

# GABA Activity Mediating Cytosolic $\text{Ca}^{2+}$ Rises in Developing Neurons Is Modulated by cAMP-Dependent Signal Transduction

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In the majority of developing neurons, GABA can exert depolarizing actions, thereby raising neuronal  $\text{Ca}^{2+}$ .  $\text{Ca}^{2+}$  elevations can have broad consequences during development, inducing gene expression, altering neurite outgrowth and growth cone turning, activating enzyme pathways, and influencing neuronal survival. We used fura-2 and fluo-3  $\text{Ca}^{2+}$  digital imaging to assess the effects of inhibiting or activating the cAMP signal transduction pathway on GABA activity mediating  $\text{Ca}^{2+}$  rises during the early stages of *in vitro* hypothalamic neural development. Our experiments stemmed from the finding that stimulation of transmitter receptors shown to either activate or inhibit adenylyl cyclase activity caused a rapid decrease in  $\text{Ca}^{2+}$  rises mediated by synaptically released GABA.

Both the adenylyl cyclase activator forskolin and the inhibitor SQ-22,536 reduced the  $\text{Ca}^{2+}$  rise elicited by the synaptic release of GABA. Bath application of the membrane-permeable cAMP analogs 8-bromo-cAMP (8-Br-cAMP) or 8-(4-chlorophenylthio)-cAMP (0.2–5 mM) produced a rapid, reversible, dose-dependent inhibition of  $\text{Ca}^{2+}$  rises triggered by synaptic GABA release. Potentiation of GABAergic activity mediating  $\text{Ca}^{2+}$  rises was observed in some neurons at relatively low concentrations of the membrane-permeable cAMP analogs (20–50  $\mu\text{M}$ ). In the presence of tetrodotoxin (TTX), postsynaptic  $\text{Ca}^{2+}$  rises triggered by the bath application of GABA were only moderately depressed (13%)

by 8-Br-cAMP (1 mM), suggesting that the inhibitory effects of 8-Br-cAMP were largely the result of a presynaptic mechanism.

The protein kinase A (PKA) inhibitors H89 and Rp-3',5'-cyclic monophosphothioate triethylamine also caused a large reduction (>70%) in  $\text{Ca}^{2+}$  rises triggered by synaptic GABA release. Unlike the short-term depression elicited by activation of the cAMP signal transduction pathway,  $\text{Ca}^{2+}$  depression elicited by PKA inhibition persisted for an extended period (>30 min) after PKA inhibitor washout. Postsynaptic depression of GABA-evoked  $\text{Ca}^{2+}$  rises triggered by H89 (in the presence of TTX) recovered rapidly, suggesting that the extended depression observed during synaptic GABA release was largely through a presynaptic mechanism. Long-term  $\text{Ca}^{2+}$  modulation by cAMP-regulating hypothalamic peptides may be mediated through a parallel mechanism.

Together, these results suggest that GABAergic activity mediating  $\text{Ca}^{2+}$  rises is dependent on ongoing PKA activity that is maintained within a narrow zone for GABA to elicit a maximal  $\text{Ca}^{2+}$  elevation. Thus, neuromodulator-mediated changes in the cAMP-dependent signal transduction pathway (activation or inhibition) could lead to a substantial decrease in GABA-mediated  $\text{Ca}^{2+}$  rises during early development.

**Key words:** mediobasal hypothalamus; calcium; GABA; GABA excitation; protein kinase A; cAMP; development; digital imaging

The role of GABA as an excitatory neurotransmitter during the early stages of neural development has been documented over the past several years (for review, see Cherubini et al., 1991). Several studies have shown that GABA triggers neural excitability by activating the GABA<sub>A</sub> receptor (Ben-Ari et al., 1989; Chen et al., 1996). Because of a relatively depolarized  $\text{Cl}^-$  reversal potential (Chen et al., 1996), opening of the GABA<sub>A</sub> receptor allows  $\text{Cl}^-$  to efflux from the neuron, triggering membrane potential depolarization, activation of voltage-sensitive  $\text{Ca}^{2+}$  channels, and, as a result, an increase in intracellular  $\text{Ca}^{2+}$  (Yuste and Katz, 1991; Yamashita and Fukuda, 1993; Obrietan and van den Pol, 1995). Although glutamate receives a lot of attention regarding its ability to elevate cytosolic  $\text{Ca}^{2+}$  levels in developing neurons, we found that many developing neurons show a greater  $\text{Ca}^{2+}$  elevation in response to GABA than to an equimolar concentration of gluta-

mate (Obrietan and van den Pol, 1995). As neurons mature, GABA becomes a predominantly inhibitory transmitter that decreases cytosolic  $\text{Ca}^{2+}$  levels (Obrietan and van den Pol, 1995). GABA has been shown to possess many of the effects of other, better characterized, fast excitatory neurotransmitters known to increase intracellular  $\text{Ca}^{2+}$  during development. For example, GABA<sub>A</sub> receptor activation can trigger BDNF induction (Berninger et al., 1995) and alter the neural phenotype (Marty et al., 1996). In addition, DNA synthesis in cortical neural progenitor cells can be blocked by specifically inhibiting GABA<sub>A</sub> receptor activity (LoTurco et al., 1995). GABA can increase  $\text{Ca}^{2+}$  levels in neurites and growth cones (Obrietan and van den Pol, 1996a), which may alter growth cone motility and the rate of neurite extension. In addition, GABA-mediated  $\text{Ca}^{2+}$  influx in growth cones leads to an increase in GAP43 and MARCKS protein phosphorylation (Fukura et al., 1996). These results suggest that the ability of GABA to raise intracellular  $\text{Ca}^{2+}$  levels may be central to its functional role during development.

The cAMP signal transduction pathway is a potent regulator of synaptic neural physiology (for review, see Anholt, 1994; Cooper et al., 1994). A primary mechanism by which cAMP regulates neural excitability is through the activation of cAMP-dependent

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protein kinase A (PKA). The neuromodulatory actions of PKA have been documented extensively and include altering ion channel activity (Nagel et al., 1992; Johnson et al., 1994; Surmeier et al., 1995), gene expression (Impey et al., 1996), and neurotransmitter release (Sciancalepore and Cherubini, 1995; Huang et al., 1996). Although a considerable amount of information is known about the effects of PKA at the molecular and single channel levels, much remains to be determined about how its actions at a variety of sites may affect cytosolic Ca<sup>2+</sup> regulation in synaptically active neurons. For example, PKA has been shown to reduce the amplitude of the GABA<sub>A</sub> receptor conductance (Moss et al., 1992), to decrease voltage-gated Na<sup>+</sup> channel currents (Smith and Goldin, 1996), and to potentiate kainate-induced currents (Gu and Moss, 1996). A question of particular interest is how GABAergic activity mediating Ca<sup>2+</sup> elevations may be regulated during early development by PKA.

Similar to GABA, PKA has also been shown to play an important role during development. The cAMP signal transduction pathway has been implicated in a variety of developmentally regulated processes, including cell cycle regulation (Grieco et al., 1996), neural migration (Behar et al., 1995), and gene induction (Zhang et al., 1993). Additionally, many neurotransmitters and neuropeptides that affect neural physiology through the regulation of adenylyl cyclase activity, including neuropeptide Y (NPY), dopamine, glutamate, and serotonin, are expressed in early development and could serve to modulate the Ca<sup>2+</sup>-elevating actions of GABA (Rajaofetra, 1989; Belin et al., 1991; Marti et al., 1992; van den Pol et al., 1995, 1996a; Lieb et al., 1996). Based on these findings, we studied the modulation of GABAergic Ca<sup>2+</sup> elevating activity by the cAMP-mediated signal transduction pathway.

Here we report that Ca<sup>2+</sup> rises regulated by synaptic GABA release during early development are dramatically influenced by activation or inhibition of the cAMP-dependent signal transduction cascade.

## MATERIALS AND METHODS

**Tissue culture.** The mediobasal hypothalamus was removed from embryonic day 18 Sprague Dawley rats. The tissue was enzymatically digested in a mild protease solution (10 U/ml papain and 0.2 mg/ml L-cysteine in Earl's balanced salt solution) for 30 min. Next, the tissue was pelleted, and the protease solution was removed. Tissue was then suspended in standard tissue culture medium (glutamate- and glutamine-free DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin, and 6 gm/l glucose) and then triturated into a single-cell suspension. Cells were washed and pelleted an additional three times. The single-cell suspension was plated onto 22 mm<sup>2</sup> glass coverslips that had been coated with high-molecular-weight (540,000 Da) poly-D-lysine. High-density cultures (200,000/cm<sup>2</sup>) were used for all experiments. Hypothalamic neural cultures were maintained in a Napco 3600 incubator (37°C and 5% CO<sub>2</sub>) until they were ready for use. To limit non-neuronal cell proliferation, cytosine arabinofuranoside (1 μM) was added to the tissue culture medium 1 d after plating.

**Fura-2 and fluo-3 Ca<sup>2+</sup> digital imaging.** Cells were loaded for 20 min with either 5 μM fura-2 AM or fluo-3 AM in standard perfusion solution (137 mM NaCl, 25 mM glucose, 10 mM HEPES, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 3 mM CaCl<sub>2</sub>, pH 7.4). The cells were then washed and allowed to recover for 15 min before the start of the experiment. Coverslips were then loaded into a laminar style perfusion chamber. Solutions rapidly moved as a straight wave through the perfusion chamber, and complete washout of the chamber occurred in ~5 sec. For fura-2, neurons were imaged using a 40× Olympus objective with high 340/380 nm transmittance on a Nikon Diaphot 300 inverted microscope. Fluo-3 experiments were performed using a 100× Olympus objective. Unless noted otherwise, Ca<sup>2+</sup> digital recordings were made from the cell soma. All experiments were performed at room temperature.

A 486 PC clone was used to collect data, run Ca<sup>2+</sup> analysis software (Fluor; Universal Imaging Corporation, West Chester, PA), and control

the Lambda-10-filtered wheel driver (Sutter Instruments). Sixteen (500 msec) digital frames of data were collected every 3 sec. Excitation light came from a 150 W Xenon lamp. The equation  $[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R)$  was used to convert fura-2 ratiometric fluorescent Ca<sup>2+</sup> values to free Ca<sup>2+</sup> concentrations.  $R$  is the ratio of the two fluorescence intensities,  $R_{min}$  is the ratio in the absence of Ca<sup>2+</sup>, and  $R_{max}$  is the ratio in a saturating concentration of Ca<sup>2+</sup>. The  $K_d$  for binding of Ca<sup>2+</sup> to fura 2 was taken to be 224 nM (Grynkiewicz et al., 1985). Data for fluo-3 fluorescence are represented on a 0–255 U scale. As standard protocol for both fura-2 and fluo-3 imaging, background fluorescence values were subtracted.

To determine the effects of different receptor agonists or signal transduction modulators on Ca<sup>2+</sup> rises triggered by the synaptic release of GABA, the mean Ca<sup>2+</sup> rise from the Ca<sup>2+</sup> level in the presence of the GABA<sub>A</sub> receptor antagonist bicuculline was determined over a 15 sec period just before the application of a receptor agonist or signal transduction modulator. The mean Ca<sup>2+</sup> rise was then determined over a 15 sec period 90 sec after administration of the receptor agonist or signal transduction modulator. Data for Ca<sup>2+</sup> rises for the two conditions are reported as a mean (pooled) Ca<sup>2+</sup> rise of all responsive neurons ± SEM. Thirty to 50% of the neurons exhibited a Ca<sup>2+</sup> rise on bicuculline removal. For assays that evoked a Ca<sup>2+</sup> response, either through agonist administration to the perfusion solution or through electrical stimulation, the maximal Ca<sup>2+</sup> rise for individual neurons was determined by subtracting the mean basal Ca<sup>2+</sup> level for a 15 sec period just before stimulation from the peak-evoked Ca<sup>2+</sup> rise. Ca<sup>2+</sup> responses from a total of 1165 neurons were recorded in the course of these experiments. Modulation of GABA-related activity could refer to either a presynaptic or postsynaptic site of action, whereas modulation of GABA-evoked Ca<sup>2+</sup> rises refers to a postsynaptic site of action.

**Electrical stimulation.** Electrodes from a Grass SD9 stimulator were placed at both ends of the perfusion chamber. Ca<sup>2+</sup> rises were stimulated by passing 2–5 V/cm<sup>2</sup>, at 20 Hz frequency and 2 msec duration, through the chamber for 6 sec. Ca<sup>2+</sup> rises were visible immediately and usually peaked 4 sec after current application. At these voltages, Ca<sup>2+</sup> rises were inhibited by either the application of the voltage-dependent Na<sup>+</sup> channel blocker tetrodotoxin (TTX; 1 μM) or the administration of fast excitatory neurotransmitter antagonists. At voltages higher than those used in this paper (>6 V/cm<sup>2</sup>), TTX did not block the Ca<sup>2+</sup> response, suggesting an additional nonsynaptic mechanism for the induction of Ca<sup>2+</sup> rises.

**Immunostaining.** Neurons were fixed for 1 hr in cold (–20°C) methanol and then treated for 30 min with 3% BSA and 0.1% Triton X-100 in PBS. Neurons were then washed and incubated with mouse anti-synapsin 1 antibody (1:100) (Chemicon, Temecula, CA). The synapsin antiserum recognized a band of the correct weight on Western blots, and preabsorption with synapsin antigen blocked staining (Smith et al., 1994). Rat anti-α-tubulin antibody (Sera Lab) was used at 1:200 for 30 min. Neurons were washed and incubated with FITC- and Texas Red-labeled secondary antibodies (Jackson Laboratory, Bar Harbor, ME) for 30 min. After washing, cell fluorescence was visualized with the appropriate filter sets. In the fluorescent microscope, synapsin-immunostained boutons were green, and tubulin immunoreactivity was red.

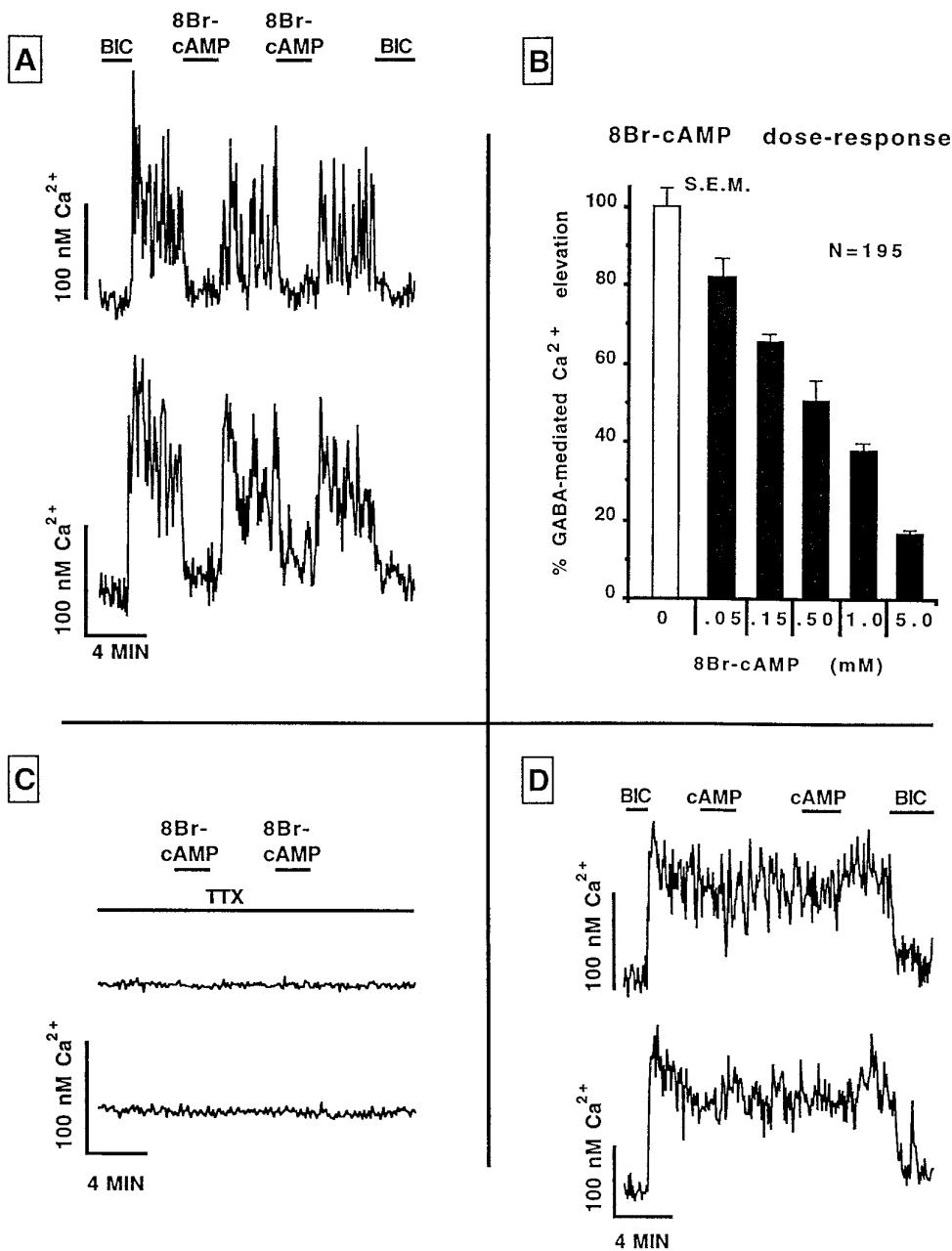
**Reagents.** SKF-38393, McN-A-343, cytosine arabinofuranoside, GABA, cAMP, 8-bromo-cAMP (8-Br-cAMP), 8-(4-chlorophenylthio)-cAMP (4-Cl-cAMP), and poly-D-lysine were acquired from Sigma (St. Louis, MO). SQ-22,536, forskolin, (±)-2-amino-5-phosphonopentanoic acid (AP5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), bicuculline, nimodipine, and TTX were acquired from Research Biochemicals (Natick, MA). NPY was acquired from Peninsula Labs. Papain was acquired from Worthington (Freehold, NJ); DMEM was from Life Technologies (Gaithersburg, MD); and fura-2 AM and fluo-3 AM were from Molecular Probes (Eugene, OR).

## RESULTS

### cAMP signal transduction pathway activation

To address the possibility that activating the cAMP signal transduction pathway affects GABA activity that mediates Ca<sup>2+</sup> elevations, membrane-permeable cAMP analogs that mimic the cellular effects of cAMP were administered to synaptically active hypothalamic neurons. Figure 1A shows that the withdrawal of the GABA<sub>A</sub> receptor antagonist bicuculline (20 μM) from the perfusion solution elicited a rapid and stable Ca<sup>2+</sup> rise, indicating that cytosolic Ca<sup>2+</sup> was raised by synaptically released GABA.

### Inhibition of GABA-mediated Ca<sup>2+</sup> rises by membrane permeable cAMP analog

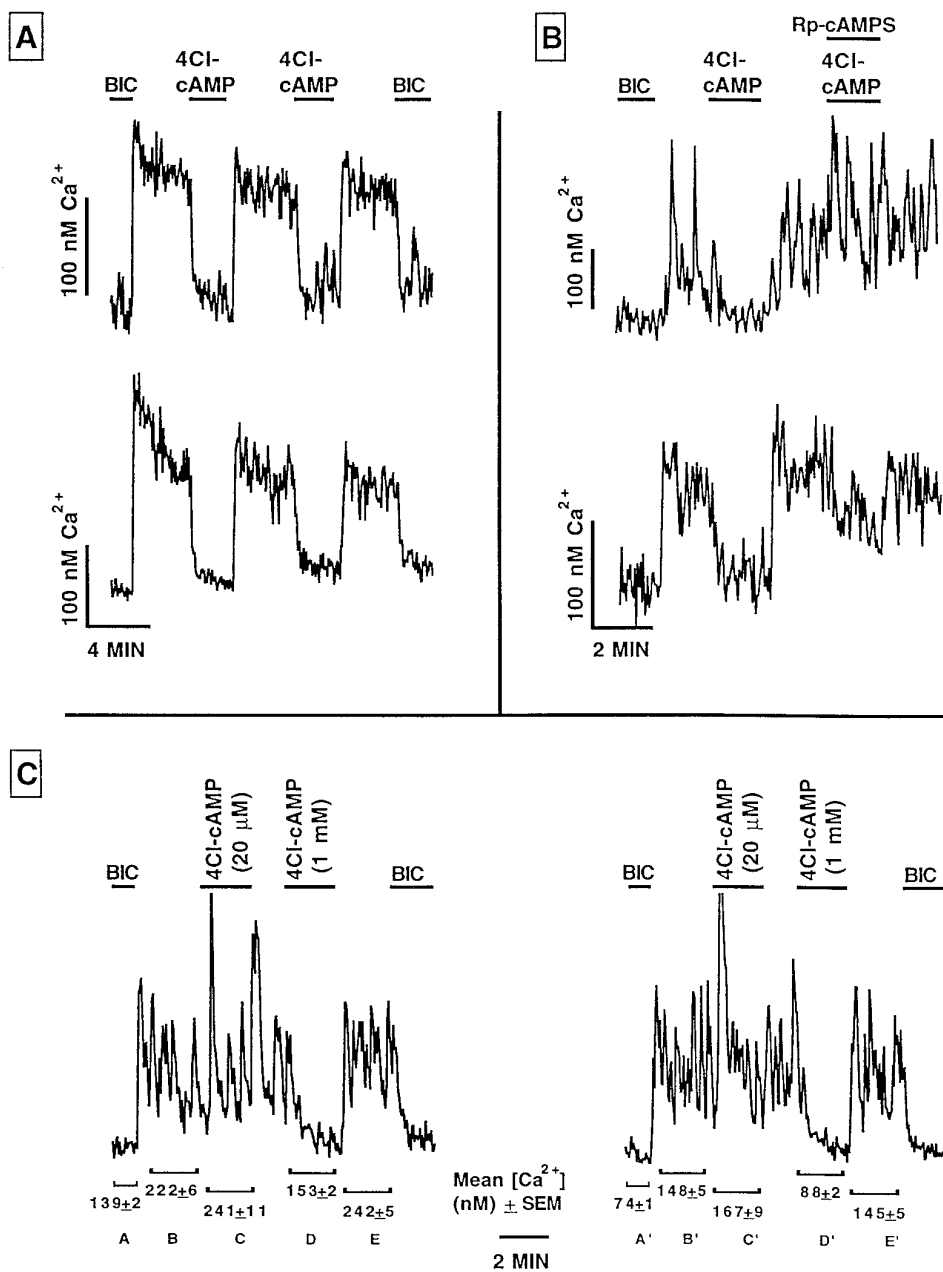


**Figure 1.** Membrane-permeable analogs of cAMP trigger reversible Ca<sup>2+</sup> depression. *A*, The removal of bicuculline (BIC, 20  $\mu$ M) from the perfusion solution triggered a rapid and sustained Ca<sup>2+</sup> rise in the two representative neurons. Repeated application of 8-Br-cAMP (1 mM) induced a rapid depression of the GABA-related Ca<sup>2+</sup> rises that lasted only as long as 8-Br-cAMP was applied. *B*, Dose-response effect of 8-Br-cAMP on the synaptically released GABA-mediated Ca<sup>2+</sup> level. The Ca<sup>2+</sup> rise from a bicuculline-defined baseline was determined over a 15 sec period just before and 90 sec after 8-Br-cAMP application. The white bar (set at 100%) is the normalized Ca<sup>2+</sup> rise just before 8-Br-cAMP application. Error bars indicate SEM. *N*, Total number of neurons assayed. *C*, 8-Br-cAMP had no effect on neuronal Ca<sup>2+</sup> levels if synaptic GABA release was inhibited by the administration of the Na<sup>+</sup> channel blocker TTX (1  $\mu$ M). Neurons with only small baseline Ca<sup>2+</sup> fluctuations were shown, so that any effect of 8-Br-cAMP on basal Ca<sup>2+</sup> might be observed. *D*, Membrane-impermeable cAMP (1 mM) had little extracellular effect on GABA-mediated Ca<sup>2+</sup> levels. All experiments were performed in the presence of AP5 (100  $\mu$ M) and CNQX (10  $\mu$ M). All synaptic release experiments were performed with embryonic day 18 neurons after 5 DIV. In *A*, *C*, *D*, the bar along the x-axis shows time in min, and the bar to the left of each Ca<sup>2+</sup> trace is the calibrated cytosolic Ca<sup>2+</sup> value for each neuron.

Addition of 8-Br-cAMP (1 mM) caused a rapid reduction in Ca<sup>2+</sup> levels. The mean  $\pm$  SEM Ca<sup>2+</sup> rise after removal of bicuculline was  $73 \pm 6$  nM. After the addition of 8-Br-cAMP the Ca<sup>2+</sup> rise decreased to  $28 \pm 5$  nM, representing a statistically significant ( $p < 0.0001$ , two-tailed  $t$  test) reduction in GABA-mediated Ca<sup>2+</sup> rise of 62% ( $n = 68$ ). Figure 1*B* shows that mean inhibition of GABA activity regulating Ca<sup>2+</sup> rises by 8-Br-cAMP was dose-dependent. GABA-mediated Ca<sup>2+</sup> levels were increased in some neurons, but only at low 8-Br-cAMP concentrations. Specifically, the Ca<sup>2+</sup> level in 6 of 20 neurons was raised by 50  $\mu$ M extracellular 8-Br-cAMP, and at 150  $\mu$ M 8-Br-cAMP, the Ca<sup>2+</sup> level was raised in 4 of 30 neurons. However, at higher concentrations (500  $\mu$ M and 1 and 5 mM) 8-Br-cAMP only depressed Ca<sup>2+</sup> levels and did not elevate Ca<sup>2+</sup> levels in any neurons ( $n = 90$ ).

Another membrane-permeable cAMP analog, 4-Cl-cAMP (2 mM), also triggered a rapid Ca<sup>2+</sup> depression (Fig. 2*A*). The effectiveness of 4-Cl-cAMP was also dose-dependent; at 2 mM, 4-Cl-cAMP caused a statistically significant ( $p < 0.0001$ , two-tailed  $t$  test) 81% Ca<sup>2+</sup> depression ( $n = 29$ ), whereas 200  $\mu$ M 4-Cl-cAMP reduced the GABA-mediated Ca<sup>2+</sup> rise by a statistically significant ( $p < 0.001$ , two-tailed  $t$  test) 39% ( $n = 25$ ). Figure 2*B* shows that the coadministration of the potent PKA inhibitor Rp-3',5'-cyclic monophosphothioate triethylamine (Rp-cAMPS) largely blocked the effects of 4-Cl-cAMP, suggesting that the actions of membrane-permeable cAMP analogs are the result of PKA activation. Application of 4-Cl-cAMP (1 mM) triggered a 78% decrease in GABA-mediated Ca<sup>2+</sup> levels, whereas 4-Cl-cAMP (1 mM) administration, in the presence of Rp-cAMPS (200

### Modulation of GABA-mediated $Ca^{2+}$ rises by membrane permeable cAMP analog



**Figure 2.** Modulation of GABA activity mediating  $Ca^{2+}$  rises. *A*, The membrane-permeable analog of cAMP, 4-Cl-cAMP (2 mM), elicited a rapid and reproducible  $Ca^{2+}$  depression. *B*, Coadministration of the protein kinase A inhibitor Rp-cAMPS (200  $\mu$ M) largely blocked the  $Ca^{2+}$  depression elicited by 4-Cl-cAMP (1 mM). *C*, Two representative neurons show that administration of a low concentration of 4-Cl-cAMP (20  $\mu$ M) triggered a  $Ca^{2+}$  increase, whereas a high concentration of 4-Cl-cAMP (1 mM) triggered a  $Ca^{2+}$  depression. Numbers under each bracket refer to the mean  $\pm$  SEM  $Ca^{2+}$  concentration for the period within the bracketed region. Twenty time points (3 sec interval) were used to determine each  $Ca^{2+}$  concentration, except for the beginning of the experiment [bicuculline (BIC) administration], at which 10 time points were used. Letters under brackets identify each bracketed region.

$\mu$ M), resulted in a 46%  $Ca^{2+}$  decrease ( $n = 42$ ). As with low concentrations of 8-Br-cAMP, the administration of relatively low levels of 4-Cl-cAMP triggered  $Ca^{2+}$  rises that were not observed at higher concentrations. Figure 2C shows two neurons treated to both low (20  $\mu$ M) and high (1 mM) concentrations of 4-Cl-cAMP. 4-Cl-cAMP at a concentration of 20  $\mu$ M triggered an enhancement in both the mean  $Ca^{2+}$  concentration ( $Ca^{2+}$  concentrations shown for each bracketed region  $\pm$  SEM) and, in some neurons, the size of GABA-mediated  $Ca^{2+}$  transients. By comparing the mean  $Ca^{2+}$  rise during bracketed region B (after bicuculline withdrawal) with the mean  $Ca^{2+}$  rise during bracketed region C (during 20  $\mu$ M 4-Cl-cAMP), we found that 4-Cl-cAMP triggered a >20% increase in GABA-mediated  $Ca^{2+}$  rises in 17 of 57 neu-

rons. This effect is in striking contrast to the large  $Ca^{2+}$  decrease observed on administration of 1 mM 4-Cl-cAMP. These neurons were cultured on embryonic day 18 and maintained for 5 d *in vitro* (DIV). Figures often show data records from two or more neurons recorded simultaneously. This serves both to validate subtle, but consistent, effects of pharmacological manipulations and to show the heterogeneity of neural response characteristics.

To remove any complicating effects of synaptically released glutamate on  $Ca^{2+}$  rises, neurons were constantly perfused with the ionotropic glutamate receptor antagonists AP5 (100  $\mu$ M) and CNQX (10  $\mu$ M). In the presence of glutamate receptor blockers AP5 and CNQX, basal  $Ca^{2+}$  levels did not change when neurons were switched between perfusion solutions containing bicuculline

(20  $\mu\text{M}$ ) or the Na<sup>+</sup> channel blocker TTX (1  $\mu\text{M}$ ), suggesting that in the absence of glutamatergic neurotransmission, GABA was the sole transmitter responsible for increasing neuronal Ca<sup>2+</sup> levels (data not shown).

The Na<sup>+</sup> channel blocker TTX (1  $\mu\text{M}$ ) inhibited Ca<sup>2+</sup> transients mediated by the synaptic release of GABA (Fig. 1C). 8-Br-cAMP (1 mM) had no independent effect on basal Ca<sup>2+</sup> levels (Fig. 1C). As an additional control we added cAMP (1 mM) to the perfusion solution. Because cAMP is membrane-impermeable, it should not mimic the intracellular effects of 8-Br-cAMP or 4-Cl-cAMP. Figure 1D shows that cAMP had very little effect on GABA-mediated Ca<sup>2+</sup> rises, suggesting that 8-Br-cAMP and 4-Cl-cAMP were acting primarily through an intracellular mechanism to depress Ca<sup>2+</sup> levels rather than at an extracellular receptor. The Ca<sup>2+</sup> rise before the addition of cAMP was  $101 \pm 10$  nM; during cAMP application, the Ca<sup>2+</sup> rise decreased to  $87 \pm 10$  nM, representing a statistically insignificant ( $p > 0.05$ , two-tailed  $t$  test) 14% cAMP-dependent decrease ( $n = 38$ ).

### 8-Br-cAMP inhibits GABA activity through presynaptic and postsynaptic mechanisms

To determine whether activation of the cAMP-dependent signal transduction cascade inhibits GABA-related Ca<sup>2+</sup> rises through a presynaptic or postsynaptic mechanism, we compared the effects of 8-Br-cAMP on postsynaptic Ca<sup>2+</sup> rises elicited by bath application of GABA with the effects of 8-Br-cAMP on electrically stimulated Ca<sup>2+</sup> rises dependent on presynaptic GABA release. Figure 3A shows Ca<sup>2+</sup> rises elicited by bath application of GABA (10  $\mu\text{M}$ ). Interestingly, Ca<sup>2+</sup> rises were either inhibited (*top trace*) or potentiated (*bottom trace*) by 8-Br-cAMP (1 mM). Relative to the control Ca<sup>2+</sup> rise immediately preceding 8-Br-cAMP administration, 8-Br-cAMP depressed the Ca<sup>2+</sup> rise by >20% in 32 of 109 neurons and potentiated the GABA-evoked Ca<sup>2+</sup> rises in 19 of 109 neurons by >20%. In the absence of 8-Br-cAMP, repeated peak Ca<sup>2+</sup> rises evoked by GABA did not vary by >20% in any of the 109 neurons assayed. Figure 3C is a scatter plot analysis of the modulatory effects of 8-Br-cAMP administration on GABA-evoked Ca<sup>2+</sup> rises. Each point (neuron) is a representation of the first Ca<sup>2+</sup> rise in the presence of 8-Br-cAMP divided by the control Ca<sup>2+</sup> rise immediately preceding 8-Br-cAMP administration for each of the 109 neurons assayed. Data are displayed as percentages. Zero percent on the y-axis signifies that the peak rise in the presence of 8-Br-cAMP was equivalent to the control peak Ca<sup>2+</sup> rise (no modulation); values <0% represent depression, and values >0% represent response potentiation. Of note is the dual effects (both potentiation and depression) of 8-Br-cAMP on GABA-evoked Ca<sup>2+</sup> rises. To ensure that the GABA-evoked Ca<sup>2+</sup> rise was purely postsynaptic in nature, the neurons were continuously perfused with TTX (1  $\mu\text{M}$ ) to block action potential-dependent synaptic release of neurotransmitters.

To determine that GABA was released by an action potential-dependent mechanism at this early stage of development, a series of experiments was performed on neurons at 4 DIV. Electrical stimulation caused a large Ca<sup>2+</sup> rise in both neural cell bodies and neurites. Neurites loaded with fluo-3 (Fig. 4A) show a large electrically evoked Ca<sup>2+</sup> rise localized to small regions, suggesting dendrite segments postsynaptic to axonal boutons (Fig. 4D). This Ca<sup>2+</sup> rise was reduced by bath application of either TTX (Fig. 4B) or bicuculline (Fig. 4C). These results suggest that action potential-mediated presynaptic release of GABA was responsible for the Ca<sup>2+</sup> rise. Ca<sup>2+</sup> rises occurred in the presence of the

glutamate receptor antagonists AP5 (100  $\mu\text{M}$ ) and CNQX (10  $\mu\text{M}$ ), ruling out synaptic glutamate release as the cause of the Ca<sup>2+</sup> rise. To demonstrate further that synapses were formed at this developmental stage, cells were immunostained with synapsin antiserum. Highly localized, punctate synapsin staining is shown in Figure 4E. For control purposes, these cells were also immunostained for the structural protein  $\alpha$ -tubulin (Fig. 4F). In contrast to the punctate synapsin staining, tubulin immunoreactivity was found throughout neurons and glia.

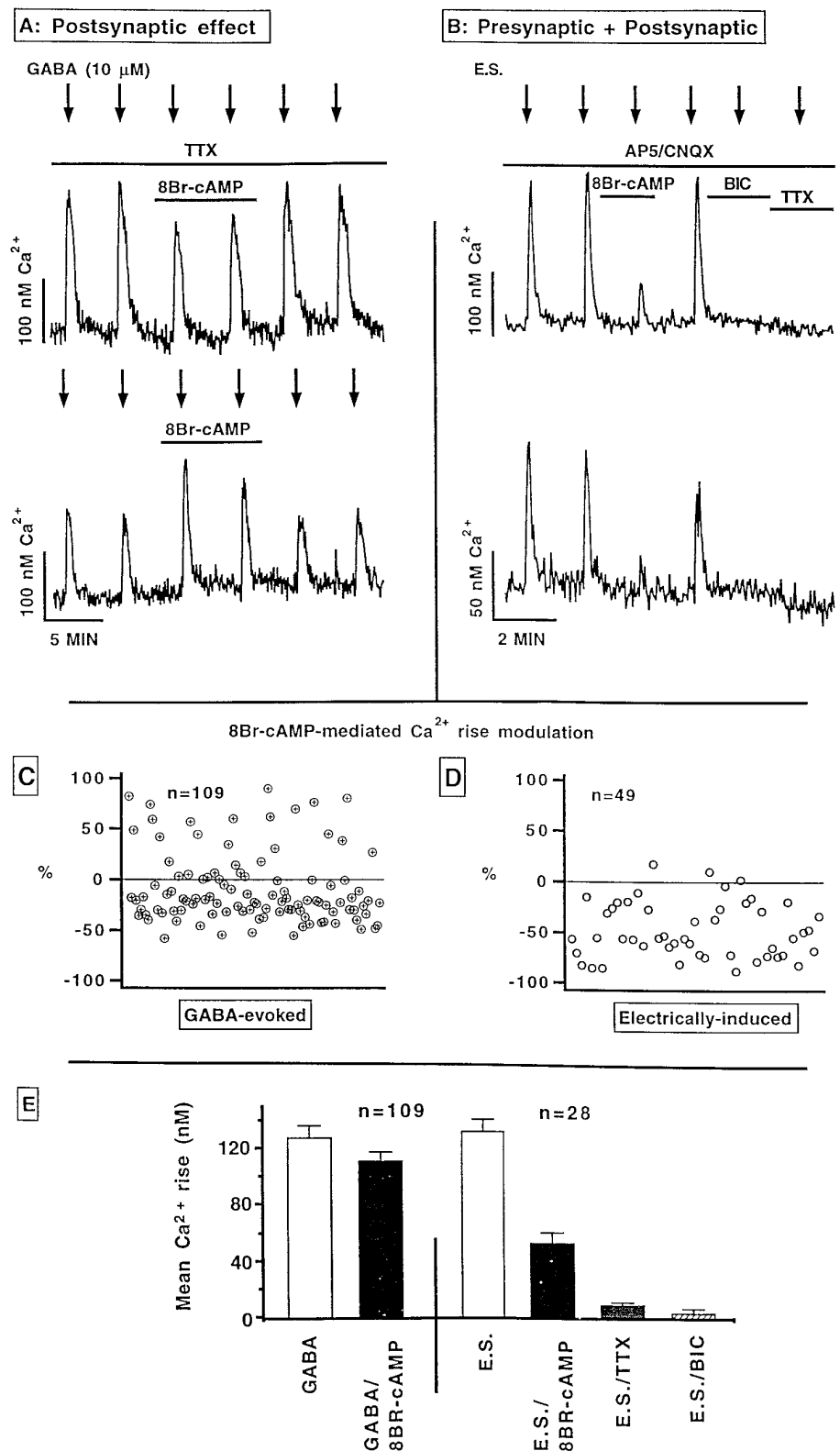
Figure 3B shows that electrical stimulation triggered reproducible Ca<sup>2+</sup> rises in the neural cell soma. The addition of either TTX (1  $\mu\text{M}$ ) or bicuculline (20  $\mu\text{M}$ ) to the perfusion solution blocked the rise. As in neurites, Ca<sup>2+</sup> rises occurred in the presence of the glutamate receptor antagonists AP5 (100  $\mu\text{M}$ ) and CNQX (10  $\mu\text{M}$ ). Under this condition, the addition of 8-Br-cAMP (1 mM) caused a large depression in the electrically evoked Ca<sup>2+</sup> rise. Relative to the control electrically stimulated Ca<sup>2+</sup> rise immediately preceding 8-Br-cAMP administration, 8-Br-cAMP depressed the Ca<sup>2+</sup> rise by >20% in 40 of 49 neurons. No neurons exhibited a >20% potentiation of the peak evoked Ca<sup>2+</sup> response. A scatter plot analysis of the effects of 8-Br-cAMP on electrically evoked Ca<sup>2+</sup> rises in 49 neurons is shown in Figure 3D. Of note is the largely inhibitory actions of 8-Br-cAMP on electrically evoked Ca<sup>2+</sup> rises. This is in contrast with data for purely postsynaptically evoked Ca<sup>2+</sup> rises (Fig. 3C), suggesting that 8-Br-cAMP also affects GABA activity mediating Ca<sup>2+</sup> rises through presynaptic regulation of GABA release.

Figure 3E compares the modulatory effects of 8-Br-cAMP on Ca<sup>2+</sup> rises elicited by bath-applied GABA with electrically evoked release of presynaptic GABA. For a comparison of effectiveness of 8-Br-cAMP on postsynaptic (GABA-evoked) and presynaptic plus postsynaptic (electrically induced GABA release) responses, data for Figure 3E were collected from neurons that exhibited a peak Ca<sup>2+</sup> rise from 120 to 140 nM during the second (control) GABA-evoked Ca<sup>2+</sup> rise. Ca<sup>2+</sup> rises of approximately equivalent levels were used so that a presynaptic component of 8-Br-cAMP-mediated Ca<sup>2+</sup> modulation could be fairly subtracted from the purely postsynaptic modulation of GABA-evoked Ca<sup>2+</sup> rises. Of interest was the finding that the mean Ca<sup>2+</sup> rise evoked by bath application of GABA was only slightly depressed (13%; statistically insignificant,  $P > 0.05$ , two-tailed  $t$  test) by 8-Br-cAMP, whereas electrically induced Ca<sup>2+</sup> rises dependent on synaptic GABA release were highly significantly ( $p < 0.0001$ , two-tailed  $t$  test) reduced (61%). This difference in 8-Br-cAMP depression of electrically versus GABA-evoked Ca<sup>2+</sup> rises (48%) was probably the result of inhibited GABA release. Unlike the other experiments, which used 5 DIV cultures, 4 DIV cultures were used for electrical stimulation experiments. At this time point during development, neurons were synaptically connected, yet the Ca<sup>2+</sup>-elevating action mediated by the spontaneous release of GABA was relatively low. Taken together, these data suggest that cAMP-dependent processes regulate Ca<sup>2+</sup> rises by decreasing the amount of presynaptic GABA release and to a lesser extent by altering postsynaptic Ca<sup>2+</sup> responsiveness to GABA.

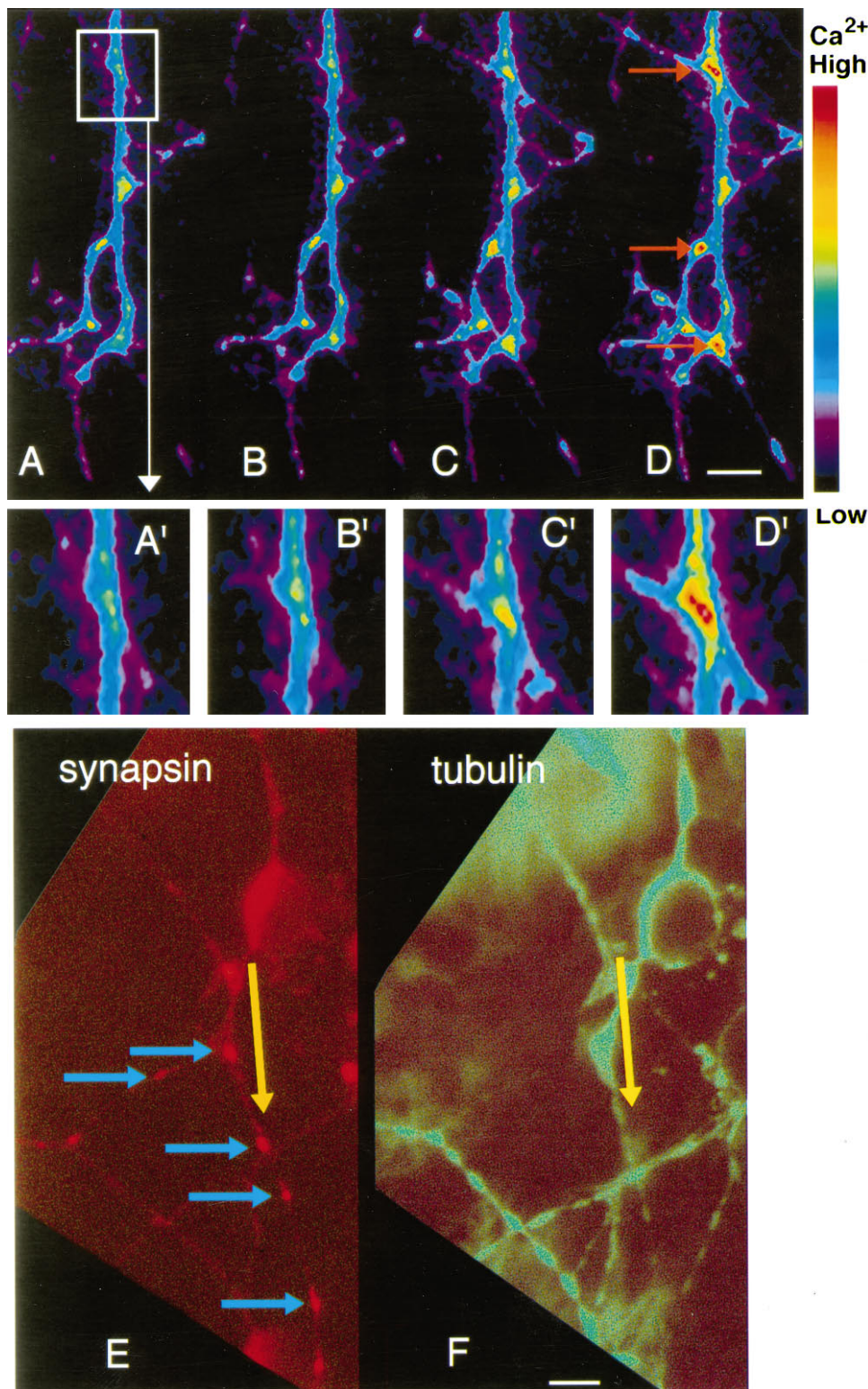
### Modulation of adenylyl cyclase activity

To determine whether altering endogenous cAMP levels would modulate GABA synaptic activity mediating Ca<sup>2+</sup> rises, neurons were treated either with forskolin (an adenylyl cyclase activator) to increase cAMP levels or with SQ-22,536 (an adenylyl cyclase

## 8Br-cAMP modulation of presynaptic and postsynaptic effects



**Figure 3.** 8-Br-cAMP reduces presynaptic release of GABA. *A*, Brief (15 sec) repeated exogenous administrations of GABA (10  $\mu$ M) (arrows) to the perfusion solution elicited rapid and reproducible postsynaptic  $Ca^{2+}$  rises. 8-Br-cAMP (1 mM) coadministration was capable of either enhancing or depressing the amplitude of GABA-evoked  $Ca^{2+}$  rises (see text). To ensure that GABA-evoked  $Ca^{2+}$  rises were exclusively postsynaptic in nature, the neurons were continuously perfused with TTX (1  $\mu$ M) to block action potential-dependent presynaptic transmitter release. *B*, Repeated electrical stimulation (E.S.) triggered reproducible  $Ca^{2+}$  rises triggered by presynaptic GABA release. Administration of TTX (1  $\mu$ M) or bicuculline (BIC, 20  $\mu$ M) blocked the  $Ca^{2+}$  rise, suggesting that electrical stimulation triggered action potential-dependent synaptic release of GABA. Administration of 8-Br-cAMP (1 mM) largely blocked the GABA activity. This experiment was performed in the constant presence of AP5 (100  $\mu$ M) and CNQX (10  $\mu$ M) to block the  $Ca^{2+}$  rise elicited by glutamate receptor activation. *C*, *D*, Analysis of the modulatory effects of 8-Br-cAMP administration on GABA-evoked  $Ca^{2+}$  rises (*C*) and on electrically evoked  $Ca^{2+}$  rises (*D*). Circles represent the first  $Ca^{2+}$  rise in the presence of 8-Br-cAMP divided by the control  $Ca^{2+}$  rise immediately preceding 8-Br-cAMP administration; each circle represents the response of a single neuron. Results are displayed as percentages of the control  $Ca^{2+}$  rise. The 0% point on the y-axis signifies that the rise in the presence of 8-Br-cAMP was equivalent to the control  $Ca^{2+}$  rise. Values  $>0\%$  represent response potentiation; values  $<0\%$  represent depression. *E*, The effects of 8-Br-cAMP on bath application of GABA and electrically stimulated synaptic release of GABA on  $Ca^{2+}$  rises were compared. Only neurons with control evoked  $Ca^{2+}$  rises (second  $Ca^{2+}$  rise, just before 8-Br-cAMP) from 120 to 140 nM were used to allow for an equitable comparison. See Results for details. Error bars indicate SEM. The mean GABA-evoked  $Ca^{2+}$  rise was slightly depressed by the administration of 8-Br-cAMP (postsynaptic effect), whereas the electrically evoked rise was dramatically depressed by 8-Br-cAMP administration (presynaptic plus postsynaptic). The electrically evoked  $Ca^{2+}$  rise was virtually abolished by TTX or bicuculline. Electrical stimulation experiments were performed after 4 DIV, a period when synaptic connections are found in most neurons.

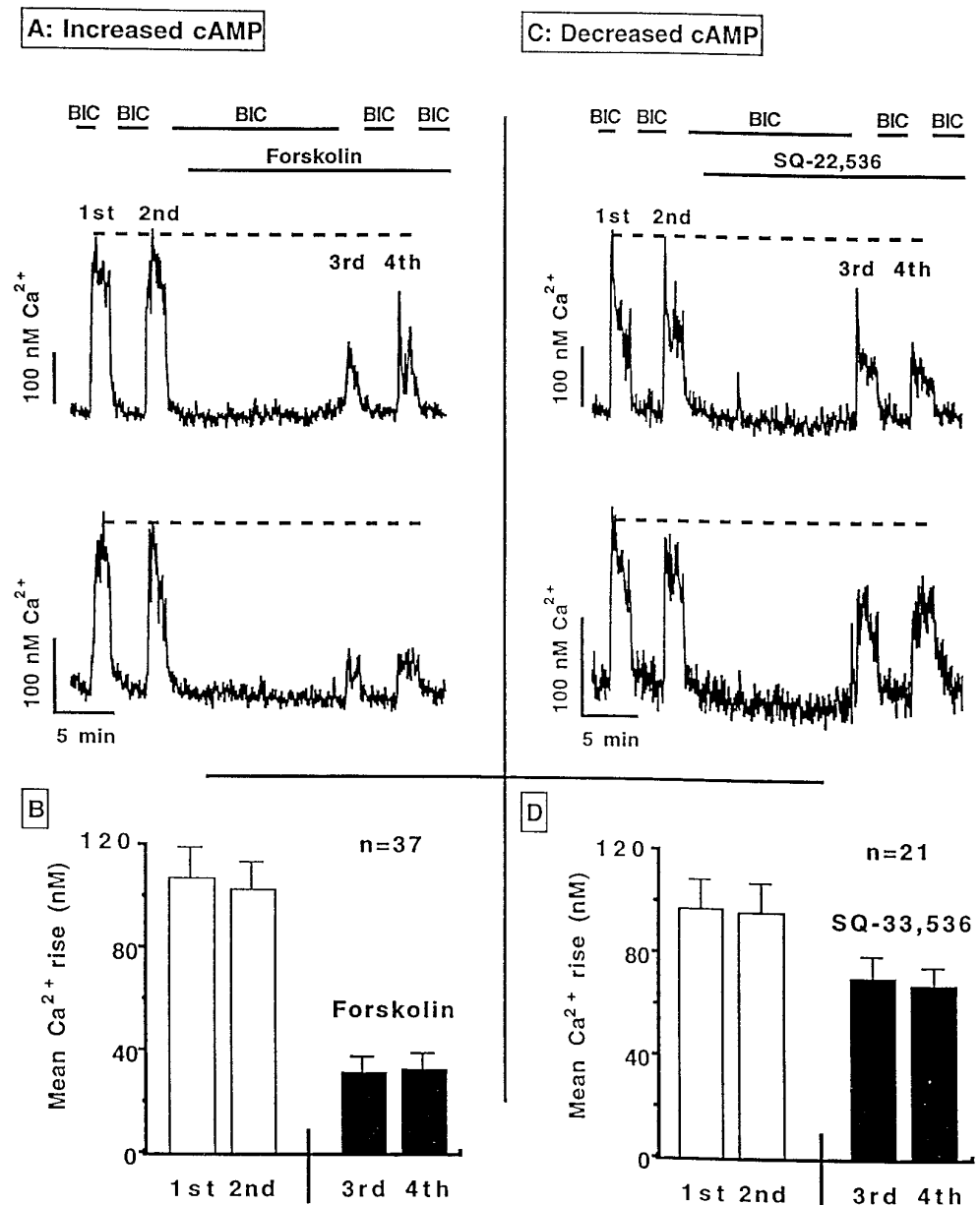


**Figure 4.** Electrical stimulation triggers GABA-mediated  $\text{Ca}^{2+}$  rises in neurites. *A*, Fluo-3-loaded neurites are shown under control conditions (no electrical stimulation). *B*, In the presence of TTX (1  $\mu\text{M}$ ), electrical stimulation does not elicit a  $\text{Ca}^{2+}$  rise. *C*, Electrically induced  $\text{Ca}^{2+}$  rise is also largely inhibited by bath application of bicuculline (20  $\mu\text{M}$ ). *D*, In the absence of bicuculline and TTX, large and localized (red arrows)  $\text{Ca}^{2+}$  rises were triggered by electrical stimulation. These experiments were performed in the presence of AP5 (100  $\mu\text{M}$ ) and CNQX (10  $\mu\text{M}$ ). *A'–D'*, High magnification of areas from *A–D* shown by the box in *A*. The color bar shows color codes of low and high  $\text{Ca}^{2+}$  levels. Scale bar, 2.5  $\mu\text{m}$ . *E*, Neurons immunostained for synapsin I. Blue arrows indicate punctate staining, corresponding to synapse location. *F*, Same region immunostained for  $\alpha$ -tubulin. Yellow arrows identify the same neurite in both micrographs. Scale bar, 8  $\mu\text{m}$ .

inhibitor) to decrease cAMP levels. Figure 5*A* shows two representative neurons exhibiting four  $\text{Ca}^{2+}$  rises: two  $\text{Ca}^{2+}$  rises before and two  $\text{Ca}^{2+}$  rises after a 15 min application of forskolin (20  $\mu\text{M}$ ). The administration of forskolin greatly depressed (>70%) the  $\text{Ca}^{2+}$  rises elicited by bicuculline withdrawal. The effects of forskolin on GABA-mediated  $\text{Ca}^{2+}$  rises are quantified in Figure 5*B*; 1st and 2nd refer to the two  $\text{Ca}^{2+}$  rises elicited by bicuculline withdrawal before forskolin administration, whereas

3rd and 4th refer to the  $\text{Ca}^{2+}$  rises after forskolin administration. A comparison of the first two with the second two  $\text{Ca}^{2+}$  rises shows that the effects of forskolin were statistically significant ( $p < 0.0001$ , two-tailed *t* test).

Interestingly, SQ-22,536 (100  $\mu\text{M}$ ) also depressed the  $\text{Ca}^{2+}$  rise elicited by bicuculline withdrawal (Fig. 5*C*). As with forskolin, a 15 min pretreatment caused a statistically significant ( $p < 0.05$ , two-tailed *t* test) decrease (>29%) in the mean  $\text{Ca}^{2+}$  rise initiated

Adenylyl cyclase modulation reduces GABA-mediated Ca<sup>2+</sup> rises

**Figure 5.** Adenylyl cyclase modulation alters GABA Ca<sup>2+</sup> rises. *A*, Before the addition of adenylyl cyclase modulators, withdrawal of bicuculline (BIC, 20  $\mu$ M) elicited rapid, reproducible Ca<sup>2+</sup> responses. *A*, After a 15 min administration of the adenylyl cyclase activator forskolin (20  $\mu$ M), GABA-mediated Ca<sup>2+</sup> rises elicited by the removal of bicuculline from the perfusion solution were significantly depressed, relative to Ca<sup>2+</sup> rises elicited before forskolin administration. *B*, Bar graph representation of the mean Ca<sup>2+</sup> rises triggered by the four bicuculline withdrawals; 1st and 2nd refer to the Ca<sup>2+</sup> rises before forskolin administration, and 3rd and 4th refer to the Ca<sup>2+</sup> rises after forskolin administration. *C*, Relative to the initial two Ca<sup>2+</sup> rises, the Ca<sup>2+</sup> rises elicited after the administration of the adenylyl cyclase inhibitor SQ-22,536 (100  $\mu$ M) were significantly depressed. *D*, Graphical representation of the mean Ca<sup>2+</sup> rises triggered by the four bicuculline withdrawals; 1st and 2nd refer to the two Ca<sup>2+</sup> rises before SQ-22,536 administration, and 3rd and 4th refer to the two Ca<sup>2+</sup> rises after SQ-22,536 administration. Error bars indicate SEM. All experiments were performed in the presence of AP5 (100  $\mu$ M) and CNQX (10  $\mu$ M).

by bicuculline withdrawal (Fig. 5D). Together, these results indicate that both increasing and decreasing cAMP levels through modulating adenylyl cyclase activity reduces GABA-mediated Ca<sup>2+</sup> rises.

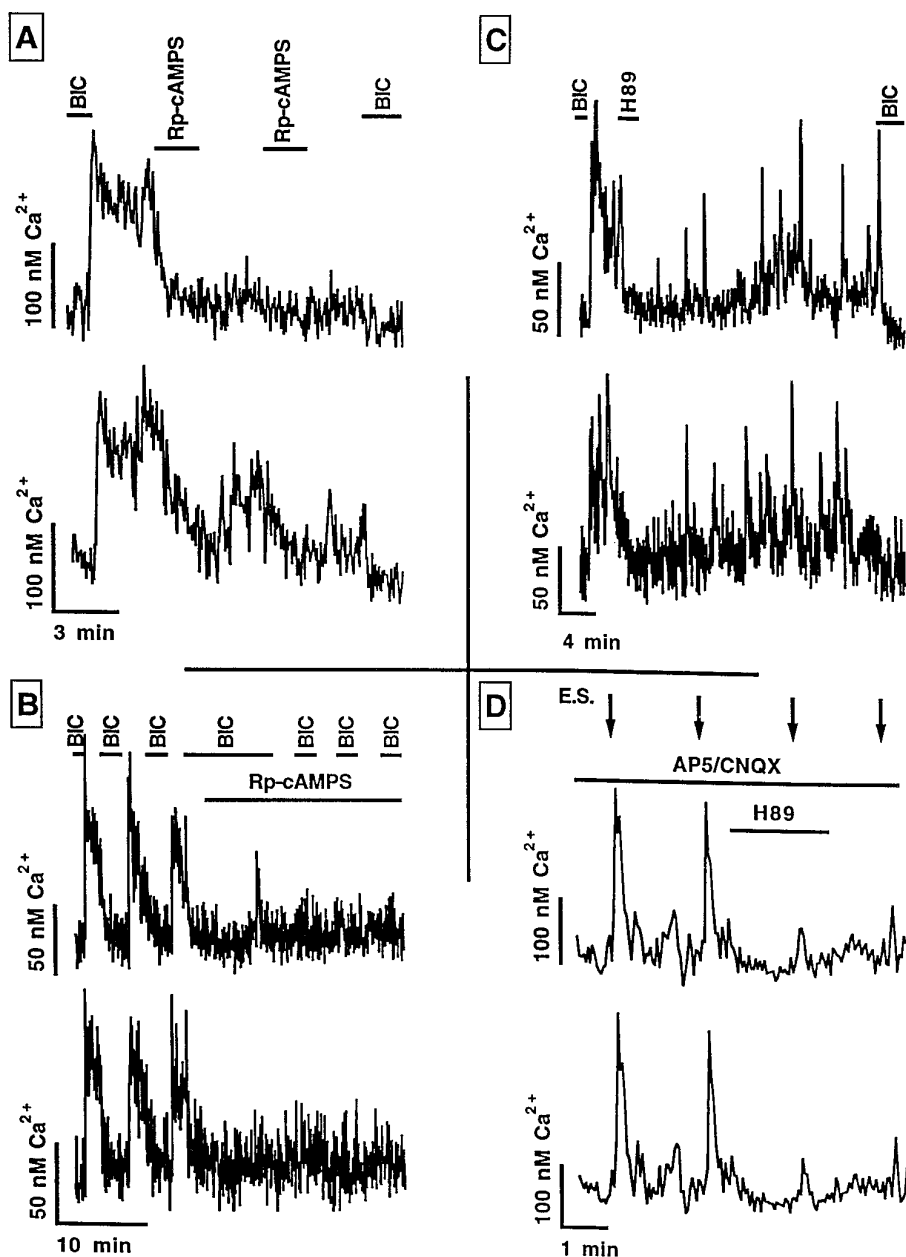
### Protein kinase A inhibition

To determine whether a basal level of PKA-mediated phosphorylation plays a role in maintaining the level of GABA Ca<sup>2+</sup> rises, we assessed the effect of the potent PKA inhibitors H89 and Rp-cAMPS. Figure 6A shows that the administration of Rp-cAMPS (200  $\mu$ M) to synaptically active neurons initiated a rapid Ca<sup>2+</sup> depression. The removal of bicuculline caused a mean Ca<sup>2+</sup> rise of 70  $\pm$  6 nM. Addition of Rp-cAMPS decreased the Ca<sup>2+</sup> rise to 18  $\pm$  2 nM, representing a statistically significant ( $p < 0.0001$ , two-tailed  $t$  test) 74% decrease in Ca<sup>2+</sup> activity ( $n = 45$ ). Of interest was the finding that the Ca<sup>2+</sup>

depression triggered by Rp-cAMPS persisted for an extended period even after the Rp-cAMPS was washed out. Fifty-eight percent of the neurons assayed (26 of 45) did not recover >50% of their pre-Rp-cAMPS Ca<sup>2+</sup> level during any time after Rp-cAMPS withdrawal. In control experiments, none of 35 unstimulated neurons showed >50% reduction in GABA Ca<sup>2+</sup> rises over an identical period.

Rp-cAMPS also inhibited the induction of Ca<sup>2+</sup> rises mediated by the synaptic release of GABA (Fig. 6B). The two representative neurons in Figure 6B initially exhibited three robust Ca<sup>2+</sup> rises, whereas after Rp-cAMPS pretreatment, bicuculline withdrawal did not initiate a Ca<sup>2+</sup> rise. As did Rp-cAMPS, a short, 2 min application of H89 (15  $\mu$ M) triggered a rapid Ca<sup>2+</sup> depression (Fig. 6C). H89 reduced the GABA-mediated Ca<sup>2+</sup> rise from 44  $\pm$  5 to 12  $\pm$  1 nM ( $n = 14$ ). The level of H89-mediated depression



Protein kinase A inhibition reduces GABA-mediated Ca<sup>2+</sup> elevations

**Figure 6.** GABA Ca<sup>2+</sup> levels are reduced by protein kinase A inhibitors. *A*, Administration of the protein kinase A inhibitor Rp-cAMPS (200 μM) caused a rapid reduction in the Ca<sup>2+</sup> level. In the *top* neuron, the Ca<sup>2+</sup> level remained depressed for an extended period after the removal of Rp-cAMPS from the perfusion solution. *B*, Pretreating neurons with Rp-cAMPS (200 μM) blocked GABA-mediated Ca<sup>2+</sup> rise induction elicited by bicuculline (BIC, 20 μM) removal. *C*, Another protein kinase A inhibitor, H89 (15 μM), also rapidly depressed GABA-mediated Ca<sup>2+</sup> levels. As with Rp-cAMPS, the Ca<sup>2+</sup> depression triggered by H89 persisted for an extended period after H89 withdrawal. *D*, The administration of H89 (15 μM) depressed Ca<sup>2+</sup> rises triggered by electrical stimulation (E.S., arrows) of GABA release. Electrically induced Ca<sup>2+</sup> rises could be blocked by tetrodotoxin or bicuculline (not shown here). As with its effects on spontaneous GABA release, the effects of H89 persisted even after it was withdrawn from the perfusion solution.

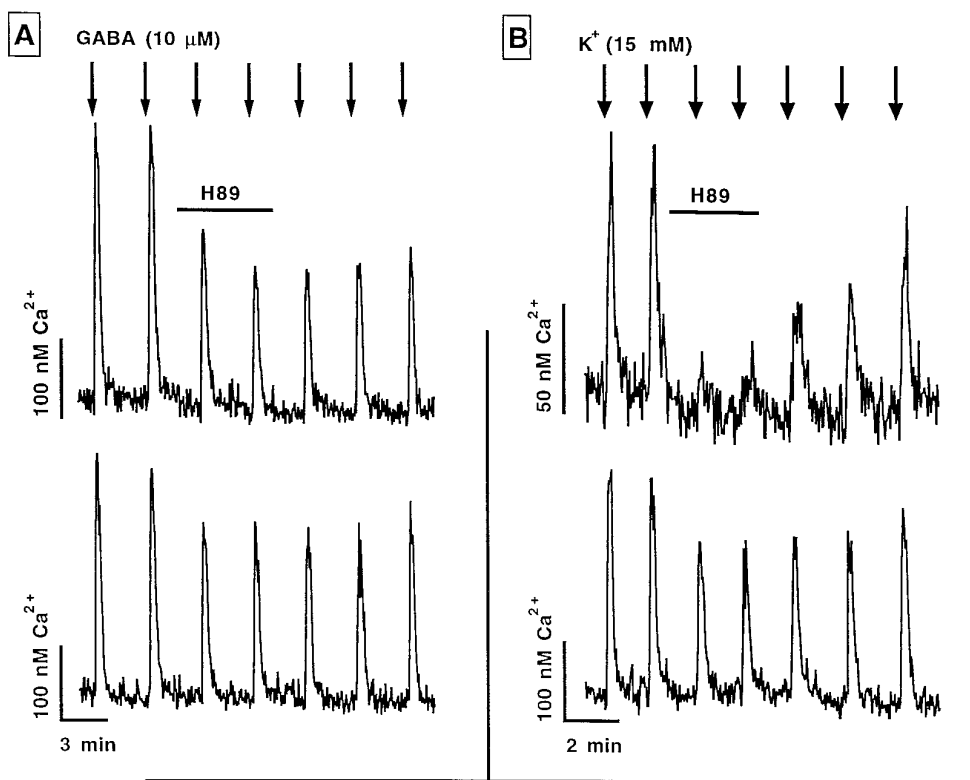
was statistically significant ( $p < 0.0001$ , two-tailed  $t$  test). As a measure of the long-term effectiveness of H89, only 4 of the 14 neurons assayed recovered  $>50\%$  of their pre-H89 Ca<sup>2+</sup> level 30 min after H89 withdrawal.

H89 also depressed GABA activity triggered by electrical stimulation. Figure 6*D* shows that the brief administration of H89 (15 μM) resulted in a statistically significant ( $p < 0.0001$ , two-tailed  $t$  test) depression in the Ca<sup>2+</sup> rise. Electrical stimulation triggered a mean Ca<sup>2+</sup> rise of  $441 \pm 26$  nM. Addition of H89 reduced the Ca<sup>2+</sup> rise to  $170 \pm 12$  nM ( $n = 78$ ). As in the endogenous activity assays described above, inhibition initiated by H89 persisted long after it was washed from the perfusion chamber in some neurons. The addition of TTX or bicuculline largely blocked the electrically evoked Ca<sup>2+</sup> rise.

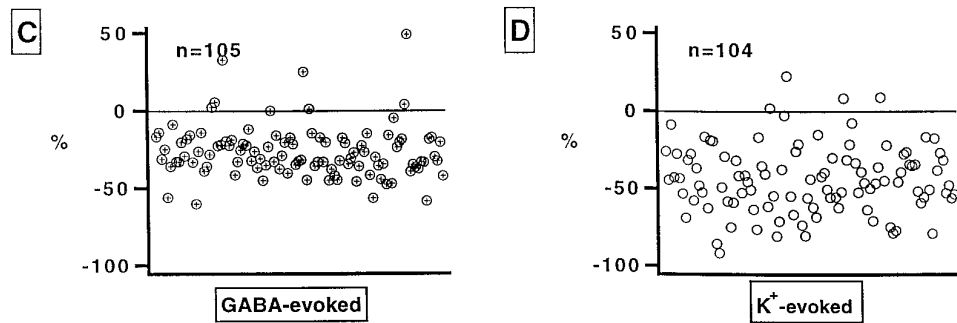
PKA and voltage-dependent Ca<sup>2+</sup> channels

In these experiments, we addressed the mechanism of postsynaptic PKA actions. In the constant presence of TTX (1 μM), the bath application of GABA (10 μM) triggered a rapid Ca<sup>2+</sup> rise that was depressed by the H89 (15 μM) administration (Fig. 7*A*). After H89 withdrawal from the perfusion solution, neuronal Ca<sup>2+</sup> responsiveness to GABA slowly recovered toward pre-H89 levels. Because GABA elicits a Ca<sup>2+</sup> rise through the activation of voltage-activated Ca<sup>2+</sup> channels (VACCs), the direct effect of H89 on high K<sup>+</sup> (15 mM)-induced Ca<sup>2+</sup> rises triggered by VACC activation was assessed. Similar to its effects on GABA, H89 administration rapidly depressed Ca<sup>2+</sup> rises mediated by VACCs (Fig. 7*B*). After H89 withdrawal the recovery of Ca<sup>2+</sup> responsiveness had a general appearance that was very similar to the recovery of

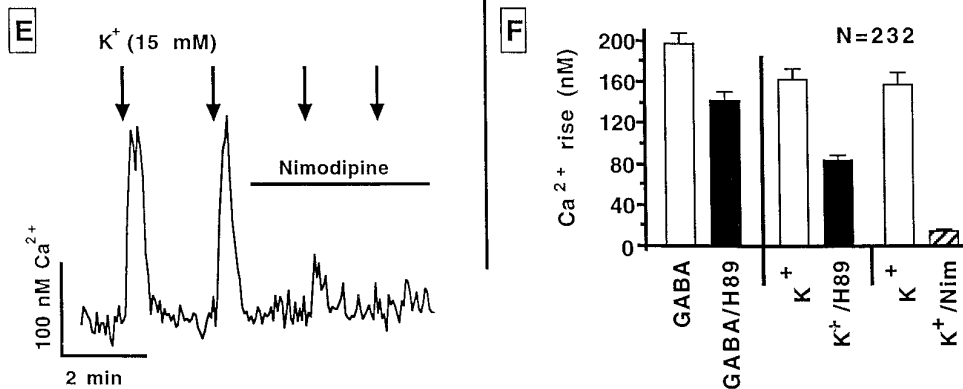
Postsynaptic depression of evoked Ca<sup>2+</sup> rises



H89 modulation of Ca<sup>2+</sup> rise



**Figure 7.** PKA inhibition reduces evoked Ca<sup>2+</sup> responsiveness. *A*, GABA (10 μM) (arrows) elicited rapid Ca<sup>2+</sup> rises that were depressed by the administration of the PKA inhibitor H89 (15 μM) in two neurons. *B*, H89 also depressed Ca<sup>2+</sup> rises initiated by high K<sup>+</sup> (15 mM) (arrows) administration. *C*, *D*, Analysis of the modulatory effects of H89 on GABA-evoked Ca<sup>2+</sup> rises (*C*) or high K<sup>+</sup>-evoked Ca<sup>2+</sup> rises (*D*). Circles are percentage representations of the first Ca<sup>2+</sup> rise in the presence of H89 divided by the control Ca<sup>2+</sup> rise immediately preceding H89 administration; each circle represents the response of a single neuron. The 0% point on the y-axis signifies that the evoked rise in the presence of H89 was equivalent to the evoked Ca<sup>2+</sup> rise. Values >0% represent potentiation; values <0% represent depression. *E*, The L-type Ca<sup>2+</sup> channel blocker nimodipine (1 μM) largely blocked Ca<sup>2+</sup> rises elicited by high K<sup>+</sup>. Both GABA and high K<sup>+</sup> were applied for 15 sec. *F*, Graphical representation of the mean Ca<sup>2+</sup> rises elicited either by GABA or high K<sup>+</sup> before (white bars) or during the coadministration of H89 (black bars) or nimodipine (striped bar). *N*, Total number of neurons assayed. Error bars indicate SEM.



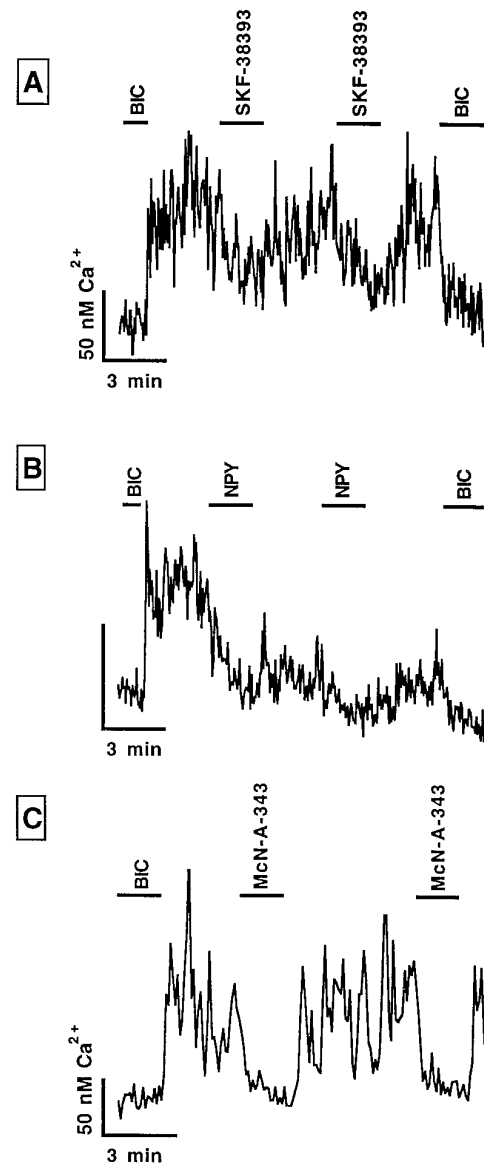
GABA responsiveness, suggesting that the H89-mediated depression of GABA responses may result, in part, from VACC inhibition. Figure 7, *C* and *D*, shows scatter plot analyses of the effects of H89 on GABA-evoked ( $n = 105$ ) or high K<sup>+</sup>-evoked ( $n = 104$ ) Ca<sup>2+</sup> rises, respectively. Single-cell values were determined by dividing the first evoked Ca<sup>2+</sup> rise in the presence of H89 by the control-evoked Ca<sup>2+</sup> rise immediately preceding H89 administration. Values are expressed as percentages of the control Ca<sup>2+</sup> rise. A Ca<sup>2+</sup> rise in the presence of H89 that was larger than control rises was >0%, whereas a rise of smaller peak height than the control rise was <0%; rises of equal peak height were 0%. Of note, the overall level of Ca<sup>2+</sup> depression elicited by H89 was greater for K<sup>+</sup>-evoked Ca<sup>2+</sup> rises than for GABA-evoked Ca<sup>2+</sup> rises. Figure 7*E* shows that high K<sup>+</sup>-induced Ca<sup>2+</sup> rises were largely suppressed by the administration of the L-type Ca<sup>2+</sup> channel blocker nimodipine (1 μM). A graphical representation of mean Ca<sup>2+</sup> rises in response to GABA and high K<sup>+</sup> before and during H89 or nimodipine application is shown in Figure 7*F*. The mean Ca<sup>2+</sup> depressions triggered by H89 and nimodipine were statistically significant ( $p < 0.0001$ , two-tailed *t* test). These findings suggest that tonic PKA-mediated Ca<sup>2+</sup> channel phosphorylation is required for GABA to elicit a robust Ca<sup>2+</sup> rise.

### Modulation of GABA Ca<sup>2+</sup> rises in adenylyl cyclase-coupled neurotransmitter receptor systems

The experiments above used pharmacological tools to directly alter different stages of intracellular pathways that affect kinase-mediated phosphorylation. We include the experiments below to demonstrate that activating neurotransmitter receptors that have previously been shown to modulate cAMP levels (either increase or decrease) exert actions parallel to those occurring when agents that act downstream of the receptor are activated (i.e., experiments described above).

We tested whether transmitter receptors shown to be either positively or negatively coupled to cAMP production alter GABA Ca<sup>2+</sup> rises. Toward this end, we chose the well characterized dopamine D1 receptor. The D1 receptor has been shown to be coupled to an increase in cAMP levels (Shultz et al., 1987; Steffy et al., 1991; Liu et al., 1992; Lovenberg et al., 1991). Administration of the dopamine D1 receptor-specific agonist SKF-38393 (5 μM) triggered a rapid and reproducible depression in the GABA-mediated Ca<sup>2+</sup> rise (Fig. 8*A*). The withdrawal of bicuculline from the perfusion solution triggered a mean Ca<sup>2+</sup> rise of  $86 \pm 4$  nM from the basal Ca<sup>2+</sup> level in the presence of bicuculline. Addition of SKF-38393 caused the Ca<sup>2+</sup> rise to decrease to  $56 \pm 3$  nM, representing a statistically significant ( $p < 0.0001$ , two-tailed *t* test) 35% depression in the GABA-mediated Ca<sup>2+</sup> rise ( $n = 79$ ). Previously, we found that NPY (100 nM) caused a large (>70%) depression in the Ca<sup>2+</sup> rise elicited by synaptically released GABA (Obrietan and van den Pol, 1996b). An example of the Ca<sup>2+</sup>-depressing actions of NPY is shown in Figure 8*B*. Several studies have shown that NPY receptor stimulation triggers inhibitory G-protein activation, leading to a decreased cAMP level (McAuley et al., 1991; Bleakman et al., 1992; Larhammar et al., 1992; Zhu et al., 1992). Additionally, administration of the muscarinic acetylcholine receptor agonist McN-A-343 (100 μM) triggered a Ca<sup>2+</sup> depression (Fig. 8*C*). The withdrawal of bicuculline from the perfusion solution elicited a mean Ca<sup>2+</sup> rise of  $96 \pm 7$  nM from the basal Ca<sup>2+</sup> level. McN-A-343 administration caused the Ca<sup>2+</sup> rise to decrease to  $65 \pm 5$  nM, representing a statistically significant ( $p < 0.0001$ , two-tailed *t* test) 32% depression in the GABA-mediated Ca<sup>2+</sup> rise ( $n = 52$ ). A large number of studies

### GABA-mediated Ca<sup>2+</sup> rises reduced by stimulating putative adenylyl cyclase-coupled receptors



**Figure 8.** Ca<sup>2+</sup> rises mediated by GABA activity are depressed by selective activation of neurotransmitter receptors coupled to adenylyl cyclase regulation. The removal of bicuculline (BIC, 20 μM) from the perfusion solution triggered a rapid and sustained Ca<sup>2+</sup> rise in the three representative neurons. *A*, Stimulation of the D1 receptor by the administration of SKF-38393 (5 μM) reduced the amplitude of the spontaneous GABA-mediated Ca<sup>2+</sup> rise. *B*, NPY (100 nM) triggered a rapid Ca<sup>2+</sup> depression. Blockade of the GABA<sub>A</sub> receptor with bicuculline at the end of the experiment reduced Ca<sup>2+</sup> to basal levels. *C*, Stimulation with the muscarinic acetylcholine receptor agonist McN-A-343 (100 μM) also triggered Ca<sup>2+</sup> depression. The ionotropic glutamate receptor antagonists AP5 (100 μM) and CNQX (10 μM) were perfused throughout the experiment to block synaptically released glutamate from triggering a Ca<sup>2+</sup> rise.

have shown that the muscarinic acetylcholine receptors modulate cAMP levels (McKinney et al., 1991; Schwarz et al., 1993; Burford et al., 1995; Migeon et al., 1995). Interestingly, in 13 of 58 neurons, a lower McN-A-343 concentration (15 μM) increased GABA-mediated Ca<sup>2+</sup> levels by >10%. In contrast, only 1 of 52

neurons treated with the higher concentration of McN-A-343 (100  $\mu$ M) showed a >10% increase in the GABA-mediated Ca<sup>2+</sup> rise. These results support the hypothesis that receptor systems coupled to either an increase or a decrease in cAMP production may alter GABA Ca<sup>2+</sup> rises significantly.

## DISCUSSION

Activation of the GABA<sub>A</sub> receptor, either through exogenous agonist application or synaptic GABA release, elicits a Ca<sup>2+</sup> rise in the majority of developing neurons from many brain regions (Obrietan and van den Pol, 1995). In the present study, we characterized the functional role of the cAMP signal transduction pathway in terms of its ability to regulate GABA-related Ca<sup>2+</sup> rises. Our data suggest that either activating or inhibiting the cAMP signal transduction pathway significantly depressed the GABA activity detected with Ca<sup>2+</sup> imaging. The results of affecting the cAMP signal transduction pathway were similar to the effects of stimulating transmitter receptors thought to be coupled to either a decrease or an increase in cAMP levels.

PKA activation or inhibition, as described in this paper, may cause a variety of different mechanisms to work together or in opposition to one another, with the general effect being a decrease in the Ca<sup>2+</sup> rise initiated by GABA-mediated neurotransmission. To our knowledge, ours is the first report characterizing the regulation of GABA-mediated Ca<sup>2+</sup> rises by direct modulation of the cAMP signal transduction pathway. These effects were observed with agents that either activated (membrane-permeable cAMP analogs and forskolin) or inhibited (PKA inhibitors and SQ-22536) the cAMP signal transduction pathway at several different points along the pathway.

### Postsynaptic effects

Blocking PKA activity with H89 or Rp-cAMPS depressed peak Ca<sup>2+</sup> rises elicited by the bath application of GABA. Because the presynaptic release of GABA-containing vesicles was blocked with TTX, the observed inhibitory effect of PKA was postsynaptic in nature. A number of possible mechanisms could account for the depression of GABA-mediated Ca<sup>2+</sup> levels observed when the cAMP signal transduction cascade was altered. A decrease in GABA<sub>A</sub> receptor activity resulting from an inhibition of steady-state GABA<sub>A</sub> receptor phosphorylation could explain the depression resulting from PKA inhibition. Along these lines, several studies have shown that GABA<sub>A</sub> receptor-mediated activity is regulated by phosphorylation. GABA<sub>A</sub> currents were decreased by >90% if cells were ATP-depleted (Chen et al., 1990), suggesting that a basal level of phosphorylation was required for maintenance of optimal GABA<sub>A</sub> receptor channel function. In addition, GABA<sub>A</sub> receptor conductance has been shown to be directly affected by PKA-mediated phosphorylation (Moss et al., 1992). In the hippocampus, the frequency of GABA-mediated giant depolarizing potentials is modulated by 8-Br-cAMP or forskolin and inhibited by Rp-cAMPS (Strata et al., 1995). Within this context, our results suggest that tonic postsynaptic PKA-mediated phosphorylation is required for optimal Ca<sup>2+</sup> responsiveness.

Ca<sup>2+</sup> responses triggered by the direct activation of VACCs (largely L-type) were also suppressed by H89. Because the ability of GABA to elicit a Ca<sup>2+</sup> rise in hypothalamic neurons is dependent on L-type Ca<sup>2+</sup> channel activation (Obrietan and van den Pol, 1995), these results suggest that H89-mediated depression of GABA-evoked Ca<sup>2+</sup> rises may result, in part, from the inhibition of L-type VACC activity. A basal level of phosphorylation may be required for optimal L-type Ca<sup>2+</sup> channel activity; Ca<sup>2+</sup> currents in HEK-293 cells transfected with the L-type Ca<sup>2+</sup> channel gene

were depressed by administration of PKA inhibitors (Perez-Reyes et al., 1994). Additionally, forskolin increased the L-type Ca<sup>2+</sup> current in ferret ventricular myocytes, and this increase was blocked by H89 (Yuan and Bers, 1995). Our results in neurons are consistent with these findings. This does not rule out a possible additional effect of phosphorylation on K<sup>+</sup> channels that could, in turn, influence VACCs.

### Presynaptic effects

The cAMP signal transduction pathway appeared to act at both presynaptic and postsynaptic sites to affect GABA-related Ca<sup>2+</sup> rises. Ca<sup>2+</sup> rises triggered by electrical stimulation of presynaptic GABA release were depressed to a much greater extent by 8-Br-cAMP than postsynaptic Ca<sup>2+</sup> rises elicited by the bath application of GABA. Additionally, postsynaptic Ca<sup>2+</sup> rises in a subpopulation of neurons were potentiated by 8-Br-cAMP, whereas potentiation was not observed when Ca<sup>2+</sup> rises were elicited by electrically induced presynaptic GABA release. These differential effects suggest that the cAMP signal transduction pathway may inhibit presynaptic GABA release, as well as modulate postsynaptic GABA responsiveness. This conclusion is consistent with the view that PKA has a spatially limited action and may exert independent or opposing effects in presynaptic axons compared with the postsynaptic somatodendritic complex.

PKA activators 8-Br-cAMP or 4-Cl-cAMP decreased GABA-related Ca<sup>2+</sup> rises, although we also noted that low concentrations of 8-Br-cAMP or 4-Cl-cAMP increased Ca<sup>2+</sup> levels in a subpopulation of neurons during endogenous activity assays. These Ca<sup>2+</sup> modulatory effects of 8-Br-cAMP and 4-Cl-cAMP were largely through a presynaptic mechanism. For a presynaptic receptor coupled to adenylyl cyclase activation (which would result in increased PKA activity), there have been a variety of reports describing either potentiation or inhibition of transmitter release. For example, activation of the dopamine D1 receptor (coupled to cAMP production) facilitates neurotransmission in the hippocampus (Imperato et al., 1993) and in the ventral tegmental area (Cameron and Williams, 1993), whereas D1 receptor stimulation has been shown to decrease neurotransmission in the basal forebrain (Momiya et al., 1996) and in the shell region of the nucleus accumbens (Pennartz et al., 1992).

The exclusive effect of PKA inhibitors on Ca<sup>2+</sup> rises elicited by presynaptic GABA release was inhibitory. Receptors coupled to adenylyl cyclase inhibition (which would result in decreased PKA activity), including the GABA<sub>B</sub> receptor (Dittman and Regehr, 1996), the adenosine A1 receptor (Potier and Dutar, 1993), and the NPY receptor (Bleakman et al., 1992), have been shown to decrease transmitter release. These findings suggest that the effects of PKA on transmitter release may depend on the presynaptic expression of a variety of phosphorylation targets that may be differentially expressed in different brain regions, developmental stages, or neural phenotypes. This is consistent with the presynaptic localization of many hypothalamic neuromodulatory receptors that act through G-proteins to regulate cAMP (Chen and van den Pol, 1996).

### Long-term Ca<sup>2+</sup> depression

In assays in which Ca<sup>2+</sup> rises were elicited via synaptic GABA release, inhibition of PKA by H89 or Rp-cAMPS triggered a rapid and long-term Ca<sup>2+</sup> depression (>30 min) in a large number of neurons (58%). Based on the differential postsynaptic effects of PKA inhibitors, long-term Ca<sup>2+</sup> depression seems to be mediated through a largely presynaptic mecha-

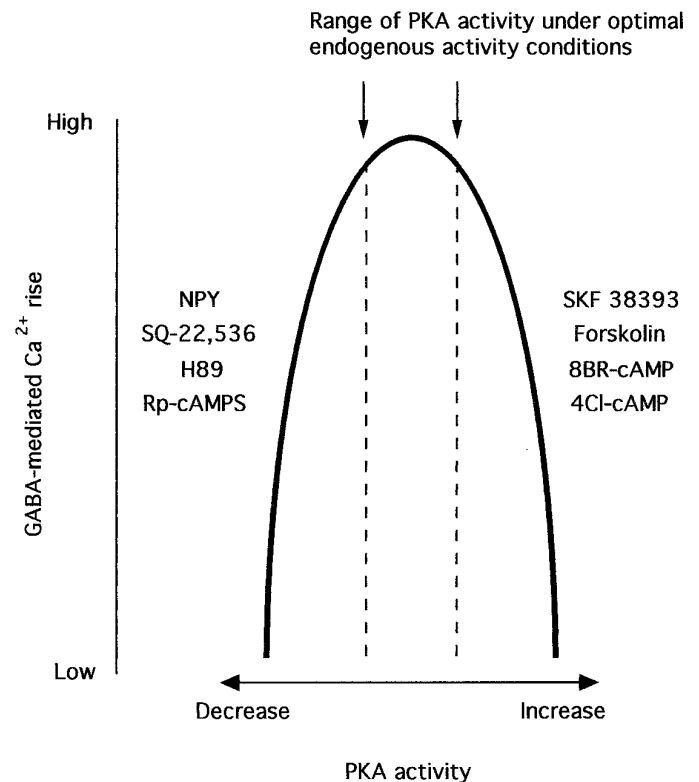
nism. Interestingly, membrane-permeable cAMP analogs triggered short- but not long-term Ca<sup>2+</sup> depression also through a largely presynaptic mechanism. These results suggest that the cellular mechanisms that inhibit PKA-mediated phosphorylation may be uniquely positioned to depress neural activity over extended periods.

NPY has both presynaptic and postsynaptic actions on GABA activity in developing hypothalamic neurons. Whereas NPY triggered a predominately brief postsynaptic depression of GABA-evoked Ca<sup>2+</sup> rises (Obrietan and van den Pol, 1996b), presynaptic actions of NPY on transmitter release were often long-term (Obrietan and van den Pol, 1996b; van den Pol et al., 1996c). In addition, NPY receptor stimulation depressed the Ca<sup>2+</sup> rise in a similar percentage of neurons and for a similar duration as did direct PKA inhibition. NPY has been shown both to decrease cAMP levels in neurons (Harfstrand et al., 1987; McAuley et al., 1991) and to act through a G<sub>i</sub>/G<sub>o</sub>-protein-coupled mechanism to reduce Ca<sup>2+</sup> rises elicited by synaptic GABA release (Bleakman et al., 1992; Obrietan and van den Pol, 1996b; van den Pol et al., 1996c). Activation of a G<sub>i</sub>-protein-coupled mechanism could result in decreased adenylyl cyclase activity and, in turn, decreased PKA activity, an effect similar to adding Rp-cAMPS or H89 to the perfusion solution. These results indicate that the long-term effects of NPY receptor activation on GABA-mediated Ca<sup>2+</sup> rises may be the result of decreased PKA activity. Other receptor systems coupled to adenylyl cyclase regulation have been shown to trigger extended depression of neural activity, including the GABA<sub>B</sub> receptor (Yang et al., 1994; Wagner and Alger, 1995) and metabotropic glutamate receptor (Bolshakov and Siegelbaum, 1994; O'Mara et al., 1995). Immunohistochemical analysis has revealed a high level of adenylyl cyclase expression both in postsynaptic densities and in presynaptic axon terminals (Mons and Cooper, 1995), suggesting that adenylyl cyclase may play an important role both as a regulator of postsynaptic membrane ion conductance and presynaptic neurotransmitter release.

### Biphasic effects of phosphorylation on GABA transmission

GABA-related Ca<sup>2+</sup> rises were depressed by both strong activation and inhibition of the cAMP signal transduction system. This biphasic response could be explained if the effects of PKA on GABA-mediated Ca<sup>2+</sup> rises were characterized by an inverted U-shape function (Fig. 9). During GABA neurotransmission, a basal level of tonic PKA-mediated phosphorylation results in a near maximal Ca<sup>2+</sup> level. This condition would put PKA activity near the top of the inverted U-shape function. When PKA activity is radically altered, either through large increases (D1 receptor activation, forskolin, and cAMP analogs) or decreases (NPY receptor activation, SQ-22,536, Rp-cAMPS, and H89), PKA activity moves out of the region of the inverted U-shape function that provides maximal GABA-mediated Ca<sup>2+</sup> rises.

Low extracellular concentrations of 8-Br-cAMP (50–150 μM) or 4-Cl-cAMP (20 μM) increased GABA-mediated activity in some neurons, whereas high concentrations of 8-Br-cAMP (≥500 μM) or 4-Cl-cAMP (≥200 μM) only decreased Ca<sup>2+</sup> levels. This is consistent with the hypothesis that the basal level of PKA activity in some neurons is just below that needed to provide a maximal level of GABA-mediated Ca<sup>2+</sup> rises. As described by an inverted U-shape response, this effect would result in a movement toward the apex of the inverted U. The data fitted to this model indicate that GABA-mediated Ca<sup>2+</sup> rises depend on ongoing PKA activ-



**Figure 9.** Model of biphasic effect of PKA activity on GABA transmission. Proposed model describing how increases or decreases in PKA activity result in a reduction in GABA-mediated Ca<sup>2+</sup> rises. Arrows and dashed lines indicate the range of endogenous PKA activity required for maximal GABA-mediated Ca<sup>2+</sup> rises. See Discussion for details.

ity, and that PKA activity must be maintained within a narrow zone for GABA to elicit a maximal Ca<sup>2+</sup> rise.

### Functional role of GABA-mediated Ca<sup>2+</sup> increases during early development

Changes in cytosolic Ca<sup>2+</sup> levels affect a variety of developmentally regulated neural processes. Induction of Ca<sup>2+</sup> influx was primarily thought to be through the activation of classic membrane-depolarizing transmitters such as glutamate. Recent data have shown that GABA can exert a similar depolarizing action in developing neurons. This Ca<sup>2+</sup>-elevating action of GABA is not restricted to hypothalamic neurons but was found in the majority of developing neurons from eight brain regions, including hippocampus, spinal cord, cortex, olfactory bulb, and striatum (Reichling et al., 1994; Obrietan and van den Pol, 1995), and after severe neural injury (van den Pol et al., 1996b). The ability of GABA to increase Ca<sup>2+</sup> levels suggests that it may have an important role during nervous system development. Along these lines, the GABA agonist muscimol can upregulate BDNF mRNA expression in rat hippocampal neurons, and this effect is blocked by the L-type Ca<sup>2+</sup> channel blocker nifedipine (Berninger et al., 1995). GABA secretion decreased DNA synthesis in cortical progenitor cells (LoTurco et al., 1995). GABA induces motility of embryonic cortical neurons through an increase in intracellular Ca<sup>2+</sup> (Behar et al., 1996). Other GABA-mediated actions during neural development include altering neurite outgrowth (Barbin et al., 1993), triggering chemokinesis (Behar et al., 1994), inducing GABA receptor expression (Meier et al., 1984), and regulating neural phenotype (Marty et al., 1996). We have recently reported

that GABA triggers localized Ca<sup>2+</sup> increases in developing neurites and growth cones (Obrietan and van den Pol, 1996a), suggesting a possible Ca<sup>2+</sup>-dependent role for GABA in altering the directionality of growth cone motility and the rate of neurite outgrowth. Based on these reports and the findings in our paper, during early development the activation of neurotransmitter receptors (such as NPY, glutamate, dopamine, serotonin, melatonin, and acetylcholine) coupled to the cAMP signal transduction pathway could substantially alter GABA-related Ca<sup>2+</sup> rises. This, in turn, could affect the myriad of Ca<sup>2+</sup>-dependent developmental processes described above.

Depression of Ca<sup>2+</sup> rises attributable to an inhibitory action of presynaptic neuromodulators on GABA release is only found during early development. Because GABA does not raise Ca<sup>2+</sup> in older neurons, but may instead decrease it (Obrietan and van den Pol, 1995), neuromodulatory inhibition of GABA release in mature neurons would be expected to exert the opposite effect on Ca<sup>2+</sup>. Thus, in developing neurons, modulatory inhibition of GABA release reduces cytosolic Ca<sup>2+</sup> but in mature neurons would raise Ca<sup>2+</sup>.

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