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EFFECTS OF LOW TEMPERATURE AND TERMINAL MEMBRANE POTENTIAL ON QUANTAL SIZE AT FROG NEUROMUSCULAR JUNCTION

BY I. S. COHEN AND W. VAN DER KLOOT

From the Department of Physiology and Biophysics, Health Science Center, SUNY at Stony Brook, NY 11794, U.S.A.

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SUMMARY

1. Two mechanisms proposed for the quantal release of acetylcholine (ACh) are: (i) that the quanta are pre-packaged in vesicles and released by exocytosis and (ii) that the ACh is released from the cytoplasm of the nerve terminal by the opening of an ACh channel. Our experiments were designed to test aspects of these hypotheses.

2. Miniature end-plate currents (m.e.p.c.s) were reversibly decreased in amplitude and increased in duration as the temperature was decreased between 15 and 6 °C. The amplitude decreased with a Q_{10} of 2·4 between 15 and 11 °C, and then with a Q_{10} of 3 between 11 and 6 °C. The half-decay time increased with a Q_{10} between 4 and 5 over the entire temperature range.

3. The effect of temperature on the end-plate current (e.p.c.) in response to ionophoretically applied ACh was also studied. The e.p.c. in response to a set, sustained dose of ACh was 30 % larger at 11 °C than at 15 °C, and about 10 % larger at 6 °C than at 15 °C.

4. The difference in the end-plate response to brief pulses of ACh (m.e.p.c.s) and to sustained application of ACh was analysed by Dionne & Stevens (1975). The amplitude of the sustained response depends on both the number of channels opened in the end-plate, and the length of time they stay open. When our temperature data are analysed in this way, it appears that the amount of ACh/quanta acting on the end-plate is altered by less than 25 % over the temperature range 15–6 °C.

5. From the experiments in which the nerve terminal membrane potential was shifted by external currents it was concluded that miniature end-plate potential amplitude was independent of the terminal membrane potential (Cooke & Quastel, 1973). This conclusion was confirmed by measuring m.e.p.c.s in low Ca^{2+} Ringer solution containing 2·0 and 22·0 mM-KCl; there was no consistent change in m.e.p.c. amplitude.

6. The effects of both temperature and terminal membrane potential are more readily interpreted by the vesicle than by the channel hypothesis, since for the channel hypothesis the duration of channel opening should be temperature-sensitive and the efflux of ACh^+ through the terminal membrane should be potential-dependent.

INTRODUCTION

Almost 30 years after the demonstration of quantal transmission at the neuromuscular junction, there is still uncertainty about how the quanta are generated (Katz, 1978). One possibility is that the ACh is pre-packaged in membrane-bounded vesicles, which release their contents by exocytosis or some similar mechanism. This idea is supported by a substantial body of morphological evidence. Another hypothesis is that there is a high concentration of ACh in the motor nerve terminals, and that

TABLE 1. Temperature effects on membrane channels

Channel*	Q_{10} for closing rate constant	Conductances	Current at $T°$/ Current at $T+10°$
Squid axon:			
Voltage-gated Na^+ (*a*)	~ 3·0	~ 1·0	~ 3·0
Voltage-gated K^+ (*a*)	~ 3·0	~ 1·0	~ 3·0
Xenopus nerve			
Voltage-gated Na^+ (*b*)	2·8	1·3	2·1
Voltage-gated K^+ (*b*)	2·8	1·2	2·3
Frog end-plate:			
ACh-gated (*c*) (−80 mV)	2·8	~ 1·0	~ 2·8
Frog extrajunctional			
ACh-gated (*d*) (−80 mV)	2·1	1·3	1·6
Locust muscle			
Glutamate-gated (*e*)	1·4	2·2	0·6

* References: (*a*) Hodgkin & Huxley (1952), (*b*) Frankenhauser & Moore (1963), (*c*) Anderson & Stevens (1973), (*d*) Neher & Sakemann (1976), (*e*) Crawford & McBurney (1976).

ACh leaves, flowing down an electrochemical gradient, when an ACh-specific channel in the terminal membrane opens. This idea is supported by biochemical evidence suggesting that there is a substantial concentration of free ACh in the terminals, and that newly synthesized ACh is released by stimulation in preference to vesicular ACh (Marchbanks, 1975; Israel & Dunant, 1975; Dunant, Jones & Loctin, 1982).

No one has devised a conclusive experiment to decide between the alternatives, and there is even substantial disagreement about the results of both morphological and chemical investigations. The best course seems to be that of trying a number of different approaches, to accumulate gradually a body of evidence favourable to one or the other interpretation.

For example, the channels that have been studied so far usually remain open longer at low temperature. The Q_{10} for their closing rate constant is usually about 3 (Table 1). In most instances the channel conductance has a low Q_{10}: a value of 1·3 is typical. For most of the known channels a fall in temperature results in an increased charge transfer with each channel opening. If the hypothetical ACh channel obeyed this rule, a fall in temperature would be expected to produce more ACh/quanta, and therefore, if the end-plate sensitivity remained constant, to lead to larger miniature end-plate currents (m.e.p.c.s).

If ACh flows through a membrane channel as a cation, the membrane potential should influence the efflux rate. Experiments in which the nerve terminal potential

was shifted with extrinsic electrical currents suggested that miniature end-plate potential (m.e.p.p.) amplitudes are independent of nerve terminal polarization, but the conclusion depends on a correction to take into account the extracellular field potential (Cooke & Quastel, 1973). Therefore, we decided to check this conclusion by measuring m.e.p.c.s in preparations in different external K^+ concentrations.

A short account of this work was presented to the Physiological Society (Cohen & Van der Kloot, 1980).

METHODS

The experiments were done on the sciatic nerve–sartorius muscle preparation from the frog, *Rana pipiens*. The preparations were pinned on a silicone rubber platform in a chamber cooled with a Peltier device. The usual bathing solution contained (in mM): NaCl, 120–150; KCl, 2; $CaCl_2$, 2·5, *N*-tris-(hydroxymethyl)methyl-2-aminoethane sulphonic acid (TES) (at pH = 7·4), 4 and tetrodotoxin (TTX), 5×10^{-4}. The NaCl concentration was adjusted to provide a convenient frequency of m.e.p.c.s.

The 2 mM-K^+ Ringer solution contained (in mM): NaCl, 120; KCl, 2; $CaCl_2$, 0·1; TES, 4; (at pH = 7·4), TTX, 5×10^{-4} and sucrose 40. The 22 mM-K^+ Ringer contained: NaCl, 120; KCl, 22; $CaCl_2$, 0·1; TTX, 5×10^{-4}, and TES (at pH = 7·4), 4. The $CaCl_2$ was reduced so that the m.e.p.p. frequency did not rise to levels that made measurements difficult in the depolarized preparations and to minimize the possibility that there might be substantial changes in the amount of ACh/quanta when the release rate is high.

End-plates were located by inserting a glass micro-electrode until fast rising m.e.p.p.s were recorded. Then a second micro-electrode was inserted within 50 μm of the first. The electrodes were filled with 3 M-KCl, were bevelled (Ogden, Citron & Pierantoni, 1978) and had d.c. resistances of between 3 and 8 MΩ. A Degan voltage clamp was used. Usually m.e.p.c.s were caught on a Gould digital oscilloscope, which can display $\frac{1}{4}$ of the sweep before the triggering event, so the entire upstroke was captured. The digitized m.e.p.c. was then stored on a magnetic disc, until it was analysed with a DEC 11/03 computer. Half-times were measured by fitting a third degree polynomial to the decay phase of the m.e.p.c.s. Usually twenty-five m.e.p.c.s were measured at each end-plate at each temperature.

For ACh ionophoresis a third electrode containing 1 M-AChCl was positioned near the end-plate. The current through the ACh pipette was controlled with a WP micro-iontophoresis programmer, which provided a small, braking potential to decrease ACh diffusion from the electrode between tests as well as a regulated current for ejecting the ACh.

RESULTS

Temperature and m.e.p.c. amplitude

For most of the channels for which data are available, a decrease in temperature will increase the total amount of charge transferred at each opening. If ACh is released from a similar channel, then m.e.p.c. amplitude should increase with falling temperature. Therefore we measured m.e.p.c. amplitudes at single end-plates at a series of different temperatures between 30 and 4 °C. Usually fifteen to forty m.e.p.c.s were recorded and then the temperature shifted to the next value. The shift usually took less than 5 min. After the conclusion of the experiment the electrode was withdrawn and the sequence of temperature changes repeated to follow any changes in the electrode potentials. The change in the electrode potentials over the temperature range studied was never more than 3 mV. A representative result is shown as Fig. 1; essentially the same result was obtained in nine additional experiments. In each case the amplitude of m.e.p.c.s declined only slightly at temperatures between 30 and 15 °C, while declining much more substantially at temperatures below 15 °C.

Since the largest effects of temperature on m.e.p.c. amplitude were observed at the lowest temperatures, we performed a second series of experiments to examine this effect in more detail, focusing on 15, 11, and 6 °C (Fig. 2). The amplitude of the m.e.p.c.s is reduced 1·4 fold between 15 and 11 °C; an additional 1·7 fold reduction occurs between 11 and 6 °C. The Q_{10} for the reduction in amplitude from 15 to 6 °C is 2·9.

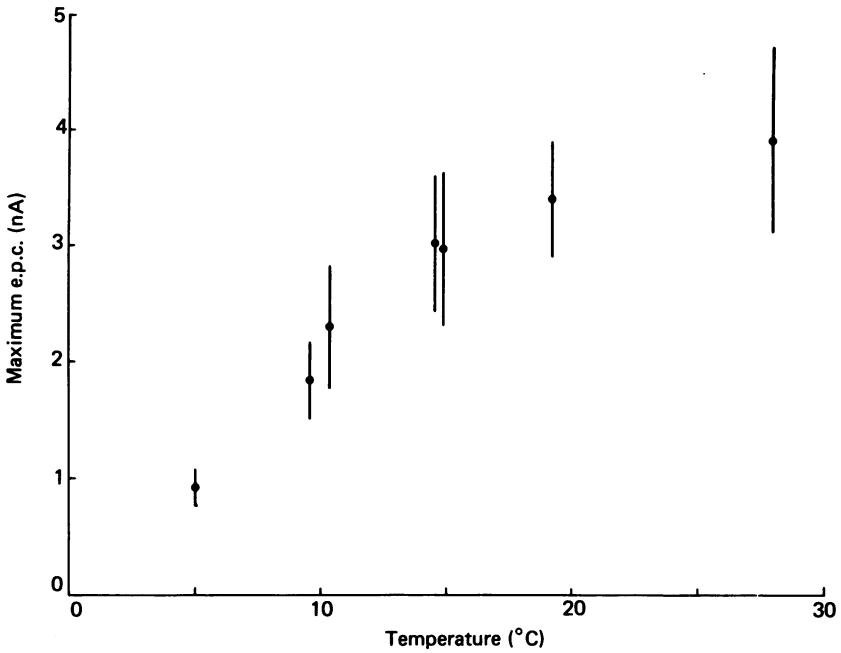

Fig. 1. The effect of temperature on the maximum amplitude of the m.e.p.c. at an end-plate. In this example the holding potential was −132 mV. Each point shows the mean peak m.e.p.c. amplitude. The vertical bars show ± a S.E. of the mean.

We also measured the half-decay time of the same m.e.p.c.s at 15, 11, and 6 °C (Fig. 2). The half-decay time was 4·4 fold longer at 6 °C than at 15 °C. The Q_{10} for the closing rate constant over this temperature range is therefore 5·1.

To interpret these results it is necessary to know whether temperature changes alter the ACh sensitivity of the end-plate. To explore this point, a third micro-electrode filled with 1 M-AChCl was positioned near the voltage-clamped end-plate. When the ACh-electrode was positioned properly, the removal of a small negative bias in the pipette, or the application of a small positive d.c. potential to the pipette, elicited an end-plate current (e.p.c.). Once the ACh-electrode was properly positioned, a steady current of ACh was ionophoresed from the pipette while the end-plate current was recorded on a slowly moving ink-writer. Then the ACh current was turned off, to make certain that the e.p.c would return to the initial value. Then the temperature was shifted, and the ionophoresis repeated. Representative records, and the complete results from another experiment are shown as Fig. 3. It is clear that there is little change in the e.p.c. with a large fall in temperature.

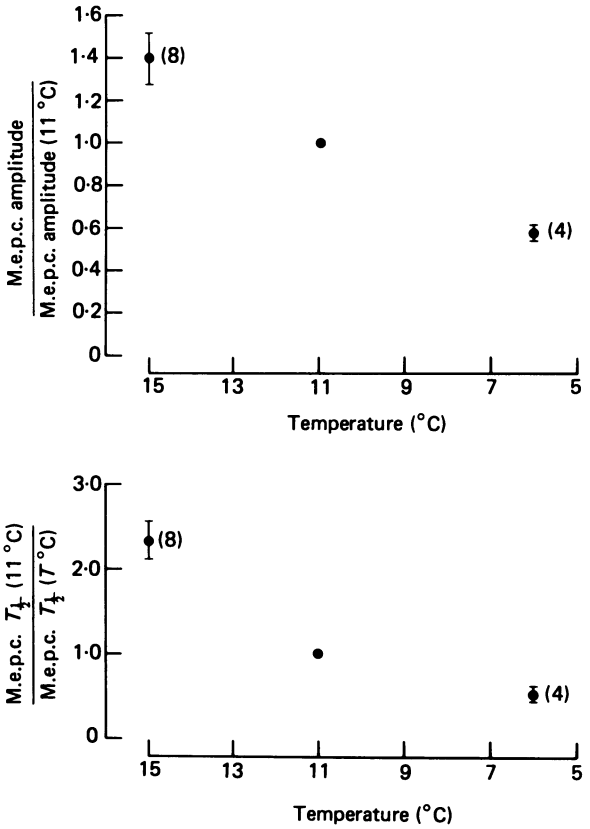

Fig. 2. Upper panel: the effects of temperature on the amplitude of m.e.p.c.s at 15, 11 and 6 °C. The ordinate shows the amplitude at each of the temperatures divided by the amplitude at 11 °C. The bars around each point indicate ± the S.E. of the mean. The number of experiments is shown next to each point. Lower panel: the effects of temperature on the half-time of decay at 15, 11 and 6 °C. The ordinate shows the $T_{\frac{1}{2}}$ at 11 °C divided by the $T_{\frac{1}{2}}$ at each of the temperatures studied.

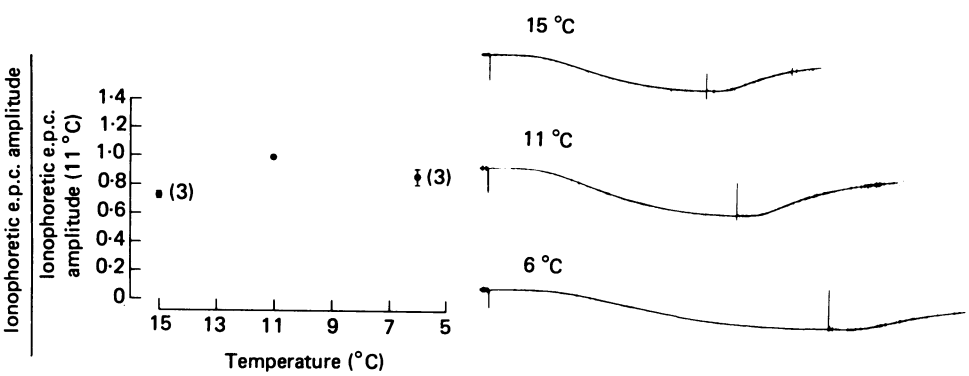

Fig. 3. Left panel: the effects of temperature on the amplitude of e.p.c.s in response to set doses of ionophoretically applied ACh at 15, 11 and 6 °C. All results are normalized to the value at 11 °C. The S.E. of the mean and the number of experiments are indicated by the bars and the numbers in parentheses. Right panel: sample records showing the e.p.c.s elicited by a set dose of ionophoretically applied ACh at an end-plate at 15, 11 and 6 °C.

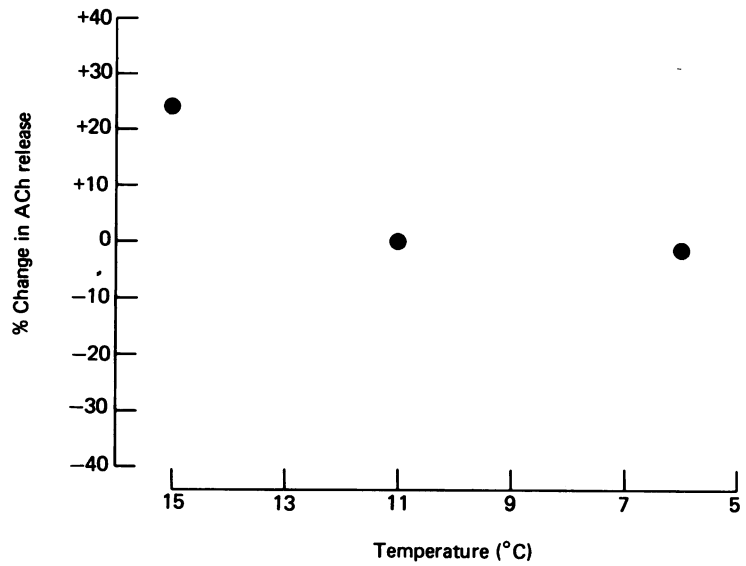

Fig. 4. The change in the amount of ACh released in a quantum required to account for the amplitude of the e.p.c. elicited by ionophoretically applied ACh by using the estimates of channel opening and closing rates obtained from m.e.p.c.s. The results are normalized to 11 °C, where it is assumed that the e.p.c. in response to ionophoretically applied ACh can be correctly predicted from the m.e.p.c. amplitude and decay time. For further explanation see the text.

In interpreting these results it is important to remember that the ACh acts by opening ionic channels in the end-plate, and that the current flowing across the end-plate at any given moment depends both on the rate at which channels are opening and on how long they remain open (Dionne & Stevens, 1975). The magnitude of an end-plate current, induced by ACh ionophoresis, I_0, is related to the rates of channel opening and closing as follows.

$$I_0 \propto \frac{\text{rate of channel opening}}{\text{rate of channel closing}}. \quad (1)$$

A m.e.p.c. is generated by ACh acting briefly on the end-plate membrane; the current rapidly reaches a peak and then decays. The peak is reached rapidly compared to the channel lifetime; therefore the channel opening rate is important in determining the amplitude, while the closing rate has no significant effect (Dionne & Stevens, 1975).

$$I_{\text{m.e.p.c}} \propto \text{rate of channel opening}. \quad (2)$$

According to equns. (1) and (2), the effect of temperature on the amplitude of the ionophoretically generated e.p.c.s should be predictable from the effects of temperature on the amplitude and the half-decay time of the m.e.p.c.s. The amplitude of the m.e.p.c.s is proportional to the opening rate (equn. 2), while the half-decay time is inversely proportional to the closing rate. Fig. 4 shows the results of calculations

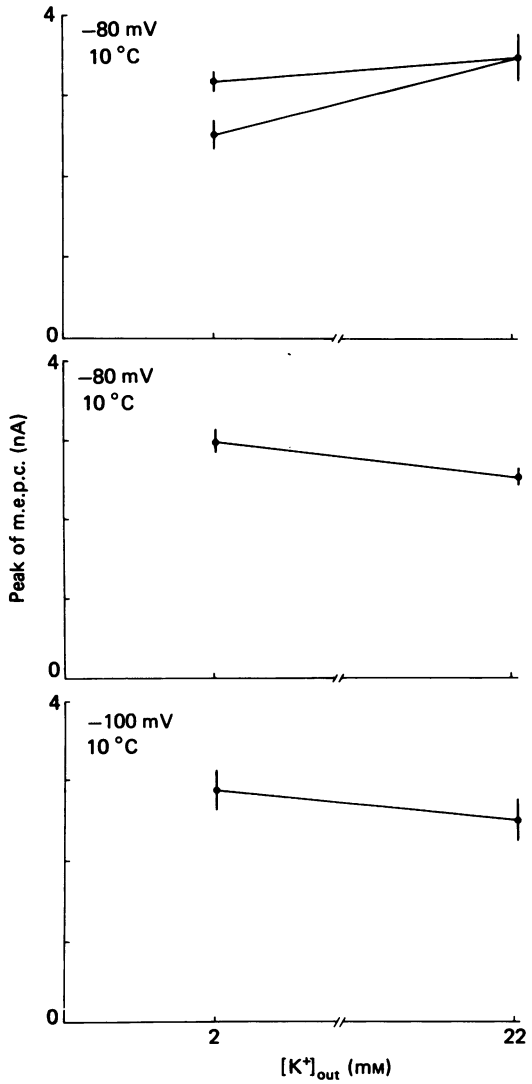

Fig. 5. Three examples of the effects of changing $[K^+]_{out}$ on the mean m.e.p.c. amplitudes. Each point shows the mean value, the vertical bars show ± S.E. of the mean. The holding potentials from each example are shown on the Figure. In the example in the upper panel the m.e.p.c.s were first recorded at 2·0 mM-extracellular K^+, then at 22 mM-extracellular K^+ and then once again at 2·0 mM extracellular K^+. There is no consistent, significant change in the m.e.p.c. amplitudes, although there must be a substantial change in the membrane potential of the nerve terminal.

in which the m.e.p.c. characteristics were used to estimate the ionophoretically generated e.p.c. All results are plotted relative to 11 °C, the differences between prediction and observation are illustrated as changes in ACh release.

Fig. 4 shows that, although the m.e.p.c. amplitude changes by a factor of almost 3 owing to the 9 °C temperature change, this is almost entirely accounted for by

changes in end-plate sensitivity, revealed by the ionophoresis experiments. Considering the limited accuracy of the experimental methods and the approximations in the simple theory, there seems little reason to believe that temperature changes the amount of ACh/quantum.

$[K^+]_{out}$ and m.e.p.c. amplitude

M.e.p.c.s were recorded in solutions containing 2 and 22 mM-K^+. The results of three representative experiments are shown in Fig. 5. There was no consistent, statistically significant change in m.e.p.c amplitude as a result of the change in $[K^+]_{out}$. This conclusion was confirmed in five additional experiments.

The rise in $[K^+]_{out}$ would be expected to increase slightly the m.e.p.c. amplitude, because the reversal potential for the ACh channel would become more positive, increasing the driving force for net ion movement, and also because substitution of K^+ for Na^+ elevates single channel conductance (Gage & Van Helden, 1979). Therefore, the effect of the elevated K^+ on the end-plate current would add to, rather than counteract, an increase in the e.p.c. caused by enhanced ACh release. We conclude that depolarizing the motor nerve terminal does not produce a detectable increase in the amount of ACh/quanta.

If ACh were released as a cation through a channel, and if it obeyed the constant field assumptions, the reduction of the membrane potential from -90 to -30 mV would increase the efflux rate almost five-fold. However, the depolarization might also shorten the mean channel lifetime (Magleby & Stevens, 1972), if it behaved like the ACh-gated channel in the end-plate, so this would decrease the total amount of ACh liberated.

DISCUSSION

Vesicles or gates?

The results make two points clear. First, the quantity of ACh in a quantum is scarcely changed with a 9 °C shift in the temperature. Secondly, the quantity of ACh in a quantum is not appreciably changed by a substantial alteration in the potential across the membrane of the motor nerve terminal. Both results are difficult to account for in a gated-channel model for ACh release.

If the ACh passes through an open channel as a cation, the flux should be notably increased by a decrease in the nerve terminal membrane potential. It is difficult to conceive of a mechanism by which the ACh could pass un-ionized through a channel, combined with an anion, and then rapidly dissociate from its strong combination with the unidentified anion. Of course it is conceivable that the change in the electrochemical gradient is by chance balanced by changes in channel lifetime due to the nerve terminal depolarization. At present we cannot rule out this alternative.

One can imagine a channel in which a fall in temperature produces two antagonistic effects: an increase in the open time and a decrease in the channel conductance. The two effects might balance out so that a temperature decrease has little effect on the total charge passing out of the open channel. It is somewhat implausible that the two effects should be so precisely balanced that a 9 °C temperature change produces little or no detectable effect.

Although the effects of temperature and membrane potential changes are difficult to account for by a channel model for ACh release, they are readily and obviously

accounted for by a model in which the ACh is pre-packaged in a vesicle and is released by exocytosis. Similarly, the quantal size is not changed by abrupt shifts in the osmotic pressure of the Ringer solution which would be expected to change the volume of the nerve terminal by a factor of 2 (Van der Kloot, 1978). The volume change would be expected to abruptly change the [ACh] in the cytoplasm of the nerve terminal. If the quantal ACh is coming out of a channel, the change in concentration surely should change quantal size.

Other models for quantal release

It is not easy to think of alternative models that are also insensitive to changes in temperature, [ACh] and membrane potential. Israel, Dunant & Manaranche (1979) considered a model in which there is a membrane protein that binds a fixed number of ACh molecules, which are then carried through the membrane to be exchanged on the outer face for cations from the extracellular solution. Such models face the problem that a carrier protein of such a size would almost surely have an appreciable electrical charge. The energy required to move an ion with a valence of two or three through a lipid bilayer is already high (Laüger & Neumcke, 1973), particles with still higher valences would face almost insuperable energetic difficulties.

Problems faced by the vesicle hypothesis

On the other hand, the biochemical evidence is strongly against the idea that all of the vesicles observed in the nerve terminal are equally likely to release their contents of transmitter, since the most recently labelled ACh seems to be released preferentially. This could be accounted for by the 'operative vesicle' model of Israel *et al.* (1979). A subpopulation of vesicles that fill rapidly with cytoplasmic ACh, release their contents, and then are rapidly reformed and reloaded. The remainder of the vesicles might contain a reserve store of transmitter.

From the electrophysiological viewpoint, we believe that the principal discrepancy between observation and the idea of exocytotic release is the occasional presence of 'monstrous' m.e.p.c.s (Crawford & McBurney, 1976; Cohen & Van der Kloot, 1980, Colmeus, Gomez, Molgo & Thesleff, 1982), with slow rise times and often lasting much longer than usual. It is difficult to see how the slow rises could be generated if the ACh is released by exocytosis almost instantly into the synaptic cleft (Rosenberry, 1979; Wathey, Nass & Lester, 1979). The 'monstrous' m.e.p.c.s will be discussed further (W. Van der Kloot & I. S. Cohen, in preparation).

Low temperature and end-plate ACh sensitivity

The results show that the amount of ACh/quantum is almost unaffected by reducing the temperature from 15 to 6 °C, but the m.e.p.c amplitude is decreased by a factor of 2·5. Gage & McBurney (1975) examined m.e.p.c. amplitudes over the range from 26 to 10 °C. Their Q_{10} was 1·5. Our results over the range 30 to 15 °C agrees with a Q_{10} of about 1·5 (Fig. 1). The surprising finding is the high Q_{10} of 2·9 over the range 15–6 °C. The fall in response to ACh could be owing to changes in the affinity of the receptor to ACh, to changes in the opening rate constant for the channel, or to a reduction in the actual number of channels available at low temperature. The data of Neher & Sakmann (1976) suggest that the single channel currents are not changed sufficiently by a fall in temperature to account for the result.

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