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Presynaptic K^+ Channels, Vesicular Ca^{2+}/H^+ Antiport—Synaptotagmin, and Acetylcholinesterase, Three Mechanisms Cutting Short the Cholinergic Signal at Neuromuscular and Nerve–Electroplaque Junctions

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Abstract In neuromuscular and nerve–electroplaque junctions, nerve impulses can be transmitted at high frequencies. This implies that transmission of individual impulses must be very brief. We describe three mechanisms which curtail the time course of individual impulses at these synapses: (1) opening of presynaptic K^+ channels (delayed rectifier) efficiently curtails the presynaptic action potential. Inhibition of K^+ channel by aminopyridines transforms the normally brief postsynaptic potential (2–3 ms) to a long-lasting "giant" potential (exceeding half a second); (2) a low-affinity Ca^{2+}/H^+ antiport ensures rapid Ca^{2+} sequestration into synaptic vesicles, curtailing the calcium signal and thereby the duration of transmitter release. Indeed vesicular Ca^{2+}/H^+ antiport inhibition by bafilomycin or Sr^{2+} prolongs the duration of the postsynaptic potential. We recently showed that synaptotagmin-1 is required for this antiport activity; thus the vesicular Ca^{2+}/H^+ antiport might be synaptotagmin itself, or regulated by it; and (3) it is recalled that, in these junctions, acetylcholinesterase is highly concentrated in the synaptic cleft and that anticholinesterases lengthen the endplate time course. Therefore, at three different steps of synaptic transmission, an efficient mechanism curtails the local synaptic signal. When one of these three mechanisms is inhibited, the duration of individual impulses is prolonged, but the synapse loses its faculty to fire at high frequencies.

Keywords Rapid cholinergic transmission · Presynaptic potassium channels · Aminopyridines · Vesicular Ca^{2+}/H^+ antiport · Synaptotagmin · Acetylcholinesterase

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Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
AP	Action potential
AmPy	Aminopyridine
EPP	Endplate potential or electroplaque potential
MEPPs	Miniature endplate or miniature electroplaque potentials
NEJ	Nerve–electroplaque junction
NMJ	Neuromuscular junction
V-ATPase	Vacuolar H^+-transporting adenosine triphosphatase
VOCCs	Voltage-operated calcium channels

Introduction

Although cholinergic transmission in central and peripheral nervous systems seems to rely on the same general mechanisms, there are considerable differences from one synapse to another. Time is the critical factor in this diversity. Certain cholinergic modulations in the central nervous system (CNS) last seconds, minutes, or more (Descarries et al. 1997; Dunant et al. 2010). Neuro-neuronal synapses in mammalian sympathetic ganglia transmit excitatory signals within 10–20 ms, while in neuromuscular junctions (NMJ), transmission is achieved in less than 2 ms, even in poikilothermic animals. If transmission of individual impulses were not as fast, NMJs could not fire at frequencies up to 100 Hz. During a sustained muscle contraction, the interval between successive impulses is of the order of 10 ms.

The present study aims at describing molecular mechanisms which make neuromuscular transmission so fast. Cholinergic transmission is chemical in nature, and we recall here that the velocity of a chemical reaction is inversely related to

affinity. This was elegantly formulated by Bernard Katz (1989): "Time is gained at the expense of sensitivity". Therefore, the processes which are directly involved in fast synaptic transmission must rely on low-affinity reactions, enabling both fast "on" and "off" reaction rates.

This article is based on ancient and recent observations made on the neuromuscular junctions of various species as well on the *Torpedo* electric organ. Embryologically, the electric organ is a neuromuscular system. Like in NMJ, transmission is cholinergic and quantal at the nerve–electroplaque junction (NEJ). The physiological, pharmacological and molecular parameters of transmission are qualitatively and quantitatively close in the two systems. Particularly, the size of evoked ACh quanta and that of the main population of spontaneous quanta is the same (6,000–10,000 ACh molecules) (Kuffler and Yoshikami 1975; Dunant and Muller 1986). In addition, a population of small quanta (or subquanta, 600–1,000 ACh molecules) becomes prominent at high release rates and under specific experimental conditions (Kriebel and Gross 1974; Girod et al. 1993). A *Torpedo* electric organ (weight 100–300 g) contains myriads of cholinergic synapses (approximately $4–6\times10^{11}$ per organ), which operate with an astonishing synchronicity. Even at 15 °C, the duration of a single electric discharge is 2–4 ms, and the fish can deliver repetitive discharges at frequencies of 100–200 Hz, for attack or defence. Nicotinic ACh receptors of NEJs are structurally and functionally similar to those of NMJs; they display even a slightly shorter open time (0.6 ms; Sakmann et al. 1985).

The electric organ offer many advantages to investigators interested in rapid cholinergic transmission: ACh release can be monitored in real-time on the same tissue samples using electrophysiology, biochemistry and morphology (rapid freezing); vesicular ACh and cytoplasmic ACh can be determined at any moment during the course of stimulation; the tissue structure and function display a high degree of homogeneity throughout the electric organ. While several tricks are used in NMJ to avoid inconveniences due to muscle contraction (low Ca^{2+}–high Mg^{2+} solutions, curare, etc.), these are not required with the *Torpedo* electric organ, which does not contract on activity. It is thus possible at the NEJ to elicit and record EPPs over a high range of quantal contents. Moreover, in *Torpedo,* the tissue electrical resistance is very low during the discharge. As a consequence, the time course of electroplaque currents (EPCs) is identical to the time course of electroplaque potentials (EPPs). Also, record traces are not biased by phenomena like non-linear summation (Girod et al. 1993).

In addition to cholinergic synapses, we shall take examples from a few reports related to other rapid synaptic preparations, particularly CNS glutamatergic synapses, and invertebrate junctions, such as the squid giant synapse, a preparation where presynaptic and postsynaptic electrical currents can be recorded simultaneously with intracellular electrodes.

Results and Discussion

Figure 1 illustrates the time course of three essential steps of rapid synaptic transmission: (1) the presynaptic depolarisation, (2) the local Ca^{2+} signal and (3) the postsynaptic depolarisation due to transmitter action on receptors. Calcium ions enter nerve terminals through voltage-operated calcium channels (VOCC) at the end of the presynaptic action potential (AP). Ca^{2+} promptly reaches a high concentration there in restricted "microdomains" situated close to the inner mouth of VOCCs. This local Ca^{2+} signal (the "Ca^{2+} spark") is extremely brief; it decays with an initial time constant in the order of 300–600 μs. Soon, very soon after, postsynaptic receptors start opening under the action of transmitter, depolarising the downstream cell. What is really amazing is the brevity of the delay separating the presynaptic Ca^{2+} spark from the postsynaptic current, only 50–200 μs (Yazejian et al. 2000; Llinas et al. 1981, 1992; Sabatini and Regehr 1996;

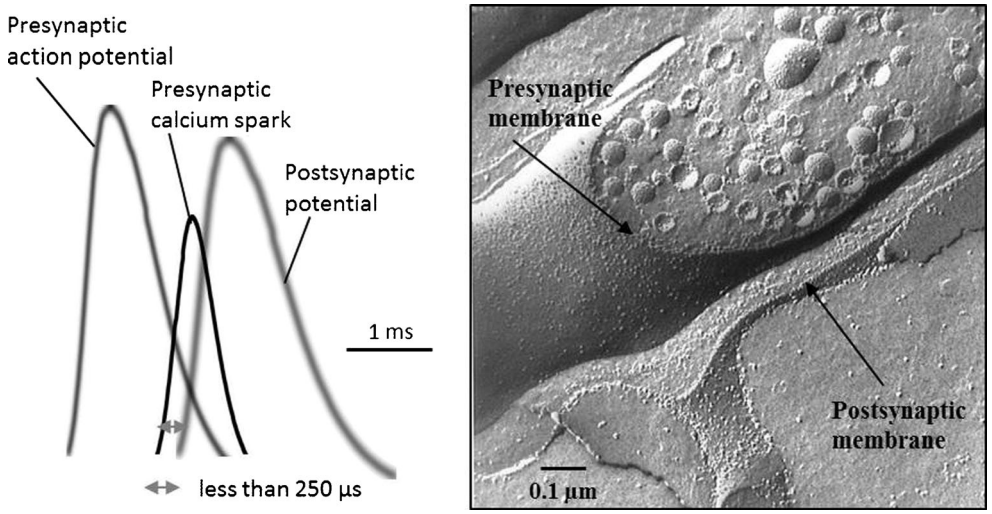

Fig. 1 Time course of the three main events in rapid synaptic transmission (schematic drawing taken from the references cited in the text). At the end of the presynaptic action potential, Ca^{2+} concentration raises abruptly in "micro-domains" situated at the inner face of the presynaptic membrane. An extremely short delay (less than 250 μs) separates the Ca^{2+} signal from the postsynaptic potential. *Right*: freeze fracture replica from a *Torpedo* nerve–electroplaque synapse (modified from Garcia-Segura et al. (1986))

Roberts 1994; Roberts et al. 1990). Within this time lapse, the releasing mechanism (whatever it is) has to be activated by Ca^{2+}; ACh has to cross the presynaptic membrane, to diffuse in the synaptic cleft and to activate the postsynaptic receptors. Our purpose here is to describe three specific "abbreviating" mechanisms which operate at these three stages of transmission. Each of them efficiently curtails the time course of transmission as seen by the fact that, when they are absent or inhibited, the time course of individual impulses is prolonged, and consequently the synapse loses its ability to fire at a high frequency.

Voltage-Gated K^+ Channels Curtail the Presynaptic Action Potential

Generation of a presynaptic action potential (AP) roughly follows the same rules as the generation of axonal or muscular APs. A local small depolarisation opens voltage-operated Na^+ channels, which explosively recruit neighbour channels, depolarising the fibre. However, the cell is then rapidly repolarised (clearly before inactivation of the Na^+ channels) by occurrence of an outward K^+ current (the so-called delayed rectifier), rapidly rising to a maximum and then declining slowly (Hodgkin and Huxley 1952). Presynaptic APs were recorded with intracellular electrodes at the squid giant axon (Katz and Miledi 1966; Bloedel et al. 1966) and with extracellular electrodes at neuromuscular and other cholinergic junctions (Katz and Miledi 1969; Dunant 1972; Brigant and Mallart 1982). The voltage-operated, outward K^+ current (delayed rectifier) is particularly vigorous in axon terminals. Additional presynaptic K^+ currents (I_{KCa}, I_{KATP}, etc.) occur in axon terminals. However, these do not seem to contribute to the rapid restoration of membrane potential in the course of the presynaptic AP. Rather, they were proposed to protect nerve terminals from a sustained depolarisation consecutive to ischemia or other factors.

Several chemicals are able to block the rapid outward K^+ current (delayed rectifier): caesium and uranyl ions, tetraethylamonium, tacrine or even ACh. More specific blockers are members of the aminopyridine family (AmPy): particularly 4-aminopyridine, 3-aminopyridine and the more potent 3-4-diaminopyridine (Van der Kloot and Molgo 1994). At the squid giant synapse, AmPy increase the duration of the presynaptic AP. This results in an increase in transmitter release as measured by monitoring the postsynaptic potential. The potentiation of transmission by AmPy was found to arise purely from the prolongation of the presynaptic AP, consecutive to K^+ channel blockage (Llinas et al. 1976). At neuromuscular junctions, AmPy drugs provoke a dramatic potentiation of the amount of transmitter released by isolated impulses. The EPP is enlarged in both amplitude and duration. The synaptic delay is prolonged, which is explained by the longer duration of the presynaptic depolarisation, delaying the onset of the Ca^{2+} spark. The Ca^{2+} current itself is also enhanced and prolonged. Spontaneous miniature potentials (MEPPs) are not affected by AmPy, suggesting that the giant evoked response results from the release of an increased number of normal quanta. However, MEPPs of irregular shape, of small or of oversized amplitude occur frequently in AmPy-treated endplates (Katz and Miledi 1979; Molgo and Thesleff 1982; Van der Kloot and Molgo 1994).

The effects of AmPy were thoroughly investigated in the *Torpedo* electric organ. These drugs dramatically potentiate the transmission of isolated impulses. Treatment with 100 μM 4-AP transforms the relatively brief EPP (2–3 ms) into a giant EPP whose amplitude is moderately increased but whose duration is enormously lengthened (Fig. 2). Such giant EPPs exhibit a complex time course, with a pronounced rebound at about 300 ms after the stimulus. It abates only after 500–600 ms. If a second stimulus is applied 3–5 s after a giant EPP, the second response will be much briefer, resembling a normal EPP. About 15 min is required for the synapse to recover and generate a

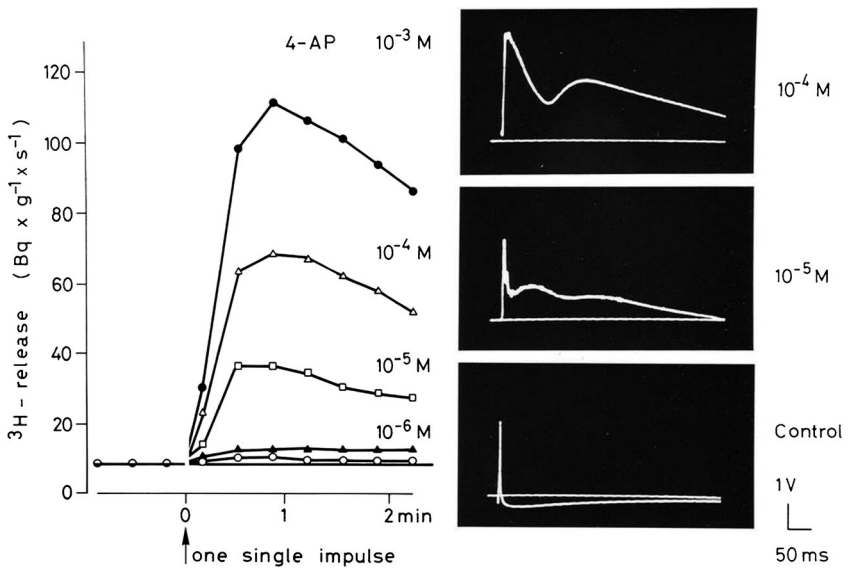

Fig. 2 Four-aminopyridine (*4-AP*) enormously prolongs the duration of the EPP (electroplaque potential, a postsynaptic potential) in the *Torpedo* electric organ. This arises from a large increase in the amount of ACh released in response to a single nerve stimulus. In the *graph* on the *left*, *open circles* show the amount of ACh overflowing from the tissue in response to the nerve stimulus applied in the absence of 4-AP (modified from Dunant et al. (1980) and Corthay et al. (1982))

second giant EPP similar to the first one. The AmPy effect is dose-dependent and purely presynaptic. As a matter of fact, we measured the amount of ACh overflowing from the tissue when a giant impulse was generated. With 4-AP concentrations ranging from 1 to 100 μM, the augmentation of ACh release closely correlated with the enlargement of the EPP area (Fig. 2; Dunant et al. 1980; Corthay et al. 1982).

Focal recording of a small group of synapses confirmed that AmPy derivatives do not alter spontaneous MEPPs' size or time course and that of the giant EPP if composed of a sustained released of standard quanta (Muller 1986).

In the absence of stimulation, AmPy do not provoke a pronounced increase in spontaneous ACh release in the NEJ. Nevertheless, resting ACh turnover is increased by 4-AP. Eventually, the amount of tissue ACh decreased, both in synaptic vesicles and in the cytosolic compartment. Therefore, the enormous potentiation of ACh release seen in an isolated impulse takes place in spite of reduced ACh stores. Advantage was taken from AmPy to analyse vesicular and cytosolic ACh during the course of a single giant impulse, using rapid freezing techniques. Cytosolic ACh exhibited an initial fall and then a transient increase at 300 ms, corresponding in time with the rebound of the giant EPP. In contrast, vesicular ACh remained stable for the whole giant EPP duration. Using compartment analysis with radioisotopes, we did not observe any significant transfer from cytosolic ACh to vesicular ACh during the course of a giant EPP (Corthay et al. 1982).

Ultra-rapid freezing followed by freeze fracture was used to capture the morphological changes accompanying the transmission of single cholinergic impulses, both at the frog NMJ (Heuser et al. 1979; Torri-Tarelli et al. 1985) and at the *Torpedo* NEJ (Garcia-Segura et al. 1986; Muller et al. 1987). Under control conditions, i.e., in the absence of any drug, the same phenomenon was observed in both systems. Transmission of a single nerve impulse is accompanied by the occurrence of a population of large intramembrane particles in the presynaptic membrane. In the *Torpedo*, we could fetch the precise time course of the phenomenon. It lasts no more than 2–3 ms (Figs. 3 and 4). With AmPy, however, the picture is differed. At the frog NMJ, vesicle openings were caught already at the beginning of the EPP, and they continued to occur after the end of the EPP. The effect correlated with the degree of transmission potentiation by AmPy (Heuser et al. 1979). In the AmPy-treated electric organ, in contrast, stimulation evoked an increase in the number of intramembrane particles like in untreated junctions. The particle change

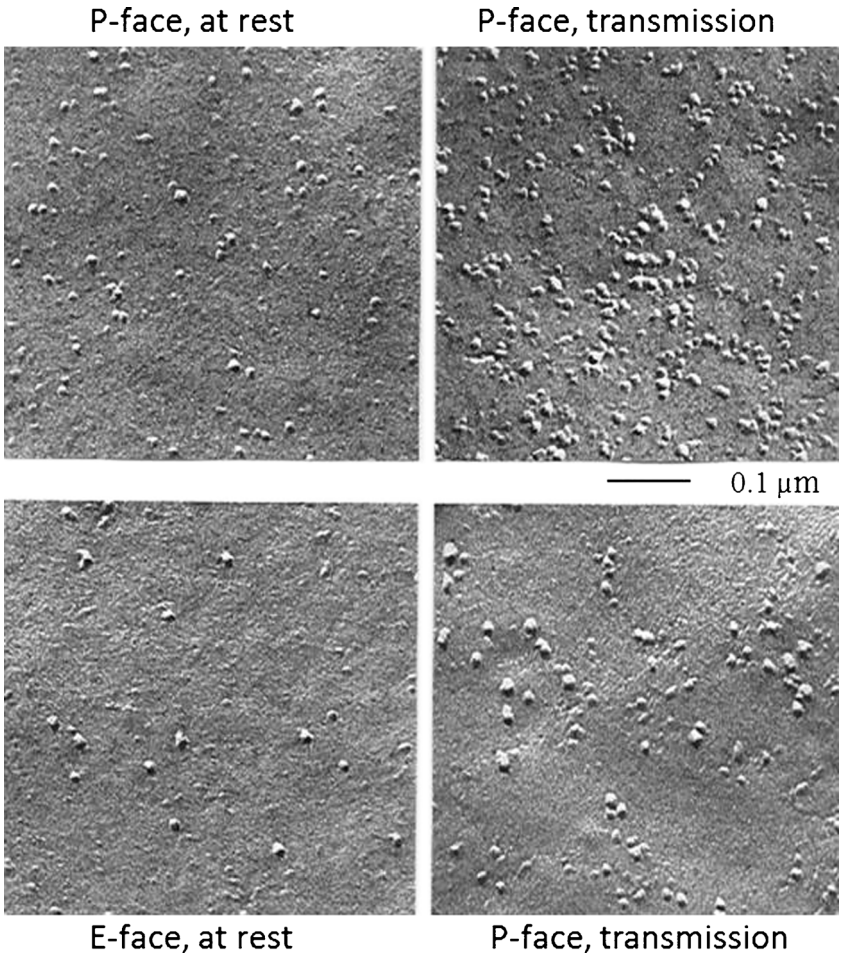

Fig. 3 Freeze fracture replicas of the presynaptic membrane in the *Torpedo* electric organ. Samples were rapidly frozen at rest and at 60 ms after a single stimulus, which was applied in the presence of 4-aminopyridine (100 μM). Transmission of the giant impulse is accompanied by a marked increase in the density of large intramembrane particles in both the protoplasmic (*P*) and the external (*E*) face of the membrane (modified from Garcia-Segura et al. (1986)). In the absence of 4-AP, the phenomenon is identical but lasts for only 2–3 ms (see Fig. 4 and Muller et al. 1987)

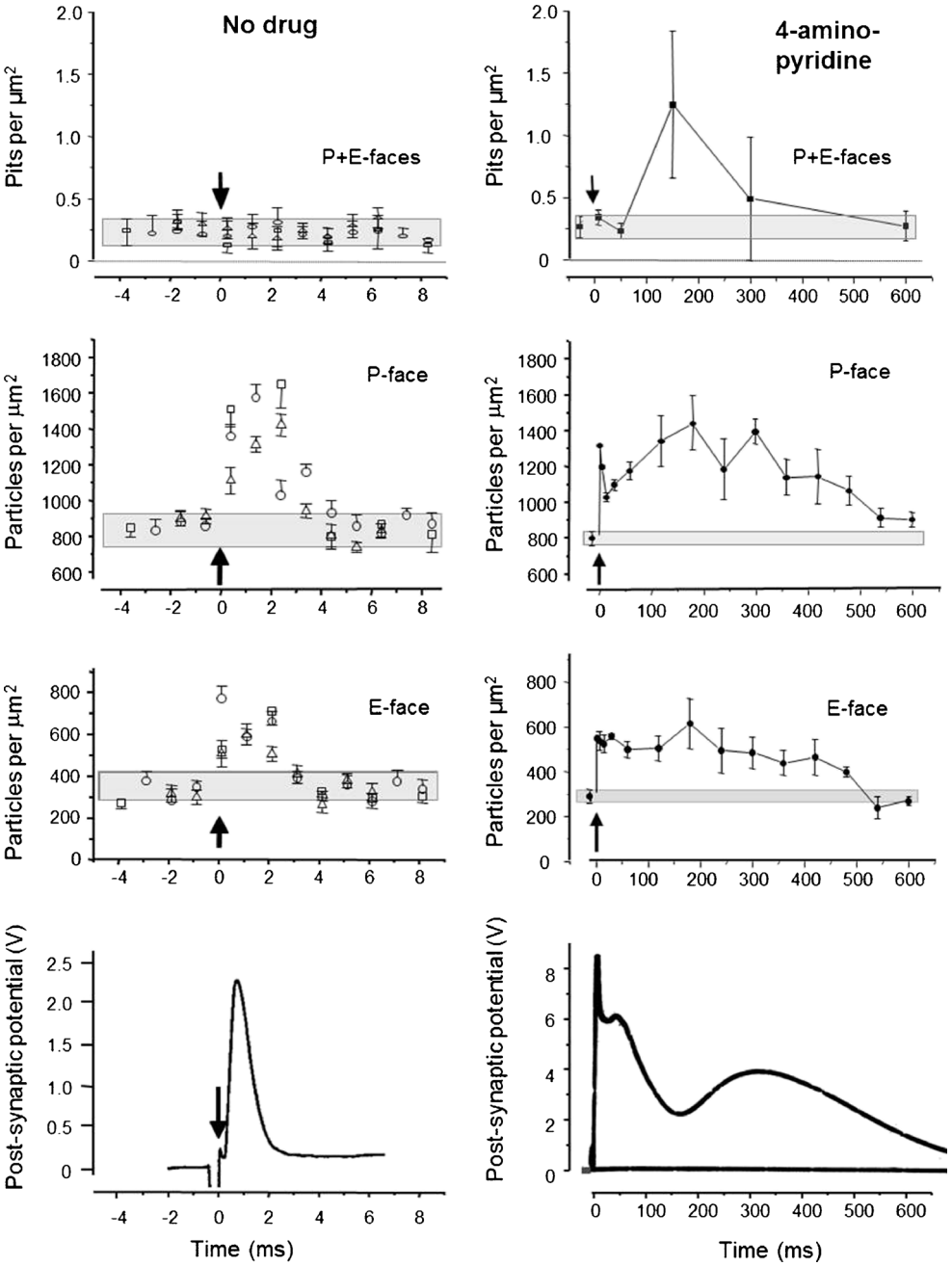

Fig. 4 Morphological changes in the presynaptic membrane accompanying the transmission of a single cholinergic impulse in the *Torpedo* electric organ in the absence or presence of 4-aminopyridine. Morphological data are plotted at the same time scale as the electrical records, shown in *bottom traces*. In controls, an abrupt (2–3 ms) increase in the number of intramembrane particles signals the transmission of a single impulse. No vesicle openings (pits) were observed during this period of time. With 4-AP, the post-synaptic potential (EPP) is transformed in a giant discharge exceeding half a second. The change in intramembrane particles follows an identical time course, whereas some vesicle openings occur with a significant delay with regard to the electrophysiological signal (modified from Garcia-Segura et al. (1986); Muller et al. 1987)

faithfully follows the time course of the giant EPP. When vesicle openings occur, this is only irregularly, at the late phase of discharge (Garcia-Segura et al. 1986; Figs. 3 and 4).

Conclusions Voltage-gated K^+ channels present in axon terminals are essential for curtailing presynaptic APs in rapid cholinergic transmission. When these are blocked by aminopyridines or other drugs, the time course of isolated impulse is greatly prolonged, and the capability of synapses to generate a high-frequency activity is impaired due to ion gradient collapse, to desensitisation of release and to transmitter and energy exhaustion (consecutive to the first giant impulse generation). Cholinergic transmission potentiation by AmPy has been attempted as a therapeutic approach in affections where ACh release is severely weakened (certain myasthenic syndromes, botulism, etc.).

The Ca^{2+}/H^+ Antiport (Synaptotagmin) Curtails the Presynaptic Ca^{2+} Spark

Different processes contribute to reduce Ca^{2+}concentration in nerve terminal active zones after an AP: diffusion, protein

binding, Ca^{2+}/Na^{+} exchange, as well as Ca^{2+} pumps (ATPases) present in the plasmalemma, reticulum and synaptic vesicles (Israël et al. 1980; Michaelson et al. 1980; Castonguay and Robitaille 2001; Villalobos et al. 2002; Rizzuto and Pozzan 2006; Rusakov 2006; Desai-Shah and Cooper 2009; Parekh 2008). Among them, several mechanisms operate at a relatively high affinity. They were therefore poor candidates for high-speed reactions. More recently, Gonçalves et al. (1998, 2000) demonstrated that calcium is also taken up by mammalian brain synaptic vesicles via a low-affinity Ca^{2+}/H^{+} antiport, which is activated only in the presence of high Ca^{2+} concentrations ($K_{0.5}$=217 μM; maximal activity at 500–600 μM). This raised the following question: could this vesicular Ca^{2+}/H^{+} antiport abbreviate the duration of the Ca^{2+} spark in rapid transmission? If so, its inhibition should result in a momentarily persistent Ca^{2+} in the active zone, which would result in a prolongation of the release time and thereby in EPP lengthening.

This is the case indeed, as illustrated in Fig. 5 from experiments carried out on the *Torpedo* NEJ (Cordeiro et al. 2011). The vesicular Ca^{2+}/H^{+} antiport can be blocked directly by strontium ions or indirectly by bafilomycin A1, a specific inhibitor of V-ATPase. Bafilomycin A1 dissipates the H^{+} gradient of synaptic vesicles, thereby annihilating the Ca^{2+}/H^{+} antiport driving force. In electrophysiology experiments, bafilomycin A1 significantly prolongs the duration of the EPP in individual impulses (Fig. 5a). A biochemical assay for ACh release and experiments with synaptosomes confirmed that the effect of bafilomycin A1 is presynaptic. Also, bafilomycin A1 augments the amount of calcium accumulating in nerve terminals following a short tetanic stimulation and delays subsequent calcium extrusion.

Strontium ions are not transported by Ca^{2+}/H^{+} antiport (Gonçalves et al. 1999), while they activate transmitter release at concentrations one degree of magnitude higher than Ca^{2+} does. In the presence of Sr^{2+}, the time course of the electroplaque potential is clearly prolonged (Fig. 5b). Bafilomycin and Sr^{2+} effects are better seen on isolated impulses. On repetitive stimulation, on the contrary, transmission exhibits a rapid "fatigue", most probably due to steady divalent cation elevation in nerve terminals and to the consecutive desensitisation of transmitter release (Adams et al. 1985; Katz and Miledi 1969; Israël et al. 1987).

We recently found that *synaptotagmin-1* is necessary for the vesicular Ca^{2+}/H^{+} antiport activity. Synaptotagmin-1 is a vesicular protein interacting with membranes upon low-affinity Ca^{2+} binding. It plays a major role in excitation–release coupling by synchronizing calcium entry with fast neurotransmitter release. We measured Ca^{2+}/H^{+} antiport activity in vesicles and granules of pheochromocytoma PC12 cells by three methods: (1) Ca^{2+}-induced dissipation of the vesicular H^{+} gradient, (2) bafilomycin-sensitive

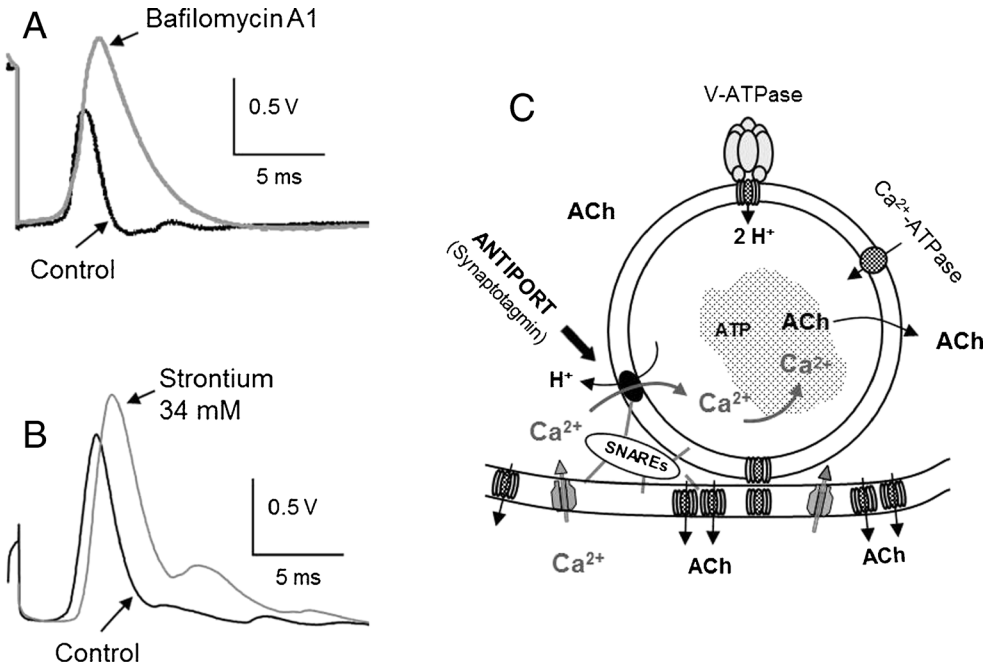

Fig. 5 A vesicular Ca^{2+}/H^{+} antiport curtails cholinergic transmission in the *Torpedo* electric organ. **a** This antiport can be inhibited indirectly with 2 μM bafilomycin-A1, a specific blocker of V-ATPase. As a result, with Ca^{2+} being less rapidly removed from the presynaptic active zone, the duration of ACh release is prolonged, as seen by the prolonged EPP. **b** A similar EPP lengthening is obtained by replacing calcium with an equipotent concentration of Sr^{2+}. Strontium ions not being transported by the vesicular Ca^{2+}/H^{+} antiport remain longer at active zones and continue to activate transmitter release. **c** Schematic drawing illustrating how Ca^{2+}, which entered via voltage-gated calcium channels, is rapidly sequestered by the vesicular Ca^{2+}/H^{+} antiport to be exchanged against ACh and ATP on the intra-vesicular matrix (Reigada et al. 2003). It was recently shown that synaptotagmin-1 is required for expression of the vesicular Ca^{2+}/H^{+} antiport (Cordeiro et al. 2013)

calcium accumulation and (3) pH-jump-induced calcium accumulation. The results were congruent and highly significant: Ca^{2+}/H^{+} antiport activity is detectable only in acidic organelles expressing functional synaptotagmin-1. In contrast, synaptotagmin-1-deficient cells—and cells where transgenically encoded synaptotagmin-1 had been acutely photo-inactivated—are devoid of any Ca^{2+}/H^{+} antiport activity (Cordeiro et al. 2013). Therefore, the vesicular Ca^{2+}/H^{+} antiport is either synaptotagmin-1 itself or a transporter whose expression is strictly dependent on synaptotagnin-1.

Other Synapses Synaptotagmin is the most abundant protein of brain synaptic vesicles (Takamori et al. 2006). So far, the vesicular Ca^{2+}/H^{+} antiport has been demonstrated in sheep and rat brain vesicles as well as in PC12 cells (Gonçalves et al. 1998; Cordeiro et al. 2013). In CNS synapses, Xu-Friedman and Regehr (2000) showed that Sr^{2+} ions activate transmitter release during a longer period than Ca^{2+} due to a longer persistence in nerve terminals after the stimulus. Also, in NMJ and other synapses where synaptotagmin-1 is either absent or inactivated, transmission of individual impulses shows either a prolonged time course or a reduced amplitude that can be interpreted by the lack of the vesicular Ca^{2+}/H^{+} antiport control of secretion timing and the consecutive increase of intraterminal $[Ca^{2+}]$ (see references in Cordeiro et al. (2013)).

Conclusions The physiological role of the vesicular Ca^{2+}/H^{+} antiport function seems therefore to curtail the Ca^{2+} spark in the nerve terminal active zones and thereby to abbreviate the "phasic" phase of transmitter release. Ca^{2+}/H^{+} antiport inhibition prolongs the time course of transmitter release in individual impulses but impairs the ability of synapses to fire repetitively.

Acetylcholinesterase Curtails the Postsynaptic Potential

A Famous Scientific Controversy It is recalled that the presence of highly concentrated AChE in the synaptic cleft is a particularity of NMJs and NEJs. This point was the quid pro quo of a famous historical controversy, namely, the battle of "sparks" against "soups". Between 1920 and 1949, most electrophysiologists (the "sparks") estimated that synaptic transmission was much too fast to rely on chemical reactions. Fascinated by the recent development of electrical techniques which enabled to record action potentials in real time, the "sparks" were not much receptive to biochemical or pharmacological arguments. On the other side, biochemists and pharmacologists (the "soups", among them H. Dale, O. Loewi, W. Feldberg, Z.M. Bacq and others) had put forward strong arguments in favour of the chemical nature of transmission, but they had difficulty to follow the speed of electrophysiologists' observations (for a vivid relation of this saga, see Z.M. Bacq (1974, 1975)). Cholinergic transmission at the NMJ and in sympathetic ganglia was at the centre of the controversy. J.C. Eccles, the file leader of the electrophysiologists' camp, had built a subtle theory based on "eddy currents", which should transfer excitation from the pre- to the post-synaptic membrane. After some time, however, Eccles began to admit that ACh could transmit impulses at the NMJ, realizing that eserine (or physostigmine, a cholinesterase inhibitor) prolongs the time course of the EPP, an observation which was made by Feng and Shen (1937) and then confirmed in Eccles' laboratory in a work carried out with B. Katz and S. Kuffler (Eccles et al. 1942). However, in Eccles' opinion, the hypothesis of "eddy currents" remained valid for the rapid phase of transmission in the neuro-neuronal synapses of sympathetic ganglia since eserine did not modify the time course of the excitatory postsynaptic potential (EPSP). Eventually, Eccles made a spectacular conversion and joined the chemical transmission camp, just in time for getting the Nobel Prize.

The discrepancy between eserine action on NMJs and sympathetic ganglia was elucidated much later. Although sympathetic ganglia contain significant amounts of AChE, the enzyme is not concentrated in the synaptic cleft of these synapses. Therefore, the decay of the ganglion EPSP is not governed by ACh hydrolysis but by diffusion away from the cleft (Gisiger et al. 1977, 1978).

Cholinesterase Inhibition at the Neuromuscular Junction and in Electric Organ It has long been known that AChE is highly concentrated in the synaptic cleft of NMJ (Couteaux 1955). AChE localisation is very similar at the *Torpedo* NEJ (Fig. 6, upper picture; see also Gautron (1970)). In vertebrate NMJs, reversible and irreversible cholinesterase inhibitors greatly lengthen the decay phase of EPP and potentiate the local response of the muscle to externally applied ACh (Feng and Shen 1937; Eccles et al. 1942). Cholinesterase inhibitors similarly affect the time course of spontaneous MEPPs (Fatt and Katz 1952). Anticholinesterase drugs were decisive tools for demonstrating the cholinergic nature of transmission in the *Torpedo* electric organ (Feldberg et al. 1940). The record traces in Fig. 6 illustrate the effects of eserine at the NEJ: (a) eserine increases moderately but significantly the amplitude of the stimulus-evoked EPP, while it greatly prolongs the EPP decay phase; (b) The time course of spontaneous MEPPs is similarly affected and (c) in response to repetitive nerve stimulation, the EPP amplitude rapidly falls down. At first potentiated by eserine, transmission cannot face with sustained repetitive activity. Unexpectedly, AChE inhibitors markedly reduce the amount of transmitter released per impulse. In the presence

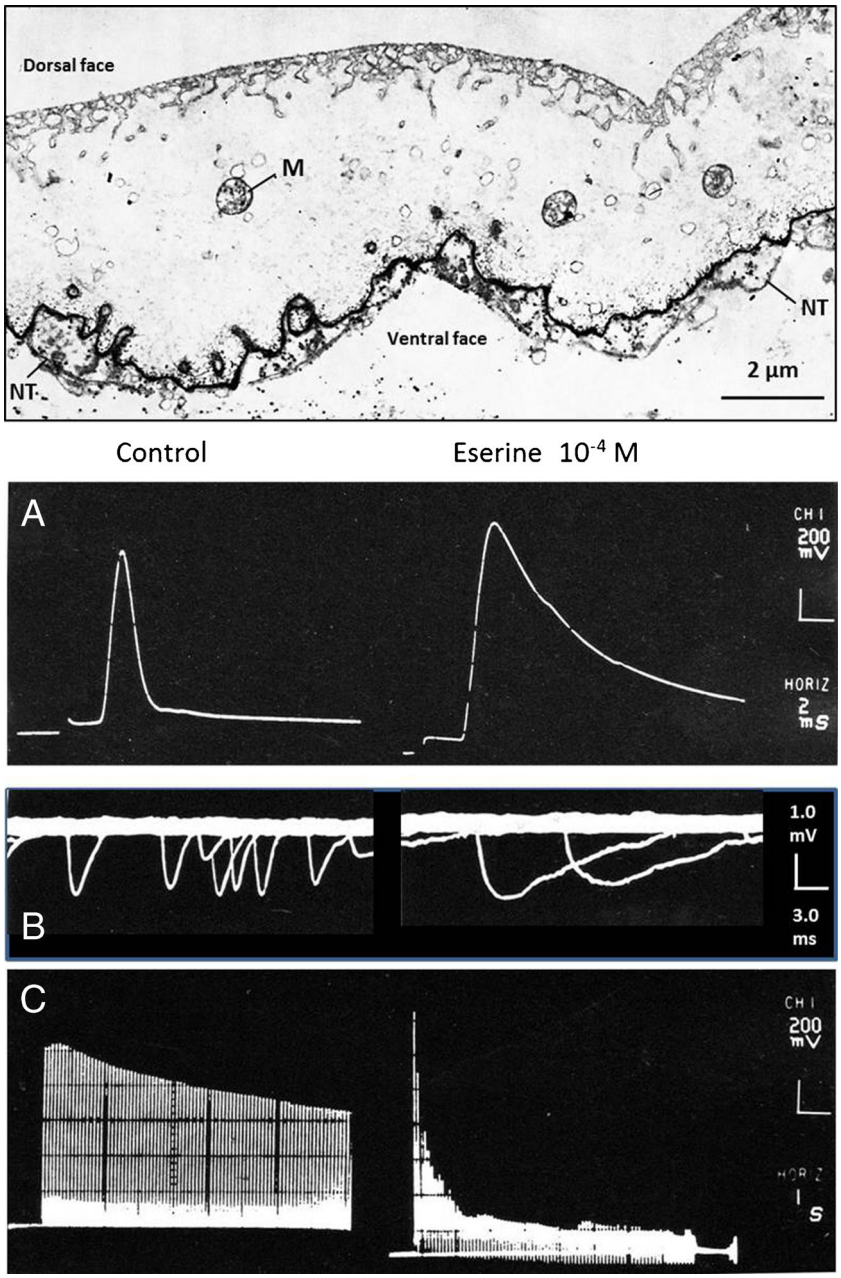

Fig. 6 *Top* histochemical labelling of acetylcholinesterase in a thin section of a *Torpedo* electroplaque. Like in neuromuscular junctions, AChE is concentrated along the postsynaptic membrane. *M* mitochondria, *NT* nerve terminals (courtesy from the late Jean Gautron). *Bottom* action of eserine on nerve–electroplaque transmission in the *Torpedo* electric organ. **a** Cholinesterase inhibition provokes a marked lengthening of the post-synaptic potential (EPP) evoked by a single volley applied to fibres innervating a fragment of electric organ. **b** The time course of miniature electroplaque potential is similarly prolonged by eserine. **c** When the synapses of a control specimen are stimulated at a frequency of 10 Hz, the EPP amplitude exhibits an initial facilitation, followed by a moderate decay. When acetylcholinesterase is inhibited by eserine, the amplitude of the first EPP is slightly enhanced, but synapses are no longer able to face with repetitive activity (from Y.D., unpublished records)

of eserine, the release of less ACh generates a larger EPP, even in an isolated impulse. This depression of ACh release by cholinesterase inhibitors is mediated by activation of presynaptic muscarinic auto-receptors (Dunant and Walker 1982).

The Characteristic Profile of Key Processes Involved in Rapid Neurotransmission: Fast Opening, Fast Closure and Slower Desensitisation

Voltage-gated ion channels provide typical examples of this behaviour. They have a brief open time in response to a rapid potential change. Most of them undergo desensitisation if the electrical stimulus is maintained. Many *ligand-gated channels* have the same behaviour. At the NMJs, an abrupt jet of transmitter opens nicotinic acetylcholine receptors (AChRs) in the postsynaptic membrane. However, if the neurotransmitter is not rapidly removed, the receptors will desensitize, that is, they will no longer open under the action of ACh (Katz and Thesleff 1957). The mean open time of JNM AChR is about 1 ms; that of *Torpedo* NEJ AChRs is even shorter (0.6 ms; Sakmann et al. 1985). Similarly, mediatophores exhibit fast activation, brief action and slower desensitisation. *Mediatophore* is a proteolipid complex forming clusters at the presynaptic active zones of NEJs and NMJs. Mediatophore is a homo-oligomer composed of several copies of a 15–16-kDa proteolipid which is very similar, if not

identical, to the c-subunit of the membrane sector (V_0) of V-ATPase. When reconstituted in liposomes, oocytes or deficient cell lines, mediatophore mimics physiological ACh release, including the emission of ACh quanta (Israël et al. 1986; Dunant and Israël 2000; Dunant et al. 2009). Mediatophores release ACh in response to a sudden elevation of $[Ca^{2+}]_i$; desensitisation occurs when $[Ca^{2+}]_i$ remains elevated for several seconds or minutes (Israël et al. 1987). It should be recalled that AChRs and mediatophore are only activated by a high concentration of ligand. As we mentioned before, high speed has a price: low affinity.

Conclusions

Without the three "curtailing" mechanisms described in this paper, transmission at NMJ and electric organs would be a relatively slow process with postsynaptic potentials lasting 10–50 ms or more, like in autonomic synapses. The choice of Nature (or of evolution) was apparently to build a robust, but not extremely rapid, apparatus and to provide several additional mechanisms for curtailing the signal at synapses where a high speed is required. Without this curtailment, a cholinergic synapse would be unable to sustain transmission at high frequencies.

A fascinating question is what about CNS and other rapid synapses, particularly those using glutamate as transmitter. They are certainly provided with similar "curtailing" processes. As a matter of fact, the vesicular Ca^{2+}/H^+ antiport was first described using isolated synaptic vesicles from mammalian brain (Gonçalves et al. 1998). Therefore, curtailing the Ca^{2+} signal is most probably crucial for rapid glutamatergic synapses as well. Synaptotagmin-1, being required for Ca^{2+}/H^+ antiport expression experiments where synaptotagmin-1 is absent or damaged, can bring some information on this question (see references in Cordeiro et al. 2013). Obviously, further investigations addressing Ca^{2+}/H^+ antiport control on CNS rapid transmission are desirable.

Another issue already exploited is to take advantage of curtailing processes for the treatment of conditions where transmission is too weak either because the amount of transmitter released per impulse is too low (myastenic syndromes, botulism, etc.) or because the density of available AChRs is reduced (myasthenia gravis). Cholinesterase inhibitors are classical drugs to achieve this. As for the inhibitors of presynaptic K^+ channels, they have been proposed in the case of transmitter release failure. Conceptually, inhibition of the vesicular Ca^{2+}/H^+ antiport should boost neurotransmission at central and peripheral synapses, yet no drug that specifically targets this function has been identified. The problem here is to support transmission in a given group of weakened synapses without perturbing other rapid junctions. Actually, such a dilemma is regularly encountered in neuropharmacology.

References

Adams DJ, Takeda K, Umbach JA (1985) Inhibitors of calcium buffering depress evoked transmitter release at the squid giant synapse. J Physiol Lond 369:145–159

Bacq ZM (1974) Les transmissions chimiques de l'influx nerveux. Gauthier-Villard, Paris Bruxelles Montréal

Bacq ZM (1975) Chemical transmission of nerve impulses. a historical sketch. Pergamon, Oxford

Bloedel J, Gage PW, Llinas R, Quastel DM (1966) Transmitter release at the squid giant synapse in the presence of tetrodotoxin. Nature 212: 49–50

Brigant JL, Mallart A (1982) Presynaptic currents in mouse motor endings. J Physiol Lond 333:619–636

Castonguay A, Robitaille R (2001) Differential regulation of transmitter release by presynaptic and glial Ca^{2+} internal stores at the neuromuscular synapse. J Neurosci 21:1911–1922

Cordeiro JM, Goncalves PP, Dunant Y (2011) Synaptic vesicles control the time course of neurotransmitter secretion via a Ca(2)+/H+ antiport. J Physiol 589:149–167

Cordeiro JM, Boda B, Goncalves PP, Dunant Y (2013) Synaptotagmin 1 is required for vesicular Ca(2+)/H(+)-antiport activity. J Neurochem 126:37–46

Corthay J, Dunant Y, Loctin F (1982) Acetylcholine changes underlying transmission of a single nerve impulse in the presence of 4-aminopyridine in *Torpedo*. J Physiol Lond 325:461–479

Couteaux R (1955) Localization of cholinesterase at neuromuscular junctions. Int Rev Cytol 4:355–375

Desai-Shah M, Cooper RL (2009) Different mechanisms of Ca^{2+} regulation that influence synaptic transmission: comparison between crayfish and *Drosophila* neuromuscular junctions. Synapse 63:1100–1121

Descarries L, Gisiger V, Steriade M (1997) Diffuse transmission by acetylcholine in the CNS. Prog Neurobiol 53:603–625

Dunant Y (1972) Some properties of the presynaptic nerve terminals in a mammalian sympathetic ganglion. J Physiol Lond 221:577–587

Dunant Y, Israël M (2000) Neurotransmitter release in rapid synapses. Biochimie 82:289–302

Dunant Y, Muller D (1986) Quantal release of acetylcholine evoked by focal depolarisation at the *Torpedo* nerve–electroplaque junction. J Physiol Lond 379:461–478

Dunant Y, Walker AI (1982) Cholinergic inhibition of acetylcholine release in the electric organ of *Torpedo*. Eur J Pharmacol 78:201–212

Dunant Y, Eder L, Servetiadis-Hirt L (1980) Acetylcholine release evoked by single or a few nerve impulses in the electric organ of *Torpedo*. J Physiol Lond 298:185–203

Dunant Y, Cordeiro JM, Goncalves PP (2009) Exocytosis, mediatophore, and vesicular Ca^{2+}/H^+ antiport in rapid neurotransmission. Ann N Y Acad Sci 1152:100–112

Dunant Y, Bancila V, Cordeiro JM (2010) Ultra-fast versus sustained cholinergic transmission: a variety of different mechanisms. J Mol Neurosci 40:27–31

Eccles JC, Katz B, Kuffler SW (1942) Effect of eserine on neuromuscular transmission. J Neurophysiol 5:211–230

Fatt P, Katz B (1952) Spontaneous subthreshold activity at motor nerve endings. J Physiol Lond 117:109–128

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 97:3P

Feng TP, Shen SC (1937) Studies on the neuro-muscular junction. III. The contracture in eserinized muscle produced by nerve stimulation. Chin J Physiol 11:51–70

Garcia-Segura LM, Muller D, Dunant Y (1986) Increase in the number of presynaptic large intramembrane particles during synaptic

transmission at the *Torpedo* nerve–electroplaque junction. Neuroscience 19:63–79

Gautron J (1970) Localisation des cholinestérases au niveau de la jonction nerf-électroplaque de l'organe électrique de la Torpille marbrée. C R Acad Sci Paris 271:714–717

Girod R, Corrèges P, Jacquet J, Dunant Y (1993) Space and time characteristics of transmitter release at the nerve–electroplaque junction of Torpedo. J Physiol Lond 471:129–157

Gisiger V, Gautron J, Dunant Y (1977) Differences in acetylcholinesterase of neuro-muscular and sympathetic ganglia. Experientia 33:804

Gisiger V, Vigny M, Gautron J, Rieger F (1978) Acetylcholinesterase of rat sympathetic ganglion: molecular forms, localization, and effect of denervation. J Neurochem 30:501–516

Gonçalves PP, Meireles SM, Gravato C, Vale MG (1998) Ca^{2+}–H^{+}-antiport activity in synaptic vesicles isolated from sheep brain cortex. Neurosci Lett 247:87–90

Gonçalves PP, Meireles SM, Neves P, Vale MG (1999) Ionic selectivity of the Ca^{2+}/H^{+} antiport in synaptic vesicles of sheep brain cortex. Mol Brain Res 67:283–291

Gonçalves PP, Meireles SM, Neves P, Vale MG (2000) Distinction between Ca(2+) pump and Ca(2+)/H(+) antiport activities in synaptic vesicles of sheep brain cortex. Neurochem Int 37:387–396

Heuser JE, Reese TS, Dennis MJ, Jan Y, Jan L, Evans L (1979) Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. J Cell Biol 81:275–300

Hodgkin AL, Huxley AF (1952) A quantitative description of the membrane current and its application to conduction and excitation in nerve. J Physiol Lond 117:500–544

Israël M, Manaranche R, Marsal J, Meunier FM, Morel N, Frachon P, Lesbats B (1980) ATP-dependent calcium uptake by cholinergic synaptic vesicles isolated from *Torpedo* electric organ. J Membr Biol 54:115–126

Israël M, Morel N, Lesbats B, Birman S, Manaranche R (1986) Purification of a presynaptic membrane protein that mediates a calcium-dependent translocation of acetylcholine. Proc Natl Acad Sci U S A 83:9226–9230

Israël M, Meunier FM, Morel N, Lesbats B (1987) Calcium-induced desensitization of acetylcholine release from synaptosomes or proteoliposomes equiped with mediatophore, a presynaptic membrane protein. J Neurochem 49:975–982

Katz B (1989) Looking back at the neuromuscular junction. In: Sellin LC, Libelius R, Thesleff S (eds) Neuromuscular junction. Elsevier Science, Amsterdam, pp 3–9

Katz B, Miledi R (1966) Input–output relation of a single synapse. Nature 212:1242–1245

Katz B, Miledi RB (1969) Tetrodotoxin-resistant electric activity in presynaptic terminals. J Physiol Lond 203:459–487

Katz B, Miledi RB (1979) Estimates of quantal content during "chemical potentiation" of transmitter release. Proc R Soc Lond B 205:369–378

Katz B, Thesleff S (1957) A study of the "desensitization" produced by acetylcholine at the motor Endplate. J Physiol Lond 138:63–80

Kriebel ME, Gross CE (1974) Multimodal distribution of frog miniature endplate potentials in adult, denervated and tadpole leg muscle. J Gen Physiol 64:85–103

Kuffler SW, Yoshikami D (1975) The number of transmitter molecules in a quantum: an estimate from iontophoretic application of acetylcholine at the neuromuscular synapse. J Physiol Lond 251:465–482

Llinas R, Walton K, Bohr V (1976) Synaptic transmission in the squid giant synapse after potassium conductance blockage with external 3- and 4-aminopyridine$_a$ino. Biophys J 16:83–96

Llinas R, Steinberg IZ, Walton K (1981) Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. Biophys J 33:323–352

Llinas R, Sugimori M, Silver RB (1992) Microdomains of high calcium concentration in a presynaptic terminal. Sci USA 256:677–679

Michaelson DM, Ophir I, Angel I (1980) ATP-stimulated Ca^{2+} transport into cholinergic *Torpedo* synaptic vesicles. J Neurochem 35:116–124

Molgo J, Thesleff S (1982) 4-Aminoquinoline-induced "giant" endplate potentials at mammalian neuromuscular junctions. Proc R Soc B 214:229–247

Muller D (1986) Potentiation by 4-aminopyridine of quantal acetylcholine release at the *Torpedo* nerve electroplaque junction. J Physiol Lond 379:479–493

Muller D, Garcia-Segura LM, Parducz A, Dunant Y (1987) Brief occurrence of a population of large intramembrane particles in the presynaptic membrane during transmission of a nerve impulse. Proc Natl Acad Sci U S A 84:590–594

Parekh AB (2008) Ca^{2+} microdomains near plasma membrane Ca^{2+} channels: impact on cell function. J Physiol 586:3043–3054

Reigada D, Diez-Perez I, Gorostiza P, Verdaguer A, Gomez DAI, Pineda O, Vilarrasa J, Marsal J, Blasi J, Aleu J, Solsona C (2003) Control of neurotransmitter release by an internal gel matrix in synaptic vesicles. Proc Natl Acad Sci U S A A100:3485–3490

Rizzuto R, Pozzan T (2006) Microdomains of intracellular Ca^{2+}: molecular determinants and functional consequences. Physiol Rev 86: 369–408

Roberts WM (1994) Localization of calcium signals by a mobile calcium buffer in frog saccular hair cells. J Neurosci 14:3246–3262

Roberts WM, Jacobs RA, Hudspeth AJ (1990) Colocalization of ion channels involved in frequency selectivity and synaptic transmission at presynaptic active zones of hair cells. J Neurosci 10:3664–3684

Rusakov DA (2006) Ca^{2+}-dependent mechanisms of presynaptic control at central synapses. Neuroscientist 12:317–326

Sabatini BL, Regehr WG (1996) Timing of neurotransmission at fast synapses in the mammalian brain. Nature 384:170–172

Sakmann B, Methfessel C, Mishina M, Takahashi T, Takai T, Kurasaki M, Fukuda K, Numa S (1985) Role of acetylcholine receptor subunits in gating of the channel. Nature 318:538–543

Takamori S, Holt M, Stenius K, Lemke EA, Gronborg M, Riedel D, Urlaub H, Schenck S, Brugger B, Ringler P, Muller SA, Rammner B, Grater F, Hub JS, De Groot BL, Mieskes G, Moriyama Y, Klingauf J, Grubmuller H, Heuser J, Wieland F, Jahn R (2006) Molecular anatomy of a trafficking organelle. Cell 127:831–846

Torri-Tarelli F, Grohovaz F, Fesce R, Ceccarelli B (1985) Temporal coincidence between synaptic vesicle fusion and quantal secretion of acetylcholine. J Cell Biol 101:1386–1399

Van der Kloot W, Molgo J (1994) Quantal acetylcholine release at the vertebrate neuromuscular junction. Physiol Rev 74:899–991

Villalobos C, Nunez L, Montero M, Garcia AG, Alonso MT, Chamero P, Alvarez J, Garcia-Sancho J (2002) Redistribution of Ca^{2+} among cytosol and organelles during stimulation of bovine chromaffin cells. FASEB J 16:343–353

Xu-Friedman MA, Regehr WG (2000) Probing fundamental aspects of synaptic transamission with strontium. J Neurosci 20:4414–4422

Yazejian B, Sun XP, Grinnell AD (2000) Tracking presynaptic Ca^{2+} dynamics during neurotransmitter release with Ca^{2+}-activated K+ channels. Nat Neurosci 3:566–571