Role of Presynaptic L-Type Ca\(^{2+}\) Channels in GABAergic Synaptic Transmission in Cultured Hippocampal Neurons

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INTRODUCTION

GABA (\(\gamma\)-aminobutyric acid) is the major inhibitory neurotransmitter in the mammalian CNS, where it acts on postsynaptic GABA\(_A\) receptors (Macdonald and Olsen 1994) and GABA\(_B\) receptors (Misgeld et al. 1995) resulting in fast and slow inhibitory postsynaptic potentials (IPSPs), respectively. The magnitude of GABAergic inhibition depends on the history of synaptic activation, and it has been established that paired-pulse depression of IPSPs is caused by GABA acting on presynaptic GABA\(_B\) autoreceptors (Davies et al. 1993; Deisz and Prince 1989), or by depletion of vesicles at the active zones (Jensen et al. 1998). We have recently reported that inhibitory postsynaptic currents (IPSCs) in cultured neurons are potentiated following tetanic activation of the GABAergic neuron (posttetanic potentiation, PTP) (Jensen et al. 1998). Following brief tetanization, IPSCs are enhanced by \(\sim\)60%, which declines to baseline over the course of 1 min. Further investigations indicated that PTP of IPSCs is caused by accumulation of Ca\(^{2+}\) ions in the GABAergic boutons, which raises the probability of release during the posttetanic period (Jensen et al. 1998).

Following excitation of the GABAergic neuron, GABA is released as a consequence of a highly localized and transient Ca\(^{2+}\) influx through N-type and P/Q-type voltage-dependent Ca\(^{2+}\) channels (VDCCs) in the presynaptic terminal (Ohnoshosaku et al. 1994). L-type VDCCs are not thought to participate in the control of transmitter release at GABAergic (Doze et al. 1995; Ohnoshosaku et al. 1994) or other synapses mediating fast synaptic transmission (Dutar et al. 1989; Horne and Kemp 1991; Wheeler et al. 1994). However, secretion of hormones or neuromodulators has been reported to depend on L-type VDCCs (Miller 1987; Perney et al. 1986). In the present report we have made dual whole cell recordings from cultured hippocampal neurons and elicited monosynaptic GABAergic IPSCs. We evoked PTP of the IPSCs and studied the effect of decreasing presynaptic Ca\(^{2+}\) influx using low Ca\(^{2+}\) \(1\)o, and the GABA\(_B\) receptor agonist, baclofen. Low [Ca\(^{2+}\)]\(_o\) caused a greater reduction in PTP than baclofen, which prompted us to hypothesize that L-type VDCCs deliver Ca\(^{2+}\) to the boutons during repetitive activity and thereby participate in the control of GABA release, which would represent a novel presynaptic regulatory mechanism at fast CNS synapses.

METHODS

Hippocampal culture preparation

Cultures of rat hippocampal neurons were prepared by standard techniques. Briefly, pregnant Sprague-Dawley rats were anesthetized by pentobarbital sodium (50 mg/kg ip) at gestational day 17–18. Fetuses were removed and decapitated, and the hippocampi were dissected free. The tissue was triturated mechanically in a HEPES-Fetuses were removed and decapitated, and the hippocampi were dissected free. The tissue was triturated mechanically in a HEPES-Fetuses were removed and decapitated, and the hippocampi were dissected free. The tissue was triturated mechanically in a HEPES-Fetuses were removed and decapitated, and the hippocampi were dissected free. The tissue was triturated mechanically in a HEPES-Fetuses were removed and decapitated, and the hippocampi were dissected free. The tissue was triturated mechanically in a HEPES-Fetuses were removed and decapitated, and the hippocampi were dissected free. 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CO₂-10% O₂ at 37°C (Brewer and Cotman 1989). Plating medium was fully replaced by 2 ml feeding medium after 1 day in vitro, and thereafter 1 ml was exchanged twice weekly. Feeding medium had the same composition as plating medium except that FCS was omitted and HS was reduced to 5%. The mitosis inhibitors 5'-fluoro-2'-deoxyuridine (FUDR; 15 µg/ml) and uridine (35 µg/ml) were added after 3–4 days when cultures showed a confluent background.

**Electrophysiology**

Coverslips with the cultured cells (10–30 days in vitro) were placed in a stainless steel chamber mounted on an inverted Nikon Diaphot 200 microscope, where individual neurons were visualized through ×200 Normarski optics. The chamber was perfused (1 ml/min) with an extracellular (control) medium containing (in mM) 140 NaCl, 3.5 KCl, 2.5 CaCl₂, 2.5 MgCl₂, 10 glucose, and 10 HEPES, adjusted with NaOH to pH 7.35 and 305 mosM/kg at 22°C. Patch-clamp electrodes (3–6 MΩ) were fabricated from borosilicate glass (1.2 mm OD) on a Flaming/Brown P-97 puller (Sutter Instruments). The presynaptic electrode contained (in mM) 130 CsCl₂, 1 CaCl₂, 2 MgCl₂, 11 EGTA, 10 HEPES, 0.10 leupeptin, and 2 MgATP, adjusted with CsOH to pH 7.3, 285 mosM/kg. The postsynaptic patch-electrode contained (in mM) 140 NaCl, 140 KOH, 1 CaCl₂, 2 MgCl₂, 11 EGTA, 10 HEPES, 0.10 leupeptin, and 2 MgATP, adjusted with methanesulfonic acid to pH 7.3, 285 mosM/kg. The postsynaptic patch-electrode contained (in mM) 130 CsCl₂, 1 CaCl₂, 2 MgCl₂, 11 EGTA, 11 EGTA, 10 HEPES, 0.10 leupeptin, and 2 MgATP, and 5 QX-314, adjusted with CsOH to pH 7.3, 285 mosM/kg. Dual whole cell recordings were made using Axopatch 200 and 200A amplifiers in the voltage-clamp mode at a h of −70 mV. Excitatory synaptic interactions between neurons were blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 µM) and dl-2-amino-5-phosphonovaleric acid (dL-APV; 50 µM). GABAergic neurons were located by screening cultures with whole cell recordings and investigating the presence of autaptic IPSCs. Single control stimulation pulses (to 0 mV for 3 ms) were delivered by a pulse-generator (Master 8, AMPI) to the presynaptic neuron at 0.2 Hz, and stimulus trains were delivered at 40 or 80 Hz for 1 or 2 s. Whole cell currents were low-pass filtered at 10 kHz, monitored on a penwriter (Servogor 220), digitized using an AD-converter (Instrutech VR 100 B) and stored on a VTR and a Pentium PC equipped with Clamplx (pClamp v. 6.0, software, Axon Instruments).

**Drug application**

Active substances were dissolved as stock solutions in distilled water at 1,000 times the final concentration and frozen. These were diluted in extracellular medium just before use and perfused through the bath (exchange time 2–3 min). Nifedipine was prepared as a stock solution dissolved in DMSO. When added to the control solution at its final concentration of 0.1%, DMSO had no effect on the synaptic transmission. (−)BayK 8644 and isradipine were prepared as stock solutions in 50% ethanol. Isradipine was applied from a three-barrel gravity-feed pipette (tip opening ~200 µm), which allowed application directly onto the neurons with exchange between control and isradipine solutions in ~1 s. Perfusion of 10 µM bicuculline from the third barrel rapidly abolished evoked IPSCs, showing that the perfusion covered the synaptic field completely. Experiments with dihydropyridines were performed in the dark. All changes in [Ca²⁺], were compensated by changes in [Mg²⁺], to keep the sum of divalent cations at 5.0 mM.

Drugs and chemicals were purchased from Sigma, except CNQX and dL-APV (Tocris Cookson), (−)BayK 8644 (RBI), and (±)-baclofen (a gift from Prof. Povl Krogsgaard-Larsen, Royal Danish School of Pharmacy). Culturing media were purchased from GIBCO, except for FUDR, uridine and poly-d-lysine, which were purchased from Sigma.

**Analysis**

IPSC amplitudes were measured on-line in Clampex, and off-line using Clamppit (pClamp program suite, Axon Instruments). All IPSCs were inspected visually and rejected if spontaneous activity disturbed the measurements. Tetanic depression of IPSCs during train stimulation was calculated as the percentage by which the plateau level was depressed with respect to the peak, which in numerical terms is given by: 100 − (Plateau/Peak) × 100. The peak amplitude was taken as the average of 10 single pretetanic IPSCs, including the 1st in the train. The plateau was calculated as the average GABA_A current during the train in the interval spanning from 300 to 400 ms for stimulation at 80 Hz, and 800 to 1,000 ms for stimulation at 40 Hz (indicated by heavy bars in the figures). PTP is presented as the percentage change in the amplitude of single IPSCs following the train in relation to the pretetanic level. All data are presented as means ± SE, with n indicating the number of pairs of neurons tested. Paired t-tests were used to assess differences between control and drug groups, and changes were considered to be significant at P-values < 0.05.

**RESULTS**

Paired whole cell recordings were used to record monosynaptic GABAergic IPSCs in cultured hippocampal neurons in the presence of CNQX (10 µM) and dL-APV (50 µM). The presynaptic GABAergic neuron and a postsynaptic neuron were both clamped at −70 mV (V_h). IPSCs were evoked by stimulating the GABAergic neuron with a step to 0 mV for 3 ms. In spite of inclusion of high-energy phosphates (MgATP) in the intracellular media, minor rundown of IPSC amplitudes was seen. We have measured rundown to be ~10% during the first 20 min of recording at low-frequency stimulation (unpublished observation).

**Effects of baclofen on tetanic depression and posttetanic potentiation of IPSCs**

The release of GABA from cultured hippocampal neurons is modulated by presynaptic tetanic stimulation (Jensen et al. 1998). During the tetanus, IPSCs display a use-dependent decrease in amplitude (tetanic depression). Following the train, GABA release evoked by low-frequency stimulation (0.2 Hz) is enhanced for ~60 s (PTP). We examined the effect of lowering the probability of release on tetanic depression and PTP of IPSCs in four pairs of neurons (Fig. 1). Tetanic stimulation (80 Hz for 1 s) of the presynaptic neuron was delivered in control solution and subsequently during bath perfusion of baclofen (10 µM). Baclofen depressed single IPSCs by 68.5 ± 4.7%. Tetanic depression was 20.0 ± 12% in the presence of baclofen, which was significantly less than for the control (55.4 ± 11.8%; P < 0.05). When IPSCs were normalized to the pretetanic baseline level, PTP was found to be 116.9 ± 10.6% in the presence of baclofen, which is 3.3 times larger than in control solution (35.9 ± 10.4%, P < 0.01; (Fig. 1Bb).

**Effects of changed [Ca²⁺]o on PTP**

Because baclofen inhibits Ca²⁺ influx through N- and P/Q-type Ca²⁺ channels (Hirata et al. 1995), we tested the effect of changing [Ca²⁺]o, which will alter presynaptic Ca²⁺ influx through all VDCCs. Perfusion of 1.2 mM Ca²⁺ (low Ca²⁺) depressed the pretetanic IPSCs by 71%, whereas PTP was enhanced from 55.4 ± 15% to 97.1 ± 12% (Fig. 2A, P < 0.05, n = 5). Upon perfusing 4.0 mM Ca²⁺ (high Ca²⁺), the prete-
tanic IPSCs were enhanced by 16%, whereas PTP was reduced from 51.8 ± 7% to 29.4 ± 11% (Fig. 2B, $P < 0.05, n = 5$). These results demonstrate an inverse relationship between release probability and PTP. Figure 2C shows a comparison between the effects of baclofen and low Ca$^{2+}$ on IPSCs and PTP. Both treatments depressed pretetanic IPSCs to the same extent ($P > 0.05$), indicating a similar depression of Ca$^{2+}$ influx through the Ca$^{2+}$ channels coupled to the rapid vesicle release. However, PTP was enhanced three times more by baclofen than by low Ca$^{2+}$ ($P < 0.05$). In addition to N- and P/Q-type channels, low Ca$^{2+}$ will decrease the influx through L- and T-type VDCCs. Because the voltage dependency and kinetic properties of T-type channels (Nooney et al. 1997) make it unlikely that these would make a marked contribution to presynaptic Ca$^{2+}$ influx during repetitive activity, we speculated that L-type VDCCs could be involved.

Effects of L-type Ca$^{2+}$ channel modulators on PTP

The selective L-type VDCC antagonist nifedipine (10 μM) had no effect on pretetanic IPSCs (which decreased by 3.9 ± 7% and is comparable with the usual run-down), but tended to reduce tetanic depression, which was 56.9 ± 9% in nifedipine, and 38.1 ± 14% in control ($P = 0.08, n = 6$, Fig. 3). Nifedipine reduced maximal PTP to 46.8 ± 11% compared...
antagonist, isradipine, which has been reported to have different effects on hippocampal synaptic plasticity than nifedipine (Christie et al. 1997; Mulkey and Malenka 1992). Isradipine (5 μM) had no effect on pretetanic IPSCs, which shows that neither nifedipine nor isradipine had nonspecific blocking effects on N- and/or P/Q-type VDCCs. When local perfusion with isradipine was started before the tetanic stimulation, the PTP curve area was depressed by 65.3 ± 7% (P < 0.01, n = 5, Fig. 4Cb), which was similar to nifedipine. This effect was due to a reduction in peak PTP and a shortening of PTP duration. Although it is quite likely that L-channel–mediated influx of Ca$^{2+}$ occurs during the train stimulation, this was ascertained by applying isradipine immediately after the train. Isradipine then had no effect on PTP compared with the control (P > 0.05, n = 2, Fig. 4Cb).

Perfusion of isradipine during the tetanic stimulation caused tetanic depression to increase to 63.4 ± 5% compared with 45.0 ± 8% for the control (P < 0.01, n = 5). When results from isradipine and nifedipine experiments were pooled, L-channel blockade was found to enhance tetanic depression by 46% (from 41.2 ± 8% in control, to 60.0 ± 5% in the presence of L-channel blockers, P < 0.005).

Finally, the effect of the L-type Ca$^{2+}$ channel “agonist” (−)BayK 8644 was tested on eight pairs of neurons. (−)BayK 8644 (4 μM) had no effect on pretetanic IPSCs, or on PTP evoked by a train of 40 Hz for 2 s (n = 3, not shown). Because the process underlying PTP is nearly saturated when evoked by 80 pulses (Jensen et al. 1998), we halved the tetanization to 40 Hz for 1 s (40 pulses), which evoked PTP of 31.6 ± 25%. (−)BayK 8644 then enhanced peak PTP to 60.2 ± 24% (P < 0.05, n = 5), without causing major changes in its time course (Fig. 5B).

**DISCUSSION**

**PTP depends on presynaptic Ca$^{2+}$ influx**

During high-frequency stimulation, IPSCs show tetanic depression, which is probably due to depletion of GABA-containing vesicles at the active zones (Jensen et al. 1998). Upon cessation of the tetanus, there was sometimes an increased rate of spontaneous IPSCs that lasted for 1–2 s (e.g., the neuron in Fig. 4A). This component of release has been analyzed in detail elsewhere (Jensen et al. 1998) and is caused by increased [Ca$^{2+}$]i in the boutons (Cummings et al. 1996). The increased [Ca$^{2+}$]i raises the probability of vesicle release and results in PTP of the GABAergic IPSCs (Jensen et al. 1998), similar to that seen at other central (Cummings et al. 1996; Griffith 1997) synapses. PTP is related to the pretetanic release probability and is correspondingly modulated by changes in [Ca$^{2+}$]i; in low Ca$^{2+}$, single pretetanic IPSCs are depressed relatively more than the posttetanic IPSCs, thereby enhancing PTP (Fig. 2A). The reverse is found on increasing [Ca$^{2+}$]i, which enhances single IPSCs and reduces PTP (Fig. 2B). Presynaptic GABA$_B$ receptors are negatively coupled by a G-protein mechanism to Ca$^{2+}$ channels that trigger vesicle release (Lambert and Wilson 1996; Thompson et al. 1993). When these are activated by baclofen, a qualitatively similar effect to that of low Ca$^{2+}$ was observed. These results demonstrate that PTP of IPSCs is inversely related to the probability of release.
In neuronal somata, baclofen preferentially depresses N-type Ca\(^{2+}\) channels (Lambert and Wilson 1996; Scholz and Miller 1991), whereas the release-depressant action of baclofen on GABAergic nerve cells involves an inhibition of N- and P/Q-type Ca\(^{2+}\) channels located at the secretory apparatus (Doze et al. 1995; Ohno-Shosaku et al. 1994). Our experiments on L-type VDCCs were prompted by the quantitative discrepancy between the effect of low Ca\(^{2+}\) and baclofen on PTP (Fig. 2C), which indicated that baclofen-insensitive Ca\(^{2+}\) channels also participate in PTP. That presynaptic L-type VDCCs contribute to the generation of PTP was demonstrated by the finding that both nifedipine and isradipine reduced peak PTP to a similar extent and shortened its duration. Neither blocker affected pretetanic IPSCs, confirming that L-type channels do not contribute to the Ca\(^{2+}\) influx responsible for transmitter release in response to a single stimulus (Wheeler et al. 1994). Accordingly, it has been suggested that L-type Ca\(^{2+}\) channels are located at some distance from the neurotransmitter release site (Miller 1987). We have also shown that the Ca\(^{2+}\) influx through L-channels supporting PTP occurs during, and not after, the train. This correlates well with the finding that the L-channels blockers increased tetanic depression.

The involvement of L-type channels was further substantiated by the finding that the selective L-type VDCC “agonist,” BayK 8644 increased PTP. BayK 8644 increases the probability of L-type channel openings, causes a negative shift in the voltage dependency so that channels open at more hyperpolarized potentials, and increases the number of available channels (Fisher et al. 1990). BayK 8644 increased PTP evoked by a submaximal stimulation (40 pulses), but not PTP evoked by a stronger stimulation (80 pulses). A likely explanation is that PTP evoked by 80 pulses is already saturated (Jensen et al. 1995).

**Involvement of presynaptic L-type Ca\(^{2+}\) channels in PTP**

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L-type Ca$^{2+}$ channels therefore make a substantial contribution to the Ca$^{2+}$ influx during tetanic stimulation. Despite the presence of endogenous and exogenous [i.e., EGTA or bis-(o-aminoophenxy)-N,N,N',N'-tetraacetic acid (BAPTA)] Ca$^{2+}$ buffers in the presynaptic neuron (unpublished observations), this raises the Ca$^{2+}$ level in the boutons and thereby the subsequent probability of release. L-type Ca$^{2+}$ channels have a relatively large unitary conductance (25 pS) (Fisher et al. 1990), and, although these channels seem to be located mainly at neuronal somata and dendrites (Westenbroek et al. 1990), a small number of channels at or near the synaptic boutons could give rise to an appreciable increase in [Ca$^{2+}$], because of the high surface area:volume ratio.

Because hippocampal GABAergic terminals are not accessible for direct patch-clamp recording, it is not possible to record single L-type Ca$^{2+}$ channel activity at this location. However, our data would indicate that functional L-type channels are present in GABAergic boutons and indeed play an important role in the regulation of transmitter release. Our results also indicate that presynaptic L-type VDCCs are not, to any large extent, inhibited by baclofen, because we found that baclofen-insensitive Ca$^{2+}$ channels participated in PTP.

Other evidence supporting the presence of L-type VDCCs in cultured nerve terminals is that stimulus-evoked Ca$^{2+}$ rises are only reduced by 40–70% by a combination of omega-conotoxins MVIIIC, omega-conotoxin NIVA, and omega-conotoxin MVIIA, which together block N-, P-, and Q-type channels (MacKenzie et al. 1996). If L-type channels make a substantial contribution to the residual 30–60% of the Ca$^{2+}$ rise, L-type antagonists would cause a corresponding reduction in the total Ca$^{2+}$ influx. We are currently using high resolution imaging techniques to see whether this can be detected.

Conclusions

The data presented here indicate that L-type Ca$^{2+}$ channels contribute to Ca$^{2+}$ accumulation in the presynaptic terminals during high-frequency activity in cultured GABAergic neurons, and thereby enhance transmitter release in posttetanic period. This represents a novel presynaptic regulatory mechanism at a fast CNS synapse. At the ultrastructural level, it will be interesting to search for L-type Ca$^{2+}$ channels at GABAergic and glutamatergic synaptic boutons.

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