ELSEVIER

European Journal of Pharmacology 447 (2002) 163–171

www.elsevier.com/locate/ejphar

Quantal GABA release in hippocampal synapses: role of local Ca^{2+} dynamics within the single terminals

S.A. Fedulova *, N.S. Veselovsky

Bogmoletz Institute of Physiology, Bogomoletz Street 4, Kiev-24, GSP 252601, Ukraine

Accepted 15 April 2002

Abstract

Results of recent studies dedicated to the mechanisms of neurotransmission at a single inhibitory synaptic terminal in cultured neurones support the hypothesis that multiple quanta of neurotransmitter are released during excitation of inhibitory and excitatory central synapses. This is an important consideration as previous less direct measurements have suggested that a synapse can release no more than one quantum. Neurotransmitter release during long stimuli may occur at certain times with maximal probability, keeping the mean inter-release interval constant. This interval is not determined directly by vesicle depletion and moreover, each release event is independent of previous ones. The recent data also suggest that constant Ca^{2+} influx is an important determinant of neurotransmitter release. It is speculated that the neurotransmitter release is regulated by a superposition of two processes: a continuous homogeneous process, (i.e. background Ca^{2+} influx), and a periodic process that acts as a synchronizing factor of the release at definite moments.

Keywords: Synaptic transmission; Synaptic terminal; Ca^{2+} influx; Neurotransmitter release

1. Introduction

Communication between neurones within the brain, achieved through synaptic transmission, is fundamental to the ability of the brain to process and store information. Such communication relies upon the release of chemical signals, known as neurotransmitters. In this review, we will dwell on mechanisms of multiquantal release of γ-aminobutyric acid (GABA) in the inhibitory synapses. The inhibitory postsynaptic currents (IPSC) activated by GABA arise from the opening of Cl^--selective ionic channels associated with $GABA_A$ receptors (Edwards et al., 1990). $GABA_A$ receptors are the major inhibitory receptors in the central nervous system and their functional properties have been extensively studied in the somatic membrane of neurones in culture and in acute brain slices with the patch clamp technique (Edwards et al., 1990; Fedulova et al., 1999, 2000; Vautrin et al., 1993a,b). However, the precise statistical analyses of quantal GABA release in single central synapses have been complicated by technical limitations. Some difficulties specific for central neurones, such as widely distributed inputs of neurones, difficulties with identification of pre- and postsynaptic cells, noise of various origin, a very high probability of transmitter release, etc. (Bekkers, 1994; Isaacson and Walmsley, 1995; Korn and Faber, 1987, 1991; Tong and Jahr, 1994) have precluded reliable recording and analyses of data. In the present paper, we will focus on recent experimental results that unveil new insights in the mechanisms of quantal release of GABA in central synapses.

It is generally accepted that the initial event, which triggers neurotransmitter release, is associated with Ca^{2+} influx through the voltage-gated Ca^{2+} channels, which begins within 200 μs after the arrival of the action potential at the synaptic terminal (Dell Castillo and Katz, 1954). Vesicle fusion is evoked by localized elevation of Ca^{2+} concentra-tion near a synaptic active zone (Llinas and Moreno, 1998; Augustine et al., 1991). The quantity of neurotransmitter released from each presynaptic nerve terminal is directly proportional to the increase in intraterminal Ca^{2+} concentration evoked by each invading action potential.

* Corresponding author. Tel.: +380-44-2562423; fax: +380-44-2530701.

E-mail address: fedulova@serv.biph.kiev.ua (S.A. Fedulova).

0014-2999/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0014-2999(02)01840-X

2. Multivesicular release at cultured central neurones

2.1. Techniques

The modern experimental approach to investigate synaptic transmission at the level of a single presynaptic terminal in cultured central neurones (Prange and Murphy, 1999; Liu and Tsien, 1995; Vogt et al., 1995; Forti et al., 1997; Tong and Jahr, 1994; Fedulova et al., 1999, 2000; Kirischuk et al., 1999a,b; Fedulova and Veselovsky, 2001) allows direct investigation of neurotransmitter release mechanisms. Investigations in cultured neurones have an important advantage such as precise visual control of presynaptic terminal and postsynaptic neurones. Several laboratories recently succeeded with visualization of single nerve terminals using low-density (35,000 cells/cm^2) primary cultures of rat hippocampal neurones (Fedulova et al., 1999; Kirischuk et al., 1999a,b). To obtain the best possible control of the intracellular potential and to reduce "dendritic filtering", the IPSC recordings were performed only from the terminals located on, or close to, the soma of postsynaptic neurone (not farther than 10–20 μm). The stimulus-evoked IPSCs reversed at the Cl^- equilibrium potential and the voltage–current dependence of IPSCs was linear. Therefore, by controlling voltage on the postsynaptic membrane, dendritic filtration can be avoided, resulting in a sufficient voltage gradient on the postsynaptic membrane and in accurate description of the IPSC kinetics (Fedulova et al., 1999).

To achieve precise control over transmitter release from single presynaptic terminals, local electrical stimulation can be used to great advantage. Regenerative action potentials can be excluded by constantly bathing the preparation in 1 μM tertrodotoxin, allowing gradual control over the presynaptic membrane potential. After establishing a whole-cell recording from a postsynaptic neurone, a single synaptic terminal innervating this neurone can be precisely defined (Fig. 1).

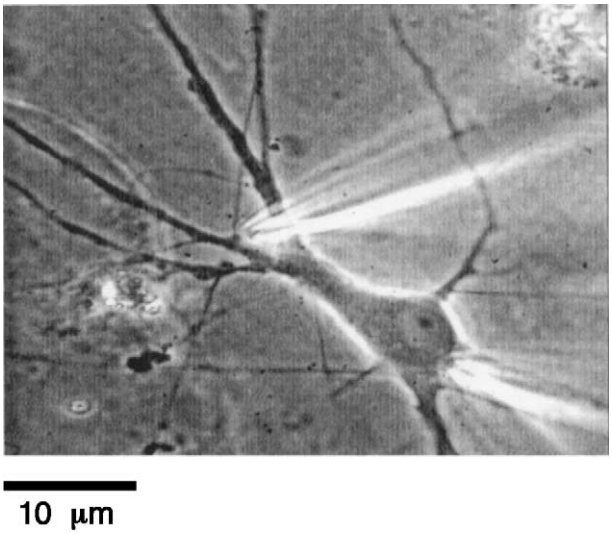

Fig. 1. Local extracellular stimulation of individual terminal. Phase contrast image of a hippocampal neurone showing axo-dendritic synapses. Upper pipette is for local electrical stimulation of the single presynaptic terminal, whereas the lower one is for recording postsynaptic currents.

2.2. Quantal properties of IPSC

Visual control of investigated neurones and nerve terminals requires high optical magnification, usually around 1000× (Fedulova et al., 1999; Kirischuk et al., 1999a,b). Optical resolution of such systems at the visible spectrum wavelength (650 nm) is approaching 350 nm that is sufficient to identify different types of nerve terminals. Usually, only two to three neurones can be seen in the field of observation (diameter—200 μm) and visual identification of single synaptic connections is not difficult. Evoked IPSCs (eIPSC) recorded under voltage-clamp conditions are revealed as a response to short extracellular depolarizing voltage pulses in close vicinity to a single presynaptic terminal. The eIPSC amplitudes vary from a measurable minimum of about 7–8 pA to more than 200 pA when the cell is held at −75 mV (Fig. 2A, inset). The stimulus-evoked IPSCs fluctuates with regard to the discrete aliquot values of peak amplitude (Fedulova et al., 1999). Amplitude distributions reveal clearly distinguished, regularly spaced, multiple peaks (Fig. 2A). Fitting histograms with several Gaussian curves, assuming independent superposition of quantal events, results in equally spaced (quantal) successive peaks. Assuming that the space between the peaks is one quantum (or quantal unit) of an integral IPSCs (Edwards et al., 1990), the mean value of one quantum equals ~20 pA at the holding potential of −75 mV. To adequately describe a random process, which is the principal feature of spontaneous and evoked transmitter release, Poisson statistics can be successfully applied (Dell Castillo and Katz, 1954; Bekkers, 1994; Isaacson and Walmsley, 1995; Korn et al., 1993).

Poisson statistics describe the accidental number of the appearance of a given event at a given time at a given place. The probability $P(x)$ that a given event is observed exactly x times in a package of N observations or the probability $P(x)$ that a given response is made up of 1, 2, 3, ...x quantal components is given by $P(x) = e^{-m}m^x/x!$, $x = 0, 1, 2, \ldots$. The mathematical expectation of Poisson statistics has to be a constant value, m. m is the average number of units responding to one impulse, or the mean quantal content. This description implies a low probability of the event $P(x)$ (less than 0.1) and a large number of N observations in a package (Dell Castillo and Katz, 1954; Isaacson and Walmsley, 1995; Korn and Faber, 1987, 1991). To use Poisson statistics for the analysis of experimental data arising from single-terminal experiments, the continuous amplitude distribution should be converted to a discrete distribution of quantal units. To construct the distribution of the number of quantal units in each response, a single quantal response (quantal unit) has to be distinguished from multiquantal events. The distribution of eIPSC amplitudes gives the value

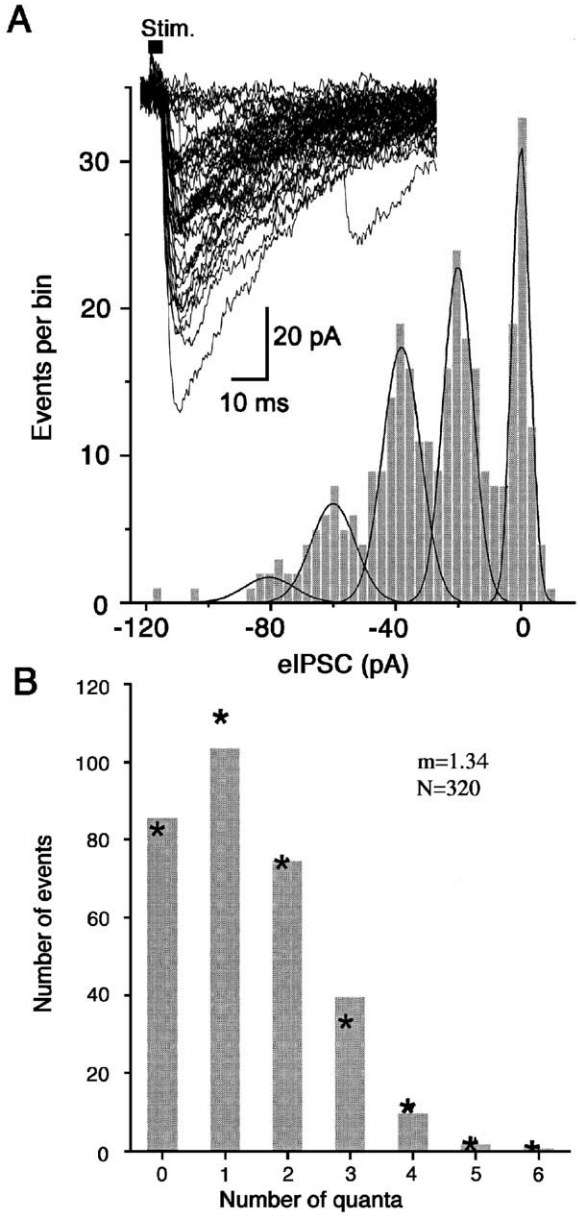

Fig. 2. Quantal origin of evoked inhibitory postsynaptic currents (eIPSC) amplitudes distribution. (A) Amplitude histogram of stimulus evoked IPSCs ($N=320$). Dotted line represents the sum of five underlying Gaussian distributions (smooth line) assuming independent superposition of quantal events, failures being included. Inset—first 50 traces out of 320 evoked IPSCs. Here and thereafter, a trace of averaged failures ($n=3$) was subtracted to avoid stimulating artifacts. (B) Stimulus-evoked transmitter release from single central synaptic terminal follows Poisson statistics. Experimentally obtained numbers of failures, singles, doubles, etc. of quantal units (columns) and predicted corresponding values from Poisson's statistics (asterisks) for the value of one quantum 20 pA. Here and thereafter, $N=$ total number of traces, the mean quantal content $m=$ mean amplitude of IPSC/mean value of one quanta.

of one quantum, equal to 20 pA. To construct a Poisson distribution, we took the bin size as, equal to the value of one quantum (20 pA). The range for an event consisting of one quantum was between 8 and 28 pA, two quanta ranged between 28 and 48 pA and so on. The dependence of the number of quantal units on the number of their realizations in each multiquantal event is shown in Fig. 2B.

To test the applicability of Poisson's law, the m value should be calculated in two ways.

The first:

$$m = \text{mean amplitude of IPSC} \,/\text{mean value of one quantum.}$$

The mean amplitude was obtained as the algebraic sum of all amplitudes divided by total number of traces.

The second way can be obtained from Poisson's law for $x=0$ or failure of response

$$m = \ln(\text{number of observations } N \,/\text{number of failures in the package } N_0),$$

where the number of N observations is the total number of traces, so

$$\text{mean amplitude of IPSC}/\text{mean value of one quanta} = \ln(N/N_0).$$

The graphic representation of this equation should be satisfactorily fitted by a linear function with a slope of 45°, as the criterion of applicability of Poisson statistics for random processes. Poisson statistics adequately described the amplitude distributions of IPSCs evoked by release from a single presynaptic terminal without artificial lowering of the probability of release by pharmacological agents (Isaacson and Walmsley, 1995).

The basic idea of the quantal model is that neurotransmitter is released from presynaptic terminales in discrete units thought to be equal to the amount of neurotransmitter packaged within a single presynaptic vesicle (Katz, 1969). Each vesicle contains one quantum (several thousand molecules) of transmitter. The vesicles fuse to the inside surface of the presynaptic terminal at specific release sites. The membrane opens transiently to allow the vesicle to extrude its entire contents into the extracellular space of the synaptic cleft. The number of release sites associated with an axon has been identified with the total number of releasable vesicles, the number of terminals, and the number of active zones (Jack et al., 1981; Korn and Faber, 1987, 1991; Neale et al., 1983; Pierce and Lewin, 1994; Pierce and Mendell, 1993; Pun et al., 1986; Redman, 1990; Redman and Walmsley, 1983; Schikorski and Stevens, 1997; Walmsley, 1991; Walmsley et al., 1985; Zucker, 1973).

The number of morphologically distinguishable active zones in one terminal of central neurones is defined as one, sometimes two (Forti et al., 1997; Schikorski and Stevens, 1997) and rarely three (Schikorski and Stevens, 1997) in contrast to the neuro-muscular junction with high quantal content and correspondingly large number of presynaptic active zones, e.g. 200–300 at the frog neuromuscular junction and about 10,000 at the squid giant synapse (McLachlan, 1978; Propst and Ko, 1987).

In our experimental configuration, this means that a visible morphological structure, i.e. the terminal of a nerve ending, has to be identical to one active zone. The number of release sites is generally accepted as corresponding to the number of active zones, although without definite evidence, which could mean that the terminal is identical to one active zone with one release site for only one vesicle.

The mean diameter of a vesicle measured for brain synapses is 35.2 ± 3.5 nm (Schikorski and Stevens, 1997). Even this tiny variability in vesicle dimension may provide a significant difference in entire vesicular content or in the size of one quantum that could result in fluctuation of IPSCs caused by the release of each vesicle. However, such fluctuations have to vary randomly from release to release around some mean value, and such a probabilistic process is described by a normal distribution (Gaussian) with one peak. Histograms for the amplitude distribution of IPSCs have regularly spaced peaks (Fig. 2). This allows one to suggest that transmitter release occurs in discrete, approximately equal portions, which could be added and result in multi-peak distributions of eIPSCs, because the amplitude of the postsynaptic response is proportional to the quantity of neurotransmitter released. The number of peaks varied from 2 to 6, for different terminals, excluding the first peak around zero. For the terminal with six peaks under our experimental conditions, this could mean that one terminal has (i) one active zone with six release sites or (ii) two active zones with three release sites each, or (iii) three active zones with two release sites each, because the presence of the sixth peak in the histogram indicates the simultaneous release of six vesicles. These assumptions were confirmed by a morphological study which showed the presence of a few active zones as well as multiple docked vesicles per one active zone in single terminals of mouse hippocampus (Schikorski and Stevens, 1997). The possibility of multivesicular release was considered for excitatory synapses of cultured hippocampal neurones under controlled conditions and upon incubation with baclofen, 4-AP and adenosine, which, by increasing intraterminal $[Ca^{2+}]$ may enhance the probability of multivesicular release (Tong and Jahr, 1994). A similar assumption was also applied to inhibitory synapses in cerebellar stellate and basket cells (Auger et al., 1998).

3. Neurotransmitter release and intraterminal Ca^{2+}

Neurotransmitter release from presynaptic terminals is triggered by local Ca^{2+} influx. The vesicular release of neurotransmitter develops when a certain concentration of cytosolic Ca^{2+} is attained to provide for vesicle fusion and subsequent transmitter release (Augustine et al., 1991; Zucker, 1993; Katz and Miledi, 1967). This Ca^{2+} influx results in local microdomains of elevated $[Ca^{2+}]_i$ in the immediate vicinity of a presynaptic active zone. These $[Ca^{2+}]_i$ microdomains are the universal expression of Ca^{2+} influx independent of its source. The microdomain model presumes that simultaneous opening of Ca^{2+} channels results in local accumulation of Ca^{2+} near a Ca^{2+} sensor (Llinas, 1997; Llinas and Moreno, 1998). The relationship between presynaptic Ca^{2+} influx and neurotransmitter release has been described by different power functions at the squid giant axon (Llinas et al., 1981) crayfish neuromuscular junction (Propst and Ko, 1987) and in the rat cerebellar synapse (Smith et al., 1989) as well as at a single terminal in cultured collicular rat embryos neurones (Kirischuk et al., 1999a,b). It is also worth noting that exocytosis of synaptic vesicles requires high Ca^{2+} concentrations, with a threshold of 20–50 μM and half-maximal activation at 190 μM (Heidelberger et al., 1994; Von Gersdorff and Matthews, 1994).

Ca^{2+} triggers transmitter release by participating in a reaction that catalyses vesicle fusion. Vesicle fusion and all other forms of vesicle trafficking involve the coordinated activity of many proteins in reactions that are regulated in both space and time. One of the crucial issues in understanding neuronal transmission is to define the identity and function of these proteins. Among the many Ca^{2+}-binding proteins found in neurones, the leading candidate for the presynaptic Ca^{2+} receptor is synaptotagmin. It is thought that synaptotagmin may be involved in converting a presynaptic Ca^{2+} rise into the signal for vesicle fusion (Nishiki and Augustine, 2001; Augustine, 2001; Tokumaru et al., 2001).

Previous studies on the neuromuscular junction with voltage clamping of the presynaptic membrane (Heuser and Reese, 1981; Von Gersdorff and Matthews, 1994) indicated that the voltage-dependent Ca^{2+} current triggered the release of synaptic transmitter, so that averaged EPSC amplitude increased and decreased directly proportionally to Ca^{2+} current amplitude and duration in the presynaptic terminal. In experiments on a single presynaptic terminal, the neurotransmitter release conditions might be affected by changes in the intensity of the stimulating current. The linear increase of the stimulating pulse amplitude causes non-linear bell-shape changes of the averaged eIPSC amplitude (Fig. 3). During small increases of the stimulating pulse, the amplitude of averaged eIPSC increases until it reaches some maximal value. A further linear increase of stimulating pulse intensity results in the reversible decrease of the averaged eIPSC amplitude (Fig. 3B, top panel) (Fedulova et al., 1999). Accordingly, the dependence of averaged eIPSC on the stimulating current is similar to that for the relationship between EPSCs and Ca^{2+} current amplitude (Heuser and Reese, 1981; Von Gersdorff et al., 1998; Llinas et al., 1981; Yazejian et al., 1997; Kirischuk et al., 1999a,b). As in the case with a controlled potential on the presynaptic membrane (Llinas et al., 1981), our experiments indicate that a change in stimulation amplitude alters the Ca^{2+} influx into the terminal. These data also mean that (i) local extracellular stimulation did not evoke any action potential-like generation on the presynaptic membrane, as

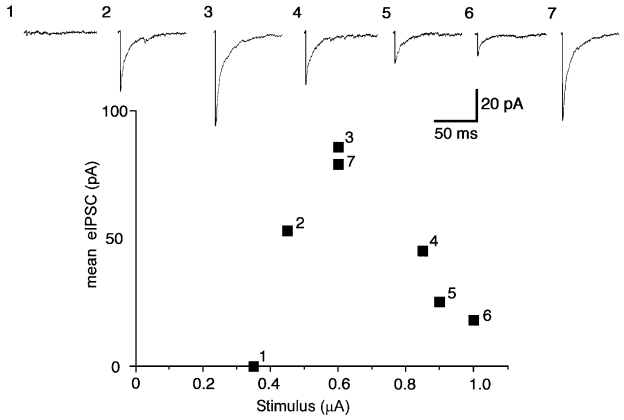

Fig. 3. Effect of extracellular presynaptic stimulating current on stimulus-evoked IPSCs. Top traces—the averaged traces of IPSCs evoked by 3-ms stimulus pulses of different intensity are shown. Each trace represents the average of 40 eIPSCs at fixed amplitudes of extracellular pulse. Stimulus amplitude was increased linearly (traces 1–6), the seventh trace is a control experiment with stable recording. Bottom graph—dependence of the mean amplitude of eIPSCs on stimulus intensity.

otherwise one would get saturation of the stimulator-response dependence of the averaged eIPSCs, and (ii) even very short (3–5 ms) stimulus are enough to get activation of Ca^{2+} currents that evoke neurotransmitter release.

Postsynaptic currents arising after short stimuli are termed "off" responses. Postsynaptic currents evoked by a long stimulating pulse are classified as "on" responses. "On" and "off" events occur independently. Application of long stimulating pulses to a single presynaptic terminal results in the appearance of typical fluctuating "on" postsynaptic currents (Fig. 4A) (Fedulova et al., 2000). The real level of postsynaptic potentials remains unknown because the postsynaptic membrane is partly involved in the area of potential change. After stimulus termination, the amplitude of "on" eIPSC is altered by changing postsynaptic potential. Both direction and averaged amplitude of the "on" eIPSCs are altered in accordance with the linear current–voltage dependence for GABA-mediated Cl^- currents. In the experiments described here, the holding potential was equal to the Cl^- equilibrium potential (approximately −20 mV) for given intra- and extracellular concentrations of Cl^- to avoid contamination by visible "off" responses (Fedulova et al., 2000). During each depolarization, the postsynaptic response occurred in the form of several superimposed successive events, which appeared after a delay (Fig. 4A,B). Delays were measured as the time elapsed from the start of the depolarizing stimulus to each successive event (Fig. 4C). In all neurones investigated, the delay distributions showed clearly distinct multiple peaks. Fitting a histogram with several Gaussian curves, assuming superposition of independent events occurring at different times during voltage shift on presynaptic membrane, resulted in equally spaced successive peaks (Fig. 5). The number of peaks depended on stimulus duration and increased with its length. Such distributions indicate that neurotransmitter release from presynaptic terminals during low-amplitude long (10–20 ms) depolarization occurs with maximal probability at definite times, irrespective of the success or failure of the previous event. The times corresponding to peaks on delay distributions presented the most probable times for release. In the delay histograms with equidistant peaks, the distance between the peaks was about 3 ms, irrespective of the number of peaks (Fedulova et al., 2000). The absence of a significant correlation between the amplitude of the first and second events demonstrated the independence of the succeeding release from the preceding one during long stimulation. This means that vesicle depletion cannot be regarded as the main reason for an interruption of the releases. We also cannot suppose a high refilling rate of vesicles at an inter-release interval of about 3 ms, since the maximal refilling rate of the readily releasable pool at excitatory hippocampal synapses nearly doubles during high frequency action potential stimulation (Stevens and Wesseling, 1998).

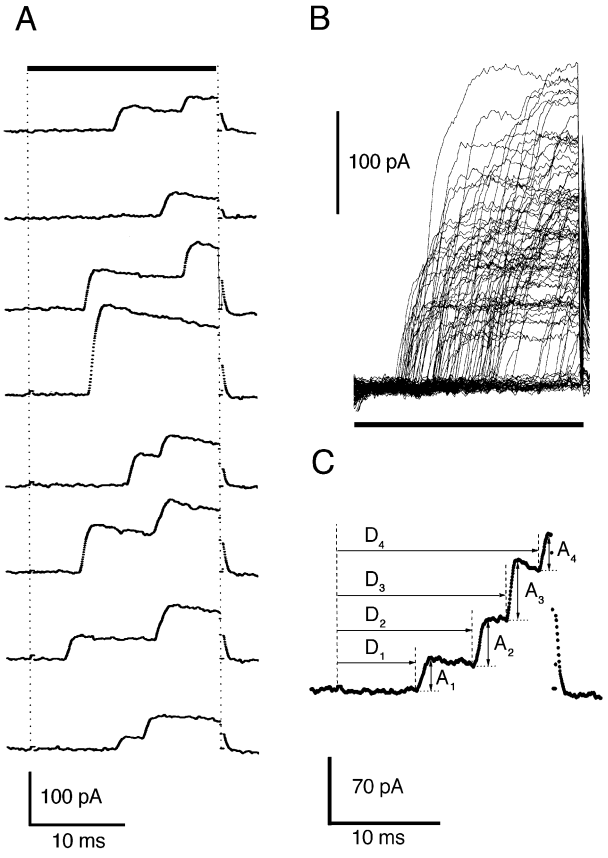

Fig. 4. Examples of stimulus-evoked "on" responses of single inhibitory terminal. (A) Selected individual traces of possible realizations with various combinations of consecutive single events and different amplitudes. The scale bars refer to all traces. (B) The first 50 IPSC traces evoked by long stimulus. (C) The sample of postsynaptic "on" response, as the superposition of four successive independent random events with different amplitudes (A_1, A_2, A_3, A_4) shown by vertical arrows. Delays of these events (D_1, D_2, D_3, D_4) were measured as the time elapsed from the start of depolarizing stimulus to the corresponding successive events, as shown by horizontal arrows.

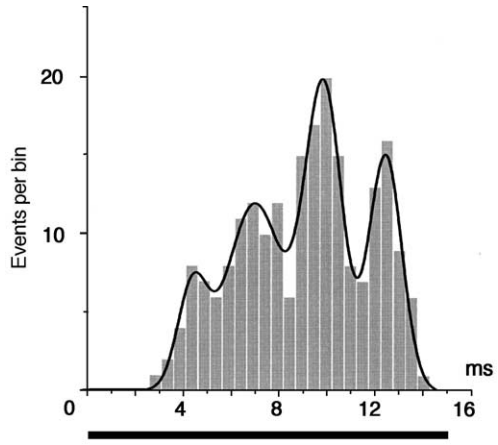

Fig. 5. Delay distributions of eIPSC for selected single inhibitory synapse. Experimental delay histogram of the low-intensity stimulus-evoked IPSCs ($N=214$). Envelope line represents the sum of four underlying Gaussian distributions.

Obviously, a low-amplitude depolarizing pulse applied to a presynaptic terminal induces continuous Ca^{2+} flow, which, presumably, follows the stochastic process of Ca^{2+} channels activation and increases the amount of Ca^{2+} influx. These experiments shown that low-intensity depolarizing pulse initiates the neurotransmitter release, which was not a continuous stochastic process progressing up to complete vesicle depletion, but occurred at certain times, keeping the mean inter-release interval constant at 2.97 ± 0.86 ms (Fig. 5). Thus, a small Ca^{2+} influx is necessary to reveal the discontinuous, but regular feature of release.

One may conclude that constant Ca^{2+} influx is an important determinant regulating neurotransmitter release. This presumption is substantiated by investigations of GABAergic axo-dendritic contacts in cultured collicular rat embryo neurones loaded with the Ca^{2+} indicator, Oregon Green 488 BAPTA-1. These experiments demonstrated that by varying the strength of the stimulating currents a wide amplitude range of both presynaptic $[Ca^{2+}]_i$ transients and postsynaptic conductance changes (up to 2–3 nS) were produced. Transmitter release retained its probabilistic character throughout the whole range of intraterminal Ca^{2+} concentrations measured. In any single terminal tested, maximal eIPSCs occurred in association with the largest $[Ca^{2+}]_i$ transients, but failures were present at any $[Ca^{2+}]_i$ (Kirischuk et al., 1999a,b). Two kinetically distinct and prone to saturation components of neurotransmitter release evoked by long depolarization have been shown for the glutamate release mediated currents (Heuser and Reese, 1981; Steyer et al., 1997; Von Ruden and Neher, 1993) and such asynchronous release was not directly parallel, or with dependent on the amount of Ca^{2+} influx (Barrett and Stevens, 1972; Gleason et al., 1994). The difference between Markov's process of Ca^{2+} channels closure and neurotransmitter release was also observed earlier (Von Gersdorff et al., 1998), when asynchronous release after Ca^{2+} channel closure was not copious and decayed sharply within 300 ms or less, in parallel with the Ca^{2+} influx.

An increase in stimulating pulse amplitude (e.g. change of Ca^{2+} influx) caused the decrease in the number of peaks in delay distributions with no change in the average distance between peaks, while the peaks still differed reliably. These experiments in which change of stimulating pulse amplitude affected the rate of Ca^{2+} entry showed that the mean interval between releases depended on Ca^{2+} influx in a

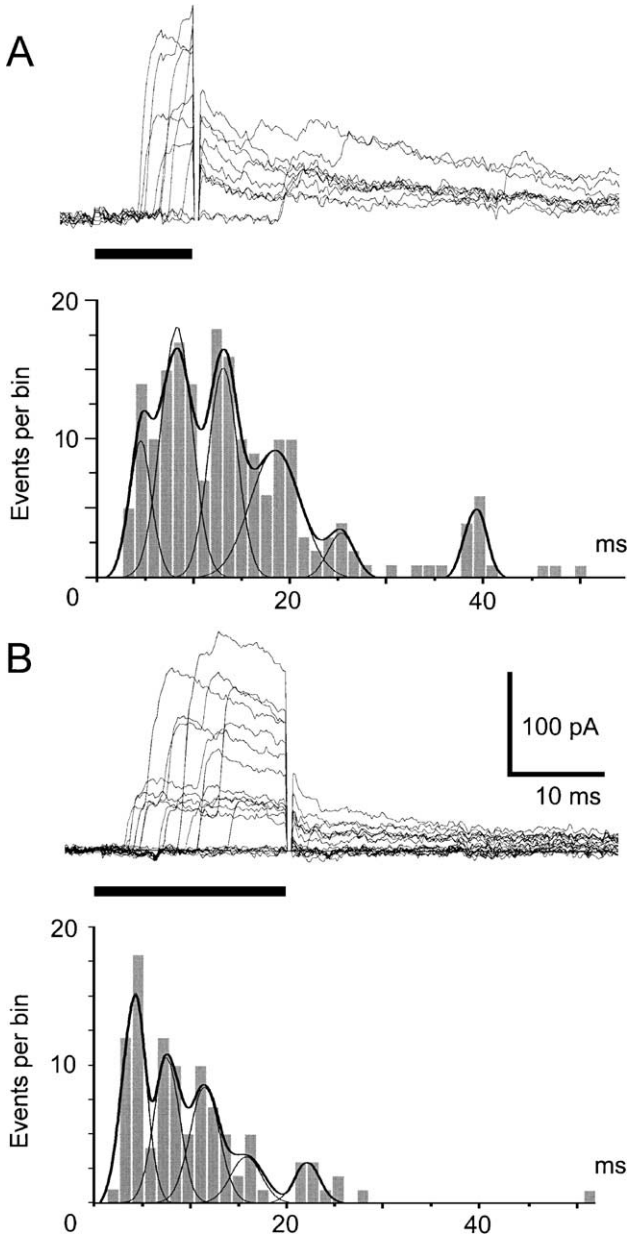

Fig. 6. Regularity of successive releases after termination of stimuli with two different durations for the same synaptic terminal. (A, B, top) Selected traces of evoked "on" and "off" IPSC during and after 10- and 20-ms stimuli, respectively. (A, B, bottom) Histograms of delay distributions of inhibitory postsynaptic currents evoked by 10- and 20-ms stimuli. Bold line represents the sum of five underlying Gaussian distributions assuming independent superposition of quantal events. Positions of peaks were at 4.7, 8.3, 13.2, 18.4, 25.2 and 39.2 (A) and at 4.4, 7.8, 11.4, 16.7 and 22.1 (B) in accordance with the ordinal number of the peak.

single presynaptic terminal and, presumably, on the time of attaining the threshold level for release by intraterminal Ca^{2+} concentration. A small increase in depolarizing pulse did not bring about an appreciable reduction of the mean interval between releases but increased the probability of release at the first most probable time. Thus, the existence of most probable times of release, when a single synapse was studied could be revealed only at low-intensity stimulus and, presumably, could be explained by a slow increase in $[Ca^{2+}]_i$, consistent with data demonstrating that a high Ca^{2+} concentration is necessary for exocytosis (Augustine and Neher, 1992; Llinas et al., 1995; Stenley, 1993).

When extracellular Ca^{2+} was increased to 5 mM, the mean interval between releases remained within the range of the standard error. The after-stimulus releases were observed in most synapses and occurred at definite, most probable times (Fig. 6). The delay distributions had equally spaced peaks within the stimulus duration, but after stimulus termination the intervals between most probable times of release increased (Fedulova et al., 2000). An increase in the mean interval between successive releases observed after stimulus termination must undoubtedly reflect the reduction of Ca^{2+} influx after a depolarizing stimulus. The decrease in Ca^{2+} influx after stimulus termination is explainable by deactivation of Ca^{2+} channels of the presynaptic terminal. The existence of most probable times of release following stimulus termination may indicate the coupling of releases following stimulus with residual Ca^{2+} level (Kirischuk et al., 1999a,b).

Thus, the interval between most probable times of vesicle release depends on Ca^{2+} influx into the presynaptic terminal and, presumably, on the time of $[Ca^{2+}]_i$ reaching the threshold level for release. Dependence of the mean interval between releases on $[Ca^{2+}]_i$ levels cannot explain the fact, that, after failure at the first probable time of release, the next successive release can occur only at the next probable time, but not at any time, when $[Ca^{2+}]_i$ reaches its critical value.

Furthermore, these studies demonstrated that each event of neurotransmitter release does not depend on the previous event and occurs only at definite, most probable times. This is possible when the release is caused by a continuous homogeneous process, such a Ca^{2+} influx and by a secondary, periodic, process that re-enforces the synchronizing factor of the release at definite moments when there is coincidence of some necessary parameters of the first and second processes. This dual effect of dependence of release on Ca^{2+} could be compared with a double-locked safe. The key is Ca^{2+} influx, but the lock has a secret inner machinery. This machinery periodically primes the terminal for neurotransmitter release. If the key (e.g. Ca^{2+}) fits the lock in this particular instance, synaptic transmission event will take place. If the key approaches the lock at any other time, release would not happen and the failure will be recorded. It also seems, that the period between primed conditions depends on Ca^{2+} flux as well.

Our experiments showed (Figs. 4 and 6) that the synchronizing factor depends on Ca^{2+} influx and might be initiated by turned-on and -off depolarizing stimuli, but that its existence is independent of depolarization of the pre- and postsynaptic membrane. With increased stimulating depolarization, the neurotransmitter was released at the first most probable time of release, which seems a result of fast Ca^{2+} entry into terminal (Catterall, 1998; Llinas et al., 1995; Stenley, 1993).

When Ca^{2+} entry is slow, release becomes intermittent and can be effected only after accumulation of a sufficient amount of Ca^{2+}. The modern concept adopts the intrinsic synaptic vesicle protein, synaptotagmin, binds Ca^{2+} and interacts with syntaxin in a Ca^{2+}-dependent manner (Nishiki and Augustine, 2001; Augustine, 2001; Tokumaru et al., 2001). Ca^{2+} influx though presynaptic Ca^{2+} channels triggers exocytosis by binding to synaptotagmin and other associated presynaptic proteins (Chapman et al., 1995; Li et al., 1995). In fast synapses, exocytosis is triggered extremely rapidly by Ca^{2+}, being triggered within 60 μs after Ca^{2+} channels opening (Sabatini and Regehr, 1997). Under our conditions, when the constitutive vesicle docking/fusion machinery is regulated by slow Ca^{2+} entry, release is substantially slower. The time from the start of the stimulating pulse to the first peak in the delays histograms exceeded the value of the mean interval between releases, i.e. some transitory processes occurred during the time to the first peak and thereafter functioning of synapse had a stationary character (Catterall, 1998; Stevens and Wesseling, 1998).

4. Conclusion

In this review, we analysed a fundamental matter related to signalling between neurones in the central nervous system. The action potential invades the presynaptic terminal and causes membrane depolarisation. The opening of Ca^{2+} channels leads to a substantial increase of intraterminal Ca^{2+} concentration. The increase of $[Ca^{2+}]_i$ causes binding of Ca^{2+} ions with an intracellular Ca^{2+} sensor, the identity of which remains enigmatic. Activation of the Ca^{2+} sensor leads to activation of synaptic vesicle fusion, which in turn leads to the quantal release of the neurotransmitter. Each of these stages includes the coordinated activity of many proteins that is regulated in both space and time. As summary, we may repeat the words of Sir Bernard Katz, "The story of the nerve endings is in itself a most fascinating chapter of physiology and full of mysteries" (Meir et al., 1999).

Acknowledgements

This research was supported by Wellcome Trust grant to NV (ref 060095), CRDF grant to SF (ref UB1-2420-KV-02).

References

Auger, C., Kondo, S., Marty, A., 1998. Multivesicular release at single functional synaptic sites in cerebellar stellate and bascet cells. J. Neurosci. 18, 4532–4547.

Augustine, G.J., 2001. How does calcium trigger neurotransmitter release? Curr. Opin. Neurobiol. 11, 320–326.

Augustine, G.J., Neher, E., 1992. Calcium requirements for secretion in bovine chromaffin cells. J. Physiol. (London) 450, 247–271.

Augustine, G.J., Adler, E.M., Charlton, M.P., 1991. The calcium signal for transmitter secretion from presynaptic nerve terminal. Ann. N.Y. Acad. Sci. 11, 1496–1507.

Barrett, E.F., Stevens, C.F., 1972. Quantal independence and uniformity of presynaptic release kinetics at the frog neuromuscular junction. J. Physiol. (London) 227, 665–689.

Bekkers, J.M., 1994. Quantal analysis of synaptic transmission in the central nervous system. Curr. Opin. Neurobiol. 4, 360–365.

Catterall, W.A., 1998. Structure and function of neuronal Ca^{2+} channels and their role in neurotransmitter release. Cell Calcium 24, 307–323.

Chapman, E.R., Hanson, P.I., An, S., Jahn, R., 1995. Ca^{2+} regulates the interaction between synaptotagmin and syntaxin 1. J. Biol. Chem. 270, 23667–23671.

Dell Castillo, J., Katz, B., 1954. Quantal component of the end-plate potential. J. Physiol. (London) 124, 560–573.

Edwards, F.A., Konnerth, A., Sakmann, B., 1990. Quantal analysis of inhibitory synaptic transmission in the dentate gyrus of rat hippocampal slices: a patch-clamp study. J. Physiol. (London) 430, 213–249.

Fedulova, S.A., Veselovsky, N.S., 2001. Multiquantal transmission at single inhibitory synapse in cultured rat hippocampal neurons. In: Morad, M., Kostyuk, P.G. (Eds.), Calcium Signaling. IOS Press, Amsterdam, pp. 190–195.

Fedulova, S.A., Vasilyev, D.V., Isaeva, E.V., Romanyuk, S.G., Veselovsky, N.S., 1999. Possibility of multiquantal transmission at single inhibitory synapse in cultured rat hippocampal neurons. Neuroscience 92, 1217–1230.

Fedulova, S.A, Vasilyev, D.V., Veselovsky, N.S., 2000. Temporal regularity of neurotrasmitter release at single terminal in cultured hippocampal neurons. Neuroscience 100, 229–239.

Forti, L., Bossi, M., Bergamaschi, A., Villa, A., Malgaroli, A., 1997. Loose-patch recordings of single quanta at individual hippocampal synapses. Nature 388, 874–878.

Gleason, E., Borges, S., Wilson, M., 1994. Control of transmitter release from retinal amacrine cells by Ca^{2+} influx and efflux. Neuron 13, 1109–1117.

Heidelberger, R., Heinemann, C., Neher, E., Matthews, G., 1994. Calcium dependence of the rate of exocitosis in synaptic terminal. Nature 371, 513–515.

Heuser, J.E., Reese, T.S., 1981. Structural changes after transmitter release at the frog neuromuscular junction. J. Cell Biol. 88, 564–580.

Isaacson, J.S., Walmsley, B., 1995. Counting quanta: direct measurements of transmitter release at a central synapse. Neuron 15, 875–884.

Jack, J.J., Redman, S.J., Wong, K., 1981. The components of synaptic potentials evoked in cat spinal motoneurones by impulses in single group Ia afferents. J. Physiol. (London) 321, 65–96.

Katz, B., 1969. The Release of Neural Transmitter Substances. Liverpool Univ. Press, Liverpool.

Katz, B., Miledi, R., 1967. The release of acetylcholine from nerve endings by graded electric pulses. Proc. R. Soc. 167, 23–38.

Kirischuk, S., Veselovsky, N., Grantyn, R., 1999a. Relationship between presynaptic calcium transients and postsynaptic currents at single γ-aminobutyric acid (GABA)ergic boutons. Proc. Natl. Acad. Sci. U. S. A. 96, 7520–7525.

Kirischuk, S., Veselovsky, N., Grantyn, R., 1999b. Single-bouton-mediated synaptic transmission: postsynaptic conductance changes in their relationship with presynaptic calcium signals. Eur. J. Physiol. 438, 716–724.

Korn, H., Faber, D.S., 1987. Regulation and significance of probabilistic release mechanisms at central synapses. In: Edelman, G., Gall, E., Cowan, M. (Eds.), Synaptic Function. Wiley, Chichester, NY, pp. 57–108.

Korn, H., Faber, D.S., 1991. Quantal analysis and synaptic efficacy in the CNS. Trends Neurosci. 14, 439–445.

Korn, H., Bausela, F., Charpier, S., Faber, D., 1993. Synaptic noise and multiquantal release at dendritic synapses. J. Neurophysiol. 70, 1249–1254.

Li, C., Ullrich, B., Zhang, J.Z., Anderson, R.G., Brose, N., Sudhof, T.C., 1995. Ca^{2+}-dependent and-independent activities of neural and non-neural synaptotagmins. Nature 375, 594–599.

Liu, G., Tsien, R.W., 1995. Properties of synaptic transmission at single hippocampal synaptic boutons. Nature 375, 404–408.

Llinas, R., 1997. Calcium and neurotransmitter release in squid synapse. In: Cowan, W.M., Ferendelli, J.A. (Eds.), Society for Neuroscience Symposia, vol. 2. Society for Neuroscience, Bethesda, pp. 139–160.

Llinas, R., Moreno, H., 1998. Local Ca^{2+} signaling in neurons. Cell Calcium 24, 359–366.

Llinas, R., Steinberg, I.Z., Walton, K., 1981. Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. Biophys. J. 33, 323–351.

Llinas, R., Sugimori, M., Silver, R.B., 1995. The concept of calcium concentration microdomains in synaptic transmission. Neuropharmacology 34, 1443–1451.

McLachlan, E.M., 1978. The statistics of transmitter release at chemical synapses. Int. Rev. Physiol. Neurophysiol. 17, 49–117.

Meir, A., Ginsburg, S., Butkevich, A., Kachalsky, S.G., Kaiserman, I., Ahdut, R., Demirgoren, S., Rahamimoff, R., 1999. Ion channels in presynaptic nerve terminals and control of transmitter release. Physiol. Rev. 79, 1019–1080.

Neale, E.A., Nelson, P.G., Macdonald, R.L., Christian, C.N., Bowers, L.M., 1983. Synaptic interactions between mammalian central neurons in cell culture: III. Morphophysiological correlates of quantal synaptic transmission. J. Neurophysiol. 49, 1459–1468.

Nishiki, T.I., Augustine, G.J., 2001. Calcium-dependent neurotransmitter release: synaptotagmin to the rescue. J. Comp. Neurol. 436, 1–3.

Pierce, J.P., Lewin, G.R., 1994. An ultrastructural size principle. Neuroscience 58, 441–446.

Pierce, J.P., Mendell, L.M., 1993. Quantitative ultrastructure of Ia boutons in the ventral horn: scaling and positional relationships. J. Neurosci. 13, 4748–4763.

Prange, O., Murphy, T.H., 1999. Analysis of multiquantal transmitter release from single cultured cortical neuron terminals. J. Neurophysiol. 81, 1810–1817.

Propst, J.W., Ko, C.P., 1987. Correlations between active zone ultrastructure and synaptic function studied with freeze-fracture of physiologically identified neuromuscular junctions. J. Neurosci. 7, 3654–3664.

Pun, R.Y., Neale, E.A., Guthrie, P.B., Nelson, P.G., 1986. Active and inactive central synapses in cell culture. J. Neurophysiol. 56, 1242–1256.

Redman, S., 1990. Quantal analysis of synaptic potentials in neurons of the central nervous system. Physiol. Rev. 70, 165–198.

Redman, S., Walmsley, B., 1983. Amplitude fluctuations in synaptic potentials evoked in cat spinal motoneurones at identified group Ia synapses. J. Physiol. (London) 343, 135–145.

Sabatini, B.L., Regehr, W.G., 1997. Control of neurotransmitter release by presynaptic waveform at the granule cell to Purkinje cell synapse. J. Neurosci. 17, 3425–3435.

Schikorski, T., Stevens, C.F., 1997. Quantitative ultrastructural analysis of hippocampal excitatory synapses. J. Neurosci. 17, 5858–5867.

Smith, S.M., Zorec, R., McBurney, R.N., 1989. Conductance states activated by glycine and GABA in rat cultured spinal neurons. J. Membr. Biol. 108, 45–52.

Stenley, E.F., 1993. Single calcium channel and acetylcholine release at a presynaptic nerve terminal. Neuron 11, 1007–1011.

Stevens, C.F., Wesseling, J.F., 1998. Activity-dependent modulation of the rate at which synaptic vesicles become available to undergo exocytosis. Neuron 21, 415–424.

Steyer, J.A., Horstmann, H., Almers, W., 1997. Transport, docking and

exocytosis of single secretory granules in live chromaffin cells. Nature 388, 474–478.

Tokumaru, H., Umayahara, K., Pellegrini, L.L., Ishizuka, T., Saisu, H., Betz, H., Augustine, G.J., Abe, T., 2001. SNARE complex oligomerization by synaphin/complexin is essential for synaptic vesicle exocytosis. Cell 104, 421–432.

Tong, G., Jahr, C.E., 1994. Multivesicular release from excitatory synapses of cultured hippocampal neurons. Neuron 12, 51–59.

Vautrin, J., Schaffner, A.E., Fontas, B., Barker, J.L., 1993a. Frequency modulation of transmitter release. J. Physiol. (Paris) 87, 51–73.

Vautrin, J., Schaffner, A.E., Barker, J.L., 1993b. Quantal and subquantal GABAergic transmissions in cultured rat hippocampal neurons. Hippocampus 3, 93–101.

Vogt, K., Luscher, H.-R., Streit, J., 1995. Analysis of synaptic transmission at single identified boutons on rat spinal neurons in culture. Pflugers Arch. 430, 1022–1028.

Von Gersdorff, H., Matthews, G., 1994. Dynamic of synaptic vesicle fusion and membrane retrieval in synaptic terminals. Nature 367, 735–739.

Von Gersdorff, H., Sakaba, T., Berglund, K., Tachibana, M., 1998. Submillisecond kinetics of glutamate release from a sensory synapse. Neuron 21, 1177–1188.

Von Ruden, L., Neher, E., 1993. A Ca-dependent early step in the release of catecholamines from adrenal chromaffin cells. Science 262, 1061–1065.

Walmsley, B., 1991. Central synaptic transmission: studies at the connection between primary afferent fibers and dorsal spinocerebellar tract (DSCT) neurons in Clarke's column of the spinal cord. Prog. Neurobiol. 36, 391–423.

Walmsley, B., Wieniawa-Narkiewicz, E., Nicol, M.J., 1985. The ultrastructural basis for synaptic transmission between primary muscle afferents and neurons in Clarke's column of the cat. J. Neurosci. 5, 2095–2106.

Yazejian, B., DiGregorio, D.A., Vergara, J.L., Poage, R.E., Meriney, S.D., Grinnell, A.D., 1997. Direct measurements of presynaptic calcium and calcium-activated potassium currents regulating neurotransmitter release at cultured Xenopus nerve-muscle synapses. J. Neurosci. 17, 2990–3001.

Zucker, R.S., 1973. Changes in the statistics of transmitter release during facilitation. J. Physiol. (London) 229, 787–810.

Zucker, R.S., 1993. Calcium and transmitter release. J. Physiol. (London) 87, 25–36.