ELSEVIER

European Journal of Pharmacology 306 (1996) 41–50

ejp

The use of invertebrate peptide toxins to establish Ca^{2+} channel identity of CA3-CA1 neurotransmission in rat hippocampal slices

Janet M. Nooney *, David Lodge

Lilly Research Centre, Erl Wood Manor, Windlesham, Surrey GU20 6PH, UK

Received 18 September 1995; revised 19 February 1996; accepted 1 March 1996

Abstract

The relative contribution(s) of different Ca^{2+} channel subtypes to synaptic transmission between Schaffer collaterals of hippocampal CA3 pyramidal cells and CA1 pyramidal cell dendrites has been assessed using the synthetic invertebrate peptide toxins ω-conotoxin GVIA to block N-type Ca^{2+} channels, ω-agatoxin-IVA to block P-type Ca^{2+} channels and ω-conotoxin MVIIC to block N-, P- and Q-type Ca^{2+} channels. ω-Agatoxin-IVA, ω-conotoxin GVIA and ω-conotoxin MVIIC all produced dose-dependent inhibitions of the excitatory post-synaptic field potential (fEPSP) recorded from the CA1 region of transverse hippocampal slices. Application of 300 nM ω-conotoxin GVIA generally produced no further inhibition to that observed with 100 nM, resulting in a maximal 50% inhibition of the fEPSP. By contrast, 30 nM ω-agatoxin-IVA reduced the fEPSP slope by only 4.6 ± 11.1% (mean ± S.D., $n = 3$), suggesting the lack of involvement of classical P-type Ca^{2+} channels, whereas 300 nM ω-agatoxin-IVA reduced the fEPSP slope by 85.7 ± 15.3% ($n = 3$) at the end of 44 min application. Similar applications of 100 and 300 nM ω-conotoxin MVIIC reduced the fEPSP slope by 30.9 ± 6.6% and 79.7 ± 5.7% respectively. Application of 30 nM ω-agatoxin-IVA together with ω-conotoxin GVIA (300 nM) produced no greater inhibition of the fEPSP than that observed with ω-conotoxin GVIA alone, suggesting that the ω-agatoxin-IVA-sensitive and ω-conotoxin MVIIC-sensitive component presents a pharmacology similar to the reported Q-type Ca^{2+} channel. The inhibition produced by ω-conotoxin GVIA and ω-conotoxin MVIIC showed no recovery with prolonged washing (1–2 h) whereas that produced by ω-agatoxin-IVA was slowly reversible. The observation that ω-agatoxin-IVA, which does not effect N-type Ca^{2+} channels (Mintz et al. (1992a) Neuron 9, 85), is capable of completely suppressing the fEPSP suggests that, whilst N-type Ca^{2+} channels may contribute to normal synaptic transmission at Schaffer collateral-CA1 synapses, they are not capable of supporting transmission when Q-type channels are blocked.

Keywords: Hippocampus; Ca^{2+} channel; Conotoxin; ω-Agatoxin-IVA

1. Introduction

Functional distinctions between subtypes of voltage-dependent Ca^{2+} channels rely on differences in biophysical (e.g. low-voltage activated versus high-voltage activated; rate of inactivation etc.) and pharmacological properties (e.g. dihydropyridine sensitivity). The biophysical properties of the Ca^{2+} channels underlying transmitter release at central synapses are poorly accessible. Thus the relative contributions of different Ca^{2+} channel subtypes to neurotransmitter release has been assessed pharmacologically using synthetic toxins: i.e. ω-conotoxin GVIA to block N-type Ca^{2+} channels (Olivera et al., 1985; McCleskey et al., 1987), ω-agatoxin-IVA to block P-type Ca^{2+} channels (Mintz et al., 1992b) and ω-conotoxin MVIIC to block N-, P- and Q-type calcium channels (Hillyard et al., 1992).

Previous studies have used these toxins to examine neurotransmitter release directly from synaptosomes or slices, using endogenous transmitter or tissue preloaded with tritiated transmitter(s) of interest (Turner et al., 1992; Adams et al., 1993; Burke et al., 1993; Luebke et al., 1993; Gaur et al., 1994). These biochemical studies generally involve the use of high K-evoked depolarizations to trigger release. Whilst yielding important information, they lack the temporal resolution of electrophysiological studies (see Luebke et al., 1993). In addition, bathing the tissue in high K solution effectively introduces a 'voltage clamp' which may lead to the recruitment of different channel types (e.g. Momiyama and Takahashi, 1994; cf. Takahashi and Momiyama, 1993) or may bias their relative contribu-

* Corresponding author. Department of Pharmacology, University of Edinburgh, 1 George Square, Edinburgh EH8 9JZ, UK. Tel.: (44) 131-650 8490; fax: (44) 131-650 6530.

PII S0014-2999(96)00195-1

tions (Luebke et al., 1993) compared to those active during 'normal' synaptic transmission.

Electrophysiological studies of neurotransmission in brain slices provide an indirect measure of transmitter release able to circumvent the above issues. They also have the advantage of being able to focus on smaller synaptic regions than can be obtained from biochemical studies with, for example, whole brain synaptosomes (Adams et al., 1993) or even hippocampal slices or synaptosomes (Burke et al., 1993; Gaur et al., 1994). However, the slow onset rate of toxins (Luebke et al., 1993; Sather et al., 1993; Wheeler et al., 1994a) becomes an issue for the 'on-line' protocols typically used in electrophysiological experiments. For this reason, many studies (e.g. Takahashi and Momiyama, 1993; Wheeler et al., 1994a; Wu and Saggau, 1995) have used high concentrations of toxin to produce a rapid block. However, as discussed below, this raises questions of toxin selectivity, or rather, the lack thereof.

To minimise this complication, the present study has used long (44 min) applications of low concentrations of the two *Conus* toxins and ω-agatoxin-IVA to examine the Ca^{2+} channels involved in neurotransmission at the synapse between hippocampal CA3-Schaffer collaterals and CA1 pyramidal cell dendrites. Some of these results have been presented in abstract form (Nooney and Lodge, 1995a,b).

2. Materials and methods

Female Wistar rats (120–150 g) were killed by decapitation following halothane anaesthesia. Transverse hippocampal slices (400 μm) were obtained from parasagittal slices prepared using a Vibroslice (Campden Instruments, UK). Hippocampal slices were submerged in, and continuously superfused (2.3 ml · min^{-1}) with, Krebs solution (in mM: 124 NaCl, 3 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgSO_4$, 1.25 NaH_2PO_4, 10 D-glucose) equilibrated with 95% O_2/5% CO_2.

Toxins were applied via the bath perfusate (2.3 ml · min^{-1}). Toxins were prepared as 10^{-4} M stock solutions in distilled H_2O and stored as aliquots at −20°C. Immediately before use, toxins were diluted in Krebs solution to the stated concentrations for application via the superfusate. Application times were taken from the time the toxin solution reached a 0.5 ml reservoir continuous with the recording bath; total bath volume (including this reservoir) was estimated to be 1.5 ml. ω-Agatoxin-IVA was obtained from N. Saccamano, Pfizer Research, USA and Peptide Institute, Japan. ω-Conotoxin GVIA and ω-conotoxin MVIIC were obtained from Bachem, UK. All other reagents were AR grade, obtained from Fisons, UK.

Excitatory post-synaptic field potentials (fEPSPs) were recorded with 2–5 MΩ electrodes filled with 3 M NaCl, in response to Schaffer collateral nerve stimulation every 30 s (20 μs; 10–40 V) with a bipolar tungsten electrode. All experiments were performed at room temperature.

2.1. Data analysis

Data were digitized, captured (10 kHz sampling rate) and analysed using Spike 2 software (CED, UK). Neurotransmission was quantified by calculating the slope of the rising phase of the averages of four fEPSPs. For a given experiment, 25–75% limits were fitted, by eye, to the rising phase of the first averaged fEPSP. The slope of the rising phase of this, and all subsequent averaged fEPSPs, was then calculated between these limits, using a least-squares regression routine supplied by the Spike 2 soft-

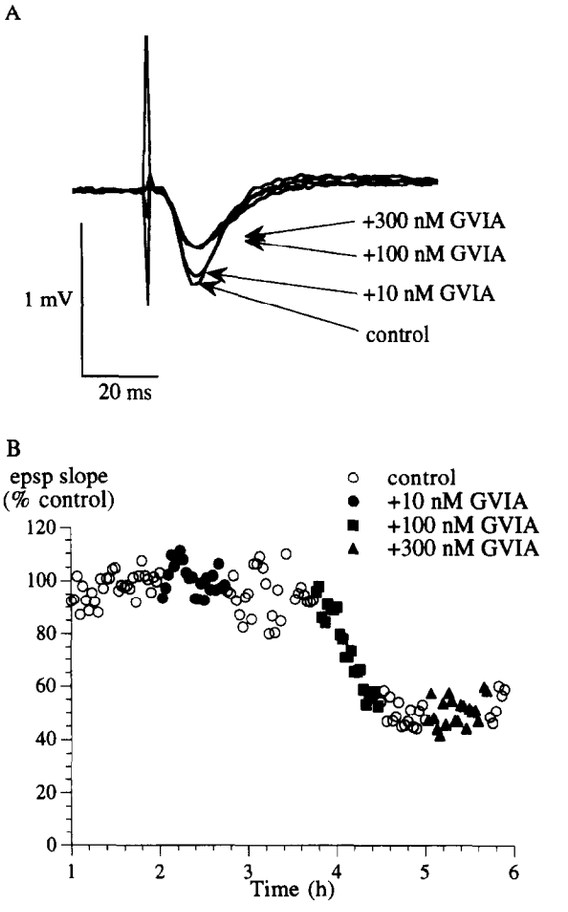

Fig. 1. ω-Conotoxin GVIA produces a partial inhibition of the CA1 fEPSP. A: Signal averaged traces ($n = 4$) illustrate fEPSP recordings from the CA1 region of the hippocampus, under control conditions and in the presence of 10, 100, and 300 nM ω-conotoxin GVIA (GVIA), from the experiment depicted in B. B: Representative experiment showing time course of ω-conotoxin GVIA effects on fEPSP slope. Note that 300 nM ω-conotoxin GVIA does not induce any greater inhibition than that induced by 100 nM ω-conotoxin GVIA. Data points represent the average fEPSP slope ($n = 4$) expressed as % of control immediately preceeding the first ω-conotoxin GVIA application. Open symbols in the presence of control Krebs solution, filled symbols during superfusion with toxin.

ware. No difference was found between data analysed in this way and those in which the slopes of individual responses were calculated and then averaged.

Data were normalised to control values obtained for 10 min immediately prior to first toxin application and are presented as mean ± S.D. from n slices. Statistical significance was assessed using 2-tailed t tests.

3. Results

A salient feature of the present study was the slow, though concentration-dependent, onset rate of the Ca^{2+} channel toxins to block synaptic transmission (see also Luebke et al., 1993; Sather et al., 1993; Wheeler et al., 1994a). The toxin-induced reduction in the fEPSP was estimated to have reached steady state by 44 min of drug application. With ω-agatoxin-IVA and ω-conotoxin GVIA this was generally the case, although with ω-conotoxin MVIIC further block frequently occurred during the initial stages of washout. Longer application times were not used because of the cost of the toxins.

ω-Conotoxin GVIA (30–300 nM), a selective N-type Ca^{2+} channel antagonist, reduced the amplitude and slope of the rising phase of the fEPSP (Fig. 1A). Dose dependency of ω-conotoxin GVIA action was more apparent in the rate of block than in the degree of block (cf. Fig. 1B and 6A): thus 100 nM ω-conotoxin GVIA produced 29.9 ± 26.1% inhibition ($n = 5$) of the fEPSP slope at the end of 20 min application. A further decrease of the fEPSP was often seen, however, in the initial stages of washout (data not shown). Consequently, 44 min applications were used thereafter, since this appeared to be sufficient to obtain a steady-state block at most of the concentrations tested. Following this more prolonged application time, 45.5 ± 4.3% ($n = 4$) steady-state inhibition was observed with 100 nM ω-conotoxin GVIA. Fig. 1 shows the time course of a typical experiment in which ω-conotoxin GVIA was applied to a hippocampal slice at increasing concentrations, for 44 min periods separated by 45 min wash, and

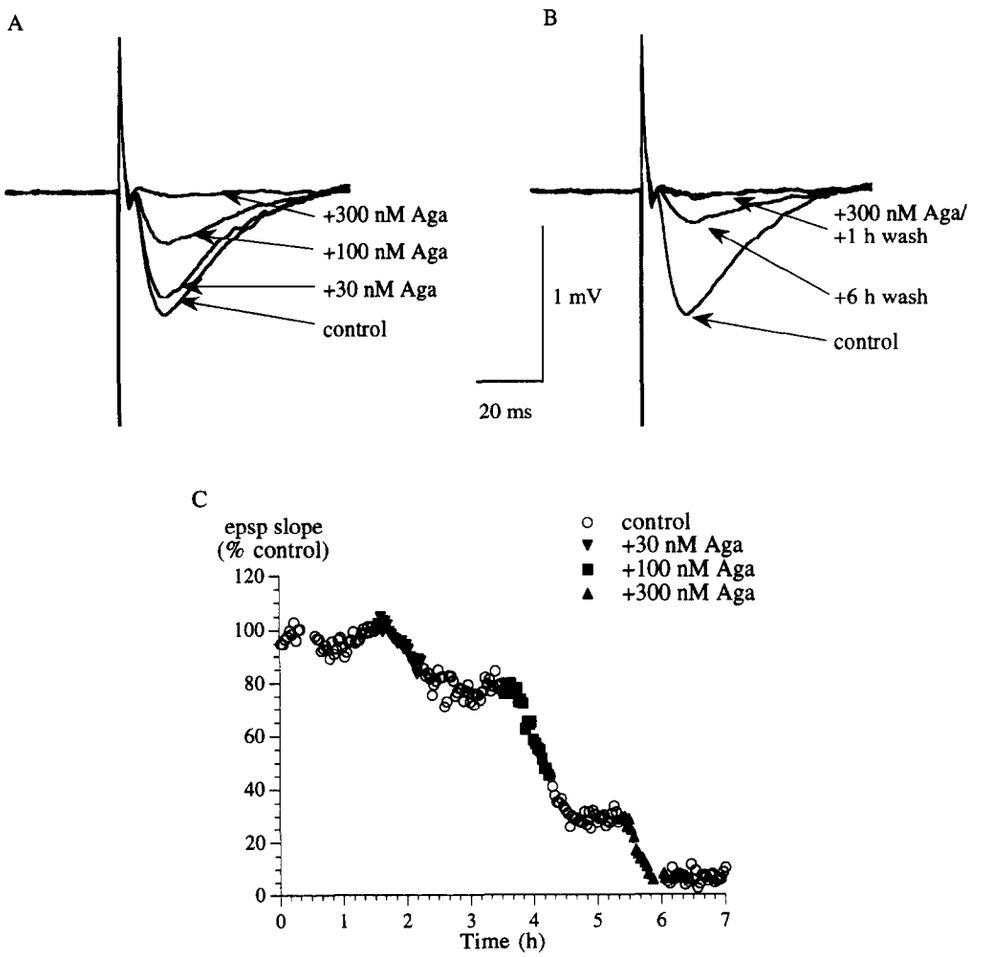

Fig. 2. ω-Agatoxin-IVA completely inhibits the CA1 fEPSP. A, B: Signal averaged traces ($n = 4$) illustrating control responses (A, B), responses remaining after 44 min applications of 30, 100, and 300 nM ω-agatoxin-IVA (Aga, A) and partial recovery of response after 6 h wash (B). C: Time course plot of ω-agatoxin-IVA-induced reduction of the fEPSP slope, same experiment as in A, B.

illustrates that application of 300 nM ω-conotoxin GVIA generally produced no further inhibition to that observed with 100 nM (Fig. 1) resulting in a maximal 50% decrease in the fEPSP. No recovery was seen with prolonged washout (1–2 h). ω-Conotoxin GVIA (300 nM) produced a similar inhibition when applied to naïve slices (45.4 ± 3.2%, $n = 3$; $P > 0.05$) to that observed when several lower doses of ω-conotoxin GVIA had been applied to the same slice (51.3 ± 2.4% reduction, $n = 3$). Consequently, data from naïve slices were pooled together with data from slices from which quasi-cumulative dose-response relationships were obtained. The resulting dose-response curve is shown in Fig. 4.

ω-Agatoxin-IVA (30–300 nM) induced a dose-dependent reduction in the fEPSP (Fig. 2) such that 100 nM ω-agatoxin-IVA reduced the fEPSP slope by 34.3 ± 39.0% ($n = 3$). Following subsequent application of 300 nM ω-agatoxin-IVA, the fEPSP was virtually eliminated in two of three slices (96 and 93% reductions; Fig. 2A cf. Fig. 3A). From the three slices examined in this way, 14.3 ± 15.3% of the control fEPSP remained after 44 min of 300 nM ω-agatoxin-IVA application (Fig. 4). A slow and variable recovery could be observed following 300 nM ω-agatoxin-IVA applications (to 30 and 100% of control after 6 h wash, $n = 2$; Fig. 2B and C). No such recovery was observed with the other toxins.

The broad spectrum Ca^{2+} channel toxin ω-conotoxin MVIIC produced an irreversible dose-dependent inhibition of synaptic transmission (Fig. 3), with 100 and 300 nM producing 30.9 ± 6.6% and 79.7 ± 5.7% ($n = 3$) steady-state inhibitions, respectively, at the end of 44 min application. By contrast, upon application to naïve slices, 300 nM ω-conotoxin MVIIC reduced the fEPSP slope by 44.9 ± 13.1% ($n = 3$, $P < 0.01$). This difference appeared to be related to the slow onset of ω-conotoxin MVIIC effects, since a further 23.7 ± 6.7% reduction was observed on washout of ω-conotoxin MVIIC, to produce a total 68.6 ± 8.0% inhibition ($n = 3$) which was not significantly different from that observed during the dose-response experiments ($P > 0.1$).

The dose-response relationships for the three toxins (Fig. 4) and their respective selectivities for Ca^{2+} channel subtypes thus suggest the presence of at least two subtypes of Ca^{2+} channels at Schaffer collateral-CA1 synapses: an ω-conotoxin GVIA-sensitive N-type channel and a P- and/or Q-type channel blocked by ω-agatoxin-IVA and ω-conotoxin MVIIC.

3.1. P- vs. Q-type Ca^{2+} channels

The selectivity of ω-agatoxin-IVA for P-type versus Q-type Ca^{2+} channels at high nM concentrations has been questioned (Wheeler et al., 1994a; Sather et al., 1993; Randall and Tsien, 1995; see Section 4). To determine whether glutamate release from Schaffer collateral terminals involved a P-type Ca^{2+} channel component, slices

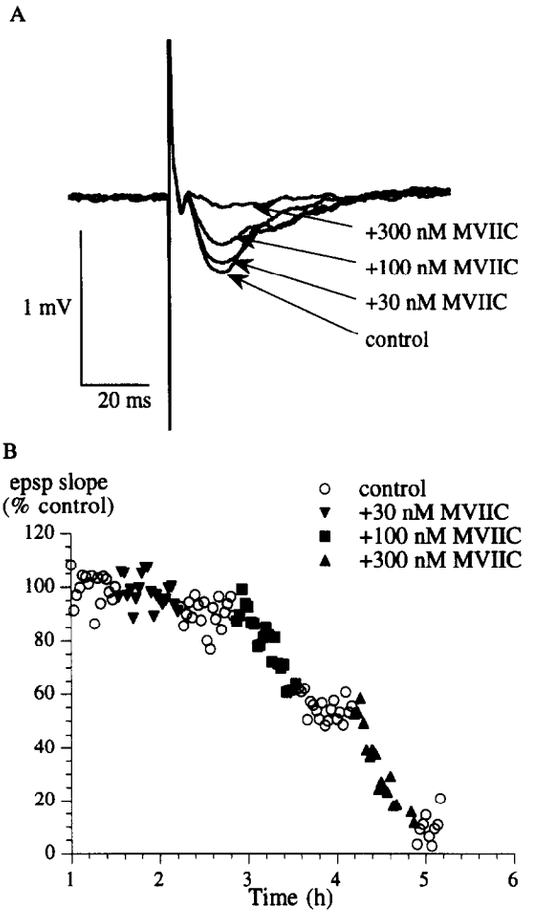

Fig. 3. ω-Conotoxin MVIIC inhibits the CA1 fEPSP. A: Signal averaged traces ($n = 4$) illustrating control responses and in the presence of ω-conotoxin MVIIC (MVIIC), after 44 min applications of 30, 100, and 300 nM respectively. Note the small residual fEPSP remaining in the presence of 300 nM ω-conotoxin MVIIC. B: Plot of the fEPSP slope, in the presence (▼, ■, ▲) and absence (○) of ω-conotoxin MVIIC, versus time for the same experiment as in A.

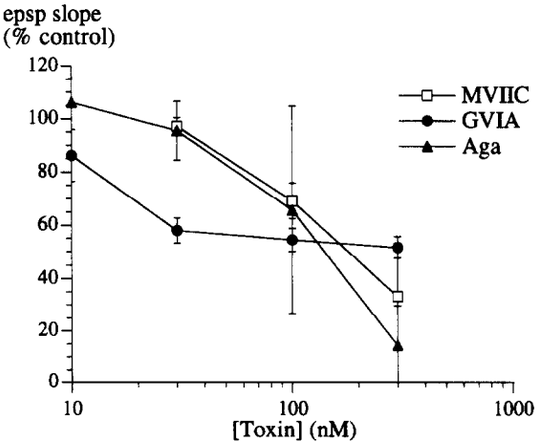

Fig. 4. Log dose-response relations for toxin-induced reduction of fEPSP slope for ω-conotoxin MVIIC (MVIIC, □), ω-conotoxin GVIA (GVIA, ●) and ω-agatoxin-IVA (Aga, ▲). Data points represent mean ± S.D. (where appropriate and larger than the symbol) from $n = 2$–6 hippocampal slices.

were co-exposed to ω-agatoxin-IVA and ω-conotoxin GVIA at a concentration of ω-agatoxin-IVA that would be expected to block only P-type Ca^{2+} channels (Fig. 5; see Section 4). Application 30 nM ω-agatoxin-IVA together with 300 nM ω-conotoxin GVIA produced no greater inhibition (39.5 ± 5.7, $n = 4$) of the fEPSP than that observed with ω-conotoxin GVIA (300 nM) alone (45.4 ± 3.2% reduction, $n = 3$, $P > 0.1$; Fig. 5C).

3.2. ω-Conotoxin MVIIC selectivity

To determine whether ω-conotoxin MVIIC was acting on N- as well as Q-type Ca^{2+} channels in this preparation, slices were exposed sequentially to 300 nM ω-conotoxin GVIA, to fully block N-type channels, and 300 nM ω-conotoxin MVIIC, which alone blocked 50–80% of the fEPSP. Fig. 6A and B show examples of two such experiments. Application of ω-conotoxin MVIIC (300 nM) following exposure to ω-conotoxin GVIA (300 nM) reduced the fEPSP slope by a further 27.3 ± 4.6% of control, from 54.6 ± 3.2% control following ω-conotoxin GVIA application to 27.3 ± 6.1% control after ω-conotoxin MVIIC application, to give a final block of 72.7 ± 6.1% ($n = 3$). In the reverse experiment, ω-conotoxin GVIA (300 nM) reduced the fEPSP slope by a further 18.5 ± 5.2% control after washout of ω-conotoxin MVIIC, to yield a final block of 87.1 ± 10.9% ($n = 3$; Fig. 6C; $P > 0.1$).

4. Discussion

The present study was aimed at elucidating the identity, and the relative contributions, of the Ca^{2+} channels responsible for the release of glutamate during synaptic

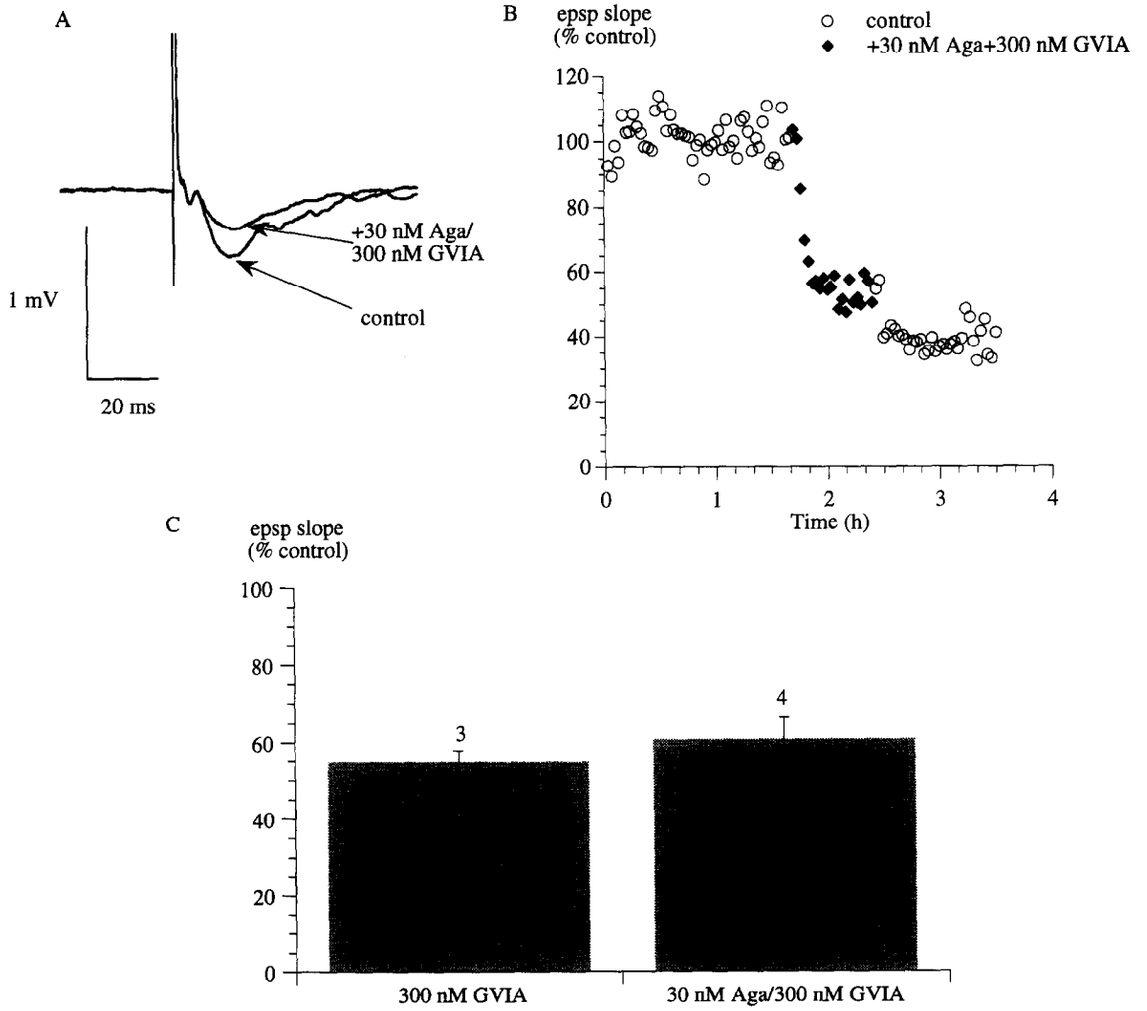

Fig. 5. Low concentrations of ω-agatoxin-IVA plus ω-conotoxin GVIA do not produce a greater block than ω-conotoxin GVIA alone. A: Representative signal averaged traces ($n = 4$) illustrating the CA1 fEPSP before (control) and in the presence of ω-agatoxin-IVA (Aga) plus ω-conotoxin GVIA (GVIA). B: Time course of a representative experiment showing the effects of application of ω-agatoxin-IVA (30 nM) plus ω-conotoxin GVIA (300 nM) on the CA1 fEPSP slope recorded from a single hippocampal slice. C: Histogram comparing the degree of block produced by ω-agatoxin-IVA plus ω-conotoxin GVIA ($n = 4$) to that produced by ω-conotoxin GVIA (300 nM) alone ($n = 3$).

neurotransmission between pyramidal cells of the CA3 and CA1 regions of the rat hippocampus. Two points emerge from the present observations: firstly, more than one channel type contributes to synaptic transmitter release and secondly, the sum of the components appears greater than 100%. The first point emerges from the different selectivities of the toxins employed, for the known subtypes of Ca^{2+} channels. The second is explained by the power function relating Ca^{2+} influx to transmitter release.

4.1. Relationship between Ca^{2+} influx and transmitter release

At the neuromuscular junction, neurotransmitter release is not linearly related to Ca^{2+} influx, but increases more steeply as the external Ca^{2+} concentration is raised (Dodge and Rahaminoff, 1967). Likewise, at squid giant synapse, release is proportional to $[Ca^{2+}]^4$ (Augustine et al., 1985; Augustine and Charlton, 1986). A similar relationship has also been assumed to be the case at mammalian central synapses (e.g. Takahashi and Momiyama, 1993) and has been demonstrated directly in guinea pig hippocampal slices (Wu and Saggau, 1994a,b, 1995) when Ca^{2+} influx was blocked by Cd^{2+}, ω-conotoxin GVIA, ω-agatoxin-IVA or ω-conotoxin MVIIC. In the latter studies, a full block of transmission was obtained when Ca^{2+} influx was reduced by ~40%. This implies that there must be a substantial threshold of Ca^{2+} influx required before neurotransmitter release can be triggered.

A similar power relationship is likely to exist in the

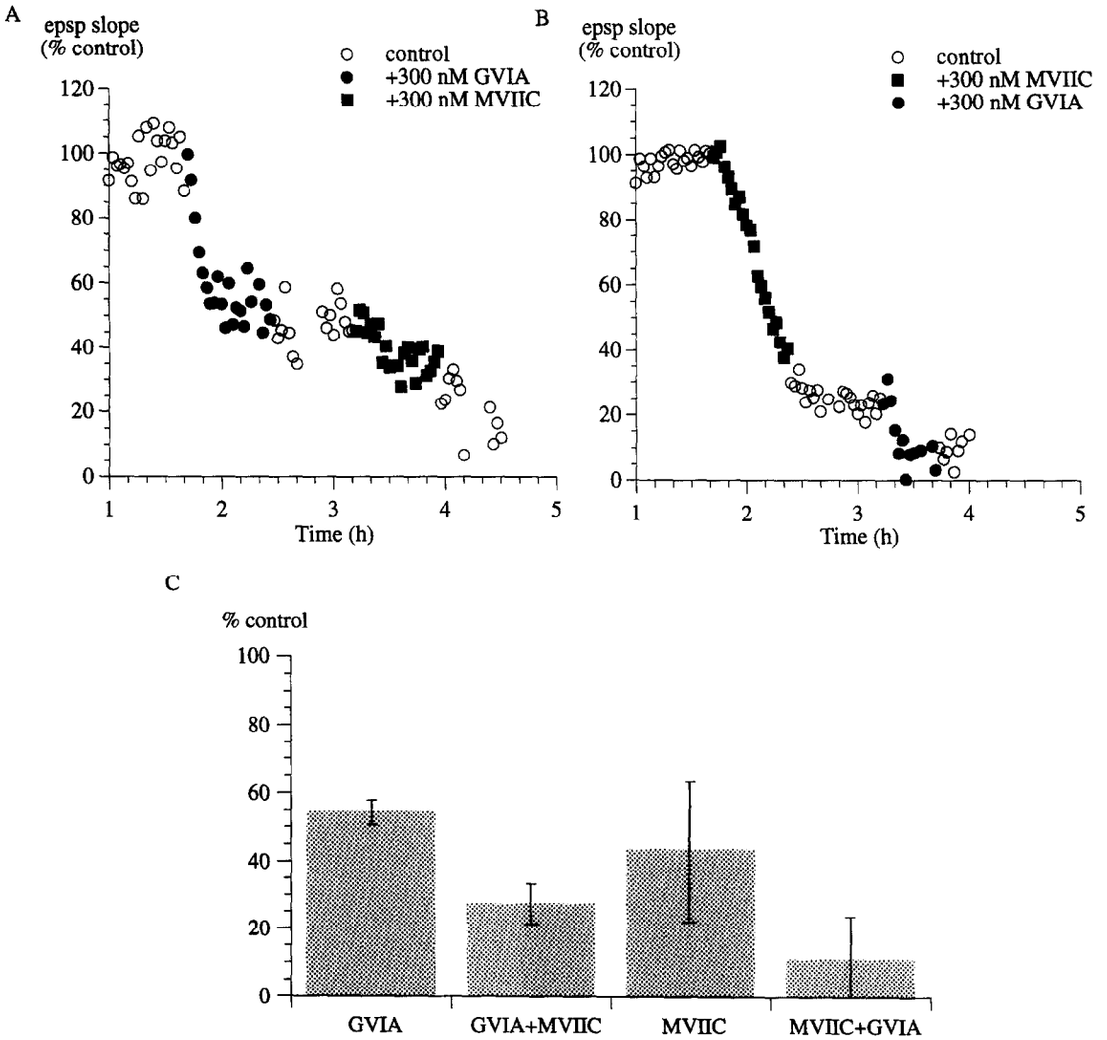

Fig. 6. Effects of sequential toxin application on CA1 fEPSP slope. A, B: Representative plots depicting the fEPSP slope, as % control obtained immediately prior to first toxin application, in control saline (○) and in the presence of 300 nM ω-conotoxin GVIA (GVIA, ●) either followed (A) or preceeded (B) by 300 nM ω-conotoxin MVIIC (MVIIC, ■). Note the difference in speed of onset of ω-conotoxin MVIIC effects compared to ω-conotoxin GVIA, even though ω-conotoxin MVIIC induces a more pronounced reduction in the fEPSP slope when applied first. C: Histogram illustrating the effects of ω-conotoxin GVIA (300 nM) or ω-conotoxin MVIIC (300 nM) applications to naïve slices and the further block induced by subsequent application of ω-conotoxin MVIIC or ω-conotoxin GVIA after 45 min wash ($n = 3$).

CA1 region of rat hippocampal slices, as used in the present study. Thus any compound or toxin able to reduce Ca^{2+} influx by $\geq 40\%$ would be predicted to induce a full block of synaptic transmission whether it is acting on a single population of channels or on a combination of channel subtypes. This power relationship between Ca^{2+} influx and transmitter release thus needs to be taken into consideration in any attempt to assign contributions of individual Ca^{2+} channel subtypes to transmitter release.

4.2. Toxin-sensitive components of neurotransmission

The inhibition of the fEPSP by the Ca^{2+} channel toxins ω-agatoxin-IVA, ω-conotoxin GVIA and ω-conotoxin MVIIC suggests the presence of at least two subtypes of Ca^{2+} channels at Schaffer collateral-CA1 synapses: an ω-conotoxin GVIA-sensitive N-type channel and a P- and/or Q-type channel blocked by ω-agatoxin-IVA and ω-conotoxin MVIIC.

4.2.1. Comparison with other studies

The present observations of a maximal 50% irreversible reduction of the fEPSP by ω-conotoxin GVIA are in keeping with other electrophysiological studies in the CA1 region of hippocampus. Thus, ω-conotoxin GVIA ($\geq$ 100 nM) typically produces an irreversible, partial (44–76%) block of CA3 to CA1 synaptic transmission when assessed by population (field) excitatory post synaptic potentials (fEPSPs) (Horne and Kemp, 1991; Wheeler et al., 1994a; Wu and Saggau, 1994b). Similar, though more variable, inhibitions have been observed on single cell excitatory post synaptic potentials (epsp; Dutar et al., 1989; Horne and Kemp, 1991; Potier et al., 1993) or currents (epsc; Takahashi and Momiyama, 1993; Luebke et al., 1993) from the same region, with a full block of transmission being obtained at some sites and, in one study, a more pronounced inhibition after pharmacological block of inhibitory pathways (Horne and Kemp, 1991). ω-Conotoxin GVIA also produces a partial, but irreversible block of synaptic transmission in other brain regions, including: fEPSPs at mossy fibre and association-commisural fibre synapses with hippocampal CA3 pyramidal cells (Kamiya et al., 1988; Castillo et al., 1994), fEPSPs and epsps at corticostriatal synapses (Lovinger et al., 1994), epscs from Purkinje cells of the cerebellum (Regehr and Mintz, 1994) and ipscs from inhibitory synapses within the deep cerebellar nuclei (Takahashi and Momiyama, 1993).

By contrast, the observations with both ω-agatoxin-IVA and ω-conotoxin MVIIC, although similar to some studies (Wheeler et al., 1994b), nonetheless show a tendency to a more complete block at slightly lower concentrations than those previously reported. Thus, although monosynaptic epsps or epscs may be inhibited 80–100% by 200 nM ω-agatoxin-IVA (Luebke et al., 1993; Takahashi and Momiyama, 1993; Dunlap et al., 1994; Wheeler et al., 1994b), fEPSP recordings have generally proved less sensitive to blockade, with 1 μM ω-agatoxin-IVA producing 60–85% inhibition (Wheeler et al., 1994a; Wu and Saggau, 1994b, 1995). Likewise a 63–70% decrease in the fEPSP has been observed with 20 min application of 500 nM ω-conotoxin MVIIC, with $\geq$ 1 μM ω-conotoxin MVIIC being required to elicit a 100% inhibition (Wheeler et al., 1994a; Wu and Saggau, 1995).

Interestingly, the present study finds the blockade of the fEPSP by ω-agatoxin-IVA to be partially reversible, as are ω-agatoxin-IVA-sensitive currents in central neurones (Mintz et al., 1992a) and α_{1A}-transfected oocytes (Sather et al., 1993). Conversely, the ω-conotoxin MVIIC-induced inhibition was found to be irreversible in the present study. This contrasts with the observations of Wheeler et al. (1994a) in hippocampal slices and the actions of ω-conotoxin MVIIC on α_{1B} Ca^{2+} channels expressed in HEK 293 cells (Grantham et al., 1994).

4.3. Identity of Ca^{2+} channels underlying glutamate release

4.3.1. Toxin selectivity

ω-Conotoxin GVIA (10 μM) blocks N- and L-type Ca^{2+} currents in dorsal root ganglion neurones (McCleskey et al., 1987), and N-type Ca^{2+} currents in isolated hippocampal CA1 pyramidal cells but not P-type channels in Purkinje cells (Regan et al., 1991). Conversely, ω-agatoxin-IVA blocks whole cell Ca^{2+} current in Purkinje cells (representing > 90% P-type current) with an IC_{50} of 2–10 nM (Mintz et al., 1992b) but not N- or L-type currents in rat dorsal root ganglion neurons or N-type currents in rat sympathetic neurones (Mintz et al., 1992a,b). Furthermore, upto 1 μM ω-conotoxin GVIA does not displace ^{125}I-ω-agatoxin-IVA binding to rat whole brain membranes (Adams et al., 1993). From this, it is clear that ω-conotoxin GVIA and ω-agatoxin-IVA target different Ca^{2+} channel types, namely N- and P-/Q-type Ca^{2+} channels respectively.

4.3.2. Involvement of N-type Ca^{2+} channels

The depression of the fEPSP by ω-conotoxin GVIA has previously been taken as evidence for the involvement of N-type Ca^{2+} channels in synaptic neurotransmission. The present observation, however, that ω-agatoxin-IVA (albeit at the 'high' concentration of 300 nM) is capable of completely suppressing the CA1 fEPSP suggests that, whilst N-type Ca^{2+} channels may contribute to normal synaptic transmission at Schaffer collateral-CA1 synapses, they are not capable of supporting transmission when non-N-type channels are blocked. This might be explained by non-N-type channels being located more closely to release sites than N-type channels (e.g. Miller, 1987). Together with the threshold of Ca influx apparently required for neurotransmitter release (see above), this would suggest that Ca^{2+} influx through non-N-type channels acts in some way to prime the release mechanism. Further

Ca^{2+} influx through either N- or non-N-type Ca^{2+} channels enhances this release, consistent with the predictions of the domain hypothesis (see Augustine et al., 1991).

4.3.3. Involvement of P- and/or Q-type Ca^{2+} channels

Although ω-agatoxin-IVA blocks P-type Ca^{2+} channels at low nM concentrations in cerebellar Purkinje cells (Mintz et al., 1992b), the concentrations that are effective in other preparations are generally one or two orders of magnitude higher (Mintz et al., 1992a), at which ω-agatoxin-IVA has also been suggested to block Q-type Ca^{2+} channels (Sather et al., 1993; Randall and Tsien, 1995).

To address the question of whether the inhibition of the fEPSP by ω-agatoxin-IVA was due to the involvement of P- and/or Q-type channels, experiments were performed at a concentration of ω-agatoxin-IVA (30 nM) that is likely to be selective for P-type channels (Wheeler et al., 1994a; Randall and Tsien, 1995). Application of ω-agatoxin-IVA (30 nM) alone produced no significant reduction in the fEPSP slope. However, toxin-sensitive components of transmitter release have been revealed in biochemical experiments using subthreshold concentrations of ω-agatoxin-IVA and ω-conotoxin GVIA which produce synergistic inhibitions of high K-evoked transmitter release when used in combination (Luebke et al., 1993). In analogous experiments in the present study, application of ω-agatoxin-IVA (30 nM) together with a maximal concentration of ω-conotoxin GVIA produced no greater inhibition of the fEPSP than that observed with ω-conotoxin GVIA alone, suggesting that the ω-agatoxin-IVA-sensitive component does not represent a P-type Ca^{2+} channel as exemplified by cerebellar Purkinje cells (Mintz et al., 1992b). Whether this channel is a distinct Q-type channel or merely a P-type channel with low affinity for ω-agatoxin-IVA remains to be determined (Dunlap et al., 1995).

4.3.4. Observations with ω-conotoxin MVIIC

The selectivity of ω-conotoxin MVIIC is not clearcut. ω-Conotoxin MVIIC displaces ^{125}I-ω-conotoxin GVIA binding, albeit with a 100-fold lower affinity than unlabelled ω-conotoxin GVIA, and 10 μM ω-conotoxin MVIIC occludes the inhibitory effects of 5 μM ω-conotoxin GVIA on the Ca^{2+} current recorded from isolated hippocampal CA1 pyramidal cells (Hillyard et al., 1992). These observations suggest that high concentrations of ω-conotoxin MVIIC are able to target N-type Ca^{2+} channels.

The ability of low concentrations of ω-conotoxin MVIIC to occlude ω-conotoxin GVIA effects was examined in the present study by sequential applications of the two toxins. ω-Conotoxin GVIA (300 nM) produced a clear reduction in the fEPSP slope in slices that had previously been exposed to 300 nM ω-conotoxin MVIIC. Thus although ω-conotoxin MVIIC is able to block fully the Ca^{2+} current in HEK293 cells transfected with the human α_{1B-1},α_{2b}, β_{1-2}, N-type Ca^{2+} channel construct, with an IC_{50} of < 10 nM (Grantham et al., 1994), in rat hippocampal slices, 300 nM ω-conotoxin MVIIC occludes only part of the ω-conotoxin GVIA-sensitive component.

If the above power relationship between $[Ca^{2+}]_i$ and release holds in its simple form then the total block produced by sequential applications of ω-conotoxin GVIA and ω-conotoxin MVIIC should be independent of the order of toxin application. Only the proportion of inhibition produced by each toxin will vary because of any action of ω-conotoxin MVIIC on N-type channels. The differences in total block were surprising therefore: exposure to ω-conotoxin MVIIC followed by ω-conotoxin GVIA produced a slightly greater inhibition than when the toxins were applied in the reverse order, although this did not reach significance. This implies that ω-conotoxin GVIA may restrict access of ω-conotoxin MVIIC to some Q channels.

ω-Conotoxin MVIIC is able to inhibit > 90% of the predominantly P-type Ca^{2+} current in cerebellar Purkinje cells (Llinás et al., 1989), with an IC_{50} between 1 and 10 μM. Consequently an inhibition of a P-type Ca^{2+} channel by the 300 nM ω-conotoxin MVIIC (highest concentration) used in the present study is unlikely to contribute substantially to the observed reduction in the fEPSP. Thus the block observed in the presence of ω-conotoxin MVIIC is likely to be due predominantly to a block of Q-type channels (Sather et al., 1993; Randall and Tsien, 1995) with a minor component through a blockade of N-type channels.

4.4. Contributions of N- and Q-type channels to glutamate release

Since ω-conotoxin GVIA-induced inhibition reached a plateau at 50% inhibition in the present experiments, it would appear from the release $\propto [Ca^{2+}]_i^4$ power function (see above) that N-type Ca^{2+} channels probably contribute 15 to 20% of the Ca^{2+} influx responsible for glutamate release at Schaffer collateral-CA1 synapses. This in turn would suggest that 80–85% of the Ca^{2+} influx occurs through non-N-type channels.

The partial block by ω-conotoxin GVIA may be explained by non-N-type channels priming the release mechanism (see above) or by a lower density of N-type Ca^{2+} channels at all release sites, so that these contribute a smaller fraction of Ca^{2+} influx than do non-N-type channels. Alternatively, a differential distribution of ω-conotoxin GVIA-sensitive N-type Ca^{2+} channels may exist at different synapses. Consistent with this, experiments with the fluorescent dye FM-143 suggest that exocytosis may be completely blocked by ω-conotoxin GVIA at some release sites but only partially blocked at others (Reuter, 1995).

Since ω-agatoxin-IVA can fully block transmission, the Q(P)-type channel is likely to contribute at least 40% of the total Ca^{2+} influx. This is unlikely to be a maximum

figure however, as higher concentrations of both ω-agatoxin-IVA and ω-conotoxin MVIIC may further reduce Ca^{2+} influx even after the fEPSP has been fully blocked (see Wu and Saggau, 1995). The exact contribution of Q-type channels to Ca^{2+} influx at rat Schaffer collateral-CA1 synapses cannot therefore be determined precisely with the present experiments. It would appear, however, that they represent a larger proportion than those contributing to glutamate release at the same synapse in the guinea pig hippocampus since Wu and Saggau (1994b, 1995) found an apparently maximal $61 \pm 5\%$ decrease of the fEPSP with 1 μM ω-agatoxin-IVA.

In conclusion, the present results suggest that calcium channels with an N-type and Q-type, rather than P-type, pharmacology cooperate in the release of glutamate at the CA3-CA1 synapse in the rat hippocampus.

Acknowledgements

The authors wish to thank Dr. N. Saccamano, Pfizer Research Inc., USA, for the gift of ω-agatoxin-IVA.

References

Adams, M.E., R.A. Myers, J.S. Imperial and B.M. Olivera, 1993, Toxityping rat brain calcium channels with ω-toxins from spider and cone snail venoms, Biochemistry 32, 12566.

Augustine, G.J. and M.P. Charlton, 1986, Calcium dependence of presynaptic calcium current and post-synaptic response at the giant squid synapse, J. Physiol. 381, 619.

Augustine, G.J., M.P. Charlton and S.J. Smith, 1985, Calcium entry and transmitter release at voltage-clamped nerve termianls of squid, J. Physiol. 367, 163.

Augustine, G.J., E.M. Adler and M.P. Charlton, 1991, The calcium signal for transmitter secretion from presynaptic nerve terminals, Ann. N.Y. Acad. Sci. 635, 365.

Burke, S.P., M.E. Adams and C.P. Taylor, 1993, Inhibition of endogenous glutamate release from hippocampal tissue by Ca^{2+} channel toxins, Eur. J. Pharmacol. 238, 383.

Castillo, P.E., M.G. Weisskopf and R.A. Nicoll, 1994, The role of Ca^{2+} channels in hippocampal mossy fiber synaptic transmission and long-term potentiation, Neuron 12, 261.

Dodge, F.A. and R. Rahaminoff, 1967, Cooperative action of calcium ions in transmitter release at the neuromuscular junction, J. Physiol. 193, 419.

Dunlap, K., J.I. Luebke and T.J. Turner, 1994, Identification of calcium channels that control neurosecretion, Science 266, 828.

Dunlap, K., J.I. Luebke and T.J. Turner, 1995, Exocytotic Ca^{2+} channels in mammalian central neurons, Trends Neurosci. 18, 89.

Dutar, P., O. Rascol and Y. Lamour, 1989, ω-Conotoxin GVIA blocks synaptic transmission in the CA1 field of the hippocampus, Eur. J. Pharmacol. 174, 261.

Gaur, S., R. Newcomb, B. Rivnay, J.R. Bell, D. Yamashiro, J. Ramachandran and G.P. Miljanich, 1994, Calcium channel antagonist peptides define several components of transmitter release in the hippocampus, Neuropharmacology 33, 1211.

Grantham, C.J., D. Bowman, C.P. Bath, D.C. Bell and D. Bleakman, 1994, ω-Conotoxin MVIIC reversibly inhibits a human N-type calcium channel and calcium influx into chick synaptosomes, Neuropharmacology 33, 255.

Hillyard, D.R., V.D. Monje, I.M. Mintz, B.P. Bean, L. Nadasdi, J. Ramachandran, G. Miljanich, A. Azimi-Zoonooz, J.M. McIntosh, L.J. Cruz, J.S. Imperial and B.M. Olivera, 1992, A new conus peptide ligand for mammalian presynaptic Ca^{2+} channels, Neuron 9, 69.

Horne, A.L. and J.A. Kemp, 1991, The effect of ω-conotoxin GVIA on synaptic transmission within the nucleus acumbens and hippocampus of the rat in vitro, Br. J. Pharmacol. 103, 1733.

Kamiya, H., S. Sawada and C. Yamamoto, 1988, Synthetic ω-conotoxin blocks synaptic transmission in the hippocampus in vitro, Neurosci. Lett. 91, 84.

Llinás, R., M. Sugimori, J.-W. Lin and B. Cherksey, 1989, Blocking and isolation of a calcium channel from neurons in mammals and cephalopods utilizing a toxin fraction (FTX) from funnel-web spider poison, Proc. Natl. Acad. Sci. USA 86, 1689.

Lovinger, D.M., A. Merrit and D. Reyes, 1994, Involvement of N- and non-N-type calcium channels in synaptic transmission at corticostriatal synapses, Neuroscience 62, 31.

Luebke, J.I., K. Dunlap and T.J. Turner, 1993, Multiple calcium channel types control glutamatergic synaptic transmission in the hippocampus, Neuron 11, 895.

McCleskey, E.W., A.P. Fox, D.H. Feldman, L.J. Cruz, B.M. Olivera, R.W. Tsien and D. Yoshikami, 1987, ω-Conotoxin: Direct and persistent blockade of specific types of calcium channels in neurons but not muscle, Proc. Natl. Acad. Sci. USA 84, 4327.

Miller, R.J., 1987, Multiple calcium channels and neuronal function. Science 235, 46.

Mintz, I.M., M.E. Adams and B.P. Bean, 1992a, P-Type calcium channels in rat central and peripheral neurons, Neuron 9, 85.

Mintz, I.M., V.J. Venema, K.M. Swiderek, T.D. Lee, B.P. Bean and M.E. Adams, 1992b, P-Type calcium channels blocked by the spider toxin ω-Aga-IVA, Nature 355, 827.

Momiyama, A. and T. Takahashi, 1994, Calcium channels responsible for potassium-induced transmitter release at rat cerebellar synapses, J. Physiol. 476, 197.

Nooney, J.M. and D. Lodge, 1995a, The use of *Conus* toxins to establish calcium channel identity of CA3-CA1 neurotransmission in rat hippocampal slices, Br. J. Pharmacol. 115, 117P

Nooney, J.M. and D. Lodge, 1995b, The use of calcium channel toxins to establish the identity of calcium channels underlying CA3-CA1 neurotransmission in rat hippocampal slices, Soc. Neurosci. Abstr. 21, 340.

Olivera, B.M., W.R. Gray, R. Zeikus, J.M. McIntosh, J. Varga, J. Rivier, V. Santos and L.J. Cruz, 1985, Peptide neurotoxins from fish-hunting cone snails, Science 230, 1338.

Potier, B., P. Dutar and Y. Lamour, 1993, Different effects of ω-conotoxin GVIA at excitatory and inhibitory synapses in rat CA1 hippocampal neurons, Brain Res. 616, 236.

Randall, A. and R.W. Tsien, 1995, Pharmacological Dissection of multiple types of Ca^{2+} channel currents in rat cerebellar granule neurons, J. Neurosci. 15, 2995.

Regan, L.J., D.W. Sah and B.P. Bean, 1991, Ca^{2+} channels in rat central and peripheral neurons: High-threshold current resistant to dihydropyridine blockers and ω-conotoxin, Neuron 6, 169.

Regehr, W.G. and I.M. Mintz, 1994, Participation of multiple calcium channel types in transmission at single climbing fiber to purkinje cell synapses, Neuron 12, 605.

Reuter, H., 1995, Measurements of exocytosis from single presynaptic nerve terminals reveal heterogeneous inhibition by Ca^{2+}-channel blockers, Neuron 14, 773.

Sather, W.A., T. Tanabe, J.-F. Zhang, Y. Mori, M.E. Adams and R.W. Tsien, 1993, Distinctive biophysical and pharmacological properties of class A (BI) calcium channel α_1 subunits, Neuron 11, 291.

Takahashi, T. and A. Momiyama, 1993, Different types of calcium channels mediate central synaptic transmission, Nature 366, 156.

Turner, T.J., M.E. Adams and K. Dunlap, 1992, Calcium channels coupled to glutamate release identified by ω-Aga-IVA, Science 258, 310.

Wheeler, D.B., A. Randall and R.W. Tsien, 1994a, Roles of N-type and Q-type Ca^{2+} channels in supporting hippocampal synaptic transmission, Science 264, 107.

Wheeler, D.B., R.W. Tsien and A. Randall, 1994b, Identification of calcium channels that control neurosecretion – Reply, Science 266, 830.

Wu, L.G. and P. Saggau, 1994a, Presynaptic calcium is increased during normal synaptic transmission and paired-pulse facilitation, but not in long-term potentiation in area CA1 of hippocampus, J. Neurosci. 14, 645.

Wu, L.G. and P. Saggau, 1994b, Pharmacological identification of two types of presynaptic voltage-dependent calcium channels at CA3-CA1 synapses of the hippocampus, J. Neurosci. 14, 5613.

Wu, L.G. and P. Saggau, 1995, Block of multiple presynaptic calcium channel types by ω-conotxin-MVIIC at hippocampal CA3 to CA1 synapses, J. Neurophysiol. 73, 1965.