The Journal of Neuroscience, May 1, 1996, 16(9):2912–2923

Upregulation of $GABA_A$ Current by Astrocytes in Cultured Embryonic Rat Hippocampal Neurons

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Embryonic rat hippocampal neurons were cultured on poly-D-lysine (PDL) or a monolayer of postnatal cortical astrocytes to reveal putative changes in neuronal physiology that involve astrocyte-derived signals during the first 4 d of culture. GABA-induced Cl^- current (I_{GABA}) was quantified using outside-out and whole-cell patch-clamp recordings beginning at 30 min, when cells had become adherent. The amplitude and density (current normalized to membrane capacitance) of I_{GABA} in neurons grown on astrocytes became statistically greater than that recorded in neurons grown on PDL after 2 hr in culture (HIC). Although the current density remained unchanged in neurons on astrocytes, that in neurons on PDL decreased and became statistically lower beginning after 2 HIC. The differences in amplitude and density of I_{GABA} in the two groups of neurons were maintained during the 4 d experiment. The upregulation effect of astrocytes on neuronal I_{GABA} required intimate contact between the neuronal cell body and underlying astrocytes. Suppression of spontaneous Ca_c^{2+} elevations in astrocytes by bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid that was loaded intracellularly decreased their modulatory effects on I_{GABA}. I_{GABA} in all cells was blocked completely by bicuculline and exhibited virtually identical affinity constants, Hill coefficients, and potentiation by diazepam in the two groups. Outside-out patch recordings revealed identical unitary properties of I_{GABA} in the two groups. More channels per unit of membrane area could explain the astrocyte enhancement of I_{GABA}. The results reveal that cortical astrocytes potentiate I_{GABA} in hippocampal neurons in a contact-dependent manner via a mechanism involving astrocyte Ca_c^{2+} elevation.

Key words: $GABA_A$ receptor; ion channels; neuronal development; astrocyte; intracellular calcium; hippocampus; rat

Astrocytes can directly influence neuronal electrical activity by regulating extracellular ions (mainly K^+) and neurotransmitter concentrations (such as GABA and glutamate) in the extracellular compartment that bathes these cells (Schon and Kelly, 1974; Lieberman et al., 1989; Hertz, 1990). Astrocytes also have significant effects on the neuronal expression and distribution of ion channels and neurotransmitter receptors (for review, see Barish, 1995). For example, experiments by Bostock et al. (1981), Shrager (1988), Ritchie et al. (1990), Joe and Angelides (1992), and Waxman and Ritchie (1993) suggest that astrocytes in close contact with axonal membranes might influence the distribution of Na^+ and K^+ channels in the latter. Wu and Barish (1994) found that direct contact with astrocytes induces the appearance of transient A-type K^+ currents and depresses that of sustained D-type K^+ current in embryonic mouse hippocampal neurons when the two cells are grown in co-culture. These effects of astrocytes on voltage-dependent K^+ currents seem to involve a surface- or extracellular matrix-associated mechanism, rather than a free diffusion of soluble factors in the extracellular space (Wu and Barish, 1994). Intimate contact between axon and vital physiologically intact astrocytes and not simply the plasma membrane of the astrocyte are required for these effects (Joe and Angelides, 1992; Wu and Barish, 1994). Glial regulation of transient K^+ current density in chick lumbar sympathetic ganglion neurons also occurs via direct contact, but the glial membrane surface remaining in nonvital cells suffices (Raucher and Dryer, 1994). In rat sympathetic neurons, astrocyte regulation of K^+ currents can be mimicked by astrocyte-conditioned culture medium or by ciliary neurotrophic factor implicating a soluble substance or substances (McFarlane and Cooper, 1993). The exact mechanisms and factors involved in astrocyte regulation of neuronal excitability, the intercellular communication, if any, between neurons and astrocytes, and the role such intercellular signaling plays in the codifferentiation of the two cell types remain to be elucidated. These initial results suggest heterogenous forms of communication and signal transduction.

In the present study, we have compared GABA-induced Cl^- currents (I_{GABA}) in embryonic rat hippocampal neurons cultured on poly-D-lysine (PDL) and on confluent monolayers of postnatal rat cortical astrocytes. We found that relative to PDL cultures, cortical astrocytes upregulate, or maintain, the neuronal expression of $GABA_A$ receptor/Cl^- channels. This modulatory effect requires intimate contact between neuronal cell bodies and astrocytes, indicating the involvement of astrocyte membrane- or extracellular matrix-associated factors and/or ultra-short-range soluble factors. Suppression of spontaneous intracellular Ca^{2+} fluctuations in astrocytes by bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid (BAPTA) loaded intracellularly significantly reduced these effects on I_{GABA}, suggesting that they involve elevation in cytosolic Ca^{2+} (Ca_c^{2+}).

Received Dec. 15, 1995; revised Feb. 12, 1996; accepted Feb. 14, 1996.

We thank the Instrumentation and Computer Section, National Institute for Neurological Disorders and Stroke, National Institutes of Health, for fabricating the nine-channel perfusion controller complete with solenoid valves. We thank Atto Instruments, Bethesda, Maryland, and Carl Zeiss, Inc., Thornwood, New York for letting us use their Attofluor RatioVision digital imaging system.

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Parts of this paper have been published previously (Liu et al., 1995).

MATERIALS AND METHODS

Preparation of astrocyte monolayers. Cortices, free of hippocampi and striata, were removed from 3-d-old rat pups, cleaned of meninges, and placed in 10 ml L-15 medium with 50 U/ml gentamicin. The tissue was triturated through a 5 ml pipette, dissociated mechanically through a series of small gauge (G) needles (3 × 19–20 G, 3 × 22–23 G, 1 × 25 G), and passed through 62 μm Nitex. Cells were centrifuged at low speed, resuspended in plating medium consisting of DMEM supplemented with 10% fetal calf serum (FCS) and 50 U/ml gentamicin, and plated at the equivalent of two brains per flask in 75 cm^2 flasks that had been pretreated with 5 μg/ml PDL (30–70 K; Sigma, St. Louis, MO). Medium was changed completely after 72 hr and twice weekly thereafter. When a confluent monolayer was present (after ~1 week), the flasks were capped tightly and placed overnight on a rotary shaker at 180 rpm at 37°C. The supernatant, containing microglia, loosely adherent O2A progenitor cells, and debris, was removed rapidly and completely after ~12 hr. Cultures were rinsed once with L-15 or DMEM and then refed with plating medium. To select further for astrocytes in the culture by removing any surviving neurons and O2A progenitor cells, the flasks were subjected to complement-mediated lysis of cells expressing A2B5 surface antigen, as described by Armstrong et al. (1992). Briefly, the cultures were incubated with a 1:50 dilution of A2B5 ascites in DMEM with 1% FCS or full-strength A2B5 culture supernatant for 1 hr at 37°C. Cultures were then rinsed twice in DMEM-1% FCS and treated for 1 hr at 37°C with rabbit complement diluted 1:8 in DMEM-1% FCS. To reduce the amount of antibody necessary and expose a greater surface area for antibody binding, the cells were trypsinized off the flask and resuspended in a small (1–2 ml) volume. A2B5 is a trypsin-resistant surface antigen. After cytolysis, cultures were rinsed twice in DMEM-1% FCS and refed with plating medium. The cultures were then trypsinized, and the cells were transferred into 35 mm culture dishes precoated with 5 μg/ml PDL (30–70 K, Sigma). Astrocytes for calcium imaging were plated in 35 mm culture dishes with glass coverslip bottoms (MatTek Corporation, Ashland, MA). When cells reached confluence they were exposed to 10 μM cytosine arabinoside for 2 d and then maintained in minimal essential medium (MEM) with 5% horse serum before being used. Cultures prepared in this way contained ≥95% type-1 astrocytes as determined by glial fibrillary acidic protein (GFAP) (positive) and A2B5 (negative) immunocytochemistry and morphological examination.

Preparation and culture of hippocampal neurons. Hippocampal neurons were dissociated from embryonic day 18 rat embryos by papain digestion, according to the method of Huettner and Baughman (1986). Briefly, the hippocampal tissues were cut carefully into small pieces, transferred into 5 ml Earle's balanced salt solution containing 20 U/ml papain, 0.01% DNase (both from Boehringer Mannheim, Indianapolis, IN), 0.5 mM EDTA, and 1 mM L-cysteine, and kept in an incubator for 35–40 min at 37°C. Single neurons, obtained by triturating the tissue with a Pasteur pipette, were washed twice with culture medium containing 90% MEM (Gibco, Grand Island, NY), 5% FCS, and 5% horse serum (Biofluid, Rockville, MD). The neurons were then plated at a density of ~5 × 10^5 cells/dish in 35 mm plastic culture dishes coated with high molecular weight PDL (Sigma) and with or without a monolayer of astrocytes. In some experiments, astrocytes were exposed to culture medium containing 10 μM BAPTA-acetoxymethyl ester compound (BAPTA-AM) for 30 min at 37°C and then washed twice before neurons were plated. Astrocytes were loaded with BAPTA to suppress Ca_c^{2+} elevations (Furuya et al., 1994; Gu and Spitzer, 1995). The cultures were kept at 37°C in a water-saturated atmosphere containing 10% CO_2. Cells were studied initially at 30 min in culture, the minimum time when they adhered firmly enough to be useful for electrophysiological study. Neurons with relatively large cell bodies bearing visible processes were selected for study. Neurons cultured on PDL were recorded only if they were not in visible contact with astrocytes, which after a variable delay spread progressively and limited useful study of astrocyte-dependent changes in neurophysiology to the first 4 d.

Current recording and analysis. Before recording, dishes were removed from the incubator, and the culture medium was replaced with either a solution containing (in mM) 142 NaCl, 8.1 CsCl, 1 $CaCl_2$, 6 $MgCl_2$, 10 Glucose, 10 HEPES-CsOH, pH 7.3, and 310 mOsm for single-channel recordings in outside-out patch-clamp mode, or Tyrode's solution containing (in mM) 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgCl_2$, 10 glucose, 10 HEPES-NaOH, pH 7.4, and 310 mOsm for whole-cell recordings. Standard patch-clamp recordings (Hamill et al., 1981) were made with pipettes pulled in three stages from 1.5 mm outer diameter glass capillary tubes (WPI, Sarasota, FL) with a computer-controlled pipette puller (BB-CH-PC, Mecanex SA). These pipettes had a resistance of 3–5 MW when filled with internal solution. Pipettes for single-channel recordings were fire-polished, coated with Sylgard-184, and filled with a solution containing (in mM) 153 CsCl, 1 $MgCl_2$, 5 EGTA, 10 HEPES-CsOH, pH 7.3, and 290 mOsm. For whole-cell recordings, the pipettes were used without being coated or fire-polished and were filled with a solution composed of (in mM) 145 CsCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 5 HEPES, 5 ATP, 5 phosphocreatine, pH 7.2, and 290 mOsm. Both whole-cell and single-channel currents were recorded using an L/M EPC-7 patch-clamp amplifier (Medical Systems, Greenvale, NY) at different gains: 5 mV/pA for whole-cell and 200 mV/pA for single-channel recordings. Series resistance was compensated for >70% in whole-cell recordings. Current signals were filtered at 10 KHz and stored on videocassettes via a videocassette recorder (VCR) and a VR-100 digital recorder (Instrutech) for later off-line analysis. Whole-cell currents also were recorded simultaneously on a pen recorder (Gould, Glen Burnie, MD). Total membrane capacitance was determined by integrating capacity transients evoked by 10 mV, 15 msec hyperpolarizing pulses (from a holding potential of −80 mV), which were recorded immediately after entering the whole-cell configuration. Input resistance was determined from the steady-state current recorded after the capacitive transients had settled. Single-channel currents were played back from the VCR system as analog signals, filtered at 2 KHz, digitized with a Labmaster-TL-1 DMA interface (Axon Instruments, Burlingame, CA), and then sampled (10 KHz) and analyzed with Pclamp V.6.02 program (Axon Instruments) on a 486 personal computer. Openings and closings of the channels were detected by applying a 50% threshold criterion (Colquhoun and Sigworth, 1983). Openings and closings were considered valid only if their durations were greater than twice the rise time of the system. GABA-activated Cl^- channels exhibit multiple current levels (Bormann et al., 1987; Smith et al., 1989). In the present study, only the dominant openings to the 30 pS conductance level were analyzed. Records that contained infrequent double openings (<5% of open events) were used for kinetic analysis. All recordings were carried out at room temperature (22–25°C) on a Nikon inverted microscope. Recorded cells and cell-free membrane patches were superfused continuously with a perfusion system composed of a locally made perfusion controller and miniature electric solenoid valves (The Lee Company, Essex, CT) that allows fast switching (<200 msec complete solution exchange time) among different solutions. Nine inputs converge into a common channel positioned 100–350 μm away from the recorded cell. The perfusion rate (~0.3–0.5 ml/min) was controlled by the air pressure applied to the solution reservoir.

Calcium imaging. Astrocytes were loaded with the calcium indicator dye Fura-2 by exposure to 4 μM Fura-2 AM (Molecular Probes, Eugene, OR) in standard bath solution (in mM: 145 NaCl, 10 HEPES, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgCl_2$, titrated to pH 7.4 with NaOH, and osmolarity adjusted to 330 mOsm with sucrose) for 30 min at 37°C, washed, and then maintained at 37°C for 45 min for ester hydrolysis. Fluorescence microscopy at room temperature (22–24°C) was used for measuring the fluorescence of a chosen field of cells (at 40×, using a Zeiss inverted microscope) with an Attofluor RatioVision digital imaging system (Atto Instruments, Bethesda, MD, and Carl Zeiss, Thornwood, NY). Images using excitation wavelengths of 340 and 380 nm were captured and stored every 2 sec. The ratio of fluorescence at the two exciting wavelengths was calculated for each pixel within a cell boundary to index free intracellular Ca^{2+} levels.

Statistical tests. Two-tailed *t* tests for paired and unpaired data were used to assess significance. Differences were considered significant if $p < 0.05$ (indicated by *) or $p < 0.01$ (indicated by **).

RESULTS

Astrocytes modulate progressive changes in steady-state membrane properties of hippocampal neurons

Embryonic rat hippocampal neurons plated on both PDL and confluent cortical astrocytes survived and differentiated into visible networks during the experimental period (Fig. 1). Some neurons retained remnants of processes after papain digestion as shown in cultures of 2 hr (Fig. 1, *left*). These process-bearing neurons were selected for electrophysiological study. During the 4 d period in culture, noticeable differences in the apparent

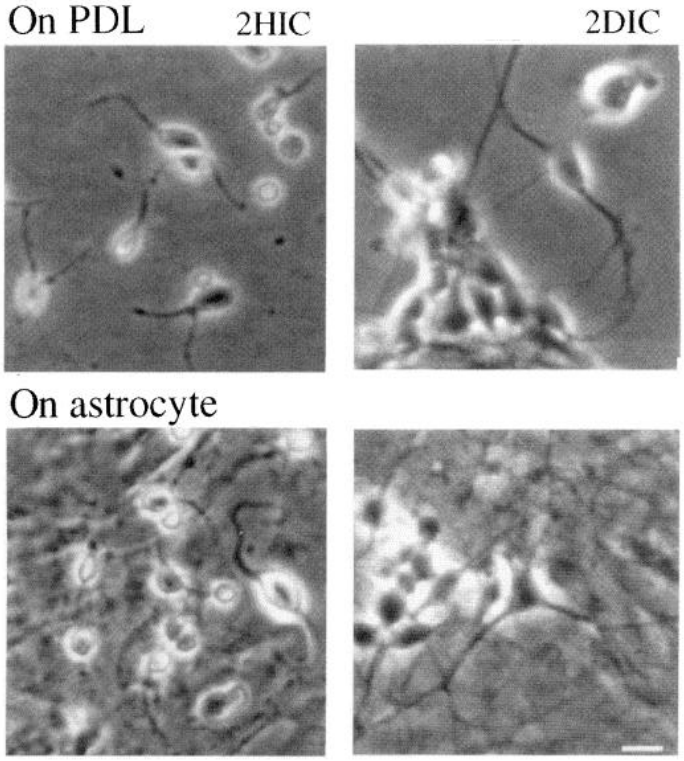

Figure 1. Neurons differentiate in a visibly similar manner on PDL and astrocytes. Phase-contrast micrographs illustrate morphologies typical of embryonic rat hippocampal neurons growing on either poly-D-lysine (*On PDL*) at 2 hr in culture (*2HIC*) or 2 d in culture (*2DIC*) or on a monolayer of confluent cortical astrocytes (*On astrocyte*). The fields show that cells survive and differentiate complex morphologies on both PDL and astrocytes with few, if any, obvious differences in their complexities. Scale bar, 20 μm.

diameters of cell bodies or the extent and numbers of associated processes were not detected using light microscopy. Hence, neurons survived as well on PDL as on astrocytes. After 2–3 d, stellate and epithelioid $GFAP^+$ astrocytes began to appear in the culture of cells growing on PDL (not shown), because the plated population was heterogenous. This limited our investigation of the neuronal properties altered by confluent cortical astrocytes with those persisting in the neuronal population grown on PDL in the presence of progenitor cells and immature, nonconfluent hippocampal astrocytes.

Resting membrane properties recorded at −80 mV in the whole-cell mode including membrane capacitance (C_m), input resistance (R_{in}), and specific conductance were quantified in all neurons tested for GABA-evoked current responses to investigate possible astrocyte-derived contributions to steady-state membrane properties. C_m and R_{in} values were 8.5 ± 0.6 pF and 4.2 ± 0.6 gW ($n = 7$), respectively, after 0.5 HIC for neurons grown on PDL, and 8.4 ± 1.7 pF and 4.9 ± 0.8 gW ($n = 7$) in neurons cultured on astrocytes (Fig. 2*A,B*). There were no significant differences in the C_m and R_{in} values between the two groups of neurons ($p > 0.05$).

After 1 d in culture (DIC), C_m increased and R_{in} decreased significantly compared with 2 HIC in neurons grown on both astrocytes ($p < 0.01$) and PDL ($p < 0.01$). Furthermore, differences between neurons grown on PDL and on astrocytes in C_m and R_{in} values recorded at 1 DIC were statistically significant ($p < 0.01$ for both parameters). C_m continued to increase in both groups of neurons, but values recorded in neurons on astrocytes

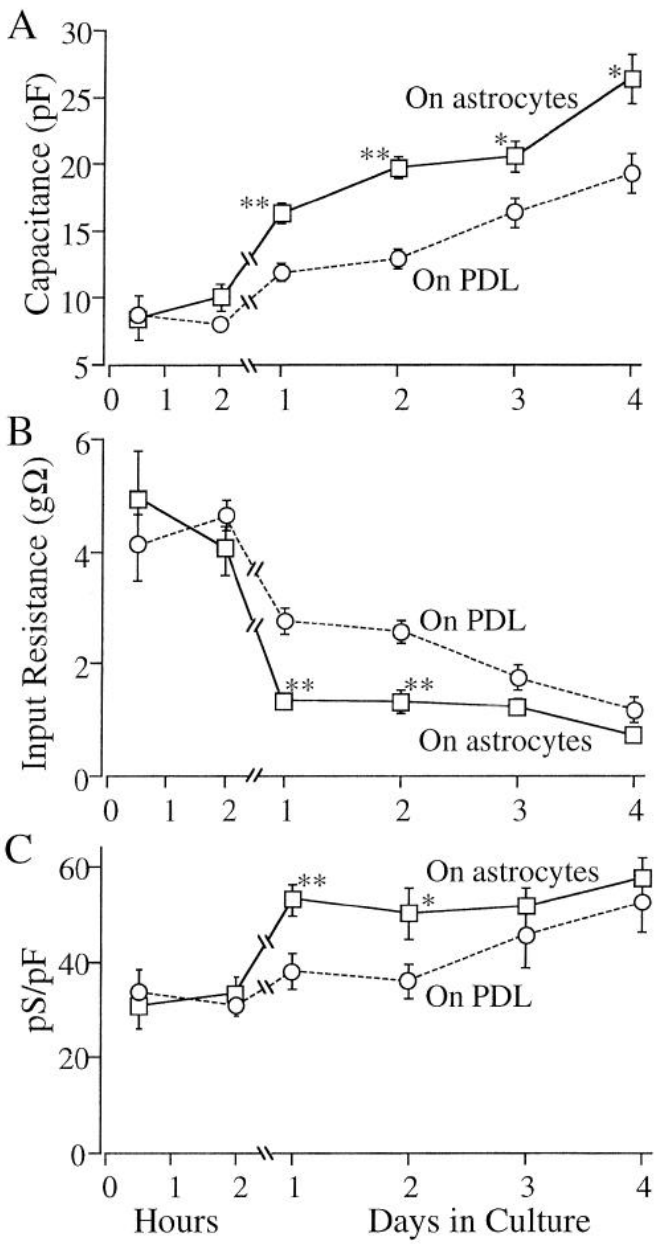

Figure 2. Steady-state electrical properties of neurons grown on PDL and astrocytes differ significantly at 1 DIC. Whole-cell recordings were used to clamp neurons at −80 mV and quantify their steady-state properties. There are no significant differences in whole-cell membrane capacitance (C_m; *A*), input resistance (R_{in}; *B*), or specific membrane conductance (*pS/pF*; *C*) between neurons grown on PDL and those on astrocytes during the first 2 hr in culture. C_m increases with time in both groups of neurons, but the change recorded at 1 DIC in neurons on astrocytes is significantly greater than that recorded in neurons on PDL. R_{in} decreases in both groups, but the change recorded on neurons on astrocytes is greater than that on PDL, so that R_{in} is significantly lower in neurons on astrocytes at 1 DIC. The reciprocal of R_{in} was used to quantify resting membrane conductance (pS), and then C_m was factored in as an index of surface area to generate specific membrane conductance (pS/pF). Although pS/pF increases in both groups, the change is greater in neurons on astrocytes, which makes values significantly different at 1 and 2 DIC. Data are mean ± SEM of 7–48 cells. $^*p < 0.05$ compared with that on PDL and $^{**}p < 0.01$.

remained significantly higher during the 4 d study (Fig. 2*A*). The differences between the two groups of neurons in R_{in} remained significant at 2 DIC but became insignificant thereafter (Fig. 2*B*). Specific steady-state membrane conductances were both ~30

pS/pF during the initial 0.5–2 HIC. At 1 DIC, specific membrane conductance had increased in neurons on astrocytes to ~50 pS/pF, which was significantly different from corresponding values recorded in neurons on PDL that had increased modestly (Fig. 2C). Thus, the decrease in R_{in} recorded at 1–2 DIC, which was significantly different between the two experimental groups, was independent of progressive increases in C_m. After 2 DIC, the difference in specific conductance, like that in R_{in}, became statistically insignificant.

Astrocytes increase the amplitude and density of GABA-activated Cl^- current

GABA activated I_{GABA} responses in all tested neurons cultured from 0.5 hr to 4 d on either PDL (n = 167) or astrocytes (n = 182) and recorded in Cl^--loaded cells at a holding potential of −80 mV. Peak current amplitudes evoked by brief applications of GABA in neurons cultured for 0.5 hr on astrocytes (1331.7 ± 287.3 pA; n = 7) were already ~20% greater than those cultured on PDL (1088.6 ± 119.6; n = 7), but this difference was not statistically significant ($p > 0.05$; Fig. 3A). After just 2 HIC, however, I_{GABA} had increased to an average of 1736.8 ± 153.2 pA in neurons on astrocytes (n = 20; $p > 0.05$), whereas I_{GABA} recorded in neurons on PDL had decreased to 839.9 ± 87.5 pA (n = 27; $p > 0.05$). This difference between mean values of I_{GABA} was highly significant ($p < 0.01$). During the following 2 d (1 and 2 DIC), there were few or modest changes in the average amplitudes of I_{GABA} recorded in neurons on PDL, and they were not different significantly from I_{GABA} recorded initially ($p > 0.05$ compared with that at 0.5 HIC). In marked contrast, I_{GABA} recorded in neurons on astrocytes became significantly greater at 1 and 2 DIC when compared with that at 0.5 HIC: 2219.6 ± 95.5 pA (n = 48) and 3177.5 ± 322.1 pA (n = 19), respectively ($p < 0.01$ compared with that at 0.5 HIC). At 1 and 2 DIC, the differences in average amplitudes of I_{GABA} recorded in the two experimental conditions were highly significant ($p < 0.01$ for both days). This difference was maintained throughout the whole period of observation (Fig. 3A).

To account for differences in current amplitude that could be correlated with growth in membrane surface area, specific current densities (I_{GABA} divided by whole-cell membrane capacitance as an index of membrane surface area) were calculated and compared (Fig. 3B). The average current density recorded at 0.5 HIC in neurons on astrocytes was ~20% greater (157.8 ± 11.7 pA/pF; n = 7) than that in neurons on PDL (131.1 ± 16.2 pA/pF; n = 7); however, this difference was not statistically significant ($p > 0.05$). The average current density did not change significantly during the 4 d period in neurons cultured on astrocytes, because both C_m and I_{GABA} increased in parallel. The current density decreased in neurons grown on PDL during the first 2 hr, continued to decrease during the first 2 d, and then increased slowly thereafter, yet it never reached the level recorded at 0.5 HIC. The rapid and lasting decrease in the density of I_{GABA} in those neurons led to statistically significant differences between the two groups beginning at 2 HIC. This was sustained for the experimental period.

Astrocyte modulation involves direct contact at the cell body level

In some dishes with a layer of confluent astrocytes, there were regions devoid of astrocyte covering (Fig. 4A). We took advantage of this to compare I_{GABA} in neurons whose cell bodies were growing on or off astrocytes in the same dishes to test whether the astrocyte effects required direct contact (Fig. 4B1,B2). In many

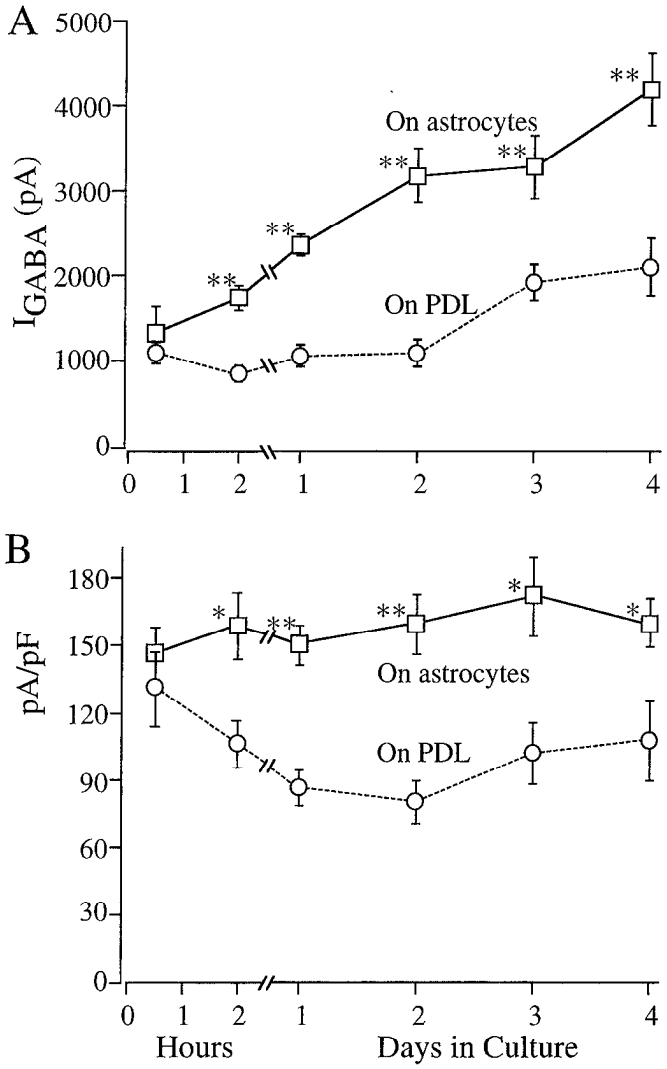

Figure 3. Astrocytes facilitate I_{GABA}. Cells were clamped at −80 mV, and currents activated by brief (1–2 sec) pulses of 10 μM GABA were recorded at short- and long-term periods of culture. *A*, In neurons grown on PDL, I_{GABA} at 2 DIC is virtually identical to that recorded at 0.5 HIC ($p > 0.05$). In neurons on astrocytes, I_{GABA} at 2 DIC is more than two times greater than that recorded at 0.5 HIC ($p < 0.01$). A significant difference in I_{GABA} between the two groups appears by 2 HIC ($p < 0.01$), which is maintained throughout the experiment. *B*, The density of I_{GABA} (I_{GABA} normalized to C_m values plotted in Fig. 2) is also significantly greater in neurons grown on astrocytes beginning at 2 HIC, and this is sustained for the duration of the study ($p < 0.05$ at 2 HIC and 3 and 4 DIC and $p < 0.01$ at 1 and 2 DIC). Data shown are mean ± SEM of 7–48 cells.

cells, the "off-astrocyte" neurons contacted astrocytes through processes. In nine neurons whose cell bodies were growing on astrocytes for 2 d, I_{GABA} averaged 2150.9 ± 255.7 pA, significantly greater than that recorded in neurons in the same dishes whose cell bodies were growing off astrocytes (992.1 ± 164.1 pA; n = 8; $p < 0.05$; Fig. 4B,C). Furthermore, I_{GABA} in off-astrocytes neurons was not significantly different from that in neurons cultured on PDL ($p > 0.05$). The density of I_{GABA} in these off-astrocyte neurons was also significantly less than that in on-astrocyte neurons (92.4 ± 15.8 pA/pF and 157.6 ± 19.1 pA/pF, respectively; $p < 0.05$) (Fig. 4D). The results demonstrate that direct contact of the neuronal cell body with astrocytes is a prerequisite for the modulatory effects of the latter on I_{GABA}.

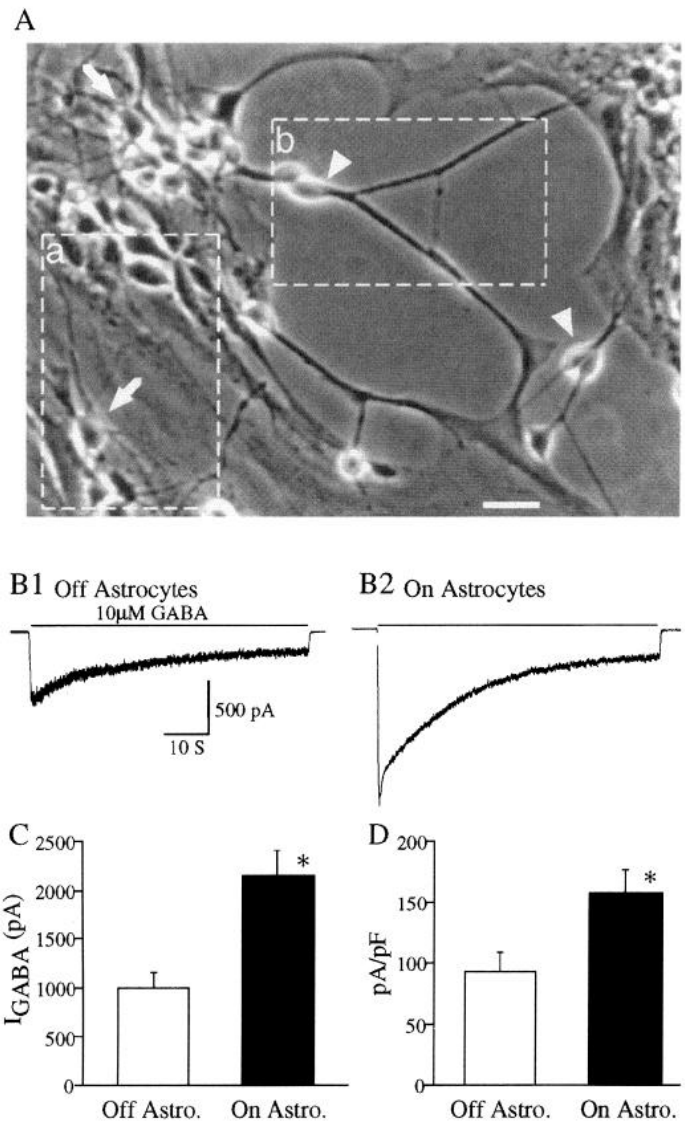

Figure 4. I_{GABA} is greater in neurons whose cell bodies contact astrocytes. GABA-induced currents were recorded in neurones voltage-clamped at −80 mV at 2 DIC. *A*, The phase-contrast micrograph shows neuronal cell bodies growing on (*arrows*) and off (*arrowheads*) astrocytes in the same field. *Boxed areas* outline a monolayer of confluent astrocytes (*a*) and a region devoid of them (*b*). I_{GABA} (*B1*, *B2*, *C*) and I_{GABA} normalized to C_m (*D*) are significantly greater in neurons grown on astrocytes ($n = 9$) than corresponding values recorded in neurons off astrocytes ($n = 8$). Scale bar, 40 μm.

BAPTA-loaded astrocytes do not modulate I_{GABA}

Spontaneous changes in Ca_c^{2+} levels in cultured astrocytes have been reported in various studies (Cornell-Bell and Finkbeiner, 1991; Fatatis and Russell, 1992), and BAPTA-AM loaded intracellularly has been used to suppress spontaneous and evoked changes in Ca_c^{2+} elevations in astrocytes and in many other cell types (Martin et al., 1992; Ballerini et al., 1993; Koyama et al., 1993; Furuya et al., 1994; Gu and Spitzer, 1995). To test whether astrocytes have spontaneous Ca^{2+} changes in our culture conditions and whether the modulatory effects of astrocytes on I_{GABA} involve elevations in astrocyte Ca_c^{2+}, we compared astrocytes unloaded and preloaded with BAPTA-AM in terms of intracellular Ca^{2+} signals and their effects on neuronal I_{GABA}. In 25 unloaded astrocytes, 15 exhibited spontaneous intracellular Ca^{2+} elevations during a 600 sec recording period (Fig. 5, *left*). When loaded with BAPTA-AM and examined for Ca^{2+} signals immediately thereafter, no astrocyte showed spontaneous Ca_c^{2+} elevations (data not shown). Furthermore, only 4 of 28 astrocytes that were exposed to BAPTA-AM for 30 min and then washed and cultured for 20 hr showed spontaneous intracellular Ca^{2+} elevations (Fig. 5, *right*). Hence, there were clear and persistent differences between the two groups of astrocytes in terms of spontaneous Ca_c^{2+} signals. The frequency of spontaneous Ca_c^{2+} signals was the same or higher in unloaded astrocytes (ranging from one to seven fluctuations) than in BAPTA-AM-treated astrocytes (one elevation in each of four astrocytes). I_{GABA} and its density in neurons grown on BAPTA-treated astrocytes for 20 hr (1000.5 ± 185.8 pA and 79.2 ± 7.2 pA/pF; $n = 11$) were both significantly less than corresponding values recorded in neurons on untreated astrocytes (1909.6 ± 84.6 pA and 118.5 ± 7.9 pA/pF; $n = 14$; $p < 0.01$) (Fig. 6*A*,*C*). These results suggest that spontaneous Ca_c^{2+} spikes and/or waves are important for the direct contact observed effects of astrocytes on neuronal $GABA_A$ receptor/Cl^- channels. Neurons grown on BAPTA-treated astrocytes also had lower C_m on average (13.6 ± 1.4 pF) compared with that recorded in neurons grown on unloaded astrocytes (17.0 ± 0.8 pF; $p < 0.05$) (Fig. 6*B*). No significant differences were detected between either R_{in} or specific steady-state conductance measured in the two groups. The effects of BAPTA-AM are unlikely to be attributable to generation of toxic metabolites, because treatment with calcein-AM that does not respond to Ca^{2+} did not produce similar effects.

Pharmacological properties of I_{GABA} are identical in neurons cultured on PDL and astrocytes

I_{GABA} was highly sensitive to bicuculline (Fig. 7*A*) and picrotoxin (data not shown), antagonists at $GABA_A$ receptor/Cl^- channels, in both groups of neurons. Ten micromolar bicuculline blocked I_{GABA} by 95.3% (from 1.36 ± 0.26 nA to 0.06 ± 0.01 nA; $n = 6$; $p < 0.01$) in neurons on PDL and by 96.4% (from 2.75 ± 0.37 to 0.10 ± 0.03 nA; $n = 7$; $p < 0.01$) in neurons on astrocytes. Fifty micromolar bicuculline blocked GABA-induced current responses recorded in neurons on both PDL ($n = 6$) and astrocytes ($n = 7$) in a virtually complete manner. The results indicate that all of the I_{GABA} involves bicuculline-sensitive currents characteristic of $GABA_A$-type receptors.

Current responses could be detected consistently with brief pulses of 1–3 μM GABA in neurons grown on either PDL ($n = 7$) or astrocytes ($n = 6$) for 2 d. I_{GABA} increased in a sigmoidal fashion with GABA concentration. When experimental values in the two groups were normalized to the maximum response, the two curves virtually superimposed and were well fitted with the continuous theoretical curves calculated from the following equation:

$$I = I_{max} * ([GABA]^n / ([GABA]^n + K_d^n))$$

where I is the amplitude of I_{GABA}, I_{max} is the maximum current, [GABA] is the concentration of GABA, K_d is the dissociation constant of GABA with its receptors, and n is the Hill coefficient (Fig. 7*B*). The Hill coefficients and K_ds were virtually identical for neurons grown on PDL (1.65 and 9.9 μM) and on astrocytes (1.56 and 9.1 μM). Thus, at the macroscopic level, I_{GABA} recorded in the two sets of neurons exhibited indistinguishable [GABA]-dependent properties.

I_{GABA} evoked in neurons cultured on PDL and astrocytes were all assumed to be Cl^--dependent because they reversed polarity at ~0 mV, the equilibrium potential for Cl^- under these recording

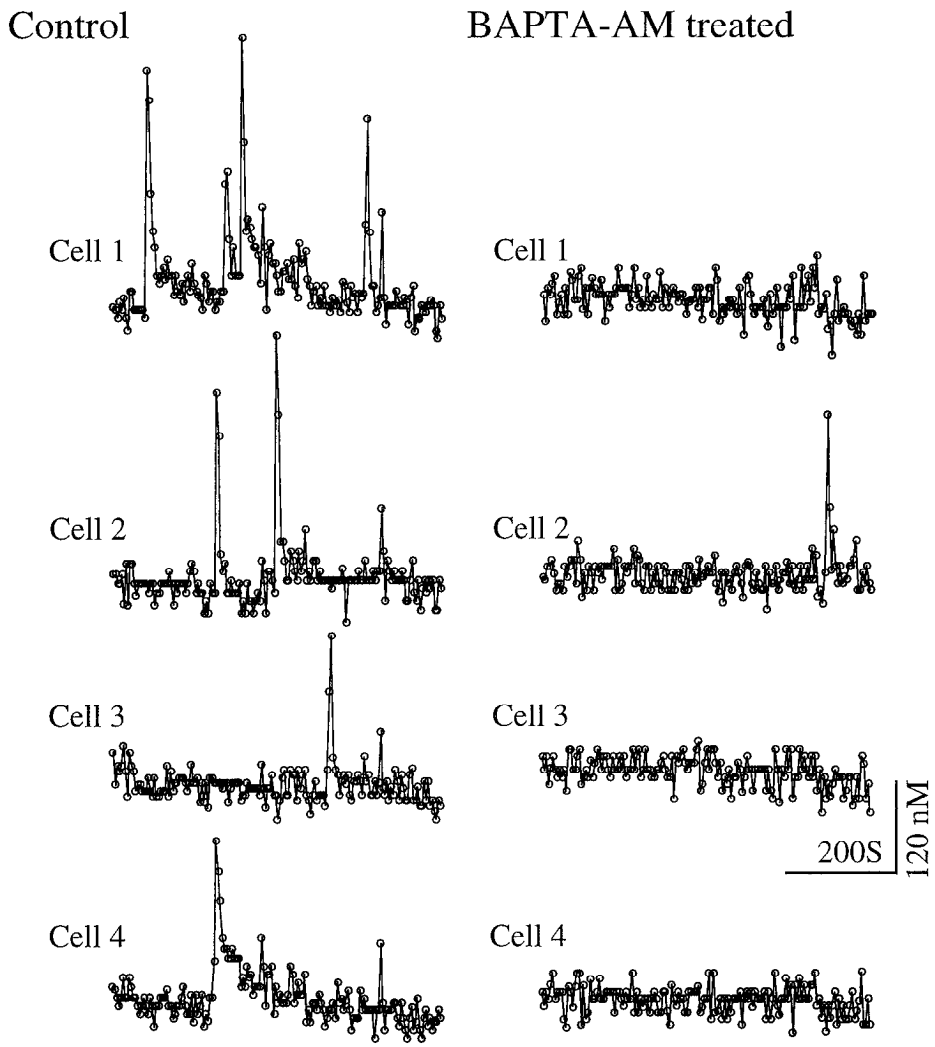

Figure 5. Brief exposure to BAPTA-AM persistently suppresses spontaneous Ca_c^{2+} transients in astrocytes. Astrocytes were cultured on glass-bottomed culture dishes and loaded with Fura-2. Some astrocytes were exposed to BAPTA-AM for 30 min and then washed and cultured for an additional 20 hr before being loaded with Fura-2. More than half (15 of 25) of the astrocytes not pretreated with BAPTA-AM showed one to seven spontaneous Ca_c^{2+} transients during the 600 sec recording period at room temperature (22–24°C). Few (4 of 28) astrocytes exposed to BAPTA-AM 20 hr previously exhibited spontaneous Ca_c^{2+} transients, and these were of uniformly low frequency (only one elevation in each of the four astrocytes in 10 min).

conditions (Fig. 7*C*). The slope conductance at the peak of the response (normalized according to whole-cell C_m) was significantly greater in neurons grown on astrocytes (1964.5 ± 202.6 pS/pF; $n = 5$) than in neurons on PDL (1249.4 ± 74.5 pS/pF; $n = 3$; $p < 0.05$).

We studied some of the pharmacological properties of the $GABA_A$ receptor/Cl^- channels expressed at 2 HIC and 24 HIC, using a clinically relevant drug that affects $GABA_A$ receptor/Cl^- channels (diazepam) and a naturally occurring divalent cation (Zn^{2+}). Diazepam increased I_{GABA} by 158.8 ± 28.8% after 2 HIC ($n = 8$; $p < 0.01$) and by 140.9 ± 20.5% after 24 HIC ($n = 7$; $p < 0.01$) in neurons on PDL, and I_{GABA} was increased by 109.9 ± 7.3% after 2 HIC ($n = 5$; $p < 0.01$) and 181.9 ± 61.4% after 24 HIC ($n = 5$; $p < 0.01$) in neurons on astrocytes. The differences in the enhancing effects of diazepam in neurons cultured on PDL and astrocytes were not significant for both 2 HIC and 24 HIC data sets ($p > 0.05$ for both groups).

After 2 HIC, 100 μM Zn^{2+} blocked 25.4 ± 3.2% of the GABA-evoked current in 13 neurons on PDL ($p < 0.01$) and 23.6 ± 3.2% in 12 neurons on astrocytes ($p < 0.01$). The differences in the blocking effects of Zn^{2+} between the two groups of neurons was not significant ($p > 0.05$). After 24 HIC, Zn^{2+} blocked 15.9 ± 2.2% of I_{GABA} in neurons on PDL ($n = 11$; $p < 0.01$) and 36.5 ± 2.9% in neurons on astrocytes ($n = 12$; $p < 0.01$). The difference was significant ($p < 0.01$) between the two groups.

I_{GABA} decay involves both redistribution of Cl^- and conductance decay

The current response to GABA recorded at negative holding potentials decreased with time (Fig. 8*A*) during continuous applications. I_{GABA} recorded in neurons grown on astrocytes decayed faster and to a greater extent than that recorded in neurons grown on PDL (Fig. 8, *inset*). In 15 neurons cultured on astrocytes, I_{GABA} decayed by 87.3 ± 1.1% with a half-decay time ($T½$) of 3.6 ± 0.2 sec, whereas that recorded in 11 neurons grown on PDL decayed by 78.4 ± 2.1% ($p < 0.01$) with a $T½$ of 7.9 ± 1.5 sec ($p < 0.01$) during 1 min GABA applications.

The current decay could be attributable to $GABA_A$ receptor desensitization at the level of the receptor/channel complex (decrease in the activation of receptor-coupled conductance) and/or by Cl^- ion redistribution across the membrane (indicated by a shift in the reversal potential of the current response) (Akaike et al., 1987; Frosch et al., 1992). To test the contributions of conductance decay and Cl^- ion redistribution to the decay of I_{GABA}, fast ramp voltage-commands (1 S, 100 mV/S) were applied every ~10 sec during the 1 min applications of GABA to generate data (Fig. 8*B*, *insets*) on reversal potential (Fig. 8*B,C*) and maximum slope conductance (g_{Cl}) (Fig. 8*D*). g_{Cl} was measured over the positive potential range when Cl^- ions moved into the cell from an infinite reservoir. The reversal potentials of the peak currents

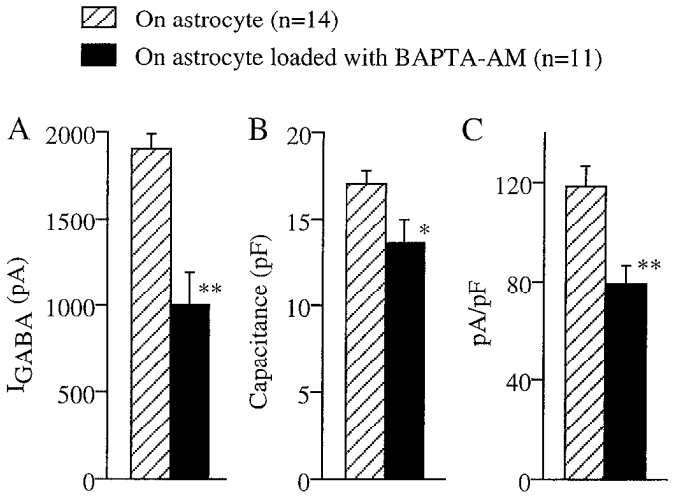

Figure 6. BAPTA-loaded astrocytes are ineffective in promoting I_{GABA}. Neurons were plated on astrocytes that had been incubated previously in culture medium containing 10 μM BAPTA-AM for 30 min at 37°C and then washed twice. Recordings were made 20 hr later and compared with results in neurons cultured on astrocytes that had not been loaded with BAPTA (control). I_{GABA} (*A*), its density (*C*), and its membrane capacitance (*B*) are significantly smaller in neurons cultured on astrocytes treated with BAPTA than values measured in neurons on untreated astrocytes.

recorded during the first second were ~0 mV in neurons grown on both PDL (−0.1 ± 1.5 mV; $n = 6$) and astrocytes (−0.6 ± 0.5 mV; $n = 5$; $p > 0.05$), whereas those measured ~10 sec later had shifted consistently to negative potentials in both groups. The mean values in both groups were fitted adequately with bi-exponential functions with time constants of 8.3 S and 111.3 S in neurons on astrocytes and 3.0 S and 498.1 S in neurons on PDL. The maximal extent in the shift was significantly greater in neurons grown on astrocytes than in neurons on PDL (Fig. 8*B,C*). In six neurons grown on astrocytes, the reversal potential revealed by the ramp command applied just before the end of GABA application averaged −24.6 ± 3.5 mV, whereas that in five neurons grown on PDL was −10.9 ± 1.2 mV ($p < 0.01$). Furthermore, the cumulative shift in reversal potential closely correlated with the peak current amplitude ($p < 0.01$). g_{Cl} in both groups decayed in a bi-exponential manner during the prolonged GABA application (Fig. 8*D*). The mean time constants were 5.1 S and 84.1 S in neurons on astrocytes and 2.1 S and 69.6 S in neurons on PDL. The extent of decrease in g_{Cl} was virtually identical in the two groups: 67.1% in neurons on astrocyte (from 60.5 ± 7.8 nS at the peak current to 19.9 ± 2.5 nS just before the end of the GABA application) and 67.3% in neurons on PDL (from 40.1 ± 2.8 nS to 13.1 ± 1.9 nS) ($p > 0.05$). The results indicate that the faster and more complete decay of I_{GABA} in neurons grown on astrocytes reflects a greater degree of rapid Cl^- ion redistribution rather than a faster rate of desensitization.

Unitary properties of GABA-activated Cl^- channels do not differ

The enhancement of GABA-activated Cl^- current by astrocytes may involve detectable changes in the biophysical properties of $GABA_A$ receptor/Cl^- channels. To investigate these possibilities further, Cl^- channels activated by GABA were recorded in outside-out patches obtained from neurons cultured for 2 d on PDL or astrocytes. Application of 3 μM GABA evoked transitions in microscopic currents, which occurred as discrete single openings or interrupted bursts (Fig. 9*A*) and were highly sensitive to bicuculline (not shown). Only the main conductance (~30 pS) was studied further. The reversal potentials of single-channel currents were ~0 mV, the equilibrium potential for Cl^- under these recording conditions (data not shown). In four patches in neurons on PDL, the mean value of open channel conductance was estimated to be 29.4 ± 0.9 pS, which was not significantly different from that derived from three patches in neurons on astrocytes (29.9 ± 1.8 pS; $p > 0.05$). These values are similar to those reported previously for GABA-activated channel openings recorded from cultured embryonic hippocampal, spinal cord, and cortical cells (Ozawa and Yuzaki, 1984; Bormann et al., 1987; Smith et al., 1989; Orser et al., 1994).

The open-time histograms were fitted adequately with bi-exponential functions having time constants of 0.58 ± 0.08 and 5.37 ± 0.62 msec in neurons on PDL ($n = 4$) and 0.64 ± 0.13 and 6.04 ± 0.10 msec in neurons on astrocytes ($n = 3$; $p > 0.05$ for both time constants) (Fig. 9*B*). The mean open times were 3.05 ± 0.41 msec in neurons on PDL and 3.35 ± 0.46 msec in neurons on astrocytes ($p > 0.05$). These values are similar to mean open-time durations reported previously for GABA-activated Cl^- channels (Mienville and Vicini, 1989; Ma et al., 1994; Orser et al., 1994). The closed-time histograms were fitted adequately with the sum of three exponential functions (Fig. 9*C*). The closed-time constants were 1.71 ± 0.11, 8.83 ± 1.55, and 48.63 ± 10.34 msec, and the mean closed time was 24.9 ± 8.9 msec in neurons on PDL, which were not significantly different from those in neurons on astrocytes (2.42 ± 0.58, 9.15 ± 2.9, and 58.46 ± 8.53 msec in time constants and 23.6 ± 13.7 msec in mean closed time; $p > 0.05$). Furthermore, the open probabilities were also not significantly different between neurons on PDL (0.08 ± 0.02) and astrocytes (0.12 ± 0.04; $p > 0.05$).

DISCUSSION

Differences in I_{GABA} in neurons cultured on astrocytes and PDL occur before changes in cell capacitance. The results show that embryonic rat hippocampal neurons cultured for hours/days on a monolayer of confluent cortical astrocytes express I_{GABA} responses that are consistently greater in amplitude during brief exposure and faster in decay during sustained application than those recorded in neurons cultured in the same medium under identical conditions on PDL. A statistically significant difference in I_{GABA} was recorded as early as 2 HIC, before differences in C_m became significant. Direct contact of neuronal cell bodies with astrocytes was essential for a difference in I_{GABA} because "off-astrocyte" neurons recorded in the same fields exhibited significantly smaller I_{GABA}, even though they visibly contacted astrocytes via their processes (Fig. 4). I_{GABA} normalized to C_m, which may be an index of the specific density of $GABA_A$ receptor/Cl^- channels, and remained relatively stable (~150 pA/pF) in neurons cultured on astrocytes, but decreased significantly during the first 2 d in culture in neurons on PDL and never recovered to its original value recorded at 0.5 HIC (Fig. 3). The delayed increase in GABA-activated Cl^- current density in PDL dishes coincided with the gradual appearance of $GFAP^+$ astrocytes (not shown).

Differences in I_{GABA} cannot be accounted for by obvious differences in pharmacological and biophysical properties

Structure–function studies of $GABA_A$ receptor/Cl^- channels composed of different subunit proteins expressed in recombinant

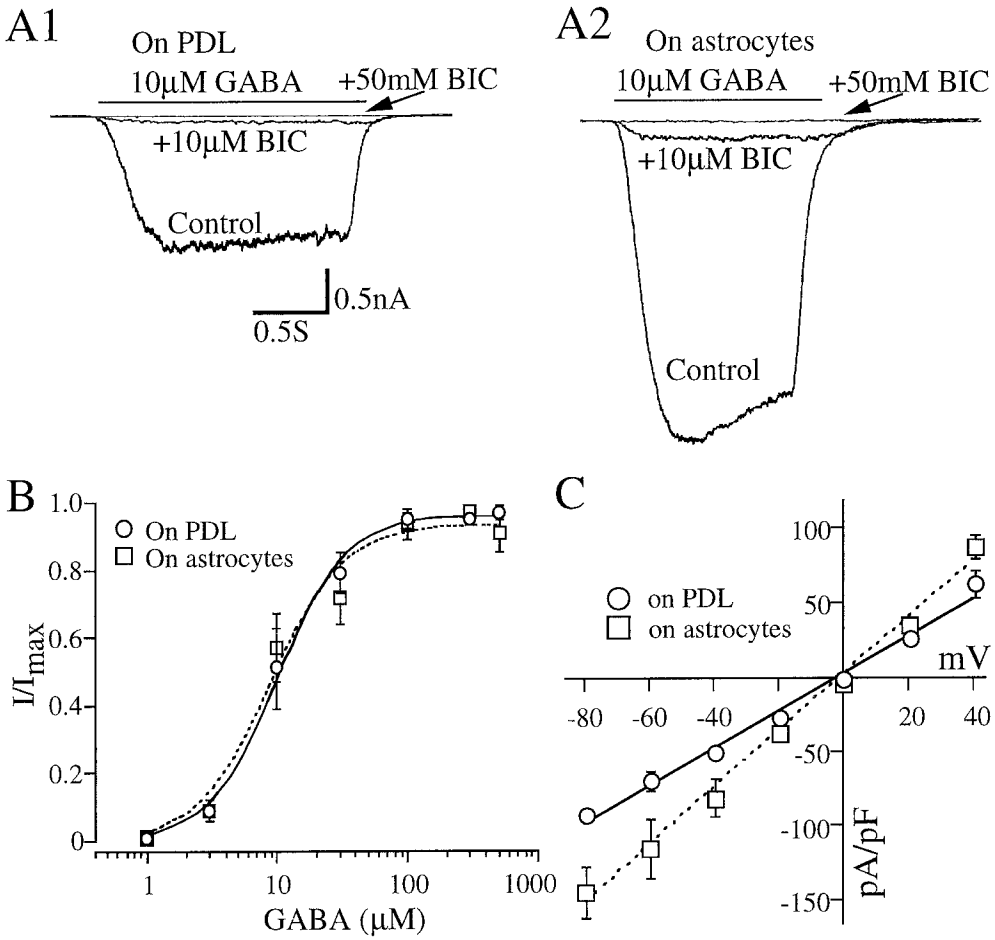

Figure 7. I_{GABA} in neurons on both astrocytes and PDL is primarily $GABA_A$ current. Neurons were cultured for 2 d. *A*, Bicuculline completely blocks I_{GABA} in all neurons. Ten micromolar bicuculline (*BIC*) blocks >90% of I_{GABA} in both sets of neurons, whereas 50 μM bicuculline blocks I_{GABA} almost completely in both groups (*A1*, *A2*). Recordings were made at −80 mV. *B*, Normalized dose–response curves of I_{GABA} recorded in the two groups of neurons superimpose. I_{GABA} was recorded at −80 mV at different GABA concentrations by brief (~1 sec) applications of 1–500 μM GABA at 2 min intervals. The peak current amplitude evoked at each concentration was normalized to the maximal response recorded in each cell, and the pooled results were plotted as a function of GABA concentration. *C*, I_{GABA} reverses polarity at ~0 mV, the equilibrium potential for Cl^- in these recording conditions, for both sets of neurons. When the current is normalized with respect to membrane capacitance and then plotted against membrane potential, the normalized slope conductance (conductance per unit whole-cell capacitance) is significantly greater for neurons grown on astrocytes. Neurons were cultured for 2 d. Each point in *B* and *C* is the mean ± SEM.

systems have revealed variabilities in biophysical and pharmacological properties associated with different subunits (Burt and Kamatchi, 1991; Burt, 1994). For example, γ_2-subunits influence the enhancement by benzodiazepines (Puia et al., 1991) and depression by Zn^{2+} (Smart et al., 1991); single-channel conductance and kinetics differ with different subunit combinations (Verdoorn et al., 1990; Angelotti and Macdonald, 1993). Although definitive experiments involving *in situ* hybridization and immunocytochemical detection of different subunits have not been performed in the present set of experiments, there were few differences in the pharmacological and biophysical properties of I_{GABA} recorded in neurons on PDL and astrocytes. They were enhanced by diazepam and blocked by bicuculline. Dose–response curves in the two groups virtually superimposed when normalized to the maximum response (Fig. 7*B*), indicating similar if not identical affinity constants and Hill coefficients. The single-channel conductance and kinetics were also similar if not identical in membrane patches obtained from cell bodies (Figs. 9). I_{GABA} in neurons on astrocytes was significantly more Zn^{2+}-sensitive at 1 DIC. Thus, the differences in properties recorded in the two groups of neurons were subtle and modest at best, and at present do not explain the approximately twofold difference in I_{GABA}. Because the elementary properties are similar, if not identical, we infer that their subunit composition is similar. It is possible that more $GABA_A$ receptor/Cl^- channels with identical properties are present at the neuronal cell surface in neurons in contact with astrocytes.

Cl^- ion redistribution rather than desensitization explains the difference in I_{GABA} decay

An important phenomenon, which seems to be intrinsic to functional combinations of $GABA_A$ receptor subunits, is marked decay in response to prolonged application of GABA that recovers over a few minutes (Numann and Wong, 1984; Thalmann and Hershkowitz, 1985; Akaike et al., 1987; Oh and Dichter, 1992; Celentano and Wong, 1994). In most cases, this decay is the mixed result of desensitization of $GABA_A$ receptor and a decrease in the transmembrane Cl^- ion gradient, because of Cl^- redistribution (Huguenard and Alger, 1986; Akaike et al., 1987; Frosch et al., 1992). It is not yet clear how E_{Cl} could change so dramatically during a prolonged exposure to GABA when Cl^- ions are present in a virtually infinite supply in both the patch pipette and extracellular solutions. The dramatic, hyperpolarizing shift of E_{Cl} at negative holding potentials (as in our case) could be attributable to local depletion of intracellular Cl^- and/or local accumulation of extracellular Cl^- in the immediate vicinity of the orifices of $GABA_A$ receptor/channels. Perhaps unstirred layers adjacent to the intracellular and extracellular openings of the channel transiently form discrete compartments with E_{Cl} then being determined by the Cl^- concentrations in these compartments. Cl^- in these undisturbed compartments may have a limited exchange rate with extracellular or cytosolic and, by inference, pipette Cl^- so that during prolonged activation of $GABA_A$ receptor/channels, Cl^- concentrations change locally, resulting in the observed shift in E_{Cl}.

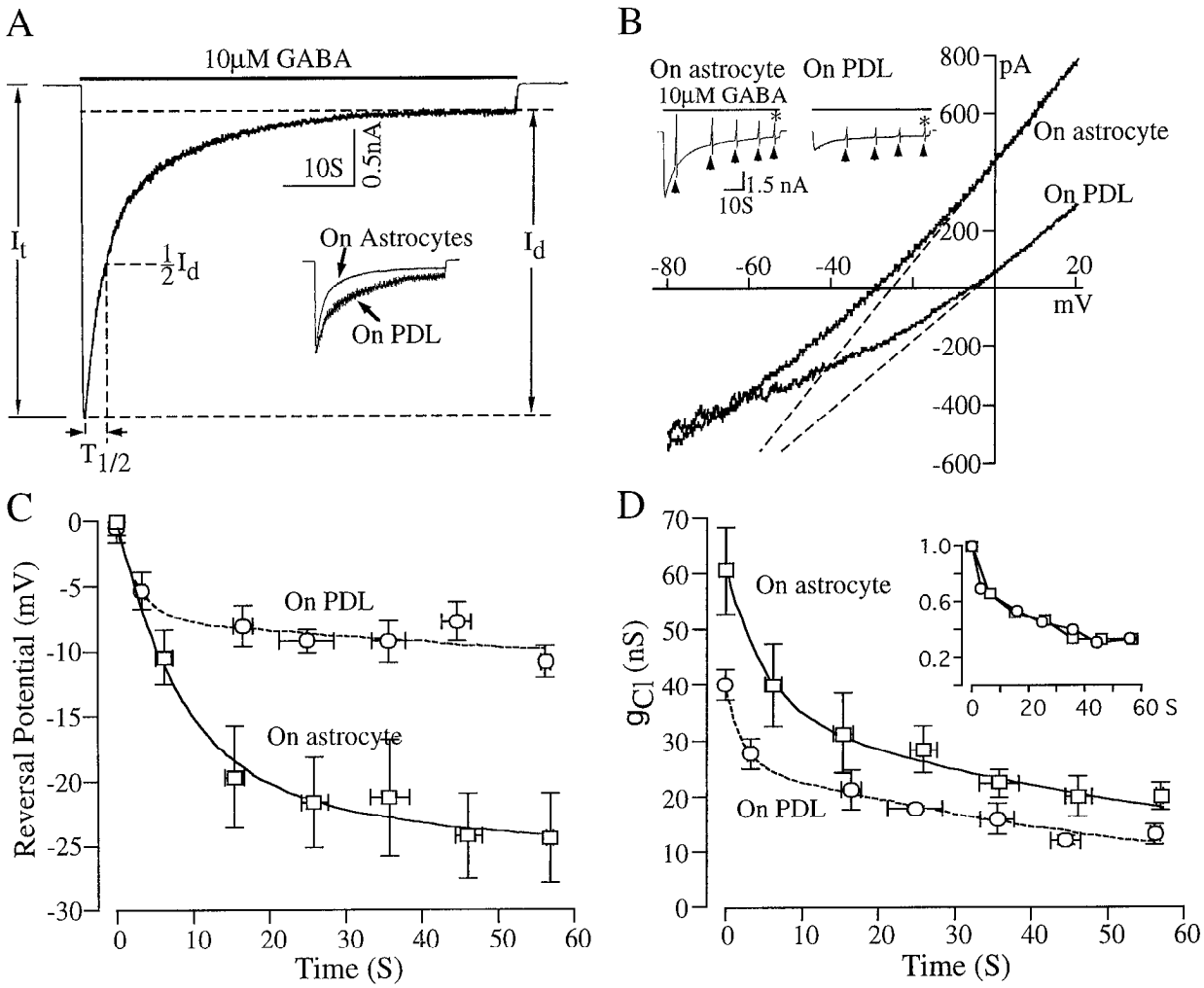

Figure 8. Both desensitization and redistribution of Cl^- contribute to I_{GABA} decay in neurons grown on astrocytes and PDL. Neurons cultured for 1 d were voltage-clamped at −80 mV and exposed to 10 μM GABA for 1 min. *A*, I_{GABA} in a neuron on astrocytes decayed to a fraction of its initial value within 1 min. *Inset* compares two current traces recorded in neurons cultured on astrocytes and PDL, respectively, and normalized to peak values to reveal the difference in their time course of decay. I_{GABA} recorded in neurons on astrocytes decays more completely and rapidly. *B*, Ramp commands of 1 sec were applied at different times during the current response to GABA to monitor the slope conductance and the potential at which the current reverses. The continuous current traces during the last ramp (indicated by * in *insets*) have been plotted against membrane potential. *Dashed lines* are fits of the linear parts of the currents and indicate maximal slope conductance. The reversal potential of the GABA-induced current response in the neuron on astrocytes at the end of the response is ~−28 mV, whereas that in the neuron on PDL is ~−6 mV. *Insets* show I_{GABA} induced by 10 μM GABA in neurons on astrocytes and PDL. *C*, Reversal potentials measured at different times have been plotted to show the time course of the shift in reversal potential during the decay of I_{GABA}. The shift is greater in neurons on astrocytes than on PDL. The mean values in both groups were fitted adequately with a bi-exponential function (shown as a *dashed line* for neurons on PDL, $r = 0.970$, and as a *solid line* for neurons on astrocytes, $r = 0.995$). *D*, g_{Cl} (derived from measurements of maximum slope) decreases in parallel and to a similar degree in the two groups of neurons. The mean values were fitted with bi-exponential functions (*dashed line* for neurons on PDL, $r = 0.995$, and *solid line* for neurons on astrocytes, $r = 0.993$). *Insets* show that normalized g_{Cl} decay in the two groups of neurons superimpose.

The molecular mechanisms of desensitization of $GABA_A$ receptors also are not entirely clear, but seem to be influenced by $GABA_A$ receptor phosphorylation state (Moss et al., 1992) and subunit composition (Verdoorn et al., 1990; Moss et al., 1992). The greater hyperpolarizing shift in the reversal potential of I_{GABA} in neurons on astrocytes (Fig. 8*B,C*), coupled with the fact that GABA-activated maximal slope conductance decays to the same extent and in a parallel manner in both groups (Fig. 8*D*), indicates that the faster, more complete degree of I_{GABA} decay in neurons on astrocytes is the result of faster and more complete Cl^- redistribution. The statistically significant correlation between the extent of I_{GABA} decay and the initial peak amplitude is consistent with the hypothesis that GABA rapidly moves the intracellular Cl^- ions out of the cell, thus depleting Cl^- ions in the immediate vicinity of $GABA_A$ receptor/channels and shifting the reversal potential of I_{GABA} in a hyperpolarizing direction.

Astrocyte modulation of I_{GABA} occurs in parallel with increases in C_m

It is well known that astrocytes support neuronal survival and neurite extension (Lindsay, 1979; Noble et al., 1984; Fallon, 1985; Manthorpe et al., 1986; Alliot et al., 1988; Le Roux and Reh, 1994). After 1 DIC, neurons on astrocytes exhibited measurably more C_m than those on PDL, although in both groups of neurons C_m increased significantly when compared with initial values (Fig. 2*A*). One explanation for larger-amplitude I_{GABA} recorded in

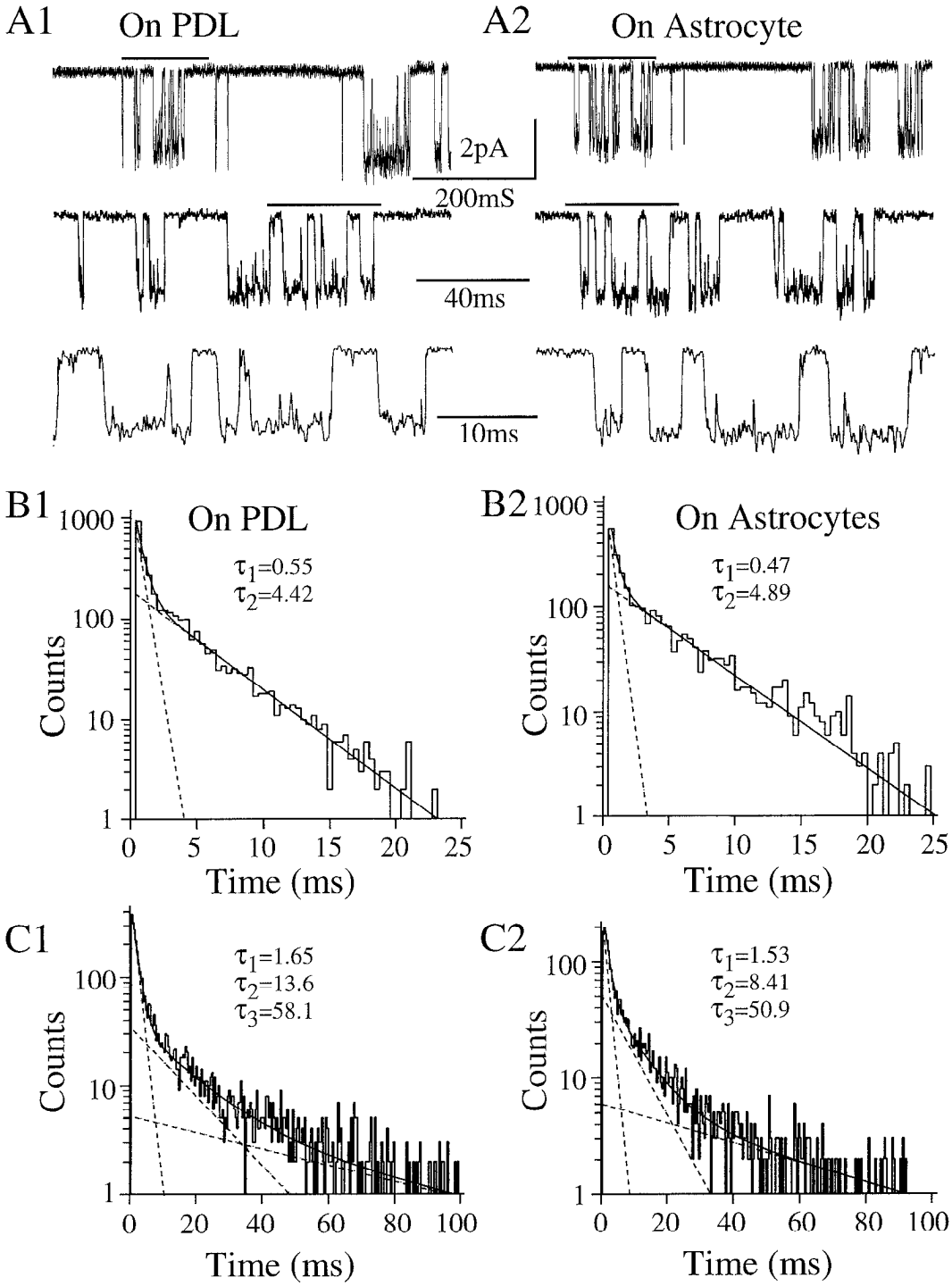

Figure 9. Single-channel properties are identical in patches excised from neurons grown on astrocytes or PDL. Single-channel currents activated by 3 μM GABA were recorded at −80 mV in outside-out patches excised from 2 DIC neuron cell body. *A*, GABA-induced channel activity is shown at different time scales (*A1*, *A2*). The portion of the current trace under the horizontal line is shown on an expanded time scale in the trace below. *B*, The open-time histograms have been fitted adequately with a bi-exponential function. The open-time constants in neurons on PDL are 0.55 and 4.42 msec (*B1*), which are virtually identical to those in neurons on astrocytes (0.47 and 4.89 msec, *B2*). *C*, The closed-time histograms are fitted adequately with a tri-exponential function with similar closed-time constants in the two groups of neurons (1.65, 13.6, and 58.1 msec, and 1.53, 8.41, and 50.9 msec for neurons on PDL and astrocytes, respectively).

neurons cultured on astrocytes would be that functional $GABA_A$ receptor/channels are inserted as new membrane differentiates, so that neurons with more plasma membrane and greater C_m will exhibit greater I_{GABA}. In fact, the specific density of GABA-activated Cl^- current never changed significantly in neurons grown on astrocytes (Fig. 3*B*). In neurons on PDL, I_{GABA} remained unchanged during the first 2 d in culture, whereas whole-cell capacitance increased by >60%, so that density of the current

decreased (Figs. 2*A*, 3). Despite morphological differentiation (Fig. 1) and an increase in plasma membrane in neurons cultured on PDL, the rate of insertion of new receptors at the cell surface is less, compared with the rate in those on astrocytes. Alternatively, without the support of astrocytes, $GABA_A$ receptors are degraded faster so that although new receptors are inserted at a constant rate, their density actually decreases. Independent assessment of receptor turnover rate will help to reveal how this is related to the observed phenomenology.

Mechanisms of the astroglial modulation of neuronal I_{GABA}

Astrocytes synthesize neurotrophic factors, including some extracellular matrix macromolecules and soluble substances that are released into the extracellular space (Lindsay, 1979; Banker, 1980; Hatten and Mason, 1986; Manthorpe et al., 1986; Pixley et al., 1987; Alliot et al., 1988; Sanes, 1989; Le Roux and Reh, 1994). These neurotrophic factors may affect neuronal survival, migration, differentiation, neurite extension, and, as recently reviewed by Barish (1995), expression, distribution, and function of ion channels. No factor or factors that generally influence ion channels have been defined clearly yet. For example, the induction of A-type transient potassium current and depression of D-type potassium current in mouse hippocampal pyramidal neurons differentiating in culture required active synthesis of a factor or factors transmitted by direct contact or short-range diffusion (Wu and Barish, 1994), whereas diffusible factors were believed to influence the potassium currents in rat sympathetic ganglion neurons (McFarlane and Cooper, 1993). Similarly, no specific factors have been identified yet for the contact-mediated astrocyte modulation of GABA-activated Cl^- current in embryonic rat hippocampal neurons. It is most likely that either factors associated with cell surface or extracellular matrix or those that diffuse only a very limited distance are involved. Furthermore, the effect of astrocytes may not be specific to $GABA_A$ receptors but rather may be part of a more general change in the physiology of cultured hippocampal cells involving other (such as glutamate) receptors. Cultured astrocytes show spontaneous Ca_c^{2+} elevations (Cornell-Bell and Finkbeiner, 1991; Fatatis and Russell, 1992). The fact that suppression of spontaneous Ca_c^{2+} elevation in astrocytes by loading BAPTA-AM intracellularly significantly reduced their modulatory effects on I_{GABA} (Fig. 6) suggests that either active Ca_c^{2+}-dependent secretion of regulatory factors from astrocytes or their elaboration on the cell surface is involved in the modulation of I_{GABA}.

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