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γ-Aminobutyric acid increases intracellular Ca^{2+} concentration in cultured cortical neurons: role of Cl^- transport

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Abstract

The effect of γ-aminobutyric acid (GABA) on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured prenatal rat cortical neurons was investigated using fluorescence imaging. GABA or muscimol, but not baclofen, increased $[Ca^{2+}]_i$ in a dose-dependent manner. The $GABA_A$ receptor antagonists, bicuculline and picrotoxin, inhibited the GABA response. Furosemide, an inhibitor of the $Na^+/K^+/2Cl^-$ cotransporter, inhibited the GABA response in a noncompetitive manner. Ethacrynic acid, an inhibitor of an ATP-dependent Cl^- pump, also inhibited the GABA-induced increased in $[Ca^{2+}]_i$. These results suggest a role for Cl^- transport processes in the GABA response. The coapplication of GABA and high K^+ led to a non-additive increase in the GABA response. The GABA response was also inhibited by nifedipine, a voltage-gated Ca^{2+} channel blocker, and abolished by the absence of extracellular Ca^{2+}. Results indicate that the GABA response shares a common pathway of Ca^{2+} movement with the high K^+-induced response. These observations suggest that the stimulation with GABA results in Ca^{2+} influx through voltage-gated Ca^{2+} channels, and that these effects are dependent on Cl^- transport systems.

Keywords: GABA (γ-aminobutyric acid); Cortical neuron; Ca^{2+} influx; Cl^- transport system; Furosemide; Ethacrynic acid

1. Introduction

γ-Aminobutyric acid (GABA), a major inhibitory neurotransmitter in the adult mammalian central nervous system, interacts with two pharmacologically distinct types of receptors. Interaction of GABA with the type A GABA receptor ($GABA_A$ receptor), which activates a chloride ion (Cl^-) conductance and hyperpolarizes the cell membrane, and the type B GABA receptor ($GABA_B$ receptor), which activates a K^+ conductance, leads to a reduction in neuronal excitability (see review by Sivilotti and Nistri, 1991). However, several electrophysiological studies have shown that GABA can also induce depolarization (Segal and Barker, 1984; Mueller et al., 1984; Ben-Ari et al., 1989) and that GABA increases the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in some immature neurons (Connor et al., 1987; Yuste and Katz, 1991; Segal, 1993; Horvath et al., 1993). Although the functional significance of excitatory effects of GABA is unclear, it has been speculated that such actions play a role in developmental growth, differentiation, and synaptogenesis of neurons (Michler, 1990; Barbin et al., 1993) as well as a role in hormone secretion (Horvath et al., 1993; Hales et al. 1994). Although the GABA-induced increase in $[Ca^{2+}]_i$ in immature neurons is thought to result from depolarization, the mechanism remains unclear. We have now investigated the mechanism of the GABA-induced increase in $[Ca^{2+}]_i$ in primary cultures of rat cortical neurons with the fluorescent Ca^{2+} indicator 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)ethane-N,N,N′,N′-tetra-acetic acid (fura-2).

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2. Materials and methods

2.1. Cell culture

Primary cortical cell cultures were prepared according to a modification of the method described by Reynolds and Miller (1989). Wistar rats at embryonic day 18 were removed from the mother under ether anesthesia. Cortical tissue was dissected out and incubated with 0.25% trypsin and 0.02% deoxyribonuclease I for 20 min at 37°C on a shaker, after which the enzymes were inactivated. The cortical cells were mechanically dispersed by repetitive pipetting and rinsed twice with culture medium, comprised of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 5% horse serum, 2 mM glutamine, 1 mM sodium pyruvate, penicillin G (50 U/ml), and streptomycin sulfate (50 μg/ml). The cells were plated at a density of 10^6/ml on poly-L-lysine-coated wells (1.77 cm^2) containing a monolayer of cultured rat cortical astrocytes, and were maintained in culture medium for 5–7 days under a humidified atmosphere of 10% CO_2 in air at 37°C.

2.2. Intracellular Ca^{2+} measurements in single cells

The $[Ca^{2+}]_i$ was measured by microspectrofluorimetry with the Ca^{2+}-sensitive indicator fura-2. Cortical cells were rinsed with a balanced salt solution (BSS) containing 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 5.5 mM glucose, and 20 mM Hepes (pH 7.4), and then incubated with 5 μM fura-2 acetoxymethyl ester in BSS for 60 min at 25°C. Fura-2-loaded cells were perifused with BSS warmed to 37°C at a flow rate of 1.5 ml/min on the stage of a fluorescence microscope-video camera system (Nikon, Tokyo, Japan). The fluorescence intensity of fura-2 was measured with excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Fluorescence was received at 6-s intervals by a charge-coupled device camera (C-2400; Hamamatsu Photonics, Hamamatsu, Japan). The ratio (340/380 nm) of the emitted fluorescence intensities was digitized by a color image processor (Argus 50; Hamamatsu Photonics) and converted to $[Ca^{2+}]_i$, which was calibrated from a standard curve constructed with 10 μM fura-2 (free acid) and known amounts of free Ca^{2+}.

2.3. Materials

GABA and picrotoxin were obtained from Nakarai Tesque (Kyoto, Japan); 5-(aminosulfonyl)-4-chloro-2-[(2-furanylmethyl)amino]benzoic acid (furosemide), 3-hydroxy-5-aminoethylisoxazole hydrobromide (muscimol hydrobromide), 3-amino-2-(4-chlorophenyl)propylphosphonic acid (phaclofen), 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid methyl ester (Bay K 8644), and 5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclo-hepten-5,10-iminemaleate (MK-801) from Research Biochemicals (MA, USA); 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (nifedipine), [*R*-(*R**,*S**)]-5-(6,8-dihydro-8-oxofuro[3,4-*e*]-1,3-benzodioxol-6-yl)-5,6,7,8-tetrahydro-6,6-dimethyl-1,3-dioxolo[4,5-*g*]isoquindinium iodide [(−)bicuculline methiodide], [4-(methylenebutyryl)-2,3-dichlorophenoxy]acetic acid (ethacrynic acid), deoxyribonuclease I, and poly-L-lysine from Sigma (MO, USA); EGTA, Hepes, fura-2, and fura-2 acetoxymethy ester from Dojin (Kumamoto, Japan); DMEM from GIBCO; fetal bovine serum and horse serum from JRH Biosciences; and trypsin from Flow Laboratories. β-Aminomethyl-4-chlorobenzenepropanoic acid (Baclofen) was donated by Daiichi Pharmaceutical (Tokyo, Japan), and penicillin and streptomycin by Meiji Seika Kaisha (Tokyo, Japan).

2.4. Statistics

Data are presented as means ± S.E.M. Comparison between two groups was performed with Student's *t*-test. Two responses of the same cell were compared with the paired *t*-test. Comparisons in Table 1 were carried out by analysis of variance followed by Duncan's multiple test. A P value of < 0.05 was considered statistically significant.

3. Results

3.1. Effects of GABA agonists on $[Ca^{2+}]_i$ in primary cultured cortical neurons

Perfusion of cortical cells with 100 μM GABA for 30 s evoked a transient increase in $[Ca^{2+}]_i$ of 156.3 ± 6.8 nM (mean ± S.E.M. of 77 cells). More than 80% of the monitored cells were responsive. In most instances, the GABA-induced Ca^{2+} response showed a single detectable peak within 30 s after the onset of GABA perfusion and returned to baseline after 1 or 2 min (Fig. 1). Therefore, in subsequent experiments, the peak amplitude within 30 s after the onset of GABA application and the average $[Ca^{2+}]_i$ baseline for 1 min before GABA application were measured. The GABA-induced increase in $[Ca^{2+}]_i$ was calculated as the difference between the peak amplitude and the average baseline.

The GABA-induced increase in $[Ca^{2+}]_i$ was dose-dependent, with a median effective concentration (EC_{50}) of 7 μM. The $GABA_A$ receptor agonist muscimol also increased $[Ca^{2+}]_i$ in a dose-dependent man-

ner. In contrast, the $GABA_B$ receptor agonist baclofen had little effect on $[Ca^{2+}]_i$ (Fig. 2).

We routinely used the response to 100 μM GABA to investigate the effects of test drugs on the GABA-induced increase in $[Ca^{2+}]_i$ in subsequent experiments. The response was reduced to 27.3 ± 2.0% (n = 56 cells)

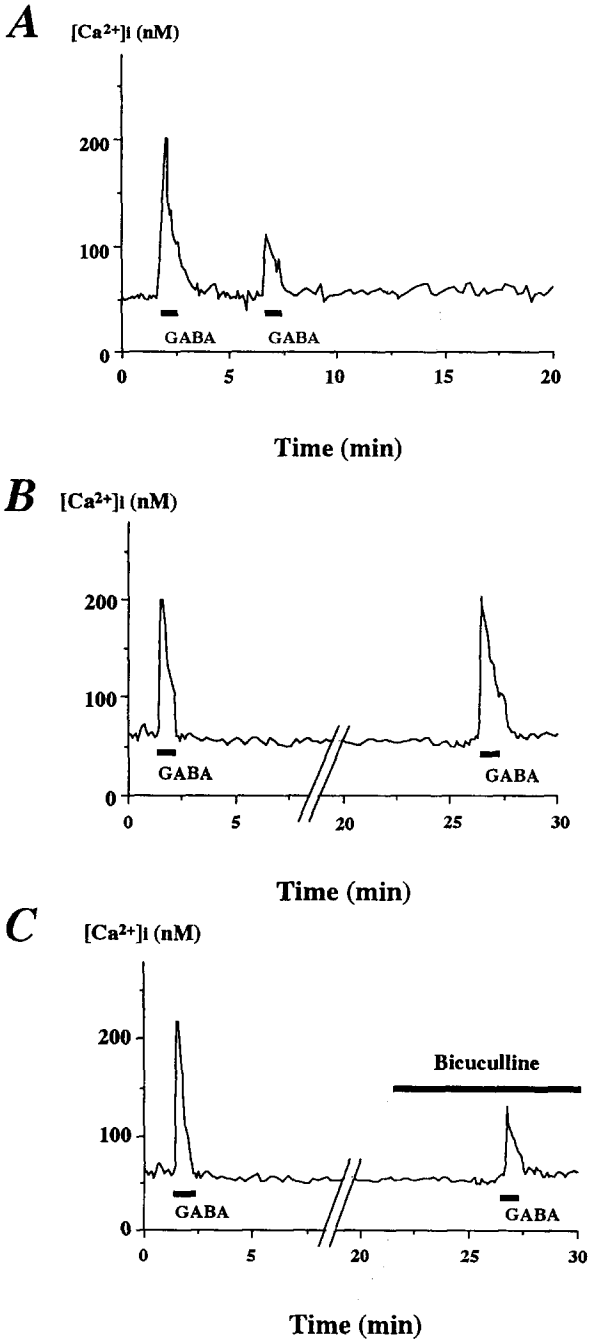

Fig. 1. Effect of GABA on $[Ca^{2+}]_i$ in primary cultured rat cortical neurons. The flurometric traces show $[Ca^{2+}]_i$ changes in typical experiments that were repeated more than three times with similar results. Cells were exposed to two 30-s pulses of GABA separated by 5 (A) or 25 (B and C) min. In (C), cells were also perifused with bicuculline 5 min before the second application of GABA. Bars represent periods of drug exposure.

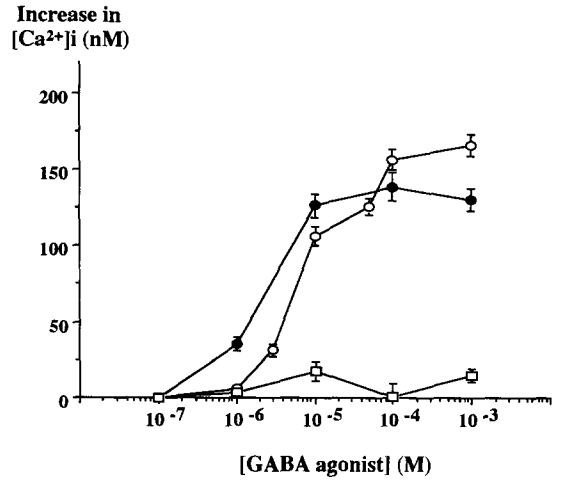

Fig. 2. Dose-response curves for the effects of GABA agonists on $[Ca^{2+}]_i$ in primary cultured cortical neurons. Values are means ± S.E.M. for the effects of GABA (○), muscimol (●), and baclofen (□) on 21–77 cells from three to 11 independent experiments.

of the first response when GABA was applied 5 min after the previous application (Fig. 1A). The cells fully recovered from this desensitization after a period of 15–20 min (Fig. 1B). Therefore, 25 min after measuring the initial response to GABA, the cells were stimulated again for 30 s in the presence of various test drugs to examine the effects of the drugs on the GABA-induced increase in $[Ca^{2+}]_i$. A typical example of the effect of the $GABA_A$ receptor antagonist bicuculline is shown in Fig. 1C.

3.2. Effect of GABA antagonists on the GABA-induced increase in $[Ca^{2+}]_i$

Cells were exposed to antagonists 5 min before the second stimulation with GABA. The $GABA_A$ receptor antagonists bicuculline and picrotoxin each inhibited the GABA-induced increase in $[Ca^{2+}]_i$ in a concentration-dependent manner; the 50% inhibitory concentration (IC_{50}) of each drug was 50 μM (Fig. 3). In contrast, the $GABA_B$ receptor antagonist phaclofen had no effect on the GABA-induced Ca^{2+} response. The response to GABA in the presence of 100 μM MK-801, an antagonist of the *N*-methyl-D-aspartate-sensitive glutamate receptor, was 91.3 ± 2.0% (n = 32 cells) of the control response. These results indicate that the $GABA_A$ receptor may mediate the GABA-induced increase in $[Ca^{2+}]_i$.

3.3. Effect of Cl^- transport inhibitors on the GABA-induced increase in $[Ca^{2+}]_i$

We investigated the role of Cl^- in the GABA-induced increase in $[Ca^{2+}]_i$ because the $GABA_A$ receptor activates a Cl^- conductance. Perfusion of cells with furosemide (10 μM to 1 mM), which blocks active Cl^-

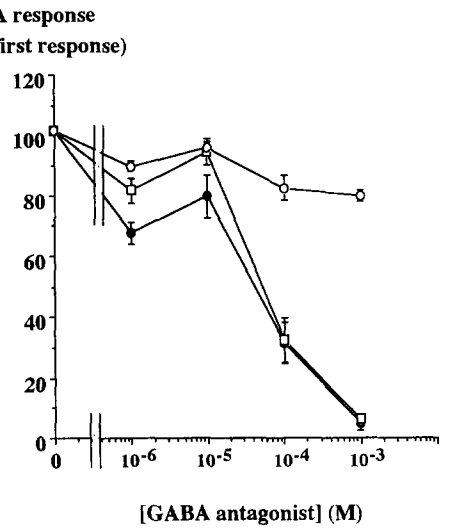

Fig. 3. Effects of GABA receptor antagonists on the GABA-induced increase in $[Ca^{2+}]_i$. The effects of picrotoxin (□), bicuculline (●), and phaclofen (○) on the GABA response were assessed as described in the legend to Fig. 1C. Values are means ± S.E.M. for 21–28 cells from three or four independent experiments.

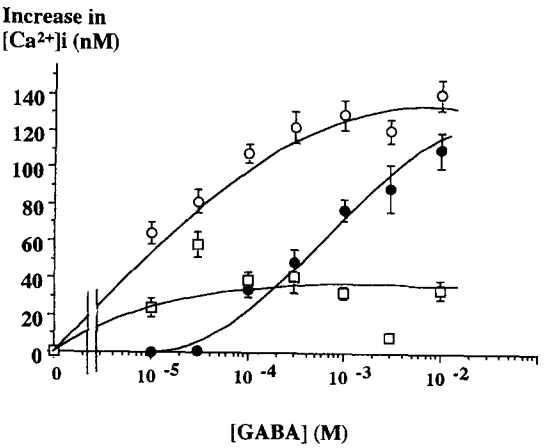

Fig. 4. Effects of bicuculline and furosemide on the concentration-response curve for the GABA-induced increase in $[Ca^{2+}]_i$. The GABA response was measured in the absence (○) or presence of 30 μM bicuculline (●) or 30 μM furosemide (□). Cells were perifused with bicuculline or furosemide 5 min before the second application of GABA. Values are means ± S.E.M. for 28 cells from four independent experiments.

transport via the $Na^+/K^+/2Cl^-$ cotransporter, 20 min before GABA application inhibited the GABA response in a concentration-dependent manner (Table 1). The inhibition by 100 μM furosemide was also dependent on the time of perfusion before GABA application [5 min, 91.0 ± 2.8%; 10 min, 62.1 ± 4.7% of the control response (n = 10–22 cells)]. Ethacrynic acid, which blocks an ATP-dependent Cl^- pump (Inoue et al., 1991), also inhibited the GABA response (Table 1). The effects of bicuculline and furosemide on the GABA concentration-response curve were investigated. Furosemide (30 μM) inhibited the GABA-induced increase in $[Ca^{2+}]_i$ in a noncompetitive manner, whereas bicuculline (30 μM) inhibited the GABA response in a competitive manner (Fig. 4). Furosemide thus did not appear to act at GABA binding sites directly. Furthermore, furosemide had no effect on the increase in $[Ca^{2+}]_i$ induced by 25 mM K^+ (Table 2).

Table 1
Effects of Cl^- transport inhibitors on the GABA-induced increase in $[Ca^{2+}]_i$. Furosemide or ethacrynic acid was added 20 min before the second GABA application. Values are means ± S.E.M. of 21–28 cells from three or four independent experiments

Treatment		GABA response (% of first response)
None (control)		99.9 ± 2.5
Furosemide	(10 μM)	64.7 ± 2.9 [a]
	(100 μM)	23.2 ± 1.9 [a]
	(1 mM)	17.8 ± 1.8 [a]
Ethacrynic acid	(100 μM)	62.0 ± 3.1 [a]
	(300 μM)	43.1 ± 3.6 [a]

[a] $P < 0.01$ vs. control.

3.4. Effect of high K^+-induced depolarization in the presence of MK-801 on the GABA-induced increases in $[Ca^{2+}]_i$

K^+ increased $[Ca^{2+}]_i$ dose dependently (25 mM K^+: 136.9 ± 7.7 nM; 50 mM K^+: 354.6 ± 8.5 nM; n = 28 cells from four independent experiments). We initially examined the effects of MK-801 on the 25 mM K^+-induced $[Ca^{2+}]_i$ response. Treatment with 50 μM MK-801 5 min before the second stimulation inhibited the high K^+ response to 82.0 ± 2.0% of the first response (n = 49 cells from seven independent experiments). This finding indicates that about 20% of the Ca^{2+} response induced by 25 mM K^+ may be associated with the NMDA receptor-induced response, probably evoked by the presynaptic-release of glutamate. In the absence of MK-801, the application of 1 mM GABA or 25 mM K^+ individually evoked an increase in $[Ca^{2+}]_i$ of 140.4 ± 7.8 and 129.4 ± 9.9 nM, respectively. The simultaneous application of the two agents induced an increase in $[Ca^{2+}]_i$ of 134.4 ± 9.5 nM (Fig. 5). When

Table 2
Effect of furosemide on the increase in $[Ca^{2+}]_i$ induced by 100 μM GABA or 25 mM K^+. Experiments with high K^+ also involved two consecutive applications, with furosemide added 20 min before the second application. Values are means ± S.E.M. for 21–28 cells from three or four independent experiments

Inhibitor	Increase in $[Ca^{2+}]_i$ (nM)	
	GABA (100 μM)	K^+ (25 mM)
None	152.2 ± 14.3	127.1 ± 11.0
Furosemide (100 μM)	38.1 ± 3.4 [a]	129.2 ± 13.2

[a] $P < 0.01$ vs. response in the absence of furosemide.

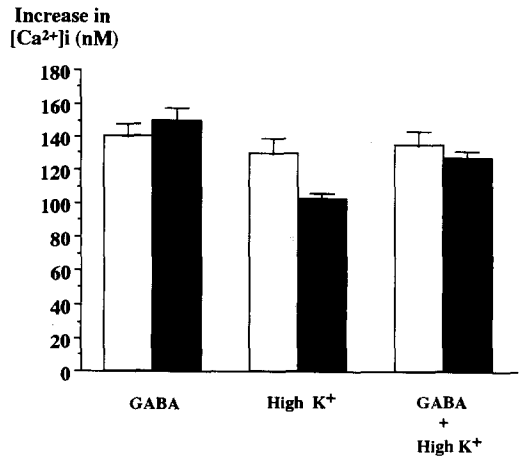

Fig. 5. Effect of high K^+ on the GABA-induced increase in $[Ca^{2+}]_i$ in the absence or presence of MK-801. The increase in $[Ca^{2+}]_i$ was measured in response to 30-s applications of 1 mM GABA, 25 mM K^+, or both in the absence (open columns) or presence (filled columns) of 50 μM MK 801, added 5 min before the second application. Values are means ± S.E.M. of 41–49 cells from six or seven independent experiments.

the administration of GABA, K^+, or their combination was preceded by 50 μM MK-801 applied 5 min beforehand, the application of 1 mM GABA or 25 mM K^+ evoked an increase in $[Ca^{2+}]_i$ of 150.7 ± 7.0 and 102.4 ± 3.5 nM, respectively. However, their simultaneous application increased the $[Ca^{2+}]_i$ to only 126.7 ± 4.5 nM (Fig. 5). The combination of the two agents thus lacked an additive effect on $[Ca^{2+}]_i$.

3.5. Effect of Ca^{2+}-related agents on GABA-induced increases in $[Ca^{2+}]_i$

The removal of $CaCl_2$ from and the addition of 1 mM EGTA to BSS completely inhibited the GABA response. Only the removal of $CaCl_2$ from BSS in the absence of 1 mM EGTA achieved the same complete inhibition (Fig. 6). The GABA-induced increase in $[Ca^{2+}]_i$ was also markedly inhibited by 10 μM nifedipine, a blocker of the L-type Ca^{2+} channels. The L-type Ca^{2+} channel agonist, BAY-K 8644, 30 μM, evoked an increase in $[Ca^{2+}]_i$ of 87.0 ± 6.5 nM. The application of 100 μM GABA increased $[Ca^{2+}]_i$ to 140.0 ± 9.2 nM, whereas application of the two agents in combination induced an increase in $[Ca^{2+}]_i$ of 149.9 ± 10.3 nM (n = 16–20 cells in three independent experiments). Thus, the combination of these two agents had a non-additive effect on $[Ca^{2+}]_i$.

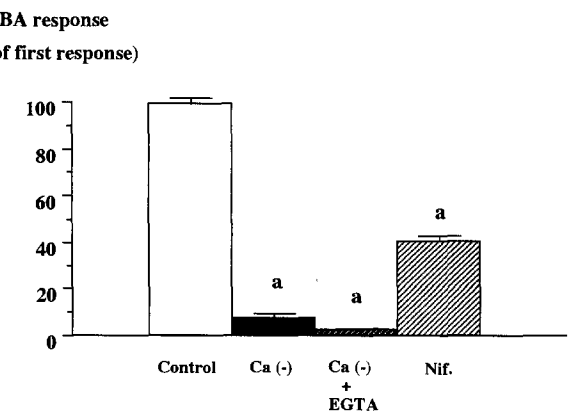

Fig. 6. Effects of Ca^{2+}-related agents on the GABA-induced increase in $[Ca^{2+}]_i$. The second GABA response was measured in the absence of EGTA with $CaCl_2$ (Control), the absence or presence of 1 mM EGTA without $CaCl_2$, or 10 μM nifedipine (Nif.), as described in the legend to Fig. 1C. Values are means ± S.E.M. of 21–28 cells from three or four independent experiments. $^aP < 0.01$ vs. control.

4. Discussion

The pharmacological characteristics revealed in our study indicate that GABA increased $[Ca^{2+}]_i$ and it seemed to be mediated by $GABA_A$ receptors. This observation is consistent with those of previous studies with immature cells such as rat cerebellar granule cells (Connor et al., 1987), rat neocortical slice cells (Yuste and Katz, 1991), rat pituitary cells (Horvath et al., 1993), and chick retinal cells (Yamashita and Fukuda, 1993). However, several studies suggest that the GABA receptor, which induces Ca^{2+} influx, is different from the conventional $GABA_A$ receptor subtype on the basis of the findings of long-lasting desensitization and the low affinity for antagonists (Segal, 1993; Sieghart, 1995). Further studies are required to clarify the molecular mechanism.

The present results of the Ca^{2+} response to agonists seems lower than those of several previous reports (Yuste and Katz, 1991; Segal, 1993). The difference may be due to variations in the tissue, types of fluorescence indicator used, and culture conditions (days in culture, etc). The effects of high K^+-induced Ca^{2+} response depend upon the stage of development of the cells in culture (Connor et al., 1987).

A high concentration of extracellular K^+ was used to elevate $[Ca^{2+}]_i$ elevation by directly depolarizing the membrane, thereby opening the voltage-gated Ca^{2+} channels. The action potential activated by 25 mM K^+ is about −38 mV when we calculate according to the Nernst equation, assuming $[K^+]_i$ as 140 mM. The value may be sufficient to start the opening of the L-type voltage-gated Ca^{2+} channels (Catterall and Striessnig, 1992). Our data suggest that the GABA-induced increase in $[Ca^{2+}]_i$ shares a common pathway of Ca^{2+} movement with high K^+-induced Ca^{2+} responses in the absence or presence of NMDA receptor antagonist (MK-801). Furthermore, the GABA-induced Ca^{2+} response also shares a common Ca^{2+} pathway with BAY-K 8644-induced Ca^{2+} responses. The failure of

GABA to increase $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} and the inhibition of the GABA response by a blocker of L-type Ca^{2+} channels suggest that GABA-induced depolarization leads to Ca^{2+} influx through the L-type voltage-dependent Ca^{2+} channels. Since the GABA-induced Ca^{2+} influx was only partly inhibited by 10 μM nifedipine, at concentrations sufficient to completely block L-type voltage-gated Ca^{2+} channels (Godfraind et al., 1986), the GABA-induced Ca^{2+} influx may occur in part via other types of voltage-gated Ca^{2+} channels and/or kainate receptors. Previous electrophysiological studies also have shown that GABA induces depolarization in immature neurons (Cherubini et al., 1990, 1991; Horvath et al., 1993; Hales et al., 1994).

The $GABA_A$ receptor is a ligand-gated Cl^- channel. Activation of the $GABA_A$ receptor therefore increases conductance to Cl^-, an ion whose permeability is normally low. In most adult central neurons, the increase in the $GABA_A$ receptor Cl^- conductance results in hyperpolarization of the cell membrane (Sivilotti and Nistri, 1991). However, activation of $GABA_A$ receptors in the dendritic regions of adult rat hippocampal pyramidal cells causes a depolarization of the cell membrane. Electrophysiological studies have revealed that the depolarizing effect of an increasing Cl^- conductance in these cells is attributable to a difference in the equilibrium potential of Cl^- (E_{Cl^-}), estimated from the intracellular ($[Cl^-]_i$) and extracellular ($[Cl^-]_o$) Cl^- concentrations (Anderson et al., 1980; Alger and Nicoll, 1982; Segal and Barker, 1984). By measuring $[Cl^-]_i$ directly with the use of a Cl^--sensitive fluorescent dye, Hara et al. (1992) demonstrated that dendritic $[Cl^-]_i$ is about 6 mM higher than perikaryonic $[Cl^-]_i$ in rat hippocampal pyramidal cells. These researchers also suggested that the uneven distribution of $[Cl^-]_i$ may be generated by furosemide- and ethacrynic acid-sensitive Cl^- transport systems. Such Cl^- transport systems have been shown to produce the active distribution of Cl^- maintain the Cl^- gradient across the cell membrane, and generate inhibitory postsynaptic potentials (Misgeld et al., 1986; Thompson et al., 1988a,b; Inoue et al., 1991). In the present study, both furosemide and ethacrynic acid inhibited GABA-induced Ca^{2+} influx, whereas furosemide had no effect on high K^+-induced Ca^{2+} influx. Our data suggest that furosemide affects the Cl^- transport system directly rather than by acting at GABA binding sites or voltage-gated Ca^{2+} channels. Thus, Cl^- transport systems appear to be required for GABA-induced Ca^{2+} influx in immature cortical neurons. GABA may therefore induce Ca^{2+} influx in immature neurons in a manner that is dependent on Cl^- transport systems, as it does in the dendritic regions of adult rat hippocampal pyrammidal cells. Indeed, studies with cultured rat hippocampal neurons suggest that $[Cl^-]_i$ is higher in the early developmental stages (Hara et al., 1992). Zhang et al. (1991) have proposed a gradual development of the outward Cl^- transport systems, which shift the E_{Cl^-} to values more negative than the resting membrane potential in more mature hippocampal neurons. It is speculated that the Cl^- gradient in immature neuron is reversed relative to that in adult neurons. One possibility is thought that the $Na^+/K^+/2Cl^-$ and ethacrynic acid-sensitive Cl^- transport systems produce active movement of Cl^- inwardly in immature neurons, so that the application of GABA induced depolarization as a result of the outward movement of Cl^-.

In conclusion, the GABA-induced increase in $[Ca^{2+}]_i$ in immature rat cortical neurons appeared to be triggered by $GABA_A$ receptors and resembled the response to high K^+-induced depolarization. Our data indicate that GABA induced Ca^{2+} influx via voltage-gated Ca^{2+} channels. These effects appeared to be dependent upon Cl^- transport systems that are sensitive to furosemide and ethacrynic acid.

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