Internal Calcium Modulates Apparent Affinity of Metabotropic GABA Receptors

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Shen, Wen and Malcolm M. Slaughter. Internal calcium modulates apparent affinity of metabotropic GABA receptors. J. Neurophysiol. 82: 3298–3306, 1999. The metabotropic GABA receptor (GABA_B) regulates calcium influx in neurons. Whole cell voltage-clamp techniques were employed to determine the effects of internal calcium on the activity of GABA_BRs. GABA_B receptor apparent affinity was maximal when bis-(o-aminophenoxy)-N,N',N'-tetraacetic acid (BAPTA) maintained internal calcium below 70 nM. Apparent affinity was reduced as internal calcium increased. EGTA did not produce similar effects, suggesting that localized increases in calcium influenced GABA_B apparent affinity. Confocal imaging disclosed relatively high internal calcium just below the plasma membrane of isolated neurons. BAPTA, but not EGTA, reduced this ring of high calcium. Heparin, dantrolene, and ryanodine increased GABA_B apparent affinity, effects similar to that of BAPTA. Calmodulin inhibitors also increased receptor apparent affinity. These results suggest that internally released calcium activates calmodulin, which reduces GABA_B apparent affinity. This identifies a reciprocal system in which the metabotropic GABA receptor can reduce calcium influx, but internal calcium can suppress this receptor pathway. Metabotropic glutamate receptors linked to inositol 1,4,5 trisphosphate (InsP3) raised internal calcium and suppressed the action of GABA_BRs. Thus negative feedback systems control the balance between excitatory and inhibitory metabotropic receptor pathways in retinal neurons.

INTRODUCTION

The balance between excitatory, glutamatergic synapses, and inhibitory, GABAergic synapses determines the excitability of many neurons in the CNS. Commonly, this interaction can be described by the relative conductances produced by these two transmitters acting at ionotropic receptors. Both transmitter systems also activate metabotropic receptors, yet little is known about interactions through second-messenger systems. Retinal ganglion cells are the final integrators of visual information in the retina, and they perform this function by combining the inputs from a number of receptors, including ionotropic and metabotropic receptors for both GABA and glutamate. Thus they are an ideal substrate for studying interactions between excitatory and inhibitory pathways.

Metabotropic GABA receptors (GABA_BRs) play a key role in the regulation of intraneuronal calcium. These receptors modulate a variety of high-voltage–activated calcium channels, most prominently the N- and L-types (Dolphin and Scott 1986; reviewed by Misgeld et al. 1995). In amphibian retinal ganglion cells, GABA_BRs reduce calcium influx by inhibiting N-type calcium channels (Zhang et al. 1997). In contrast, these ganglion cells also possess metabotropic glutamate receptors that raise internal calcium through activation of inositol trisphosphate (Akopian and Witkovsky 1996; Shen and Slaughter 1998). Thus these two metabotropic transmitter systems may have opposing effects on internal calcium.

But calcium is not a passive participant in the actions of guanine nucleotide binding proteins (G-protein) receptors. Through activation of calmodulin, calcium can cause a suppression of G-protein–coupled receptor kinases (GRKs) or influence the activity of G-proteins directly (Levay et al. 1998; Liu et al. 1997; Pronin et al. 1997). Thus the regulation of internal calcium by one metabotropic receptor may influence the properties of heterologous receptors. Therefore the effects of internal calcium on GABA_B function were examined.

The experiments indicate that elevated levels of internal calcium suppress the action of GABA_BRs in retinal ganglion cells. This suppression is initiated by local calcium release from inositol trisphosphate and ryanodine-sensitive stores, which stimulates calmodulin. Metabotropic glutamate receptor can generate this release of internal calcium (Akopian and Witkovsky 1996; Shen and Slaughter 1998) and can inhibit the action of GABA_BRs. These results indicate that there are reciprocal negative feedback pathways between excitatory and inhibitory metabotropic pathways and that the interactions may be critical in determining the relative weighting of synaptic signals.

METHODS

Retinal preparation

Experiments were performed on acutely isolated neurons from the tiger salamander retina, Ambystoma tigrinum (Kons Scientific, Germantown, WI) using methods previously described in detail (Bader et al. 1978). The procedures conformed to the guidelines set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Briefly, the animals were decapitated and pithed, then the eye was removed and the retina was isolated. The retina was incubated for ~30–60 min at room temperature (22°C) in 400 μL of enzyme solution containing papain (12U/ml papain, Worthington Biochemicals, Freehold, NJ) and 5 mM L-cysteine in amphibian Ringer solution. Subsequently, the retina was rinsed five times with amphibian Ringer solution, transferred to calcium-free Ringer solution, and shaken until the tissue dissociated. The cells were placed on a lectin-coated cover slip and stored in Ringer solution at 17°C. Experiments were performed on acutely isolated cells, usually within 5 h of dissociation.

Retinal cells were superfused at room temperature with amphibian Ringer solution consisting of (in mM) 111 NaCl, 3 KCl, 2 CaCl₂, 1
MgCl₂, 10 dextrose, and 5 HEPES buffered at pH 7.8. To examine calcium channel currents, 10 mM BaCl₂ and 40 mM tetraethylammonium chloride (TEA Cl) replaced equimolar NaCl in the Ringer solution after whole cell recordings were initiated. Voltage-activated sodium current was blocked by 1 μM TTX. Ringer and drug containing solutions were applied through a gravity-fed system to a manifold in connection with the tissue chamber. Valves controlled drug application, and there was a delay of ~2–3 s due to exchange time.

Recording pipettes were filled with (in mM) 110 K gluconate, 5 NaCl, 0.1 CaCl₂, 1 MgCl₂, 5 EGTA, and 5 HEPES and adjusted to pH 7.4 with KOH. The pipette solution also contained an “ATP regenerating cocktail” consisting of 4 mM ATP, 20 mM phosphocreatine, and 50 units/ml creatine phosphokinase. As noted in the text, bis-(o-aminophenoxy)-N,N,N′,N′-tetraacetic acid (BAPTA) sometimes replaced EGTA. Impermeant drugs were dialyzed into neurons by including them in the pipette.

Ryanodine, calmidazolium, trifluoperazine, heparin, and dantrolene were obtained from Research Biochemicals International (Natnick, MA). 1S, 3R trans aminocyclopentadine dicarboxylic acid (ACPD) was purchased from Tocris-Cookson (St. Louis, MO) and calmodulin-binding protein from Calbiochem (San Diego, CA). EGTA-AM, BAPTA-AM, and Fluo-4 were obtained from Molecular Probes (Eugene, OR). Baclofen and CGP35348 were gifts from Novartis Pharma (Basel, Switzerland). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Electrophysiological recordings

The whole cell voltage-clamp technique was employed in combination with a List EPC9 amplifier and HEKA Pulse software (ALA Scientific Instruments, Westbury, NY) running on a Macintosh Quadra computer. Igor software (WaveMetrics, Lake Oswego, OR) was used for data analysis, curve fitting, and figure illustration. Data are expressed as means ± SE.

Both voltage steps and ramps were used to monitor calcium channel currents and, because these channels inactivated slowly, both protocols gave similar results (Zhang et al. 1997). Neurons were voltage clamped to ~70 mV. In the ramp protocol, the cell was stepped to ~40 mV, then ramped from ~40 mV to +40 mV in 50 ms. The step protocol was a single step from ~70 mV to +10 mV for 10–30 ms. This voltage was chosen because it evoked the largest amplitude inward current. The inward current was carried by barium and could be blocked by external cadmium (100 μM), indicative of a calcium channel current. Filled with the internal solution, electrodes had resistances of 5 MΩ. The currents shown are raw data, not corrected for electrode junctional potentials or access resistance.

Isolated ganglion cells were identified using morphological and physiological criteria, particularly soma diameters over 15 μm and inward sodium currents over 1 nA. These characteristics do not positively exclude amacrines, but based on the number of cells studied and the consistency of the results, it is reasonable to conclude the observations are representative of ganglion cell responses.

Calcium imaging

Dissociated cells were loaded for 20 min at room temperature in the dark with 5 μM Fluo-4 AM in Ringer solution containing 0.02% pluronic acid and 0.01% dimethylsulfoxide (DMSO). Then cells were washed with Ringer solution and kept in the dark for 30 min to allow for complete dye deesterification. Fluorescent images were detected by a laser-scanning confocal Biorad MRC-1024 system and upright Nikon Optiphot microscope equipped with a ×63, 1.2 NA water immersion objective. A 488-nm argon laser was used for dye excitation. Emissions were cut off at 522 nm. Full resolution images (512 × 512 pixels) were collected at 1.4- to 4-s intervals and processed by Confocal Assistant software. When applicable, drugs were applied to the cell chamber for 15 min before images were taken. The stage of the confocal microscope was not equipped with a perfusion or drug application system. Therefore drug application was completed just before confocal imaging except where noted in the text (Fig. 6).

RESULTS

At a concentration of 10 μM, baclofen (Bowery et al. 1980) potently activated bipolar cell and ganglion cell GABA₉Rs in amphibian in situ preparations such as the intact retinal eyecup or the retinal slice (Maguire et al. 1989; Slaughter and Bai 1989; Tian and Slaughter 1994; Zhang et al. 1997). This concentration produced very small effects in isolated cells. But a high baclofen concentration applied to isolated ganglion cells produced the same effect as much lower concentrations in the slice preparation, namely a large reduction in high-voltage-activated calcium channel current (Zhang et al. 1997). This was surprising because isolated cells are usually more sensitive to low drug concentrations, partly due to the absence of perfusion barriers and uptake mechanisms. As shown in Fig. 1A, 300 μM baclofen suppressed 65% of the barium current in an isolated ganglion cell, whereas 10 μM baclofen reduced <9% of the current. Several factors indicated that baclofen was acting on the GABA₉R, despite the high concentration that was required. One was that CGP35348, a specific GABA₉R antagonist (Bittiger et al. 1990; Tian and Slaughter 1994), blocked the effect of baclofen (Fig. 1B). Another was the voltage sensitivity of the GABA receptor.
the baclofen effect (Campbell et al. 1993, 1995; Grassi and Lux 1989). Consistent with this, a prepulse to +100 mV reduced the effect of baclofen in isolated cells (Fig. 1A) and the peak inward current evoked by a voltage ramp was shifted to the right in the presence of baclofen (Fig. 1B). Therefore despite the need for a significantly higher dose, it appears that baclofen acts specifically on GABAB Rs in these isolated cells.

A possible explanation of this phenomenon came from studies of internal calcium. Under standard conditions, internal calcium was buffered with 5 mM EGTA. Under these conditions, which are referred to as “control,” 10 μM baclofen had a small effect on inward barium current and the EC50 for baclofen was 92 μM (Fig. 2). If the pipette solution contained 10 mM BAPTA, then 10 μM baclofen produced a 27% suppression of the barium current, the baclofen dose-response curve was shifted to the left, and the EC50 was reduced to 28 μM. In five cells buffered with EGTA, 10 μM baclofen suppressed 6 ± 3% of the inward current. In another six cells buffered with BAPTA, 10 μM baclofen suppressed 23 ± 3% of the current. Based on calculations using MaxChelator v6.81 (Bers et al. 1994), the change in buffers did not appreciably alter the overall internal free calcium concentration. A more likely factor is that BAPTA, a faster buffer than EGTA (Bers et al. 1994), the change in buffers did not appreciably alter the overall internal free calcium concentration. A more likely factor is that BAPTA, a faster buffer than EGTA (Naraghi and Neher 1997), reduced local calcium near internal release sites. Consequently, BAPTA interfered with calcium-dependent phenomena that were very localized within the cell.

Confocal imaging of isolated cells disclosed a ring of relatively high internal calcium near the plasma membrane. Fluo-4 AM was loaded into isolated cells, and then the neurons were scanned. Neurons with long processes were chosen, thus selectively high internal calcium near the plasma membrane. But this ring of high calcium was not observed in the presence of BAPTA (Fig. 3B).

To evaluate the effect of internal free calcium, several concentrations of BAPTA were included in the pipette solution to control internal free calcium. Baclofen’s dose-response relationship was determined and fitted to the logistic equation (Fig. 4). When internal calcium was buffered to 70 nM, the baclofen EC50 was 28 μM. Increasing the pipette’s BAPTA concentration did not lower the EC50. For example, when BAPTA was increased to a level where the calculated free calcium approached 1 pM, the dose response curve was almost identical to the curve generated for 70 nM internal calcium. The Kd of BAPTA is ~200 nM (Tsien 1980), and buffers are less effective at more than one order of magnitude away from their Kd values. Nevertheless, it is reasonable to conclude that an internal free calcium concentration of ~70 nM yielded maximal receptor apparent affinity. When internal free calcium was buffered with BAPTA to 200 nM, it produced a dose-response curve that was very similar to control (EGTA buffering) conditions. Although it is not shown in Fig. 4, BAPTA was used to buffer calcium at several concentrations between 70 and 200 nM. Each of these concentrations produced a parallel shift in the dose response curve. The curves fit between the two illustrated (70 and 200 nM), with higher calcium levels shifting the curve to the right. Thus within this range, the baclofen EC50 increased monotonically with calcium concentration. When internal calcium was buffered to 1 μM, the dose-response curve became compressed, but the EC50 increased only slightly to 100 μM. Therefore moderate concentrations of internal calcium produced a competitive suppression of the baclofen effect while high concentrations of calcium produced a noncompetitive inhibition of baclofen’s action. Because the GABAB R acts through a second-messenger pathway, the site of inhibition may not be at the receptor, but could be at any of the steps along the transduction cascade.

Although this ambiguity exists for agonists, it may not be true of receptor antagonists because they act directly at the

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**Fig. 2.** Effect of calcium chelation on the response to baclofen. Isolated retinal neurons were exposed to an external solution containing 10 mM barium and 40 mM TEA. Neurons were voltage clamped to −70 mV, stepped to −40 mV, and then the voltage was ramped from −40 to +40 mV in 50 ms. Traces show the voltage-gated current before and during application of 10 μM baclofen. A: when 5 mM EGTA was included in the whole cell pipette solution, baclofen produced a very small suppression of the barium current. B: with 10 mM BAPTA in the whole cell pipette solution, the response to baclofen was enhanced. C: dose-response curves show the normalized inhibition of the barium current by baclofen when the pipette solution contained 5 mM EGTA or 10 mM bis(o-aminophenoxy)-N,N,N’,N’-tetraacetic acid (BAPTA). Normalized currents for these neurons were fitted to \( I_{\text{exp}} = I_{\text{max}} \cdot \left( \frac{E_{\text{C50}}}{E_{\text{C50}} + [B]} \right)^d \) where \( I_{\text{exp}} \) is the barium current suppressed by baclofen, \( I_{\text{max}} \) is the maximum barium current suppressed by baclofen, and \( n \) is the Hill coefficient.
receptor and do not involve the transduction pathway. Consequently, the influence of internal calcium on the IC$_{50}$ of CGP-35348, a competitive GABA$_B$R antagonist, was explored. The effects of internal buffering with 5 mM EGTA and 10 mM BAPTA were compared. For each condition, the concentration of antagonist was varied while the concentration of agonist was kept constant. Because baclofen was more potent when cells were buffered with BAPTA, we used equivalent effective concentrations (but different absolute concentrations) of baclofen. Based on EC$_{50}$ values calculated from the dose-response curves shown in Fig. 1, 100 μM baclofen was used when buffering with EGTA, whereas 30 μM baclofen was used when buffering with BAPTA. Experiments revealed that BAPTA shifted the IC$_{50}$ of CGP35348 to the left (Fig. 5), an effect that was qualitatively similar to the effect of this buffer on baclofen’s EC$_{50}$. In seven cells buffered with 5 mM EGTA, the IC$_{50}$ of CGP35348 was 47 μM. In a different set of five cells, which were buffered with 10 mM BAPTA, the IC$_{50}$ was 20 μM. This confirms that internal calcium alters GABA$_B$R apparent affinity.

The source of internal calcium could be a standing high level of bulk calcium, but this is unlikely because of the differing effects of EGTA and BAPTA. An explanation consonant with the different actions of these two buffers is that there is a local source of internal calcium. This local source could be very close to its binding site, so that it could produce a rapid effect that EGTA was unable to buffer. One potential source is the membrane calcium channel, either one regulated by the GABA$_A$R or another type of calcium channel. Thus divalent ion influx might act to reduce the affinity of the receptor. Although

![钙离子成像的单个分离神经元加载Fluo-4 AM。A: 在控制条件下的孤立神经元的图像。B: 用EGTA-AM（左）或BAPTA-AM（右）处理的孤立神经元的图像。在每列中，亮场图像在荧光图像上方显示了同一区域。](image)

**FIG. 3.** Calcium imaging of individual isolated neurons loaded with Fluo-4 AM. **A**: image of an isolated neuron under control conditions. **B**: image of an isolated neuron treated with either EGTA-AM (left) or BAPTA-AM (right). In each column, a bright-field image is shown above a fluorescent image of the same field.
this might influence the baclofen response, it is unlikely to account for our observations. Baclofen acts at much lower doses in the slice and intact retina, where a similar inward calcium current has been observed. It seems more likely that dissociated neurons have altered internal calcium dynamics, perhaps weaker calcium buffering or greater release from internal stores. Intracellular sources of calcium could produce localized calcium increases that might be EGTA insensitive. Two internal sources were investigated: the InsP₃-sensitive and ryanodine-sensitive stores.

Heparin was included in the pipette solution to block InsP₃-stimulated calcium release (Hill et al. 1987). The baclofen dose-response curve in the presence of heparin was determined in seven cells and compared with the set of five control neurons shown in Fig. 1. All experiments were performed with 5 mM EGTA in the pipette. Heparin produced a parallel, leftward shift in the dose-response curve (Fig. 6B). The EC₅₀ was reduced from 92 to 41 μM. This suggests that calcium release from InsP₃-sensitive stores was responsible, at least in part, for the lowered affinity of the GABA_B R in isolated cells. Because heparin is membrane permeable, imaging experiments were performed to test the effect on internal calcium distribution. Confocal calcium imaging demonstrated that heparin suppressed the ring of high calcium under the plasma membrane that was observed in control cells. These imaging experiments were performed in two ways. In one protocol, cells were superfused with heparin (Fig. 6A), and the fluorescence of these cells was compared with control cells (e.g., Fig. 3A). Alternatively, cells were observed under the confocal microscope while heparin was added to the solution bathing the cells. This permitted observation of the same cells before and during heparin treatment, but a perfusion system was not available, so there was imprecise control of drug application (Fig. 6B). Both protocols demonstrated that heparin reduced the ring of high fluorescence. Again, this suggests a link between this ring of high calcium and the reduction in apparent affinity of the GABA_B R.

The ryanodine-sensitive store was also investigated. Two blockers of the ryanodine receptor were tested: dantrolene (Ohta et al. 1990) and high concentrations of ryanodine (Buck et al. 1992). Both had very similar effects (Fig. 7, A–C). In the presence of these blockers, 10 μM baclofen significantly suppressed the barium current, and both blockers shifted the baclofen dose-response curve to the left, reducing the EC₅₀ to 27 μM. The effect of each antagonist was almost identical to the maximum effect of internal BAPTA buffering.

The effect of ryanodine receptor stimulation was examined by applying baclofen alone or in the presence of caffeine (Fig. 7D). Internal calcium was buffered with 5 mM EGTA. The inward barium current was partially suppressed by baclofen alone. Application of 10 mM caffeine rapidly reduced the inward current and almost totally blocked baclofen’s effect. This could be interpreted to indicate that caffeine increased internal calcium to a range where it produced the noncompetitive inhibition of the GABA_B R described in Fig. 4. Calcium imaging of isolated cells showed that caffeine increased internal levels of free calcium (not shown). Although supporting the data obtained with ryanodine receptor antagonists, it is also possible that caffeine closed the same calcium channels that were modulated by baclofen, and thus simply occluded the baclofen effect. An important outcome of the caffeine experiments was that the calcium stores in isolated cells were far from depleted, despite the presence of elevated free calcium under the plasma membrane.

Calcium could have a direct action on the GABA_B R, or might stimulate an intracellular pathway. Because calmodulin is a ubiquitous detector of internal calcium, we examined the influence of three calmodulin antagonists: trifluperazine, calmidazolium, and the calcium binding domain peptide (CaM kinase II 290–309) (James et al. 1995; Van Belle 1981; Vandoorselaar et al. 1994). All three agents produced a parallel, leftward shift of the baclofen dose-response curve (Fig. 8). Calmidazolium and trifluperazine had similar effects, producing a shift of baclofen EC₅₀ to 36 μM. The effect of the calmodulin binding domain was slightly more pronounced, shifting the EC₅₀ to 20 μM.
Metabotropic glutamate receptors stimulate InsP$_3$-mediated increases in internal calcium in amphibian ganglion cells in *Xenopus* (Akopian and Witkovsky 1996) and salamander (Shen and Slaughter 1998). This suggests that glutamate could reduce the effectiveness of the GABA$_B$R. To test this, baclofen’s effect on barium current was measured before and after activation of metabotropic receptors. The metabotropic glutamate receptors were stimulated with ACPD. Because metabotropic glutamate receptors suppress L-type calcium channels while baclofen inhibits N-type channels (Shen and Slaughter 1998; Zhang et al. 1997), all the experiments were done in the presence of 50 μM nifedipine. ACPD could still activate the InsP$_3$ system in the presence of nifedipine, but did not produce an effect on calcium channels that might be confused with the action of baclofen. In the presence of nifedipine, 100 μM baclofen was applied and produced a suppression of the inward current (Fig. 9). However, after application of ACPD, the suppressive effect of baclofen was significantly reduced. Inward current was 47.64% suppressed by 100 μM baclofen alone, but baclofen in the presence of ACPD suppressed 29.3% of the current (n = 4).

**DISCUSSION**

Regulation of the metabotropic GABA receptor

These experiments indicate that modulation of calcium release from internal stores influences the affinity of the GABA$_B$R. This modulation can be very significant if synaptically released GABA is acting on the steep portion of the dose-response curve. For example, the equivalent of 50 μM baclofen could produce 75% of its peak inhibition if the internal calcium is 70 nM, whereas a modest increase to 200 nM internal calcium would reduce this inhibition to ~30%. An additional increase in internal calcium to 1 μM would reduce the effect of 50 μM baclofen to only 10%. These levels of
internal calcium are very likely to be within the normal physiological range of excitable cells.

The metabotropic GABA receptor appears very sensitive to modulation by internal calcium. By comparison, the $K_{1/2}$ of the photoreceptor cyclic nucleotide gated channel, which is regulated by internal calcium during dark adaptation, changes from 86 µM in the presence of 20 µM calcium to 59 µM in a calcium-free environment (Hackos and Korenbrot 1997). The GABA$_{B}$Rs go through a larger change in apparent affinity when internal calcium changes by $<100$ nM.

Internal calcium can regulate ionotropic, as well as metabotropic, GABA receptors. Rapid influx of calcium through voltage-regulated calcium channels produced a significant reduction in the apparent affinity of the GABA$_{A}$ receptor (Inoue et al. 1986). Other studies have shown that calcium release from internal stores can suppress (Brussaard et al. 1996; Desaulles et al. 1991), enhance (Llano et al. 1991), or produce a biphasic effect (Taleb et al. 1987) on the GABA$_{A}$ receptor current. In a preliminary report, Akopian and Witkovsky (1997) found that internal calcium suppressed the GABA$_{A}$ receptor in turtle ganglion cells and identified a link between internal calcium and calmodulin. Therefore both metabotropic and ionotropic GABA receptors may be concomitantly modulated by changes in internal calcium.

**Apparent or real affinity?**

It is difficult to determine whether calcium is truly modulating receptor affinity, or whether it is having an effect downstream of the receptor. To some extent this ambiguity is resolved by the observation that calcium shifts the IC$_{50}$ of GABA$_{A}$R antagonists as well as receptor agonists. The logic is that antagonists only interact with the receptor and are independent of the downstream cascade. However, IC$_{50}$ measurements clearly require agonist action. Based on published reports, at least one plausible mechanism that might act downstream of the receptor yet appear to affect affinity is the action of calmodulin on G-proteins. Calmodulin binds to the $\beta\gamma$ subunit of G-proteins (Liu et al. 1997). GABA$_{B}$Rs suppress calcium channels by promoting $\beta\gamma$ binding to the calcium channel. Thus calmodulin may inhibit this interaction. If only submaximal stimulation of GABA$_{B}$Rs is normally required for maximal suppression of calcium channels (spare receptor model), then calmodulin inactivation of $\beta\gamma$ subunits could shift the dose-response curves for both agonists and antagonists. This would appear to be a change in receptor affinity.

**Calcium-activated second-messenger pathway**

The experiments link internal calcium release with GABA$_{A}$R apparent affinity. BAPTA, but not EGTA, affected receptor apparent affinity. This suggests that local regions of high intracellular calcium influence receptor apparent affinity. Based on their distinct on-rates, it has been estimated that neither EGTA or BAPTA can alter the free calcium concentration within 20 nm of a calcium release site and that both buffers are equally effective at distances $>200$ nm from the release site. The distinct effects of BAPTA and EGTA suggest that calmodulin binding occurs within $\sim200$ nm of the calcium release site. Confocal images of isolated cells suggest that these release sites are just below the plasma membrane, although these neurons have very large nuclei resulting in a restriction of the cytoplasmic compartment.

The calcium that regulates apparent receptor affinity comes from internal stores, not from extracellular sources. Heparin, a blocker of InsP$_{3}$-sensitive stores, shifts the affinity of the receptor to almost the same extent as dantrolene and ryanodine, which are ryanodine receptor blockers. This suggests that both release sites are important in controlling intracellular calcium in isolated cells.

The effectiveness of trifluoperazine, calmidazolium, and calmodulin-binding peptidog suggests that calcium release from internal stores stimulates calmodulin, leading to a reduction of GABA$_{B}$R apparent affinity. Thus the simplest interpretation of the experimental data is that isolated retinal neurons possess leaky InsP$_{3}$ receptors that produce small, localized increases in internal calcium. This calcium signal could be amplified by ryanodine receptors, detected by calmodulin, leading to a reduced affinity of the GABA$_{B}$R. The calcium release must be close to a source of calmodulin, although not necessarily near the GABA$_{B}$R.

However, alternative interpretations remain possible. There are some reports that heparin blocks ryanodine receptors and others, indicating that there is an internal calcium pool that is sensitive to both ryanodine and InsP$_{3}$ receptor agonists (Stauderman et al. 1991; Zaccetti et al. 1991). Thus the experiments strongly suggest that internal calcium release is a key step in regulating apparent affinity; the evidence is less compelling that both release sites contribute to this mechanism. Similarly, the identification of a calmodulin-dependent step depends on the specificity of calmidazolium, trifluoperazine, and calmodulin-binding protein. The latter may be the most specific, but there is a report that phospholipase C delta contains...
a sequence that is very similar to calmodulin and that the phospholipase C could be blocked by an inhibitory calmodulin peptide (Richard et al. 1997).

Transmembrane calcium flux may regulate the GABA<sub>B</sub> receptor (Inoue et al. 1986). A similar mechanism could operate on metabotropic GABA receptors. The GABA<sub>B</sub>R regulates the N-type calcium channel by a direct G-protein interaction, implying that the channel and receptor are close to each other (Campbell et al. 1993; reviewed by Misgeld et al. 1995). If calmodulin was present near the mouth of the calcium channel, it might mediate this negative feedback. Barium was used in our experiments to avoid this complicating factor. However, in a few experiments where calcium was used as the current carrier, there was no apparent decrease in the effect of baclofen during the course of calcium influx. Therefore it appears that this calmodulin regulatory system was not near the calcium channel. However, this regulation might not have been detected in our experiments if it occurred very quickly (less than a few milliseconds) or very slowly (longer than our 30-ms voltage step). If calcium influx regulates GABA<sub>B</sub>Rs in retinal ganglion cells, it functions in parallel with the regulatory mechanism dependent on internal calcium release. The same may be true of GABA<sub>A</sub>Rs. Although Inoue et al. (1986) suggested that voltage-gated calcium channels could modulate GABA<sub>A</sub> receptor affinity, other studies have suggested GABA<sub>A</sub> responses are suppressed by internal calcium release and not by influx across the plasma membrane (Desaulles et al. 1991).

**Physiological significance**

At this point, we can only speculate about the functional significance of the interaction between metabotropic glutamate and GABA receptors. There are several sites in the retina where this interaction might occur. One is the ganglion cell, where GABA<sub>B</sub>Rs suppress a calcium-activated potassium conductance (Zhang et al. 1997). When this conductance is suppressed, bipolar cell glutamatergic input can produce a larger depolarization of the ganglion cell (Zhang et al. 1998). This added depolarization could move the ganglion cell to a voltage that produces inactivation of the sodium channel. That is, counter intuitively, the absence of the calcium-activated potassium current may reduce the spike output of the ganglion cell. Under these conditions, a suppression of the GABA<sub>B</sub> R might be advantageous because it would restore the calcium-activated potassium current, allowing for a repolarization that removes sodium channel inactivation. Therefore ganglion cell responsiveness might be improved if glutamate release from bipolar cells activated both ionotropic and metabotropic ganglion cell glutamate receptors. Strong ionotropic glutamate receptor activation would be accompanied by a metabotropic glutamate receptor stimulation that inhibited the GABA<sub>B</sub> R. This might increase the dynamic range of a ganglion cell, permitting high spike rates during strong excitatory inputs.

Glutamate, acting through metabotropic receptors to lower the affinity of the GABA<sub>B</sub> receptor, might also play a key role at the bipolar cell synaptic terminal. The bipolar presynaptic terminal releases glutamate (Tachibana and Okada 1991) and may have GABA<sub>B</sub>Rs (Maguire et al. 1989; but see Lukasiewicz and Werblin 1994). These bipolar cell terminals also contain metabotropic glutamate receptors (Brandstätter et al. 1996) and high levels of InsP<sub>3</sub> (Peng et al. 1991). (These properties are cumulative data from different species and may not be found in combination in a single species, but serves as a heuristic model.) When GABA<sub>B</sub>Rs at the terminal are active, this would reduce transmitter release during bipolar cell excitation (Maguire et al. 1989). However, if the released glutamate fed back onto high affinity, InsP<sub>3</sub>-linked metabotropic glutamate receptors, this would suppress the GABA<sub>B</sub> R inhibition and enhance glutamate release at the synapse. The net effect would be that metabotropic glutamate autoreceptors stimulate a positive feedback system that produces synaptic facilitation. This is opposite to the conventional model of autoreceptors as a negative feedback system that reduce transmitter release at the synapse.

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