Changes in Intracellular Ca\(^{2+}\) Induced by GABA\(_A\) Receptor Activation and Reduction in Cl\(^{-}\) Gradient in Neonatal Rat Neocortex

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Fukuda, Atsuo, Kanji Muramatsu, Akihito Okabe, Yasunobu Shimano, Hideki Hida, Ichiro Fujimoto, and Hitoo Nishino. Changes in intracellular Ca\(^{2+}\) induced by GABA\(_A\) receptor activation and reduction in Cl\(^{-}\) gradient in neonatal rat neocortex. J. Neurophysiol. 79: 439–446, 1998. We have studied the effects of \(\gamma\)-aminobutyric acid (GABA) and of reducing the Cl\(^{-}\) gradient on the [Ca\(^{2+}\)]i in pyramidal neurons of rat somatosensory cortex. The Cl\(^{-}\) gradient was reduced either by furosemide or by oxygen-glucose deprivation. Immature slices taken at postnatal day (P) 7–14 were labeled with fura-2, and [Ca\(^{2+}\)]i was monitored in identified pyramidal cells in layer II/III as the ratio of fluorescence intensities (RF340/F380). The magnitude of the [Ca\(^{2+}\)]i increase induced by oxygen-glucose deprivation was significantly reduced (by 44%) by bicuculline (10 \(\mu\)M), a GABA\(_A\) receptor antagonist. Under normal conditions, GABA generally did not raise [Ca\(^{2+}\)]i, although in some neurons a small and transient [Ca\(^{2+}\)]i increase was observed. These transient [Ca\(^{2+}\)]i increases were blocked by Ni\(^{2+}\) (1 mM), a blocker of voltage-dependent Ca\(^{2+}\) channels (VDCCs). Continuous perfusion with GABA did not cause a sustained elevation of [Ca\(^{2+}\)]i, but bicuculline caused [Ca\(^{2+}\)]i oscillations. After inhibition of Cl\(^{-}\) extrusion with furosemide (1.5 mM), GABA induced a large [Ca\(^{2+}\)]i increase consisting of an initial peak followed by a sustained phase. Both the initial and the sustained phases were eliminated by bicuculline (10 \(\mu\)M). The initial but not the sustained phase was abolished by Ni\(^{2+}\). In the presence of Ni\(^{2+}\), the remaining sustained response was inhibited by the addition of 2-amino-5-phosphopentanoic acid (AP5, 20 \(\mu\)M), a selective N-methyl-D-aspartate (NMDA) receptor antagonist. Thus the initial peak and the sustained phase of the GABA-evoked [Ca\(^{2+}\)]i increase were mediated by Ca\(^{2+}\) influx through VDCCs and NMDA receptor channels, respectively, and both phases were initiated via the GABA\(_A\) receptor. These results indicate that, in neocortical pyramidal neurons, a reduction in the Cl\(^{-}\) gradient converts the GABA\(_A\) receptor-mediated action from nothing or virtually nothing to a large and sustained accumulation of cellular Ca\(^{2+}\). This accumulation is the result of Ca\(^{2+}\) influx mainly through the NMDA receptor channel. Thus GABA, normally an inhibitory transmitter, may play an aggravating role in excitotoxicity if a shift in the Cl\(^{-}\) equilibrium potential occurs, as reported previously, during cerebral ischemia.

**INTRODUCTION**

\(\gamma\)-Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the neocortex. Recent reports have demonstrated that GABA\(_A\) inhibitory postsynaptic potentials/currents (IPSP/Cs) are more sensitive to anoxia than glutamatergic excitatory postsynaptic potentials/currents (EPSP/Cs) both in the hippocampus (Katchman et al. 1994; Khazipov et al. 1995) and in the neocortex (Luhmann and Heine-mann 1992; Rosen and Morris 1993). A loss of the normal Cl\(^{-}\) gradient during hypoxia was suggested as a mechanism that might underlie the reduction and/or reversal of the GABAergic IPSP/Cs (Katchman et al. 1994; Khazipov et al. 1995; Luhmann et al. 1993). Such a withdrawal and/or reversal of GABAergic inhibition could aggravate the cell deterioration caused by ischemia/hypoxia, because GABA, as well as glutamate, increases in the extracellular space during ischemia (Matsumoto et al. 1996; O’Regan et al. 1995). In a previous study, we revealed that bicuculline reduces the magnitude of the [Ca\(^{2+}\)]i increase induced by oxygen-glucose deprivation in neocortical neurons (Fukuda et al. 1998). This result indicates that GABA may act to increase [Ca\(^{2+}\)]i during ischemia, indicating a collapse or reversal of the GABAergic inhibitory system.

GABAergic inhibition serves to control the N-methyl-D-aspartate (NMDA) receptor-mediated excitatory system in the cortex (Kanter et al. 1996; Luhmann and Prince 1990a,b; Metherate and Ashe 1994, 1995). Especially during development, when the GABAergic system is relatively immature, an imbalance between the inhibitory and excitatory networks can lead to an NMDA receptor-mediated hyperexcitability of the neocortical upper layers (Luhmann and Prince 1990a,b). In addition cortical neurons at this age may be particularly fragile in terms of Cl\(^{-}\) homeostasis, because their ability to extrude Cl\(^{-}\) is not fully developed (Fukuda et al. 1993; Luhmann and Prince 1991; Owens et al. 1996; Zhang et al. 1991). Therefore, depression and/or reversal of GABAergic inhibition could occur easily during anoxia. Oxygen-glucose deprivation induces an intracellular accumulation of Ca\(^{2+}\) that is greatest in neocortical layer II/III of postnatal day (P) 7–14 rats, an effect mediated via NMDA receptors (Fukuda et al. 1998). Therefore the inadequate Cl\(^{-}\) extrusion and the resultant reduction in GABAergic inhibition during the immature period may be important factors in the generation of an NMDA receptor-mediated cellular Ca\(^{2+}\) overload (Choi 1990).

In the present study we have demonstrated that a reduction in the Cl\(^{-}\) gradient produced either by furosemide or by oxygen-glucose deprivation induces a GABA\(_A\) receptor-mediated increase in [Ca\(^{2+}\)]i, in pyramidal neurons in neocortical layer II/III. This GABA\(_A\) receptor-mediated increase [Ca\(^{2+}\)]i increase in furosemide-treated slices comprised a transient Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels (VDCCs) and a subsequent sustained [Ca\(^{2+}\)]i increase mediated via NMDA receptor channels. Some of these results were published in abstract form (Fukuda et al. 1996).
METHODS

The procedures used for the preparation and maintenance of neocortical slices and for the optical imaging of \([\text{Ca}^{2+}]_{\text{i}}\) were described in the preceding paper (Fukuda et al. 1998). Wistar rats of P 7–14 were used in the present study also. The protocols used conformed with guidelines on the conduct of animal experiments issued by Nagoya City University.

The optical images indicating the \([\text{Ca}^{2+}]_{\text{i}}\) were obtained from neocortical layer II/III of the somatosensory cortex at intervals of 30 s to 2 min. Because we noted some absorption of UV light (340 nm > 380 nm) by furosemide at a concentration of 1.5 mM, furosemide was perfused for ~30 min, then stopped immediately before the initiation of optical recording. A normalized ratio of fluorescence intensities \(R_{340/380}\) was calculated and used in the temporal analysis of \([\text{Ca}^{2+}]_{\text{i}}\) transients during the application of GABA. This enabled changes in \([\text{Ca}^{2+}]_{\text{i}}\) to be assessed over time in a given cell. Toward this end, each \(R_{340/380}\) was divided by the baseline \(R_{340/380}\) (the average of 3 values obtained before the application of GABA). Optical images were obtained using a ×40 objective lens with differential interference contrast filters (Zeiss, Achroplan, numerical aperture 0.75). It was possible to distinguish pyramidal neurons by their morphological features with the aid of Nomarski optics (see Fukuda et al. 1998).

The following drugs were used: fura-2 acetoxy methyl, dimethylsulfoxide, pluronic F127 (Molecular Probes); (-)-bicuculline methiodide, GABA, furosemide (Sigma), and DL-2-amino-5-phosphonovaleric acid (DL-AP5, Tocris Cookson). When used, bicuculline (10 μM), DL-AP5 (20 μM), and NiCl₂ (1 mM) were dissolved in perfusion medium and continuously perfused before, during, and after bath application of GABA (100–200 μM).

RESULTS

**Bicuculline reduced the oxygen-glucose deprivation-induced \([\text{Ca}^{2+}]_{\text{i}}\) increase.**

The increase in the normalized \(R_{340/380}\) of layer II/III neocortical pyramidal neurons induced by oxygen-glucose deprivation was seen to be significantly reduced by the addition of bicuculline (10 μM) when corresponding values were compared at 50 min (Fig. 1). This indicates that GABA, via the GABA\(_A\) receptor, may act to facilitate the accumulation of intracellular \([\text{Ca}^{2+}]_{\text{i}}\) induced by oxygen-glucose deprivation.

**Effects of GABA and bicuculline on \([\text{Ca}^{2+}]_{\text{i}}\), transients under normal conditions.**

The effects of GABA on \([\text{Ca}^{2+}]_{\text{i}}\), were studied under normal conditions, because GABA is known to cause increases in \([\text{Ca}^{2+}]_{\text{i}}\) in neocortical neurons through VDCCs in rats younger than P7 (Lin et al. 1994; LoTurco et al. 1995; Owens et al. 1996; Yuste and Katz 1991). In our slices, taken at P7–14, continuous perfusion of GABA (100 μM) did not induce intracellular \([\text{Ca}^{2+}]_{\text{i}}\) accumulation in neocortical neurons (Fig. 2A). However, in experiments with higher temporal resolution (see Fig. 2C1), transient increases in \([\text{Ca}^{2+}]_{\text{i}}\), induced by the brief application of GABA were revealed in 27% of neurons tested (59/219, 18 slices). Thus the GABA response was only transient, if present at all; no sustained increases were observed. The transient \([\text{Ca}^{2+}]_{\text{i}}\) increase evoked by GABA was blocked by Ni\(^{2+}\) (1 mM) in 73% of cells tested (11/15, 3 slices; Fig. 2C2). This result indicates that the occasional and transient GABA-evoked \([\text{Ca}^{2+}]_{\text{i}}\) increases that occur under normal conditions are mediated by Ni\(^{2+}\)-sensitive VDCCs.

Bicuculline is an epileptogenic agent known to induce synchronized burst discharges in neocortical slices. For this reason, we decided to study the effect of bicuculline on intracellular \([\text{Ca}^{2+}]_{\text{i}}\) accumulation. Continuous perfusion with bicuculline (10 μM) induced \([\text{Ca}^{2+}]_{\text{i}}\) oscillations in 34 of 75 cells (7 slices; Fig. 2B), indicating possible epileptiform activity associated with \([\text{Ca}^{2+}]_{\text{i}}\) influx. Thus under normal conditions, GABA may play an inhibitory role that has the effect of suppressing such activity.

To elucidate to what degree perfusion with GABA or bicuculline can cause intracellular \([\text{Ca}^{2+}]_{\text{i}}\) accumulation, either GABA (n = 6 slices) or bicuculline (n = 7 slices) was applied continuously for 45 min, and \([\text{Ca}^{2+}]_{\text{i}}\) transients monitored. GABA (100 μM) did not cause a significant accumulation of intracellular \([\text{Ca}^{2+}]_{\text{i}}\) (Fig. 3), though some neurons (25/70) showed oscillation-like \([\text{Ca}^{2+}]_{\text{i}}\), transients of smaller magnitude than those induced by bicuculline, probably because of the desensitization of GABA\(_A\) receptors (not shown). On the other hand, bicuculline (10 μM) tended to cause \([\text{Ca}^{2+}]_{\text{i}}\) oscillations (see Fig. 2B), although the magnitude of the intracellular \([\text{Ca}^{2+}]_{\text{i}}\) accumulation was not significantly different from the resting level when compared after 45 min of perfusion and was quite small by comparison with that induced by oxygen-glucose deprivation (Fig. 3; also compare Fig. 3 with Fig. 1). Thus GABA may not play a significant excitatory role in normal neocortical slices of this age, at least in terms of intracellular \([\text{Ca}^{2+}]_{\text{i}}\) accumulation.
Effect of a shift in the Cl⁻ equilibrium potential on GABA-evoked \([\text{Ca}^{2+}]\), transients

It is known that a positive shift in the Cl⁻ equilibrium potential and a resultant reduction or even reversal of GABAergic IPSP/Cs occur during anoxia in cortical slices (Katchman et al. 1994; Khazipov et al. 1995; Luhmann et al. 1993). To mimic such a loss of the Cl⁻ gradient, an inhibition of Cl⁻ extrusion was achieved by perfusing slices with furosemide (1.5 mM), an inhibitor of the Cl⁻ co-transporter, for >30 min. After perfusion with furosemide, the baseline R\(_{F340/F380}\) was higher by 20-80% (only cells showing increases of <50% are included in the present analysis). To enable comparison of data obtained before and after furosemide treatment, normalized R\(_{F340/F380}\) values were used (see METHODS). Application of GABA to nontreated slices evoked increases in \([\text{Ca}^{2+}]\) that were sometimes small and transient, sometimes almost nonexistent (Fig. 4A). Inhibition of the Cl⁻ extrusion system by perfusion with furosemide turned both types of GABA response into large and sustained increases in \([\text{Ca}^{2+}]\), (Fig. 4B). In furosemide-treated slices, GABA evoked increases in \([\text{Ca}^{2+}]\), in 75% of neurons tested (140/187, 15 slices) and the incidence of GABA-evoked \([\text{Ca}^{2+}]\), increases was significantly greater than in nontreated slices (\(P < 0.0001\), \(\chi^2\) test). The \([\text{Ca}^{2+}]\), increases were very prolonged, in contrast to those evoked in nontreated slices (see Fig. 2C1 for comparison), and the level had not returned to baseline even 10 min or so after termination of the GABA application. It usually took more than a 45 min washout to eliminate the furosemide effects on GABA-evoked \([\text{Ca}^{2+}]\), (Fig. 4C).

Mediation by the GABA\(_A\) receptor of GABA-evoked \([\text{Ca}^{2+}]\), increases in furosemide-treated slices

The GABA-evoked \([\text{Ca}^{2+}]\), increases in furosemide-treated slices usually consisted of an initial peak followed by a sustained phase (Figs. 4B, 5A, and 6A1). In fact, 80%...
of the GABA-evoked \([\text{Ca}^{2+}]\) increases showed such a biphasic form, whereas 14% were without an obvious peak and 6% were only transient. To confirm that these GABA-evoked responses are \(\text{GABA}_A\) receptor-mediated, the effects of bicuculline were studied. Both the initial and the sustained phases of the GABA-evoked increase in \([\text{Ca}^{2+}]\) in furosemide-treated slices were eliminated by application of bicuculline in 17 of 21 neurons tested in 5 slices (Fig. 5). In three of the four remaining neurons, bicuculline inhibited only the initial peak (not shown). These results suggest that both the initial and the sustained phases were primarily mediated by the \(\text{GABA}_A\) receptor.

**Secondary mediation by NMDA receptors of GABA-evoked sustained \([\text{Ca}^{2+}]\) increase**

The effects of several \(\text{Ca}^{2+}\) channel antagonists on the GABA-evoked increases in \([\text{Ca}^{2+}]\), were investigated in furosemide-treated slices. In contrast to its effect in nontreated slices, \(\text{Ni}^{2+}\) had only a partial effect. Addition of \(\text{Ni}^{2+}\) (1 mM) abolished the initial peak in 28 of 32 biphasic responses in furosemide-treated slices, whereas it suppressed the sustained phase in only 11 of them (4 slices; Fig. 6A2). In the presence of \(\text{Ni}^{2+}\), GABA evoked sustained \([\text{Ca}^{2+}]\) increases lacking initial peaks in 50 of 90 furosemide-treated neurons (18 slices; Figs. 6A2, B1); this response was abolished by the addition of AP5 (20 \(\mu\)M) in 13 of 18 neurons tested (3 slices; Fig. 6B2). In contrast, AP5 did not reduce the amplitude of the GABA-evoked transient \([\text{Ca}^{2+}]\), increase in nontreated control slices (\(n = 5, 2\) slices) although two of the responses showed an apparent shortening in duration (not shown). These results indicate that the sustained phase of the GABA response may be mediated by NMDA receptors and that such an NMDA receptor-mediated component of the GABA-evoked \([\text{Ca}^{2+}]\) increase emerges only when the \(\text{Cl}^-\) gradient is reduced by an inhibition of \(\text{Cl}^-\) extrusion.

**DISCUSSION**

The results of the present study indicate that, in layer II/III of the immature neocortex under normal conditions, GABA does not cause a prolonged increase in \([\text{Ca}^{2+}]\). However, after a reduction of the \(\text{Cl}^-\) gradient with furosemide GABA evoked a large \([\text{Ca}^{2+}]\), increase consisting of an initial peak followed by a sustained phase. The initial peak and the sustained phase were secondary to \(\text{Ca}^{2+}\) influxes through VDCCs and NMDA receptor channels, respectively, and both were preceded by \(\text{GABA}_A\) receptor activation. Thus a shift in the \(\text{Cl}^-\) equilibrium potential may be responsible for allowing bicuculline-sensitive neuronal \([\text{Ca}^{2+}]\), increases to occur during oxygen-glucose deprivation.

Connor et al. (1987) reported that a persistent \(\text{Ca}^{2+}\) elevation was induced by GABA in cultured cerebellar granule cells, whereas Segal (1993) observed a GABA-induced release of \(\text{Ca}^{2+}\).
versatile $[\text{Ca}^{2+}]$, rise with a fast desensitizing nature in cultured hippocampal neurons. Neither of these GABA-induced $[\text{Ca}^{2+}]$, increases was correlated with depolarization. Thus GABAergic effects on $[\text{Ca}^{2+}]$ as well as on the membrane potential may be mediated by diverse mechanisms in different cell types and under different conditions. In the present study, we used neocortical slices from rats of P7–14 to provide the experimental material, because an imbalance between inhibitory and excitatory systems was reported to exist in the developing rat neocortex (Luhmann and Prince 1990a,b). In fact, GABA causes depolarization (Luhmann and Prince 1991) and an influx of $\text{Ca}^{2+}$ through VDCCs in the immature neocortex (Lin et al. 1994; LoTurco et al. 1995; Owens et al. 1996; Yuste and Katz 1991). In this case, GABA depolarizes cells because the $\text{Cl}^-$ equilibrium potential is more positive than the resting potential, at least in part because of the immaturity of the $\text{Cl}^-$ extruding mechanism (Fukuda et al. 1993; Luhmann and Prince 1991; Owens et al. 1996; Zhang et al. 1991). Although Staley et al. (1995) and Perkins and Wong (1996) suggested that GABA-mediated depolarization is attributable to the permeability to $\text{HCO}_3^-$ of adult hippocampal neurons, Owens et al. (1996) proved that GABAergic depolarization in the immature neocortex was not mediated by $\text{HCO}_3^-$ conductance. In the normal course of development, GABA-evoked $[\text{Ca}^{2+}]$, increases in the neocortex disappear during P7–14 (Lin et al. 1994). Actually in the present study, GABA application caused only transient $[\text{Ca}^{2+}]$, increases in 27% of neurons and no sustained $[\text{Ca}^{2+}]$, increases under normal conditions. Thus immaturity alone cannot explain the bicuculline effect on oxygen-glucose deprivation-induced $\text{Ca}^{2+}$ accumulation.

Furosemide was used to increase $[\text{Cl}^-]$, although we did not measure $[\text{Cl}^-]$. Furosemide effectively shifts the $\text{Cl}^-$ gradient in the positive direction both in adult neocortical slices (Misgeld et al. 1986; Thompson et al. 1988a,b) and in culture (Jarolimek et al. 1996), although the direction of $\text{Cl}^-$ transport might be inward in embryonic cortical cells (LoTurco et al. 1995; Owens et al. 1996; also see Hará et al. 1992). Recently the idea that increases in $[\text{Cl}^-]$, may be induced by furosemide was elegantly confirmed in neocortical slices of similar age to those used in the present study (i.e., P7–18) by using a $\text{Cl}^-$ sensitive dye with optical imaging (Schwartz and Yu 1995). Therefore we can be confident that $[\text{Cl}^-]$, would have increased and the $\text{Cl}^-$ equilibrium potential shifted to a more positive level after treatment of slices with 1.5 mM furosemide for 30 min, as in the present experiments.

We noted 20–80% increases in baseline $R_{\text{F340/F380}}$ in furosemide-treated slices, indicating an increase in the resting $[\text{Ca}^{2+}]$, when the $\text{Cl}^-$ gradient was impaired by furosemide. The reported GABA $\text{A}$ receptor-mediated perpetual inhibitory activity in slices that is generated by spontaneous GABA release (Otis et al. 1991) would turn into depolarization when there is a positive shift in the $\text{Cl}^-$ equilibrium potential. Indeed tonic activation of the GABA $\text{A}$ receptor may contribute to a depolarizing shift in the resting potential in the embryonic cortex with a depolarized $\text{Cl}^-$ equilibrium potential (LoTurco et al. 1995, also see Cherubini et al. 1991). In addition, such a loss of GABAergic inhibition might lead to a disinhibition of the tonic activation of NMDA receptors, resulting in an enhancement of excitability in slices (LoTurco et al. 1990; Sah et al. 1989). Thus a positive shift in the $\text{Cl}^-$ equilibrium potential might be responsible for a depolarization that would allow $\text{Ca}^{2+}$ influx and an elevation of the resting $[\text{Ca}^{2+}]$, in furosemide-treated slices. Indeed furosemide treatment was shown to increase synaptic excitability leading to bursts with or without synchronized activity (Hochman et al. 1995; Jarolimek et al. 1996). That may be enough to permit an increase in $[\text{Ca}^{2+}]$, via $\text{Ca}^{2+}$ influx through both voltage-gated and NMDA receptor channels. It should be said that a glial influence on the effects of furosemide and/or GABA could exist, because glial cells...
also respond to GABA (MacVicar et al. 1989) as well as to furosemide (MacVicar and Hochman 1991) and can release glutamate, which could cause an NMDA receptor-mediated increase in neuronal $[\text{Ca}^{2+}]_i$ (Parpura et al. 1994).

Accumulating evidence suggests that GABAergic inhibitory systems strongly regulate the NMDA component of the glutamatergic EPSP in the neocortex (see Fig. 7). Indeed, a depression of GABAergic inhibition results in an accentuation of NMDA receptor-mediated EPSPs leading to bursts and/or polysynaptic activity, which could cause a synchronized discharge (Kanter et al. 1996; Luhmann and Prince 1990a,b; Metherate and Ashe 1994, 1995). In fact a collapse of the Cl$^-$ gradient can change the GABA$_A$-mediated IPSP to a depolarizing effect that is sufficient to account for the frequency modulation of synaptic NMDA receptor activation in hippocampal slices (Ling and Benardo 1995; Staley et al. 1995). Therefore the AP5-sensitive sustained $[\text{Ca}^{2+}]_i$ increases evoked by GABA after furosemide-treatment (the present study) could be the result of an accentuation of NMDA receptor-mediated Ca$^{2+}$ influx as a result of an abolition or reversal of GABAergic inhibition, in turn caused by a collapse of the Cl$^-$ gradient (Fig. 7).

A positive shift in the Cl$^-$ equilibrium potential during anoxic events was suggested in hippocampal and neocortical slices (Katchman et al. 1994; Khazipov et al. 1995; Luhmann et al. 1993). Although the mechanisms have yet to be studied in detail, Katchman et al. (1994) speculated that ATP depletion could decrease chloride transport by reducing the activity of the ATP-dependent chloride pump and cause a secondary accumulation of extracellular K$^+$ because of impairment of Na$^+$/K$^+$ ATPase. An increase in extracellular K$^+$ would reduce Cl$^-$ extrusion by the Na$^+$ and/or K$^+$/Cl$^-$ cotransporter as a result of a reduction in the driving force (Thompson et al. 1988a). Thus a positive shift in the Cl$^-$ equilibrium potential by impairment of the Na$^+$ and/or K$^+$/Cl$^-$ cotransporter during oxygen-glucose deprivation in the present experiments could have caused a reversal of the GABA$_A$ receptor-mediated responses, as observed previously (Katchman et al. 1994) (also see Fig. 1).

GABA is reported to be protective against ischemic brain damage in vivo (Sternau et al. 1989). Although our present data on Ca$^{2+}$ overload do not necessarily relate to cell death, discrepancies between the above result and ours may be explained as described below. In in vivo experiments, a GABAergic agent exerts its protective effect only when administered before the ischemic insult (Sternau et al. 1989). This may indicate that, at the time when GABA was administered, a shift in the Cl$^-$ equilibrium potential had not yet occurred, unlike the situation in the present study, and thus GABA might hyperpolarize the membrane potential. This idea is also compatible with the finding that GABA has a neuroprotective effect on delayed neuronal death induced by an excitotoxic lesion (Saji and Reis 1987), because a loss of the Cl$^-$ gradient may not occur in the case of excitotoxicity and GABA would hyperpolarize the postsynaptic membrane as normal. Thus the mechanisms by which GABA acts may differ between the normal and Cl$^-$-accumulated conditions (see Fig. 7).

These results lead us to the hypothesis detailed below. Under normal conditions, GABA plays primarily an inhibitory role, and bicuculline can increase excitability by blocking GABA$_A$ inhibitory systems. On the other hand, the impairment of Cl$^-$ extrusion that may occur during ischemia would shift the Cl$^-$ gradient to such an extent that GABA would then depolarize postsynaptic membranes. Indeed bicuculline-sensitive tonic GABAergic depolarization is induced by an increase in ambient GABA during hypoxia in hippocampal slices (Katchman et al. 1994). Such a GABA$_A$-mediated depolarization associated with an initial Ca$^{2+}$ influx through VDCCs would result in a secondary accumulation of extracellular glutamate and induce a sustained influx of Ca$^{2+}$ through NMDA receptor channels (Fig. 7). In this

![FIG. 6. Effects of Ca$^{2+}$-channel antagonists on GABA-evoked increases in $[\text{Ca}^{2+}]_i$.](image)
way, the normally inhibitory transmitter GABA could play an aggravating role in the excitotoxicity mediated by the NMDA receptor during ischemia, although this effect may be smaller in mature neurons with more efficient Cl\textsuperscript{−} extrusion mechanisms. Such a collapse and conversion of GABA inhibition to excitation may in effect switch on a pathological positive feedback loop. This may be a crucial event in the immature brain, because its imbalance between inhibitory and excitatory systems (Luhmann and Prince 1990a,b) and its higher susceptibility to NMDA receptor overstimulation (Johnston 1995; Young et al. 1991) would make it extremely vulnerable to such a pathological loop. This hypothesis would repay further investigation.

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REFERENCES


KHAZIPOV, R., CONGAR, P., and BEN-ARI, Y. Hippocampal CA1 lacuno-


