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Protein kinase A cascade regulates quantal release dispersion at frog muscle endplate

Ella A. Bukharaeva *†, Dmitry Samigullin *, Eugeny Nikolsky *† and František Vyskočil ‡§

* *State Medical University, Butlerov st. 49, Kazan, Russian Federation,* † *Institute of Biochemistry and Biophysics, Russian Academy of Sciences, PO Box 30, Kazan, Russian Federation,* ‡ *Department of Animal Physiology and Developmental Biology, Faculty of Sciences, Charles University, Viničná 7, Prague 2, Czech Republic and* § *Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 4, Czech Republic*

Uniquantal endplate currents (EPCs) were recorded simultaneously at the proximal, central and distal parts of the frog neuromuscular synapse, and their minimal synaptic latencies, latency dispersions and sensitivity to noradrenaline, cAMP and protein kinase A inhibition were measured. The latency dispersion was highest in the proximal part (P_{90} = 1.25 ms); it decreased to P_{90} = 0.95 ms in the central part and to P_{90} = 0.75 ms (60 % of the proximal part) in the distal part. In the proximal parts of the long neuromuscular synapse, stimulation-evoked EPCs with long release latencies were eliminated when the intracellular cAMP was increased by β1 activation by noradrenaline, by the permeable analogue db-cAMP, by activation of adenylyl cyclase or by inhibition of cAMP hydrolysis. This makes the evoked release more compact, and the amplitude of the reconstructed multiquantal currents increases. Protein kinase A is a target of this regulation, since a specific inhibitor, Rp-cAMP, prevents the action of cAMP in the proximal parts and increases the occurrence of long-latency events in the distal parts of the synapse. Our results show that protein kinase A is involved in the timing of quantal release and can be regulated by presynaptic adrenergic receptors.

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Corresponding author F. Vyskočil: Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 4, 142 20, Czech Republic. Email: vyskocil@biomed.cas.cz

The molecular mechanisms that underlie transmitter release are still in the realm of hypotheses rather than being a firmly established theory. To understand these mechanisms, it is useful to study two variables: the number of released vesicles (quantal content) and the release parameters as reflected by the synaptic latency distribution (Katz & Miledi, 1965; Minenko & Magazanik, 1986). In our previous study (Bukharaeva *et al.* 1999) we measured both parameters simultaneously and found that after application of noradrenaline (NA), the quantal content remained unaltered, whereas the duration of release became shorter. This suggests that different mechanisms are involved in the control of quantal content and the time course of release (Zucker, 1999). The molecular mechanisms underlying this finding were followed in two parallel lines of research.

One line was pharmacological. Inhibitor and agonist experiments showed that NA acts on the β1 receptor (Bukharaeva *et al.* 1999), which might be coupled with the cAMP cascade (Carlson *et al.* 1994; Majewski & Barrington, 1995; Sulakhe & Vo, 1995). We report here on changes in the latencies of focally recorded uniquantal endplate currents (EPCs) after the application of several agents that regulate intracellular cAMP levels, and demonstrate that the synchronizing action of NA is mediated by cAMP and protein kinase A (PKA). Interestingly, an increased fluctuation and asynchronicity of evoked release were described recently for several *Drosophila* mutants in which the synthesis and degradation of cAMP were affected (Renger *et al.* 2000).

Another aspect of our research was physiological. It is known that catecholamines facilitate neuromuscular transmission (Orbeli, 1923; Burn, 1945) by increasing EPC amplitude. This has been attributed to an increase in quantal content (Jenkinson *et al.* 1968; Hidaka & Kuriyama, 1969; Kuba, 1970; Kuba & Tomita, 1971; Wessler & Anschuetz, 1988; Vizi, 1991), an increase in quantum size (Van der Kloot & Van der Kloot, 1986) and a greater sensitivity of the subsynaptic membrane to ACh (Chen *et al.* 1991). For example, when a decrease in quantal content and longer latencies are observed, NA removes activity-induced fatigue (Ruzzier & Scuka, 1986). We showed that the larger synaptic potential may result from a more synchronized release, even though the quantal content is the same (Bukharaeva *et al.* 1999). These data were obtained by focal recording of EPCs in the proximal part of the 150- to 200-μm-long nerve terminal at the frog endplate. However, it is known that there are different patterns of spike amplitudes and time courses along the frog nerve terminal (Benoit & Mambrini, 1970; Mallart,

1984; Shakiryanova *et al.* 1994), non-uniform spontaneous and evoked release (Zefirov, 1983; Mallart, 1984; D'Alonzo & Grinnell, 1989) as well as different structural characteristics of the proximal and distal endplate regions (Davey & Bennett, 1982; Robitaille & Tremblay, 1987). We therefore estimated the latencies of EPCs simultaneously at the proximal, central and distal parts of the synapse and found substantial differences between these parts in minimal synaptic latencies, latency dispersions and sensitivity to NA, cAMP manipulations and PKA inhibition. Preliminary results with cAMP in the proximal part of the synapse have already been presented as a short report (Bukharaeva *et al.* 2000).

METHODS

Animals and drugs

The experimental preparation, bathing solutions and focal extracellular recording technique have been described in a previous paper (Bukharaeva *et al.* 1999). Briefly, experiments were carried out on the cutaneous pectoris muscle from the frog *Rana ridibunda* from November to April. Animals were anaesthetized with ether before being stunned and double-pithed. The preparations were pinned to a translucent chamber and superfused with (mM): NaCl 113.0, KCl 2.5, $CaCl_2$ 0.2, $NaHCO_3$ 3.0, and $MgCl_2$ 4.0. The pH was 7.3 at 20.0 ± 0.3 °C. The Animal Care and Use Committee of the Institute of Physiology, Czech Academy of Sciences, approved the protocol.

The following drugs were used (Sigma, St Louis, MO, USA): NA, dibutyryl cAMP, adenosine 3,5-monophosphothioate (Rp-cAMP), forskolin, and 3-isobutyl-1-methylxanthine (IBMX). The drugs were added to the superfusing solution, and the measurements were started 20 min after drug application, unless stated otherwise. In most cases, the drugs were washed out for a further 30 min before new recordings were made.

Electrophysiology

Suprathreshold stimuli of 0.1 ms duration were applied to the nerve at 2 s intervals. Nerve action potentials and extracellular EPCs were recorded using three focal Ringer-filled extracellular pipettes with a tip diameter of 2–3 μm and 1–3 MΩ resistance. Extracellular pipettes were positioned under visual control in the proximal, central and distal endplate regions of a long nerve terminal, 3–5 μm, 40–50 μm and 80–100 μm, respectively from the end of the myelinated segments of the axon. In these regions, three-component nerve spikes were recorded simultaneously with extracellular EPCs (Mallart, 1984; Shakiryanova *et al.* 1994). The recorded signals were filtered between 0.03 Hz and 10 kHz, digitized at 3 μs intervals by an analog-to-digital 9 bit converter, fed into a computer and processed by our application program package.

Uniquantal EPCs (Katz & Miledi, 1965; Barrett & Stevens, 1972) were recorded in the presence of 0.2 mM Ca^{2+} and 4.0 mM Mg^{2+}. The quantal content (m_o) of the low-quanta EPCs were determined from 5 or 6 stimulation periods (256 stimuli each) in the absence and in the presence of particular drugs. The number of failures in a series of 250–400 uniquantal responses was measured and m_o calculated as equal to $\ln N/n_o$, where N is the total number of stimuli and n_o is the number of failures (Del Castillo & Katz, 1954; Martin, 1955). The number of stimuli was therefore between 1280 and 1536 in each series of stimulation periods. Unless m_o is quite low, at least some of the responses in a series of 250–400 responses will be larger than uniquantal.

Latency was measured as the time interval between the peak of the inward presynaptic Na^+ currents and the time at which the rising phase of the concomitantly recorded quantal event reached 20 % of maximum (Fig. 1*A*). Because the amplitude of the reference Na^+ current decreases along the nerve terminal (Mallart, 1984, Shakyrianova *et al.* 1994), the distal recording site was selected so that the Na^+ peak, from which the latency was measured, was still clearly seen on averaged records from this part of the nerve terminal (e.g. Fig. 1*A*, DIST). The limit of the latency measurement was 6 ms for experiments performed at 20 °C.

The stability of the recording electrode position next to the membrane region studied was crucial during long-lasting extracellular recordings. Therefore, we monitored the amplitude of the nerve terminal action potentials throughout each data set. Only data sets in which the terminal spike changed by less than 10 % during the drug application and washout were analysed further (Bukharaeva *et al.* 1999). Statistical analyses of pre- and postsynaptic events were performed using Student's t test for paired data.

The quantitative characteristics of the change in the time course of evoked secretion can be obtained by cumulative curve analysis (Van der Kloot, 1991) and by the release probability function (Barrett & Stevens, 1972; Baldo *et al.* 1986). From our experience (Bukharaeva *et al.* 1999), both methods provide reliable information about the time course of evoked release; therefore in the present paper only cumulative curves were used (Fig. 1*C*) together with an estimation of the modal values of latency histograms (MV in Fig. 1*B*) and the minimal synaptic latencies (ML in Fig. 1*B*). Cumulative curves were constructed from latency histograms of the uniquantal EPCs. The histograms of the amplitudes of the total population of EPCs were analysed using a standard computer procedure. Uniquantal EPCs were characterized by amplitudes that fell in the first peak in the amplitude histograms (not shown).

The mean value of the shortest 5 % of the latencies in each series was taken as the minimal synaptic latency. For the comparison of dispersion histograms in different parts of the terminal, minimal synaptic latencies, which differ along the nerve terminal, were subtracted. The interval between the minimal synaptic latency and the time at which 90 % of all measured uniquantal EPCs had occurred was designated as P_{90} (Fig. 1*B*, *C*). The statistical significance of the difference between two cumulative curves was assessed by the Kolmogorov-Smirnov statistic; $P \leqslant 0.05$ was taken as significant (Bronstein & Semendjaev, 1986; Van der Kloot, 1991). Other statistical tests were performed with SigmaStat 0.1 (Jandel Corporation 1992–1994). Analyses of variance (ANOVA) of the experimental groups *versus* the control group were performed by multiple comparisons using the ANOVA Bonferroni t test. The amplitudes, rise times of EPCs (20–80 % of the maximal amplitude) and exponential decay constants (τ) of the responses are presented as means ± S.E.M. Differences between two groups were considered statistically significant at the probability level $P = 0.05$. The letter n indicates the number of endplates measured in each group.

RESULTS

Proximal to distal gradient of release dispersion

Evoked presynaptic nerve spikes recorded in three parts of the synapse were different ('NS' in Fig. 1*A*), as were the

mean quantal contents of EPCs. In a low-calcium, high-magnesium solution, m_o was 0.33 ± 0.09 (n = 12) in the proximal part, 0.25 ± 0.07 (n = 12) in the central part and 0.18 ± 0.09 (n = 12) in the distal part. In the proximal region, the nerve spikes had three components (Fig. 1*A*, PROX): an outward (positive) passive capacitance current, a large inward Na^+ current and an outward K^+ current (Mallart, 1984; Shakiryanova *et al.* 1994). The spikes recorded in the central part and the most remote distal part were characterized by two phases (Fig. 1*A*). The EPCs appeared after a synaptic latency ('L' in Fig. 1*A*; Katz & Miledi, 1965). The time courses of quantal release latencies were also different in these regions. This is documented by native records (Fig. 4), synaptic latency histograms (Fig. 1*B*) and the cumulative plots of latencies (Fig. 1*C*). The vertical arrows in Fig. 1*C* indicate the times when 90 % of the quanta have been released (P_{90}) in the distal, central and proximal parts. The dashed lines parallel to the curves are confidence limits at P = 0.05. According to the Kolmogorov-Smirnov criterion, the difference between the curves is statistically significant if the confidence limits of the two curves do not overlap. For example, significant differences between the distal and proximal parts can already be seen when about 30 % of EPCs are released.

The minimal synaptic latencies differed significantly ($P \leqslant 0.05$) and were 0.50 ± 0.01 ms in the proximal part, 0.41 ± 0.01 ms in the central part and 0.31 ± 0.01 ms in the distal part (n = 12 each). For this reason, the latency distribution histograms were shifted to the left in the central and distal parts. They were asymmetrical due to EPCs with long latencies (late release according to Barrett & Stevens, 1972). However, the latency dispersion expressed as P_{90}, corrected for minimal synaptic latency (Fig. 1*C*, *D*),

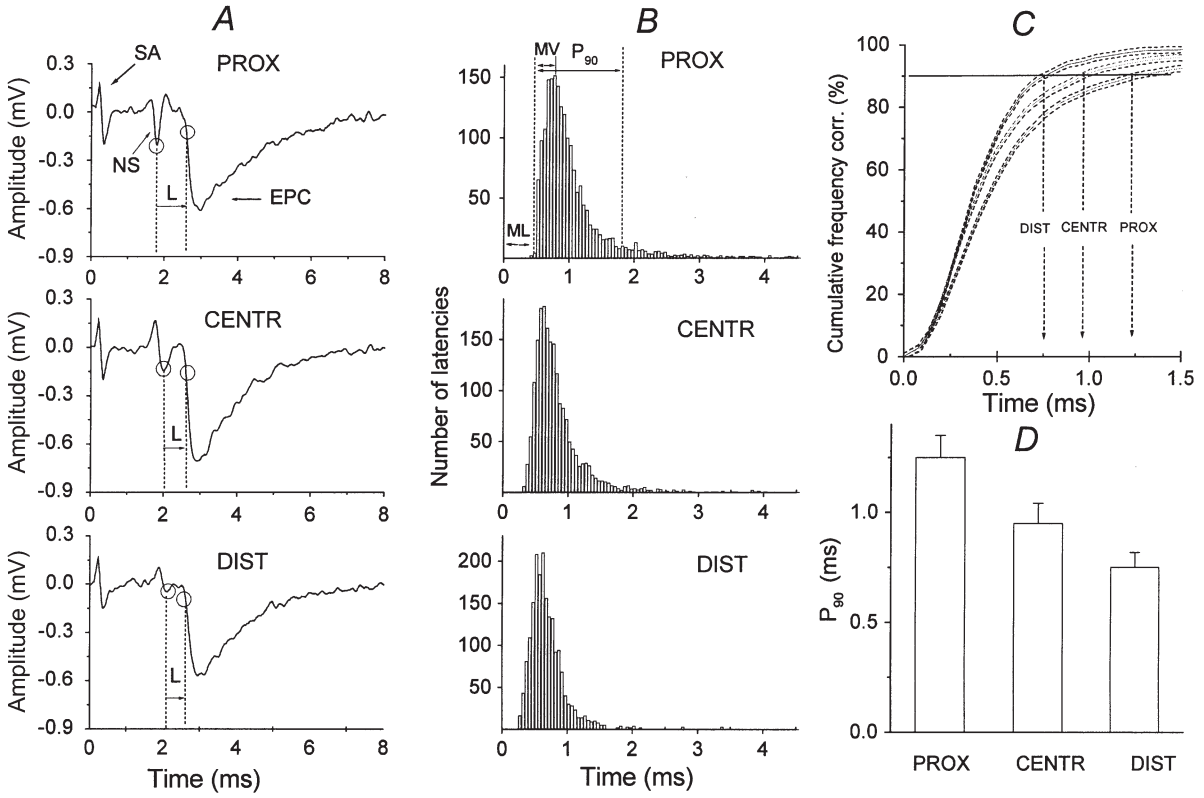

Figure 1. Latencies of quantal releases in the proximal (PROX), central (CENTR) and distal (DIST) parts of the synapse

A, extracellularly recorded presynaptic nerve spikes (NS) and individual endplate currents (EPCs). The stimulus artefact (SA) is followed by a three-phase action potential, the shape of which changes along the nerve terminal (Shakyrianova *et al.* 1994). The time interval between the peak of the inward presynaptic Na^+ current and the time at which the rising phase of the EPC reached 20 % of maximum (ovals) is defined as the release latency (L). *B*, non-corrected latency histograms. Values of minimal synaptic latencies (ML, were not subtracted) of 2268 uniquantal EPCs from 9 experiments. The bin size is 0.05 ms. The modal value (MV) of the histogram and the time at which 90 % of all measured uniquantal EPCs had occurred (P_{90}) are indicated. *C*, the cumulative plots of latencies for the same data, corrected for minimal synaptic latencies, are shown. The vertical arrows indicate P_{90} in the distal (DIST), central (CENTR) and proximal (PROX) parts. The dashed lines parallel to the curves are confidence limits at P = 0.05. *D*, corrected P_{90} (values of minimal synaptic latencies subtracted) at PROX, CENTR and DIST parts of the nerve terminal (means ± S.E.M. from 10 experiments).

was highest in the proximal part (1.25 ± 0.10 ms), decreasing to 0.95 ± 0.09 ms in the central part and 0.75 ± 0.07 ms (60 % of the proximal part) in the distal part; all differences were significant at $P \leqslant 0.05$ ($n = 12$). The modal values of the histograms, when measured from the minimal synaptic latencies in the proximal, central and distal parts (Fig. 1*B*), were similar: 0.25 ± 0.02 ms, 0.21 ± 0.02 ms and 0.28 ± 0.01 ms, respectively ($P \leqslant 0.05$, $n = 12$ each). These observations demonstrate that the highest degree of synchronization in the remote parts of the long nerve terminal were due to a small number of long-latency EPCs.

The effect of pressure on release dispersion

In five experiments, the extracellular electrode was located near the nerve ending under different pressures to check whether the differences observed along the endplate were caused mechanically while searching for the optimal recording site along the progressively narrowing nerve ending (Davey & Bennett, 1982). Figure 2 shows one of five experiments in which the effect of mechanical pressure was tested. The data on this figure demonstrate that the time course of the release (latency histograms and P_{90}, Fig. 2*B*, *C*) was not altered when greater pressure was applied and when presynaptic spikes, quantal content and EPC amplitudes increased (Fig. 2*Ab*). In particular, the increase in m_o from 0.29 ± 0.04 to 0.41 ± 0.07 without any changes in the latency dispersion pattern, provides further evidence (Parnas & Parnas, 1999) that under particular conditions, quantum content and quantal latencies are controlled independently, and that probably no presynaptic quantal interaction exists in the frog, as was suggested recently for the lobster neuromuscular junction (Bykhovska *et al.* 1999).

NA decreases the latency dispersion in the proximal part

Control quantal content m_o was 0.33 ± 0.09 ($n = 6$) in the proximal part. After application of 1×10^{-5} M NA, m_o remained unchanged at 0.39 ± 0.08 ($n = 6$, $P \geqslant 0.05$). The

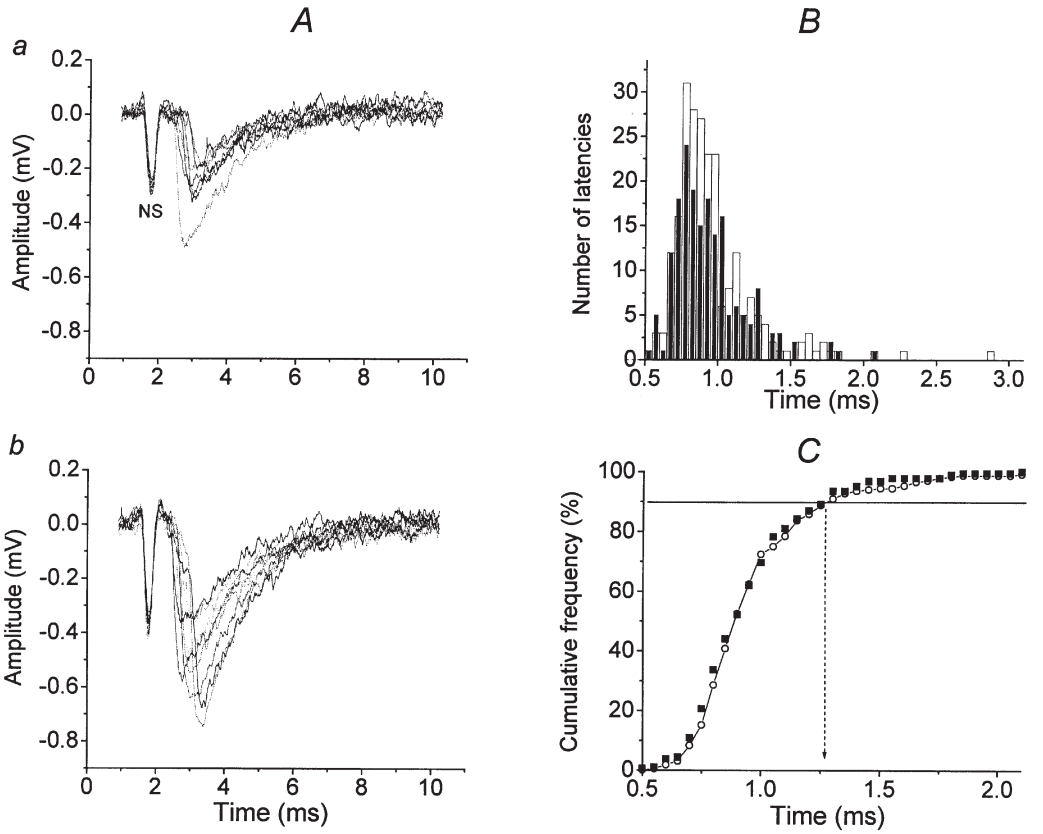

Figure 2. Effect of slight and strong microelectrode pressure on the latencies of quantal releases in the proximal part of the synapse

A, 10 superimposed randomly selected recordings from a total of 220 presynaptic NSs and extracellular EPCs before (*a*) and after the application of stronger pressure (*b*) applied by moving the recording microelectrode 20 μm vertically towards the muscle fibre. Recordings with failures were omitted. Quantum content increased from 0.32 in panel *a* to 0.60 in this case, apparently due to mechanical stretching in the release area (Turkanis, 1973; Chen & Grinnell, 1997). *B*, corrected latency histograms of 220 uniquantal EPCs from this experiment. The bin size is 0.05 ms. Filled columns, slight pressure; open columns, strong pressure. *C*, the cumulative plots of latencies for the same data before (○) and after pressure (■). The vertical dotted arrow indicates P_{90}, which is the same (1.25 ms) for slight and strong pressure of the microelectrode.

mean frequency of miniature EPCs (mEPCs) in the presence of NA (1.73 ± 0.45 s^{-1}, $n = 6$, $P \leqslant 0.05$) was also similar to that recorded in the controls before the drug was administered (1.4 ± 0.50 s^{-1}). NA did not change the presynaptic action potential, the EPC amplitude or the minimal synaptic latency, whereas the modal values of the latency histograms shifted slightly from 0.27 ± 0.02 ms to 0.18 ± 0.03 ms (Bukharaeva *et al.* 1999). NA did, however, change the latency distribution by removing long synaptic latencies in the proximal part where the latency dispersion was originally high (Fig. 3). The control value of the corrected P_{90}, 1.25 ± 0.10 ms, was significantly and reversibly reduced by 35 % (as assessed using the Kolmogorov-Smirnov criteria) to 0.81 ± 0.10 ms ($n = 6$, $P \leqslant 0.05$). On the other hand, the changes in the central parts were marginal (minimal latency was 0.41 ± 0.01 ms before and 0.40 ± 0.02 ms after NA; modal values were 0.21 ms and 0.20 ms, respectively, and P_{90} was 0.95 ms and 0.80 ms, respectively), and were not significant in the distal parts, where they were characterized by the most compact release (minimal latency was 0.31 ± 0.02 ms before and 0.31 ± 0.01 ms after NA; modal values were 0.25 ± 0.05 ms and 0.27 ± 0.04 ms, respectively, and P_{90} 0.78 ms and 0.75 ms, respectively).

db-cAMP imitates NA action

The permeable derivative of cAMP, db-cAMP (1×10^{-6} M), when present for 1 h, markedly shortened the latency distribution in a way resembling the action of NA (Fig. 4). The most pronounced effect on long-latency EPCs was found in the proximal part: the number of EPCs with longer synaptic latencies was decreased, but the minimal synaptic latency was unchanged (0.50 ± 0.02 ms in nine control experiments *versus* 0.47 ± 0.04 ms in the presence of db-cAMP for minimal latencies), whereas the modal values of the latency histograms shifted slightly (from 0.28 ± 0.04 ms to 0.20 ± 0.03 ms, respectively, $P \leqslant 0.05$). Corrected cumulative curves of the synaptic latencies resulted in a control P_{90} value of of 1.25 ± 0.10 ms, which was significantly reduced to 0.89 ± 0.10 ms ($n = 6$, $P \leqslant 0.05$, Fig. 4) in the presence of db-cAMP. The ratio between the P_{90} value in db-cAMP *versus* that in the controls was 0.71. This means that this drug shortened the release by 29 %.

The corrected P_{90} in the central part was decreased to 0.78 ± 0.05 ms in db-cAMP, *versus* 0.95 ± 0.07 ms ($n = 6$, $P \leqslant 0.05$) in controls. In the distal part, P_{90} was not significantly changed: 0.75 ± 0.07 ms in controls, and 0.78 ± 0.09 ms in db-cAMP (Fig. 4).

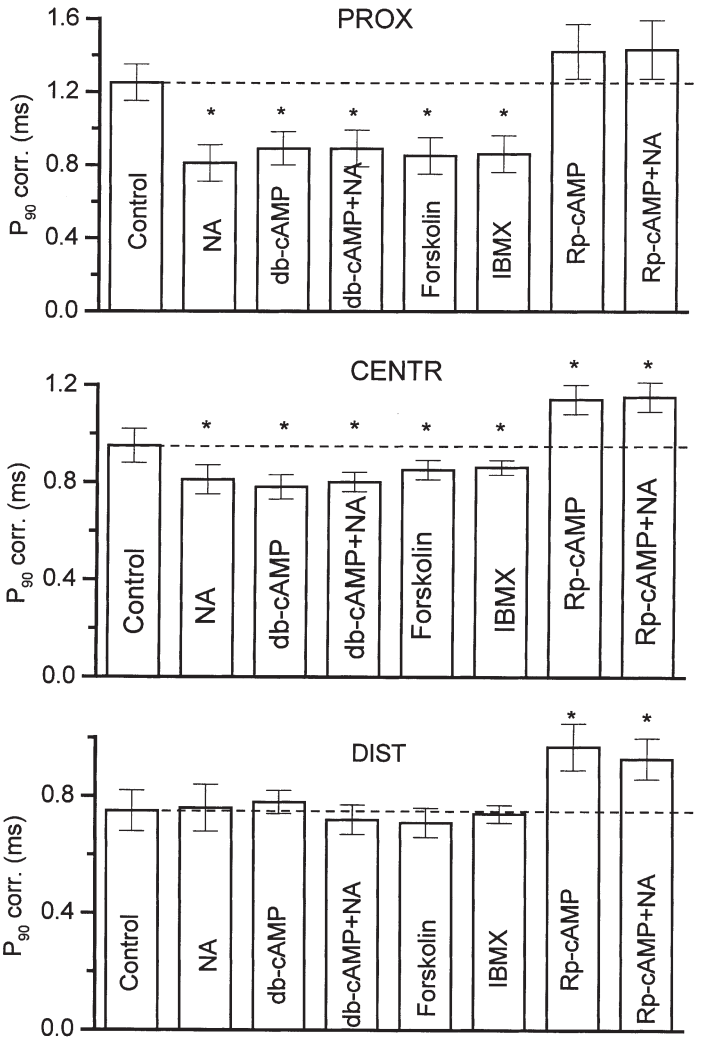

Figure 3. Effects of drugs changing cAMP levels and the protein kinase A (PKA) inhibitor Rp-cAMP on P_{90} values at PROX, CENTR and DIST parts of the endplate

Left ordinates represent the corrected (corr) P_{90} (i.e. minimal synaptic latencies were subtracted). Dashed lines indicate the control level in each group. The drugs applied were: noradrenaline (NA), 1×10^{-5} M; db-cAMP, 1×10^{-6} M; forskolin, 1×10^{-6} M; 3-isobutyl-1-methylxanthine (IBMX) 1×10^{-4} M; Rp-cAMP, 2×10^{-5} M; Rp-cAMP + NA–Rp-cAMP together with 1×10^{-5} M NA. Asterisks indicate values that were significantly different from controls at the probability level $P = 0.05$.

No other effects of 1×10^{-6} M db-cAMP were found: the quantal content in the central part was 0.34 ± 0.15 ($n = 6$) before, 0.35 ± 0.25 ($n = 6$) 1 h after db-cAMP application, and 0.36 ± 0.17 ($n = 5$) after washing out of the drug for 2 h. The mean frequency of mEPCs (0.83 ± 0.45 s^{-1}) was very similar to that recorded in the controls before application of the drug (0.74 ± 0.50 s^{-1}, $n = 5$, $P \leqslant 0.05$). It is worth mentioning that a higher concentration of the drug, in particular 1×10^{-4} M, shortened the release phase but increased the frequency of occurrence of miniature endplate potentials (mEPPs; Miyamoto & Breckenbridge, 1974) to an extent that made the latency estimation incorrect due to the unpredictable appearance of mEPPs in the latency window. Like NA, db-cAMP did not change the amplitude and decay time of mEPCs, suggesting that at a concentration of 1×10^{-6} M, it had no effect on postsynaptic receptor sensitivity.

The effects of db-cAMP and NA were not cumulative. When applied 1 h after db-cAMP, NA did not further shorten the early release phase in the proximal zone. The P_{90} was 0.89 ± 0.10 ms in the presence of 1×10^{-6} M db-cAMP and 0.85 ± 0.05 ms ($n = 5$, $P \leqslant 0.05$) when NA was added for another 60 min. The absence of an effect could be expected if the NA action were completely mediated by endogenous cAMP.

Forskolin imitates NA and db-cAMP actions

The adenylyl cyclase activator forskolin (Laurenza *et al.* 1987) was tested at a concentration of 1×10^{-6} M, which is not high enough to influence the frequency of mEPCs or postsynaptic ACh sensitivity (Khirough *et al.* 1998). The time course of evoked quantal secretion in the presence of forskolin was affected almost the same as in the presence of NA and db-cAMP. This effect is demonstrated by the reduction of P_{90} from 1.25 ± 0.10 ms in the controls to 0.85 ± 0.09 ms ($n = 6$, $P \leqslant 0.05$), which means that forskolin shortened the release by 32 %. A much smaller effect was observed in the central part, where P_{90} decreased by 11 % from 0.95 ± 0.07 ms in the controls to 0.85 ± 0.06 ms ($n = 6$, $P \leqslant 0.05$). Forskolin was almost ineffective in the distal part (Fig. 3). Other parameters, such as minimal

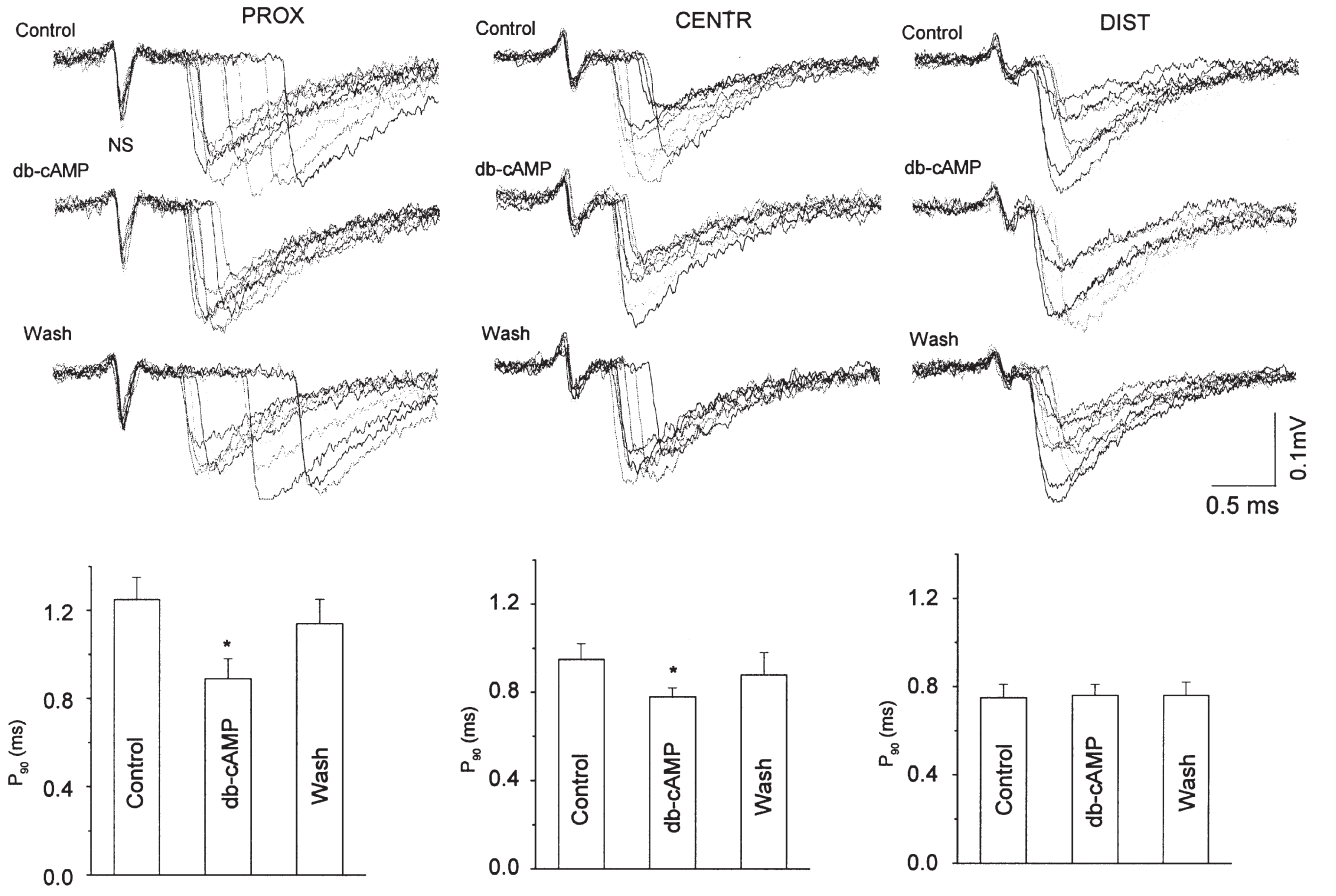

Figure 4. Effect of db-cAMP on the latencies in three parts of the synapse

Upper records are 8–10 superimposed extracellular recordings of presynaptic NSs and individual EPCs before (Control), in the presence of and after washout of 1×10^{-5} M db-cAMP (Wash). All recordings were obtained simultaneously in one experiment, by three extracellular microelectrodes located in three zones of the same endplate. Columns: P_{90} parameter (ordinates) in controls, in the presence of 1×10^{-5} M db-cAMP and after 60 min washout of the drug ($n = 6$).

synaptic latencies and modal values of latency histograms, were affected similarly, as in the case of NA and db-cAMP in all parts of the synapse (data not given).

The minimal latency in controls in the proximal part was 0.49 ± 0.02 ms, and in forskolin it was 0.50 ± 0.03 ms; the modal value in controls was 0.30 ± 0.01 ms, which slightly but significantly decreased in forskolin (0.25 ± 0.03 ms). As with db-cAMP, the effects of forskolin and NA in the proximal part were not cumulative, and P_{90} was 0.85 ± 0.11 ms ($n = 5$, $P \geqslant 0.05$) in the presence of both drugs (Fig. 3).

IBMX imitates the actions of NA, db-cAMP and forskolin

The level of cAMP and of other cyclic nucleotides in the cell is regulated not only by their production, but also by the rate of their degradation to 5′-AMP (Nestler & Greengard, 1989; Majewski & Barrington, 1995). We therefore studied the effect of 1×10^{-4} M of the phosphodiesterase inhibitor IBMX (Beavo *et al.* 1970) on latency distribution; a similar pattern of action was found as when db-cAMP and forskolin were used (Fig. 3). P_{90} was decreased by 31 % in the proximal part, a lesser shortening by 10 % was observed in the central part and no effect was seen in the distal part. NA was ineffective when applied together with IBMX in the proximal part (not shown). The above concentration of the drug slightly increased the quantal content, from 0.33 ± 0.01 before to 0.45 ± 0.03 after 1×10^{-4} M IBMX. Once again, other parameters (i.e. minimal synaptic latencies and the modal values of latency histograms) were affected similarly in all parts of the synapse (data not given), as in the case of NA and db-cAMP. The minimal latency in the proximal part in controls was 0.48 ± 0.04 ms and was unchanged in IBMX (0.49 ± 0.05 ms). The modal values in controls (0.24 ± 0.03 ms) and in IBMX (0.19 ± 0.02 ms) were not significantly different.

PKA inhibition increases latency dispersion

All hitherto presented data indicate that an increase of intraterminal cAMP can slightly shorten the early release period and substantially decrease the late phase of the release. It is known that cAMP activates the phosphorylation of many functional and regulatory proteins (Majewski & Barrington, 1995). The inhibition of a particular class of protein kinases should thus increase the latency dispersion. In release timing, the activation of PKA might be involved, since in a preliminary screening of several protein kinase inhibitors, we found that Rp-cAMP, a specific inhibitor of PKA (Dostmann *et al.* 1990), was the most effective. When applied at a concentration of 2×10^{-5} M, Rp-cAMP induced the appearance of long-latency EPCs mostly in remote release sites, originally the most synchronized, but to a lesser extent in proximal parts characterized by high dispersion (Fig. 3). Thus, P_{90} increased by 29 ± 5 % in the distal part, by 20 ± 5 % in the central part, and insignificantly by 14 ± 5 % ($n = 6$) in the proximal part. At this concentration, the drug slightly decreased the quantal content, from 0.33 ± 0.09 before to 0.24 ± 0.03 after application. No effect of NA or db-cAMP was observed in any part of the junction when they were applied together with Rp-cAMP to the endplates already treated with this PKA inhibitor (Fig. 3, the far right columns). Rp-cAMP prolonged slightly but significantly the minimal synaptic latencies in the proximal and central parts by 10 % (from 0.50 ± 0.01 ms to 0.55 ± 0.03 ms and from 0.41 ± 0.01 ms to 0.46 ± 0.02 ms, respectively) and by 12 % (from 0.31 ± 0.01 ms to 0.35 ± 0.03 ms) in the distal part ($n = 10$ each, $P \leqslant 0.05$). In contrast, cAMP manipulation, had no effect on this parameter. Nevertheless, the modal values of the histograms, when measured from the minimal synaptic latencies in each part, were similar before and after Rp-cAMP application: 0.31 ± 0.03 ms and 0.28 ± 0.04 ms, respectively, in the proximal part, 0.25 ± 0.04 ms and 0.22 ± 0.05 ms, respectively, in the central part and 0.22 ± 0.02 ms and 0.21 ± 0.03 ms, respectively, in the distal part ($P \geqslant 0.05$, $n = 12$ each). Thus, the main group of short-latency quanta (early release) remained unchanged, and the PKA inhibition increased the number of EPCs with longer latencies. The increase in P_{90} was reversible because after a 30–60 min washout of Rp-cAMP, P_{90} returned to 96 % of its control value. Such good recovery probably indicates that phosphorylation and dephosphorylation take place at sites already prepared for quantal release and not during priming and docking of the vesicles, because once docked, vesicles are very stable at the release sites (Betz *et al.* 1993).

A similar conclusion can be drawn from results with tetanic stimulation. In five experiments, neuromuscular preparations were stimulated for 60 min with a frequency of 10 Hz in a solution containing 1.8 mM Ca^{2+} and in the presence of 2×10^{-5} M Rp-cAMP. The stimulation was aimed to reach exhaustion of the immediately available quanta from the sites of release when multiquantal full-size EPCs are evoked (Ceccareli *et al.* 1972). After stimulation, the solution was again changed to one containing 0.35 mM Ca^{2+}, +4.0 mM Mg^{2+} and $+2 \times 10^{-5}$ M Rp-cAMP. Following 30 min of solution exchange, recordings of uniquantal EPCs were made every 2 s in the distal part in the presence of Rp-cAMP, for a period of 60 s (second post-tetanic recording period). P_{90}, corrected for minimal synaptic latency, was prolonged during the first post-stimulation period to 1.06 ± 0.09 ms ($n = 5$) as compared with mean values obtained in the distal zone without stimulation (Fig. 3, lower panel). After washout of the drug, P_{90} decreased to normal and was 0.75 ± 0.07 ms ($n = 5$, $P \leqslant 0.05$) during the second recording period. This represents a shift of 41 % if the washout values are taken as 100 %. In control experiments without the drug, P_{90} values were 0.76 ± 0.04 ms and 0.71 ± 0.09 ms during the first and second post-tetanic recording periods, respectively, without any

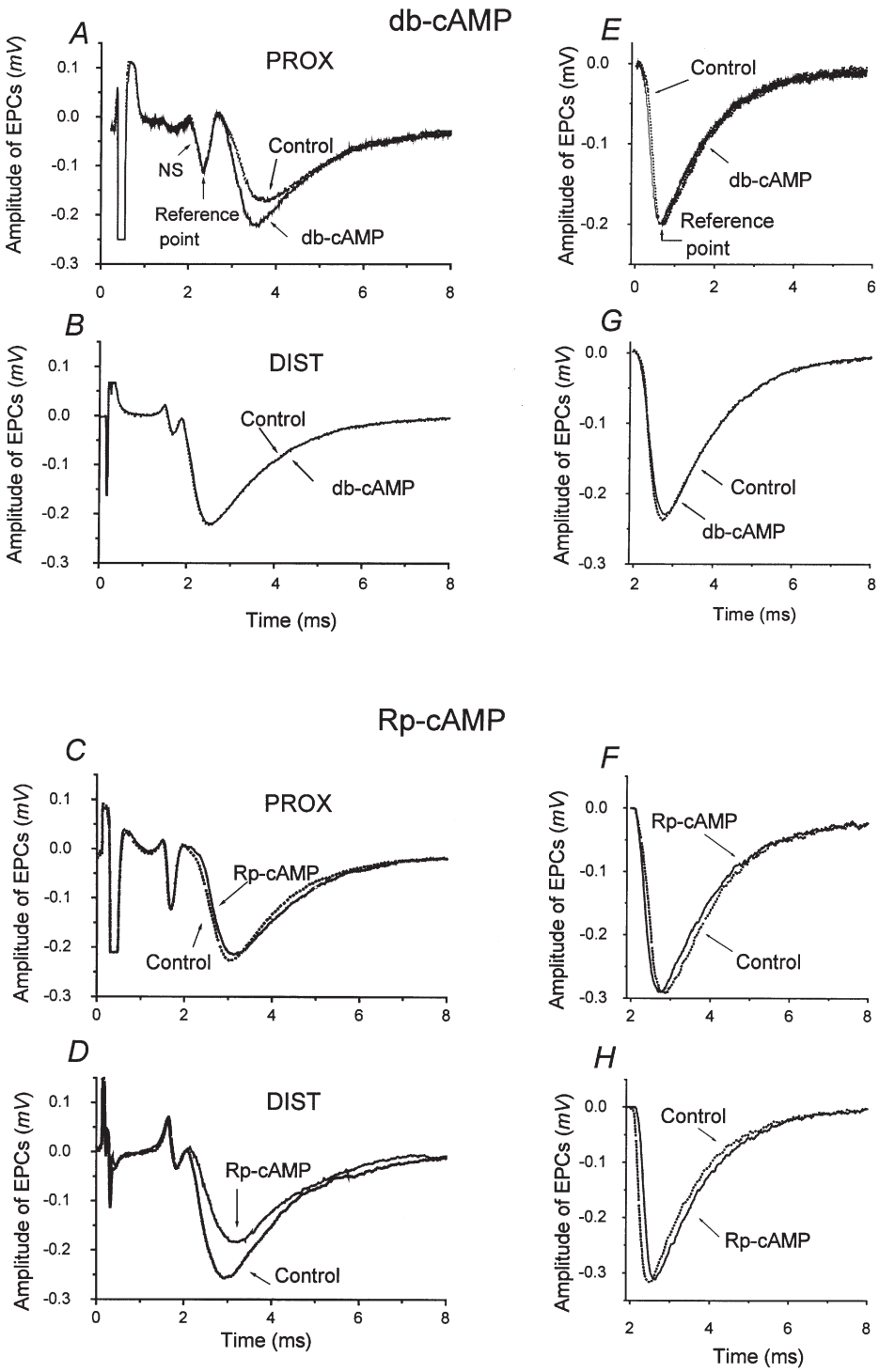

Figure 5. Multiquantal EPCs summated from uniquantal EPCs recorded in the absence (Control) and presence of 1×10^{-6} M db-cAMP (*A*, *B*) or 2×10^{-5} M Rp-cAMP (*C*, *D*)

Uniquantal EPCs used for construction were recorded in the PROX and DIST parts of the endplate. Abscissae represent time in ms, ordinates represent amplitude in mV. Summations of 50–70 uniquantal EPCs measured before (Controls, dotted lines) and 30 min after the application of either drug (continuous lines) were performed. The summations were performed according to two different reference points (arrows): either according to the downward deflection of the NS (i.e. each EPC was considered with its own latency before and during the application of the drugs; *A*, *B*, *C*, *D*), or the same uniquantal EPCs were summated and normalized according to their amplitude peaks (*E*, *F*, *G*, *H*). In these latter reconstructions, controls and drug-treated EPCs are either indistinguishable or very close.

significant difference. Minimal synaptic latencies were slightly shortened during the second recording period after the washout of Rp-cAMP (0.31 ± 0.02 ms) as compared with the first stimulation period in the presence of Rp-cAMP (0.38 ± 0.05 ms). Without Rp-cAMP, the latencies were 0.33 ± 0.04 ms and 0.34 ± 0.03 ms, respectively, in controls without the drug. The modal values of latency histograms were not influenced by stimulation and were similar to controls during both recording periods (not shown).

Construction of multiquantal EPCs

Our data have shown that an increase in cAMP makes the evoked release of single quanta more synchronous by eliminating long-latency events and that PKA inhibition leads to a more dispersed release. To assess the impact of these changes on the EPC peak amplitude, multiquantal EPCs were constructed by summation of the experimentally obtained uniquantal EPCs and their real latencies, before and after treatment with db-cAMP or Rp-cAMP (Fig. 5). In the proximal part, the amplitudes of the multiquantal EPCs constructed from uniquantal EPCs in the presence of db-cAMP, which decreased P_{90} by 29 %, were larger than those of the controls by as much as 27 % (Fig. 5*A*). The rise time to 20–80 % of the maximal amplitude decreased from 0.4 ms to 0.3 ms and the τ of the exponential decay was also slightly shortened from 2.1 ms to 2.0 ms. This is in accord with previous model studies (Soućek, 1971; Giniatullin *et al.* 1995, Zefirov & Gafurov, 1997) predicting that changes in the latency dispersion of standard-size single quanta affect mostly the amplitude and rise time and, to a lesser extent, the decay time of reconstructed EPCs, provided that standard quanta with $\tau = 1.5$ are summated. On the other hand, the summed EPCs in the distal parts were not affected by db-cAMP, as the drug had no effect on the dispersion pattern (Fig. 5*B*).

The opposite was found for the PKA inhibitor Rp-cAMP. EPCs from the proximal parts were the same in controls and after treatment with Rp-cAMP (Fig. 5*C*), apparently because there was no change in release pattern in these areas. Reconstruction based on recordings in the distal parts of the terminal was affected significantly by Rp-cAMP (Fig. 5*D*). The amplitude of the EPC reconstructed from uniquantal EPCs recorded in the presence of the PKA inhibitor Rp-cAMP (which increased P_{90} by 24 %) was lower by 28 % than in the controls, evidently due to the participation of events with longer latencies. The rise time increased from 0.35 ms to 0.50 ms, and the τ of the exponential decay was slightly increased from 1.8 ms to 2.0 ms. Parts *E*, *F*, *G*, and *H* in Fig. 5 show the amplitudes and time courses of the EPCs reconstructed from the same experimental uniquantal EPCs, normalized according to their amplitude maxima. In these reconstructions, multiquantal EPCs were virtually identical before and during the application of both drugs; this also indicates that no changes occurred in the quantum size and in postsynaptic sensitivity in the presence of either drug, and once again demonstrates the stability of the recordings.

DISCUSSION

We have demonstrated that long-latency EPCs are removed to a great extent when intracellular cAMP is increased following β1 activation by NA, by the permeable analogue db-cAMP, by forskolin activation of adenylyl cyclase or by inhibition of cAMP hydrolysis. On the other hand, the number of long-latency EPCs increased when PKA was inhibited. NA was ineffective in the presence of the PKA inhibitor Rp-cAMP.

The effects of PKA activation and inhibition depend upon the initial level of the late release along the nerve terminal. The late release is characterized by the presence of long-latency EPCs, and can be revealed by the P_{90} parameter, provided that the modal value of the early release latency histograms is not substantially changed. In the proximal part of the synapse, many EPCs are released with latencies longer than 2 ms, and they are eliminated to a great extent when the cAMP level is increased. In contrast, the inhibition of PKA had no effect. In the distal parts, EPCs were released mostly during the early release period of about 1 ms, and upon cAMP application, the P_{90} did not significantly change. The inhibition of PKA leads to the appearance of the longest latencies, which can be found in the frog muscle endplate at room temperature. In the distal parts, the EPCs have shorter minimal latencies and a more compact release than in the proximal parts. Saturating concentrations of cAMP may occur in the distal parts of the terminal, probably due to smaller axonal volumes (Robitaille & Tremblay, 1987, Pawson *et al.* 1998*a*,*b*). Other morphological differences between the proximal and distal sites do not seem to be a necessary prerequisite for the release pattern. This follows from the appearance of long-latency EPCs after PKA inhibition in the distal parts, which would not be expected if only differences in micro-morphological properties (e.g. the amount, size and shape of the active zones of release) were responsible. Even though this difference was not found in a recent freeze-fracture analysis (Pawson *et al.* 1998*b*), it may exist as indicated by the proximo-distal gradient of the minimal latencies, which are 0.5 ms in the proximal parts and only 0.3 ms in the distal parts; minimal latencies are not influenced by any changes in cAMP levels. The value of 0.3 ms is close to previous estimates (Almers, 1990), indicating that the opening of voltage-dependent Ca^{2+} channels, the entry of Ca^{2+}, the activation of the exocytotic machinery, and the release and diffusion of the neurotransmitter to the postsynaptic membrane can occur within 0.2 ms. Synaptic vesicles that undergo exocytosis must therefore be docked very close to or in physical contact with Ca^{2+} channels (Stanley, 1997; Wiser *et al.*

Table 1. A comparison of the amplitude and time parameters of short- and long-latency uniquantal endplate currents (EPCs)

	A_{EPC} (mV)	RT (μs)	τ_{dec} (ms)	A (mV ms)
Short-latency EPCs	0.26 ± 0.01	165 ± 70	1.30 ± 0.18	0.91 ± 0.11
Long-latency EPCs	0.27 ± 0.01	163 ± 80	1.00 ± 0.22	1.00 ± 0.18

150 EPCs were recorded in the proximal part of 3 synapses (50 in each). The EPCs released with latencies in the interval between 0.5 ms (minimal synaptic latencies) and 0.7 ms were considered as short-latency EPCs. Long-latency EPCs were those that exhibited latencies of 1.2 ms and longer. A_{EPCs}, EPC amplitude in mV; RT, rise time of EPC between 20 and 80 % of maximum; τ_{dec}, decay time constant; A, mean area of the EPC.

1999). This distance might, however, be larger in the proximal part than in the distal part, and this might be reflected in the longer minimal latencies.

What really determines the synaptic latencies is unknown. Minimal latencies are probably the most constant parts of the release time course and represent the quanta (probably vesicles) that are optimally located with respect to Ca^{2+} entry sites and that have their soluble *N*-ethyl-maleimide-sensitive factor (NSF)-attachment protein receptors (SNARE proteins), such as the calcium-binding protein synaptotagmin I (Fernández-Chacon *et al.* 2001), optimally assembled for release. After quanta with minimal latencies, a main pool of quanta is released and characterized by the modal value of latency histograms. This early release (Barrett & Stevens, 1972) is followed by a late release with long latencies. Are these quanta released with latencies as long as 5 ms, or even longer, and therefore forced to move to the release site from more remote docking sites? Are they not docked properly, or do they belong to another population of vesicles? A comparison of the amplitudes and time parameters (Table 1) of fast-released EPCs with latencies between 0.5 and 0.7 ms with the rest of the EPCs showed no difference in quantum size or rise and decay times. This means that they most probably belong to the same release pool. However, other parameters of docking and release are difficult to measure in real time, at least at vertebrate endplates. The molecular organization of Ca^{2+} channels and synaptic proteins are generally studied using immunobinding studies, channel expression and protein-to-protein interactions revealed by separation techniques (Wiser *et al.* 1999), but no direct method exists to reproduce experimentally the fusion process that occurs in small synapses in real time. Even such important findings as those concerning SNARE pins as essential machinery for vesicle fusion (Weber *et al.* 1998) have been obtained on a time scale of minutes.

In the present study we demonstrate the regulatory cascade NA–β1 receptors–cAMP–PKA. Is this cascade operating only after a nerve stimulus, or is it acting permanently by aligning vesicles in an optimal release position? Regulation of the time lag between stimulus and vesicle fusion would imply phosphorylation and dephosphorylation of v- and t-SNARE proteins. Many other proteins proposed for fusion regulation may also undergo phosphorylation (Pfeffer, 1996; Fergestad *et al.* 1999). However, the speed of exocytosis in fast synapses such as the motor endplate would not allow enough time for a series of sequential protein interactions and PKA-dependent hydrolysis of ATP during nerve stimulation, but would provide time only for a rapid conformational change in fusion proteins during quantum release. The present experiments in which the early releasable pool of vesicles was exhausted during tetanic stimulation were aimed at distinguishing where phosphorylation *via* PKA takes place. The number of long-latency EPCs increased after tetanic stimulation, when new vesicles are apparently primed and docked, in the presence of a PKA inhibitor. However, this effect was readily reversible, and we can therefore speculate that, similarly to the already described phosphorylation of α-soluble NSF attachment protein (α-SNAP; Hirling & Scheller, 1996), PKA can phosphorylate release proteins when vesicles are already docked at the release zone. Some observations indicate that ATP-dependent priming of secretion occurs before calcium-triggered exocytosis in chromaffin and PC12 cells (Hay & Martin, 1992; Holz *et al.* 1992). As shown, the inhibition of PKA, even without any tetanic stimulation, increases the number of long-latency EPCs. This means that previously phosporylated release protein(s) can be slowly dephosphorylated by phosphatases in the absence of functional PKA, and this disqualifies these quanta for short-latency release. Yet another remote possibility is that without PKA-mediated phosphorylation, the turnover of otherwise fixed vesicles at docking sites increases and 'fast' vesicles are then replaced by 'slow' ones. This can be tested in frog terminals (e.g. using dye-loaded vesicles; Betz *et al.* 1993). Thus, exocytotic fusion may occur and be differentially regulated by phosphorylation at several stages in the process of transmitter release (Fesce, 1999).

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