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Stimulation of High Affinity γ-Aminobutyric Acid$_B$ Receptors Potentiates the Depolarization-Induced Increase of Intraneuronal Ionized Calcium Content in Cerebellar Granule Neurons

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SUMMARY

In the treatment of spasticity, the therapeutic cerebrospinal fluid levels of (±)-baclofen, a γ-aminobutyric acid (GABA)$_B$ receptor agonist, are below 1 μM. However, the mechanism of the therapeutic action of (±)-baclofen remains unknown, because, for the most part, the action of (±)-baclofen on GABA$_B$ receptors requires micromolar concentrations. Using fura-2 fluorescence microscopy, intracellular ionized calcium was measured in cerebellar granule neurons. Stimulation of a high affinity GABA$_B$ receptor potentiated by 2–3-fold the rise in intracellular calcium observed after depolarization of the cell with a Krebs Ringer's buffered solution containing 40 mM K^+. Both GABA (100 nM) and (±)-baclofen (10–100 nM) stimulated this high affinity receptor. The potentiation of the depolarization-induced rise in intracellular calcium by (±)-baclofen (100 nM) was completely blocked by the GABA$_B$ receptor antagonist CGP 35348 (200 μM). Also, the intracellular calcium response induced by the activation of high affinity GABA$_B$ receptors was prevented by dantrolene (10 μM). The cerebellar granule neurons contained calcium-induced calcium release (CICR) stores. Caffeine (3 mM) and ryanodine (100 μM) potentiated the depolarization-induced rise in intracellular calcium, and this response to both drugs was blocked by dantrolene (10 μM). Because dantrolene does not prevent the rise in intracellular calcium after cell depolarization (this calcium originated from the influx of extracellular calcium), (±)-baclofen acting via the high affinity GABA$_B$ receptor indirectly activates the CICR stores, allowing the influx of extracellular calcium to trigger the release of calcium from these dantrolene-sensitive CICR stores. Thus, this high affinity GABA$_B$ receptor might become activated during persistent depolarization caused by pathological states and could be a mechanism to be studied for the therapeutic action of (±)-baclofen in spasticity.

Certain pathologies (e.g., spinal cord injury and multiple sclerosis) may change some physiological properties of a class of central GABA receptors termed GABA$_B$ (1). In tissues obtained from normal animals, stimulation of GABA$_B$ receptors with GABA or (±)-baclofen (*p*-chlorophenyl-GABA) typically results in an activation of potassium currents (2–5), an inhibition of voltage-dependent calcium currents (6, 7), or an inhibition of adenylyl cyclase (8, 9). Each of these responses is elicited by micromolar concentrations of (±)-baclofen or GABA. Interestingly, in the treatment of spasticity, the cerebral spinal fluid concentration of (±)-baclofen that is therapeutically active does not exceed 1 μM (10); when the concentration exceeds 1 μM, adverse effects, such as a loss of muscle tone, sleepiness, and respiratory depression, ensue (11, 12). Similarly, rats injected with baclofen show ataxia and myorelaxation reminiscent of the human untoward reactions to high doses of (±)-baclofen. In rats, the ataxia and myorelaxation caused by (±)-baclofen were antagonized by the GABA$_B$ receptor antagonist CGP 35348 (13, 14). Moreover, these doses of this antagonist given alone were without any effect.

A plausible working hypothesis to explain how doses of (±)-baclofen below those that elicit classical GABA$_B$ receptorial responses relieve human spasticity might be that a high affinity GABA$_B$ receptor, which can bind nanomolar concentrations of (±)-baclofen, becomes operative in association with specific spinal cord changes during spasticity. The experiments to be reported show that, in primary cultures of cerebellar granule cells, nanomolar concentrations of (±)-baclofen at least double the increase in intracellular free calcium triggered by cell depolarization. It is proposed that (±)-baclofen acts at a high

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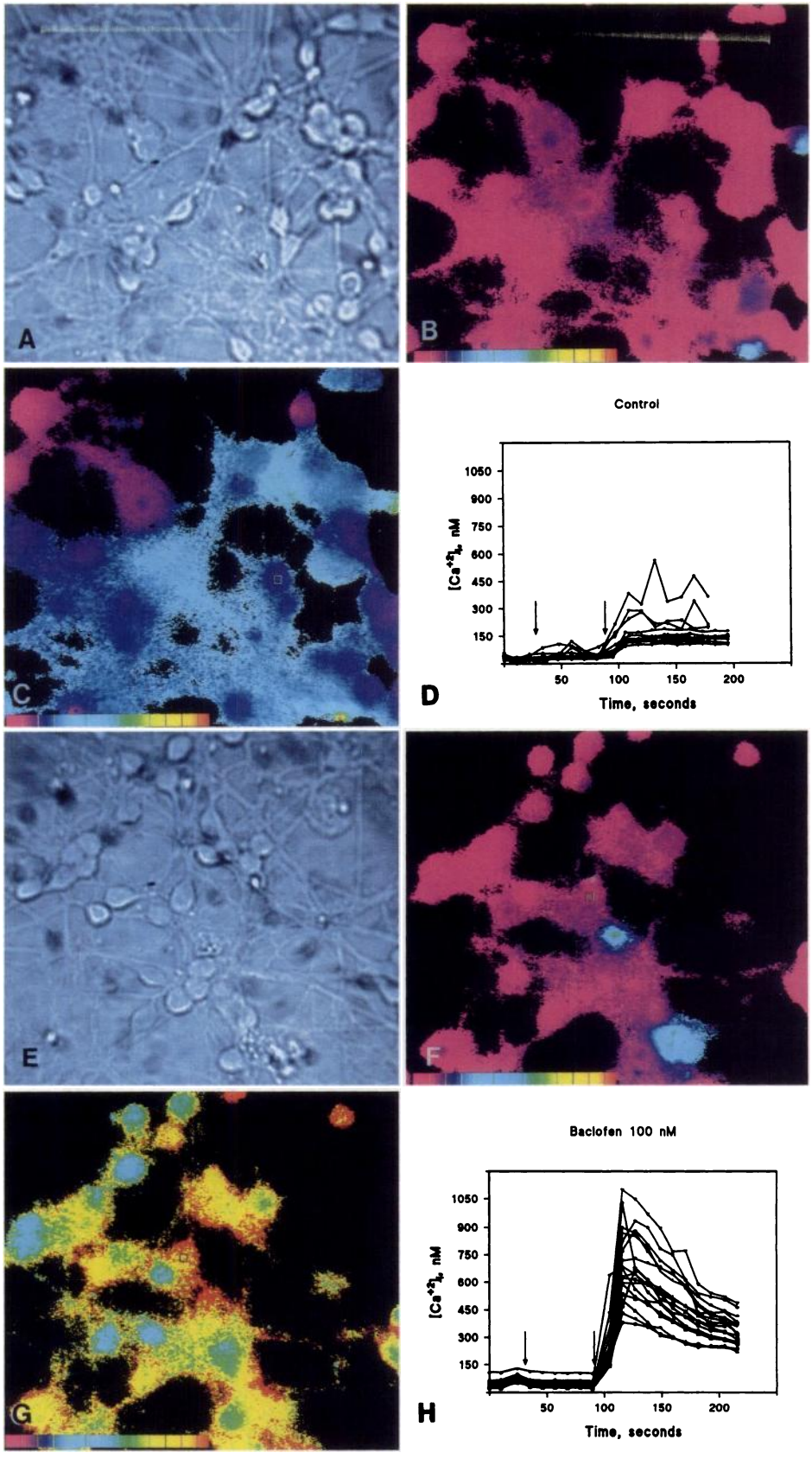
ABBREVIATIONS: GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; CGP 35348, 3-aminopropyldiethoxymethylphosphonic acid; CI-966, [1-[2-[bis(trifluoromethyl)phenyl]methoxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid; 4-AP, 4-aminopyridine; TEA, tetraethylammonium; NMDA, *N*-methyl-D-aspartate; CICR, calcium-induced calcium release.



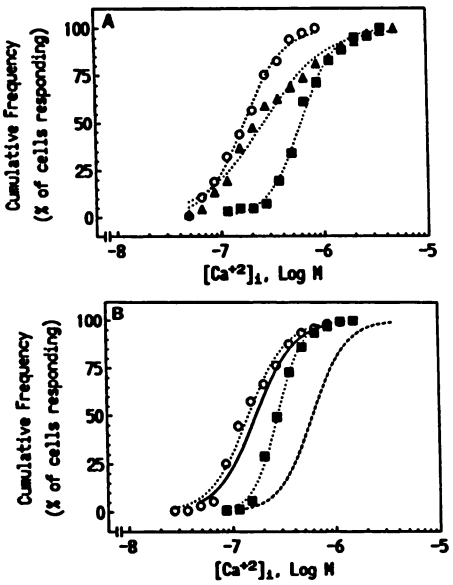


Fig. 2. (±)-Baclofen [10 nM (▲) and 100 nM (■)] (A) and GABA (100 nM) (■) (B) facilitate the depolarization-induced increase in intracellular free calcium concentration in cerebellar granule neurons. ○, Control for A. The results are represented as the percentage of cells responding (shown as a cumulative frequency plotted on the *ordinate*) to (±)-baclofen or GABA with a certain intracellular calcium concentration (*abscissa*). Neither (±)-baclofen nor GABA had any effect on intracellular calcium content when the cultures were incubated with nondepolarizing Krebs Ringer's solution (data not shown). (±)-Baclofen (10 nM) elicited its effect on intracellular calcium in only half of the cells studied. The GABA uptake inhibitor CI-966 and the $GABA_A$ receptor antagonists bicuculline (○ in B) did not alter the increase in intracellular calcium concentration resulting from cell depolarization with K^+. [In B, *solid line*, control curve from A; *dashed line without symbols*, curve for 100 nM (±)-baclofen from A.] The values shown represent measurements taken from 123 cells for the control group, 33 cells for the 0.3 nM (±)-baclofen group, 133 cells for the 10 nM (±)-baclofen group, 123 cells for the 100 nM (±)-baclofen group, 123 cells for the control group exposed to 50 μM CI-966 plus 100 μM bicuculline, and 203 cells from the 100 nM GABA plus CI-966 and bicuculline group. Typically, 15–20 cells were measured simultaneously in one microscopic field.

affinity $GABA_B$ receptor that differs from previously reported low affinity $GABA_B$ receptors.

Materials and Methods

Cerebellar granule cell cultures. Cerebellar granule neurons were prepared from cerebella dissected from 7-day-old rat pups, as previously described (15, 16). Briefly, cerebella from neonatal rats were dispersed by trypsinization, washed, and plated in basal Eagle medium with 10% fetal calf serum. Dissociated cerebellar cells, having a density of 1.5–2 × 10^6 cells, were plated onto poly-L-lysine (10 μg/ml)-coated 25-mm circular glass coverslips (thickness no. 1; Fisher Scientific) and placed in 35-mm Petri dishes (Nunc). Twenty-four hours after cell plating, the cultures were treated with cytosine-1-β-D-arabinofuranoside. Experiments were performed after 7–9 days in cell culture (37°, 6% CO_2/94% air humidified gas mixture).

Imaging of intracellular free calcium using fluorescence microscopy. On the seventh, eighth, and ninth days of primary culture, cerebellar granule cultures were preloaded with fura-2/acetoxymethyl ester (25 μM; Molecular Probes Inc.) for 15 min at 37°. After this incubation period, the remaining fura-2 was removed; cultures were washed and incubated with fresh buffer for another 15 min at 37° before the coverslips were mounted in plastic holders for the microscope. This 15-min washout period is necessary to reduce the background fluorescence. Moreover, compartmentalization of fura-2 does not appear to occur with a 15-min incubation with fura-2/acetoxymethyl ester in cerebellar granule cell cultures. The Krebs Ringer's buffer contained 136 mM NaCl, 5 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 2.6 mM $NaHCO_3$, 1.2 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.6 mM glucose, and 15 mM HEPES, pH 7.3, and the depolarizing Krebs Ringer's solution contained 40 mM K^+, with isotonicity being balanced by an appropriate decrease in the concentration of Na^+. All experiments were performed at room temperature (about 22°), and full-field images of fura-2 fluorescence at 334 nm and 390 nm were ratioed and stored every 9 sec. The intracellular concentrations of calcium were determined for the neuronal cell body. The fluorescence microscope (Atto Instruments) was previously described (17). Calibration was according to external standards of calcium and fura-2. The color scale at the bottom of each picture in Fig. 1 depicts pixel intensity of the ratioed image multiplied by a constant factor. All images of fluorescent cells are shown on the same intensity scale.

Statistical analyses. Because many cells within any given cell culture preparation responded differently to the various drugs, and cell depolarization and measurements of intracellular calcium concentrations did not show a normal distribution, typical statistical representations of these data, such as mean ± standard deviation, could not be performed. We decided to represent the various responses of the population of cells within each experiment by using the cumulative frequency of cells responding with a certain intracellular calcium concentration. For the graphs showing cumulative frequency, the numbers of cells showing an intracellular calcium concentration within an increasing 0.125-log unit increment were counted and plotted as percentages of the total number of responding cells. Thus, most of the data are presented either as measurements of intracellular calcium concentrations in cerebellar granule neurons over time or as percentages of total cells responding to the treatment (plotted as cumulative frequency on the ordinate) with a specific intracellular calcium content (abscissa), at 16–20 sec after cell depolarization.

Because the actual measurements of intracellular calcium for each group did not fit a normal distribution, statistical analyses involved

Fig. 1. (±)-Baclofen (100 nM) increases intracellular free calcium after depolarization of cerebellar granule cells in primary culture. Pseudocolor images of intracellular free calcium in cerebellar granule neurons, according to fura-2-calibrated 334/390-nm fluorescence. Data shown in each figure originated from one cell preparation (see cumulative frequency graphs shown in Figs. 2 and 4 for the combined results of many cells and many different preparations of primary cell cultures). A and E, Bright-field images of cerebellar granule neurons after 8 days in cell culture shown for B and C, and F and G, respectively (cerebellar granule neurons are 5–8 μm in diameter). Each picture corresponds to a 1000× magnification of the field. The fluorescent image of intracellular calcium concentration in cerebellar granule neurons is shown under normal resting conditions (B) and after cell depolarization with a buffer containing 40 mM K^+ for approximately 30 sec (C). Normal calcium concentrations were approximately 45 nM. After addition of 40 mM K^+, the intracellular concentration of calcium increased to approximately 200 nM (range, 150–500 nM). D, The intracellular calcium concentration in each cell was measured repetitively every 9 sec for >6 min, and these results are shown. *First arrow*, time when the incubation buffer was exchanged for fresh buffer; *second arrow*, buffer being exchanged with buffer containing 40 mM K^+ (the aforementioned buffer, except that NaCl was 101 mM). F, After the addition of (±)-baclofen (100 nM) to the culture for 40 sec, the intracellular calcium concentration did not change from the basal concentration of calcium. G, After depolarization of the neurons with 40 mM K^+ in the presence of (±)-baclofen, the increase in intracellular calcium was greater than that observed with cell depolarization alone. H, The graph of intracellular concentrations of calcium, from neurons shown in E–G, over time is shown. *First arrow*, exchange of the incubation buffer for fresh buffer containing (±)-baclofen (100 nM); *second arrow*, exchange of buffer for one containing both (±)-baclofen and 40 mM K^+.

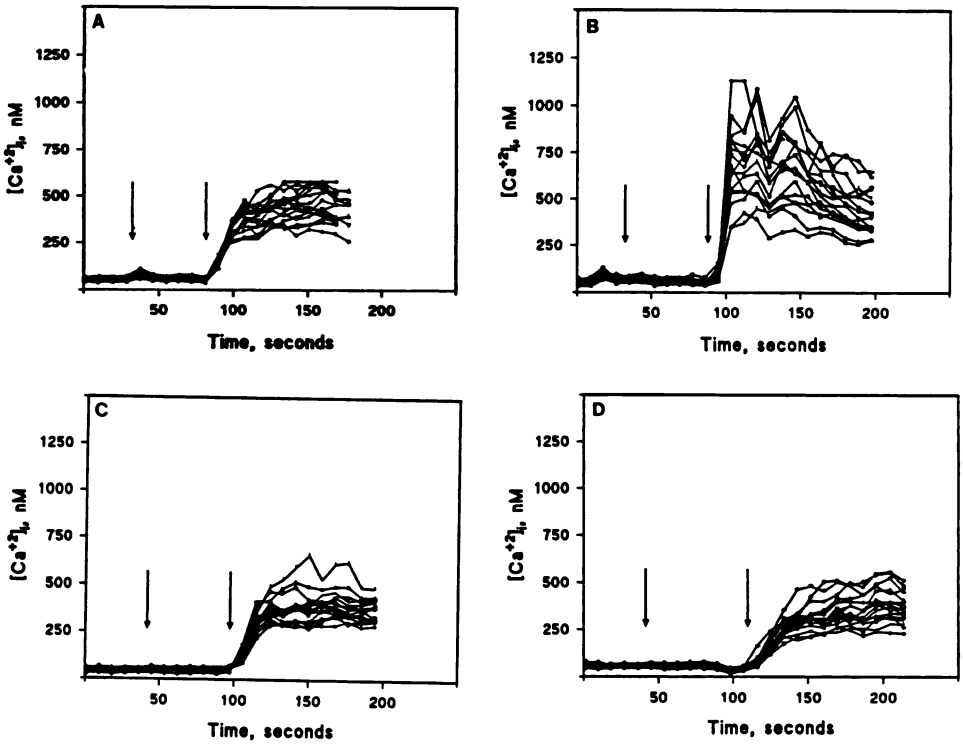


Fig. 3. A $GABA_B$ receptor antagonist blocks the actions of (±)-baclofen in cerebellar granule neurons. Concentrations of intracellular calcium measured over time, showing the effects of depolarizing concentrations of potassium (40 mM) alone (A), (±)-baclofen (100 nM), alone and in the presence of 40 mM K^+ (B), CGP 35348 (200 μM), alone and in the presence of 40 mM K^+ (C), and (±)-baclofen (100 nM) plus CGP 35349 (200 μM), both before and during cell depolarization with 40 mM K^+ (D). The experimental design was similar to that shown in Fig. 1. These data are representative of multiple experiments. The average basal concentration of intracellular calcium determined in cerebellar granule neurons during many studies with these reported $GABA_B$ receptor antagonists was 52 nM for >500 cells. The average concentration of intraneuronal calcium was 525 nM after the addition of 40 mM K^+-containing buffer to the cell culture (98 cells), 869 nM after (±)-baclofen plus 40 mM K^+ (124 cells), 299 nM after (±)-baclofen and CGP 35348 plus 40 mM K^+ (70 cells), and 430 ± 71 nM after CGP 35348 plus 40 mM K^+ (58 cells). All values were obtained from images taken approximately 28 sec after the addition of the buffer containing the appropriate drugs acting at $GABA_B$ receptors plus 40 mM K^+. These drugs did not change intracellular free calcium concentration unless the cell cultures were depolarized.

using the nonparametric Kruskal-Wallis test, with multiple comparisons between groups using the Mann Whitney *U* test (18). The level of statistical significance was determined to be $p < 0.05$. All curves shown in the figures were calculated and plotted using the computer program GraphPAD (19).

Results

From primary cultures of cerebellar granule neurons plated on glass coverslips, intracellular free calcium concentrations were quantified using fura-2 imaging, with fluorescence microscopy (17). Depolarizing concentrations of potassium increased intracellular free calcium concentrations from a resting level of about 40 nM to 250 nM (Fig. 1, A–D). If 100 nM (±)-baclofen was added to normal cultures, no effect was detected; however, when the cultures were depolarized with 40 mM potassium, the intracellular concentration of free calcium reached levels that were at least double those normally associated with the addition of depolarizing buffer alone (Fig. 1, E–H).

Because cerebellar granule neurons are glutamatergic, cell depolarization should release endogenous glutamate, which would increase intracellular free calcium by acting on either NMDA- or kainic acid-selective glutamate receptors present on these cells (20, 21). However, the response of intracellular calcium content to (±)-baclofen and cell depolarization was not diminished when all incubation buffers included maximal concentrations of 6-cyano-7-nitro-quinoxaline-2,3-dione (1 μM) and either dizocilpine (MK-801, 1 μM) or (±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphinic acid (40 μM), which antagonize kainate and NMDA receptors, respectively (results shown in Figs. 2–4 were obtained using these glutamatergic receptor antagonists). Whether the incubation buffer contained glutamate receptor antagonists or did not, (±)-baclofen potentiated the increase in intracellular free calcium induced by cell depolarization. Thus, it did not appear that a glutamate-$GABA_B$ receptor interaction was operative in mediating the function of the high affinity (nanomolar) $GABA_B$ receptor.

In the presence of cell depolarization, (±)-baclofen increased intraneuronal free calcium in a concentration-related manner (Fig. 2A). Whereas the average calcium concentration appearing in the granule neuron after depolarization with 40 mM K^+-containing buffer was slightly more than 200 nM (median concentration of intracellular calcium observed in 50% of the total cells was 171 nM), the average calcium concentration observed after addition of 100 nM (±)-baclofen and cell depolarization was about 900 nM (median value was 604 nM). In the presence of 100 nM (±)-baclofen, most of the cells responded by increasing the intracellular concentration of free calcium. In the presence of 10 nM (±)-baclofen, the average intracellular calcium content reached approximately 600 nM (median concentration was 262 nM), and the number of responding cells decreased. In depolarized neurons, the threshold concentration

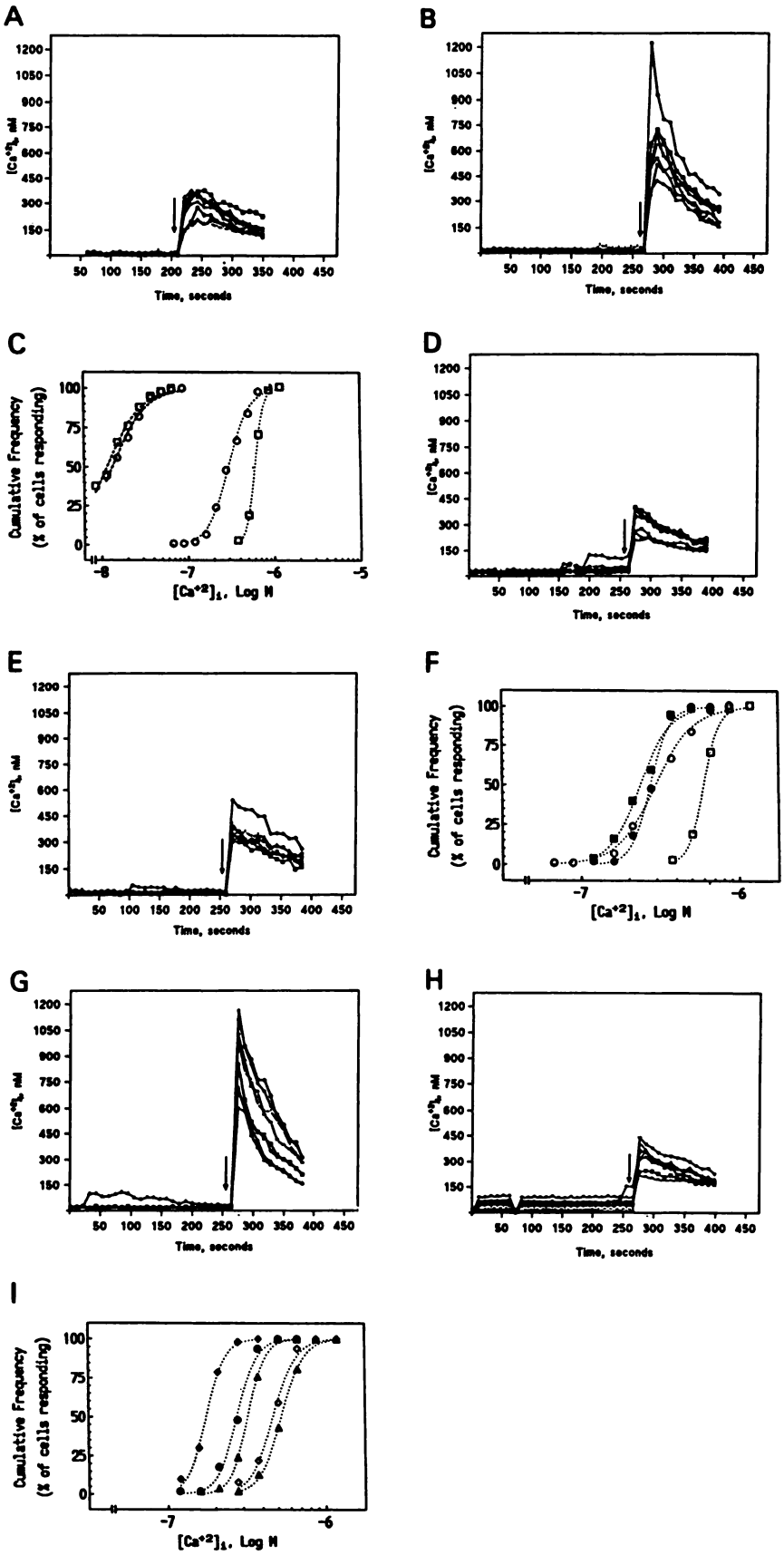


Fig. 4. Dantrolene (10 μM) blocks the action of (±)-baclofen (100 nM) plus cell depolarization. The actual effects of depolarization alone (control) (A) and depolarization in the presence of 100 nM (±)-baclofen (B), 10 μMdantrolene (D), dantrolene plus (±)-baclofen (E), 3 mM caffeine (G), and dantrolene plus caffeine (H) on intracellular calcium concentrations over time are shown. These results represent eight cells in one microscopic field from different culture dishes within one cell preparation. The same experimental design was used as described in Fig. 1, in which all drugs were added to the neuronal cell culture 1 min before cell depolarization (*arrow*, time when the normal Krebs Ringer's buffer was replaced with the buffer containing 40 mM K^+). Many additional neurons were tested, and these results are plotted as cumulative frequencies for control (n = 113 cells), (±)-baclofen (n = 58 cells), dantrolene (n = 50 cells), (±)-baclofen plus dantrolen (n = 49 cells), caffeine (n = 47 cells), and caffeine plus dantrolene (n = 54 cells) in C, F, and I. C, Graph shows cell depolarization with 40 mM K^+ (○····○) to increase intracellular calcium concentrations to an average value of 275 nM (median value where 50% of the cells responded was 297 nM), from a resting average concentration (○– – –○) of 15 nM (median value was 14 nM). In the presence of 100 nM (±)-baclofen, the resting concentration of calcium (□– – –□) did not change. However, cell depolarization in the presence of (±)-baclofen (□·····□) increased intracellular calcium to an average value of 519 nM (median value of 600 nM). F, Graph shows the cumulative frequency curves for cell depolarization alone (control) (○) and cell depolarization in the presence of 100 nM (±)-baclofen (□), dantrolene (●), and dantrolen plus (±)-baclofen (■). Eighteen seconds after cell depolarization, the average intracellular calcium concentrations for control and (±)-baclofen-treated groups were 275 nM and 519 nM, respectively (repeated from those shown in C), and the calcium concentration was 236 nM (median value was 276 nM) for the dantrolene-treated group and 208 nM (median value of 238 nM) for the (±)-baclofen plus dantrolene-treated group. I, Graph shows the cumulative frequency curves for cell depolarization in the presence of 3 mM caffeine (△) (n = 47 cells), 100 μM ryanodine (◇) (n = 49 cells), 10 μM dantrolene (●) (taken from F), dantrolene plus caffeine (▲) (n = 54 cells), and dantrolen plus ryanodine (◆) (n = 59 cells). After cell depolarization, the average intracellular calcium concentration was 448 nM (median value was 527 nM) for the caffeine group, 405 nM (median value was 467 nM) for the ryanodine group, 274 nM (median value was 324 nM) for the dantrolene plus caffeine group, and 203 nM (median value was 234 nM) for the dantrolene plus ryanodine group. The Kruskal-Wallis test, followed by the Mann-Whitney U test, showed that cell depolarization in the presence of either (±)-baclofen, caffeine, or ryanodine was statistically different from cell depolarization alone ($p < 0.001$). None of the drug treatments altered resting intracellular calcium concentrations.

of (±)-baclofen that elicited the increase in intracellular calcium appeared to be around 0.3–1 nM. The average intracellular calcium concentration observed after addition of 0.3 nM (±)-baclofen and 40 mM K^+ was about 350 nM (median value was 288 nM, from 30 measurements).

To ascertain whether $GABA_B$ receptors were operative in this (±)-baclofen-mediated response, we tested the effectiveness of GABA and attempted to block the (±)-baclofen-mediated increase in intracellular free calcium with the $GABA_B$ receptor antagonist CGP 35348 (13, 14). Similar to the effects observed with (±)-baclofen, GABA (100 nM) also increased intracellular calcium after cell depolarization with buffer containing 40 mM K^+ (Fig. 2B). A comparison of equimolar concentrations of GABA and (±)-baclofen showed GABA to be less effective than (±)-baclofen. The average intracellular calcium concentration observed after 100 nM GABA and cell depolarization was about 370 nM (median value was 278 nM). In order to prevent GABA from stimulating $GABA_A$ receptors [an action not shared by (±)-baclofen] and to inhibit neuronal GABA uptake, the experiments with GABA were performed in the presence of bicuculline (100 μM) and CI-966 (50 μM) (a GABA uptake inhibitor). Neither bicuculline nor CI-966 affected the depolarization-evoked increase in intracellular free calcium caused by GABA (Fig. 2B). The $GABA_B$ receptor antagonist CGP 35348 did not show an effect on either basal levels of intracellular calcium or the depolarization-induced increase in intracellular free calcium (Fig. 3, A and C). When CGP 35348 (200 μM) and (±)-baclofen (100 nM) were added to the neurons 1 min before cell depolarization, CGP 35348 prevented the elevation of intracellular calcium concentration induced by (±)-baclofen and cell depolarization (Fig. 3). A lower concentration of CGP 35348 (40 μM) was ineffective in blocking the action of (±)-baclofen (100 nM).

We also tested whether the increase in intracellular free calcium resulted from a (±)-baclofen-induced hyperpolarization secondary to an activation of a potassium current. In our experimental design, the (±)-baclofen-mediated hyperpolarization of the cell might occur during the 1 min before cell depolarization. Because potassium currents activated by a low affinity $GABA_B$ receptor are blocked by potassium channel inhibitors, such as 4-AP (1 mM) and TEA (10 mM) (2–5), we examined the effects of these inhibitors on the increase of calcium mediated by the high affinity $GABA_B$ receptor during cell depolarization. Our studies showed that (±)-baclofen (100 nM) increased intracellular free calcium evoked by cell depolarization even in the presence of TEA and 4-AP. The average intracellular calcium concentration observed 18 sec after depolarization for control ($n = 26$ cells), 100 nM (±)-baclofen ($n = 45$ cells), TEA and 4-AP ($n = 50$ cells), and baclofen plus TEA and 4-AP ($n = 39$ cells) groups was 166 nM, 442 nM, 264 nM, and 362 nM, respectively. Comparisons by the nonparametric Kruskal-Wallis test, followed by Mann Whitney U tests, showed only the (±)-baclofen and (±)-baclofen plus TEA and 4-AP groups to be statistically significantly different from the control depolarized group, at $p < 0.01$. There were no statistical differences observed between the calcium responses elicited by (±)-baclofen or (±)-baclofen plus TEA and 4-AP. Thus, these potassium currents may not affect the increase in intracellular calcium mediated by high affinity $GABA_B$ receptors.

If the extracellular calcium was removed from the buffer and EGTA (1.5 mM) was added 1 min before depolarization of the neurons with high potassium (40 mM), no increase in intracellular calcium resulted from either cell depolarization alone or depolarization in the presence of (±)-baclofen (100 nM). The average intracellular calcium concentration from the control group of 32 cells was 33 nM measured approximately 18 sec before depolarization and 196 nM measured approximately 18 sec after cell depolarization. The average calcium concentration observed after depolarization by 40 mM K^+ was 772 nM in the presence of 100 nM (±)-baclofen, 21 nM in the presence of a Krebs Ringer's solution containing no calcium and 1 mM EGTA, and 15 nM in the presence of (±)-baclofen plus the calcium-free buffered solution. Thus, these results showed the influx of extracellular calcium to be important in the depolarization-induced increase in intracellular calcium content. Moreover, these results suggested that the action of (±)-baclofen was also dependent on the influx of extracellular calcium.

Lastly, dantrolene (10 μM), an inhibitor of calcium release from CICR stores (22–24), prevented (±)-baclofen from increasing intracellular calcium in depolarized cells (see Fig. 4, A–F, and legend for a description of statistical analyses). The increase in intracellular calcium evoked by depolarization (40 mM K^+) was unaffected in the presence of dantrolene alone. These results suggested that (±)-baclofen acts through some unknown mechanism to make dantrolene-sensitive CICR stores operational. However, CICR stores have not been described in cerebellar granule cells. These calcium stores, if similar to those described in sympathetic neuronal cultures (23) and muscle (24), are stimulated by caffeine to release calcium and are sensitive to ryanodine, which depletes calcium from CICR stores by releasing calcium and preventing subsequent calcium storage. In cerebellar granule neurons, neither 3 mM caffeine nor 100 μM ryanodine had any action on intracellular calcium concentrations under normal nondepolarizing conditions (see Fig. 4, G–I, and legend for description of statistical analyses). However, both caffeine and ryanodine increased intracellular calcium content after cell depolarization with Krebs Ringer's buffer containing 40 mM K^+, and these responses were antagonized by 10 μM dantrolene (Fig. 4, G–I). Caffeine at 10 mM was tested in a few experiments, but this concentration of caffeine also did not increase intracellular calcium by releasing stored calcium from nondepolarized cerebellar granule cells. However, cell depolarization with the Krebs Ringer's solution containing 40 mM K^+ plus 10 mM caffeine resulted in an elevated intracellular calcium content similar to that seen with cell depolarization plus 3 mM caffeine (data not shown). Dantrolene competitively blocks the CICR store, because, at 10 μM, it only partially reversed the action of 10 mM caffeine (data not shown).

Discussion

Our current hypothesis is that (±)-baclofen and GABA act at a high affinity $GABA_B$ receptor. Stimulation of this high affinity $GABA_B$ receptor potentiates the rise in intracellular calcium evoked by cell depolarization. GABA was previously shown to increase intracellular concentrations of calcium in explant cultures of cerebellar granule cells (22). However, in that report, neither the subtype of GABA receptor nor the affinity of GABA at this site was determined. In our study, the $GABA_B$ receptor-mediated response was dependent on calcium influx into the cerebellar granule neuron, presumably via volt-

age-dependent calcium channels that were triggered by cell depolarization. The enhanced calcium release observed in the presence of (±)-baclofen and cell depolarization results from the mobilization of calcium from dantrolene-sensitive CICR stores (22–24). These CICR stores in cerebellar granule cells are somewhat different from those in other cells studied, because caffeine alone does not release stored calcium unless the cells are depolarized. The molecular mechanism linking the high affinity $GABA_B$ receptor to release of intracellular calcium remains to be elucidated.

The rise in intracellular calcium resulting from cell depolarization and (±)-baclofen could appear to be contradictory to the inhibition of depolarization-induced influx of calcium-45 elicited in these cell cultures by nanomolar concentrations of (±)-baclofen (25–27). An alternative explanation could be that the reduced entry of calcium-45 into the cerebellar granule neurons elicited by baclofen results from a decrease in the concentration gradient for calcium across the plasma membrane, due to the increase of intracellular free calcium originating from the calcium release from intraneuronal stores. The high levels of intracellular free calcium may also inactivate voltage-dependent calcium channels, resulting in the reduced calcium currents seen in these cultures (26).

To date, it is unknown whether this high affinity, (±)-baclofen-sensitive, $GABA_B$ receptor found in primary cultures of cerebellar granule neurons belongs to the same receptor family that is activated by (±)-baclofen to elicit clinically beneficial effects on spasticity. Only a few studies have reported a $GABA_B$ receptor having nanomolar affinity for (±)-baclofen (25–31). $GABA_B$ receptors having both nanomolar and micromolar affinities for (±)-baclofen can be found on cerebellar granule neurons. Whereas nanomolar concentrations of (±)-baclofen facilitate the depolarization-induced increase in intracellular calcium content, via the activation of CICR stores and mobilization of stored calcium, micromolar (±)-baclofen concentrations inhibit adenylyl cyclase and glutamate release (25–27). The low affinity $GABA_B$ receptor has also been reported to inhibit the rise in intrasynaptosomal calcium concentrations evoked by depolarization (32, 33). The mechanism associated with the activation of this low affinity $GABA_B$ receptor on cortical synaptosomes is not understood, but these reports support the existence of a diversity of $GABA_B$ receptors.

In summary, we described a (±)-baclofen-mediated (calcium-dependent) rise in intracellular free calcium that results from the activation of a high affinity $GABA_B$ receptor by (±)-baclofen. Because (±)-baclofen can double the intracellular content of calcium after cell depolarization, and this effect is blocked by dantrolene, we propose that the high affinity $GABA_B$ receptor allows the mobilization of calcium from dantrolene-sensitive CICR stores to occur only after cell depolarization triggers the entry of extracellular calcium.

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