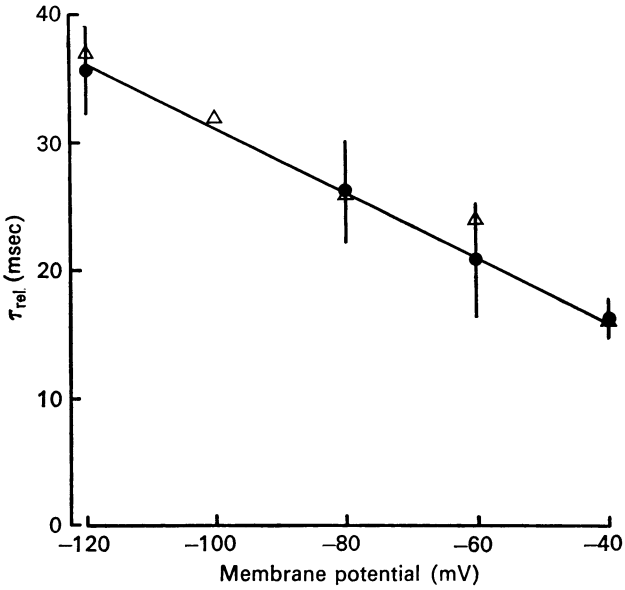

Fig. 7. Voltage dependence of τ_{rel}. △, values of τ from the experiment of Fig. 6. ●, pooled results from four cells (including that of Fig. 6). Error bars: ± S.D Temp. 12 °C.

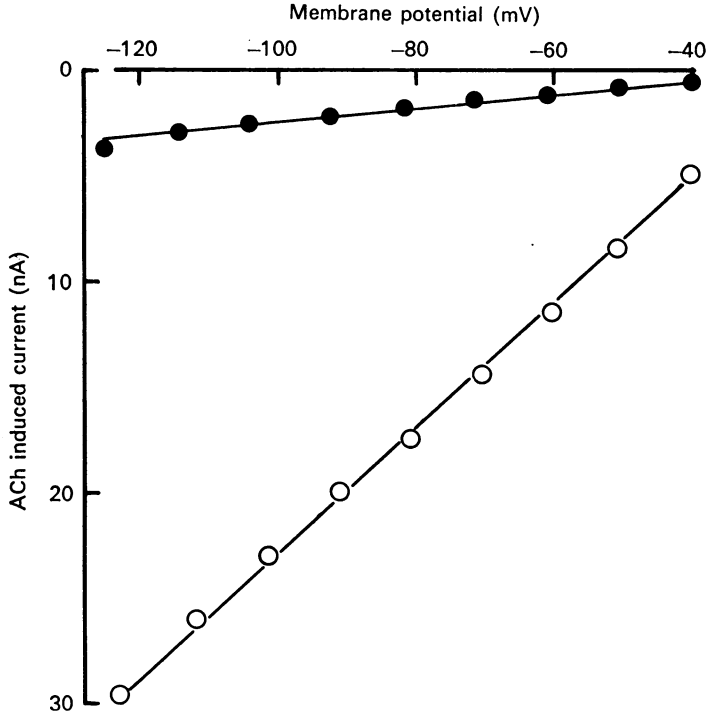

Fig. 8. Voltage dependence of the steady-state ACh induced current (I_{ss}). Data from the experiment of Fig. 6, and corresponding to the two ACh doses used. The two straight lines differ by a scaling factor of 8, and would both cross the abscissa at −23 mV.

As we can determine independently $\tau(-80)/\tau(-40)$ and $I_{ss}(-80)/I_{in}(-80)$, eqn. (6) allows the calculation of $p(-80)/p(-40)$. In the experiment of Fig. 6, $\tau(-80)/\tau(-40) = 0{\cdot}51$, $I_{ss}(-80)/I_{in}(-80) = 0{\cdot}57$, so that $p(-80)/p(-40) = 0{\cdot}9$.

The same kind of argument holds if data on i_{el} are used instead of instantaneous current analysis. Combining eqns. (2) and (3) yields

$$I_{ss} = \langle I \rangle = Np\tau i_{el}. \tag{7}$$

Fig. 8 illustrates the voltage dependence of the stationary ACh induced current I_{ss}. This voltage dependence is much steeper than that of the relation between i_{el} and V illustrated in Fig. 2. Fig. 9 shows that the ratio $I_{ss}/\tau i_{el}$ is constant between -120 and -40 mV, so that p appears again to be independent of voltage.

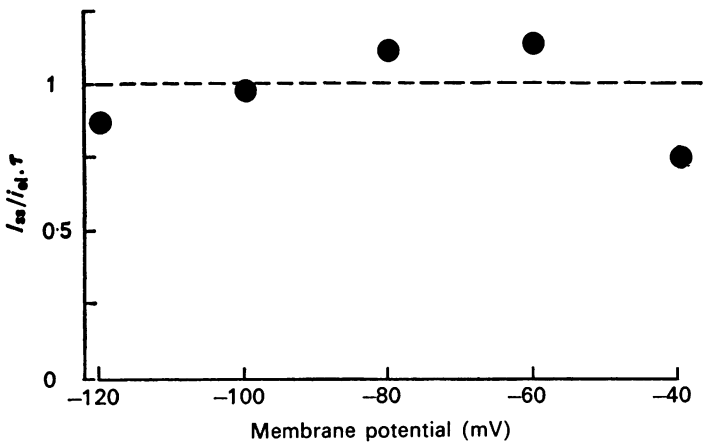

Fig. 9. Constancy of p, the probability of opening of the channels. The value of the ratio $I_{ss}/i_{el}\tau$ has been plotted as a function of membrane potential. I_{ss} was taken from the experiment of Fig. 6, i_{el} from Fig. 2 and τ from Fig. 7. The resulting ratio is about constant (dashed line), which shows according to eqn (7) that p is independent of voltage. The low value found at -40 mV may be due to a contribution of the Ca dependent K permeability (see text). The vertical scale is in arbitrary units.

It may be seen from the two sets of determinations of $I_{ss}(V)$ in the experiment of Fig. 8 that the voltage sensitivity of I_{ss} does not depend on ACh dose. This observation, plus the fact already mentioned that neither i_{el} nor τ_{noise} or τ_{rel} depend on ACh dose, confirms that the low concentration limit assumed in the theoretical part of the methods is indeed a valid approximation.

It may seem paradoxical that while both $i_{el}(V)$ and $\tau(V)$ relationships are adequately described by straight lines, with different intersects with the voltage axis, their product appears proportional to another linear function of voltage $I_{ss}(V)$ with still a different intersect with the voltage axis. For $\tau(V)$ and $I_{ss}(V)$, the observed linear relationships is obviously only an approximation, valid in a limited voltage range, and without theoretical justification. On a larger range, if $i_{el}(V)$ is linear, both $\tau(V)$ and $I_{ss}(V)$ curves should bend (in opposite directions) for voltages less negative than -40 mV. Such bendings were experimentally observed in media where relaxation experiments could be done in that range of potentials such as in Cs sea water (see below).

Carbachol and tetramethylammonium

The elementary current parameters obtained with ACh were compared with those obtained with carbachol and tetramethylammonium, two compounds which have been shown to mimick the excitatory effects of ACh on molluscan neurones (Kehoe, 1972*b*). Because of the variability of the results from one cell to another we compared the time constants for the three agonists on the same neurones. For both agonists, the relaxation was exponential and the voltage dependencies of τ_{noise} (or τ_{rel}) and of I_{ss} similar to those for ACh. As shown in Table 2, the results suggest that the time constants are equal for carbachol and tetramethylammonium, and that they are somewhat smaller (about 75 %) for these two agonists than for ACh.

TABLE 2. Comparison of i_{el} and τ for ACh, carbachol (Carb) and tetramethylammonium (TMA). The data were obtained from a total of five cells at 12 °C. No neostigmine was present. The iontophoretic pipette was changed twice during the course of each experiment

Cell	V (mV)	i_{el} Carb (pA)	i_{el} TMA (pA)	i_{el} ACh (pA)	$\frac{i_{el}\ Carb}{i_{el}\ ACh}$	$\frac{i_{el}\ TMA}{i_{el}\ ACh}$
1	−40	—	0·62	0·70	—	0·89
	−60	—	0·58	0·61	—	0·95
2	−40	0·39	0·55	0·58	0·67	0·95
	−60	0·57	0·61	0·62	0·92	0·98
	−80	0·65	0·64	0·77	0·84	0·83
3	−40	0·63	0·50	0·46	1·37	1·09
	−60	0·49	0·51	0·55	0·89	0·93
	−80	0·625	0·69	0·53	1·18	1·30
					mean 0·98 ± 0·25 (S.D.)	mean 0·99 ± 0·15 (S.D.)

Cell	V (mV)	Method of determination of τ	τ_{Carb} (msec)	τ_{TMA} (msec)	τ_{ACh} (msec)	$\frac{\tau_{Carb}}{\tau_{ACh}}$	$\frac{\tau_{TMA}}{\tau_{ACh}}$
3	−40	Noise	10·6	10·6	11·4	0·93	0·93
	−60		15·9	13·8	22·7	0·70	0·61
	−80		15·9	17·7	22·7	0·70	0·78
4	−40	Relaxation	17	18	23	0·74	0·78
	−60		23	22	26	0·88	0·85
	−80		31	21	48	0·65	0·44
5	−40	Relaxation	15	14	19	0·79	0·74
	−60		21	21	29	0·72	0·72
	−80		25	24	31	0·81	0·77
						mean 0·77 ± 0·09 (S.D.)	mean 0·74 ± 0·14 (S.D.)

The values of the elementary current are very similar for the three agonists (Table 2). The similarity of the results concerning τ and I_{ss} whether ACh or another agonist were used, suggests that a possible voltage dependence of acetylcholinesterase activity does not affect the data seriously. This is further indicated by the fact that noise or relaxation data obtained using ACh in the presence of neostigmine (5×10^{-5} M) show no difference with those obtained without an anticholinesterase agent present.

'Secondary' K currents associated with ACh action

In the experiment illustrated in Fig. 5, the 'off-relaxation' observed at the end of the hyperpolarizing pulse resembles the 'on-relaxation' and can be fitted by a single exponential. This was not found in all experiments, and in many occasions the repolarizing voltage step triggered a transient outward current which obscured the analysis of the off-relaxation. In some experiments such as those illustrated in Figs. 10 and 11, this outward current was particularly marked, and could be analysed in more detail.

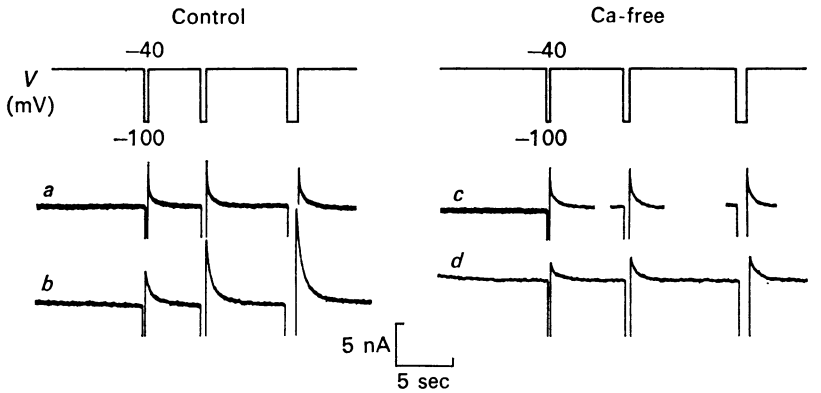

Fig. 10. Ca dependent transient outward current. A sequence of three hyperpolarizing pulses of increasing duration, illustrated in the upper trace (V) was applied 4 times: twice in control sea water (first in the absence of ACh, *a*; then during an application of ACh, *b*); twice in Ca free sea water, again in the absence of ACh (*c*) and during an ACh application (*d*). The pulses brought the membrane potential from -40 to -100 mV, for periods of 0·25, 0·5 and 1 sec. The current was recorded at low speed with a pen recorder. The currents flowing across the membrane when the potential was at -100 mV were off scale, and all records illustrate the currents recorded at -40 mV. The traces *a* and *c* show that both in normal sea water and in Ca-free sea water, in the absence of ACh, a transient outward current develops when the membrane potential is suddenly brought back from -100 to -40 mV. This transient outward current appears similar for the three successive pulses. In the presence of ACh, and in normal sea water (*b*) the transient outward current is larger than in the absence of ACh, and appears now to increase when the duration of the hyperpolarizing pulse is increased. This indicates the presence of an additional, ACh dependent, transient outward current. In the presence of ACh, but in Ca-free sea water (*d*) this ACh dependent transient outward current disappears; subtraction of records *d* and *c* would reveal only an 'off' relaxation similar to that illustrated in Fig. 5*A*.

In this experiment Ca^{2+} was replaced with Co^{2+}, but the same result was obtained when Ca^{2+} was simply omitted from the solution. Temp. 12 °C.

In the experiment of Fig. 10*A*, the membrane potential was stepped from a holding value of -40 to -100 mV. The three successive pulses had increasing durations. In the absence of ACh, the return to -40 mV was accompanied by a small transient outward current, which was independent of the duration of the pulse. In the presence of ACh, the transient current was markedly increased, and the more so for longer durations of the hyperpolarizing pulse. The outward current was also increased if the dose of ACh was increased, or if the membrane potential during the pulse was made more negative (not illustrated). This suggests that the outward current is determined

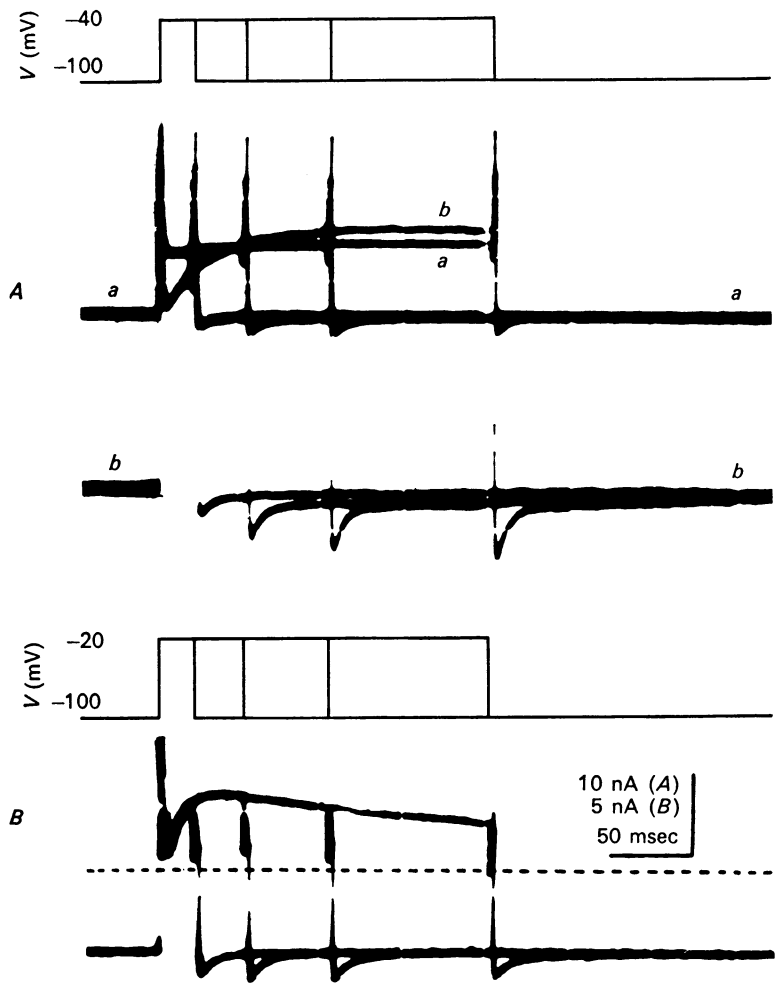

Fig. 11. Ca-dependent K current. *A*, in this experiment, the potential was temporarily brought from a holding value of 100 mV to −40 mV. Four depolarizing steps of increasing duration (20, 50, 100, 200 msec) were applied first in the absence of ACh (*a*) then during an Ach application (*b*). The Figure allows two observations. When the membrane potential was brought from −100 to −40 mV, there was no transient outward current in the absence of ACh; in the presence of ACh, on the other hand, an outward current developed progressively, which corresponded to the extra outward current described in Fig. 10. This current was so marked that after 50 msec the net ACh induced membrane current was outward. For longer depolarizing pulses (not illustrated) the traces *a* and *b* would have crossed again, as the outward current is only transient and as, at −40 mV, the steady-state ACh induced current is inward. When the membrane potential was brought back from −40 to −100 mV, rapid inward tails were observed. *B*, same series of pulses as in *A*, but the test potential was −20 mV (from another cell) and there was no ACh present. It may be seen that the outward K current (at −20 mV), and the inward tails (observed upon repolarization to −100 mV) have time courses roughly similar to the corresponding ACh-dependent currents of *A*. The dashed line indicates the steady-state value of the current at −20 mV. Temp. 12 °C. Calibrations: 50 msec; 10 nA (*A*) and 5 nA (*B*).

by the number of ions transferred during the pulse, and therefore is the result of some ionic concentration change due to the ACh induced current.

That Ca entry was the main factor involved is indicated by experiments like that illustrated in Fig. 10, which shows that the ACh induced outward current disappeared when the extracellular Ca concentration was reduced to 0. This effect was observed both when Ca was simply omitted from the sea water, or when it was replaced by Co.

The outward current is most probably carried by K. This was shown in experiments like that of Fig. 11 where, after activation of the outward current, the membrane was

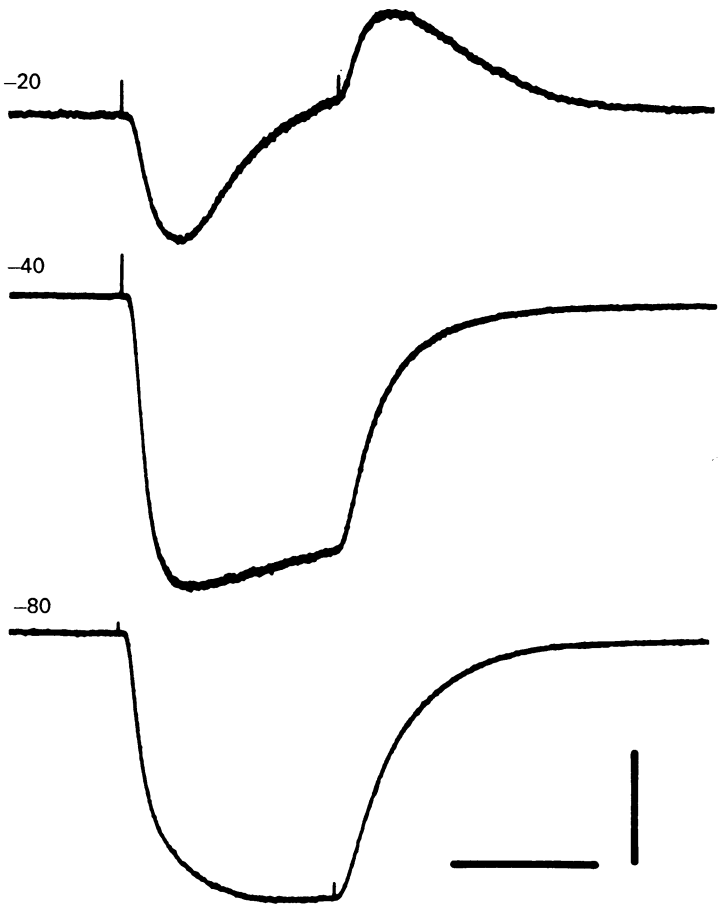

Fig. 12. Steady Ca-induced K current. Currents measured at −20 mV (upper trace) −40 mV (middle trace) and −80 mV (lower trace) during three successive and identical applications of ACh. At −80 mV the response reaches a steady plateau value. At −40 mV it shows a slow decline. At −20 mV the decline is larger and faster. It is most probably due to the development of an outward K^+ current, and not to desensitization (see text). When the ACh application is interrupted, the K^+ current appears as outward because it outlasts the 'direct' ACh induced current. This cell showed an exceptionally large Ca induced permeability. This was also apparent in the large amplitude of the transient outward currents observed after hyperpolarizing pulses in the presence of ACh. The behaviour illustrated in this Figure disappeared in Ca-free sea water. Temp. 12 °C. Calibration: 20 sec; 10 nA (−20 and −40 mV); 20 nA (−80 mV).

repolarized to various levels. In Fig. 11 the holding potential was −100 mV. The membrane potential was brought to −40 mV by depolarizing pulses of increasing duration, and then brought back to −100 mV. The depolarizing pulse triggered an outward current, and the hyperpolarizing step triggered an inward tail. The variations of this tail with the duration of the pulse are clearly parallel to the development of the outward current observed at −40 mV. The tail was not observed if the membrane was repolarized to −80 mV, which is the K equilibrium potential in most *Aplysia* neurones (Kehoe, 1972*a*; Russell & Brown, 1972). The experiment thus suggests that both the transient outward current activated by depolarization and the corresponding tails are K currents.

These experiments suggest that ACh produces an entry of Ca which can lead to a voltage dependent increase in K permeability. In the experiment of Fig. 10, the K currents, activated by depolarization, inactivate. If they inactivated completely, they would complicate the analysis of the relaxation induced by depolarizing pulses, but not that of the steady-state ACh induced currents. However, experiments like that illustrated in Fig. 12 suggest that some secondary K currents persist even when the voltage is kept steady.

Fig. 12 shows the responses to 30 sec ACh applications (measured at −80, −40 and −20 mV) in a cell which showed a marked transient outward current under the conditions of the preceding experiments (off-relaxation). At −80 mV, the ACh induced current reaches a stable plateau after about 20 sec but at −40 mV the response goes through a maximum followed by a small decrease and at −20 mV it is so severely distorted that at the end of the ACh application the current is outward. In cells in which the K current was not initially present, it could be revealed by increasing the external Ca concentration. When it was present, whether spontaneously or after increasing the external Ca concentration, noise analysis showed that at −40 and −20 mV the variance did not decrease in parallel to the total ACh induced current, in contrast to what was normally observed (Fig. 1). It thus seems that the phenomenon can be explained by the progressive development of a steady K current triggered by Ca entry through the ACh controlled channels. This current appears markedly voltage-dependent (in Fig. 12 the increase of the K driving force between −40 and −20 mV does not account for its increase); it does not inactivate, and does not seem to be associated with a noise comparable to that of the 'primary' ACh current.

The progressive reduction of the current with time observed in Fig. 12 at −40 mV should not be confused with desensitization. In a preliminary analysis of desensitization in Ca-free solutions we found that during desensitization the progressive decrease of the total ACh induced current is parallel to a decrease of the noise variance; the speed of both changes is augmented by hyperpolarization. In both respects the phenomenon resembles that observed at the frog end-plate (Anderson & Stevens, 1973; Magazanik & Vyskočil, 1976). On the contrary the progressive reduction of the total current illustrated in Fig. 12 is not associated with a decrease of the noise variance; and the phenomenon disappears at −80 mV.

Reversal potential of ACh excitatory effects

We usually avoided the cells in which the secondary K currents were clearly important, but it is likely that these currents were present, with low intensity, in most

of our experiments. Indeed, their presence is the most plausible interpretation for the difference between the two evaluations of the reversal potential of ACh currents: that using the relation between i_{el} and V, and that using the relation between the instantaneous currents, I_{in}, and V. The apparent value of i_{el} measured at levels less negative than $E_K(-80\,mV)$ can be expected to be higher than the true value if at these potentials, as indicated above, the K current reduces the apparent ACh induced current without modifying the noise variance. The error will increase with increasing depolarization. Inversely the apparent value of i_{el} measured at levels more hyperpolarized than E_K will be smaller than its effective value. This will lead to an overestimate of E_{rev} measured by extrapolation of the $i_{el}(V)$ relation. For the instantaneous currents, on the other hand, the contribution of a secondary K current will lead to an underestimate of E_{rev}. We think, therefore, that the value of the reversal potential for ACh induced currents is situated between the values obtained by the two different methods of extrapolation. In view of these data we assume that its value is close to $+20\,mV$.

Selectivity of the ionic channels opened by ACh

The proposed value of $+20\,mV$ for the ACh reversal potential is more positive than that found at the frog end-plate (usually less than 0 mV). In our first report, we found an even higher value ($+80\,mV$) because of an underestimate of i_{el} at hyperpolarized levels (see Methods). Our first interpretation of these positive values of E_{rev} (Marty *et al.* 1976) was that the ionic channels opened by ACh in *Aplysia* neurones were more selective for Na than those of the frog neuromuscular junction. This interpretation was based mainly on the extreme sensitivity of the ACh excitatory responses to changes in external Na, as reported by Blankenship *et al.* (1971) in *Aplysia* and by Levitan & Tauc (1972) in *Navanax*. However, the experiments reported below indicate that ACh controlled channels in *Aplysia* are far from ideally selective for Na ions, and actually display a weak selectivity between cations. This new conclusion is based on the observation that appreciable inward currents can be induced by ACh in Na free solutions where Na has been replaced by Cs, Mg or Ca, and on a reinterpretation of the dramatic reduction of the ACh response after partial Na-Tris substitutions.

Cs–Na substitution

When all the external Na was replaced by Cs, significant inward currents could still be obtained at membrane potentials from -20 to $-100\,mV$. Variance measurements, made on eight cells, gave values of i_{el} larger than the values found in normal sea water (Table 3). The increase of i_{el} was comparable at 12 and at 21 °C, at -40 and at $-80\,mV$.

Noise power spectra and relaxation experiments made on six cells at $-80\,mV$ indicated that τ was increased in Cs sea water by a factor close to 2 (mean values: 1·8 for the noise analysis; 2·0 for relaxation experiments).

Relaxation experiments also allowed the evaluation of the ACh reversal potential by comparing instantaneous and steady currents as illustrated in Fig. 5. The value obtained ranged between -7 and $+17\,mV$.

The value of τ observed in Cs sea water at $-80\,mV$ is approximately equivalent to that predicted at $-140\,mV$ in Na sea water by extrapolating the $\tau(V)$ curves of Figs. 4 or 7. This could suggest that the effect of Cs sea water is due to a large surface potential change. This is made unlikely, however, by additional observations con-

cerning the relative amplitude of the steady-state and instantaneous ACh induced currents in Cs sea water.

Fig. 13 illustrates an experiment in which we took advantage of the fact that Cs sea water increases the membrane resistance at depolarized membrane levels and suppresses the transient outward current. A relaxation was induced by a voltage step from 0 to -40 mV. The ratio of $I_{ss}(-40)$ to $I_{in}(-40)$ is close to 5·6. As discussed previously (p. 187) this ratio should be equal to the ratio $\tau(-40)/\tau(0)$ if, in the model used,

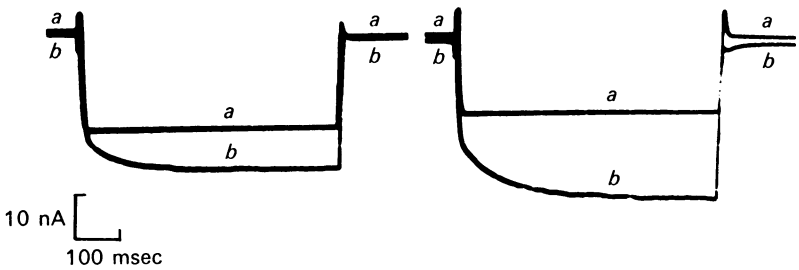

Fig. 13. Relaxations in Cs sea water. Two unusual voltage steps were used: from 0 to -40 mV (left records) and from -20 to -60 mv (right records). The traces labelled (*a*) correspond to the records obtained in the absence of ACh; the traces labelled (*b*) correspond to the records obtained in the presence of ACh. At 0 mV (left) and -20 mV (right) the steady-state ACh induced currents are barely detectable; accordingly hyperpolarization to -40 mV (left) and -60 mV (right) only triggers a small ohmic (instantaneous) component; but then a large relaxation develops. Temp. 12 °C. Neostigmine 5×10^{-5} M. Calibration: 10 nA, 100 msec. $\tau(-40) = 36$ msec. $\tau(-60) = 47$ msec.

TABLE 3. Values of i_{el} and τ in Na-free sea water. *Elementary current*, i_{el}. The value of $i_{el,Na}$ used in the first column was either the value measured in the same cell before replacing Na with its substitute, or the mean values of Fig. 2. The membrane potential at which i_{el} was measured varied from -40 to -80 mV. The temperature was 12 °C or 22 – 24 °C. In this last case the reference values of i_{el} were those of Fig. 2 at 21 °C. *Time constants*, τ. The values of τ_{rel} and τ_{noise} were pooled together. The value of $\tau(-80, Na)$ was taken from the data of Fig. 4. The number of independent determinations is indicated in parentheses

Ion replacing Na	$\frac{i_{el}}{i_{el,Na}}$	$\tau(-80)$ (msec) (12° C)	$\frac{\tau(-80)}{\tau(-80,Na)}$ (12° C)	$\tau(-80)$ (msec) (22° C)	$\frac{\tau(-80)}{\tau(-80,Na)}$ (22° C)
Cs	1·68 ± 0·36 (S.D.) (9)	52 ± 6 (4)	1·9	—	—
Mg	0·29 ± 0·12 (7)	92 (2)	3·4	21 ± 4 (3)	3·9
Ca	0·13 ± 0·04 (3)	270 (1)	10	34 (1)	6·3

p is independent of the membrane potential. A shift of 60 mV of the function $\tau(V)$ would predict that $\tau(-40)/\tau(0)$ in Cs sea water should be close to $\tau(-100)/\tau(-60)$ in Na sea water, i.e. 1·7. Therefore, the effects of Cs sea water on τ do not appear to imply only a shift of the $\tau(V)$ relationship.

Mg–Na substitution

The ability of *Aplysia* neurones to sustain depolarizing responses to ACh in Mg sea water was first reported by Sato *et al.* (1968). We confirmed that ACh induced currents may be observed under these conditions and were further able to measure the parameters of the 'elementary event'.

The elementary current, i_{el}, was measured in four cells at membrane potentials ranging from -40 to -80 mV, at room temperature (22–24 °C). It was smaller than in Na sea water (the mean ratio was 0·29, with extreme values 0·16 and 0·46).

Relaxation experiments were performed on six cells, at 12 °C and at 22 °C. They revealed a dramatic lengthening of τ which at -80 mV was increased by a factor of 2–4 (Table 3). Furthermore this increase of τ appeared to be voltage dependent, and this peculiar effect was submitted to a more detailed analysis.

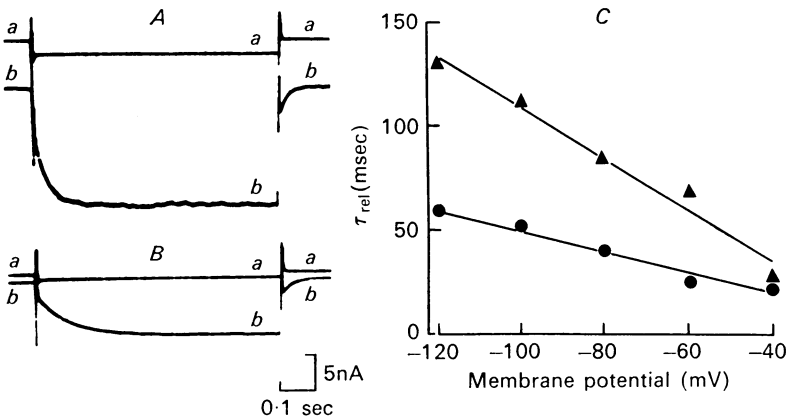

Fig. 14. A relaxation experiment in Mg sea water. *A*, Na sea water. *B*, Mg sea water. The two records are from the same cell, at 12 °C. The position of the ACh pipette and the iontophoretic current were the same in *A* and *B*, and in both cases the voltage step was from -40 to -80 mV. Notice that in going from *A* to *B* (1) I_{ss} is smaller, (2) the noise disappears on the ACh current trace, (3) the relaxation becomes slower ($\tau(-80) = 38$ msec in *A*, 85 msec in *B*), (4) the ratio $I_{ss}(-80)/I_{in}(-80)$ increases. The reduction of the current from *A* to *B* corresponds roughly to what is expected from the differences in i_{el} (see Table 3) and τ. Thus, Na-Mg substitution does not appear to affect the opening probability p of the channels. Calibration: 5 nA, 100 msec. The relaxation time constants obtained in *A* (Na sea water: ●) and *B* (Mg sea water: ▲) have been plotted as a function of membrane potential. Lines drawn by eye. Notice that the τ (V) relationship is steeper in Mg sea water.

Fig. 14 illustrates an experiment made at 12 °C. The lengthening of τ is clearly visible at -80 mV: $\tau(-80)$ is 2·1 times larger in Mg sea water than in normal sea water. But this lengthening is much less striking in the off relaxation at -40 mV. Fig. 14*C* shows a more complete analysis of the voltage dependence of τ on the same cell. The relationship between τ and V is approximately linear both in Na and in Mg sea water, but its slope increases in Mg sea water. The ratio $\tau(-120)/\tau(-40)$ goes from 2·7 in Na sea water to 4·8 in Mg sea water.

A second effect observed in Mg sea water is an increase of $I_{ss}(-80)/I_{in}(-80)$. In the

experiment of Fig. 14, this ratio, close to 2 in Na sea water, increases to 3·7 in Mg sea water. This increase is what is expected if the ratio of the two currents is equal to the ratio of the time constants at the test and holding potentials, and thus confirms that the relation between τ and V becomes steeper in Mg sea water. The equality between the ratios $I_{ss}(-80)/I_{in}(-80)$ and $\tau(-80)/\tau(-40)$ holds only if p is independent of the membrane potential (eqn. (6)). The validity of this last condition over the whole voltage range from -40 to -120 mV was checked by measuring the ratio of I_{ss} over $(i_{el}\tau)$ (eqn. (7)); taking $E_{rev} = -5$ mV from instantaneous currents analysis, this ratio was found constant within 10 % between -40 and -120 mV.

Ca–Na substitution

Significant inward currents can be elicited by ACh in Ca sea water. However, whereas at membrane potentials more negative than -80 mV the current can be sustained for several tens of seconds, at more depolarized potentials it decreases rapidly, and often turns outward. This behaviour, similar to that illustrated in Fig. 12 at -20mV is probably due to an accentuation of the effects seen in this Figure, i.e. to the development of a K current following a large entry of Ca ions. The importance of this effect prevented relaxation experiments performed in the usual way, i.e. from a holding potential of -40 mV. However, it was still possible to measure $\tau(-80)$ by using a holding potential of -80 mV, the K equilibrium potential, and by applying a depolarizing pulse and reading τ after the repolarizing step to -80 mV. In two experiments where this was done $\tau(-80)$ was found to be much larger than in Na sea water (Table 3). Measurements of i_{el} (2 at -80 mV, 1 at -60 mV) gave values considerably smaller than in Na sea water (Table 3).

The lengthening effect of Ca ions on τ was also observed in less extreme conditions where the proportions of Ca and Mg ions were changed while the total number of divalent ions was kept the same as in normal sea water. In such an experiment at 21 °C, $\tau_{rel}(-80)$ was found to be 35 msec in a 50 Ca: 10 Mg sea water; it was too fast to be measured by the relaxation technique in a 0 Ca: 60 Mg sea water. For comparison, the mean of $\tau_{noise}(-80)$ in normal sea water (10 Ca: 50 Mg) is 5·4 msec at this temperature. Thus, it appears that Ca ions have a more powerful lengthening effect on τ than Mg ions, as already suggested by the data of Table 3.

It appears on the whole that the characteristics of the elementary event are similar in Mg and Ca rich sea-waters; the main difference between the two situations being that Mg cannot replace Ca in triggering an indirect K permeability.

Tris–Na substitution

We found a striking reduction of the ACh response in Tris substituted sea water.

Fig. 15 illustrates the reduction of the ACh induced current as a function of the Tris concentration. This reduction is the same for the total current and for i_{el}.

The extent of the reduction of I_{ss} and i_{el} in Tris sea water is such that a specific blocking action of Tris ions must be involved. This also explains the different values of i_{el} obtained in Tris and bicarbonate buffered sea water (Fig. 2).

The effects of Tris on i_{el} are more pronounced at -80 mV than at -40 mV (Fig. 15). This is even more apparent if the data are corrected for the presence of 10 mM-Tris

in the 'normal' sea water: according to the data of Fig. 2, this leads to an additional reduction of i_{el} by 7 and 15% at -40 and -80 mV respectively.

The Tris substitution did not affect the value of τ, whether obtained from noise power spectra or voltage step induced relaxations (Table 4; see also Fig. 4).

From eqn. (6), it appears that since the reduction of I_{ss} is entirely accounted for by the reduction of i_{el}, p is not changed by Tris.

In conclusion, Tris substitution reduces i_{el} without modifying p or q. As will be discussed below, this suggests that Tris does not interfere with the ACh receptor interaction, but rather acts on the channel itself.

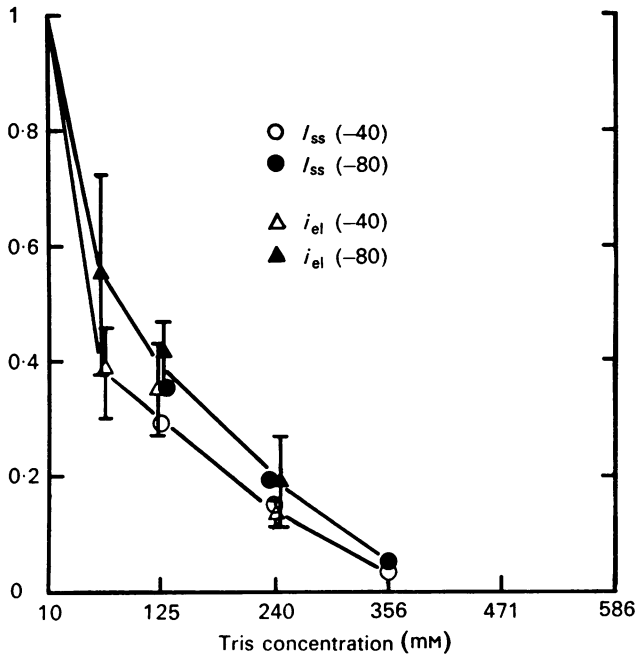

Fig. 15. Effects of Tris on the steady-state (I_{ss}) and the elementary (i_{el}) ACh induced currents. The vertical scale measures 'normalized' values of I_{ss} and i_{el} obtained by dividing the value of these currents in Tris substituted sea water by their control values. The horizontal scale indicates the degree of substitution between the control sea water (480 mM-Na, 10 mM-Tris) and the fully substituted sea water (0 mM-Na, 586 mM-Tris). At -40 mV, the steady-state current (○) and the elementary current (△) decrease in parallel when the Tris concentration increases. For a partial Tris substitution they are less reduced than the steady-state current (●) and the elementary current (▲) measured at -80 mV. Error bars: ± S.E. (for i_{el}). Temp. 12 °C.

TABLE 4. Effects of Tris-Na substitution on I_{ss} and τ_{rel}. All measurements were done on the same cell at 12 °C. Membrane potential -80 mV

Tris concentration (mM)	$\frac{I_{ss}}{I_{ss\,cont}}$	τ (msec)	$\frac{\tau}{\tau_{cont}}$
10	1	26	1
125	0·37	25	1·0
240	0·06	23	0·9

Cl-isethionate substitution

Substitution of NaCl by Na-isethionate did not reveal any measurable change in I_{ss}, i_{el}, τ_{rel}, τ_{noise}. This suggests that Cl does not play an important role in the ACh induced conductance.

DISCUSSION

The results presented above suggest that there is no major difference between the 'elementary events' (Katz & Miledi, 1972) underlying the excitatory effects of ACh on *Aplysia* neurones and those on frog muscle. Quantitatively, however, differences exist. The values of τ are larger in *Aplysia* whereas the value of the elementary conductance is smaller. The effects of voltage may not be exactly the same. We will discuss below a few of these differences, as well as some results from *Aplysia* which are without equivalents at the frog neuromuscular junction.

Voltage dependence of τ. As pointed out by Dionne & Stevens (1975) it is important to rule out that the increase in conductance during a hyperpolarizing pulse is not due to a local increase in the ACh concentration resulting from ACh iontophoresis across a series resistance located in the external medium. A first argument against this hypothesis is that neither i_{el} nor τ was affected by the ACh dose, whereas they should be according to the series resistance hypothesis (Dionne & Stevens, 1975). A second argument is that the input resistance of the cell during ACh application was in most experiments larger than 5MΩ, which is certainly much higher than the access resistance to the neurones we used. Therefore, an interpretation of the voltage sensitivity based on a series resistance effect appears even less tenable than in the frog, and we will assume in the following that the ACh concentration at the level of the membrane is independent of the membrane potential.

We have supposed at the beginning of this article, as did previous authors (see Neher & Stevens, 1977) that the behaviour of the ACh sensitive channel is governed by a transition between two states, open and closed. Our results show that a description of the system based on constant transition probability densities between these two states adequately accounts for all noise and relaxation data, and that the low ACh concentration limit is a valid approximation in our experimental conditions. In addition, it appears that the probability density for opening of the channel, p, depends on the ACh concentration but not on the membrane potential, while the probability density for closing, q (or $1/\tau$), does not depend on the ACh concentration but depends on the membrane potential.

Our conclusions concerning p resemble those of Neher & Sakmann (1975) who found no indication of voltage sensitivity for the opening reaction (see also Sheridan & Lester, 1975). Our results on the voltage sensitivity of q agree qualitatively with those of previous authors (Anderson & Stevens, 1973; Neher & Sakmann, 1975, 1976). Quantitatively the ratio $\tau(-120)/\tau(-80)$ which we found ($\approx 1{\cdot}4$ (Figs. 4 and 7)) resembles that found by Neher & Sakmann (1975, 1976) for ACh induced currents in muscle. The change of τ with membrane potential does not seem to be exponential in our experiments (Figs. 4 and 7) contrary to what has been observed by Anderson & Stevens (1973) or Sheridan and Lester (1975). However, the voltage range we studied was much smaller than in the previous studies and should be extended before it can be said that there is a true difference between the two systems.

The conclusion that τ does not depend on the ACh concentration only holds at low ACh doses. At higher doses, an 'inverse' component appears in relaxation experiments, leading to a situation which can no more be described by a single time constant, and which resembles that described in the following paper (Ascher *et al.* 1978).

The changes in τ observed when Na was replaced by Mg, Ca or Cs raise interesting problems. An explanation based on a change of surface potential appears difficult to defend, because such a change would only produce a shift of the relation $\tau(V)$ (see for example, Muller & Finkelstein, 1972). The experiments illustrated in Figs. 13 and 14 indicate that the changes in τ are associated with a change of the slope of the relation $\tau(V)$. We would like to suggest that these changes can be explained by assuming that Cs, Ca, Mg and, more generally, all permeant ions, interfere directly with the opening and/or closing processes. More specifically, it is possible to imagine that permeant ions stabilize the channel in the open configuration. This hypothesis could not only account for the dependence of τ on the permeant cation, but also for part or all of the dependence of τ on membrane potential. In the extreme case neither the opening nor the closing of the channels would depend on voltage in the absence of permeant ions. In the presence of permeant ions, the opening probability would not be altered, but the closing probability would be decreased because of ion binding inside the channel. Recent data on artificial bilayers (Kolb & Bamberg, 1977) showing an increase of τ with the ionic concentration, may also be interpreted in this way. In a simple model with one binding site located between two unequal energy barriers, if the larger energy barrier is more internal than the binding site, the probability of occupancy by a permeant cation increases at negative membrane potentials. This in turn increases τ. It is of interest that according to this model one would expect the probability of occupancy, and therefore τ, to have a steeper dependence on voltage for divalent cations (as Mg) than for monovalent cations (as Na). Such predictions are not easily drawn from other current interpretations of the voltage dependence of τ (see Gage, 1976; Adams, 1977).

The Ca induced K current. The evidence showing that the ACh induced current can cause a 'secondary' K current comes from two types of experiments. Those illustrated in Figs. 10 and 11 show a transient outward current activated by depolarizing steps in the presence of ACh. Those illustrated in Fig. 12 indicate that one can also observe a steady outward current. Both the transient and the steady outward currents appear to be K currents. The corresponding conductances seem voltage dependent, since the currents increase more with depolarization than is expected from the change in driving force.

It is not clear whether the transient outward current and the steady-state K current correspond to two different conductances, or to a single conductance which inactivates only partially after depolarization. It is also not clear to which of the various K currents described in molluscan neurones (see Thompson, 1977) these currents are related. Their dependence on Ca entry suggests a link with the Ca dependent K currents studied by Meech and Standen (1975) and Heyer & Lux (1976). However, the experiments of Meech (1974) do not suggest that the Ca dependent K conductance is sensitive to voltage, and it is therefore possible to consider other interpretations. In particular, the similarity between the K currents observed at -40 mV in the presence of ACh and those observed at -20 mV in the absence of ACh (Fig. 11) may suggest an alternative explanation in which the Ca ions, having entered the cell through the ACh

induced channels, would bind close to voltage dependent K channels of the Hodgkin–Huxley type, and create a positive change of the internal surface potential. This would result in a transient shift to the left of the activation curve of these channels. From the experiments of Fig. 11 *A* and *B*, it appears that this shift need only to be of the order of 20 mV to account for the data. Since Mg, which is also permeant, does not induce a K current, the shift would have to be specific for Ca, and thus would imply genuine binding rather than screening.

The value of the ACh reversal potential E_{rev}. The voltage dependence of τ excludes an evaluation of E_{rev} based on the extrapolation of the relation between the stationary current (I_{ss}) and the membrane potential. The extrapolations of the $i_{el}(V)$ or $I_{in}(V)$ relationship should be more reliable. Although both are subject to the error introduced by the Ca dependent K permeability, it has been shown in the Results section that this error affects the two types of evaluations in opposite directions. The corrected value of +20 mV appears to be a good approximation of the reversal potential, as long as we can assume that the relationship between i_{el} and V remains linear above −40 mV.

This value leads to an elementary conductance, γ, of $8 \cdot 10^{-12}\ \Omega^{-1}$ (at 12 °C) in Tris-buffered sea water. The value would be higher in the absence of Tris.

The value of +20 mV for the ACh reversal potential does not agree with the values previously published for molluscan neurones. Our initial evaluation based on the extrapolation of $i_{el}(V)$ relationship, was +80 mV (Marty *et al.* 1976). This value was too high both because of the technical problems discussed in the Methods section, and because we had not taken into account the Ca induced K current. By contrast all the evaluations of other authors were much lower than +20 mV. We think that in many cases (e.g. Blankenship *et al.* 1971; Levitan & Tauc, 1972) this is due to the fact that the authors extrapolated the curve describing the total ACh induced current, and therefore were exposed to errors due to the voltage dependence of τ. As for the authors who have claimed to have actually observed a reversal of the ACh excitatory effects below 0 mV, we have discussed elsewhere (Ascher & Kehoe, 1975) the possibility that they were simultaneously activating an excitatory and a Cl response. We have ourselves observed such reversals in some *Aplysia* neurones, at about −10 mV, using K_2SO_4 electrodes. But in all cases, hexamethonium (10^{-3} M) revealed the presence of an inhibitory component. In the pleural neurones studied in the present paper, hexamethonium did not reveal any inhibitory component, and no sign of an inversion was observed up to −10 mV. Above this level the membrane conductance became so high that it was not possible to detect any additional ACh induced conductance.

Ionic selectivity of the ACh controlled channels. The persistence of appreciable ACh-induced inward currents in solutions where Na had been replaced by Cs, Mg or Ca implies that the ionic channels opened by ACh are permeable to these ions. It is even possible that the channels are permeable to anions: the fact that the Cl isethionate substitution does not modify τ or i_{el} only indicates that Cl and isethionate are equivalent; it does not indicate if both are permeant, or impermeant.

Since our experiments involved only massive ionic substitutions, one may wonder if the fact that a given ion can cross a channel when it is the dominant species in the extracellular solution implies that the same species contributes an appreciable current in normal sea water. We cannot answer this question because we cannot evaluate precisely the ACh reversal potential, and even less the contribution of each ionic

species to the total current in normal sea water. In the case of Ca, the presence of a secondary K current in normal sea water suggests that the ACh induced Ca current exists in these conditions. However the magnitude of this Ca current does not need to be large to account for the observed effects. Meech & Standen (1975) have calculated that in snail an increase of $[Ca]_i$ from 10^{-7} to 10^{-6} M triggers an appreciable increase in g_K. If we assume the same for our neurones, as well as an initial level of $[Ca]_i$ of 10^{-1} M, a diameter of 60μm (i.e. a volume of about 10^{-10} l.) and no fast extrusion of Ca, the rise of $[Ca]_i$ to 10^{-6} M would take 100 msec for a current of 10^{-10} A. As the total ACh induced current was often of the order of 10^{-8} A, the above calculation is compatible with a g_{Na}/g_{Ca} of about 100. Smaller values of this ratio certainly cannot be ruled out.

The hypothesis that all the cationic species contribute to the ACh induced current would make the ACh induced channel of *Aplysia* resemble that of the frog neuromuscular junction, where it is known that not only Na and K (Takeuchi & Takeuchi, 1960), but also Ca (Takeuchi, 1963) and Mg (Katz & Miledi, 1969) are permeant. In this case, how can we account for the difference between the positive value of E_{rev} in *Aplysia* and the values (close to 0 mV or negative) found in the frog? A possible interpretation could involve the ratio of ionic concentrations in the external and internal solutions. The internal K concentration of *Aplysia* neurones can be estimated at 240 mM (Kehoe, 1972*a*) and there are not many other diffusible cations in the intracellular solution. This contrasts with the high concentration of cations outside – 480 mM for Na only. Therefore, even if the ACh induced channels had no intercationic selectivity, one could expect the reversal potential to be slightly positive. In frog muscle, on the other hand, the ratio of extracellular over intracellular diffusible cations is closer to 1 (cf. Zachar, 1971).

Effects of Tris. Wilson, Clark & Pellmark (1977) have recently reported that Tris is an antagonist of ACh responses in *Aplysia* neurones. These authors did not analyse the noise in the presence of Tris but, at the frog neuromuscular junction, Stevens (quoted in Dreyer, Walther & Peper, 1976) has observed a reduction of the elementary current similar to that described in the present paper. In the case of *Aplysia* neurones, the reduction of elementary current appears to account entirely for the reduction of the total ACh induced current. Tris does not modify p and q, the probabilities of opening and of closing of the channels. The simplest interpretation is that Tris ions obstruct the channel but do not interfere with the receptor. However, it seems important to consider whether other interpretations can account for the data, and in particular for the progressive reduction of i_{el} by increasing concentrations of Tris. If the elementary event is assumed to be a square pulse of current of an average duration of 30 msec in Tris-free sea water (at – 80 mV and 12 °C), it may become in Tris sea water a pulse of the same duration and of reduced height, or alternatively it may be cut into a high frequency train of shorter pulses. For example, ta a concentration of 70 mM, which reduces i_{el} to about one half, the elementary event could be a 30 msec train of pulses lasting 10 μsec, having the same height as in the absence of Tris and separated by an average interval of 10 μsec. The total charge transferred during these trains would be only half that transferred in normal conditions.

In principle, this should lead to a biphasic power spectrum. However, the 'fast' component of this spectrum would occur mostly in a frequency range far outside the range in which we worked; thus this component, although matching in area the slow

component, would have such a low amplitude that one could not expect to detect it and that it would not contribute appreciably to the elementary current measured. It is only if the internal frequency inside the train of pulses became about 10 times slower than in the example considered that one could expect a visible change in the spectra.

The hypothesis considered above has the advantage that it can be easily formalized into a 'cyclic' model which is the following. Even in the presence of Tris, the receptor-channel complex is supposed to have only two states – R (resting) and R_A (activated-conducting). Tris is assumed to bind both to R and R_A, leading to RT and R_AT, both non-conducting. To account for the absence of change in the spectra and in the relaxation in the presence of Tris, one has to suppose that RT and R_AT interconvert at the same rates (p and q) as R and R_A. This implies that the equilibrium constant of the reaction $R+T \rightleftarrows RT$, K_T, is equal to that of the reaction $R_A+T \rightleftarrows R_AT$. The model can thus be written

$$\begin{array}{ccc} R & \overset{p}{\rightleftarrows} & R_A \\ + & {\scriptstyle q} & + \\ T & & T \\ K_T \uparrow\downarrow & & \uparrow\downarrow K_T \\ RT & \underset{q}{\overset{p}{\rightleftarrows}} & R_AT. \end{array}$$

If $p \ll q$ the concentration of Tris (70 mM), which reduces the total current by half, corresponds to K_T. This constant is the ratio of two rate constants which we cannot evaluate independently. However, if we take for the forward rate constant a value close to those calculated for various ACh antagonists in the following paper (Ascher *et al.* 1978), e.g. $10^6\ \text{M}^{-1}\,\text{sec}^{-1}$, then the backward rate constant is $7 \times 10^4\,\text{sec}^{-1}$, and the average duration of the fast pulses inside the train is about 14 μsec. If we take for the forward rate constant a value closer to that calculated for Na entry in the channel (Ascher *et al.* 1978) e.g. $10^7\ \text{M}^{-1}\,\text{sec}^{-1}$, then the duration of the fast pulses falls to 1·4 μsec.

To account for the effects of Tris it is possible to imagine either that the binding of Tris triggers a conformation change which leads to a less conducting or non-conducting state, or that Tris obstructs the channel. In the type of interpretation developed above (a fast 'flutter' of the channel) the high speed at which Tris would have to open and close the channel seems too high to be easily attributed to a series of conformation changes and is therefore more in favour of a direct block of the channel. We think that binding of Tris to the channel is also likely to be the best hypothesis if the reduction of i_{el} occurs in a graded manner rather than as considered above, in particular because it is difficult to see how the binding of Tris to the receptor instead of the channel would lead to a *continuous* variation of i_{el} when the Tris concentration is increased. Finally, the voltage dependence of the effect of Tris (Fig. 15) is also consistent with an action on the channel. It may be noted that a voltage dependent blockade of the elementary conductance does not necessarily require an entry of the blocker into the channel (Bamberg & Laüger, 1977).

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REFERENCES

ADAMS, P. R. (1975). Kinetics of agonist conductance changes during hyperpolarization at frog end-plates. *Br. J. Pharmac.* **53**, 308–310.

ADAMS, P. R. (1977). Relaxation experiments using bath-applied suberyldicholine. *J. Physiol.* **268**, 271–289.

ANDERSON, C. R. & STEVENS, C. F. (1973). Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. *J. Physiol.* **235**, 655–691.

ASCHER, P. & KEHOE, J. S. (1975). Amine and amino-acid receptors in Gastropod neurones. In *Handbook of Psychopharmacology*, vol. 4, ed. IVERSEN, L. L., IVERSEN, S. D. & SNYDER, S. H., pp. 265–310. New York: Plenum .

ASCHER, P., MARTY, A. & NEILD, T. O. (1978). The mode of action of antagonists of the excitatory response to acetylcholine in *Aplysia* neurones. *J. Physiol.* **278**, 207–235.

BAMBERG, E. & LAÜGER, P. (1977). Blocking of the gramicidin channel by divalent cations. *J. Membrane Biol.* **35**, 351–375.

BLANKENSHIP, J., WACHTEL, H. & KANDEL, E. R. (1971). Ionic mechanisms of excitatory, inhibitory and dual synaptic actions mediated by an identified interneuron in abdominal ganglion of *Aplysia*. *J. Neurophysiol.* **34**, 76–92.

DIONNE, V. E. & STEVENS, C. F. (1975). Voltage dependence of agonist effectiveness at the frog neuromuscular junction: resolution of a paradox. *J. Physiol.* **251**, 245–270.

DREYER, F., WALTHER, C. & PEPER, K. (1976). Junctional and extra-junctional receptors in normal and denervated frog muscle fibres. *Pflügers Arch.* **366**, 1–9.

GAGE, P. W. (1976). Generation of end-plate potentials. *Physiol. Rev.* **56**, 177–247.

GORMAN, A. L. F. & MIROLLI, M. (1972). The passive electrical properties of the membrane of a molluscan neurone. *J. Physiol.* **227**, 35-49.

HEYER, C. B. & LUX, H. D. (1976). Control of the delayed outward potassium currents in bursting pace-maker neurones of the snail, *Helix pomatia*. *J. Physiol.* **262**, 349–382.

KATZ, B. & MILEDI, R. (1969). Spontaneous and evoked activity of motor nerve endings in calcium Ringer. *J. Physiol.* **203**, 689–706.

KATZ, B. & MILEDI, R. (1972). The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol.* **224**, 665–699.

KATZ, B. & MILEDI, R. (1973). The characteristics of end-plate noise produced by different depolarizing drugs. *J. Physiol.* **230**, 707–717.

KATZ, B. & MILEDI, R. (1975). The effect of procaine on the action of acetylcholine at the neuromuscular junction. *J. Physiol.* **249**, 269–284,

KEHOE, J. S. (1972*a*). Ionic mechanisms of a two component cholinergic inhibition in *Aplysia* neurones. *J. Physiol.* **225**, 85–114.

KEHOE, J. S. (1972*b*). Three acetylcholine receptors in *Aplysia* neurones. *J. Physiol.* **225**, 115–146.

KEHOE, J. S. (1972*c*). The physiological role of three acetylcholine receptors in synaptic transmission in *Aplysia*. *J. Physiol.* **225**, 147–172.

KOLB, H. A. & BAMBERG, E. (1977). Influence of membrane thickness and ion concentration on the properties of the gramicidin A channel. Autocorrelation, spectral power density, relaxation, and single channel studies. *Biochim. biophys. Acta* **464**, 127–141.

LEVITAN, H. & TAUC, L. (1972). Acetylcholine receptors: topographic distribution and pharmacological properties of two receptor types on a single molluscan neurone. *J. Physiol.* **222**, 537–558.

MAGAZANIK, L. G. & VYSKOČIL, F. (1976). Desensitization at the neuromuscular junction. In *Motor Innervation of Muscle*, ed. THESLEFF, S., pp. 151–176. New York: Academic Press.

MARTY, A. (1978). Noise and relaxation studies of acetylcholine induced currents in the presence of procaine. *J. Physiol.* **278**, 237–250.

MARTY, A., NEILD, T. O. & ASCHER, P. (1976). Voltage sensitivity of acetylcholine currents in *Aplysia* neurones in the presence of curare. *Nature, Lond.* **261**, 501–503.

MEECH, R. W. (1974). The sensitivity of the *Helix aspersa* neurones to injected calcium ions. *J. Physiol.* **237**, 259–278.

MEECH, R. W. & STANDEN, N. B. (1975). Potassium activation in *Helix aspersa* neurones under voltage clamp: a component mediated by calcium influx. *J. Physiol.* **249**, 211–239.

MULLER, R. U. & FINKELSTEIN, A. (1972). The effect of surface charge on the voltage-dependent conductance induced in thin lipid membranes by monazomycin. *J. gen. Physiol.* **60**, 285–306.

NEHER, E. & SAKMANN, B. (1975). Voltage dependence of drug-induced conductance in frog neuromuscular junction. *Proc. natn. Acad. Sci., U.S.A.* **72**, 2140–2144.

NEHER, E. & SAKMANN, B. (1976). Noise analysis of drug induced voltage clamp currents in denervated frog muscle fibres. *J. Physiol.* **258**, 705–729.

NEHER, E. & STEVENS, C. F. (1977). Conductance fluctuations and ionic pores in membranes. *Ann. Rev. Biophys. Bioeng.* **6**, 345–381.

RUSSELL, J. M. & BROWN, A. M. (1972). Active transport of potassium by the giant neuron of the Aplysia abdominal ganglion. *J. gen. Physiol.* **60**, 519–533.

SACHS, F. & LECAR, H. (1977). Acetylcholine-induced current fluctuations in tissue cultured muscle cells under voltage clamp. *Biophys. J.* **17**, 129–143.

SATO, M., AUSTIN, G., YAI, H. & MARUHASHI, J. (1968). The ionic permeability changes during acetylcholine induced responses of *Aplysia* ganglion cells. *J. gen. Physiol.* **51**, 321–345.

SHERIDAN, R. E. & LESTER, H. A. (1975). Relaxation measurements on the acetylcholine receptor. *Proc. natn. Acad. Sci., U.S.A.* **72**, 3496–3500.

STEINBACH, J. H. & STEVENS, C. F. (1976). Neuromuscular transmission. In *Neurobiology of the Frog*, ed. LLINAS, R. & PRECHT, W., pp. 33–92. Berlin: Springer.

TAKEUCHI, N. (1963). Effects of calcium on the conductance change of the end-plate membrane during the action of transmitter. *J. Physiol.* **167**, 141–155.

TAKEUCHI, A. & TAKEUCHI, N. (1960). On the permeability of end-plate membrane during the action of transmitter. *J. Physiol.* **154**, 52–67.

TAUC, L. & GERSCHENFELD, H. M. (1962). A cholinergic mechanism of inhibitory synaptic transmission in a molluscan nervous system. *J. Neurophysiol.* **25**, 236–262.

THOMPSON, S. H. (1977). Three pharmacologically distinct potassium channels in molluscan neurones. *J. Physiol.* **265**, 465–488.

WILSON, W. A., CLARK, M. T. & PELLMAR, T. C. (1977). Tris buffer attenuates acetylcholine responses in *Aplysia* neurones. *Science, N.Y.* **196**, 440–441.

ZACHAR, J. (1971). *Electrogenesis and Contractility in Skeletal Muscle Cells.* Baltimore: University Park Press.