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Modulation of γ-aminobutyric acid-mediated inhibitory synaptic currents in dissociated cortical cell cultures

(rat cortex/patch–clamp/benzodiazepines)

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ABSTRACT **Inhibitory γ-aminobutyric acid-mediated synaptic currents were studied in dissociated primary cultures of neonatal rat cortex with the whole-cell patch–clamp technique. Immunocytochemical staining of the cultures showed the presence of a large number of glutamic acid decarboxylase-containing neurons, and electrical stimulation of randomly selected neurons produced in many cases chloride-mediated and bicuculline-sensitive inhibitory synaptic currents in postsynaptic cells. The amplitude and decay time of the inhibitory synaptic currents were increased by flunitrazepam and decreased by the β-carboline derivative methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate, two high-affinity ligands for the allosteric regulatory sites of γ-aminobutyric acid receptors. The imidazobenzodiazepine Ro 15-1788, another high-affinity ligand of the γ-aminobutyric acid receptor regulatory sites that has negligible intrinsic activity, blocked the action of flunitrazepam and β-carboline. However, Ro 15-1788 also increased the decay rate of the inhibitory synaptic currents. This might suggest that an endogenous ligand for the benzodiazepine-β-carboline binding site is operative in γ-aminobutyric acid-mediated synaptic transmission.**

The development of the patch–clamp technique (1) has greatly facilitated the study of receptor function in terms of specific ion currents. The intracellular perfusion characteristic of this technique (2) allows manipulation of the ionic environment that is useful for distinguishing and characterizing different ion currents.

Among the functional synaptic connections that have been described in primary neuronal cultures from fetal rat cortex (3) are those mediated by γ-aminobutyric acid (GABA), an inhibitory neurotransmitter that is synthesized, released, and taken up by these cells (4, 5). Voltage-clamp analysis of GABA-mediated inhibitory postsynaptic currents (IPSCs) has been reported on various synapses. The best characterized is the neuromuscular junction of the crayfish and locust (6–10). IPSCs were also recorded in hippocampal slices (11) and in primary cultures of hippocampal neurons in which diazepam, a ligand for the allosteric modulatory site of GABA receptors, potentiated the IPSCs (12).

Another anxiolytic benzodiazepine, flunitrazepam, and an anxiogenic β-carboline derivative, methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), potentiate and reduce, respectively, the increase in chloride conductance induced by direct application of GABA on the cell bodies of spinal cord neurons (13). We studied the action of these drugs and of the imidazobenzodiazepine Ro 15-1788 (ethyl 5,6-dihydro-8-fluoro-5-methyl-6-oxo-4*H*-imidazo-[1,5a][1,4]-benzodiazepine-3-carboxylate), a virtually inert ligand of the benzodiazepine recognition site that antagonizes the actions of flunitrazepam and DMCM (14), on the amplitude and the decay of the postsynaptic currents.

MATERIALS AND METHODS

Primary Culture. Primary neuronal cultures were obtained from 1-day-old rat cerebral cortices by the dissociation procedure described (15) for neonatal rat cerebellar cultures. Cells were plated (2×10^6 cells per dish) on 35-mm Nunc dishes coated with poly (L-lysine) (10 μg/ml; M_r, 70–150 × 10^3) and were cultured in basal Eagle's medium/10% (vol/vol) fetal bovine serum (both from GIBCO)/25 mM KCl/2 mM glutamine (Sigma)/gentamycine at 100 μg/ml (GIBCO). After 24 hr *in vitro*, the medium was replaced, and 1 μM cytosine arabinofuranoside was added to inhibit replication of nonneuronal cells. The cultures were used from the 7th to the 20th day after plating.

Immunocytochemistry. These cultures were washed with 0.1 M phosphate-buffered saline (Standard Sörensen, PBS, pH 7.4) and then fixed with 4% (wt/vol) paraformaldehyde in PBS for 10 min at 25°C. The cells were first incubated for 20 min with a 1:10 dilution of normal swine serum and then incubated for 2 hr at 25°C in PBS containing 0.1% Saponin (Sigma) with anti-glutamic acid decarboxylase antiserum (1:100 dilution) (a gift from I. Kopin, National Institutes of Health) (16). The incubation was terminated by several washings and followed by incubation with the secondary antibody (1:50 dilution) in PBS plus Saponin for 15 min. Secondary antibody was affinity-purified swine anti-sheep IgG conjugated with rhodamine isothiocyanate (Boehringer Mannheim). To block axonal transport some dishes were treated with 1 μM colchicine (Sigma) added to the culture medium at 37°C for the 3 hr preceding immunostaining. After staining, the cells were mounted with buffered glycerol [50% (vol/vol) PBS, pH 7.4/50% (vol/vol) glycerol] and viewed with a fluorescence microscope (Olympus, Japan). The specimens were photographed on Tri-X film (Kodak, 400 ASA). Control dishes were incubated with preimmune serum in the same condition as the specific antiserum. No staining was observed in this case. The omission of the link antiserum or of the specific antiserum completely prevented the labeling. Four scratches made on the bottom of the culture dish close to each electrophysiologically examined neuron allowed their identification after the immunostaining procedure.

Electrophysiology. Cells were bathed in standard saline medium at room temperature (21–23°C). This solution was 145 mM NaCl/5 mM KCl/2 mM $CaCl_2$/5 mM Hepes·NaOH, pH 7.4. Osmolarity was adjusted to 325 mosM with sucrose. The pipette contained 145 mM potassium gluconate, 1.1 mM EGTA, 2 mM $MgCl_2$, 10 mM Hepes·KOH, pH 7.2 and 315

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Abbreviations: GABA, γ-aminobutyric acid; IPSC, inhibitory postsynaptic current; DMCM, methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate.

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mosM. Liquid junction potential was corrected using a saline bridge connected to the ground that contained the two solutions. The whole-cell tight-seal technique (1) was employed to record spontaneous and evoked synaptic currents with a patch–clamp amplifier (EPC7, List Electronics, Darmstadt, F.R.G.). Pressure application onto the presynaptic cell of 15 μM L-glutamate lasting 10–100 ms, or current injection to elicit action potential while recording with another patch amplifier, triggered synaptic transmission.

No significant differences in the synaptic currents generated by these two procedures were observed, provided a short enough glutamate pulse was used to trigger only one synaptic event. Glutamate applications facilitated the testing of many different synaptic inputs so that this procedure was preferred. Synaptic currents, after sampling at 2.5 kHz and filtering at 500 Hz (8-pole, low-pass Bessel), were stored in a LSI 11/23 computer (Indec System, SunnyVale, CA) for further analysis. Exponential fit of the initial part of the decay phase of IPSCs was performed with a nonlinear, least-square fitting routine between two cursors, the first set on the peak of the current and the second set approximately at the beginning of the slower phase. A more precise evaluation of the two components, using a double-exponential fitting routine, gave similar values for the fast decay.

The benzodiazepine and β-carboline derivatives were dissolved in bath solution containing dimethyl sulfoxide at a final concentration of 0.01% and pressure applied in the proximity of the cell body with a micropipette (the tip being slightly larger than in a patch pipette). Flunitrazepam and Ro 15-1788 were from Hoffmann–LaRoche (Nutley, NJ) and DMCM from Ferrosan (Copenhagen).

RESULTS

GABAergic Neurons in Culture. More than 120 inhibitory synaptic connections between presynaptic and postsynaptic cortical neurons of neonatal rat in culture (Fig. 1*A*) were studied with the patch–clamp technique. Immunocytochemical staining for the enzyme glutamic acid decarboxylase, a marker of GABAergic neurons, after short incubation in colchicine, documented presence of the enzyme in cells generating inhibitory synaptic currents (Fig. 1*B*). Data were collected from four different culture dishes each with 3–4 cells identified as capable to generate IPSCs. Since in cultured cortical cells there is a very good correlation between glutamic acid decarboxylase staining and GABA uptake (17), the enzyme-containing cells were considered to be GABAergic.

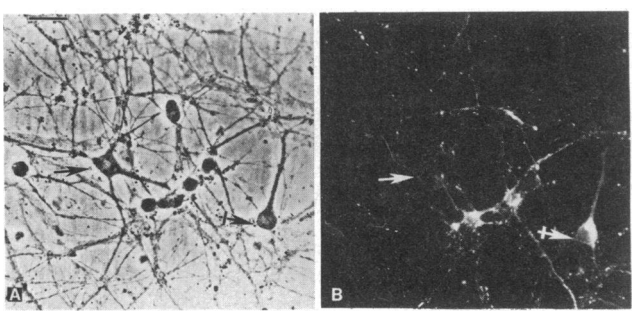

FIG. 1. Cortical neurons from neonatal rat brain after 14 days *in vitro*. (*A*) Phase-contrast micrograph of synaptically paired cells. The crossed arrow points to the presynaptic neuron, and the other arrow points to the postsynaptic neuron. (*B*) Fluorescent micrograph of the same cells stained for rhodamine-conjugated glutamic acid decarboxylase antibodies. The scratches to identify the cell are out of the field since they were done at a certain distance to minimize damage to the neurites of the cell. (Bars = 20 μm.)

The resting membrane potential ranged between −50 and −60 mV, and, at the ages tested (7–20 days in culture), all generated action potentials upon electrical or chemical stimulation. The putative GABAergic neurons showed a strong tendency to repetitive firing. Stimulation of the presynaptic neuron at 0.08 Hz produced outward currents in the postsynaptic cell held at −40 mV. The average amplitude of the IPSCs in 130 neurons at this holding potential was 213 ± 160 pA (mean ± SD). Most of these synaptic currents decayed in a biphasic exponential fashion (Fig. 2). The initial fast phase was followed by a delayed component, slower by a factor of 2–3, accounting for 30–40% of the total amplitude at the peak of the current. From the 130 connections tested, the average fast decay time constant was 24 ± 10 ms (mean ± SD) at room temperature. The slow phase and the large standard deviation of the fast component could reflect differences in the reuptake system, the poor space clamp of the dendritic tree (18), or the activation of channels with different kinetics. Due to the complexity of the system, we have focused our attention only on the pharmacological modifications of the fast component of the current decay without further investigating the slow phase. Application of GABA on the cell body of the postsynaptic cell just after the recording of evoked IPSCs (Fig. 3*A*) showed the same reversal potential (Fig. 3 *C* and *D*) for the two events, which averaged −64 ± 8 mV (mean ± SD), $n = 25$. Pressure application of the $GABA_A$ receptor antagonist bicuculline methiodide (25 μM) (Fig. 4*A*) abolished the evoked synaptic response reversibly.

The slope conductances from the current–voltage relationship were different in the outward from the inward direction either when they were derived from the peak amplitude of synaptic current (Fig. 3*C*) or from the directly evoked GABA

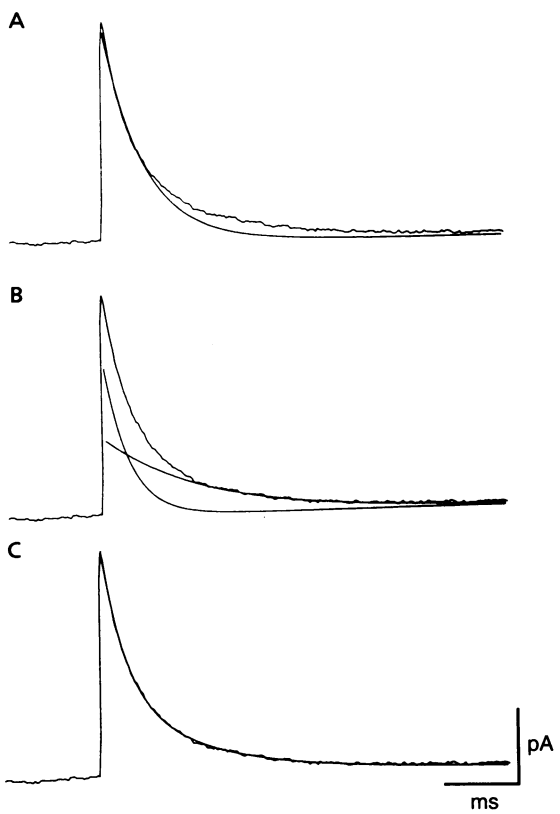

FIG. 2. Inhibitory postsynaptic currents recorded from a cortical neuron were fitted by a single-exponential curve in *A*, and by the sum of two exponential curves in *C*. These two curves are shown separately in *B*. In the example shown the fast time constant was 16 ms, the slow one was 52 ms with a 40% contribution to the peak amplitude. (Bars = 50 ms and 50 pA.)

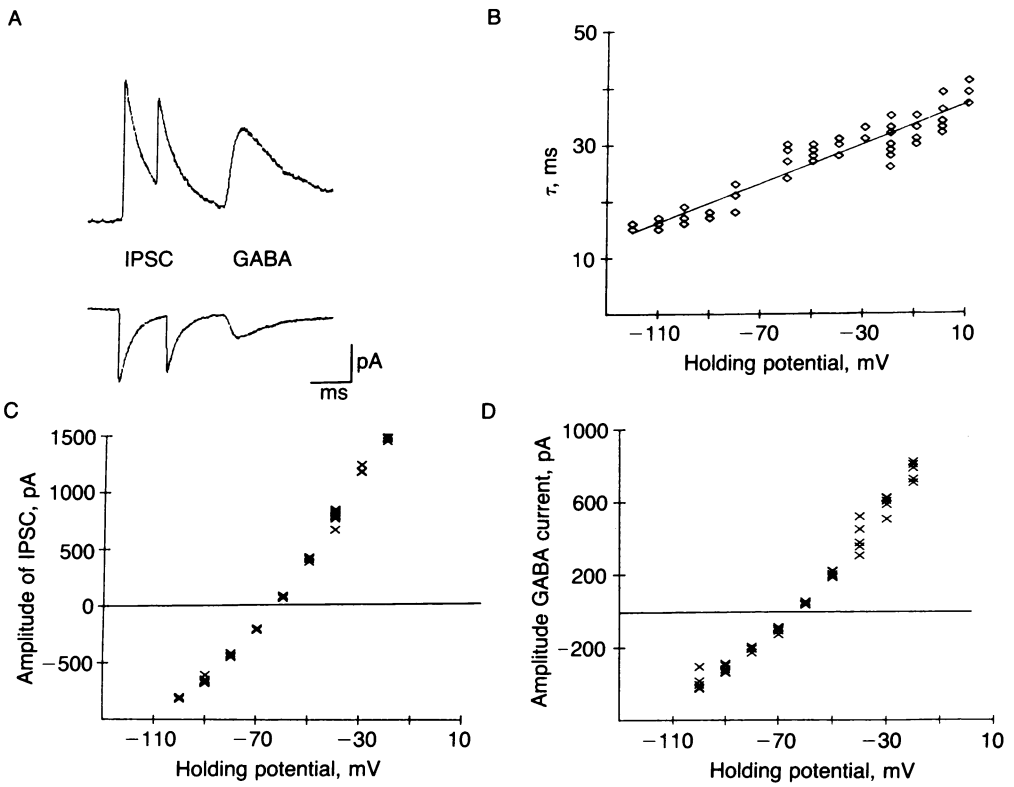

FIG. 3. Recordings of GABA-mediated presynaptically evoked IPSCs (*A*) followed by chloride currents induced by direct application of GABA on the cell body of the postsynaptic cell held at −40 mV (upper trace) and −80 mV (lower trace). Outward currents are shown upward. (Horizontal bar = 50 ms. Vertical bar = 50 pA.) Reversal potential in this case was −63 mV as shown from the current–voltage relationship (*C* and *D*) of the two responses. Each point represents a single IPSC. Voltage dependence of the decay time constant τ of synaptic currents from one cell is reported (*B*).

response (Fig. 3*D*). The fast decay of the current was voltage dependent (Fig. 3*B*) becoming faster with hyperpolarized potentials. Using the relation reported for the GABA-mediated crayfish neuromuscular junction (8) $\tau = ae^{AV}$, where τ is the decay time constant and V is the applied voltage, we derived values for A of 0.008 ± 0.001 mV^{-1} and a of 38 ± 9 ms (mean ± SD, n = 3).

Modulation of the Synaptic Currents. When 4 μM flunitrazepam was pressure ejected for 5 s during low-frequency repetitive stimulation of the presynaptic cell, a transient increase (Fig. 4*B*) followed by a slight depression without full recovery in the current amplitude was observed. Application of the β-carboline DMCM (Fig. 4*C*) reduced the current in a fully reversible manner. Flunitrazepam slowed down the rapid current decay by a factor of 2 (Fig. 5*C*), while DMCM slightly accelerated it (Fig. 5*D*). Coapplication of Ro 15-1788 masked the effects of either flunitrazepam or DMCM (Fig. 5 *E* and *F*). When Ro 15-1788 was applied alone it decreased the time course of IPSCs (Fig. 5*B*). It should be mentioned that

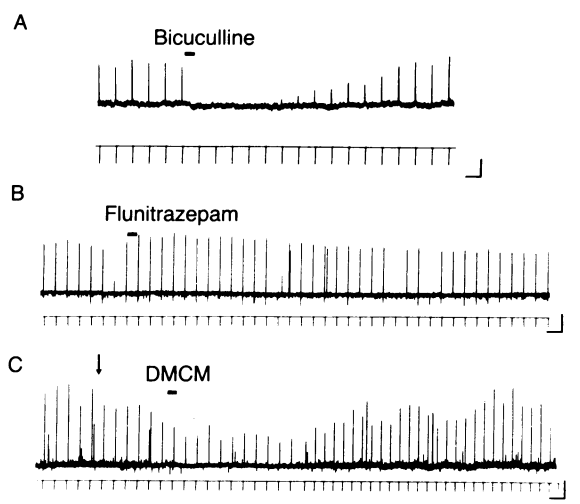

FIG. 4. Pressure application of 25 μM bicuculline methiodide abolished (*A*) outward IPSCs that 15 μM glutamate evoked in the postsynaptic cell held at −40 mV, as shown on this slow time base chart recording. Flunitrazepam (4 μM) slightly potentiated the amplitude of inhibitory currents recorded from another couple of neurons (*B*) without full recovery. DMCM (10 μM) reduced IPSCs in a reversible fashion (*C*). Decrease of the response was already present as soon as the drug-containing pipette was brought close to the recorded cell (arrow) due to leakage of the DMCM-containing pipette. [Horizontal bars = 7.5 s (*A*), 10 s (*B* and *C*). Vertical bars = 50 pA.]

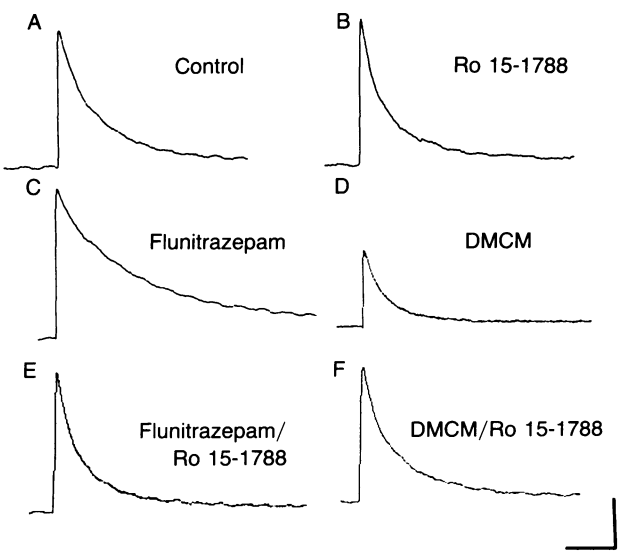

FIG. 5. Inhibitory synaptic outward currents recorded from cortical neurons at −40 mV holding potential. Control current (*A*), IPSCs after application of 10 μM Ro 15-1788 (*B*), 4 μM flunitrazepam (*C*), 10 μM DMCM (*D*), and flunitrazepam and DMCM in combination with Ro 15-1788 (*E* and *F*). (Horizontal bar = 50 ms. Vertical bar = 200 pA.)

in 20–30% of the cells tested no significant effect was observed. A summary of the drug-induced modifications of IPSCs, obtained from all the synaptically connected neurons studied, is reported in Table 1. In one series of experiments, application of a solution containing 0.5 mM $CaCl_2$ and 2 mM $MgCl_2$ to reduce transmitter release, decreased the amplitude without affecting the current decay constant (n = 3). Application of Ro 15-1788 during repetitive direct pressure ejection of GABA on the postsynaptic cell failed to modify the chloride current produced (n = 4) (Fig. 6).

FIG. 6. Chloride currents induced by pressure application of GABA directly on the cell body of the postsynaptic cell held at −40 mV were not affected by application of 20 μM Ro 15-1788. (Horizontal bar = 0.5 sec. Vertical bar = 100 pA.)

DISCUSSION

The cortical neurons studied for this report appear to be an appropriate model to investigate the physiological and pharmacological regulation of the GABAergic synapse. While a great deal of information is available on the physiology of nicotinic cholinergic transmission, much less is known about GABA-mediated synaptic transmission. Most information is derived from studies of the crayfish and locust inhibitory synapses (6–10). In good agreement with data from these preparations and from rat hippocampal slices (11), we observed a voltage-dependent decay of the IPSCs recorded from rat cortical cells. From analysis of GABA-induced current noise (10), it seems that the voltage dependence of postsynaptic channel kinetics underlies this phenomenon. The nonlinear current–voltage relationship was also seen in other GABA-mediated IPSCs (6, 8, 9, 11). Sometimes the amplitude of our IPSCs decreased to a plateau without recovery. This might be explained by changes in chloride equilibrium potential, desensitization, loss of unknown intracellular components essential for the cell physiology or a depletion of GABA stores located in the presynaptic terminal. We did not investigate which of these factors was operative, and we included data only from recordings in which a stable amplitude was maintained throughout the experiment. Other preparations (6–10) showed currents with a single exponential decay faster than the one we measured. Only the rate of the current decay of IPSCs from hippocampal GABAergic neurons in culture compares to the fast phase of the current decay of cortical IPSCs (12). It is possible that the kinetics of the GABA chloride channel might be particularly slow in cultured neurons.

In agreement with other reports (12), flunitrazepam slows down the decay time of IPSCs and to a smaller extent increases the current amplitude. A slight reduction of the IPSC persisted after flunitrazepam application. A spontaneous decrease of the current amplitude was sometimes present in control conditions; however, the current was always smaller after flunitrazepam. Whatever mechanism is responsible for the spontaneous decline of the GABA-mediated currents, the benzodiazepine appears to facilitate it. A simultaneous increase of the open time and frequency of openings of the chloride channel has been proposed to explain the benzodiazepine effect on GABA-mediated synaptic current and GABA-induced current fluctuations (12, 19). DMCM decreased the current amplitude by half and its decay time by 20%. Since it has been shown that this compound reduces the response to direct application of GABA (13, 20), this reduction of the amplitude may reflect a decrease in the number of channels activated during synaptic transmission and in either the single-channel conductance or the frequency of openings. The decrease of IPSC duration following DMCM was similar to that obtained with Ro 15-1788 application. This faster time course of the IPSC could be due to a direct drug action on the postsynaptic channel. The decrease in amplitude caused by DMCM and the prolongation of the decay by flunitrazepam were antagonized by Ro 15-1788 suggesting that the three compounds may act after binding to the same allosteric regulatory site of GABA receptor. There has been a report on the action of Ro 15-1788 on responses elicted by ionophoresed GABA (13, 21) suggesting that the imidazobenzodiazepine may be a weak partial agonist. Applications of Ro 15-1788 failed to change chloride currents induced by GABA application. Perhaps the resolution of our technique may not have been adequate to detect small variations. In hippocampal slices (22, 23), reduction of spontaneous and evoked inhibitory postsynaptic potential has been reported using a concentration of Ro 15-1788 lower by a factor of 10 than we used; however, with such concentrations we failed to detect any modification of IPSCs.

Unlike DMCM, Ro 15-1788 never changed IPSC amplitude, but both compounds decreased the synaptic current decay. In contrast, flunitrazepam increased the IPSC decay time. Other investigators (7, 10, 12) suggested that the rate-limiting step of the IPSC decay is the mean channel-bursting time, but one cannot exclude that a drug can modify the reuptake system, the enzymatic cleavage of GABA, or the mechanism of transmitter release. The application of low Ca/high Mg solution to decrease synaptic release of transmitter, failed to change the time course of IPSC. However, these experiments do not completely exclude a drug action on transmitter release and reuptake. Even though Ro 15-1788 failed to change GABA-elicited currents, in the absence of more direct experiments, we cannot yet rule out an action of the imidazobenzodiazepine on the postsynaptic channel. In fact one cannot dismiss the possibility that Ro 15-1788 displaces an endogenous modulator that binds to the benzodiazepine recognition site and mimics the benzodiazepines in prolonging the IPSCs duration. A final conclusion on the action of Ro 15-1788 must depend on a careful study of its action on GABA-activated channel kinetics in membrane patches from these cortical cells. *In vivo* evaluation of the action of Ro 15-1788 at present is difficult (see ref. 24 for review). In conclusion, the present report cannot exclude the existence of a putative endogenous ligand or ligands to the allosteric modulatory site of the GABA receptor.

Table 1. Variation from control values of amplitude and fast decay phase of IPSC following applications of various ligands of benzodiazepine binding site

Compound	Conc, μM	Relative amplitude	SD	n	Relative τ value	SD	n
Flunitrazepam	4	116	14	5	174	36	5
DMCM	10	65	6	3	82	6	3
Ro 15-1788	4	91	8	7	78	9	7
	10	98	10	3	79	6	3
	20	87	28	3	63	11	3
Flunitrazepam/Ro 15-1788	4/50	94	15	6	67	6	6
DMCM/Ro 15-1788	10/20	99	7	3	88	8	3
Vehicle		97	6	4	102	6	4

Control values before drug applications, 100; n, number of cells; conc, concentration.

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