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Effect of changes in action potential shape on calcium currents and transmitter release in a calyx-type synapse of the rat auditory brainstem

J. G. G. Borst and B. Sakmann

Max-Planck-Institut für Medizinische Forschung, Abt. Zellphysiologie, Jahnstr. 29, D-69120 Heidelberg, Germany (*borst@sunny.mpimf-heidelberg.mpg.de*)

We studied the relation between the size of presynaptic calcium influx and transmitter release by making simultaneous voltage clamp recordings from presynaptic terminals, the calyces of Held and postsynaptic cells, the principal cells of the medial nucleus of the trapezoid body, in slices of the rat brainstem. Calyces were voltage clamped with different action potential waveforms. The amplitude of the excitatory postsynaptic currents depended supralinearly on the size of the calcium influx, in the absence of changes in the time-course of the calcium influx. This result is in agreement with the view that at this synapse most vesicles are released by the combined action of multiple calcium channels.

Keywords: calcium current; presynaptic terminal; voltage clamp; calcium domain; action potential; calyx of Held

1. INTRODUCTION

(a) *General considerations*

The intracellular Ca^{2+} concentration decreases steeply with distance from an open calcium channel (reviewed in Neher 1998). If the open calcium channel is modelled as a point source in a planar boundary, in the absence of mobile calcium buffers the steady-state Ca^{2+} concentration is inversely proportional to distance. In the presence of mobile calcium buffers (Neher 1986) or if the channel openings are brief (Pape *et al.* 1995), as is the case during action potentials, the concentration gradients are even steeper. The highly localized increase in intracellular Ca^{2+} concentration in the immediate vicinity of an open calcium channel is called a 'calcium domain' (Chad & Eckert 1984; figure 1*a*). A calcium domain rapidly dissipates on closure of the calcium channel.

Transmitter release depends strongly on the extracellular Ca^{2+} concentration, which indicates that calcium ions cooperate to trigger transmitter release (Dodge & Rahamimoff 1967; Katz 1969). High intracellular concentrations of Ca^{2+} are needed to activate the calcium sensor that triggers phasic release. Therefore, release sites and calcium channels need to be colocalized (reviewed in Bennett & Brain 1997; Neher 1998; Schweizer *et al.* 1995; Zucker 1996). The present paper deals with the question whether a single calcium channel controls the release of a vesicle (figure 1*a*), or whether calcium domains normally overlap at release sites and most vesicles are released by the action of Ca^{2+} flowing in through several calcium channels (figure 1*b*).

The extent to which calcium domains will overlap depends on the distance between open calcium channels, on how far a calcium ion can diffuse before it is captured by a calcium buffer and on the duration of the calcium channel openings (Neher 1998). More overlap is expected if the distance between calcium channels is small, if Ca^{2+} can diffuse far and if the channels open for a long time. Whether the low-affinity calcium sensor will be activated by calcium originating from a single or multiple calcium channels depends on the extent of calcium domain overlap and on the distance to the calcium sensor. The further away the calcium sensor is, the more important it becomes that multiple calcium channels act together to raise the Ca^{2+} concentration sufficiently high to release a vesicle.

The question whether domains do or do not overlap cannot be answered by a simple 'yes' or 'no'. Even if release is gated by single calcium domains, there will still be a strong dependence on distance. Conversely, if a vesicle is located in the immediate vicinity of an open channel it is likely to be released, even if vesicles are generally sensing overlapping domains. The question that we will focus on is what happens during action potentials to most synaptic vesicles at release sites.

(b) *Overview of techniques used to study domain overlap*

The different approaches have been divided into three different groups.

(i) *Anatomical*

The localization of individual calcium channels gives an indication on the extent to which domains of adjacent calcium channels can overlap. Several types of studies have indicated that the density of calcium channels at

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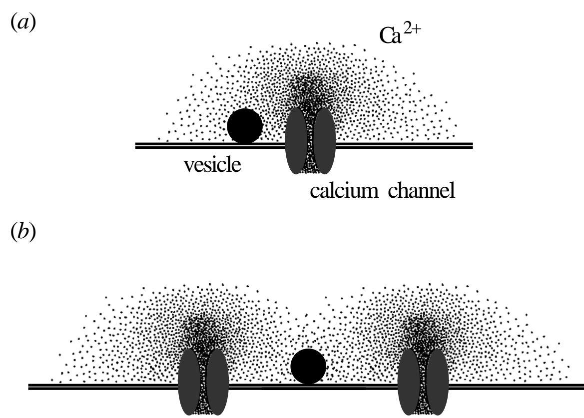

Figure 1. Schematic view of the single calcium domain and the overlapping calcium domain schemes. (*a*) The localized increase in the Ca^{2+} concentration in the vicinity of an open calcium channel is called a calcium domain. If the vesicle is very close to an open calcium channel the concentration is likely to be high enough to trigger release. (*b*) If the vesicle is further away from the calcium channels, multiple calcium channels have to cooperate to trigger release.

active zones can be quite high. Following freeze-fracture of synapses, large intramembrane particles concentrated at active zones in the presynaptic membrane have been suggested to represent the calcium channels (Heuser *et al*. 1974; Pumplin *et al*. 1981), although definitive proof is still lacking. At many active zones the distance between these particles is quite small, only tens of nanometres. Calcium channels have been labelled with specific toxins to which either fluorescent markers or gold particles were attached (Haydon *et al*. 1994; Robitaille *et al*. 1990). Their localization was studied with the light microscope or the atomic force microscope. Both in the frog neuromuscular junction and in the chick ciliary ganglion, the calcium channels were concentrated at the active zones (Haydon *et al*. 1994; Robitaille *et al*. 1990). The results from cell-attached recordings of calcium channels at the release face have also suggested that the density of calcium channels at active zones can be quite high (Stanley 1991). Because of the poor accessibility of the release face of terminals, this has not been generally studied.

Interestingly, calcium channels bind to proteins that are involved in the last steps before a vesicle fuses, such as syntaxin (Bennett *et al*. 1992; Yoshida *et al*. 1992). This again suggests a tight colocalization of calcium channels and vesicles.

(ii) *Changing the calcium influx*

A change of the extracellular Ca^{2+} concentration will affect the calcium influx through all open calcium channels. This does not necessarily change the extent of the domain overlap between calcium channels greatly. The dependence of the transmitter release on the third or fourth power of the calcium influx, that was often observed following a change in the extracellular Ca^{2+} concentration, more likely reflects the cooperative action of Ca^{2+} on the calcium sensor (reviewed in Wu & Saggau 1997). However, if the calcium influx through a subset of the calcium channels is changed, the likelihood that calcium domains do or do not overlap can be altered (Augustine *et al*. 1991; Mintz *et al*. 1995; Yoshikami *et al*. 1989). The calcium influx has been changed by changing the shape of the action potential or by blocking calcium channels with toxins. By examining the effect that this change has on release, an indication can be obtained about the degree of domain overlap that is present under physiological conditions.

Broadening the action potential with potassium channel blockers will increase the calcium influx. Depending on the gating properties of calcium channels, this may lead to an increase in the number of functional calcium domains and/or the duration of the calcium influx per calcium channel (Augustine 1990; Zucker *et al*. 1991). In contrast to rat cerebellar synapses (Sabatini & Regehr 1997), in the squid giant synapse a linear relationship between the increases in calcium influx and in transmitter release has been inferred from a simulation of the calcium currents (Augustine 1990). This result suggests that calcium domains at the squid giant synapse do not overlap during action potentials (Augustine *et al*. 1991).

Calcium influx has also been changed using calcium channel type-specific toxins. Two different approaches have been taken. The frog neuromuscular junction contains N-type calcium channels, which are sensitive to the blocker ω-conotoxin MVIIA. The shape of the dose–response curve of this toxin was qualitatively different from the dose–response curve measured with the inorganic calcium channel blocker Cd^{2+} (Yoshikami *et al*. 1989). Relatively high concentrations of the toxin were necessary to completely block transmission. Apparently, even if only a few calcium channels opened, they could still trigger release. The conclusion that calcium channels do not act cooperatively to trigger transmitter release at the frog neuromuscular junction was supported by model calculations (Yoshikami *et al*. 1989). An alternative interpretation, namely that a fraction of the N-type calcium channels at this terminal are less sensitive to ω-conotoxin MVIIA (Olivera *et al*. 1987) cannot be ruled out, since the presynaptic calcium channels at the frog neuromuscular junction were subsequently shown to be heterogeneous in their sensitivity to another toxin, the funnel web spider toxin (Katz *et al*. 1995).

Most nerve terminals in the central nervous system express a mixture of calcium channel types. The effect on release of blocking calcium channel types can be tested. In these experiments it is important to rule out non-specific effects of the calcium channel toxins (e.g. Katz *et al*. 1995; Luebke *et al*. 1993; Zengel *et al*. 1993), to make sure that the toxins target only one type of calcium channels (Mintz *et al*. 1995; Wu & Saggau 1994) and that release and calcium influx are measured from the same (population of) terminals. A general finding is that if the effect of blocking different types of calcium channels on synaptic transmission is tested in separate experiments, the sum of the blocking effect on transmitter release exceeds 100% (e.g. Luebke *et al*. 1993; Mintz *et al*. 1995; Takahashi & Momiyama 1993; Wu & Saggau 1994). As first pointed out by Mintz *et al*. (1995), this indicates that some of the vesicles sense Ca^{2+} entering via different types of calcium

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channels, suggesting that domains overlap at these release sites.

In conclusion, manipulations of the calcium influx have provided evidence for a tight coupling between release and calcium influx. In some preparations evidence has been obtained for domain overlap, whereas in other preparations the coupling may be so tight that most vesicles are released by the influx of calcium via a single calcium channel.

(iii) *Competition with exogenous intracellular calcium buffers*

When Ca^{2+} enters the terminal via an open calcium channel, most of it is rapidly captured by intracellular buffers (Neher 1995). The speed of capturing depends on the concentration of the buffer and the binding on- and off-rates. Assuming that the buffer concentration remains constant, the characteristic binding time constant (τ) is

$$\tau = 1/(k_{on}[\text{buffer}_{total}] + k_{off}),$$

where k_{on} and k_{off} are the forward and reverse rate constants of the binding reaction of Ca^{2+} to the buffer. The effect of calcium buffers with different on- and off-rates for Ca^{2+} on transmitter release has been compared by injecting them into the giant presynaptic terminal of the squid (Adler *et al.* 1991). The results suggested that the time window that is available for the exogenous calcium buffers to intercept calcium ions before they bind to the calcium sensor to trigger release is only a few microseconds. Since Ca^{2+} does not diffuse very far in a few microseconds, the calcium sensor has to be located less than a few hundred nanometres from the open calcium channels in the squid giant synapse (Adler *et al.* 1991). The slow buffer EGTA was remarkably ineffective. Even at concentrations as high as 80 mM release was not affected (Adler *et al.* 1991). The low effectiveness of EGTA suggests that release in the squid giant synapse is controlled by single calcium channels (Schweizer *et al.* 1995).

For these types of experiments it is important that the concentration of the buffer and the kinetics of Ca^{2+} binding inside the cell are known. However, even if the absolute concentration is not known, an estimate of the relative distances between calcium channels and, for example, the calcium sensor or the calcium-dependent potassium channels can be made (Robitaille *et al.* 1993).

(c) *Domain overlap at the calyx of Held*

In this paper we present experimental evidence that at release sites of a calyx-type terminal in the rat brainstem, calcium domains overlap during action potentials. The synapse we have used to study this question is a large axosomatic synapse in the medial nucleus of the trapezoid body (MNTB). Each principal cell in the MNTB is innervated by a large terminal, called the calyx of Held. The calyx can elicit suprathreshold glutamatergic excitatory postsynaptic potentials in the postsynaptic cells (Banks & Smith 1992; Forsythe & Barnes-Davies 1993). The MNTB is part of an auditory pathway that is involved in the localization of sound (Oertel 1997). It is possible to voltage clamp both the pre- and the postsynaptic part of this synapse in whole-cell recordings (Borst *et al.* 1995; Borst & Sakmann 1998). In voltage clamp it becomes possible to manipulate the time-course and size of the presynaptic calcium current and thus to study the relationship between calcium influx and release in a more controlled way. We compared the effect on release of different action potential waveforms that evoked calcium currents with similar time-courses but different amplitudes. The aim was to vary the number of calcium channels that opened during an action potential, while keeping the duration or amplitude of the calcium influx per open calcium channel constant. With this technique, we also tested the effect on release of a change in the shape of the action potential that is observed during high-frequency trains (Borst *et al.* 1995).

2. EXPERIMENTAL METHODS

(a) *Simultaneous pre- and postsynaptic voltage clamp recording*

Eight-to-ten-day-old Wistar rats were decapitated and 200 μm parasagittal slices were cut with a vibratome in ice-cold saline, containing (in mM): 125 NaCl, 2.5 KCl, 4 $MgCl_2$, 0.1 $CaCl_2$, 25 dextrose, 1.25 NaH_2PO_4, 0.4 ascorbic acid, 3 *myo*-inositol, 2 sodium pyruvate, 25 $NaHCO_3$. Slices were equilibrated for 30 min at 37 °C in the same solution, except that the Ca^{2+} and Mg^{2+} concentrations were 2 and 1 mM, respectively. Afterwards, slices were transferred to a recording chamber, and during recordings of the calcium current, they were perfused with a solution containing (in mM): 105 NaCl, 20 TEA-Cl (Fluka, Buchs, Switzerland), 0.1 3,4-diaminopyridine (Sigma), 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 25 dextrose, 1.25 NaH_2PO_4, 0.4 ascorbic acid, 3 *myo*-inositol, 2 Na-pyruvate, 0.001 tetrodotoxin (TTX, Research Biochemicals International, Natick, MA), 0.05 D-2-amino-5-phosphonovalerate (D-APV, Tocris Neuramin, Bristol, England), 25 $NaHCO_3$, pH 7.4 when bubbled with 95% O_2, 5% CO_2. Bath temperature was 22–24 °C.

Presynaptic electrodes (4–6 MΩ) were filled with a solution containing (in mM): 125 Cs-gluconate, 20 CsCl, 10 Na_2-phosphocreatine, 4 MgATP, 0.3 GTP, 0.05 fura-2 (Molecular Probes, Portland, OR, USA), 10 HEPES (pH 7.2 with CsOH). Postsynaptic pipettes (2.5–3.5 MΩ) were filled with (in mM): 125 K-gluconate, 20 KCl, 10 Na_2-phosphocreatine, 4 MgATP, 0.3 GTP, 0.5 EGTA, 10 HEPES (pH 7.2 with KOH).

Simultaneous pre- and postsynaptic whole-cell recordings were made with two Axopatch 200 A amplifiers. Presynaptic and postsynaptic series resistance (typically 15 MΩ and 8 MΩ, respectively) were compensated to 90% (lag < 8 μs, prediction when applicable 60%). Holding potential was -80 mV. Potentials were corrected for a liquid junction potential of -11 mV. Subtraction of passive responses was with the P/5 or the P/-5 method. Currents were filtered at 5 kHz and digitized at 50 kHz with a 16-bit analogue-to-digital converter (ITC-16, Instrutech, Greatneck, NY). Interpulse interval was 15–60 s. Voltage clamping terminals with action potential waveforms was done as described previously (Borst & Sakmann 1996).

Previously, we observed that during 100 Hz trains of action potentials, the amplitude of the action potentials and their halfwidth decrease (Borst *et al.* 1995). In the series of ten experiments performed at 23–24 °C that were reported in Borst *et al.* (1995), the amplitude decreased from 117 ± 2 mV for the first action potential of a 0.5 s, 100 Hz train to 95 ± 2 mV for the last action potential in the train. The maximal rate of rise decreased from 584 ± 21 V s^{-1} to 318 ± 23 V s^{-1}. The halfwidth increased from 0.54 ± 0.02 ms to 0.74 ± 0.03 ms. To study the influence of the change in the shape, the first and last action

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potential of one of these trains were taken as the command potential in the voltage clamp experiment. The amplitudes of the first and last action potential of this particular train were 116 and 88 mV, maximal rate of rise 654 and 306 V s^{-1} and half-width were 0.49 and 0.71 ms, respectively.

(b) *Fluorescence measurements*

The presynaptic volume-averaged Ca^{2+} concentration was measured by forming ratios between continuous recordings of fluorescence at a calcium-insensitive (357 nm), followed by a calcium-sensitive wavelength (380 nm), after background subtraction (Helmchen *et al.* 1997; Wu *et al.* 1998).

Fura-2 was excited using a polychromatic illumination system (T.I.L.L. Photonics, Munich, Germany). Excitation light was coupled into an upright microscope (Axioskop, Zeiss) via a light guide. The microscope was equipped with a 40 × water-immersion objective (NA 0.75, Zeiss), a dichroic mirror (500 nm) and a long pass (520 nm) emission filter. Two photodiodes (Hamamatsu, area: 1.1 × 1.1 mm), situated in the image plane were used for signal and background subtraction (Borst & Helmchen 1998). Photodiode signals were filtered at 20 Hz (8-pole Bessel filter). Excitation light was generally attenuated by 60%, reducing bleaching of fura-2 (or the background fluorescence) to less than 1% per second. When necessary, fluorescence signals were corrected for bleaching by linear interpolation.

At a concentration of 50 μM, fura-2 will already outcompete the endogenous calcium buffers in the calyx of Held and for small changes in volume-averaged Ca^{2+} concentration, such as those occurring in figure 3, fluorescence changes are proportional to calcium influx (Helmchen *et al.* 1997; Wu *et al.* 1998).

Minimal and maximal fluorescence ratios (R_{min} and R_{max}) were measured in terminals using intracellular solutions containing 20 mM EGTA and 13.3 mM CaEGTA plus 6.7 mM EGTA (Helmchen *et al.* 1997). R_{min} was 0.56 ± 0.04 ($n = 4$) and R_{max} was 5.9 ± 0.5 ($n = 3$). K_d for calcium of fura-2 was assumed to be 273 nM (Helmchen *et al.* 1997).

(c) *Acquisition and analysis*

Acquisition and analysis were done with Pulse Control 4.7 (Herrington & Bookman 1994), combined with Igor (WaveMetrics, Lake Oswego, OR) macros.

The calcium current integrals in the experiments illustrated in figure 3 were quantified as the difference between the maximum and minimum of the calcium current integral in the time window from 0.7 ms before to 0.7 ms after the peak amplitude of the calcium current.

Data are presented as average ± s.e.m.

3. RESULTS

(a) *Simultaneous pre- and postsynaptic voltage clamp*

The calyx of Held and its postsynaptic partner, the principal cells of the MNTB (figure 2*a*), were simultaneously voltage clamped with patch pipettes in the whole-cell recording configuration and calcium currents were isolated pharmacologically. To study the relationship between the shape of the action potential and transmitter release using voltage clamp techniques, good voltage control is important (reviewed in Borst & Helmchen 1998). We previously observed that the quality of the spatial clamp in the presynaptic terminal depends mostly on the length of the preterminal axon. If the axon is short, spatial clamp can be quite good (Borst & Sakmann 1998). In the present series of experiments, preterminal axon lengths were on average less than 100 μm and passive responses were generally well described by a single exponential function (figure 2*b*). In contrast, two exponential functions were needed for the postsynaptic passive response (figure 2*c*). The total postsynaptic capacitance was clearly larger than for the presynaptic terminal (44.7 ± 4.8 pF versus 22.5 ± 2.5 pF; $n = 5$).

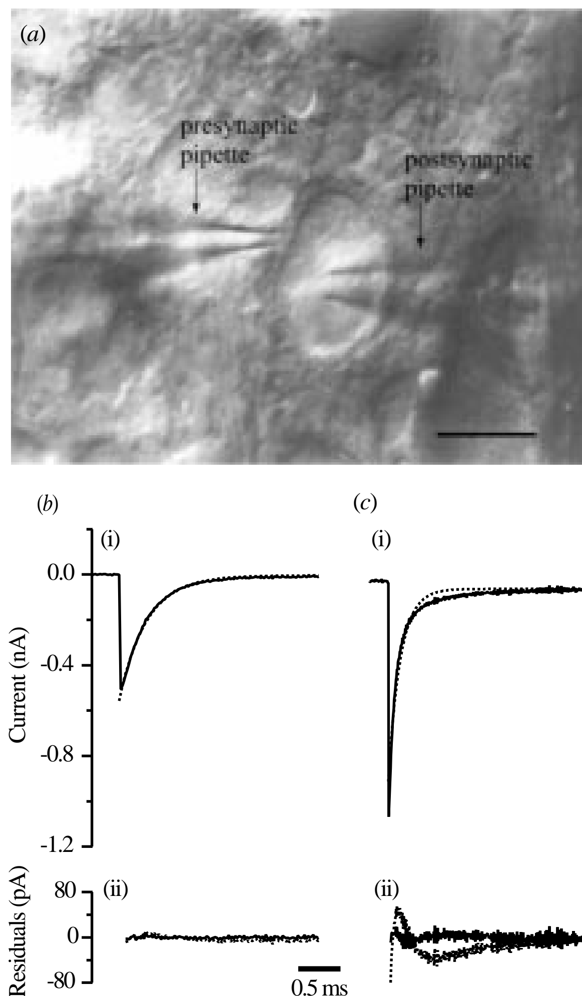

Figure 2. Passive properties of calyx of Held and a principal cell in the MNTB in a brain slice during simultaneous voltage clamp recordings. (*a*) Infrared differential interference contrast video image of the MNTB, showing simultaneous pre- and postsynaptic recording with two patch pipettes. Scale bar 10 μm. (*b*) Passive properties of nerve terminal. (i) Current needed to hyperpolarize the calyx by 10 mV from the holding potential of −80 mV. Dotted line is fit with a single exponential function, with a time-constant of 325 μs. Calculated total capacitance was 20.8 pF, membrane resistance 3.6 GΩ. Average of 25 traces, filtered at 50 kHz. (ii) difference between the measured currents and the fit with a single exponential function (dotted trace) or the sum of two exponential functions. (*c*) Passive properties of postsynaptic cell. (i) Dotted line is fit with a single exponential function, with a time-constant of 440 μs. Calculated total capacitance was 52.8 pF, membrane resistance 310 MΩ. (ii) Residuals for the fit with a single (dotted trace) or the sum of two exponential functions (solid line).

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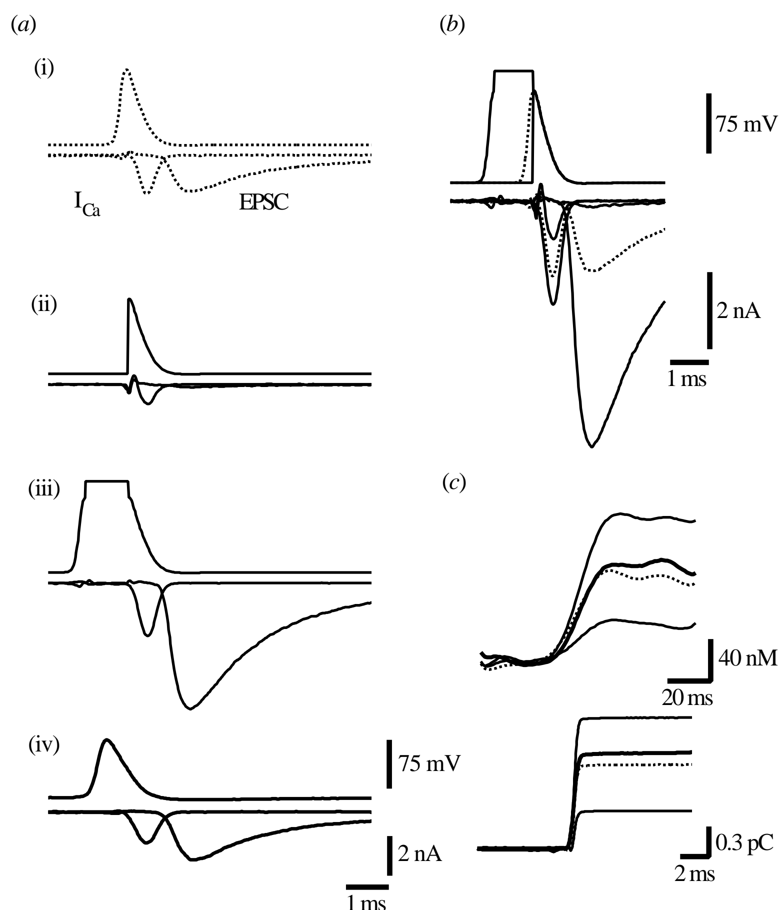

Figure 3. Effect of the shape of an action potential on EPSC amplitude. (*a*) Effect of four different command potentials (V_{pre}) on calcium current (I_{Ca}) and postsynaptic current (EPSC). Traces are from the same experiment as displayed in figure 2*b*,*c*. Action potential waveforms are (from top to bottom): (i) control AP (dotted traces), (ii) control AP with a stepwise depolarization phase, (iii) control AP with a plateau phase and (iv) broad AP (bold traces). (*b*) Traces displayed in (*a*) overlaid, except for the broad AP. Dotted traces are again the control AP. (*c*) Top: changes in volume-averaged Ca^{2+} concentration measured by fura-2, evoked by the calcium currents shown in (*a*). Bottom: calcium current integrals of the traces shown in (*a*).

(b) *Relation between shape of the action potential and transmitter release*

We tested the effect of four different action potential-like waveform shapes on the size of the excitatory postsynaptic currents (EPSCs) in simultaneous pre- and postsynaptic voltage clamp recordings (figure 3*a*). One of the four waveforms was the first action potential ('control AP') of a 0.5 s, 100 Hz train recorded previously in current clamp, another one the last action potential ('broad AP') of that train. In addition, terminals were also voltage clamped with the control AP but with a fast depolarization time-course ('step AP') and with the same action potential but with an added plateau of 1 ms to 60 mV ('plateau AP'). The effect of adding a plateau phase is that calcium channels will have sufficient time to open (Borst & Sakmann 1998), whereas if the depolarization is a step, followed by a normal repolarization, they will have less time to open during the action potential waveform. Since the repolarization time-course of these action potentials was not changed, the shape of the calcium current was similar in all three waveforms that were based on the control AP, but the amplitude of the calcium current was very different (figure 3*b*). The calcium current evoked by the broad AP was somewhat smaller and broader than the current induced by the control AP. For both the control AP and the broad AP, the peak calcium current was reached at a membrane potential of around −30 mV, measured with a separate electrode in two-electrode voltage clamp recordings ($n=10$, not shown). Calcium influx was quantified in three different ways, as the peak amplitude of the presynaptic calcium current, as the integral of the calcium current or as the change in the volume-averaged Ca^{2+} concentration measured with fura-2 (figure 3*c*). Release was quantified by measuring the peak amplitude of the EPSC. EPSCs began around the time when the calcium current ended, in agreement with earlier results from separate pre- and postsynaptic recordings (Borst & Sakmann 1996).

The size of the postsynaptic currents depended strongly on the size of the calcium influx. Excluding the broad AP, the slope of the regression line of calcium against EPSC amplitude plotted on a double logarithmic scale was around 3.5 ($n=5$), 3 ($n=5$) and 3.1 ($n=2$) for amplitudes (figure 4*a*), integrals (figure 4*b*) or fluorescence (not shown), respectively. If the results obtained with the broad AP were included, EPSC amplitudes were predicted better by the increases in volume-averaged Ca^{2+} concentration or the integrated calcium current than by the peak amplitude of the calcium current. In the latter case, data obtained with the broad AP were clear outliers (figure 4*a*). If the broad AP was excluded, average Pearson correlation coefficients for the linear regression of the relationship between calcium and EPSC plotted on a double logarithmic scale were 0.979 ± 0.008, 0.986 ± 0.008 and 0.987 ± 0.011 for amplitude, integral

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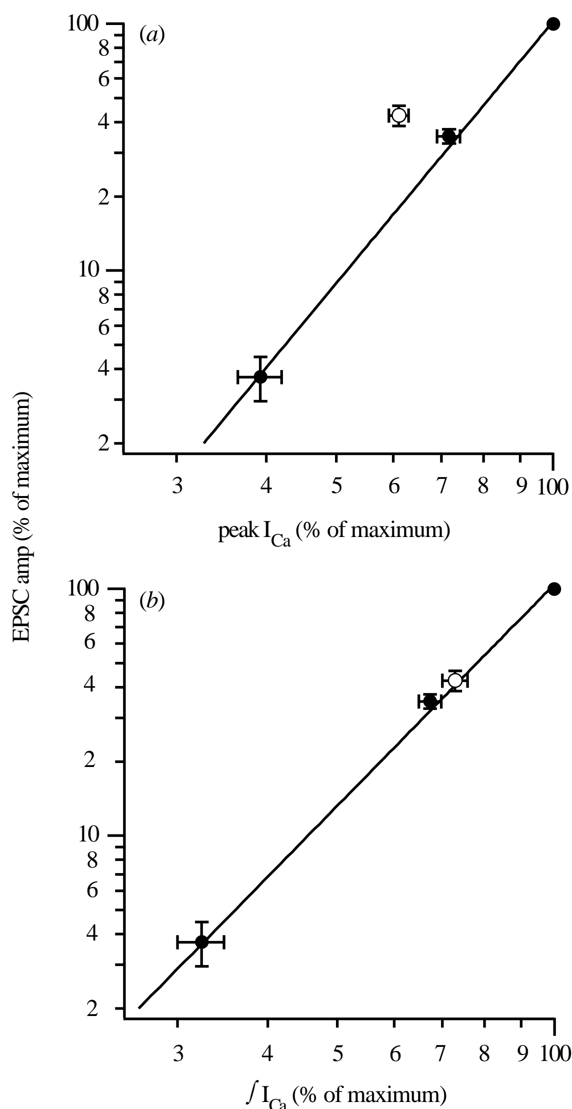

Figure 4. Relationship between calcium influx and transmitter release. (*a*) Relationship between EPSC amplitude and the peak amplitude of the calcium current that was evoked by the different action potential waveforms shown in figure 3*a* ($n = 5$). Before averaging, currents were normalized to the calcium current or to the EPSC that was evoked with the plateau action potential waveform, which were on average -2.9 ± 0.2 and -6.9 ± 1.8 nA ($n = 5$), respectively. Open symbols represent the responses obtained with the broad AP. Solid line has a slope of 3.5. (*b*) Relationship between EPSC amplitude and integral of the calcium current that was evoked by the four different action potential waveforms ($n = 5$). Responses obtained with the plateau action potential, which was on average 1.5 ± 0.2 pC for the calcium current integral, were defined as 100%. Slope of the solid line is 3.

and fluorescence, respectively. If the broad AP was included the values for r were 0.935 ± 0.009, 0.986 ± 0.008 and 0.985 ± 0.009, respectively.

4. DISCUSSION

(a) *Effect of changing the AP shape on release in the MNTB*

An advantage of voltage clamping both the pre- and the postsynaptic membranes of a synapse is that it becomes possible to quantitatively study the effect of changes in the size and the time-course of the presynaptic calcium influx on release. In the MNTB, increasing or decreasing the calcium influx evoked by different action potential waveforms, without changing its time-course significantly, resulted in a supralinear change in the EPSC amplitude. The driving force for calcium entry was similar for each of the three action potential waveforms, in contrast to what happens when the calcium influx is modified by changing $[Ca^{2+}]_o$. This means that, most likely, the difference between the size of the calcium current evoked by the three different waveforms was due to a difference in the number of calcium channels that were opened. The time-course or amplitude of the calcium influx per individual calcium channel was probably the same in all three cases, because the calcium channels opened during the repolarization phase, which was the same for all three waveforms. This indicates that the EPSC amplitude depended supralinearly on the number of calcium domains. A linear dependence would be expected if the calcium domains did not overlap (Augustine *et al.* 1991), therefore our results support the view that calcium domains overlap at release sites during action potentials in the calyx of Held.

The size of the EPSCs depended on approximately the third power of the calcium influx. This is similar to what we recently observed during simultaneous pre- and postsynaptic recordings in which the presynaptic calcium influx during action potentials was quantified using fura-2 (2.7 ± 0.2; Wu *et al.* 1998). A previous estimate of a fourth power dependence, that was obtained from separate pre- and postsynaptic voltage clamp measurements (Borst & Sakmann 1996), was probably too high, because of a larger rundown in the postsynaptic recordings than in the presynaptic calcium current measurements. A second-power dependence has been reported by Barnes-Davies & Forsythe (1995).

During a 100 Hz train of presynaptic action potentials, the action potentials become smaller and broader and at the same time the size of the EPSCs decreases to around 10% (Borst *et al.* 1995). This change in the presynaptic action potential leads to a small decrease in the peak amplitude of the calcium current, but an increase in the current integral. This action potential evoked a somewhat larger release. The change in the shape of the action potential does not add to the ongoing depression during high-frequency signalling, because, by itself, the change would increase rather than decrease release. A systematic investigation of the relationship between halfwidth of the calcium current and release was not made, because if the action potential is broadened further, the calcium current will overlap with the EPSC, making the quantification more difficult.

The comparison of the relationship between calcium influx and transmitter release for the different waveforms suggests that when the inflow of Ca^{2+} is brief, as is the case during action potentials, the total number of calcium ions that enter determines the number of vesicles that will be released. Interestingly, a similar observation was made during longer (3–6 ms) step depolarizations in the squid giant synapse, where release was better predicted by the integral of the calcium current than by the isochronal calcium current amplitude (Augustine *et al.* 1985). In

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contrast, for jellyfish or *Xenopus* neuromuscular junctions, release appeared to depend more on the calcium current amplitude than on its integral (Pattillo *et al*. 1998; Spencer *et al*. 1989).

(b) *Other evidence for domain overlap at the MNTB*

The present results further support the view that in the calyx of Held the calcium domains do overlap at most release sites during action potentials. Additional evidence in favour of this scheme consists of the following four points.

(i) The calcium influx during an action potential is large and as many as 60 calcium channels open for each vesicle that is released (Borst & Sakmann 1996). Although this of course does not mean that a vesicle senses the contribution of 60 open calcium channels, it does mean that a sufficiently large number of channels are opening to make domain overlap a realistic possibility.

(ii) The open probability of calcium channels during an action potential is close to maximal in the calyx of Held. Adding a plateau phase to the action potential waveform, as was also done in figure 3, resulted in a maximal increase in the calcium current during the repolarization phase of around 40% (Borst & Sakmann 1998). This indicates that action potentials open calcium channels effectively. As a result, the probability that adjacent calcium channels all open during an action potential is close to maximal, increasing the likelihood that their domains will overlap.

(iii) The sum of the block of release induced by different calcium channel types in the calyx of Held is more than 100% (Wu *et al*. 1998, 1999). The P/Q-type blocker ω-agatoxin IVA blocks about half of the presynaptic calcium influx and 97% of release (Wu *et al*. 1999). This means that release of almost every vesicle depends on calcium influx via P/Q-type channels. In addition, in separate experiments, the N-type calcium channel blocker ω-conotoxin GVIA blocks around one-quarter of the presynaptic calcium influx and 36% of release (Wu *et al*. 1999), whereas the influx via R-type calcium channels was also around 25% and it contributed up to 36% to release (Wu *et al*. 1998). Many vesicles in the calyx of Held of 8–10-day-old rats therefore depend for their release not only on calcium influx via P/Q-type calcium channels, but also on N- or R-type calcium channels, in agreement with the overlapping calcium domain view.

(iv) The slow calcium buffer EGTA reduces transmitter release in the calyx of Held at relatively low concentrations (Borst & Sakmann 1996). In contrast to the situation in the squid giant synapse, where more than 80 mM EGTA did not affect transmission (Adler *et al*. 1991), EGTA already reduced transmitter release by around 30% at a concentration of 1 mM. Similar results have been obtained in synapses between pyramidal neurons of the somatosensory cortex (Ohana & Sakmann 1998). This indicates that the diffusion times for Ca^{2+} to the calcium sensor are relatively long, which is again in agreement with the overlapping domain model.

In table 1 the evidence in favour of the overlapping calcium domain model in the MNTB is summarized and it is compared with the data available from the squid giant synapse.

(c) *Domain overlap in synapses of the vertebrate nervous system*

In the calyx of Held good evidence has been obtained that multiple calcium channels contribute to the release of a vesicle. In, for example, cochlear hair cells (Roberts *et al*. 1990), cerebellar granule cells (Mintz *et al*. 1995) or hippocampal pyramidal neurons (Luebke *et al*. 1993; Takahashi & Momiyama 1993; Wu & Saggau 1994) a similar scheme may be at work. Although a single calcium channel may under certain conditions trigger the release of a single vesicle in the chick ciliary ganglion, the observed probability of release was very low (Stanley 1993). During action potentials, the probability of release may be much higher at this synapse, since the quantal content of the evoked postsynaptic currents is at least 100 (Zhang *et al*. 1996). Given the high density of calcium channels on the release face (Haydon *et al*. 1994; Stanley 1991), the large calcium current amplitudes in recordings from the calyx-type terminals and their fast activation kinetics (Stanley & Mirotznik 1997; Yawo & Momiyama 1993), it seems conceivable that during normal synaptic transmission at this synapse most vesicles are released by overlapping domains as well.

(d) *Advantages of the overlapping calcium domains scheme*

In conclusion, at most synapses that were studied, release appears to be controlled by overlapping calcium domains. Only at the squid giant synapse several lines of evidence indicate that release is controlled by single calcium domains. The single calcium domain scheme has obvious advantages over the overlapping domain scheme. The calcium load for the terminal is relatively small. Diffusion times are minimized, although diffusion times probably constitute only a small portion of the total delay between calcium channel opening and transmitter release (Adler *et al*. 1991; Borst & Sakmann 1996; Yamada & Zucker 1992). The single calcium domain scheme also fits in nicely with recent experiments in which the properties of calcium channels were found to depend on the presence of proteins that are involved in release (Bezprozvanny *et al*. 1995; Stanley & Mirotznik 1997; Wiser *et al*. 1996).

Nevertheless, the single calcium domain scheme has not become very popular in the vertebrate nervous system. Apparently, its disadvantages outweigh its advantages. What are then the advantages of calcium domain overlap? First, if the release of a vesicle depends on the opening of several calcium channel types, each calcium channel type may control a large fraction of the release. These different types may be differentially regulated by neurotransmitters that are active at presynaptic receptors. Each of these neurotransmitters may exert a strong control over transmitter release (Wu & Saggau 1997). Second, in the overlapping domain scheme, the release probability of a vesicle is less dependent on its

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Table 1. *A comparison of experiments relevant to the question whether calcium domains overlap at release sites in the MNTB and the squid giant synapse*

(Experiments in the MNTB were done with an extracellular solution containing 2 mM Ca^{2+}, 1 mM Mg^{2+}. The squid extracellular solution contains 10–11 mM Ca^{2+} and 50–54 mM Mg^{2+}.)

	MNTB	squid giant synapse
I_{Ca} during APs versus release[a]	3–3.5	1–1.5
I_{Ca} during steps versus release[b]	2	4
$P_{0,AP}$ (%)[c]	70	10–30
k_b EGTA (mM)[d]	$1 < k_b < 10$	>80
k_b BAPTA (mM)[e]	$0.05 < k_b < 1$	0.4

[a] Power relation between calcium current (I_{Ca}) during an action potential (AP) and transmitter release. In the squid this relation was directly measured by Llinás *et al.* (1982); in Augustine (1990) it was calculated based on a Hodgkin–Huxley model.
[b] A second power relation was observed for brief steps in the MNTB by Takahashi *et al.* (1996). The fourth power relation in the squid was only observed at $[Ca]_0$ below 10 mM (Augustine & Charlton 1986). At physiological $[Ca]_0$ the relation was already submaximal. The high power relation during 3–6 ms steps was interpreted as indicating that domains will start overlapping in the squid giant synapse if the calcium channels open much longer than they do during action potentials.
[c] The peak open probability of calcium channels during an action potential as a fraction of the maximum open probability ($P_{0,AP}$) was 70% in the MNTB (Borst & Sakmann 1998) and was calculated to be 10% for a 70 mV action potential in Pumplin *et al.* (1981), based on a Hodgkin–Huxley model. In figure 2 of Llinás *et al.* (1982) a $P_{0,AP}$ of around 30% was measured for a 70 mV action potential waveform, which elicited an EPSP of around 5 mV.
[d] Half-maximal block (k_b) of release by the slow calcium buffer EGTA in terminals (MNTB, Borst & Sakmann 1996; squid, Adler *et al.* 1991).
[e] Half-maximal block (k_b) of release by the fast calcium buffer BAPTA in terminals: MNTB, Borst *et al.* (1995); squid, Adler *et al.* (1991).

exact location, since activation of its calcium sensor can occur without the necessary presence of an open calcium channel within tens of nanometres. Third, at most synapses transmitter release depends on the third or the fourth power of the calcium influx. As a result the variance in calcium influx may contribute significantly to the total variance in transmitter release. However, at the squid giant synapse, with more than 10 000 active zones (Pumplin *et al.* 1981) and a quantal content of many thousands (Augustine *et al.* 1985; Augustine & Eckert 1984), the extra variance introduced by the calcium channels is unlikely to contribute much to the overall variance in the synaptic responses. In the vertebrate nervous system, where many boutons release on average less than one vesicle, minimizing the variance in transmitter release by minimizing the variance in the calcium influx during presynaptic action potentials may be much more important.

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