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POTENTIATION BY 4-AMINOPYRIDINE OF QUANTAL ACETYLCHOLINE RELEASE AT THE *TORPEDO* NERVE–ELECTROPLAQUE JUNCTION

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SUMMARY

1. The effects of 4-aminopyridine (4-AP) on electrophysiological post-synaptic responses evoked by field stimulation or evoked focally using a loose patch-clamp technique, and on radiolabelled transmitter release were studied in the *Torpedo* electric organ.

2. In this preparation, 4-AP had three major effects: it greatly potentiated the amount of acetylcholine (ACh) released by a nerve impulse, it prolonged the duration of the post-synaptic electroplaque current (e.c.) by several hundreds of milliseconds, and it increased the delay of responses triggered by a presynaptic action potential.

3. Noise analysis performed at different times during the focally recorded giant response showed that it was made of a sustained release of ACh quanta.

4. The maximum synchronous release of transmitter, expressed as the maximum number of quanta simultaneously delivered/μm^2 of presynaptic membrane, was apparently not modified by 4-AP.

5. A slightly different dose dependence was found for the effects of 4-AP on the potentiation of transmitter release and on the prolongation of the synaptic delay. The effects of tetraethylammonium (TEA) and other K^+ channel blockers on these parameters were similar to those of 4-AP.

6. Strong depolarizing pulses applied focally to a nerve ending were able to evoke a giant response even in the presence of 1 μM-tetrodotoxin (TTX). The prolongation of the discharge by 4-AP was therefore not caused by repetitive re-excitation of the nerve branches.

7. Both the amplitude and the time course of the giant response were Ca^{2+} dependent. At a low Mg^{2+} concentration, the Ca^{2+} dependence of transmitter release was identical in the presence or absence of 4-AP.

8. Paradoxically, in the presence of 4-AP, addition of 4 mM-Mg^{2+} considerably increased the Ca^{2+} dependence of release, whereas in the absence of 4-AP, Mg^{2+} blocked transmitter release by decreasing its sensitivity to Ca^{2+}. This potentiating interaction between Mg^{2+} and 4-AP was not seen with TEA or guanidine.

9. In conclusion, 4-AP potentiates ACh release in two different ways in the *Torpedo* electric organ: (i) it promotes a sustained quantal release of transmitter during several hundreds of milliseconds without any significant change in the

maximal synchronous release, (ii) it interacts with Mg^{2+} in such a manner that the sensitivity to Ca^{2+} of the nerve terminals is increased.

INTRODUCTION

The potentiating action of 4-aminopyridine (4-AP) has been used as a tool to investigate the presynaptic mechanisms of transmitter release at cholinergic synapses. In a neuromuscular junction, Heuser and colleagues showed that transmission of a nerve impulse in the presence of 4-AP was accompanied by the occurrence of endo-exocytotic images in the presynaptic membrane (Heuser, Reese, Dennis, Jan, Jan & Evans, 1979). In the *Torpedo* electric organ, another purely cholinergic preparation which shares many similarities with neuromuscular junctions (see Dunant & Muller, 1986), 4-AP was used to determine the changes in acetylcholine (ACh) content occurring in the nerve endings during and soon after transmission of a single impulse; it appeared that the release of transmitter was associated with a rapid utilization and renewal of cytoplasmic ACh (Corthay, Dunant & Loctin, 1982). More recently, rapid freezing experiments carried out in identical conditions revealed that the time course of the recorded electroplaque potential was best correlated in this preparation by the appearance of large presynaptic intramembrane particles (Garcia-Segura, Muller & Dunant, 1986). These experiments raised several questions about the nature of the release mechanism, but they also showed that the action of the drug is somewhat different in the two preparations. If a 30–40 times increase in the amount of transmitter released has been reported in both cases (Katz & Miledi, 1979; Corthay *et al*, 1982), it is both the amplitude and the duration of the end-plate response which are increased in the neuromuscular junction, but only the duration of the electroplaque potential in the electric organ.

The purpose of the present study was therefore to analyse in more detail the nature of this phenomenon in the electric organ. This appeared important not only to better understand the mechanisms of action of 4-AP in this preparation, but also to gain general information upon the mechanisms of excitation–secretion coupling in cholinergic synapses.

METHODS

Materials

The fish, *Torpedo marmorata*, were supplied by the Station de Biologie Marine, Arcachon, France. They were anaesthetized by tricaine methane sulphonate (MS 222, Sandoz, Switzerland) at a concentration of 1 g/3 l of sea water. Slices of electric organ were excised and kept in a physiological saline medium containing (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 7·1. Unless mentioned, all experiments were performed at room temperature (18–22 °C).

[1-^{14}C]acetate, specific radioactivity 19 mCi/mM, was obtained from the Radiochemical Centre, Amersham, U.K.; 4-AP chloride, tetraethylammonium chloride, guanidinium chloride, caesium chloride from Merck; tetrodotoxin from Calbiochem; verapamil from Knoll; 4-aminoquinoline was a generous gift from P. Lechat and J. Molgo, Paris.

Field simulation of whole prisms of electric organ

Prisms of electric organ, which are composed of a stack of about 500 electroplaques, were dissected and incubated for a minimum of 2 h in a large volume of saline medium. The Ca^{2+} and

Mg^{2+} concentrations of this saline medium were modified according to the conditions of the experiment. The prisms were then placed on a small nylon cloth between two stimulating platinium electrodes situated parallel to them. Recording electrodes were inserted at the dorsal and ventral ends of the prism. Field stimuli of 120 V and 0·5 ms were applied to the tissue, resulting in an electrical discharge which in this tissue is composed of the sum of many well synchronized electroplaque potentials (e.p.s.), generated by each of the superimposed electroplaques. The prisms were kept under continuous superfusion of saline medium. The drugs to be tested were than added to the superfusing medium and incubation continued for 60 min. This time was sufficient to allow complete exchange of the extracellular space by diffusion. After this period, a single stimulus was again applied to each prism to test the effects of the drug. The responses were recorded on a digital storage oscilloscope (Gould, OS 4200) and plotted on paper by an $X-Y$ recorder (Gould, 60000). The surface under the potential trace was measured with the aid of an electronic pen and a graphic tablet (Tektronix Inc., Beaverton, OR, U.S.A., type 4953) connected to an S-100 (IEE 696) microprocessor system (type 8080, Intel, Corp., Santa Clara, CA, U.S.A.).

Measurement of radiolabelled ACh release

The protocol used was that of Dunant, Eder & Servetiadis-Hirt (1980). Briefly, the tissue ACh was labelled by incubating dissected prisms with a radioactive precursor ([^{14}C]acetate; 2 μCi/ml) for 5 h. The prisms were then washed 3 times for 20 min in 100 ml physiological medium and kept overnight in a large volume of saline at 4 °C. On the next day, stimulation and recording were carried out as described above. The prisms were placed on a nylon cloth and kept under continuous superfusion of saline medium cooled to 16 °C to reduce spontaneous ACh release that can become prominent in the presence of 4-AP. The superfusing medium was collected at regular intervals before and after a single field stimulation, when 4-AP was present, or before and after five stimuli at 10 Hz in the absence of the drug. The radioactivity in these samples was then measured in a liquid scintillation counter. For each sample collected after stimulation, the mean value of the resting release of radioactivity per sample was subtracted. The amount of radiolabelled ACh released during the 100 s following stimulation was then calculated and expressed in relationship to the weight of the prism.

Focal stimulation and recording

The post-synaptic currents evoked by focal depolarization of the nerve endings were recorded by the loose patch-clamp technique described in Dunant & Muller (1986).

Noise analysis of post-synaptic currents

If the giant response recorded focally results from a sustained release of ACh quanta, noise analysis performed on segments of records is expected to reveal the amplitude and time characteristics of these quanta (see Simonneaux, Tauc & Baux, 1980, for a similar approach), in analogy to the analysis of the ACh receptor properties following iontophoretic application of ACh (Katz & Miledi, 1972; Anderson & Stevens, 1973). Noise analysis was therefore performed at different times of the giant discharge recorded focally in the presence of tetrodotoxin (TTX; 1 μM) and 4-AP (100 μM). The whole post-synaptic response was digitalized at 5 or 10 kHz and 8-bit. The variance of the noise was determined on traces of 200–500 points (20–100 ms) taken at the selected times. The amplitude (i) of the unitary quantal currents responsible for that noise was then calculated according to Cambell's theorem (Rice, 1944; Katz & Miledi, 1972) by the following relationship:

$$i = 2\Sigma/I,$$

where Σ is the variance and I the mean amplitude of the post-synaptic currents on the segment of trace considered.

The time characteristic of the unitary quantal currents was calculated by analysing power density spectra. These were obtained by applying a discrete Fourrier transform to segment of traces of 128 points sampled at 2 or 5 kHz. The power density spectra of three to ten segments were then averaged and the frequency (f) corresponding to the half-power point determined. The time characteristic of the unitary quantal events (τ) was then calculated according to the following relationship (see Katz & Miledi, 1972):

$$\tau = 1/2\pi f.$$

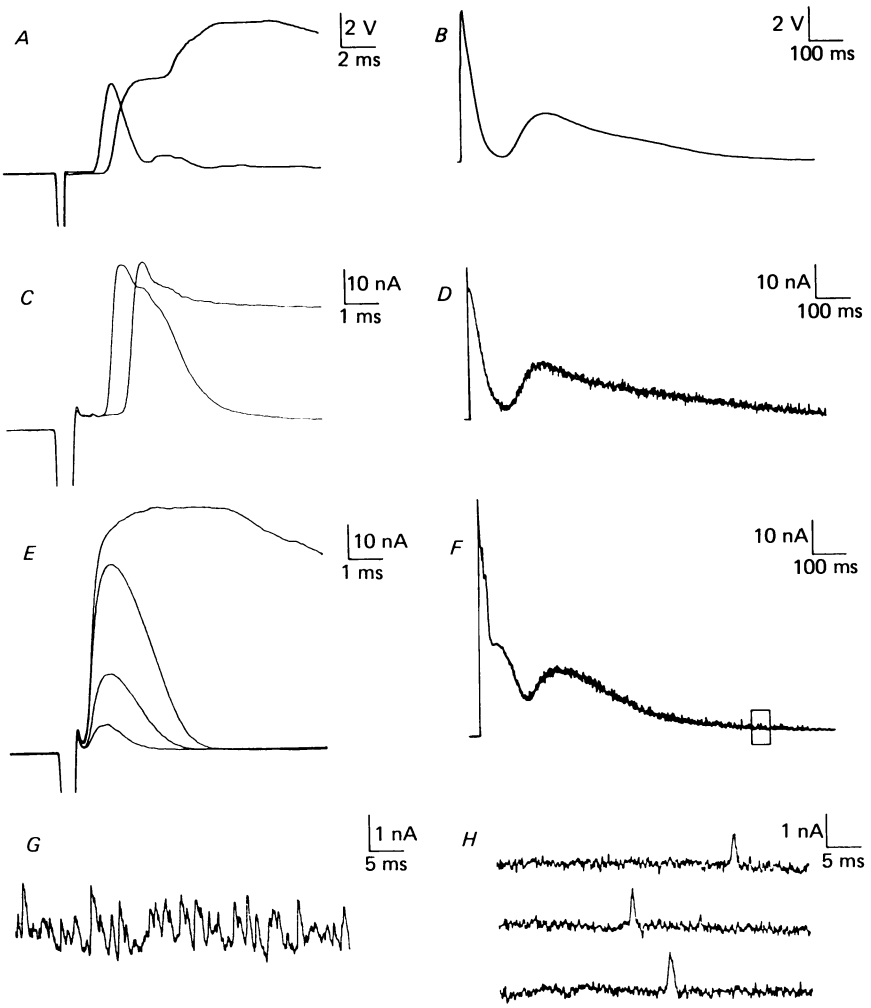

Fig. 1. Effects of 4-AP (100 μM) on the post-synaptic responses evoked by a single nerve impulse in the *Torpedo* electric organ. The electroplaque potential generated by field stimulation of a whole prism before and after application of 4-AP is shown in *A* and *B* with two different time scales. Under the action of 4-AP, its latency was increased and its duration enormously prolonged. *C*–*F* show the post-synaptic currents evoked by focal depolarization of nerve endings. For convenience, they were illustrated as upward-going signals. In *C*, an all-or-none response was evoked in the absence and in the presence of 4-AP. The drug also increased the delay and prolonged the time course (*C* and *D*), but did not increase the maximal amplitude of the post-synaptic current. The graded response in *E* was elicited in the presence of 4-AP and TTX (1 μM) by depolarizing pulses of increasing strength. These pulses generated a giant current response (*F*) only when they reached a maximal intensity. The giant responses recorded focally (*D* and *F*) were also characterized by an increase in noise. A segment of the noise recorded at the tail of a giant response (square in *F*) has been reproduced in *G* with expanded time and current scales. The noise appears to be composed of units similar in size and time course to the spontaneous m.e.c.s recorded focally at the same site (*H*).

RESULTS

Electrophysiological characteristics of the giant response

The effects of 4-AP on nerve–electroplaque transmission were studied by recording the electrical response of a whole prism of electroplaques and then by recording with focal loose patch-clamp electrodes the events occurring at restricted synaptic areas.

The response of an isolated untreated prism to a field stimulation is a well synchronized potential change, arising after a latency of about 2 ms and lasting for

TABLE 1. Effects of 4-AP on the synaptic delay and the maximal rate of ACh release

	Control	100 μM-4-AP
Latency of the electrical discharge generated by a whole prism (ms)	$2{\cdot}15 \pm 0{\cdot}03$ $n = 15$	$3{\cdot}12 \pm 0{\cdot}06$ $n = 13$
Synaptic delay of the focally evoked all-or-none response (ms)	$1{\cdot}43 \pm 0{\cdot}03$ $n = 19$	$2{\cdot}25 \pm 0{\cdot}10$ $n = 12$
Maximal synchronous release/μm^2 of presynaptic membrane (quanta/μm^2)	$1{\cdot}22 \pm 0{\cdot}10$ $n = 6$	$1{\cdot}30 \pm 0{\cdot}12$ $n = 6$

The increase in latency produced by 4-AP on the response elicited by field stimulation of a whole prism corresponds in magnitude to the prolongation of the synaptic delay of the focally evoked all-or-none response. The maximal synchronous release (expressed as the number of quanta delivered simultaneously per unit surface of presynaptic membrane) was not changed by 4-AP. Latency and synaptic delay were measured as the time between the onset of the pulse and the onset of the post-synaptic response. Results were obtained with an electrode of 7 μm in diameter and maximal quantal contents were respectively of 25·1 and 23·6 in the presence and absence of 4-AP.

2–3 ms. It is sometimes followed by low secondary responses, probably due to restimulation of some nerve branches. Addition of 100 μM-4-AP had two major effects. The latency was increased by *ca.* 1 ms, and the duration of the potential change was hugely prolonged. Usually at the beginning of this giant discharge, the 'normal' response could still be distinguished in the form of a characteristic inflexion in the rising phase of the potential (Fig. 1*A*). Later on, the giant discharge reproducibly exhibited a peculiar time course, characterized by a first giant peak followed by a late rebound. The over-all duration of the giant discharge reached several hundreds of milliseconds (Fig. 1*B*).

Using a loose patch-clamp technique, it has been shown that focal depolarization of nerve endings evokes two types of post-synaptic response in the electric organ, an all-or-none response and a graded response (Dunant & Muller, 1986). The all-or-none response results from the generation of a presynaptic action potential and resembles in many aspects the compound discharge recorded from a whole prism. Its delay, however, (measured as the time between the onset of the pulse and the onset of the post-synaptic currents) is shorter, since, in the whole prism, time is required for impulse conduction along the nerves in the tissue. The effects of 4-AP on the all-or-none response were similar to those on the whole prism. The delay was increased to nearly the same extent (Fig. 1*C*; Table 1), and the duration of the electroplaque current enormously enlarged, also exhibiting a two-peaked time course (Fig. 1*D*).

The graded response can be elicited by depolarizing pulses of graded intensity at all places where spontaneous miniature electroplaque currents (m.e.c.s) are recorded. It is TTX resistant, but Ca^{2+} dependent and is therefore thought to result from a direct activation of presynaptic Ca^{2+} channels by the depolarizing pulses (Dunant & Muller, 1986). Fig. 1*E* and *F* show graded responses evoked in the presence of 1 μM-TTX and 100 μM-4-AP. Under these conditions, a depolarizing pulse of low strength produced an electroplaque current (e.c.) whose duration was only slightly longer than that recorded in the absence of 4-AP. Also, the synaptic delay was not increased by 4-AP, in contrast to what was observed with the all-or-none response. When the pulse strength was raised, the graded response increased in amplitude and also slightly in duration, but was not followed by a giant response, unless the intensity of the depolarising pulse was sufficient to evoke a maximal graded response. In this case it produced a giant e.c. which was similar in all aspects to that produced by 4-AP on the all-or-none response (Fig. 1*F*).

Table 1 summarizes the data on latency and amplitude of the responses. The latency of the response recorded from a whole prism and the delay of the all-or-none focal response were both significantly increased by 4-AP, and both nearly to the same extent. An important observation was that 4-AP did not significantly increase the maximal synchronous release of ACh quanta, since it did not increase the maximum e.c. amplitude. It has been shown in the accompanying paper (Dunant & Muller, 1986) that in the absence of 4-AP approximately 1·3 quanta are released/μm^2 of presynaptic membrane during the passage of a nerve impulse. This value was not increased by 4-AP (Table 1). Therefore, 4-AP potentiates ACh release in this tissue by increasing the duration of release, but not the maximum number of quanta released synchronously.

Quantal nature of the giant response

As shown on Fig. 1*D* and *F*, the e.c. records, when compared to base-line traces, were characterized by an increase in noise level. When these records were reproduced with expanded time and current scales (Fig. 1*G*), the noise looked very much like a high frequency occurrence of units similar in size and time course to the m.e.c.s (Fig. 1*H*). This similarity was examined more quantitatively by noise analysis and the results are expressed in Table 2. First, it is necessary to mention that 4-AP affected neither the amplitude nor the decay time constant of spontaneous m.e.c.s. This confirmed the observation that 4-AP, at the concentration used, has no direct post-synaptic effect and did not inhibit acetylcholinesterase (Corthay *et al.* 1982). By analysing the variance and the power density spectra of the noise of focally evoked responses, we determined the amplitude and time characteristic of the unitary quantal events responsible for that noise. This was done with giant responses recorded on four different sites and at three different times during their time course: (i) in the trough after the initial peak, (ii) at the top and (iii) at the end of the late rebound. In the trough of the giant response, the amplitude of the unitary components of the noise was slightly but not significantly smaller than that of the spontaneous m.e.c.s. This decrease was significant at the top of the late rebound, but again insignificant at the tail of the giant response (Table 2). At the 3 times analysed, the time characteristic obtained by noise analysis was found to be identical to the

decay time constant of m.e.c.s (the slight increase at the top of the late rebound was not significant). These data strongly suggest that the giant response recorded in the presence of 4-AP results from a sustained quantal release of ACh.

TABLE 2. Characteristics of m.e.c.s and noise parameters of focally recorded giant responses

	Amplitude (nA)	Decay time constant (ms)
M.e.c.s in control conditions	$3{\cdot}6 \pm 0{\cdot}3$ $n = 7$	$0{\cdot}53 \pm 0{\cdot}03$ $n = 7$
M.e.c.s in the presence of 100 μM-4-AP	$3{\cdot}7 \pm 0{\cdot}4$ $n = 8$	$0{\cdot}53 \pm 0{\cdot}03$ $n = 8$
Noise parameters in the trough of the giant response	$3{\cdot}2 \pm 0{\cdot}2$ $n = 4$	$0{\cdot}55 \pm 0{\cdot}04$ $n = 4$
Noise parameters at the top of the late rebound of the giant response	$2{\cdot}1 \pm 0{\cdot}3$ $n = 4$	$0{\cdot}62 \pm 0{\cdot}04$ $n = 4$
Noise parameters at the tail of the giant response	$3{\cdot}4 \pm 0{\cdot}2$ $n = 4$	$0{\cdot}54 \pm 0{\cdot}03$ $n = 4$

The amplitude and decay time constant of m.e.c.s, recorded with and without 100 μM-4-AP, were found to correspond to the noise parameters calculated from the variance and the density power spectra of segments of focally recorded giant responses. Only a discrepancy in amplitude was observed at the top of the late rebound ($P < 0{\cdot}01$). Results are mean $\pm$ S.E. of mean; n refers to the number of recording sites in which twenty to fifty m.e.c.s and one to three giant e.c.s were analysed. The amplitude of m.e.c.s were calculated from measured data according to the values of the electrode resistance and the shunt resistance (see Dunant & Muller, 1986).

Dose dependence of the effects of 4-AP

The increase in delay produced by 4-AP has been interpreted as reflecting the prolongation of the nerve action potential by the block of K^+ channels (Benoît & Mambrini, 1970; Dunant & Muller, 1986). Since we were interested to determine if the increase in transmitter release and the generation of a giant response were also related to the block of K^+ channels, we compared the dose dependence of all these effects. As illustrated on Fig. 2, the increase in delay and the increase in radiolabelled transmitter release had similar, but not identical dose dependences. This difference might be because the two phenomena were produced by different mechanisms. We therefore compared the effects of 4-AP to those of other K^+ channel blockers (tetraethylammonium (TEA), caesium) or other drugs able to potentiate transmitter release (guanidine, 4-aminoquinoline) also probably by acting on K^+ channels (Matthews & Wickelgren, 1977). All transformed the normal discharge into a giant response characterized by a two-peaked time course, prolonged the synaptic delay and increased the release of radiolabelled transmitter. As shown in Fig. 2, TEA, although less potent, was as efficient as 4-AP in increasing ACh release and prolonging the synaptic delay. The slight discrepancy between the dose–effect relationship for the release and for the delay was also observed with TEA. It seems therefore that 4-AP and TEA exerted these two effects through the same mechanism, which is likely to be the block of the presynaptic K^+ channels.

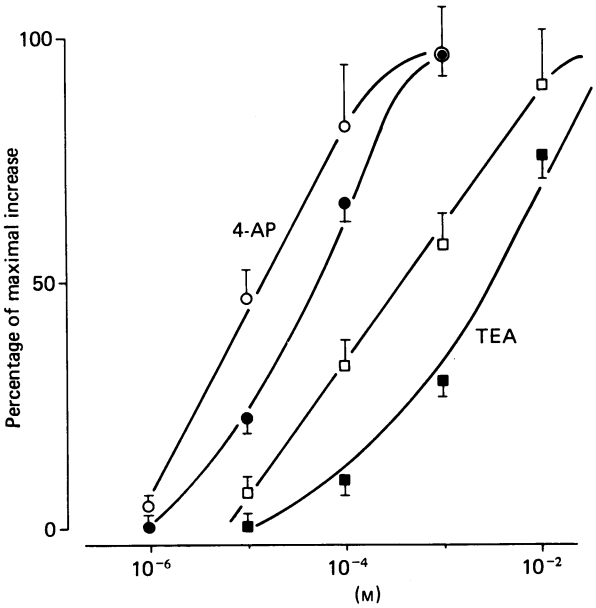

Fig. 2 Dose–response curves of the effects of 4-AP (○ and ●) and TEA (□ and ■) on nerve electroplaque transmission. Open symbols show the increase in ACh release measured by a radiochemical method, and filled symbols the prolongation of the latency of the giant discharge. Results were obtained from four different experiments and each point represent the mean ± S.E. of mean of seven to sixteen prisms. They were expressed in each experiment as percentage of the maximal changes obtained with 4-AP, which were 828 Bq/g.min and 1·6 ms respectively.

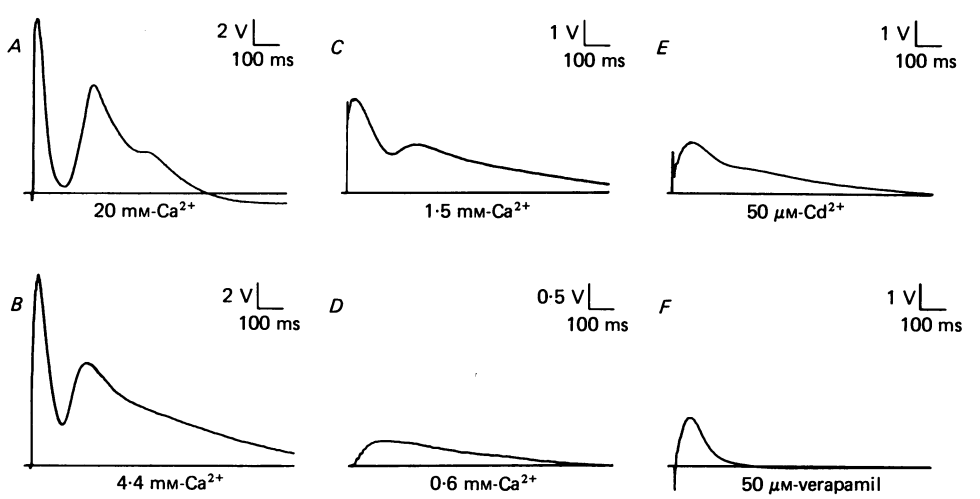

Fig. 3. Ca^{2+} dependence of the giant response. A giant response was evoked from a prism of electric organ in the presence of 20 mM-Ca^{2+} in *A*, in standard physiological saline (4·4 mM-Ca^{2+}) in *B*, 1·5 mM-Ca^{2+} in *C*, 0·6 mM-Ca^{2+} in *D*. Both the amplitude and the time course of the e.p. were dependent upon the external Ca^{2+} concentration. Results similar to those observed in low Ca^{2+} were obtained in the presence of a standard Ca^{2+} concentration when Ca^{2+} entry was prevented by adding Cd^{2+} (50 μM) in *E*. In contrast to this, verapamil (50 μM) acted by shortening the duration of the giant e.p. (*F*). 4-AP (100 μM) was present in all conditions.

Ca^{2+} dependence of the giant response

How can the block of the presynaptic K^+ channels lead to a sustained release of ACh quanta, lasting for several hundreds of milliseconds? Does Ca^{2+} enter the nerve endings during all this time? To examine this question, we analysed the Ca^{2+} dependence of the giant response. At 20 mM-Ca^{2+} (Fig. 3*A*), the amplitude of the giant e.p. was not increased over that recorded in presence of the standard 4·4 mM-Ca^{2+} concentration (Fig. 3*B*); however, its normally two-peaked time course was transformed into a nearly three-peaked one. At lower Ca^{2+} concentrations (1·5 mM, Fig. 3*C*; 0·6 mM, Fig. 3*D*), the amplitude progressively decreased and the trough between the first and the second peak disappeared, so that the giant response was finally characterized by a regular 'monophasic' time course. In all cases, the over-all duration of the giant e.p. was the same. Similar results were obtained when Ca^{2+} entry was blocked with different Cd^{2+} concentrations (20–100 μM) (Fig. 3*E*). Verapamil, however, had a different action. It depressed the late phase more than the initial part of the giant discharge, which resulted in a shortening of the electrophysiological response (Fig. 3*F*).

The Ca^{2+} dependence of the giant discharge was also analysed biochemically by measuring the amount of radiolabelled ACh released either by a single nerve impulse (in the presence of 100 μM-4-AP), or by five impulses at 10 Hz (in the absence of the drug). Results were expressed as a percentage of maximal values and compared to electrophysiological responses: the surface under the giant response (4-AP) or the amplitude of the first e.p. (no drug). First, it was observed that the biochemical and electrophysiological data were in good agreement; saturation of maximal values occurred at the same Ca^{2+} concentration and the relationships could be superimposed (see Dunant *et al.* 1980). Secondly, the relationships obtained in the presence and absence of 4-AP were not significantly different, indicating that the drug did not appreciably change the sensitivity of the release process to Ca^{2+} (it will be seen in the next section, however, that the last point is only valid at low Mg^{2+} concentrations).

These results suggest that the sustained quantal release accompanying transmission of a single nerve impulse in the presence of 4-AP is the consequence of a prolonged entry of Ca^{2+} into the nerve endings.

Interaction between 4-AP and Mg^{2+}

Mg^{2+} is known to antagonize Ca^{2+} entry into nerve endings and therefore to depress transmitter release (Katz & Miledi, 1969; Cooke, Okamoto & Quastel, 1973). This was also expected to occur in the presence of 4-AP for the giant discharge. Paradoxically, however, in the presence of 4 mM-Mg^{2+} and 100 μM-4-AP, the evoked transmitter release, whether measured by radiolabelled ACh or by the electrophysiological response, showed an increased sensitivity to Ca^{2+}, in contrast with the results obtained in the absence of 4-AP (Fig. 4*B*). This phenomenon was particularly evident at low Ca^{2+} concentrations where almost no response was obtained in the absence of 4-AP and a nearly maximal one in its presence. Comparing Fig. 4*A* to 4*B* (where the dashed line is the mean of the results of Fig. 4*A*), it can be observed that the Ca^{2+} dependence of the giant response is displaced by 4 mM-Mg^{2+} towards lower Ca^{2+}

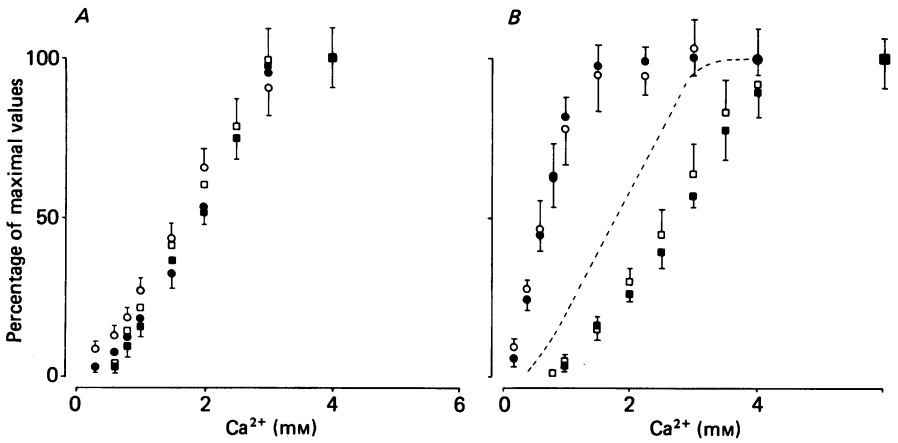

Fig. 4. Ca^{2+} dependence of transmitter release with and without 4-AP (100 μM), at low Mg^{2+} concentration (0·1) mM) in *A*, and in the presence of 4 mM-Mg^{2+} in *B*. Results were obtained from whole prisms, in the presence of 4-AP, by measuring the amount of radiolabelled ACh released by a single nerve impulse (○) and by analysing the area under the potential of the giant discharge (●). In the absence of 4-AP, the radiolabelled ACh was collected after a train of five impulses at 10 Hz (□) and compared to the amplitude of the first e.p. (■). Each point is the mean ± S.E. of mean of five to eighteen values obtained from five to eight experiments. They were expressed as a percentage of the maximal values which were respectively in *A* and *B*: 763 ± 75 and 809 ± 97 Bq/g . min for giant responses, 422 ± 47 and 487 ± 27 Bq/g . min for non-potentiated responses, 561 ± 28 and 528 ± 29 V ms for giant e.p., 3·9 ± 0·2 and 4·1 ± 0·4 V for physiological e.p. In *A* the relationships for all parameters are superimposed; in *B*, the presence of Mg^{2+} increases the Ca^{2+} sensitivity of the release process when 4-AP is added, and reduces it in its absence. The dashed line in *B* corresponds to the mean relationship observed in *A*.

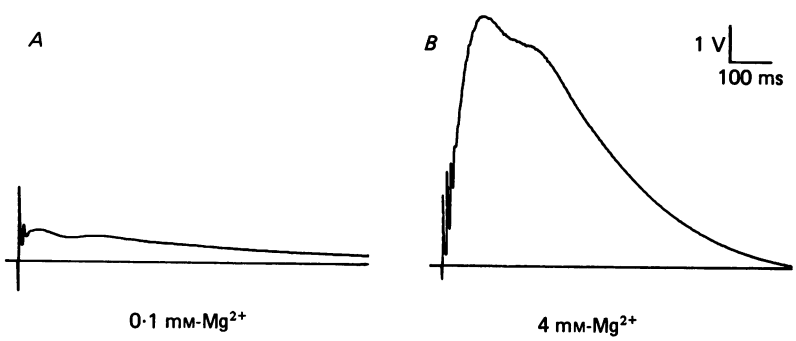

Fig. 5. Potentiation by Mg^{2+} of the giant response evoked from a prism in presence of 1 mM-Ca^{2+}, 100 μM-4-AP (100 μM-Mg^{2+} in *A*, 4 mM-Mg^{2+} in *B*). The amplitude of the giant e.p. is increased by a factor 4–6 by increasing Mg^{2+}, but no appreciable change in its time course can be observed.

concentrations. However, the maximal increase in transmitter release was not found to be different.

This paradoxical effect of Mg^{2+} is striking in Fig. 5 where a pair of giant responses was recorded in the presence of 1 mM-Ca^{2+}. The amplitude of the post-synaptic e.p. was increased by a factor of 4–6 when the Mg^{2+} concentration was increased from 0·1 to 4 mM. Its time course, however, was not appreciably modified.

This phenomenon was further analysed biochemically and electrophysiologically in Fig. 6. Here ACh release has been expressed as a function of the Mg^{2+} concentration either in the absence of any drug or in the presence of 4-AP or other K^+ channel blockers. These experiments were carried out with 1 mM-Ca^{2+}, a concentration at which the interaction between 4-AP and Mg^{2+} is the most apparent. Without 4-AP, the maximal response was observed at the lowest Mg^{2+} concentration and increasing Mg^{2+} up to 4 mM blocked ACh release, presumably by preventing Ca^{2+} entry into the nerve endings. In the presence of 4-AP, however, Mg^{2+} increasingly potentiated ACh release from 0·1 to 4 mM whereas very high concentrations depressed release.

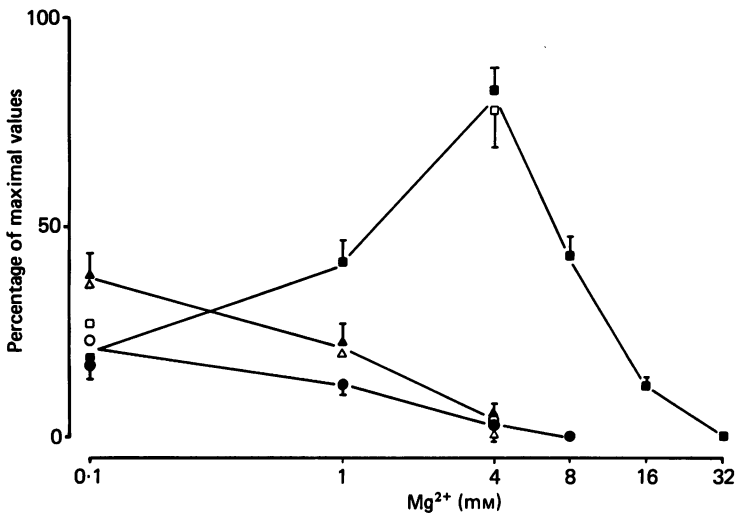

Fig. 6. Changes in transmitter release as a function of Mg^{2+} concentration: in standard saline medium (○ and ●), in the presence of 100 μM-4-AP (□ and ■), in the presence of 2 mM-TEA (△) and 2 mM-guanidine (▲). Open symbols (○□△) characterize results obtained from prisms by measuring the release of radiolabelled transmitter and filled symbols (●■▲) results obtained by analysing electrophysiological responses. Results are expressed as a percentage of maximal values which are identical to those of Fig. 4. In the presence of 4-AP, Mg^{2+} potentiates transmitter release with a maximum effect at 4 mM, higher concentrations block it. This was not observed in the presence of TEA or guanidine.

This unexpected interaction between 4-AP and Mg^{2+} was also searched for with TEA (2 mM) and guanidine (2 mM). The effects of TEA were analysed by measuring the release of radiolabelled ACh and those of guanidine by measuring the area under the potential of the giant discharge. In both cases, no interaction with Mg^{2+} was found. At 4 mM-Mg^{2+}, release was almost completely blocked in the presence of either TEA or guanidine. It seems therefore that the potentiating interaction between 4-AP and Mg^{2+} does not result from a block of K^+ channels since it is not shared by the other K^+ channel blockers.

DISCUSSION

In the *Torpedo* electric organ, 4-AP transforms the normally brief e.p. into a giant discharge of 600 ms duration. That this giant response does not result from any post-synaptic action of the drug is supported by several observations. Neither the

mean amplitude nor the time course of spontaneous m.e.c.s were modified by 4-AP. Similar results were obtained by different authors at the neuromuscular junction (Molgo, Lemeignan & Lechat, 1977; Lundh, 1978; Thesleff, 1980). The acetylcholinesterase activity measured *in vitro* is not inhibited by the drug (Corthay *et al.* 1982). Furthermore, an excellent correlation was found in the present work between the biochemical assay of ACh release and the electrophysiological data when analysing the Ca^{2+} dependence of the giant discharge, indicating that the 4-AP effect is mainly due to presynaptic changes.

By applying noise analysis to focally recorded giant e.c.s. we found in the present study that transmitter is released in a quantal manner during the whole time course of the giant response. The amplitude and time characteristic of the unitary events obtained by noise analysis were found to correlate well with the amplitude and decay time constant of spontaneous m.e.c.s, except for a slight decrease in amplitude observed at the top of the late rebound. This difference is probably due to factors other than a change in transmitter release, although this cannot be totally excluded. It is likely that at a high and sustained release rate some of the receptors are unable to bind ACh and open their ion channel because they are already in an activated or desensitized state. Similarly, any decrease in the electrical or ionic gradients consecutive to the maintained release rate, would also be accompanied by a decrease in amplitude of the unitary currents obtained by noise analysis.

We also found that the maximal synchronous release of ACh quanta/μm^2 of presynaptic membrane is not significantly increased at any time during the giant discharge. This seems to hold true at the beginning of the giant e.c., and also at the top of the late rebound, even when one considers the decrease in amplitude of the unitary currents. Thus, the increase in the amount of ACh released in 4-AP-treated preparations can only be attributed to a prolongation of release. This contrasts with the effects of 4-AP in neuromuscular preparations, where the drug increases not only the duration but also greatly the amplitude of the end-plate response, indicating that it also increases the number of quanta delivered simultaneously by the motor nerve endings (Katz & Miledi, 1979).

The block of K^+ channels by 4-AP (Yeh, Oxford, Wu & Narahashi, 1976) appears to be the primary cause of this giant response although a slight discrepancy was observed between the dose–response curves for the increase in synaptic delay and the increase in evoked release. A similar discrepancy was also reported by Datyner & Gage (1980) at a neuromuscular junction. Nevertheless, the similarity of action of 4-AP with that of other drugs known either to block K^+ channels, such as TEA or caesium, or to potentiate transmitter release, such as guanidine or 4-aminoquinoline, supports the primary role played by the block of K^+ channels.

The prolongation of release might be interpreted as resulting from a repetitive electrical activity of the nerve endings enhanced by the block of the presynaptic K^+ channels. This phenomenon has been reported in frog neuromuscular junction (Lundh, 1978; Heuser *et al.* 1979), where it was overcome by increasing the Ca^{2+} concentration or by adding TTX. However, in the electric organ, giant responses were obtained either in the presence of 20 mM-Ca^{2+} or 1 μM-TTX. Therefore the sustained quantal release evoked by transmission of one impulse does not result from repetitive Na^+-dependent action potentials in the terminals.

The present work, however, suggests that, following blockade of K^+ channels, transmission of a single nerve impulse is accompanied by a prolonged entry of Ca^{2+} into the nerve endings and this would be responsible for the sustained quantal release of transmitter. The Ca^{2+} dependence of both the amplitude and time course of the giant response as well as the inhibition produced by Cd^{2+} and verapamil are in good agreement with this. The particular effect of verapamil on the tail of the giant response probably results from the increased sensitivity of Ca^{2+} channels to the drug during prolonged depolarization (Lee & Tsien, 1983). The prolongation of Ca^{2+} entry most probably did not result from non-inactivation of Ca^{2+} channels since, in the presence of TTX, a large depolarization was required to elicit a giant response. This might suggest that a Ca^{2+} action potential was elicited by the large depolarizing pulses in the presence of TTX, as was described in the squid presynaptic terminals (Katz & Miledi, 1969). Alternatively, the present results might also be explained by a phenomenon similar to the oscillations of the membrane potential reported after a strong depolarization of the presynaptic ending of the giant synapse in the presence of 4-AP and TTX (Llinas, Walton & Bohr, 1976).

Another question concerned the characteristic two-peaked time course of release evoked in the presence of 4-AP. It was shown, in the present study, that this time course was dependent upon the amount of Ca^{2+} entering the nerve endings, but not upon the total amount of ACh released, since Mg^{2+} potentiated transmitter release without modifying the aspect of the giant e.c. This therefore makes it unlikely that during the trough, ACh release subsides because of a temporary shortage of substrate (see Corthay *et al*, 1982). Release probably stops because it transiently fails to be activated. This may be due either to a transient decrease in Ca^{2+} entry or, on the contrary, to a temporary excess of intracellular Ca^{2+} which might also inhibit the mechanism of release (Adams, Takeda & Umbach, 1985).

Finally the results obtained in the present study suggest that 4-AP potentiates transmitter release not only by blocking K^+ channels, but also by interacting with Mg^{2+}. An effect of this kind has been reported by Illes & Thesleff (1978), as a shift to the right of the Mg^{2+} dose–response curve for the amplitude of the end–plate potential; however, only Mg^{2+} concentrations greater than 4 mM were used and the effect was mimicked by TEA and guanidine. It is difficult therefore to know if both phenomena are closely related. In any case, in the present study, the paradoxical effect of Mg^{2+} occurred with 4-AP but not with TEA or guanidine; it was demonstrated as well by the electrophysiological response as by the amount of radiolabelled ACh released. Thus, the potentiating interaction between 4-AP and Mg^{2+} was not a consequence of the block of the presynaptic K^+ channels. It seems also unlikely that this interaction promoted an increased Ca^{2+} entry into the nerve endings. First, because in the absence of 4-AP, Mg^{2+} is known to act as an antagonist of Ca^{2+} entry (Katz & Miledi, 1969; Cooke *et al.* 1973); and secondly, because the time course of the giant response potentiated by Mg^{2+} in low Ca^{2+} was left unchanged, whereas increasing external Ca^{2+}, and thereby Ca^{2+} entry, gave clearly a two-peaked aspect to the giant discharge. As an alternative explanation, 4-AP and Mg^{2+} might interact with the mechanisms of Ca^{2+} sequestration and extrusion or even more directly with the process of transmitter release.

In summary, the results presented here show that in the *Torpedo* electric organ,

4-AP potentiates cholinergic transmission essentially by promoting the release of ACh quanta during several hundreds of milliseconds without modifying the maximal rate of release. This phenomenon is accompanied by a rapid utilization and renewal of extravesicular ACh in the nerve endings (Corthay *et al*, 1982), and is correlated in its time course with a large increase in the number of presynaptic intramembrane particles (Garcia-Segura *et al*. 1986). The potentiation of ACh release produced by 4-AP in this preparation is therefore of interest in analysing the mechanisms of cholinergic transmission.

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