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N-Type Calcium Channels and Their Regulation by $GABA_B$ Receptors in Axons of Neonatal Rat Optic Nerve

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Axons of neonatal rat optic nerves exhibit fast calcium transients in response to brief action potential stimulation. In response to one to four closely spaced action potentials, evoked calcium transients showed a fast-rising phase followed by a decay with a time constant of ~2–3 sec. By selective staining of axons or glial cells with calcium dyes, it was shown that the evoked calcium transient originated from axons. The calcium transient was caused by influx because it was eliminated when bath calcium was removed. Pharmacological profile studies with calcium channel subtype-specific peptides suggested that 58% of the evoked calcium influx was accounted for by N-type calcium channels, whereas L- and P/Q-type calcium channels had little, if any, contribution. The identity of the residual calcium influx remains unclear. GABA application caused a dramatic reduction of the amplitude of the action potential and the associated calcium influx. When $GABA_A$ receptors were blocked by bicuculline, the inhibitory effect of GABA on the action potential was eliminated, whereas that on the calcium influx was not, indicating involvement of $GABA_B$ receptors. Indeed, the calcium influx was inhibited by the $GABA_B$ receptor agonist baclofen. This baclofen effect was occluded by a previous block of N-type calcium channels and was unaffected by the broad-spectrum K^+ channel blocker 4-AP. We conclude that neonatal rat optic nerve axons express N-type calcium channels, which are subjected to regulation by G-protein–coupled $GABA_B$ receptors. We suggest that receptor-mediated inhibition of axonal calcium channels plays a protective role in neonatal anoxic and/or ischemic injury.

Key words: neonatal optic nerves; axons; calcium transient; $GABA_B$ receptors; G-protein; axon–glia signaling; calcium channel regulation

A transient increase in the intracellular Ca^{2+} concentration is involved in signal transduction in both excitable and nonexcitable cells (Clapham, 1995; Ghosh and Greenberg, 1995). In the CNS, activity-dependent calcium transients have been found to participate in such diverse processes as transmitter release, gene regulation, and synaptic plasticity. The generation of calcium transients usually involves voltage-dependent Ca^{2+} channels (VDCCs) (Dunlap et al., 1995).

In rat optic nerve, recent studies have revealed dynamic calcium signaling. Brief and prolonged electrical stimulation of the axons generate two types of calcium responses. For brief stimulations, Lev-Ram and Grinvald (1987) first resolved a fast calcium transient that was suggested to be caused by axonal calcium influx, and its inhibition by broad-spectrum calcium channel blockers such as Cd^{2+} suggested that it was mediated by calcium channels. More recently, when prolonged, repetitive stimulation was applied, a delayed glial response was resolved in the neonatal rat optic nerve (Kriegler and Chiu, 1993). These calcium signals are interesting, because no vesicular release events have been traditionally associated with CNS white matter, raising questions regarding the role of calcium in mediating axon–glia signaling.

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The present study focuses on the fast calcium transient and examines its regulation in CNS white matter using neonatal [postnatal day 2 (P2)–P7] rat optic nerve as the model system. The questions we are addressing consist of the following. First, where does the fast calcium transient originate, from axons or glia? Second, what are the axonal calcium channel subtypes that mediate these fast calcium transients? Third, are axonal calcium channels subjected to neurotransmitter-mediated modulation? These questions are gaining significance, because neurotransmitter-mediated signaling is now thought to occur in systems such as mammalian axonal tracts that lack the traditional vesicular means of neurotransmitter release (Chiu and Kriegler, 1994).

By devising dye-loading methods to label either glia or axons selectively, we show that the fast activity-dependent calcium transients originate from axons, thus confirming previous studies (Lev-Ram and Grinvald, 1987). Combining specific calcium channel blockers with the confocal-imaging technique, we establish that N-type calcium channels mediate most of the calcium transient with a smaller contribution possibly arising from R- and/or T-type calcium channels and/or reverse Na^+–Ca^{2+} exchange. P/Q- and L-type calcium channels do not seem to contribute to the calcium transient. Most interestingly, we show that the N-type axonal calcium channels are modulated directly by the neurotransmitter GABA via activation of $GABA_B$ receptors, raising the role of GABA in signal transduction in this nonvesicular pathway.

Parts of this paper have been published previously (Sun and Chiu, 1997, 1998).

MATERIALS AND METHODS

Animal preparation and dye loading. P2–P7 Sprague Dawley rat optic nerves were excised and laid down on the bottom of an experimental

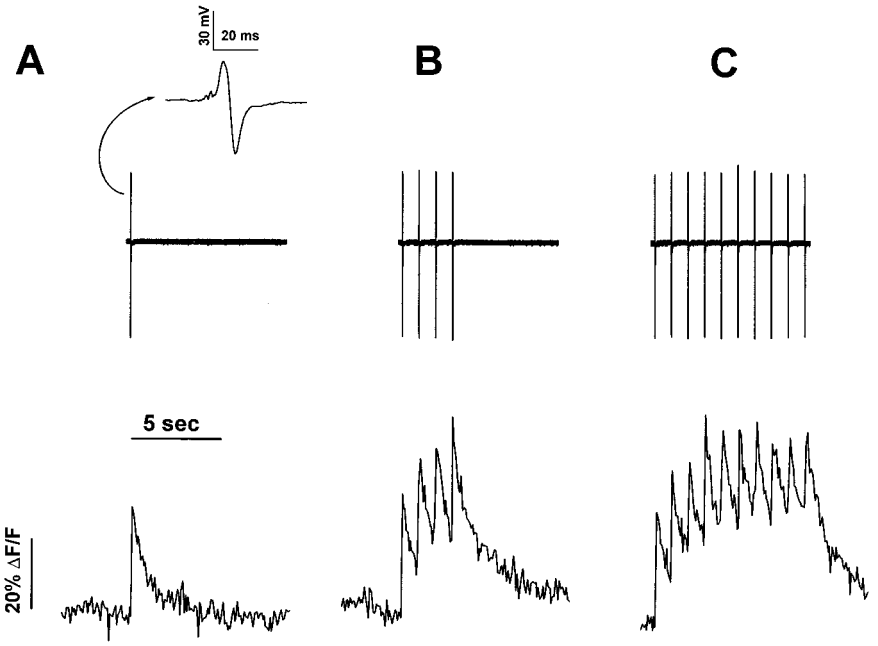

Figure 1. Calcium transients evoked by brief stimulation. *A–C*, Simultaneous recordings of action potentials (*top*) and calcium transients (*bottom*) evoked by a train of 1 (*A*), 4 (*B*), and 10 (*C*) stimulations. The calcium transient showed an abrupt rising phase followed by a slower decay to baseline with a time constant of ~2 sec. The intracellular calcium signal is calibrated as $\Delta F/F$. *A*, *Inset*, The shape of an action potential on an expanded time scale. Neonatal optic nerves were labeled with the standard whole-nerve–labeled protocol in which both axons and glial cells were stained (see Materials and Methods). Experiments were performed at room temperature for the data in this and all other figures.

perfusion chamber. The distal end of the nerve trunk was loosely sucked into a stimulating pipette, and the proximal end was sucked tightly into a recording pipette. Lukewarm agar solution (2.5%) was then carefully poured onto the nerve, forming a thin layer covering the nerve and immobilizing it during subsequent dye injection and imaging experiments. The chamber was immediately mounted on the stage of an Upright Nikon Diaphot microscope, and perfusion was then started. For dye labeling, a fine-tip glass pipette was back-filled with 50 μM calcium green-1 (AM form; Molecular Probes, Eugene, OR) made in Ringer's solution. The injection pipette was gently inserted into the middle part of the nerve trunk by piercing the outermost pial sheath, followed by injection of the dye into the extracellular space within the whole nerve by brief pulses of positive pressure delivered via a Picospritzer (General Valve, Fairfield, NJ). The entire procedure was monitored closely under the microscope. During each pressure pulse, the nerve trunk was seen to undergo a slight expansion around the injection site, and the dark-shaded solution could be seen driven in both directions along the longitudinal axis of the nerve. The injection pressure was carefully adjusted to minimize the nerve expansion associated with each injection pulse, and the nerve was judged to have recovered from this slight distention before another injection was given. The volume of solution delivered in each injection was estimated to be ~0.02 μl, and typically 15 such injections were given at approximately one injection per minute. During injections the bath containing the nerve was continuously perfused with oxygenated Ringer's solution at room temperature. After the final injection, the nerve was perfused for 90–120 min before imaging experiments began.

Confocal fluorescence imaging of intracellular calcium. After the nerve was stained with calcium indicators, calcium images could be viewed with either a 4× or 40× (Olympus Optical, Tokyo, Japan) objective lens on a Noran Odyssey confocal system (Odyssey, Noran Instruments, Middleton, WI). Calcium green-1 was excited with an argon laser at 488 nm, and confocal fluorescence images were monitored with a 500 nm long-pass emission filter. For electrical stimulation experiments, fast calcium signals were monitored near the video rate (30 Hz). Image acquisition and on-line calculations were controlled via the Metamorph software (Universal Imaging Corporation, West Chester, PA). Intracellular calcium concentration was reported as $\Delta F/F_0$ without calibration for absolute values. All experiments were done at room temperature (22–25°C).

Electrophysiology. Compound action potentials were evoked by a 125% supramaximal stimulus applied via the suction electrode to the cut end and were recorded from a second suction electrode at the other cut end. Compound action potential (CAP) data were analyzed using Pclamp 6.0 software (Axon Instruments).

Solutions and drugs. The optic nerve was normally bathed in a Ringer's solution that contained (in mM): NaCl, 129; KCl, 3; KH_2PO_4, 1.2; $CaCl_2$, 2.4; $MgSO_4$, 1.3; HEPES, 3; $NaHCO_3$, 20; and glucose, 10. Calcium-free solutions were prepared by replacing Ca^{2+} with Mg^{2+} and by adding EGTA (1 mM); pH was adjusted to 7.4 with NaOH or HCl as necessary. Nifedipine, verapamil, diltiazem, GABA, baclofen, ω-conotoxin-GVIA, and ω-conotoxin-MVIIC were purchased from Research Biochemicals (Natick, MA), and ω-Aga-IVA and ω-Aga-TK (ω-Aga-IVB) were purchased from Peptides International (Louisville, KY). All other compounds were from Sigma (St. Louis, MO). Sprague Dawley rats were from Harlan Sprague Dawley (Indianapolis, IN).

RESULTS

Calcium transients evoked by brief stimulation in neonatal optic nerves

Figure 1 shows simultaneous recordings of calcium transients and action potentials evoked by brief trains of action potentials. Brief stimulations were used to avoid eliciting a delayed glial response (Kriegler and Chiu, 1993). Figure 1*A–C* shows the calcium response triggered by a single stimulation, a train of 4 stimulations, and a train of 10 action potential stimulations, respectively. The fluorescence was collected from the whole field of view in an optic nerve stained with the calcium dyes. Even though both axons and glial cells were stained by our standard staining procedure (see Materials and Methods), the fluorescence changes evoked by brief stimulation arose primarily from axons, as shown by the selective labeling experiments below.

Figure 2*A* shows an experiment with a P6 rat optic nerve in which the axons, but not the glial cells, were selectively labeled with calcium green-1 hexapotassium salt (cell-impermeant form). Calcium indicators were introduced into the axons by transport and diffusion from the cut end via a tightly fitted suction electrode. Because the diameters of neonatal optic nerve axons are small, individual axons could not be resolved with the confocal system. Activity-dependent calcium transients were not only seen in such axon-only–labeled preparations (Fig. 2*A*), but they exhibited properties similar to those obtained from our standard whole-nerve–labeled preparations. Furthermore, the transient was reversibly blocked by 5 mM Ni^{2+}, suggesting that it may be mediated by calcium channels ($n = 3$). These results suggest that at least part of the fast calcium transients seen in our standard whole-nerve–labeled preparations originated from axons. We next selectively labeled glial cells with the cell-impermeant form of

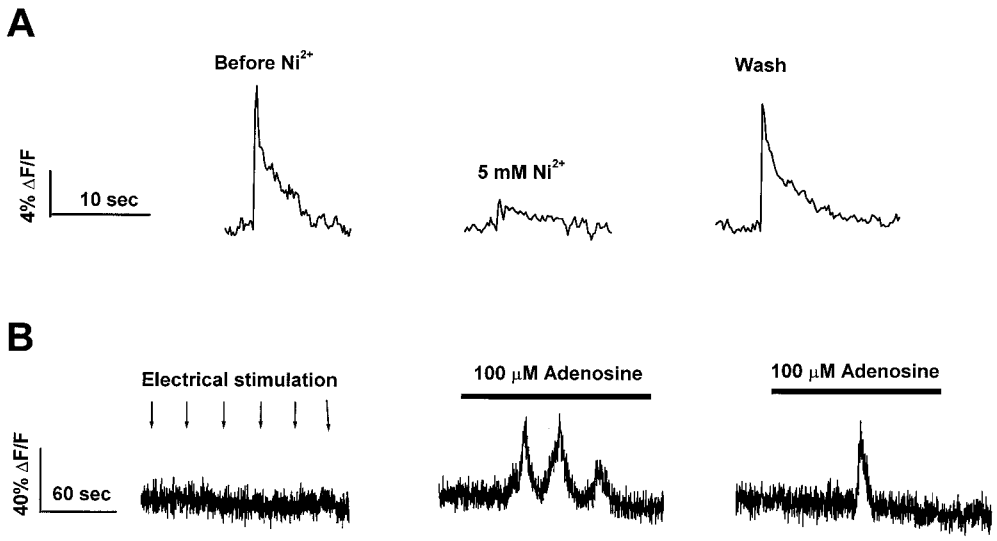

Figure 2. Selective labeling of axons and glial cells. *A*, Calcium transients evoked by single action potentials in a P6 rat optic nerve, in which the axons were selectively labeled with the calcium indicators by diffusion through the cut ends. For selective labeling of axons, the suction pipette enclosing the nerve end contained calcium green-1 hexapotassium salt (membrane impermeant) dissolved to a final concentration of 10 mM in a buffer containing (in mM): KCl, 150; $MgCl_2$, 4.6; $CaCl_2$, 0.1; EGTA, 1; HEPES, 10; Na-GTP, 0.4; and Na-ATP, 4, pH 7.3. A loading time of 4–5 hr was found to give adequate labeling of axons for detection of evoked calcium transients. In these experiments, the glial cells at the immediate vicinity of the loading pipette were stained, but unlike the axons, no transfer of the dye through glial gap junctions to the middle portion of the nerve (at which calcium imaging was performed) was seen. This may be caused either by closing of the gap junctions at the damaged end of the nerve or by a much slower diffusion rate of the dye through the gap junction channels compared with that along the axoplasm of axons. The calcium transient shows properties similar to that shown in Figure 1 (*left*), in which both axons and glial cells were labeled. The calcium transient was inhibited by Ni^{2+} (*middle*) and recovered after washing (*right*). *B*, A stimulation experiment in which only the glial cells were stained by impalement with a sharp microelectrode. Brief electrical stimulation failed to elicit a calcium transient (*left*). The sharp microelectrode contained calcium green-1 hexapotassium salt dissolved to a final concentration of 12 mM in 140 mM KCl plus 10 mM HEPES, pH 7.2. The glial cells were responsive to bath application of adenosine (*middle*, *right*).

calcium green-1 via single-cell impalement with sharp electrodes (Fig. 2*B*). Both the glial cell bodies and processes were well stained, and on several occasions dye-coupled cells were seen. However, brief nerve stimulation (one to four action potentials) evoked no fast calcium transients in either the soma or the glial processes (data shown in Fig. 2*B*, *left*, are from a glial process) (n = 10–12 cells per nerve in 4 optic nerves). The labeled glial cells appeared healthy during the experiment, as judged by their morphology, stable dye retention, and responsiveness to bath application of 100 μM adenosine [Fig. 2*B*, *middle*, *right*; experiments were separated by a 30 min wash (see Kriegler and Chiu, 1993)]. Taken together, these selective-labeling experiments indicate that the fast calcium transients in our standard whole-nerve–labeled preparations originated primarily from axons. In the remainder of this paper, the standard whole-nerve–labeling procedures were adopted, and the evoked calcium transients using brief stimulations were assumed to represent axonal signals.

The fast calcium transient is attributable exclusively to influx

The evoked calcium transient could result from either internal release and/or calcium influx. Two types of experiments demonstrated that the axonal calcium transients were caused by calcium influx. First, removing calcium from the bath solution abolished the transients (Fig. 3*A*) with little effect on the action potential (n = 4). Second, pharmacological manipulations of the internal calcium stores with ryanodine and thapsigargin had no effect on the calcium influx (data not shown).

N-type calcium channel is the major axonal channel subtype

The evoked calcium influx can be mediated by several mechanisms, one of which is axonal calcium channels. We therefore examined the effects of calcium channel blockers. Figure 3, *B* and *C*, shows that the evoked calcium transient was nearly completely eliminated by the broad-spectrum calcium channel blockers Cd^{2+} (50 μM) and Ni^{2+} (5 mM), respectively. Because these divalent cations also block the Na^+–Ca^{2+} exchanger, these results by themselves cannot distinguish between influx through axonal calcium channels and/or reverse Na^+–Ca^{2+} exchange. However, we found that the exchanger inhibitor bepridil (10, 50, and 100 μM) had no effect on the fast calcium transient (data not shown). To establish the role of axonal calcium channels further and to clarify the channel subtypes, we used more specific calcium channel blockers. Figure 3*D* shows the results of experiments with L-type calcium channel blockers (10 μM nifedipine, 50 μM diltiazem, and 50 μM verapamil). For nifedipine, varying the concentration between 5 μM (n = 2), 25 μM (n = 5), and 50 μM (n = 3) yielded similar results (data not shown). On the basis of the pharmacological profiles (summarized in Fig. 3*E*), L-type channels are probably not expressed on neonatal optic nerve axons. A more definitive pharmacological profile was obtained with specific peptide blockers (Fig. 4). Because of diffusion-barrier problems associated with bath application, we microinjected these peptides directly into the optic nerve trunk. To control for possible reduction in the evoked calcium transients caused by the trauma of the injection itself, we also performed sham injection as a control (injecting the vehicle solution only, without the peptides). We found that there was a brief (2–3 min) interval after the injection in which both the action potential and the calcium transient were depressed, presumably because of the trauma caused by the injection. However, in the sham control, both signals (action potential and the associated calcium transient) recovered to the preinjection level within 3–5 min. To eliminate potential errors attributable to the transient trauma, we compared the effects of peptide toxins at a fixed time of 15–20 min after injection.

The stimulation paradigm in all these peptide experiments is shown in Figure 4*D*, *left*. Each stimulation consisted of four

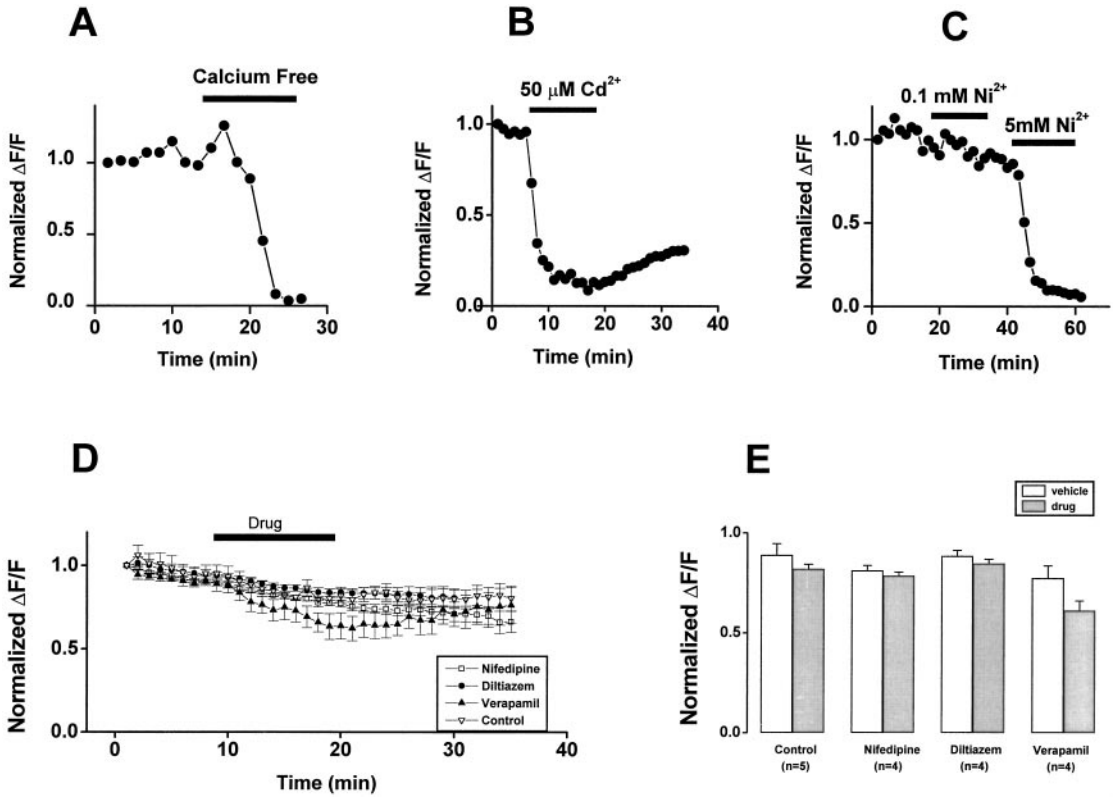

Figure 3. The evoked calcium transient is caused by influx sensitive to divalent cations but not to L-type channel blockers. *A*, The calcium transient was abolished in a calcium-free bath, indicating that it was caused by calcium influx. *B*, *C*, The inhibitory effect of divalent cations (Cd^{2+} and Ni^{2+}, respectively) on the calcium transient is shown. *D*, Experiments in which three types of L-type calcium channel blockers were tested are shown. The period during which drug was applied is indicated by the *horizontal bar*. *Symbols* represent relative calcium influx normalized to the first data point within each experiment. Stimulation frequency was 1/min in all cases. *E*, The results are summarized. The control and the drug result are not significantly different based on Student's *t* test and one-way ANOVA ($p > 0.05$). In each set of the experiments, the averaged peak calcium transient (every peak normalized to the first data point within each experiment) with and without the drug (indicated as drug and vehicle, respectively) is shown. The average value in the presence of drug was taken as the average of the last four data points during drug application just before wash. The average vehicle value was taken as an average of the last four data points before the application of the drug and the last four data points after wash, assuming a complete wash.

closely spaced action potentials. Also shown (Fig. 4*D*, *right*) is its associated calcium transient (note that the action potentials were so closely spaced that the calcium responses fused together to form a single calcium transient). As shown in Figure 4*A*, the specific N-type calcium channel blocker ω-conotoxin-GVIA (10 μM in the injecting pipette) blocked the calcium transients by ~58% ($n = 6$). Another peptide, ω-conotoxin-MVIIC (10 μM in the injecting pipette) showed a 65% block ($n = 5$) (Fig. 4*B*). The bigger effect of ω-conotoxin-MVIIC probably reflects its blockade of N-type as well as non-N-type calcium channels (Sather et al., 1993; Wheeler et al., 1994). We further tested for the presence of P-type channels by using the highly specific blockers ω-Aga-IVA (1 μM in the injecting pipette; $n = 5$) and ω-Aga-IVB (1 μM in the injecting pipette; $n = 3$). As shown in Figure 4*C*, ω-Aga-IVA had no effect (a similar result with ω-Aga-IVB was obtained; data not shown). Figure 4*E* summarizes the results. It is important to point out that in the above experiments, the peptides had little effect on the shape and amplitude of the action potentials (Fig. 4*A–C*). It has been reported that ω-conotoxin-MVIIC produced a gradual increase in the CAP area over 130 min of experiment (Fern et al., 1995). We have not observed such an effect in our experiments. The discrepancy could be caused either by the age of the animal (neonate in our case vs adult in their case) or by the duration of the experiment (45 vs 130 min). The mechanism that mediates the residual calcium transient remains to be resolved.

Regulation of axonal calcium channels

A number of studies indicates that neurotransmitter receptors are present along the main course of both nonmyelinated (Brown and Marsh, 1978; Agrawal and Evans, 1986) and myelinated (Allan et al., 1980; Morris et al., 1983; Bhisitkul et al., 1987, 1990) peripheral axons. Moreover neurotransmitter receptors have been suggested to be present on axons in the CNS (Simmonds, 1983; Sakatani et al., 1991a,b, 1992; Honmou et al., 1993). For example, Sakatani et al. (1992) have shown that in the neonatal rat optic nerve, GABA modulates the axonal impulse activity via $GABA_A$ receptors. Because voltage-dependent calcium channels are known to be modulated by receptors in the presynaptic terminals, an interesting question is whether axonal calcium channels are also subjected to such modulation. Our experiments showed that GABA (1 mM) reduced the amplitudes of both the action potentials and the evoked calcium transients (Fig. 5*A*,*B*). This is consistent with the presence of axonal $GABA_A$ receptors, activation of which shunted the action potential, causing a reduction in its amplitude, which then indirectly caused a reduction in the calcium transient (Sakatani et al., 1992). Yet two lines of evidence indicated that GABA could also exert a direct action on the

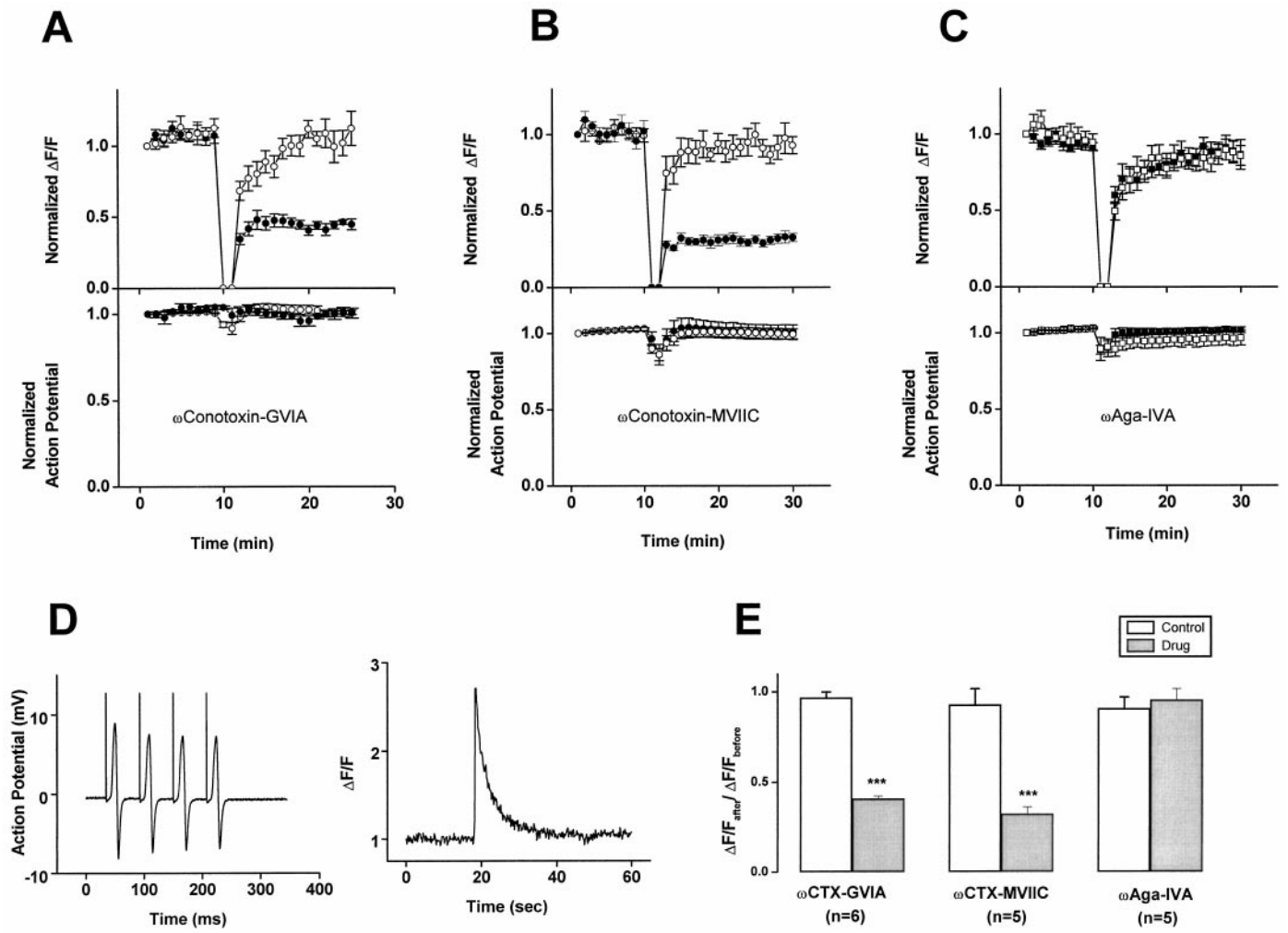

Figure 4. Effect of peptide blockers. *A*, The peak calcium transient (*top*) and the amplitude of action potentials (*bottom*) before and after injection of ω-conotoxin-GVIA (*ω-CgTX-GVIA*; *solid circles*). Control experiments with sham injections (vehicle solutions without drugs) are shown as *open circles*. *ω-CgTX-GVIA* had a significant inhibitory effect on the evoked calcium transient. *B*, *C*, Similar experiments with ω-conotoxin-MVIIC (*ω-CgTX-MVIIC*) and ω-Aga-IVA, respectively. *Open circles* represent sham controls, and *solid circles* represent peptide injections. In contrast to ω-conotoxin-GVIA (*A*) and ω-conotoxin-MVIIC (*B*), ω-Aga-IVA (*C*) had no effect on the evoked calcium in axons, indicating that P/Q-type calcium channels are probably absent. *D*, The stimulation paradigm used for the peptide studies. The nerve was stimulated every minute with a train of four action potentials (*left*). The associated calcium transient is shown on the *right*. Note that the calcium response from each action potential fused together because of the close spacing of the action potentials. *E*, Summary. *ω-CgTX-GVIA* and *ω-CgTX-MVIIC* blocked the calcium transient by 57.9 ± 1.6% (Student's *t* test, $p < 0.001$) and 65.2 ± 4.2% (Student's *t* test, $p < 0.001$), respectively. ω-Aga-IVA had no effect on the calcium transient (Student's *t* test, $p > 0.05$). $\Delta F/F_{before}$ and $\Delta F/F_{after}$ were calculated as the average of the last four data values (normalized to the first data point within each experiment) before and after the drug application.

calcium transient unrelated to the reduction in the action potential amplitude. First, as shown in Figure 5*C*, GABA would still cause a 50% reduction in the calcium transient even when the amplitude of the action potential was prevented from being shunted (reduced) by blocking the $GABA_A$ receptors with a saturating concentration of bicuculline. This reduction of calcium transient in the absence of functional $GABA_A$ receptors suggested the involvement of $GABA_B$ receptors. This was confirmed by the observation that baclofen, a specific $GABA_B$ receptor agonist, caused a reduction in the calcium transient without changing the shape or amplitude of the action potentials (Fig. 6*A*,*B*).

The dose–response curves of baclofen and GABA are shown in Figure 6*C*. The IC_{50} for baclofen was ~1 μM. This value is close to the value measured at the granule cell–Purkinje cell synapse in rat cerebellar slices [IC_{50} = 1.4 μM (Dittman and Regehr, 1996)] and to that measured at the synapse at the calyx of Held [IC_{50} = 0.77 μM (Takahashi et al., 1998)]. The IC_{50} for GABA is higher than that for baclofen, which resembles what has been found at the calyx of Held synapse (Takahashi et al., 1998).

$GABA_B$ receptor activation regulates N-type calcium channels

The above experiments suggested that axonal calcium channels were regulated by $GABA_B$ receptor activation but left unspecified the channel subtype being regulated. To examine this issue, we performed occlusion experiments with the N-type channel blocker ω-conotoxin-GVIA. Figure 7*A* shows an experiment in which baclofen was first applied to induce an ~50% inhibition of the calcium transient. After washing away baclofen, ω-conotoxin-GVIA was applied to block N-type calcium channels, which also resulted in a similar reduction in the calcium transient. Now, when baclofen was added in addition to ω-conotoxin-GVIA, little additional inhibition was observed. This experiment thus strongly

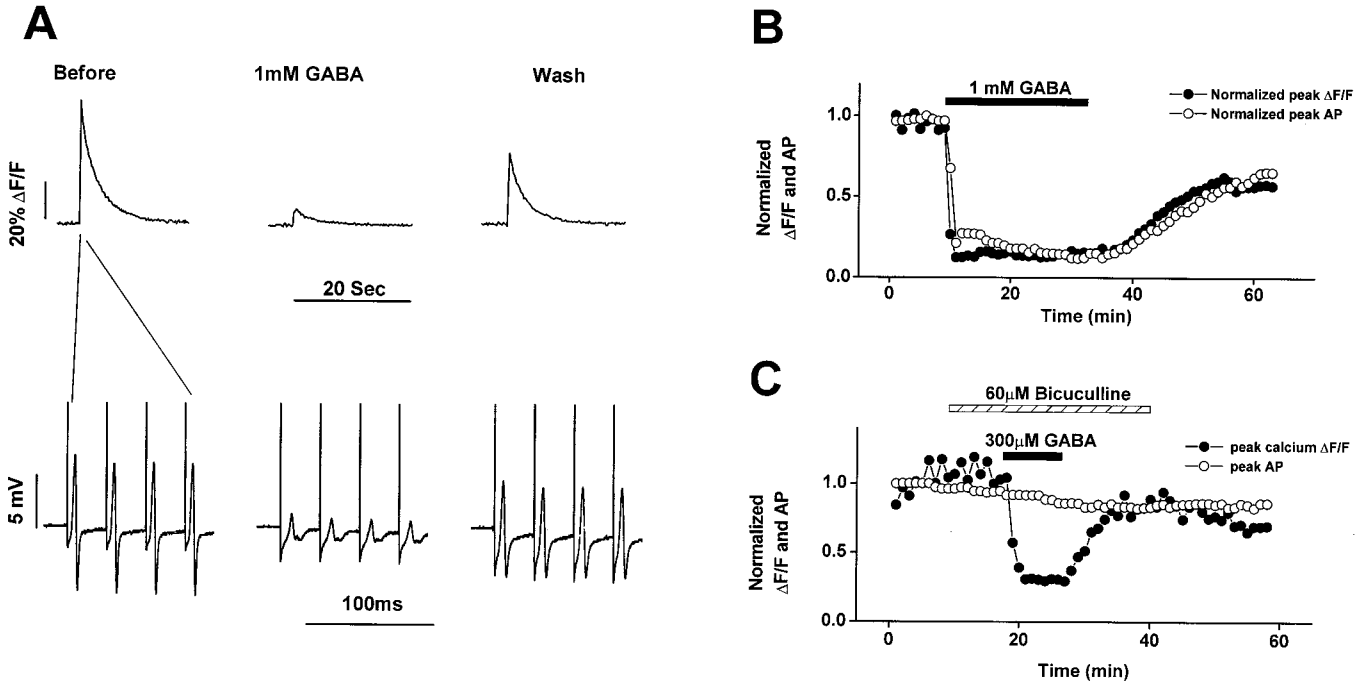

Figure 5. The action of GABA on the action potential and the evoked calcium transient. *A*, Simultaneous recordings of action potentials (*bottom*) and calcium transients (*top*) before (*left*), during (*middle*), and after (*right*) GABA (1 mM) application. The nerve was stimulated with a train of four closely spaced action potentials, resulting in a fused, single calcium transient. *B*, Normalized amplitudes of the calcium transient and the action potential during bath application of 1 mM GABA. *C*, Effects of GABA on the calcium transient when $GABA_A$ receptors were blocked by bicuculline. Bicuculline blocked the GABA-mediated reduction in the action potential but only partially relieved the inhibition of the calcium transient. This indicates possible involvement of $GABA_B$ receptors in the inhibition of the calcium transient.

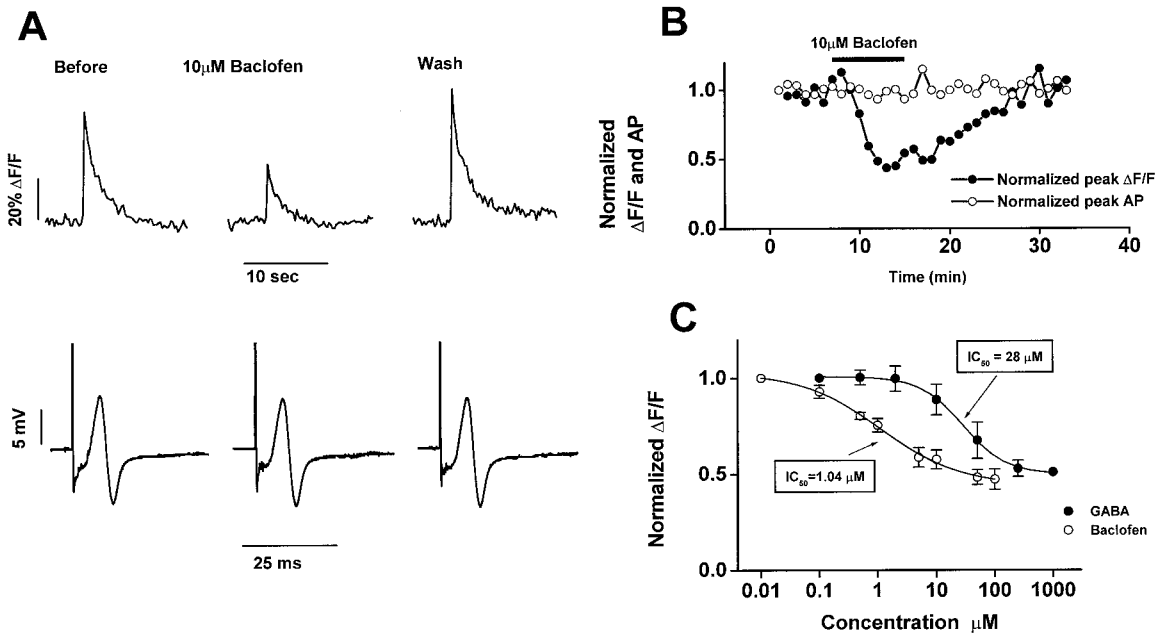

Figure 6. Baclofen inhibits the evoked calcium transient without affecting the action potentials. *A*, Simultaneous recordings of a single action potential (*bottom*) and its associated calcium transient (*top*) during baclofen application. Note that baclofen inhibited the calcium transient without affecting the waveform and amplitude of the action potential. The effect of baclofen is fully reversible. *B*, Normalized amplitude of the calcium transient and the action potential in an experiment in which 10 μM baclofen was applied. *C*, Dose response of baclofen- and GABA-mediated inhibition of the evoked calcium transient. The dose–response curve was fitted to a function of the form: $A_\infty + (100 - A_\infty)/[1 + ([\text{drug}]/IC_{50})^n]$, where $n = 1$. For baclofen, $A_\infty = 49\%$, and $IC_{50} = 1.0$ μM; for GABA, $A_\infty = 51\%$, and $IC_{50} = 28$ μM.

suggested that both baclofen and ω-conotoxin-GVIA acted on the same population of calcium channels. When this population was first maximally blocked by ω-conotoxin-GVIA, it occluded the subsequent action of baclofen. Figure 7*B* summarizes the results, showing that the baclofen-sensitive component in the calcium transient (expressed as a percent of the original total calcium transient) was significantly diminished after a previous application of ω-conotoxin-GVIA. Thus, we conclude that the major channel subtype being regulated by baclofen is the N-type calcium channel.

Evidence that baclofen directly targets N-type calcium channels on axons

How might $GABA_B$ receptor activation cause a reduction in the calcium transient? There are two categories of mechanism. The first is that $GABA_B$ receptor activation induces an increase in axonal K^+ conductance, which accelerates the action potential repolarization, thereby leading to a secondary inhibition of the calcium transient by reducing the degree of calcium channel activation. Various examples have been reported in the literature that certain K^+ channels are linked to G-protein–coupled receptors, such that activation of the receptors leads to an enhancement of K^+ channel conductance. The second mechanism is that $GABA_B$ receptor activation directly targets the calcium channels. We believe that our data are best explained by this second mechanism. Based on our data, a direct action of baclofen on K^+ channels is unlikely, because there was no change in the shape and amplitude of the compound action potential after baclofen application (see Figs. 6*B*, 8*C*). Furthermore, when a broad-spectrum K^+ channel blocker (4-AP) was applied to block axonal K^+ channels, the inhibitory effect of baclofen on the calcium transient remained unaffected (Fig. 8*A*,*B*). When tetraethylammonium chloride (TEA, 2.5 mM) was coapplied with 4-AP (1 mM) to block axonal K^+ channels further, the inhibitory action of baclofen on the calcium transient persisted ($n = 3$; data not shown). These experiments therefore suggest that calcium channels, rather than K^+ channels, are the targets of $GABA_B$ receptor activation. Evidently, $GABA_B$ receptor activation is linked to axonal calcium channel inhibition. An interesting issue is that the waveform of the action potential, which was normally insensitive to baclofen application (Fig. 8*C*), became sensitive after 4-AP treatment (Fig. 8*D*). In particular, baclofen selectively inhibited a delayed, secondary hump in the action potential that appeared after 4-AP application (Fig. 8*D*). This suggested that calcium channel activation normally does not contribute to the action potential waveform but becomes important when the action potential is prolonged after K^+ channel blockage. The delayed inward calcium current apparently contributed to the secondary hump in the 4-AP–treated action potential. This interpretation was corroborated by application of Cd^{2+} (50 μM; $n = 3$), which also inhibited the secondary hump in the 4-AP–treated action potentials (Fig. 8*E*). In addition, injection of the N-type calcium channel blocker ω-conotoxin-GVIA (10 μM in the injecting pipette) partially inhibited the secondary hump (Fig. 8*F*). When baclofen was applied after ω-conotoxin-GVIA, no significant further inhibition was observed (Fig. 8*G*), as compared with baclofen application alone (Fig. 8*D*). This is consistent with baclofen and ω-conotoxin-GVIA targeting the same population of calcium channels (N-type), as suggested previously by the calcium-imaging experiment in Figure 7. Furthermore, complete removal of bath calcium (with a calcium-free saline solution plus 1 mM EGTA) caused a significant reduction in the secondary hump (Fig. 8*H*). Interestingly, the secondary hump was not completely eliminated. One reason might be that calcium channels become markedly permeable to monovalent cations like sodium and potassium after external calcium removal (Almers and McCleskey, 1984).

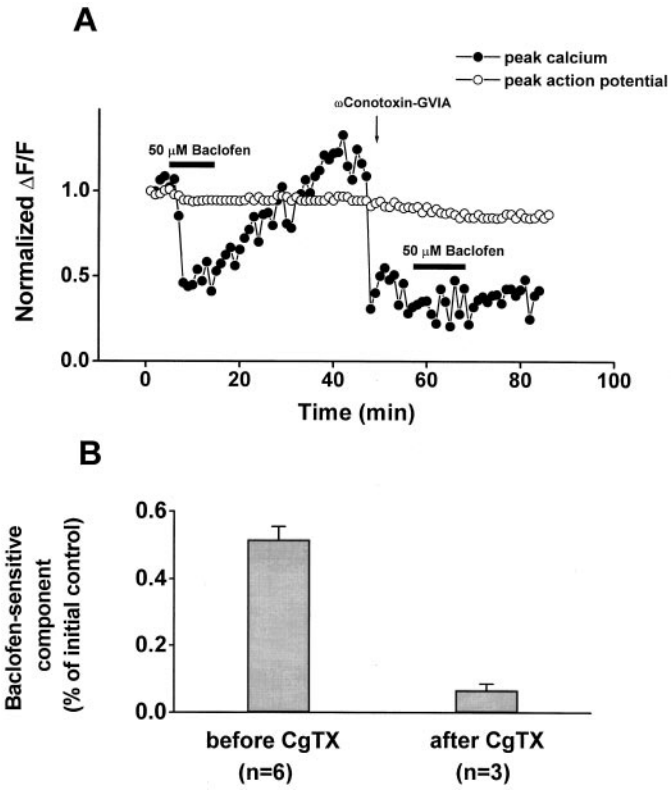

Figure 7. ω-Conotoxin-GVIA occludes the action of baclofen. *A*, Previous application of ω-conotoxin-GVIA occluded the action of baclofen. *B*, Summary of the occlusion experiments is shown. Baclofen (50 μM) alone inhibited 52% of the total calcium transient ($n = 6$). The same concentration of baclofen inhibited <7% of the total calcium transient after blockade of N-type calcium channels with ω-conotoxin-GVIA (*CgTX*).

DISCUSSION

In the present study, confocal laser-scanning microscopy has been used to investigate dynamic changes in $[Ca^{2+}]_i$ evoked by brief electrical activity in isolated neonatal rat optic nerves. By selectively labeling axons or glial cells with calcium dyes, we have demonstrated that the evoked calcium transients originate from axons. Using pharmacological profiles with various calcium channel subtype blockers, we have shown that a major portion of the axonal calcium influx is mediated by N-type calcium channels. Most interestingly, the N-type calcium channels are modulated by $GABA_B$ receptor activation. Our demonstration of a fast calcium entry mechanism (e.g., N-type calcium channels) and associated regulatory machinery along mammalian CNS axons raises important questions on the functional role of neurotransmitter-mediated signaling in nonsynaptic regions of the brain.

Axonal calcium transients induced by electrical activity

Activity-dependent calcium signals in rat optic nerves were first demonstrated by Lev-Ram and Grinvald (1987) and further studied by Kriegler and Chiu (1993). Recently, calcium-imaging studies have also been performed in other preparations such as Purkinje cell axons in rat cerebellar slices (Callewaert et al.,

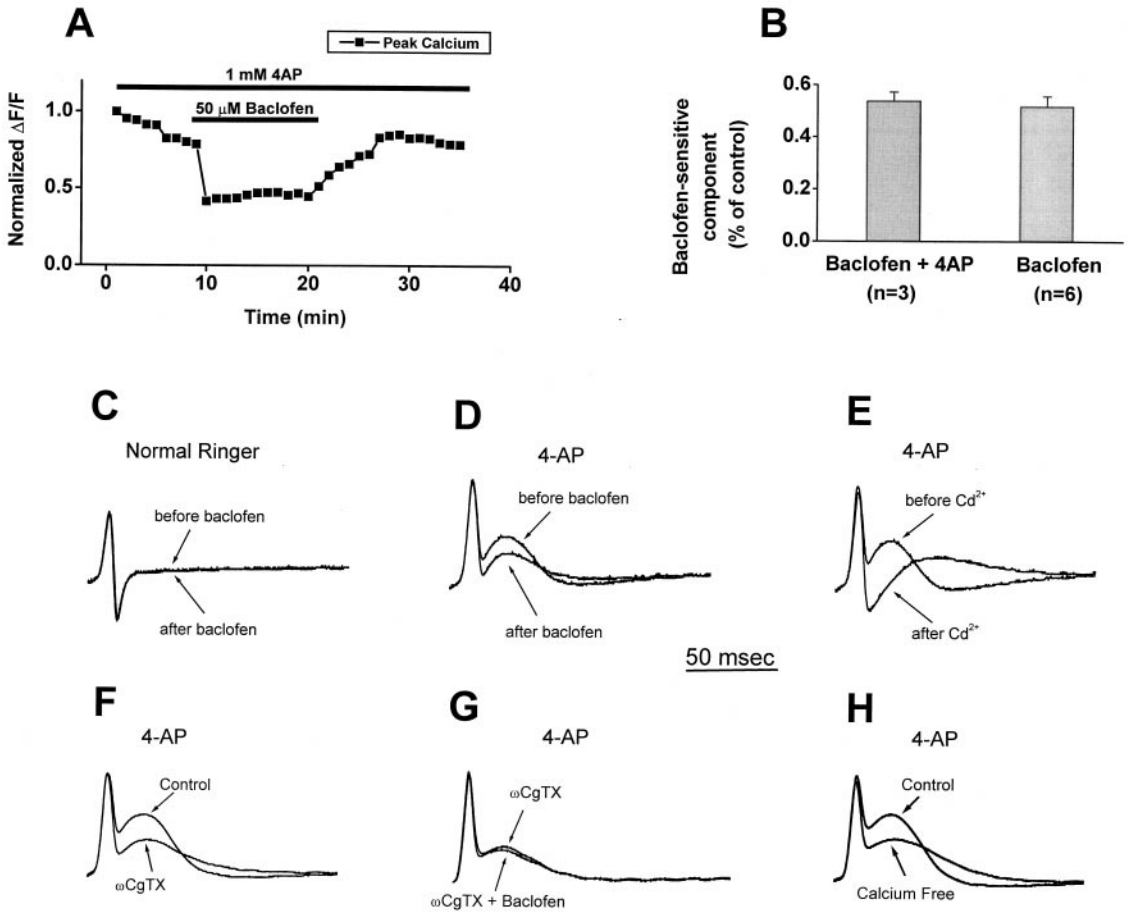

Figure 8. Blocking K^+ channels has no effect on baclofen-mediated inhibition of calcium transient. *A*, Blocking K^+ channels with the broad-spectrum blocker 4-AP (1 mM) had no effect on baclofen-mediated inhibition of calcium influx. *B*, Summary of baclofen-mediated inhibition with or without 4-AP is shown. Baclofen (50 μM) inhibited 52% of the calcium transient without 4-AP ($n = 6$) and 53% with 4-AP ($n = 3$). *C*, *D*, Effects of baclofen on the waveform of the action potential with (*D*) or without (*C*) 4-AP treatment are shown. Note that in the presence of 4-AP, a delayed secondary hump was unmasked in the action potential that was sensitive to baclofen. *E*, This secondary hump was related to calcium channel activation, because it could be blocked by cadmium. *F*, The effect of ω-conotoxin-GVIA (*ω-CgTX*) on the secondary hump of the action potential in the presence of 4-AP is shown. *G*, Baclofen application, after *ω-CgTX* treatment, had little or no effect on the secondary hump of the action potential. *H*, The effect of removal of bath calcium on the secondary hump is shown. Bath calcium was removed by perfusing a bath saline solution with 0 calcium and 1 mM EGTA for 15–60 min until the steady-state effect was observed. Note that in these experiments with 4-AP, we stained the preparation with a low-affinity calcium indicator (Oregon green 488-BAPTA-5N/AM) to avoid calcium signal saturation. This was necessary because 4-AP greatly increased the amplitude of the evoked calcium transient.

1996) and adult rat vagus nerve (Wachtler et al., 1998). The fast calcium transient in rat optic nerve has been suggested to originate from axons (Lev-Ram and Grinvald, 1987; Kriegler and Chiu, 1993). Similar results were obtained by Wachlter et al. (1998) in rat vagus nerve. In their calcium-imaging analysis of Purkinje axons, Callewaert et al. (1996) were able to resolve the Purkinje cell bodies, their axons, and dendrites by labeling single cells with patch pipettes. It was shown that the axonal calcium transient was mediated by influx through P-type calcium channels, the same channel type found at the soma. In this study, we found that N, but not P, is the main channel type on the optic nerve axon. This suggests that the expression of axonal calcium channel subtypes is differentially regulated in different brain regions.

Modulation of calcium channels by neurotransmitters

Because of the importance of calcium in the nervous system, modulation of voltage-dependent calcium channels seems to be an important means to enrich signaling diversity. There are several mechanisms by which VDCCs can be modulated, among which coupling to G-protein–coupled receptors has been the one subjected to the most study (Dolphin, 1998; Zamponi and Snutch, 1998). Although the detailed molecular pathways linking G-protein–coupled receptors to inhibition of VDCCs remain to be worked out, it is generally thought that this kind of modulation contributes significantly to important processes such as presynaptic inhibition (Wu and Saggau, 1997; Miller, 1998). Such inhibition may serve to fine tune synaptic strength, achieve synaptic depression, and prevent excessive transmitter release.

Current studies on calcium channel modulation have focused extensively on the neuronal cell soma and the nerve terminal. In contrast, little is known about calcium channel modulation on axons. Our data provide the first direct evidence that neurotransmitter-mediated regulation of calcium channels exists on axons and that the molecular mechanism of the axonal modulation is similar to that described for the synapse. Hence, the axon might be more than a passive conduit for relaying information between the cell body and the synapse and might be capable of dynamic signal integration. Furthermore, neurotransmitter-mediated inhibition of axonal calcium channels might play a

protective role against anoxic and/or ischemic injury in the CNS white matter. These physiological and pathophysiological implications of our findings are discussed below.

Calcium channels and the modulation of axonal excitability

Although the physiological functions of activity-dependent calcium influx are primarily unknown at present, several roles are possible. One hypothesis is that axonal calcium channels modulate axonal excitability via calcium-activated K^+ channels, especially under conditions of large calcium influx during repetitive stimulation. It has been shown that axonal Ca^{2+} transients regulate the frequency (Callewaert et al., 1996) and speed (Luscher et al., 1996) of propagation of action potentials, possibly by activating Ca^{2+}-dependent K^+ channels. A Ca^{2+}-dependent K^+ conductance has been reported in the rat optic nerve (Lev-Ram and Grinvald, 1986). If the rapid buildup of axonal $[Ca^{2+}]_i$ during a train of action potentials activates a Ca^{2+}-dependent K^+ conductance and thereby effectively decreases membrane excitability, then inhibition of either Ca^{2+} buildup or Ca^{2+}-dependent K^+ channels will upregulate the axonal excitability. Interestingly, norepinephrine has been shown to increase the neonatal optic axonal excitability by activating β-1 adrenoceptors in a calcium-dependent manner (Honmou and Young, 1995). Although neither apamin nor TEA blocked the norepinephrine-mediated effect, the authors pointed out that other Ca^{2+}-dependent K^+ channels might still be the possible targets. In fact, neurotransmitters such as norepinephrine, serotonin, ACh, and glutamate have all been shown to inhibit one particular type of Ca^{2+}-activated K^+ conductance (which is insensitive to TEA and apamin), leading to a reduction in spike-frequency adaptation and increased membrane excitability (Nicoll, 1988; Sah, 1996).

Another possible function of axonal calcium channels is to modulate branch point failures in an axonal tree. In an axonal tree with extensive branching, information flow to the nerve terminal can be spatially and temporally regulated by modulation of branch point failures (Swadlow et al., 1980). Recent calcium image analysis of the axonal tree of the basket cells in the cerebellum revealed local calcium hot spots at axonal branch points as well as at nerve terminals (Llano et al., 1997). It is unclear whether calcium channels are clustered at the branch points to produce the local calcium elevation. Branch points are sites of impedance mismatch (Swadlow et al., 1980; Wall, 1995), and action potential propagation there might be particularly sensitive to local excitability changes. It is possible that calcium channels, if localized at branch points, may profoundly influence the direction of information flow within an axonal tree.

With respect to neurotransmitter-mediated regulation of axonal calcium channels, where does the transmitter come from? In the neonatal rat optic nerve, it has been demonstrated that GABA immunoreactivity was present at high levels in astrocytes as well as in the axons (Sakatani et al., 1992; Rogers and Pow, 1995). Intriguingly, the GABA immunoreactivity in astrocytes declined with development (Sakatani et al., 1992). Thus, abundant glial and axonal sources of GABA are present in neonatal rat optic nerves. How might GABA be released, given the general lack of vesicular means of neurotransmitter release in axonal tracts? One release mechanism suggested by Chiu and Kriegler (1993) is reverse operation of neurotransmitter transporters. For example, most transporters are driven by Na^+ gradients and are electrogenic. During repetitive activity, large shifts in ionic gradients coupled with depolarizations might drive GABA transporters in the optic nerve to release GABA. There is evidence that three subtypes of GABA transporter are differentially expressed in both neonatal and adult rat optic nerves (Howd et al., 1997).

The role of axonal calcium channels in anoxic and/or ischemic injury in mammalian white matter

Recent studies have demonstrated that excessive calcium influx into axons during anoxia is a major cause of anoxic and/or ischemic injury in mammalian CNS white matter (Stys et al., 1992; Fern et al., 1995, 1996; Stys, 1996). Various pathways for injurious calcium influx have been suggested, including reverse operation of the Na^+–Ca^{2+} exchanger and various types of axonal calcium channels. Indeed, blockers of both calcium channels and the Na^+–Ca^{2+} exchanger have been reported to have protective effects in ischemic damage in optic nerves (Stys et al., 1992; Fern et al., 1995). Most intriguingly, neurotransmitters such as adenosine and GABA were found to be protective, and the effect of GABA was mediated by $GABA_B$ receptors (Fern et al., 1994). The protective action of these neurotransmitters is apparently related to their ability to mediate an inhibition of calcium influx into axons, but the molecular linkage between GABA and inhibition of calcium influx remains unclear. One hypothesis is that $GABA_B$ receptor activation has a downstream effect of inhibiting the Na^+–Ca^{2+} exchanger, hence prohibiting it from mediating injurious calcium influx (Fern et al., 1996). Our finding that $GABA_B$ receptor activation leads to N-type calcium channel inhibition provides a novel hypothesis for the protective effect of GABA. We speculate that GABA, which is known to be released under anoxic and/or ischemic conditions (Anden et al., 1989; Shimada et al., 1993), mediates an inhibition of N-type axonal calcium channels that confers protection to the neonatal white matter against anoxic and/or ischemic injury.

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