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QUANTAL RELEASE OF ACETYLCHOLINE EVOKED BY FOCAL DEPOLARIZATION AT THE *TORPEDO* NERVE–ELECTROPLAQUE JUNCTION

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SUMMARY

1. To analyse evoked acetylcholine (ACh) release in the electric organ of *Torpedo marmorata*, a loose patch-clamp technique was used that allowed with a single extracellular electrode both focal depolarization of nerve endings and recording of the post-synaptic currents produced by the released transmitter.

2. Two different types of post-synaptic response could be evoked by depolarizing pulses of increasing intensity: a graded response appearing with a delay of 0·6 ms (pulses of 0·2 ms duration), and an all-or-none response characterized by a mean delay of 1·4 ms. Both responses had a similar maximal amplitude and a similar rise time of 0·6 ms.

3. The graded response was evoked in all places where spontaneous miniature electroplaque currents (m.e.c.s) could be recorded. It was not modified by 1 μM-tetrodotoxin (TTX), but was Ca^{2+} dependent and was abolished by Cd^{2+} (0·2 mM) or Mg^{2+} (10 mM).

4. The all-or-none response could be evoked in only 30 % of places where m.e.c.s were recorded, it was highly TTX sensitive, Ca^{2+} dependent, and abolished by Cd^{2+} (0·2 mM) or Mg^{2+} (10 mM).

5. K^+ channel blocking agents, such as 4-aminopyridine (4-AP) or tetraethylammonium (TEA), which are known to prolong the duration of action potentials, prolonged the delay of the all-or-none response, but not that of the graded response.

6. At low strength stimulation, the graded response was clearly evoked in a quantal way, with the quantum corresponding to the amplitude of spontaneous m.e.c.s. The amplitude distribution of the evoked responses closely followed a Poisson distribution.

7. The maximum synchronous release of transmitter was found to be approximately 1·3 quanta/μm^2 of presynaptic membrane and a mean quantal size of about 7000 ACh molecules was estimated from the charge transfer of m.e.c.s.

8. The nerve terminal time constant was calculated from strength–duration curves obtained with depolarizing pulses just able to evoke either the all-or-none response or the first few quanta of the graded response. Respective mean values of 0·22 and 0·40 ms were found.

9. Increasing the duration of the depolarizing pulse had two consequences: it

differently affected the delay of the all-or-none response and that of the graded response; it increased the mean quantal content of the graded response. Both effects could not simply be accounted for by the influence of the nerve terminal time constant.

10. It is concluded that focal depolarizing pulses, applied to *Torpedo* nerve electroplaque junctions, activate Na^+ channels and generate presynaptic action potentials only at limited areas of the nerve terminal arborization. In these and in all other places, presynaptic voltage-dependent Ca^{2+} channels can be activated directly, resulting in the production of a post-synaptic response composed of a graded number of quanta. Thus, despite several differences in the morphological organization of the synapses, the electrophysiological characteristics of transmitter release in the *Torpedo* electric organ seem to be very similar to those of neuromuscular junctions.

INTRODUCTION

Although the electric organ of the fish *Torpedo marmorata* is embryologically homologous to neuromuscular systems, it differs from them in some important aspects. Morphologically, in the electric organ, the nerve endings are organized in an extremely ramified network and the synaptic vesicles have a diameter twice as large as in neuromuscular junctions. The 'active zones', that characterize the presumed sites of transmitter release in motor nerve terminal membrane (Couteaux & Pécot-Dechavassine, 1970), are not present in the electric organ. Evoked transmitter release is quantal at the neuromuscular junction (see Katz, 1969) and rapid-freezing experiments have shown that transmission of nerve impulses is accompanied by the occurrence of vesicle openings in the presynaptic plasmalemma (Heuser, Reese, Dennis, Jan, Jan & Evans, 1979). These and other observations support the view that the neurotransmitter is released from the synaptic vesicles by a process of exocytosis.

Experiments carried out on the *Torpedo* electric organ have led to a different hypothesis for acetylcholine (ACh) release. Biochemical analyses performed after and during transmitter release have suggested that vesicular ACh behaves as a rather stable pool, that is not immediately mobilized on stimulation. In contrast, there appears to be a pool of cytoplasmic ACh that is rapidly used and renewed on activity and therefore has been considered as the immediate source for transmitter release (Dunant, Gautron, Israël, Lesbats & Manaranche, 1972; Israël & Lesbats, 1981; Dunant, Jones & loctin, 1982). Moreover, transmission of a single nerve impulse at the nerve electroplaque junction is apparently not accompanied by vesicle openings, but by a transient increase of large intramembrane particles (Israël, Manaranche, Morel, Dedieu, Gulik-Krzywicki & Lesbats, 1981; Dunant, Muller, Parducz, Jones & Garcia-Segura, 1984; Garcia-Segura, Muller & Dunant, 1986). Also, in a reconstituted system from the *Torpedo* electric organ, ACh release can be elicited in the absence of synaptic vesicles (Israël, Lesbats, Morel, Manaranche, Gulik-Krzywicki & Dedieu, 1984). These observations point to a release system which is inserted in the presynaptic membrane and uses preferentially cytoplasmic ACh.

These discrepancies made it interesting to further analyse the properties of ACh release in the *Torpedo* electric organ by using electrophysiological techniques. A major difficulty in this is the high density of the synaptic innervation in this

preparation. The difficulty has been overcome in the present study by means of a loose patch-clamp technique (Stühmer, Roberts & Almers, 1983; Dudel, 1983), which has the great advantage of allowing use of a single electrode for both focal depolarization of a nerve ending by injecting a current pulse, and recording of the post-synaptic response produced by the release transmitter.

Using this approach, we have first studied the excitability properties of the nerve endings in relation to their function of releasing transmitter. Secondly, we have demonstrated, at this junction, the quantal nature of evoked ACh release and determined qualitatively and quantitatively some of its properties. Finally, we have analysed the excitation–secretion coupling characteristics of *Torpedo* nerve terminals by recording the post-synaptic responses to depolarizing pulses of various strength and duration.

The same technique was used in a second study (Muller, 1986), the aim of which was to investigate the mechanisms by which transmitter release is potentiated by 4-aminopyridine (4-AP) and other substances in this preparation.

METHODS

The fish *Torpedo marmorata* were supplied by the Station de Biologie marine, Arcachon, France. Substances used were: tetrodotoxin (TTX) obtained from Calbiochem, 4-aminopyridine (4-AP) and tetraethylammonium chloride (TEA) from Merck; echothiopate iodide (Phospholine) was a generous gift from Ayerst Laboratories.

The *Torpedo* was anaesthetized by tricaine methane sulphonate (MS 222, Sandoz, Basle, Switzerland) at a concentration of 1 g/3 l of sea water. Controls have shown that this brief anesthesia is rapidly reversible and has no effect on nerve electroplaque transmission. Slices of electric organ were excised and kept in an elasmobranch physiological medium of the following composition (in mM): NaCl, 280; KCl, 7; $CaCl_2$, 4·4; $MgCl_2$, 1·3; $NaHCO_3$, 5; HEPES, 20; urea, 300; glucose, 5·5. This medium was gassed with 95 % O_2 and 5 % CO_2; its pH was adjusted to between 7·1 and 7·3. All experiments were carried out at room temperature (18–22 °C).

Recording of post-synaptic currents

A small fragment of electric organ containing three to four prisms (stacks of electroplaques), was excised and sectioned transversely into a thin slice of tissue. It was then placed with the ventral innervated faces uppermost in a small Plexiglas chamber coated with Sylgard and maintained under continuous superfusion with physiological saline medium. Stimulation and recording were done by carefully positioning an extracellular borosilicate glass micro-electrode on the superficial electroplaque. The electrodes were produced using a BB-CH puller (Mecanex, Geneva, Switzerland). They were broken to a 20–50 μm outer diameter and the tip melted in a microforge. An optimum electrode had a tip inner diameter of 5–10 μm and a resistance of about 0·3 MΩ when filled with saline medium. The tip resistance to ground was increased to about 1 MΩ after carefully pressing the electrode against the innervated face of an electroplaque and applying a slight suction to the inside of the pipette, which considerably improved the recording stability and the signal-to-noise ratio. The positioning of the electrode was performed under visual control using a dissection binocular microscope. Since the nerve terminal arborizations could not be distinguished, experiments were carried out only at those places where spontaneous miniature electroplaque currents (m.e.c.s) were recorded. Current pulses of 0·1–10 μA amplitude and 0·1–10 ms duration were injected through the recording electrode by means of a loose patch-clamp amplifier built by the Laboratoire d'Electronique of the University of Geneva. In order to balance the residual artifact arising in the electrode after the pulse, two compensating exponential wave forms of adjustable amplitude and time constant were added to the recorded traces after the head stage of the amplifier. The compensation was usually adjusted at the beginning of an experiment and then kept identical till the end. With this procedure, the recorded traces could be faithfully interpreted as quickly as 0·2 ms after the end of the depolarizing pulse.

All traces were stored on a FM tape recorder (HP 3964A) at 19·05 cm/s, band with DC-5000 Hz. For analysis of amplitudes and synaptic delays, slowed taped recordings were fed into a microprocessor (type SSM 8080A, San Jose, CA, U.S.A.) after 8-bit digitalization at an equivalent of 30 kHz. The parameters of each trace were calculated using a program written in FORTRAN, previously tested with known artificial signals. These were stored on a floppy disk for further statistical analysis.

Estimation of the maximal rate of transmitter release

We used electrodes of different sizes to determine the number of quanta released per unit surface of presynaptic membrane during transmission of a nerve impulse. For each electrode used, the surface of presynaptic membrane involved in the release of the transmitter which was responsible for the recorded currents was estimated to represent 50 % of the surface covered by the opening of the tip of the electrode. This value was obtained from morphological pictures in two different ways. We first analysed freeze-fractured replicas of quickly frozen tissue (Garcia-Segura *et al.* 1986) in which the fracture plane was parallel to the innervated face of an electroplaque, so exposing the nerve terminal network (see Pl. 1). We found that the surface covered by presynaptic nerve endings corresponded to 47 % of the 240 μm^2 of innervated electroplaque membranes analysed. Alternatively, we also measured the area of electroplaque covered by nerve endings on conventionally fixed tissue in transverse sections of electroplaques. In this case too, we found a value of approximately 50 %. The surface under the opening of the electrodes was calculated from the inner tip diameter of each electrode, as measured in a microscope. In all the experiments, the maximum rate of transmitter release has been calculated as the ratio of the maximal evoked post-synaptic currents over the mean amplitude of spontaneous m.e.c.s.

Estimation of the charge transfer responsible for m.e.c.s

Because of the loose patch-clamp technique used, the recorded post-synaptic responses (I_e) do not actually reflect the total post-synaptic currents generated (I_t). These, however, can be calculated according to the following relationship (Neher, Sakmann & Steinbach, 1978):

$$I_t = I_e . R_t / (R_t - R_e),$$

where R_e is the electrode resistance and R_t the total resistance to ground measured once the electrode was applied on the tissue. Using this procedure we calculated the mean amplitude of spontaneous m.e.c.s before and after treatment with a cholinesterase inhibitor, and, from the integral of m.e.c.s, the post-synaptic charge transfer produced by the release of one ACh quantum. Considering a mean open time of 1·3 ms and a unitary conductance of 20 pS as single-channel characteristics in the *Torpedo* electric organ (Schindler & Quast, 1980), it was possible to estimate the number of ACh receptors activated by one quantum and therefore the number of ACh molecules presumably contained in one quantum.

Analysis of the nerve terminal time constant

We calculated the nerve terminal time constant (τ), as Datyner & Gage (1982), from strength–duration curves, according to the following equation:

$$I = I_{rh}/[1\text{-}\exp(-t/\tau)],$$

where I is the amplitude and t the duration of the depolarizing current pulse and I_{rh} the amplitude of the minimal rheobasic current, estimated as the amplitude of a 2 ms duration pulse. For each curve characterizing a single nerve ending ramification, a mean time constant was calculated from the individual values obtained for the six to ten different points of the curve.

RESULTS

Effect of graded depolarizing pulses

By applying an electrode of the type described in Methods to the superficial electroplaque of a dissected prism, it was possible, in the cases where the innervated face was on top, to record spontaneous m.e.c.s in a very reproducible way. The membrane potential of the nerve endings present under the opening of the electrode

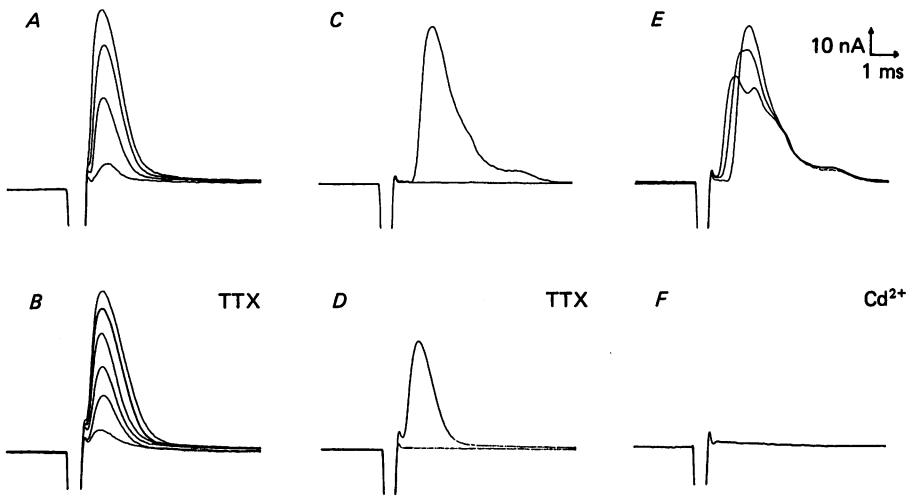

Fig. 1. Post-synaptic currents recorded in the *Torpedo* electric organ in response to focally applied depolarizing pulses. For convenience, they were illustrated as upward-going signals. Pulses of increasing intensity evoked after a short delay a graded response (*A*), which was not sensitive to 1 μM-TTX (*B*). At some places, an all-or-none response characterized by a longer delay was evoked by pulses of low intensity (*C*). This all-or-none response was abolished by 1 μM-TTX, but by increasing the pulse strength, it was always possible to obtain a graded response at the same place (*D*). In the absence of any drug, both responses could be evoked simultaneously, but the appearance of the graded response was then accompanied by a progressive decrease in the amplitude of the all-or-nothing response (*E*). Both responses were abolished in the presence of 0·2 mM-Cd^{2+} (*F*).

was then modified by injecting depolarizing current pulses through the same electrode (Katz & Miledi, 1967; Dudel, 1983), resulting in the generation of post-synaptic currents. The *Torpedo* electric organ has an advantage over neuromuscular preparations in that all responses obtained in this way reflect only transmitter release, since the *Torpedo* electroplaques are not electrically excitable (Bennett, Wurzel & Grundfest, 1961).

In the absence of any drug, two different types of response were evoked. The first type presented a graded amplitude as a function of the intensity of the depolarizing pulse. It appeared, with pulses of 0·2 ms duration, after a mean delay of $0{\cdot}61 \pm 0{\cdot}03$ ms (mean $\pm$ S.E. of mean of thirty-seven responses) after the onset of the pulse and was characterized by a mean rise time of $0{\cdot}60 \pm 0{\cdot}02$ ms (mean $\pm$ S.E. of mean of thirty-seven responses; Fig. 1*A*). The graded response was present in all places where m.e.c.s could be recorded. It was insensitive to 1 μM-TTX (Fig. 1*B*), but was Ca^{2+} dependent and abolished by either 0·2 mM-Cd^{2+} or 10 mM-Mg^{2+} (Fig. 1*F*). For these reasons, it was concluded that the depolarizing pulses responsible for this first type of response directly activated the voltage-dependent Ca^{2+} channels presumably present in the nerve terminal membranes.

The second type of response was characterized by an all-or-none behaviour. It had a constant amplitude and appeared at a very precise threshold level of current pulse intensity after a relatively long delay of $1{\cdot}43 \pm 0{\cdot}03$ (mean $\pm$ S.E. of mean of nineteen

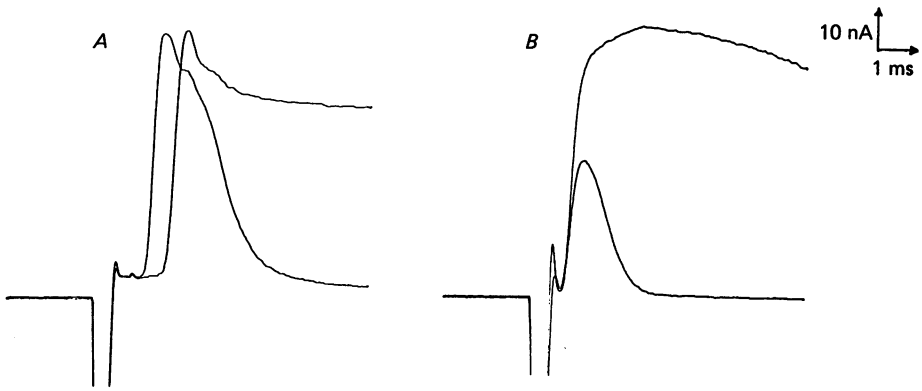

Fig. 2. Prolongation by 100 μM-4-AP of the synaptic delay of the all-or-none response (*A*) but not of the graded response (*B*). Note that the duration of both responses was greatly increased by 4-AP.

responses) regardless of the pulse duration (Fig. 1*C*). Its time to peak was similar to that of the graded response. The all-or-none response could be evoked in only 30 % of places where m.e.c.s were recorded. This proportion depended upon the size of the electrode, increasing with a larger electrode diameter. When present, the all-or-none response was usually elicited by a relatively small depolarizing pulse. By increasing further the intensity of the pulse, it was possible to elicit a graded response at the same place (Fig. 1*E*). This was accompanied by a simultaneous and progressive decrease in the amplitude of the all-or-none response. In these places, the maximum amplitude of the graded responses was usually found to be equal to the maximum amplitude of the all-or-none response, suggesting that the same number of releasing sites were activated through two different excitation mechanisms. Also different from the graded response, the all-or-none response was highly TTX sensitive. It was abolished in the presence of 1 μM-TTX, whereas the graded response could still be evoked (Fig. 1*D*). The all-or-none response was also blocked by omitting Ca^{2+} from the superfusing medium or by adding 0·2 mM-Cd^{2+}, or 10 mM-Mg^{2+} (Fig. 1*F*). All these observations support the idea that a Na^+-dependent presynaptic action potential is involved in the generation of the all-or-none response. Furthermore, that this type of response could not be evoked in all places where m.e.c.s were recorded, suggests that, in the electric organ, Na^+ channels are not present on the whole surface of the nerve ending ramifications.

Effects of 4-AP and TEA on the synaptic delay

For short depolarizing pulses, the synaptic delay, measured as the time between the onset of the depolarizing pulse and the onset of the post-synaptic currents, was found to be significantly different for the two types of response described above ($P < 0{\cdot}001$). Since these two responses differed primarily in their TTX sensitivity, the longer delay of the all-or-none response is likely to be related to the generation of a presynaptic action potential. To further test this possibility, we measured the synaptic delays in the presence of 4-AP and TEA, two K^+ channel blocking agents (Yeh, Oxford, Wu & Narahashi, 1976) which are known to prolong the duration of

the action potential in non-myelinated nerve fibres (Den Hertog, Pielkenrood, Biessels & Agoston, 1983).

As illustrated in Fig. 2, two effects were observed with 100 μM-4-AP: a potentiating action on ACh release characterized by a prolongation of the post-synaptic responses (Corthay, Dunant & Loctin, 1982; Muller, 1986) and a significant increase in the delay of the all-or-none response (from 1·4 ms to 2·2 ms, $n = 12$, $P < 0{\cdot}001$), but not of the graded response. TEA at a concentration of 1 mM had similar effects. It seems therefore that the longer delay of the all-or-none response is related to the generation of a presynaptic action potential and somehow reflects in magnitude the duration of this presynaptic action potential.

Quantal nature of evoked transmitter release

In the presence of physiological Ca^{2+} and Mg^{2+} concentrations, when adjusting the intensity of the depolarizing pulse just above the threshold level for a graded response, it was observed that the amplitude of recorded currents fluctuated from one trial to the next. These fluctuations corresponded to integral multiples of the smallest current responses evoked. This is illustrated in Fig. 3. The amplitude distribution of 178 responses obtained with a series of pulses of constant intensity showed multiple peaks at regular intervals. The amplitude and the distribution of the first peak corresponded to those of the spontaneous m.e.c.s recorded at the same place. In this experiment, the mean quantal content was 2·09 and the interval characterizing the individual evoked quanta 1·8 nA; the number and distribution of evoked responses and of failures corresponded closely to that predicted by a Poisson law (Boyd & Martin, 1956; Katz, 1969; see Table 1). Similar results were found in four different preparations.

Maximal synchronous release and size of the quantum

One way to calculate the quantal content of evoked current responses is to divide their amplitude by the mean amplitude of spontaneous m.e.c.s (see Katz, 1969). This was done in Table 2 with graded responses of maximal amplitude and with all-or-none responses. The results of both were pooled since, as stated above, no significant difference was observed between their maximal amplitude in a given preparation. As shown in Table 2, increasing the size of the electrode was accompanied by an increase in the maximal quantal content of evoked responses. We therefore expressed this quantal content in relationship with the surface of presynaptic membrane present under the opening of the electrode, as described in Methods. Doing so, we then obtained a very constant value of about 1·3 quanta/μm^2 of presynaptic membrane.

Information about the size of one quantum could also be gained by analysing the change in amplitude of m.e.c.s before and after treatment with an irreversible inhibitor of cholinesterase and by calculating the charge transfer involved in a m.e.c. from the integral of the current response. After correction of recorded responses according to the values of the shunt resistance and the electrode resistance (see Methods), we obtained the following results: (i) the mean amplitude $\pm$ S.E. of mean of m.e.c.s increased from $3{\cdot}8 \pm 0{\cdot}2$ nA (number of recording sites (n) = 32) to $5{\cdot}2 \pm 0{\cdot}3$ nA ($n = 20$) after treatment with 50 μM-Phospholine; (ii) the mean charge transfer ($\pm$ S.E. of mean) involved in a m.e.c. before treatment with Phospholine was

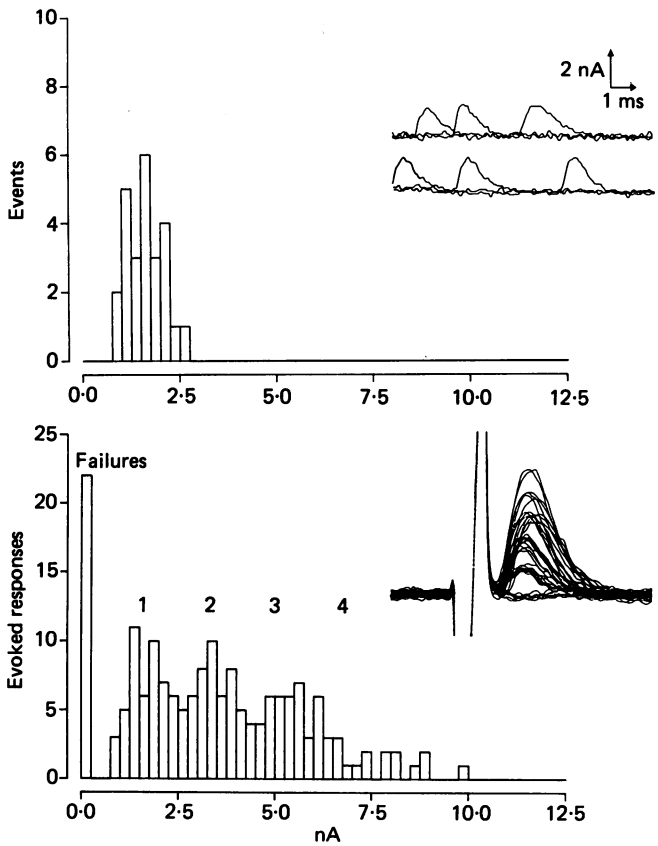

Fig. 3. Quantal release of transmitter at the *Torpedo* nerve–electroplaque junction. Upper picture: spontaneous m.e.c.s and their size distribution as recorded through the loose patch-clamp electrode. Lower picture: responses evoked in the presence of physiological Ca^{2+} and Mg^{2+} concentrations, by pulses of constant intensity near to the threshold level of the graded response. The amplitude of the post-synaptic currents varied stepwise and the magnitude of one step corresponded to the mean amplitude of spontaneous m.e.c.s. The amplitude distribution clearly shows, besides a number of failures, the presence of multiple peaks which correspond to the release of a different number of units.

TABLE 1. Statistical analysis of quantal transmitter release in the *Torpedo* electric organ

Number of quanta	Failures	1	2	3	4	5	6	7
Predicted	22	47	48	33	17	7	3	1
Observed	22	45	46	34	21	8	2	0

Observed data are from the experiment illustrated in Fig. 3. 178 responses were elicited by pulses of constant intensity. The mean quantal content of evoked responses was 2·09 and the mean amplitude of an individual quantum 1·8 nA. An excellent correlation has been found between the distribution of observed responses and that predicted by a Poisson law (see Katz, 1969).

TABLE 2. Maximum synchronous release of transmitter in the *Torpedo* electric organ

Electrode inner tip diameter	6·5 μm	8 μm	12 μm
Maximal quantal content of evoked responses	20·9 ± 0·75 $n = 9$	31·5 ± 1·5 $n = 11$	69·2 ± 5·1 $n = 9$
Maximal quantal release/ μm^2 of presynaptic membrane	1·26 ± 0·05 $n = 9$	1·25 ± 0·067 $n = 11$	1·22 ± 0·09 $n = 9$

The maximal quantal contents were calculated as the ratio of the maximal amplitude of evoked currents (both all-or-none responses and graded responses) over the mean amplitude of spontaneous m.e.c.s recorded at the same place. They were measured with three electrodes of different sizes from three different *Torpedoes*. Results were then reported to the surface of presynaptic innervating membrane, estimated to represent 50% of the surface covered by the opening of the electrode.

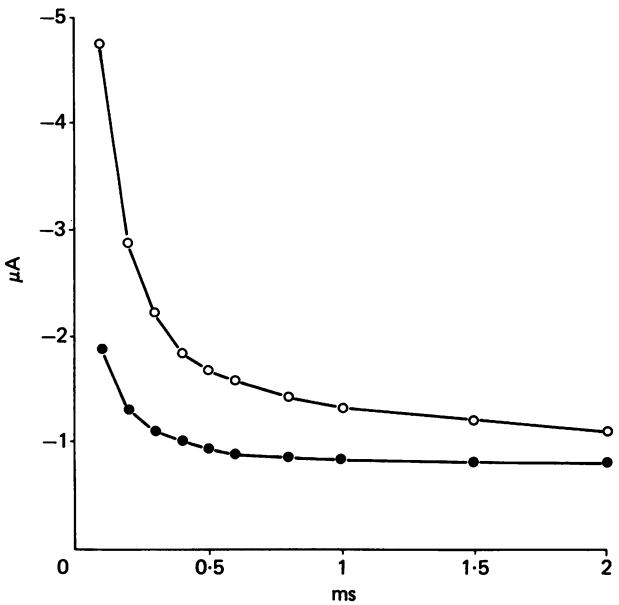

Fig. 4. Strength–duration relationships for threshold pulses just able to evoke the graded response (○) and the all-or-none response (●). The time constant of nerve terminals was calculated from these curves and found to be of 0·40 and 0·22 ms respectively.

4·7 pC. These results were used to estimate the number of ACh receptors activated by the release of one quantum and therefore estimate the number of ACh molecules contained in one quantum (see Discussion).

Time constant of presynaptic nerve terminals

The duration of the depolarizing pulses was observed to influence both the time course and the quantal content of evoked responses. To better understand these effects, it was important to first determine the nerve terminal time constant. This was done by analysing strength–duration curves obtained with pulses whose intensity was just sufficient to evoke either the all-or-none response or the first quanta of the graded response. Two examples are shown in Fig. 4. Although the curves obtained

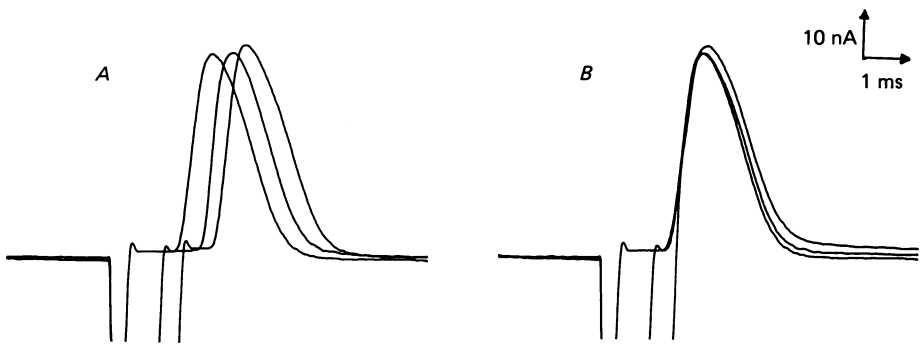

Fig. 5. Effect of pulse duration on the delay of the all-or-none response. In *A* the pulse intensity was adjusted for each duration so as to just reach the response threshold. In *B* the all-or-none response was elicited by pulses of constant intensity.

with both types of response looked very similar, they differed in three ways. The level of depolarization required to evoke the graded response was always found to be higher than that for the all-or-none response. The mean time constant obtained with the graded response ($0{\cdot}40 \pm 0{\cdot}03$ ms; $n = 9$) was significantly larger than that obtained with the all-or-none response ($0{\cdot}22 \pm 0{\cdot}02$ ms; $n = 7$, $P < 0{\cdot}001$). The rheobasic current intensity for eliciting the graded response did not reach a steady level with pulses three to five times longer than the time constant, as was observed with the all-or-none response.

Effects of pulse duration on the synaptic delay

Increasing the duration of depolarization had different effects on the delay of the all-or-none response and of the graded response. Delays were always considered as the time between the onset of the depolarizing pulse and the onset of the post-synaptic currents. As shown in Fig. 5*A* increasing the duration of the depolarizing pulse could prolong the delay of the all-or-none response, when the intensity of the pulse was adjusted for each pulse duration so as to just reach the threshold level of the presynaptic action potential. In this case, the increase in delay corresponded to the increase in pulse duration up to a maximal delay increase of 0·6–0·8 ms, that is about three times the value of the time constant obtained for the all-or-none response. On the other hand, when the intensity of the pulse was kept constant, increasing its duration did not modify the delay (Fig. 5*B*). These observations are therefore in agreement with the results shown in Fig. 4, that a nerve terminal time constant determines the time course of the presynaptic depolarization produced by the stimulating pulses.

In contrast, in the case of the graded response, an increase in delay by a prolongation of the pulse duration was observed not only when the intensity of each pulse was adjusted so as to evoke only a few quanta (Fig. 6*A*), but also when the intensity of the pulse was kept constant (Fig. 6*B*). This result therefore indicates that with pulses shorter than 1·5 ms no release could occur during the course of a depolarizing pulse.

We also noticed (see Fig. 6*B*) a constant latency of about 0·3 ms between the end

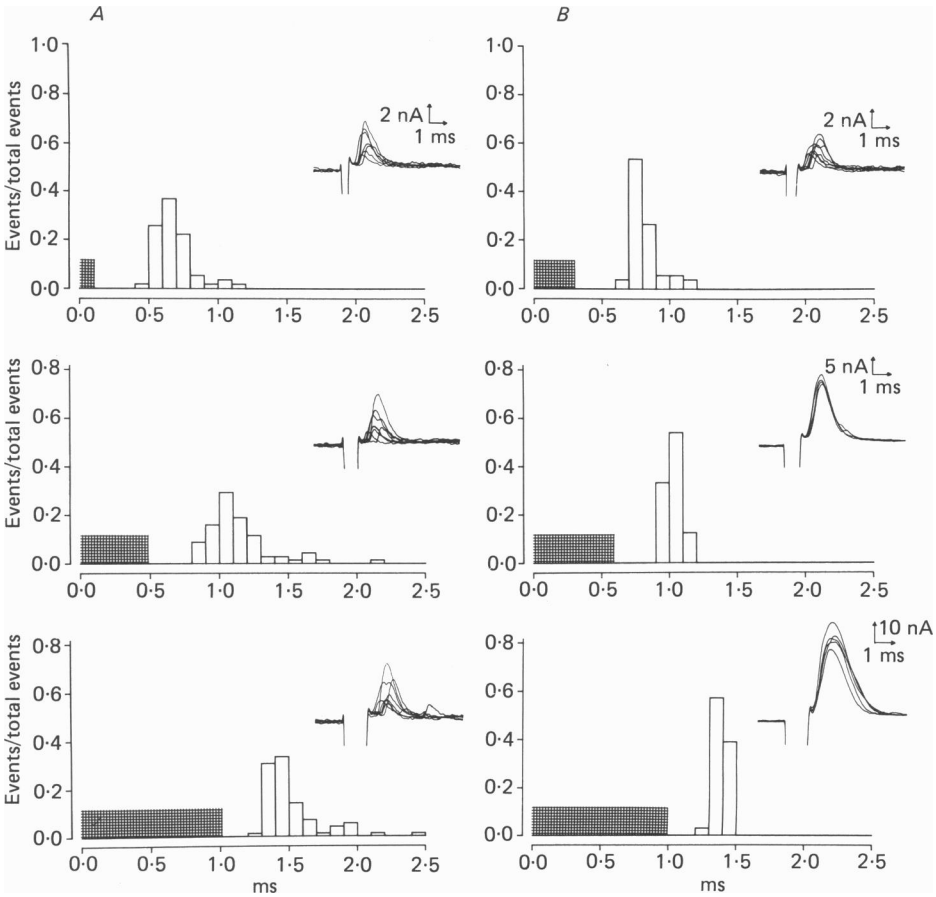

Fig. 6. Effect of pulse duration on the delay of the graded response. In *A* the pulse intensity was adjusted for each duration so as to only evoke a few quanta. In *B* the pulse intensity was kept constant. In both cases, the delay was prolonged by approximately the increase in pulse duration. In *B* an increase in the mean quantal content as well as a narrowing of the delay distribution can also be observed. Results are represented as delay histograms and expressed in each condition as the ratio over the total number of events.

of the pulse and the earliest response evoked. This latency also corresponded to the minimum delay obtained with the shortest possible pulses, and might therefore reflect, in part, the time required by the release process (Llinas, Steinberg & Walton, 1981).

Effects of pulse duration on the mean quantal content of the evoked response

The prolongation of a given depolarizing pulse was also accompanied by a considerable increase in the quantal content of the graded response (see Fig. 6*B*). This effect is illustrated in Fig. 7 for pulses of different durations, showing for each duration the relationship between pulse strength and quantal content. Results were expressed as a Hill plot and it appeared that they could be fitted by straight lines

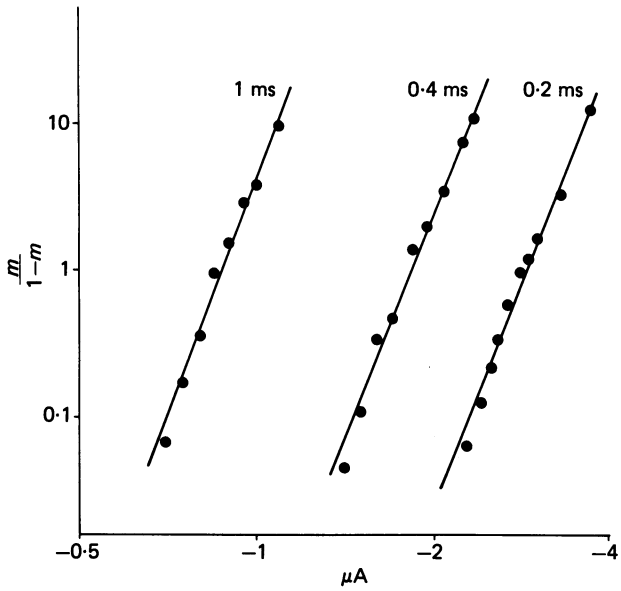

Fig. 7. Relationships between quantal content and pulse strength for pulses of different durations (0·2, 0·4, 1 ms). The quantal contents (m) were expressed as a percentage of the maximal value, which was 28 in this set of experiments. Results were obtained on the same spot and are represented as a Hill plot. The Hill coefficients were calculated by linear regression. Respective values of 10·8, 10·4 and 11 were found for the different pulse durations.

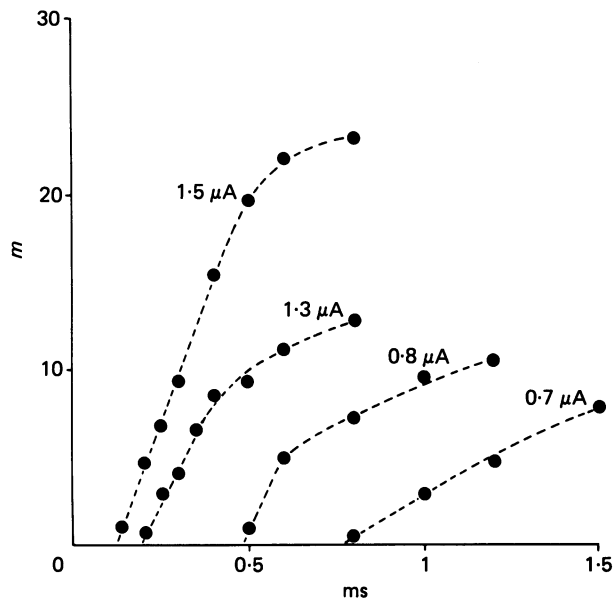

Fig. 8. Effect of pulse duration on the mean quantal content of evoked responses. Each point represents the mean ± S.E. of mean of ten to twenty-two responses.

with correlation coefficients close to 1. It can be seen that increasing the duration of depolarization shifted the relationships to the left but did not modify the Hill coefficient of these relationships. Hill coefficients ranged between 8 and 11 for the different preparations analysed, but were always found to be very close for the same nerve ending ramification. The dependence of release upon pulse duration, as expressed for pulses of different intensity in Fig. 8, shows that the greatest effect occurred with pulses shorter than 0·6–0·8 ms, that is in conditions where the nerve terminal time constant might considerably influence the efficacy of the depolarizing pulse. However, it can also be observed that pulses up to 1·5 ms or even longer, were still able to increase ACh release. It does not seem that, in this case, the time constant of nerve terminals could have played any role. A more likely possibility would be that the duration of the depolarizing pulse also directly influenced the quantal content of the graded response.

DISCUSSION

Functional organization of the presynaptic nerve terminals

Using a focal extracellular electrode both to depolarize a restricted area of the nerve ending arborization and to record the post-synaptic currents evoked in the same area, we observed two types of response in the *Torpedo* electric organ, both of them reflecting ACh release.

The first type of response was characterized by a graded amplitude as a function of the pulse intensity. Its mean delay, with pulses of 0·2 ms duration, was short (0·6 ms). It was TTX resistant, but Ca^{2+} dependent and was antagonized by Cd^{2+} or Mg^{2+}. This graded response resembles very much the post-synaptic response elicited by focal depolarization in TTX-treated neuromuscular and other synapses (Bloedel, Gage, Llinas & Quastel, 1966; Katz & Miledi, 1967; Llinas *et al.* 1981; Dudel, 1983). It most likely results from a direct activation of the voltage-dependent Ca^{2+} channels present in the nerve endings.

The second type of response was elicited by pulses of a relatively low intensity, at a very precise threshold level of depolarization. It appeared in an all-or-none fashion, was TTX sensitive, but also Ca^{2+} dependent and blocked by Cd^{2+} or Mg^{2+}. This all-or-none response was evoked after a longer delay (1·4 ms), that was further increased by K^+channel blockers such as 4-AP or TEA. It was therefore concluded that in this case, ACh release was triggered by a presynaptic action potential and that the delay of this all-or-none response reflected somehow the duration of this action potential. This might be accounted for if, as proposed by Llinas, Sugimori & Simon (1982), the Ca^{2+} currents generated by an action potential appear only during its repolarizing phase. An interesting point, also found at the crayfish neuromuscular junction (Dudel, 1983), was that the graded response could be elicited at practically all places where m.e.c.s were recorded, while the all-or-none response was present at only about 30 % of these places. Since this proportion also depended upon the size of the electrode, it is proposed that Na^+ channels are not uniformly distributed over the surface of the nerve terminal arborization and even that they might be present only in certain regions, such as the preterminal areas where the axons lose their myelin sheet. A similar conclusion was reached by Brigant & Mallart (1982) at a

mammalian neuromuscular junction; this view, however, has recently been contested (Konishi & Sears, 1984). Nevertheless, there is one argument in favour of this explanation in the electric organ. Recent work on *Torpedo* synaptosomes by Meunier (1984) has indicated that the pinched-off presynaptic nerve endings have few Na^+ channels, since their membrane potential and ACh release were very insensitive to veratridine, an agent that acts by opening Na^+ channels.

It can be concluded that, in the *Torpedo* electric organ, Ca^{2+} channels are probably distributed over the whole length of the terminal arborization, whilst Na^+ channels are present only on restricted areas.

Quantal properties of transmitter release

The graded response, elicited by pulses of constant intensity in the presence of physiological Ca^{2+} and Mg^{2+} concentrations, varied in size by steps of regular amplitude, the size of one step corresponding to the mean amplitude of spontaneous m.e.c.s. At a low release rate, it was shown that the probability of evoking 0, 1, 2, 3, or more units followed a Poisson law. Thus, despite the absence of active zones or similar differentiation, evoked transmitter release appears to be quantal in the electric organ as in many other synapses (see Katz, 1969).

By measuring the peak of the post-synaptic currents in areas of different sizes, we concluded that the maximal synchronous release was about 1·3 quanta/μm^2 of presynaptic membrane. The assumption is implied here that the post-synaptic response was not limited by receptor saturation, in which case the value of 1·3 quanta/μm^2 would have been underestimated. We feel, however, that receptor saturation did not greatly alter the responses investigated here, since a good correlation was found in the accompanying paper (Muller, 1986) between the amount of radiolabelled transmitter released and the amplitude of the electroplaque potential as a function of Ca^{2+} concentration. Also, our estimate is similar to those obtained by Kuno, Turkanis & Weakly (1971) at the neuromuscular junction and Nishi, Soeda & Koketsu (1967) at the toad sympathetic ganglion.

From the mean amplitude and charge transfer of m.e.c.s, one can estimate the size of one quantum in the *Torpedo* electric organ. The charge transfer of a m.e.c. makes it possible to calculate the approximate number of ACh receptors activated by the release of one quantum. This was done assuming a resting membrane potential of -70 mV for the electroplaques (Bennet *et al.* 1961), a mean open time of 1·3 ms and a unitary conductance of 20 pS for single *Torpedo mormorata* ACh receptors reconstituted into planar membranes (Schindler & Quast, 1980). The unitary conductance in *Torpedo* compares well with that obtained by noise analysis (Katz & Miledi, 1972; Anderson & Stevens, 1973) or by single-channel recording (Neher & Sakmann, 1976) in neuromuscular preparations. From these values, we estimated that the charge transfer of a m.e.c. corresponded to the activation of about 2600 receptors. Furthermore, the increase in m.e.c. amplitude observed after treatment with 50 μM-Phospholine indicates that there would be about 36% more receptors activated, if a fraction of the released ACh was not immediately destroyed by acetylcholinesterase. Considering these two assumptions and the requirement of two ACh molecules to activate one receptor, one can estimate that about 7000 ACh molecules are needed to open 2600 receptors, thus generating a m.e.c. The actual

number of ACh molecules released in one quantum may still be somewhat higher, since every single molecule cannot be expected to be effective in opening a receptor-linked ion channel. Our estimate, however, seems to be in good agreement with the value obtained by Kuffler & Yoshikami (1975) at the frog and snake neuromuscular junction by a different approach. This similarity, might appear somehow surprising, since in the electric organ, the synaptic vesicles have about twice the diameter or 8 times the volume of those in neuromuscular junctions.

Another important interest of these quantitative analyses arose from the possibility to compare them with the morphological results previously obtained in the electric organ by quick freezing techniques (Dunant *et al.* 1984). Transmission of a single nerve impulse was reported to be accompanied by a large, but very transient (2–3 ms) increase in the number of large presynaptic intramembrane particles. This change, estimated at about 500–600 particles/μm^2, was found, like transmitter release, to be Ca^{2+} dependent. However, considering the present results, which suggest that in those circumstances 1·3 quanta/μm^2 or about 9000 ACh molecules/μm^2 are released, it appears that, at present, no simple quantitative correlation can be established between the morphological and the electrophysiological data.

Time constant of nerve terminals and stimulation–secretion relationship

The values of the nerve terminal time constant that we obtained in the electric organ by analysing strength–duration curves were found to be about 5 times smaller than those obtained in a similar way by Datyner & Gage (1982) at a frog neuromuscular junction. This might result from differences in size or organization of the nerve terminals. The two types of response evoked in the electric organ were also characterized by two distinct time constants which might be accounted for either by a non-linear relationship between the stimulation intensity and the depolarization of the nerve terminals or by the possibility that the two different responses were elicited in distinct regions of the nerve terminal arborization.

When release was evoked by pulses of short duration, the influence of the nerve terminal time constant on the time course and the quantal content of elicited responses was considerable. However, the shift in synaptic delay observed with the graded response for pulses of constant strength, as well as the continuous increase in ACh release produced by pulses more than 3 times longer than the time constant, suggest that the duration of the depolarization by itself also influenced the time course and the quantal content of the evoked response. Similar conclusions were also reached by other authors at various synapses (Katz & Miledi, 1967; Datyner & Gage, 1982; Dudel, 1984*a*, *b*) and different explanations have been proposed (Llinas *et al.* 1981; Dudel, 1984*b*).

Finally it was also observed in these experiments, that, despite the absence of active zones, the relationship between the strength of the depolarizing pulse and the quantal content of evoked responses is as steep in the *Torpedo* electric organ as reported for neuromuscular preparations (Dudel, 1984*a*). This further supports the view that the active zones do not play a determinant role in the effectiveness of ACh release.

Properties of nerve electroplaque transmission

Despite the differences reported between the nerve electroplaque junction and neuromuscular junctions, we were surprised to find that, from an electrophysiological point of view, there is a complete similitude between the two systems. The presence of spontaneous m.e.c.s, the quantal composition of the evoked response, the rate of release per unit surface, the molecular size of the quantum, the stimulation–secretion coupling, the effects of prolonged depolarizations, all the observations reported in the present work were found to be similar to those reported for neuromuscular preparations. Another analogy, which was not mentioned in the present work, is the existence, in the electric organ too, of a subpopulation of small spontaneous miniature potentials (Muller & Dunant, 1985), as described for neuromuscular junctions (Kriebel & Gross, 1974).

The fact that, electrophysiologically, ACh release seems to operate qualitatively and quantitatively in the same way in the two systems, raises several questions: (i) what is the nature of the release mechanism? (ii) what is the functional importance of the active zones in neuromuscular preparation? (iii) how can the present electrophysiological data be correlated with the previous biochemical and morphological results obtained in the *Torpedo* electric organ? These problems will certainly be most interesting for further research on the neurotransmitter release mechanism.

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REFERENCES

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

BENNETT, M. V. L., WURZEL, M. & GRUNDFEST, H. (1961). The electrophysiology of electric organs of marine electric fishes. *Journal of General Physiology* **44**, 757–804.

BLOEDEL, J., GAGE, P. W., LLINAS, R. & QUASTEL, D. M. J. (1966). Transmitter release at the squid giant synapse in the presence of tetrodotoxin. *Nature* **212**, 49–50.

BOYD, I. A. & MARTIN, A. R. (1956). The end-plate potential in mammalian muscle. *Journal of Physiology* **132**, 74–91.

BRIGANT, J. L. & MALLART, A. (1982). Presynaptic currents in mouse motor endings. *Journal of Physiology* **333**, 619–636.

CORTHAY, J., DUNANT, Y. & LOCTIN, F. (1982). Acetylcholine changes underlying transmission of a single nerve impulse in the presence of 4-aminopyridine in *Torpedo*. *Journal of Physiology* **325**, 461–679.

COUTEAUX, R. & PÉCOT-DECHAVASSINE, M. (1970). Vésicules synaptiques et poches au niveau des 'zones actives' de la jonction neuromusculaire. *Comptes rendus hebdomadaires des séances de l'Académie des sciences* **271**, 2346–2349.

DATYNER, N. B. & GAGE, P. W. (1982). Secretion of acetylcholine in response to graded depolarization of motor nerve terminals. *Journal de physiologie* **78**, 412–416.

DEN HERTOG, A., PIELKENROOD, J., BIESSELS, P. & AGOSTON, S. (1983). The effect of some new aminopyridines on mammalian non myelinated nerve fibers. *European Journal of Pharmacology* **94**, 353–355.

DUDEL, J. (1983). Graded or all-or-nothing release of transmitter quanta by local depolarizations of nerve terminals on crayfish muscle? *Pflügers Archiv* **398**, 155–164.

DUDEL, J. (1984*a*). Control of quantal transmitter release at frog's motor nerve terminals. Dependence on amplitude and duration of depolarization. *Pflügers Archiv* **402**, 225–234.

DUDEL, J. (1984*b*). Control of quantal transmitter release at frog's motor nerve terminals. Modulation by de- or hyperpolarizing pulses. *Pflügers Archiv* **402**, 235–243.

DUNANT, Y., GASTRON, J., ISRAËL, M., LESBATS, B. & MANARANCHE, R. (1972). Les compartiments d'acétylcholine de l'organe électrique de la Torpille et leurs modifications par la stimulation. *Journal of Neurochemistry* **19**, 1987–2002.

DUNANT, Y., JONES, G. & LOCTIN, F. (1982). Acetylcholine measured at short time intervals during transmission of nerve impulses in the electric organ of *Torpedo*. *Journal of Physiology* **325**, 441–460.

DUNANT, Y., MULLER, D., PARDUCZ, A., JONES, G. J. & GARCIA-SEGURA, L. M. (1984). Augmentation très brève du nombre de particules dans la membrane présynaptique pendant la transmission d'un influx nerveux. *Comptes rendus hebdomadaires des séances de l'Académie des sciences* **299**, 547–552.

GARCIA-SEGURA, L. M., MULLER, D. & DUNANT, Y. (1986). Increase in the number of presynaptic large intramembrane particles during synaptic transmission at the Torpedo nerve-electroplaque junction. *Neuroscience* (in the Press).

HEUSER, J. E., REESE, T. S., DENNIS, M. J., JAN, J., JAN, L. & EVANS, L. (1979). Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. *Journal of Cell Biology* **81**, 275–300.

ISRAËL, M. & LESBATS, B. (1981). Continuous determination by a chemiluminescent method of acetylcholine release and compartmentation in Torpedo electric organ synaptosomes. *Journal of Neurochemistry* **37**, 1475–1483.

ISRAËL, M., LESBATS, B., MOREL, N., MANARANCHE, R., GULIK-KRZYWICKI, T. & DEDIEU, J. C. (1984). Reconstitution of a functional synaptosomal membrane possessing the protein constituents involved in acetylcholine translocation. *Proceedings of the National Academy of Sciences of the U.S.A.* **81**, 227–231.

ISRAËL, M., MANARANCHE, R., MOREL, N., DEDIEU, J. C., GULIK-KRZYWICKI, T. & LESBATS, B. (1981). Redistribution of intramembrane particles related to acetylcholine release by cholinergic synaptosomes. *Journal of Ultrastructure Research* **75**, 162–178.

KATZ, B. (1969). The release of neural transmitter substances. In *The Sherrington Lectures*. vol. x. Liverpool: Liverpool University Press.

KATZ, B. & MILEDI, R. (1967). The release of acetylcholine from nerve endings by graded electric pulses. *Proceedings of the Royal Society* B **167**, 23–38.

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

KONISHI, T. & SEARS, T. A. (1984). Electrical activity of mouse motor nerve terminals. *Proceedings of the Royal Society* B **222**, 115–120.

KRIEBEL, M. E. & GROSS, C. E. (1974). Multimodal distribution of frog miniature endplate potentials in adult, denervated and tadpole leg muscle. *Journal of General Physiology* **64**, 85–103.

KUFFLER, S. W. & YOSHIKAMI, D. (1975). The number of transmitter molecules in a quantum: an estimate from iontophoretic application of acetylcholine at the neuromuscular synapse. *Journal of Physiology* **251**, 465–482.

KUNO, M., TURKANIS, F. A. & WEAKLY, J. N. (1971). Correlation between nerve terminal size and transmitter release at the neuromuscular junction of the frog. *Journal of Physiology* **213**, 545–556.

LLINAS, R., STEINBERG, Z. & WALTON, K. (1981). Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. *Biophysical Journal* **33**, 323–352.

LLINAS, R., SUGIMORI, M. & SIMON, S. M. (1982). Transmission by presynaptic spike-like depolarization in the squid giant synapse. *Proceedings of the National Academy of Sciences of the U.S.A.* **79**, 2415–2419.

MEUNIER, F. M. (1984). Relationship between presynaptic membrane potential and acetylcholine release in synaptosomes from *Torpedo* electric organ. *Journal of Physiology* **354**, 121–137.

MULLER, D. (1986). Potentiation by 4-aminopyridine of quantal acetylcholine release at the *Torpedo* nerve–electroplaque junction. *Journal of Physiology* **379**, 479–493.

MULLER, D. & DUNANT, Y. (1985). Subminiature electroplaque potentials are present in *Torpedo* electric organ. *Experientia* **41**, 824.

NEHER, E. & SAKMANN, B. (1976). Single-channel currents recorded from membrane of denervated frog muscle fibers. *Nature* **260**, 799–802.

NEHER, E., SAKMANN, B. & STEINBACH, J. H. (1978). The extracellular patch clamp: a method for resolving currents through individual open channels in biological membranes. *Pflügers Archiv* **375**, 219–228.

NISHI, S., SOEDA, H. & KOKETSU, K. (1967). Release of acetylcholine from sympathetic preganglionic nerve terminals. *Journal of Neurophysiology* **30**, 114–134.

SCHINDLER, H. & QUAST, V. (1980). Functional acetylcholine receptor from *Torpedo* marmorata in planar membranes. *Proceedings of the National Academy of Sciences of the U.S.A.* **77**, 3052–3056.

STÜHMER, W., ROBERTS, W. M. & ALMERS, W. (1983). The loose patch clamp. In *Single-Channel Recording*, ed. SAKMANN, B. & NEHER, E., pp. 123–132. New York: Plenum Press.

YEH, Y. Z., OXFORD, G. S., WU, C. H. & NARAHASHI, T. (1976). Interactions of aminopyridines with potassium channels of squid axon membranes. *Biophysical Journal* **16**, 77–81.

EXPLANATION OF PLATE

Presynaptic network innervating the ventral face of a *Torpedo* electroplaque. On most of their surface, the nerve terminal ramifications are covered by a thin Schwann cell. The micrograph has been taken by L. M. Garcia-Segura from a quickly frozen, cryofractured prism. From such pictures, we estimated that about 50 % of the innervated face of an electroplaque is covered by presynaptic terminals. It can also be observed that the responses recorded with the type of electrode used arose from more than one nerve ending ramification. (Calibration bar: 1 μm.)