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RAPID ACETYLCHOLINE AND ADENOSINE TRIPHOSPHATE OSCILLATIONS TRIGGERED BY STIMULATION OF THE *TORPEDO* ELECTRIC ORGAN

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SUMMARY

1. When the electric organ of *Torpedo* is stimulated a large number of synchronized cholinergic synapses are activated. This permits the measurement of changes in the tissue level of ACh associated with the release process, usually recorded as an electrical discharge.

2. At 5 Hz stimulation the output per impulse and the amount of cytoplasmic (free ACh) declines for about 30 s. The output then remains constant while ACh is synthesized for about 90 s. Finally, the output and cytoplasmic ACh are exhausted after 120 s. These 'slow wave' changes in ACh represent about 50% of the total.

3. Superimposed on the 'slow wave' are rapid oscillations of 5 s period, which represent about 30% of the total ACh.

4. The amount of ATP oscillates in phase with ACh. These oscillations might result from regulation of enzymes involved in the synthesis of transmitter.

5. The amplitude of electrical discharge does not normally oscillate. Transmitter output is therefore not directly related to ACh concentration changes. The mechanism releasing ACh is a saturable process.

INTRODUCTION

The electric organ of *Torpedo marmorata* is an extremely homogeneous structure. It consists of a large number of prisms, arranged side by side, each consisting of about 500 superposed electroplaques, which are covered on their ventral surface by a rich network of nerve terminals. The cholinergic nature of transmission has been demonstrated in this organ (Feldberg, Fessard & Nachmansohn, 1940; Feldberg & Fessard, 1942). When the nerve is stimulated the terminals release acetylcholine (ACh), which acts on receptors situated at the ventral membrane of the plates. This causes a large number of synchronized post-junctional potentials which summate to generate a strong discharge (positive towards the dorsal surface of the fish). The released transmitter is then rapidly hydrolysed and choline, and also acetate (see Israël &

Tucek, 1974), are taken up into the nerve terminals where ACh is resynthesized.

Hydrolysis of the released transmitter is accomplished in a matter of milliseconds; consequently all the ACh found in the tissue when it is quenched for extraction must be regarded as intracellular and most probably intraterminal. Advantage has been taken of this to measure nerve-terminal ACh as a function of time during stimulation. In preliminary experiments the level of transmitter was found to change quickly. It was therefore necessary to improve the rapidity of the biochemical methods, which finally achieved a time resolution of 1 s. The changes of tissue ACh have been correlated, when possible, with modifications of the electrical parameters of the response.

Most of the present work has been carried out by analysing the total ACh of the tissue. Experiments were also performed to determine which ACh fractions were modified during stimulation. Bound ACh is that which remains constant after the tissue has been homogenized. Most of it is present in synaptic vesicles, which can be isolated and purified by fractionation methods (Israël, Gautron & Lesbats, 1968, 1970; Whittaker, Essman & Dowe, 1972). 'Free ACh' is the part, about half the total, which is hydrolysed by esterases when the tissue is disrupted. The term 'free' is a notional one since the subcellular localization of this fraction has not yet been elucidated (see review by Marchbanks, 1975). Works by Dunant *et al.* (1974*a*) and Israël & Dunant (1975) show that stimulation increases the turnover of 'free ACh' but not that of bound ACh. In a few experiments, however, a loss of bound (vesicular) ACh occurred, during prolonged stimulations or when the fish was stimulated out of sea water. Such a drop of vesicular ACh was also found by Zimmermann & Whittaker (1974*a*, *b*).

We have re-examined the experimental conditions leading to dramatic change in vesicular ACh. We find that this only occurs in conditions where the electrical energy of the 'electric organ generator' is not allowed to flow as it does in sea water or in a well-conducting physiological solution. In 'dry conditions' the voltage is high but the generator does not deliver current through the external resistance and only a self-electrocution can occur with consequent tissue damage (see Dunant *et al.* 1976). In recent experiments Suszkiw, Zimmerman & Whittaker (1978) have perfused and stimulated the electric organ. The main vesicular fraction VP_1 did not show significant changes. Most interesting is the finding that a heavy fraction (VP_2) contains ACh with a high specific radioactivity as it would in our free fraction. It is certainly possible that VP_2 represents a special population of vesicles. However, it is conceivable that VP_2 contains a very small amount of free cytoplasmic ACh occluded in or adsorbed on a heavier membrane fraction. The present results will only describe the evolution of free ACh, which is presumably cytoplasmic, in conditions where the vesicular pool remains constant. Under these conditions the release process is largely fed by synthesis of new transmitter.

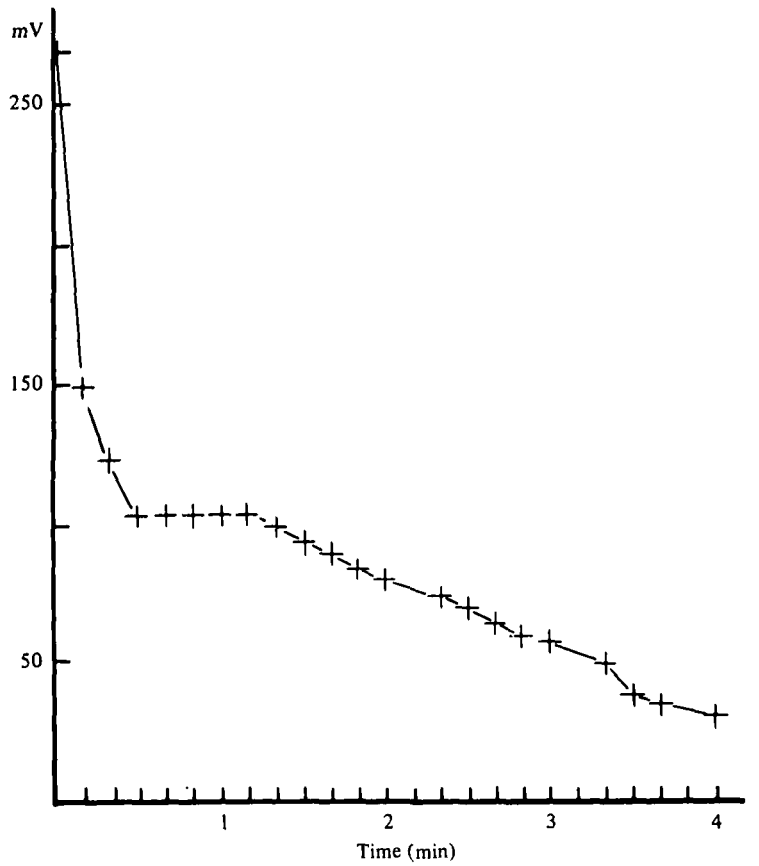

Fig. 1. Changes in amplitude of the physiological discharge during a repetitive stimulation at 5 Hz.

(1) *Acetylcholine release: a characteristic decay curve*

When two or three dissected prisms of electric tissue are submitted to a brief (1 ms) electric shock, the response-discharge recorded is separated from the stimulus artifact by an irreducible latency of 2–3 ms. Field stimulation only excites nerves and nerve terminals in the tissue directly (see Auger & Fessard, 1941; Bennett, Würzel & Grundfest, 1961). The electrical discharge is an indirect response of electroplaques to ACh released by nerve terminals: or, more precisely, is the sum of individual postjunctional potentials. Field stimulation was found very convenient in experiments where the tissue had to be incubated in solutions containing drugs or radioactive precursors, or when a high resolution with time was wanted.

The tissue was weighed and set on a piece of nylon gauze between the two stimulating electrodes parallel to the prisms. Stimulation is more efficient in this way since the field can easily reach the nerves at the edges of the electroplaque and between them (Fessard, 1947). The set-up was immersed in the physiological solution to allow the high currents produced by the tissue to flow in the low resistance of the medium and thus to protect the tissue from current effects.

Fig. 1 represents the decay curve of the electrical discharge. When the electric

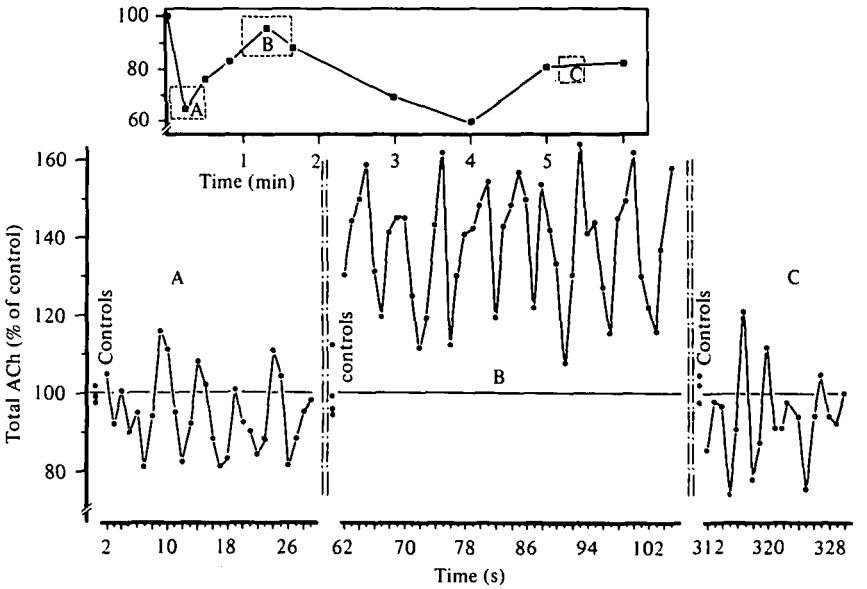

Fig. 2. Slow wave and fast oscillation of total ACh in the course of stimulation at 5/s. Inset shows a curve describing the slow ACh changes of the tissue which appeared when the sampling was performed with a low time resolution. This curve with its successive peaks has been called the 'slow wave'. When Regions A, B and C of the slow wave are analysed, with a high time resolution of sampling (every second), in the lower graphs, the rapid oscillations become apparent. They occur at different mean levels corresponding to three phases of the slow wave. The rapid oscillations were undamped and involved about 35 % of total ACh. Their period was approximately 5 sec. Lower graphs A, B and C are from separate experiments. The extraction procedure was 'hot HCl' as described in the text. The results are expressed as percentage of unstimulated samples.

tissue is stimulated at 5 Hz, the discharge becomes exhausted in about 6 min. Seventy per cent of the amplitude is lost in about 30 s, then it remains constant for approximately 60 s. After this plateau it declines in a couple of minutes to a few per cent of its initial value (Dunant *et al.* 1974*a*).

2. *Amount of acetylcholine present in the tissue in the course of stimulation*

A group of samples was continuously stimulated and at different times one of the samples was dropped into the extraction solution. The latency of this operation was about 0·25 s and could be repeated after 5 s. The resolution of the analysis was reduced to 1 s by repeating the sampling protocol, each time with a delayed origin. Two different rapid quenching procedures were used. For the first, the samples were dropped into liquid nitrogen or isopentane cooled by liquid nitrogen. After freezing, it was powdered and the powder extracted in 5 ml of 5% trichloroacetic acid (TCA).

In the second method the samples were dropped in a hot (87 °C) acidified solution. Both procedures gave equivalent results. A detailed description can be found in Dunant *et al.* (1977).

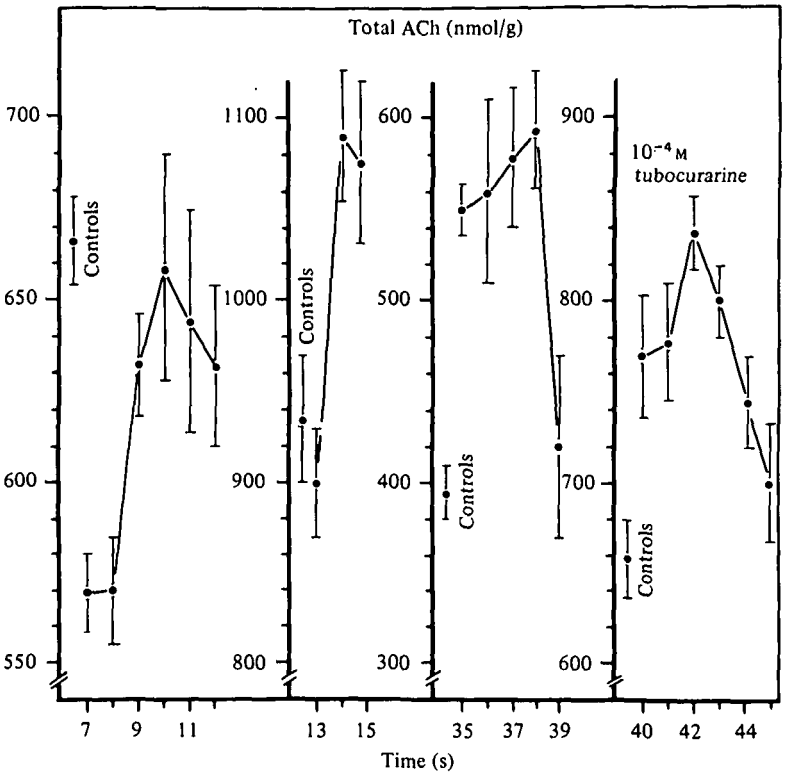

Fig. 3. Statistical evidence for the rapid ACh oscillation occurring during stimulation. In four different experiments a number (n) of samples were stimulated at 5/s for the same length of time, quenched and analysed for total ACh. Ordinate, mean (±S.E.M.) of total ACh in nmol/g of wet tissue. There is at least one point in each graph having a mean lower than a previous or subsequent point. The following means are statistically different. Graph A, 7 s point compared to 9 s, $P < 0{\cdot}01$; 7 s+8 s points compared to 9 s+10 s points, $P = 0{\cdot}001$. Graph B, 13 s point compared to 14 s, $P < 0{\cdot}001$; 13 s compared to 15 s, $P < 0{\cdot}01$. Graph C, 38 s point compared to 39 s, $P = 0{\cdot}02$. Graph D, 42 s point compared to 45 s, $P < 0{\cdot}01$; In Expt D the presence of tubocurarine (10^{-4} M) in the incubation medium abolished the electrophysiological response to stimulation but did not alter the oscillation. In B a radiochemical method was used to estimate ACh. n was 6, 10, 5 and 5 in Expts A, B, C and D respectively.

(*a*) *The slow ACh wave*

Fig. 2 (upper inset) shows that, in general, the amount of ACh in the tissue is below that of the controls during the first phase of the discharge decay curve. It then rises at the end of the plateau and finally declines. To interpret this so-called 'slow ACh wave' we can presume that at rest the release of ACh is constantly balanced by transmitter synthesis. The entry of Ca^{2+} which is triggered by stimulation will upset that equilibrium and the release of ACh is suddenly increased. This will not only tend to reduce the intraterminal ACh concentration (20 mM, see Morel, Israël & Manaranche, 1978) but will also trigger the recycling of acetate and choline, since their concentrations increase very rapidly in the small volume of the cleft. In the nerve terminal, acetate will be converted to AcetylCoA while ATP is hydrolysed. If CoA and ACh concentrations are low one might expect that choline acetylase

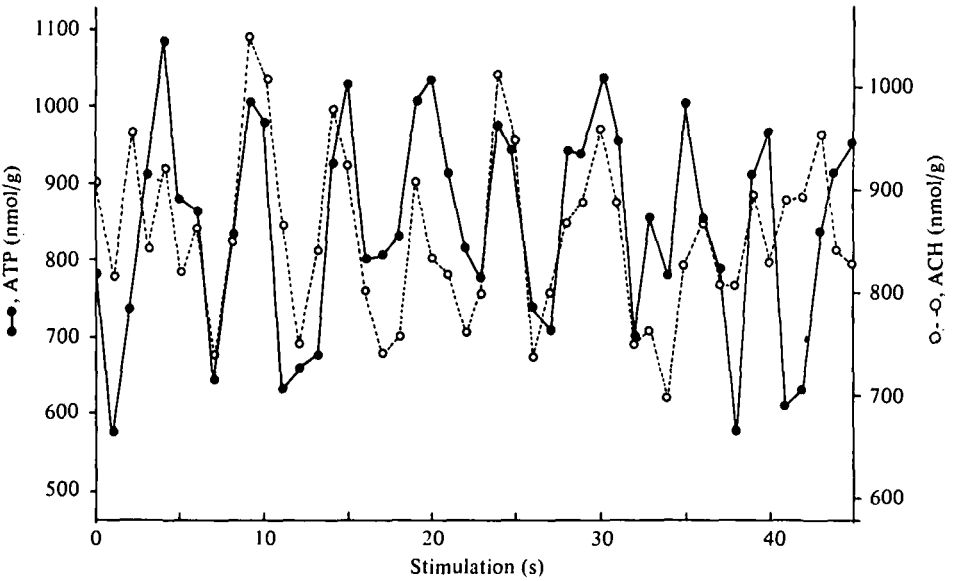

Fig. 4. Rapid in-phase oscillations of total ACh (●—●) and ATP (○--○) in the course of a 5 Hz stimulation. The period of the oscillations is about 5 s. The amplitude of the order of 40% of the total. Sampling protocol and quenching procedure (hot HCl) are described in Methods.

will not be inhibited particularly if an inward flux of choline feeds the reaction. Synthesis of ACh thus catches up the increased output triggered by stimulation. An increase of ACh and CoA concentrations will switch off synthesis and while the release of ACh continues the ACh content decreases. Such a model explains satisfactorily the 'slow wave'. It is, in fact, possible to balance from the rates of synthesis and free ACh variations the output of transmitter (Israël, Lesbats & Manaranche, 1978).

(*b*) *Rapid acetylcholine and adenosine triphosphate oscillations*

The 'slow wave' was submitted to a more detailed analysis in regions A, B and C (Fig. 2).

A rapid oscillation of total ACh with an amplitude of the order of 30% of the total and a period of 4–5 s is triggered by stimulation (Fig. 2). The mean level of this oscillation is below unstimulated controls during the initial declining phase of the electrical response (A). It will then fluctuate above the controls during the plateau (B), and will oscillate again below the controls (C) when the electrical response is exhausted (Dunant *et al.* 1977).

A more convenient way to demonstrate the rapid oscillations is to repeat the same stimulation several times, to average out the values for a given stimulation time and compare the means of points taken in an interval lower than or equal to the period (Fig. 3). It can be seen in Fig. 3 that with a time interval of less than 5 s, two points at least have statistically different means.

As ATP is essential for the synthesis of ACh we have measured the amount of ATP present in the stimulated samples.

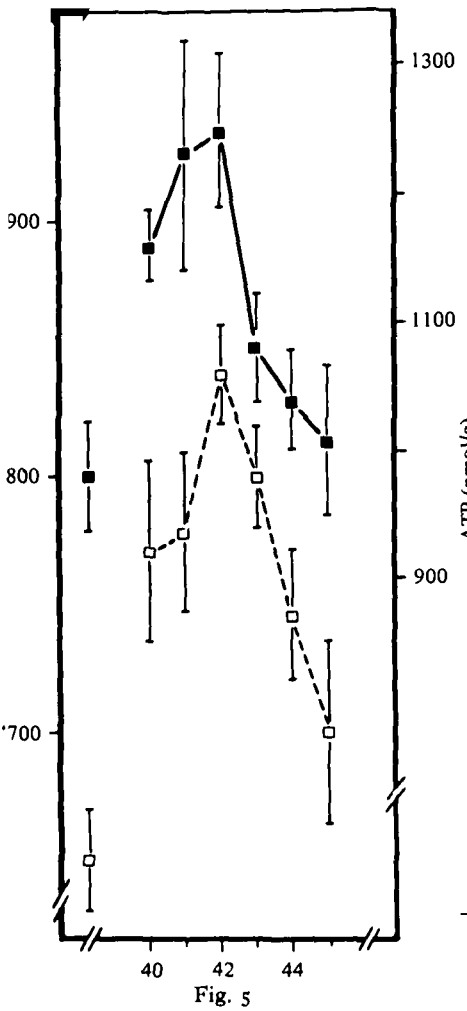

Fig. 5

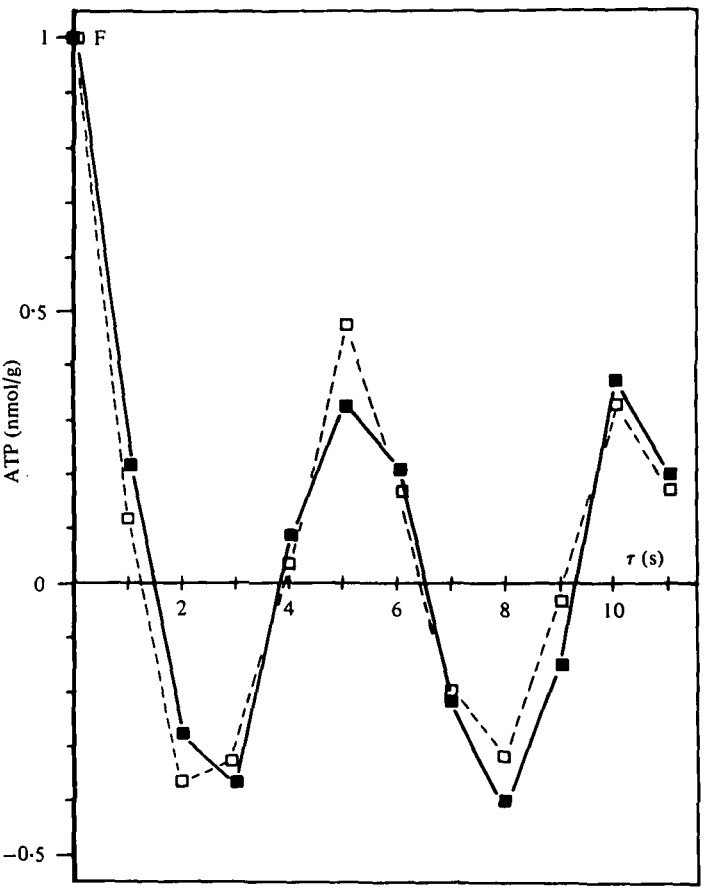

Fig. 6

Fig. 5. As for Fig. 3, the oscillation is demonstrated by comparing means of samples stimulated for the same time: this was done for ACh (□) and ATP (■). For ACh point 42 s compared to 45 s, $0{\cdot}05 > P > 0{\cdot}02$, $n = 5$, samples for each stimulation time. In this particular experiment curare (10^{-4} M) was present and abolished the electroplaque discharge. The in-phase ACh and ATP oscillations were not altered. (Liquid-nitrogen TCA extraction procedures.)

Fig. 6. Autocorrelation analysis of the rapid ACh and ATP oscillations in two different experiments in which the stimulation was delivered at 5 Hz for 1 min. Time resolution is of 1 s. ACh was analysed in one experiment during the whole 2nd minute of stimulation and ATP was measured in the other experiment during the 1st min of stimulation. The period of the oscillation is 5 s. □, ACh; ■, ATP.

Most surprisingly it was found that ATP oscillates in phase with ACh (Israël *et al.* 1975, 1977). The ACh and ATP oscillations have similar amplitudes and the same period (Fig. 4). The ATP oscillations have a mean level falling below controls after about 3–5 min of stimulation.

The ACh and ATP oscillations are superimposable (Fig. 5). Moreover curare at 10^{-4} M did not alter them, showing that they are presumably independent of post-synaptic transmitter action and likely to be presynaptic in origin. The autocorrelation

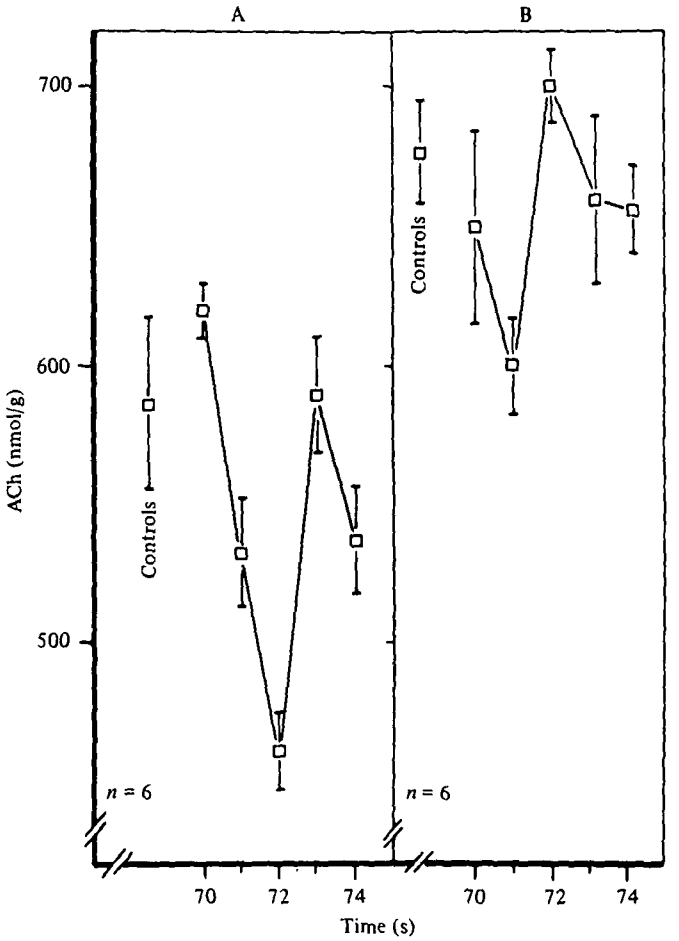

Fig. 7. Effect of hemicholinium 3 on the rapid oscillation. On the left, untreated samples. The points 70–72 s of stimulation have statistically different means, $P < 0{\cdot}001$. On the right, hemicholinium (10^{-5} M) altered the amplitude of the oscillations. The hemicholinium experiment was done 3 h after the untreated samples.

analysis (Fig. 6) of the rapid ACh and ATP oscillations clearly shows that the fluctuation is not random and that the period is 5 s.

Some other observations should also be mentioned. First, hemicholinium (a compound which inhibits choline uptake) attenuates the oscillation (Fig. 7). We also have evidence that during stimulation at 6 °C the amplitude and regularity of the oscillation is altered. The frequency of stimulation between 1 and 20 Hz did not modify the period of the oscillation (Dunant *et al.* 1977).

The possibility exists that the rapid oscillations are artifactual. However, several controls were carried out to minimize this possibility. In the absence of stimulation, the maximum dispersion between samples was not greater than 10%. For example, five controls taken in different parts of the organ had 757 ± 17 nmol/g of ACh and 766 ± 15 nmol/g of ATP. It was also shown that the oscillations were independent of the sampling protocol and that ATP did not interfere with the ACh assay and that ACh did not interfere with the ATP assay. The ACh content of a peak of the

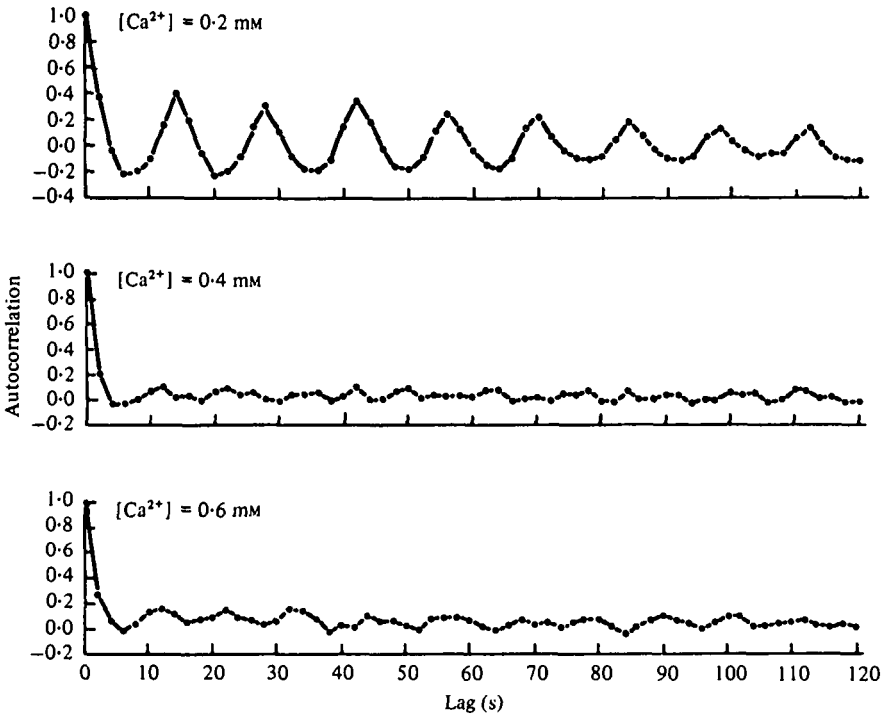

Fig. 8. Oscillations in e.p.p. amplitudes. Autocorrelations of three series of e.p.p.s. from the same end-plate in three different Ca concentrations (from Meiri & Rahamimoff, 1978).

oscillation could be hydrolysed by esterase without changing the ATP level of the sample. It was also possible to hydrolyse ATP by apyrase without changing the amount of ACh in the sample.

If we accept the existence of rapid oscillations, the lack of effect of curare indicates that they are independent of postsynaptic transmitter action and are located in the nerve terminals where ACh is synthesized. The fact that hemicholinium perturbates the ACh oscillations by presumably altering the influx of choline indicates that they might be related to the synthesis of transmitter. The enzymes (choline acetylase and acetylCoA synthetase) involved in the system do not have kinetic properties that are able to explain the oscillations. *In vitro* the rates of synthesis of the enzymes are compatible with the ACh production observed *in vivo* during the rising phase of the slow wave, but they are far too slow to account for the rising phase of the rapid oscillation. It is necessary to understand how these proteins are organized to attain such rates of transmitter production. Is such an organization part of the release mechanism?

Recently at the frog neuromuscular junction Erulkar & Rahamimoff (1976) have described an oscillation in the frequency of the miniature end-plate during stimulation. Meiri & Rahamimoff (1978) also show that at a low quantal content the amplitude of end-plate potential oscillates. The period they have found is close to the period of the ACh oscillations described in *Torpedo* electric organ. If there is a relation between the cytoplasmic concentration of ACh and the amount of transmitter released one would expect to see an oscillation of the end-plate potential, particularly

when the amount of cytoplasmic ACh is low and only fed by an oscillating synthesis. Such a situation would happen in low Ca^{2+}. It is in this condition (low quantal content) that the end-plate potential amplitude was shown to oscillate (Fig. 8). In our experiments carried out with normal $[Ca^{2+}]_0$ we have not observed an oscillating electroplaque discharge. It is possible that the oscillation appears when the release mechanism is not saturated by a high ACh concentration.

Is it necessary to suppose that kinetic systems, working far from equilibrium, are important features of synaptic activity? Or should we consider them as a curiosity and of no physiological significance? These questions will not be rapidly answered until it becomes possible to identify the oscillator and study its properties. What is the saturable process releasing ACh? The present data do not permit us to know whether it is plugged in the presynaptic membrane or in the membrane of vesicles adjacent to it.

REFERENCES

AUGER, D. & FESSARD, A. (1941). Action du curare, de l'atropine et de l'ésérine sur le prisme électrogène de la Torpille. *C. r. Séanc. Soc. Biol.* **135**, 76–78.

BENNETT, M. V. L., WÜRZEL, M. & GRUNDFEST, H. (1961). The electrophysiology of electric organs of marine electric fishes. Properties of electroplaques of *Torpedo nobiliana*. *J. gen. Physiol.* **44**, 757–804.

DUNANT, Y., GAUTRON, 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. *J. Neurochem.* **19**, 1987–2002.

DUNANT, Y., GAUTRON, J., ISRAËL, M., LESBATS, B. & MANARANCHE, R. (1974*a*). Evolution de la décharge de l'organe électrique de la Torpille et variations simultanées de l'acétylcholine au cours de la stimulation. *J. Neurochem.* **23**, 635–643.

DUNANT, Y., ISRAËL, M., JIROUNEK, P., LESBATS, B. & MANARANCHE, R. (1974*b*). Sustained oscillations of Ach during nerve stimulation. *Nature*, **252**, 485–586.

DUNANT, Y., ISRAËL, M., LESBATS, B., MANARANCHE, R. (1976). Loss of vesicular acetylcholine in *Torpedo* electric organ on discharge against high external resistance. *J. Neurochem.* **27**, 975–977.

DUNANT, Y., ISRAËL, M., LESBATS, B., MANARANCHE, R. (1977). Oscillation of acetylcholine during nerve activity in the *Torpedo* electric organ. *Brain Res.* **125**, 123–140.

ERULKAR, S. D. & RAHAMIMOFF, R. (1976). Periodic fluctuations in transmitter release at the frog neuromuscular junction. *J. Physiol., Lond.* **256**, 20–21*P*.

FELDBERG, W. & FESSARD, A. (1942). The cholinergic nature of the nerves of the electric organ of Torpedo (*Torpedo marmorata*). *J. Physiol., Lond.* **101**, 200–215.

FELDBERG, W., FESSARD, A. & NACHMANSOHN, D. (1940). The cholinergic nature of the nervous supply to the electric organ of the Torpedo (*Torpedo marmorata*). *J. Physiol., Lond.* **97**, 3*P*.

FESSARD, A. (1947). Recherches sur le fonctionnement des organes électriques. I. Analyse des formes de décharges obtenues par divers procédés d'excitation. *Archs Int. Physiol.* **55**, 1–26.

ISRAËL, M. & DUNANT, Y. (1975). Dynamics of acetylcholine compartments at rest and during nerve activity. In *Metabolic Compartmentation and Neurotransmission* (ed. S. Berl, D. D. Clarke and D. Schneider), pp. 621–639. New York: Plenum.

ISRAËL, M., GAUTRON, J. & LESBATS, B. (1968). Isolement des vésicules synaptiques de l'organe électrique de la Torpille et localisation de l'acétylcholine à leur niveau. *C. r. hebd. Séanc. Acad. Sci., Paris* **266**, 273–275.

ISRAËL, M., GAUTRON, J. & LESBATS, B. (1970). Fractionnement de l'organe électrique de la Torpille: localisation subcellulaire de l'acétylcholine. *J. Neurochem.* **18**, 1441–1450.

ISRAËL, M., LESBATS, B. & MANARANCHE, R. (1978). Quantitative description of acetylcholine release and fluctuations in nerve terminals of *Torpedo* electric organ submitted to stimulation. *Pflügers Arch.* **377**, 117–118.

ISRAËL, M., LESBATS, B., MANARANCHE, R., MARSAL, J., MASTOUR-FRACHON, P. & MEUNIER, F. M. (1977). Related changes in amount of ACh and ATP in resting and active *Torpedo* nerve electroplaque synapses. *J. Neurochem.* **28**, 1259–1267.

ISRAËL, M., LESBATS, B., MARSAL, J. & MEUNIER, F. M. (1975). Oscillations des taux tissulaires d'acétylcholine et d'adénosine triphosphate au cours de la stimulation de l'organe électrique de la Torpille. *C. r. hebd. Séanc. Acad. Sci., Paris* **280**, 905–908.

ISRAËL, M., TUCEK, S. (1974). Utilization of acetate for the synthesis of acetylcholine in the electric organ of *Torpedo*. *J. Neurochem.* **22**, 487–491.

MACINTOSH, F. C. & PERRY, W. L. M. (1950). Biological estimation of acetylcholine. *Meth. Med. Res.* **3**, 78–92.

MARCHBANKS, R. M. (1975). Biochemistry of cholinergic neurons. In *Handbook of Psychopharmacology* (ed. L. L. Iversen, S. D. Iversen and S. H. Snyder), chapter 5, pp. 247–326. New York: Plenum.

MEIRI, H. & RAHAMIMOFF, R. (1978). Clumping and oscillations in evoked transmitter release at the frog neuromuscular junction. *J. Physiol., Lond.* **278**, 513–523.

MOREL, N., ISRAËL, M. & MANARANCHE, R. (1978). Determination of ACh concentration in *Torpedo* synaptosomes. *J. Neurochem.* **30**, 1553–1557.

SUSZKIW, J. B., ZIMMERMANN, H. & WHITTAKER, V. P. (1978). Vesicular storage and release of acetylcholine in Torpedo electroplaque synapses. *J. Neurochem.* **30**, 1269–1280.

WHITTAKER, V. P., ESSMAN, W. B. & DOWE, G. H. C. (1972). The isolation of pure cholinergic synaptic vesicles from the electric organs of elasmobranch fish of the family Torpidinae. *Biochem. J.* **128**, 833–846.

ZIMMERMANN, H. & WHITTAKER, V. P. (1974*a*). Effect of electrical stimulation on the field and composition of synaptic vesicles of the electric organ of *Torpedo*: A combined biochemical, electrophysiological and morphological study. *J. Neurochem.* **22**, 435–450.

ZIMMERMANN, H. & WHITTAKER, V. P. (1974*b*). Different recovery rates of the electrophysiological, biochemical and morphological parameters in the cholinergic synapses of the *Torpedo* electric organ after stimulation. *J. Neurochem.* **22**, 1109–1114.