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High Intracellular Cl^- Concentrations Depress G-Protein-Modulated Ionic Conductances

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Numerous G-protein-modulated ionic conductances are present in central neurons and play major roles in regulating neuronal excitability. Accordingly, endogenous factors that alter the operation of these conductances may have profound effects on neuronal function. We now report that several G-protein-modulated ionic conductances in hippocampal neurons are very much altered when Cl^- is the predominant anion in the recording electrode. We used both sharp-electrode and whole-cell techniques in rat hippocampal slices to determine whether hippocampal CA1 pyramidal cell properties are altered by KCl-filled, as compared with KCH_3SO_3- or K-gluconate-filled, electrodes. We studied the effects of the anions on synaptically evoked $GABA_B$ responses and baclofen- and serotonin-induced currents as well as on a voltage-activated cation current, I_h. High intracellular concentrations of chloride ($[Cl^-]_i$) depressed all the responses without altering resting cell properties. Intermediate $[Cl^-]_i$ reduced baclofen-induced currents as well as I_h in a dose-dependent manner. In KCH_3SO_3-filled cells, equimolar substitution of GTPγS for Tris-GTP results in activation of a K^+ conductance that hyperpolarizes cells and lowers their input resistance. These effects of GTPγS were blocked in KCl-filled cells. In view of the tight coupling between the G-protein and activation of the $GABA_B$-activated K^+ conductance, the effect of Cl^- ions is likely to be exerted either on the G-protein or the K^+ channel itself. We observed substantial effects of Cl_i^- at concentrations that are believed to exist during development in the CNS as well as during pathological conditions, such as spreading depression. Thus, the results we describe must be taken into consideration during such physiological and pathological conditions as well as in experimental studies of G-protein-modulated conductances.

Key words: $GABA_B$; baclofen; serotonin; I_h; spreading depression; anions

We have noticed that large $GABA_B$ responses are rare in CA1 pyramidal cells when KCl is the major constituent in the recording electrode solution (compare with Fig. 1 in Pitler and Alger, 1992; Pham and Lacaille, 1996). Chloride-dependent $GABA_A$ responses are reversed and very large when intracellular chloride concentration ($[Cl^-]_i$) is high, so it appears that the $GABA_B$ response is reduced selectively. Although several explanations are conceivable, it could be that high $[Cl^-]_i$ affects $GABA_B$ responses. However, no thorough study of this issue has been performed.

Intracellular recording techniques offer many advantages for the study of neuronal function. However, it has been known since the earliest studies using intracellular techniques (Coombs et al., 1955) that the ions present in the electrolyte solution in the intracellular electrode diffuse into the cell being studied and can affect cellular properties. Whole-cell voltage clamp is a very powerful and widely used technique that has many advantages over traditional intracellular recording. Access to, and control over, the internal milieu as well as improved clamp control are major advantages of large-bore patch pipettes over traditional high-resistance intracellular electrodes. However, alterations of normal cellular constituents can compromise cellular functioning drastically. Classic studies performed on the squid giant axon established early on the variable ability of different anions to restore action potential amplitude (Tasaki et al., 1965). Although often overlooked, high intracellular concentrations of anions (Cl^-, F^-, gluconate$^-$, et cetera) can alter various electrophysiological characteristics of excitable cells (Baker et al., 1962; Adams and Oxford, 1983; Nakajima et al., 1992; Zhang et al., 1994).

Because the normal intracellular concentration of Cl^- is ~8 mM (McCormick, 1990) and Cl^--based patch electrode solutions often contain ~150 mM Cl^-, it is quite possible that these abnormally high concentrations could affect the cell adversely. High intracellular concentrations of KCl ($[KCl]_i$) can modify G-proteins (Nakajima et al., 1992) and K^+ channels (Adams and Oxford, 1983). Because these studies were performed on cardiac atrial cells and the squid giant axon, respectively, and used very high (≥400 mM) $[Cl^-]_i$, we wanted to determine whether KCl affected mammalian central neurons at concentrations that commonly are used in patch pipette solutions. Zhang et al. (1994) reported that certain anions attenuate the slow Ca^{2+}-dependent K^+ conductance in hippocampal neurons but that this could be explained by an effect on intracellular Ca^{2+} handling. If high $[Cl^-]_i$ does affect G-protein-linked responses, such as those mediated by $GABA_B$ receptor activation, then conditions in which $[Cl^-]_i$ is high, such as during development, and during pathological conditions, such as spreading depression (Lux et al., 1986), will affect those responses. We have undertaken the present experiments to determine whether hippocampal CA1 pyramidal

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cell properties are altered by KCl-filled, as compared with KCH_3SO_3- or K-gluconate-filled, electrodes. Our results support the hypothesis that $[Cl^-]_i$ attenuates in a dose-dependent manner both $GABA_B$- and serotonin-mediated currents in CA1 neurons as well as a voltage-activated cation current, I_h. Moreover, the $[Cl^-]_i$ effects are exerted at the level of the G-protein-linked pathway.

A preliminary report of this work has appeared in abstract form (Lenz et al., 1994).

MATERIALS AND METHODS

Preparation of slices. Adult male Sprague Dawley rats (125–300 gm, 30–60 d) were anesthetized deeply with halothane and decapitated. Both hippocampi were removed and placed on agar blocks in a slicing chamber containing oxygenated, partially frozen saline. A Vibratome (Technical Products International) was used to cut transverse slices at 400 μm intervals. Slices were transferred to a holding chamber where they were maintained at the interface of physiological saline and humidified 95% O_2/5% CO_2 atmosphere at room temperature. Slices were allowed at least 1 hr to recover before being transferred to a submerged perfusion-type chamber (Nicoll and Alger, 1981) where they were perfused with saline (29–31°C) at 0.5–1 ml/min.

Solutions. The bath solution contained the following (in mM): 124 NaCl, 25 $NaHCO_3$, 3.5 KCl, 2.5 $CaCl_2$, 2 $MgSO_4$ or 2 $MgCl_2$, 1.25 $NaHPO_4$, and 10 glucose. When monosynaptic $GABA_B$ responses were studied, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μM), 2-amino-5-phosphonovaleric acid (APV; 50 μM), and bicuculline (20 μM) were present in the saline to block ionotropic glutamate- and $GABA_A$-mediated responses, respectively. CGP 35348 (1 mM) was used in some experiments to antagonize $GABA_B$ receptors. This concentration blocked the synaptic $GABA_B$ response completely as well as that mediated by a 2 min bath application of baclofen (5 μM). Serotonin (10 μM) was bath-applied for 2 min.

Whole-cell patch electrodes had resistances of 3–6 MΩ and were filled with one of three solutions (in mM): (1) 150–160 KCH_3SO_3, 10 HEPES, 2 BAPTA, 0.2 $CaCl_2$, 1 MgATP, 1 $MgCl_2$, and 0.3 Tris-GTP, pH 7.25; (2) 150–160 KCl, 10 HEPES, 2 BAPTA, 0.2 $CaCl_2$, 1 MgATP, 1 $MgCl_2$, and 0.3 Tris-GTP, pH 7.25; (3) 150 $KC_6H_{11}O_7$ (K-gluconate), 10 KCl, 10 HEPES, 2 BAPTA, 0.2 $CaCl_2$, 1 MgATP, and 0.3 Tris-GTP, pH 7.25. For the experiments performed with intermediate $[Cl^-]_i$ (see Fig. 5), the electrode solution contained either 45 KCl and 120 KCH_3SO_3 or 65 KCl and 100 KCH_3SO_3 with 10 HEPES, 2 BAPTA, 0.2 $CaCl_2$, 1 MgATP, 1 $MgCl_2$, and 0.3 Tris-GTP, pH 7.25. In a few experiments, as noted, the nonhydrolyzable analog of GTP, GTPγS (0.3 mM), was substituted for Tris-GTP. Intracellular recordings also were performed with sharp electrodes having resistances of 40–100 MΩ and filled with either 3 M KCl or 2 M KCH_3SO_3.

CNQX was purchased from Research Biochemicals International (Natick, MA), and BAPTA was purchased from Molecular Probes (Eugene, OR). CGP 35348 was a generous gift from CIBA-Geigy (Basel, Switzerland). All other drugs and chemicals were obtained from Sigma Chemical (St. Louis, MO).

Whole-cell and intracellular recordings and data analysis. CA1 pyramidal cell recordings were obtained either with conventional intracellular or the "blind" whole-cell patch-clamp recording technique (Blanton et al., 1989). Cells obtained with the whole-cell technique were voltage-clamped near their resting potential soon after break-in. Acceptable cells had resting potentials equal to or greater than −55 mV and input resistances >35 MΩ (except those cells recorded with GTPγS; see below). Series resistance was <12 MΩ at the beginning of an experiment and was compensated by 60–70%. Cells were discarded if series resistance increased to >30 MΩ during an experiment. Bipolar concentric stimulating electrodes (Rhodes Electronics) were positioned in stratum radiatum (s. radiatum) to allow orthodromic activation of CA1 pyramidal cells. Liquid junction potentials between the three intracellular solutions and the extracellular solution were measured according to the method of Neher (1992). These junction potentials were small—KCl (3 mV), KCH_3SO_3 (4 mV), K-gluconate (11 mV)—and were not corrected for.

An Axoclamp-2 (Axon Instruments, Foster City, CA) was used for all experiments. Evoked synaptic currents or potentials were elicited at 0.2 Hz and were filtered at 2 kHz with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA) and digitized at 5 kHz by a Digidata 1200 analog-to-digital converter (Axon Instruments). Data also were stored on a VCR-based tape recorder system (Neuro-corder DR-484, Neuro Data Instruments) and played into a computer for off-line analysis with pCLAMP 6.0 software (Axon Instruments). The effects of the three intracellular solutions on various responses were assessed by one-way ANOVA, followed by unpaired Student's *t* tests (SigmaStat, Jandel Scientific, Corte Madera, CA). The significance level chosen was $p < 0.05$, and all data are reported as mean ± SEM.

RESULTS

When evoking synaptic responses in hippocampal CA1 neurons, we often observed that $GABA_B$-mediated IPSPs were small or nonexistent when recorded with KCl-filled electrodes. To test the hypothesis of an interaction between high $[Cl^-]_i$ and $GABA_B$-mediated responses, we began by examining synaptically evoked $GABA_B$ IPSPs under different recording conditions.

High intracellular chloride ($[Cl^-]_i$) inhibits synaptic $GABA_B$ responses

In cells recorded with KCH_3SO_3-filled high-resistance intracellular electrodes, a multiphasic synaptic response is reliably observable when stimulation is given in s. radiatum (Fig. 1; Davies et al., 1990). To prevent the occurrence of an afterhyperpolarization (AHP), which might contaminate the synaptic response, we used stimulus intensities that produced EPSPs that were just subthreshold for action potential initiation in the recorded cell. The initial depolarizing potential (truncated) is the CNQX-sensitive EPSP, which is followed immediately by a rapidly rising $GABA_A$-mediated IPSP (labeled *f* for fast). The prolonged hyperpolarization (labeled *s* for slow) is mediated by the activation of $GABA_B$ receptors and can be blocked by the $GABA_B$ receptor antagonist CGP 35348 (Dutar and Nicoll, 1988; Olpe and Karlsson, 1990). The response in CGP 35348 (*middle traces*) consists of the EPSP, followed by the $GABA_A$ IPSP. Subtraction of the synaptic response obtained in CGP 35348 (*middle traces*) from the response recorded in control saline (*left-hand column*) reveals the $GABA_B$-mediated IPSP in isolation (*right-hand column*). The slow IPSP had a latency to peak of 190 msec and was blocked by 1 mM CGP 35348 (*middle trace*), thereby confirming that it is a $GABA_B$-mediated response.

When the recording electrode contained 3 M KCl, the $GABA_B$ component of the synaptic response was attenuated (Fig. 1, *bottom traces*). The $GABA_A$-mediated fast IPSP is depolarizing in KCl-filled cells because the normal inward driving force for Cl^- is reversed in these cells. Addition of 1 mM CGP 35348 (*middle trace*) blocked the small $GABA_B$-mediated slow IPSP. The subtracted traces obtained from both KCl-filled and KCH_3SO_3-filled cells are expanded and superimposed to demonstrate that the CGP-35348-sensitive component in the KCl-filled cell is clearly smaller than that recorded in the KCH_3SO_3-filled cell. The KCl-filled cell illustrated in Figure 1 displayed the largest $GABA_B$ response of the four KCl-filled cells that we examined in this way.

Because these initial experiments were performed with high-resistance (40–100 MΩ) intracellular electrodes and because the diffusion of small molecules from such electrodes is linearly related to the access resistance, the diffusion of the electrode solution into the cell might have been incomplete (Pusch and Neher, 1988). Moreover, cells could be voltage-clamped more effectively with low-resistance electrodes. Therefore, we used the whole-cell patch-clamp technique to insure maximal dialysis of the neuron with the electrode solution and to improve clamp control. To isolate the evoked $GABA_B$ response, we added CNQX (20 μM), APV (50 μM), and bicuculline (20 μM) to the bathing solution to block ionotropic glutamate and $GABA_A$ re-

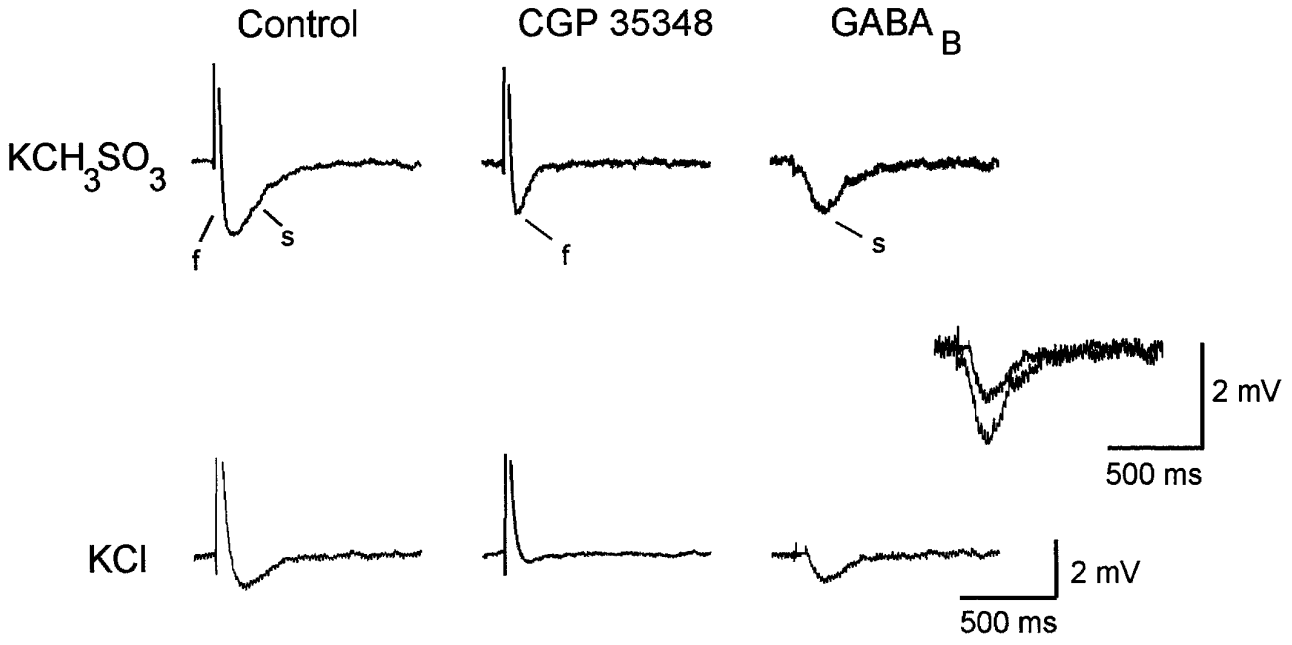

Figure 1. Synaptically evoked $GABA_B$ responses are attenuated in cells filled with KCl. While recording intracellularly with high-resistance electrodes in CA1 pyramidal cells, we elicited synaptic responses by electrical stimulation in the CA1 s. radiatum. The synaptic response recorded from a KCH_3SO_3-filled cell consists of a depolarizing EPSP, followed by an IPSP with an initial rapid rise and slow phase (*left trace*). The initial rapid phase of the IPSP (*f*) is mediated by activation of $GABA_A$ receptors and can be blocked by bicuculline (not shown). The slow phase of the IPSP (*s*) is mediated by activation of $GABA_B$ receptors and is blocked by 1 mM CGP 35348 (*middle trace*). The *right trace* is a subtraction of the CGP 35348 trace from the control trace and represents the $GABA_B$-mediated slow IPSP in isolation. The EPSPs recorded during control and in the presence of CGP 35348 are 12 mV in amplitude and were truncated for display purposes. In the KCl-filled cell (*bottom traces*), the $GABA_A$-mediated IPSP is depolarizing, as is the EPSP, which together result in a 10 mV depolarization in control and in the presence of CGP 35348. Subtracted traces illustrating the $GABA_B$-mediated response are superimposed and expanded to demonstrate that the $GABA_B$ response in KCl-filled cells is smaller than in KCH_3SO_3-filled cells. Resting membrane potentials were −62 mV in the KCH_3SO_3-filled cell and −60 mV in the KCl-filled cell. Similar results were seen in three other KCH_3SO_3-filled and three other KCl-filled cells.

ceptors, respectively. Orthodromic activation of CA1 neurons was achieved by a stimulating electrode placed within s. radiatum on the CA3 side of the recording electrode, but no more than 0.5 mm from it. For each cell the maximum $GABA_B$ response was obtained by stimulating at intensities up to 800 μA for 70 μsec or until further increases in intensity did not result in an increased current. Each cell was voltage-clamped at −55 mV to minimize contributions of different driving forces to the magnitude of the synaptic current. Low-frequency (0.2 Hz) high-intensity stimulation invariably elicited a monosynaptic $GABA_B$ response when we recorded from a KCH_3SO_3-filled cell (Fig. 2*A*; Davies et al., 1990). This synaptic current displayed paired-pulse depression, was occluded by baclofen application, and was blocked completely by 1 mM CGP 35348, thus indicating it was a $GABA_B$-mediated current (data not shown). However, when KCl was the main electrolyte in the electrode solution, the monosynaptic $GABA_B$ responses were much smaller. The mean maximum monosynaptic $GABA_B$ IPSC from seven KCl-filled cells (25.3 ± 6.9 pA) was significantly less than the mean response from eight KCH_3SO_3-filled cells (64.4 ± 8.1 pA) (Fig. 2*B*; $p < 0.005$).

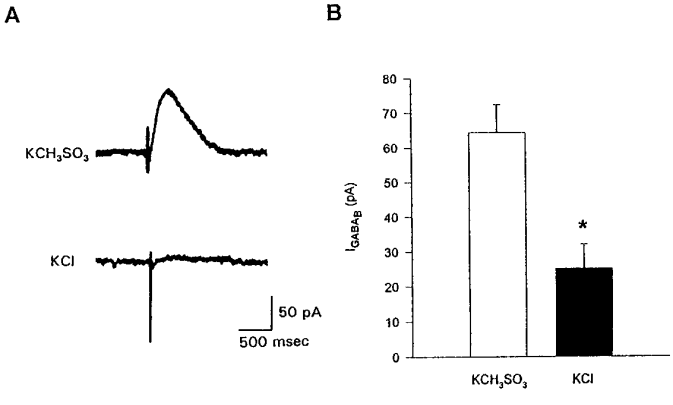

Figure 2. Monosynaptically evoked $GABA_B$ responses recorded under whole-cell voltage clamp are greatly reduced in cells containing high intracellular $[Cl^-]$. *A*, Monosynaptic $GABA_B$ responses were elicited by electrical stimulation in s. radiatum in the presence of 20 μM CNQX, 50 μM APV, and 20 μM bicuculline. Traces are from two cells, one recorded with a patch electrode solution containing 155 mM KCH_3SO_3 (*open bar*) and the other with a solution containing 155 mM KCl (*filled bar*). *B*, Bar graph showing that the average peak monosynaptic $GABA_B$ response recorded from KCl-filled cells (25.3 ± 6.9 pA, $n = 7$) is significantly smaller than from KCH_3SO_3-filled cells (64.4 ± 8.1 pA, $n = 8$; $p < 0.005$).

Baclofen and serotonin responses are reduced in KCl-filled cells

To determine whether high $[Cl^-]_i$ affected responses mediated by extrasynaptic as well as synaptic $GABA_B$ receptors and to insure that we were activating maximal numbers of $GABA_B$ receptors in all cells, we bath-applied baclofen for brief periods. Bath application of baclofen directly hyperpolarizes cells by activating $GABA_B$ receptors (Newberry and Nicoll, 1984), which activate an outward current carried by K^+ ions (Gahwiler and Brown, 1985). Figure 3*A* illustrates that a 2 min bath application of 5 μM baclofen causes a large outward current in KCH_3SO_3-filled cells. However, the same application of baclofen to a KCl-filled cell voltage-clamped at the same resting potential (−60 mV) results in

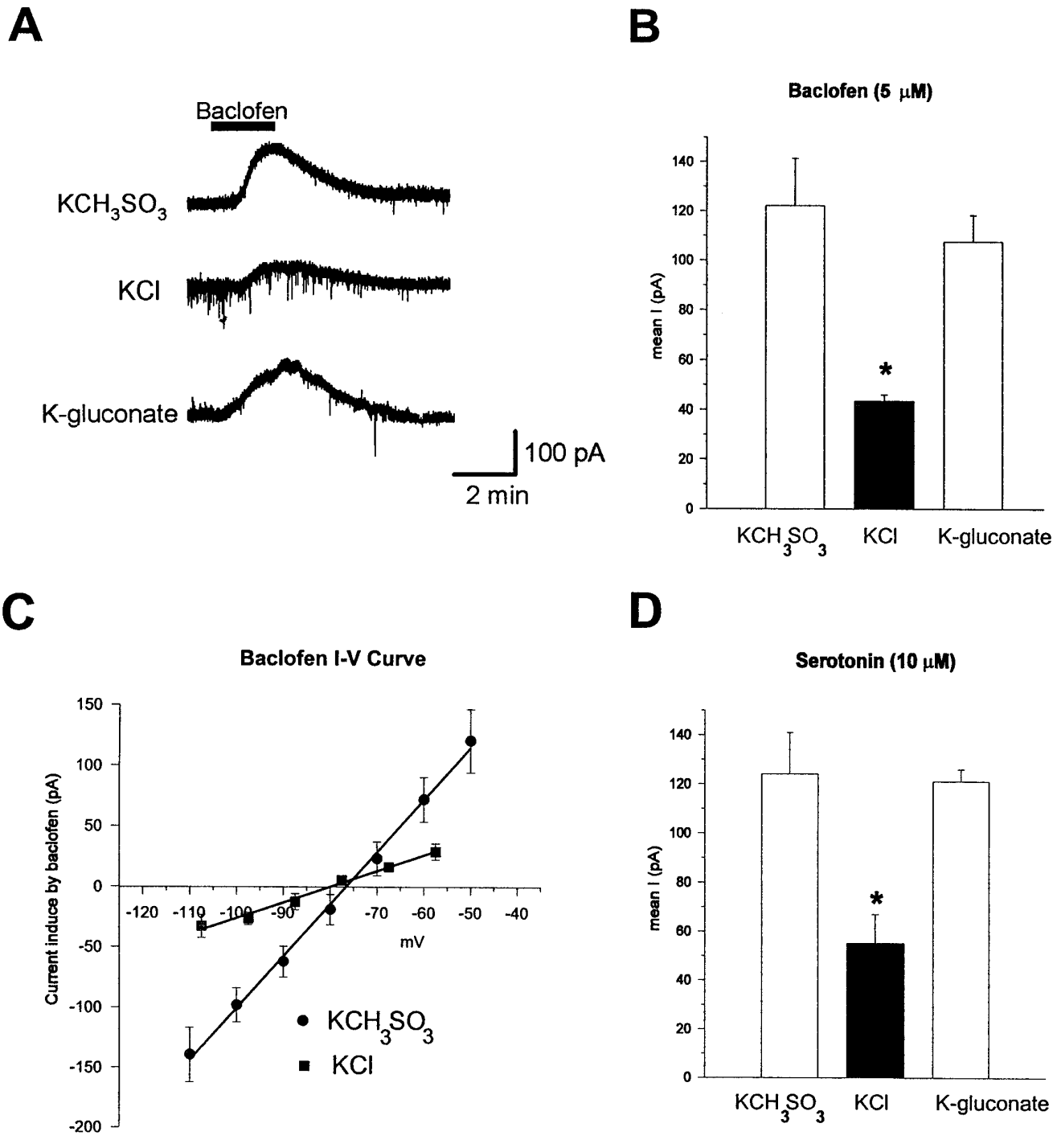

Figure 3. Baclofen and serotonin responses are reduced in KCl-filled cells. *A*, Traces of outward currents elicited by a 2 min bath application of 5 μM baclofen recorded under whole-cell voltage clamp. The three traces are from three separate cells recorded with intracellular solutions, based on three different salts: KCH_3SO_3 (155 mM), KCl (155 mM), and K-gluconate (150 mM). All cells were voltage-clamped between −58 and −60 mV. Downward deflections in the KCl trace are spontaneous IPSCs. *B*, Group data showing peak baclofen responses recorded with the three different intracellular solutions. Baclofen responses recorded in KCl-filled cells (*filled bar*) are significantly reduced, as compared with those in either KCH_3SO_3-filled (*open bar*) or K-gluconate-filled (*open bar*) cells ($p < 0.005$), whereas responses in KCH_3SO_3-filled and K-gluconate-filled cells were not different ($p = 0.62$). *C*, Baclofen *I–V* plot for four KCl-filled and five KCH_3SO_3-filled cells. The line is fit to the data points by linear regression analysis. Baclofen conductance was obtained by averaging the slopes of the linear portions of the *I–V* plots from each cell. *D*, Bath application of 10 μM serotonin for 2 min elicited an outward current similar in amplitude and duration to baclofen, which was greatly reduced in Cl^--filled cells. Serotonin responses from four KCl-filled cells are significantly less than those measured in five KCH_3SO_3-filled and six K-gluconate-filled cells ($p < 0.02$). Serotonin responses from KCH_3SO_3-filled and K-gluconate-filled cells were not different ($p > 0.7$).

a substantially smaller current. As is shown in Figure 3*B*, the mean baclofen current measured from KCl-filled cells (43 ± 2.7 pA, $n = 10$) is significantly less than that measured in KCH_3SO_3-filled cells (122 ± 19.2 pA, $n = 15$; $p < 0.005$). These results confirm that $GABA_B$ responses are smaller in KCl-filled cells. We determined the conductance of the baclofen response by a series of 200 msec voltage steps between −50 and −110 mV before and during the peak baclofen response. Subtraction of the conductance obtained during the control period from the conductance during the peak baclofen response gave the baclofen conductance. As shown in Figure 3*C*, baclofen conductance in KCl-filled cells was significantly smaller (1.5 ± 0.18 nS, $n = 4$) than the baclofen conductance measured in KCH_3SO_3-filled cells (4.2 ± 0.49 nS, $n = 5$; $p < 0.005$). There was no significant difference in the reversal potentials of the baclofen currents of KCl-filled, as compared with KCH_3SO_3-filled, cells.

To determine whether high $[Cl^-]_i$ was responsible for the decreased $GABA_B$-mediated currents, we repeated the baclofen application to cells filled with a K-gluconate-based intracellular solution. Baclofen responses measured from K-gluconate-filled cells (107 ± 10.8 pA, $n = 7$) were not statistically different from those measured from KCH_3SO_3-filled cells ($p > 0.5$). However, the mean baclofen response in KCl-filled cells was significantly smaller than the response obtained from cells filled with K-gluconate ($p < 0.005$). Thus it appears that the Cl^- ion per se causes the decrease in $GABA_B$-receptor-mediated responses.

The reduced $GABA_B$ response recorded from cells with high $[Cl^-]_i$ could be produced by Cl^- acting at any one of several sites within the cell. The chloride ions could interact with the $GABA_B$ receptor specifically, which could result in a decreased ability of an agonist to activate the receptor, or they could interact with the G-protein or the K^+ channel to which the receptor is coupled. To address the possibility that high $[Cl^-]_i$ interacts specifically with the $GABA_B$ receptor, we briefly bath-applied (2 min) 10 μM serotonin (5-HT) to cells filled with the different electrode solutions. $GABA_B$ and $5\text{-}HT_{1a}$ receptors appear to be coupled via G-proteins to the same K^+ channel (Andrade et al., 1986). If the effects of Cl^- are specific to the $GABA_B$-receptor-mediated response, then the outward current elicited by activation of serotonin receptors should be of similar magnitude irrespective of the electrode solution. As illustrated in Figure 3*D*, this is not the case. Serotonin produced a similar current in both KCH_3SO_3-filled (121 ± 4.8 pA, $n = 6$) and K-gluconate-filled (124 ± 16.9 pA, $n = 5$; $p > 0.5$) cells, whereas in KCl-filled cells the mean 5-HT response was reduced significantly (55 ± 11.9 pA, $n = 4$; $p < 0.05$). These data support the idea that high $[Cl^-]_i$ mediates its effects, not via specific interaction with the $GABA_B$ receptor per se, but rather via interaction either with the G-protein involved in coupling the receptors to the K^+ channel or with the K^+ channel itself.

I_h is greatly reduced in Cl^--filled cells

To determine whether high $[Cl^-]_i$ affects currents other than those mediated by neurotransmitter receptors, such as $GABA_B$ and 5-HT, we investigated the effects of various intracellular solutions on the hippocampal I_h. The I_h is a hyperpolarization-activated inward cationic current found in hippocampal CA1 pyramidal cells (Halliwell and Adams, 1982; Maccaferri et al., 1993). This slowly activating current is thought to be mediated by a nonspecific, monovalent cationic conductance and is highly regulated by numerous neurotransmitters that act via G-proteins (Bobker and Williams, 1989; Jiang et al., 1993; Maccaferri and McBain, 1996). Figure 4*A* illustrates that a 20 mV, 1 sec hyperpolarizing voltage step from −60 mV given ~5 min after break-in produces an inwardly relaxing current associated with a membrane conductance increase, I_h, that is greatly reduced in cells with high $[Cl^-]_i$. The group data in Figure 4*B* demonstrate that the I_h measured in KCH_3SO_3- and K-gluconate-filled cells did not differ (KCH_3SO_3: 169.2 ± 11.2 pA, $n = 28$; K-gluconate: 175.0 ± 17.1 pA, $n = 9$; $p > 0.7$), whereas the I_h from KCl-filled cells was significantly smaller than either (82.3 ± 5.7 pA, $n = 20$; $p < 0.001$).

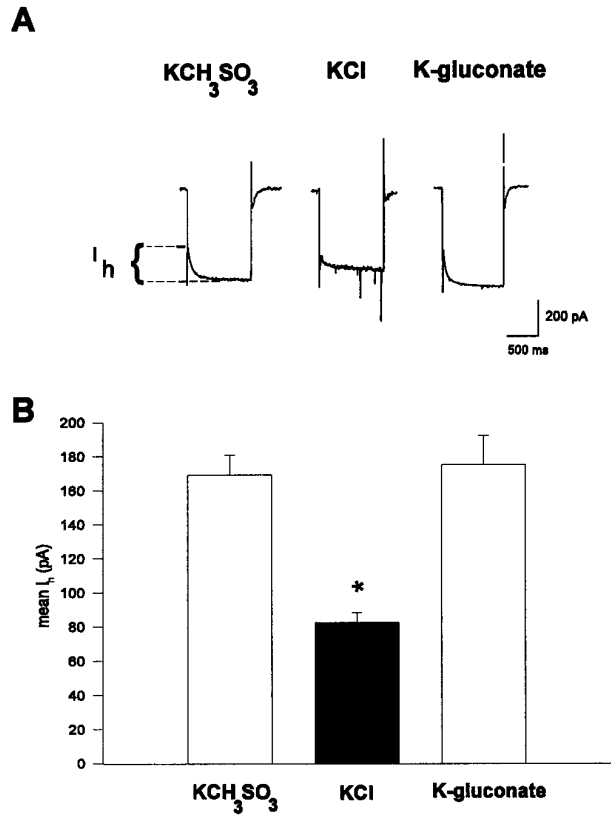

Figure 4. I_h is reduced in cells with high $[Cl^-]_i$. *A*, I_h was elicited by giving a 1 sec, 20 mV hyperpolarizing voltage step from rest shortly after breaking into the cell. Magnitudes of I_h recorded from three different cells with whole-cell patch electrodes filled with three different solutions are displayed as the slowly activating inward current. I_h from the illustrated traces are KCH_3SO_3, 160 pA; KCl, 60 pA; and K-gluconate, 180 pA. *B*, Group data showing the mean I_h from cells filled with KCH_3SO_3 ($n = 28$), KCl ($n = 20$), and K-gluconate ($n = 9$). The I_h measured in Cl^--filled cells is significantly smaller than that measured in either the KCH_3SO_3-filled or K-gluconate-filled cells ($p < 0.0001$). All cells were voltage-clamped between −55 and −58 mV.

To determine whether high $[Cl^-]_i$ reduced the maximal I_h or whether it shifted the voltage dependence of activation to more negative potentials, we maximally activated I_h by giving a series of 2 sec hyperpolarizing voltage steps from −57 to −117 mV in 10 mV increments (data not shown). The conductance of the I_h between −117 and −67 mV was determined from linear regression of the slope of the linear portion of the current versus voltage (*I–V*) plot. The conductance of I_h measured from KCl-filled cells was significantly smaller (5.89 ± 0.84 nS, $n = 7$) than that measured in KCH_3SO_3-filled cells (17.2 ± 1.5 nS, $n = 7$; $p < 0.0005$). Furthermore, linear extrapolation of the averaged data in the *I–V* plot from both KCl-filled and KCH_3SO_3-filled cells intersected the ordinate at the same voltage, indicating that the

voltage dependence of activation was not changed. Together these indicate that high $[Cl^-]_i$ reduced the maximal I_h.

Because it was apparent that high concentrations of Cl^- were quite effective at reducing both $GABA_B$-mediated current and the G-protein-modulated I_h, we wanted to determine the effects of intermediate $[Cl^-]_i$ on these currents. Cells filled with 45 or 65 mM KCl displayed reduced baclofen-induced currents and I_h, as compared with KCH_3SO_3-filled cells. Figure 5 is a graphical representation of these data, which were fit by a computer-generated hyperbolic equation of the form % block = (% max. block)([KCl])/(EC_{50} + [KCl]). Assuming that maximal block occurred at 155 mM Cl^- and that no block was present with 0 mM Cl^-, the EC_{50} was 42 mM for block of I_h and was 58 mM Cl^- for block of the baclofen-induced current. Thus, $[Cl^-]_i$ reduces $GABA_B$-mediated currents as well as the voltage-activated, G-protein-modulated I_h in a dose-dependent manner.

Cl^- effects on GTPγS

Because the depressant effects of high $[Cl^-]_i$ were not restricted to a single G-protein-linked neurotransmitter receptor or ion channel type, we considered the possibility that high $[Cl^-]_i$ might interfere with the G-protein pathway more directly. To do so, we investigated cells to which the hydrolysis-resistant analog of GTP (guanosine 5′-*O*-13-thiotriphosphate, GTPγS), an activator of G-proteins, was applied internally. It has been suggested that GTPγS activates the same K^+ channels that are activated by both baclofen and 5-HT (Andrade et al., 1986). Indeed, we found that application of either baclofen or 5-HT had no additional effect on cells recorded with GTPγS-filled electrodes ($n = 2$; data not shown), as expected if the neurotransmitter-linked channels already had been opened by the GTP analog. In agreement with previous reports (Andrade et al., 1986), we observed that, in KCH_3SO_3-filled cells, equimolar substitution of GTPγS for Tris-GTP resulted in a significantly more negative resting potential (GTP: -60 ± 0.8 mV, $n = 21$; GTPγS: -74 ± 2.0 mV, $n = 5$; $p < 0.0001$) and low input resistance (GTP: 62 ± 3.6 MΩ, $n = 24$; GTPγS: 25 ± 2.3 MΩ, $n = 5$; $p < 0.0001$) (Fig. 6). However, we found that, in KCl-filled cells, substitution of GTPγS for Tris-GTP did not result in significant differences in either membrane potential (GTP: -60 ± 0.9 mV, $n = 13$; GTPγS: -63 ± 2.0 mV, $n = 8$; $p > 0.08$) or input resistance (GTP: 67 ± 3.2 MΩ, $n = 20$; GTPγS: 60 ± 4.6 MΩ, $n = 8$; $p > 0.2$). Application of baclofen to KCl-filled cells containing GTPγS produced only a small outward current (30 ± 7.6 pA, $n = 3$), which decayed approximately three times more slowly than that in Tris-GTP-containing cells. Thus, high $[Cl^-]_i$ blocks the effects of GTPγS on input resistance and resting membrane potential.

DISCUSSION

The results of this study show that high $[Cl^-]_i$ significantly reduces G-protein-modulated currents in CA1 neurons. We found that monosynaptic $GABA_B$ currents in KCl-filled cells are greatly reduced, as compared with those in KCH_3SO_3-filled cells. Furthermore, the responses to brief applications of both baclofen and 5-HT were smaller in cells filled with high $[Cl^-]$. Interestingly, the effects of Cl^- ions were not limited to neurotransmitter-activated K^+ currents. The voltage-dependent, nonspecific cation current, I_h, was reduced as well. Finally, high $[Cl^-]_i$ blocked the effects of GTPγS on resting membrane potential and input resistance that normally are seen in KCH_3SO_3-filled cells. We conclude that high $[Cl^-]_i$ affects cellular properties by interacting with G-protein-modulated ionic conductances.

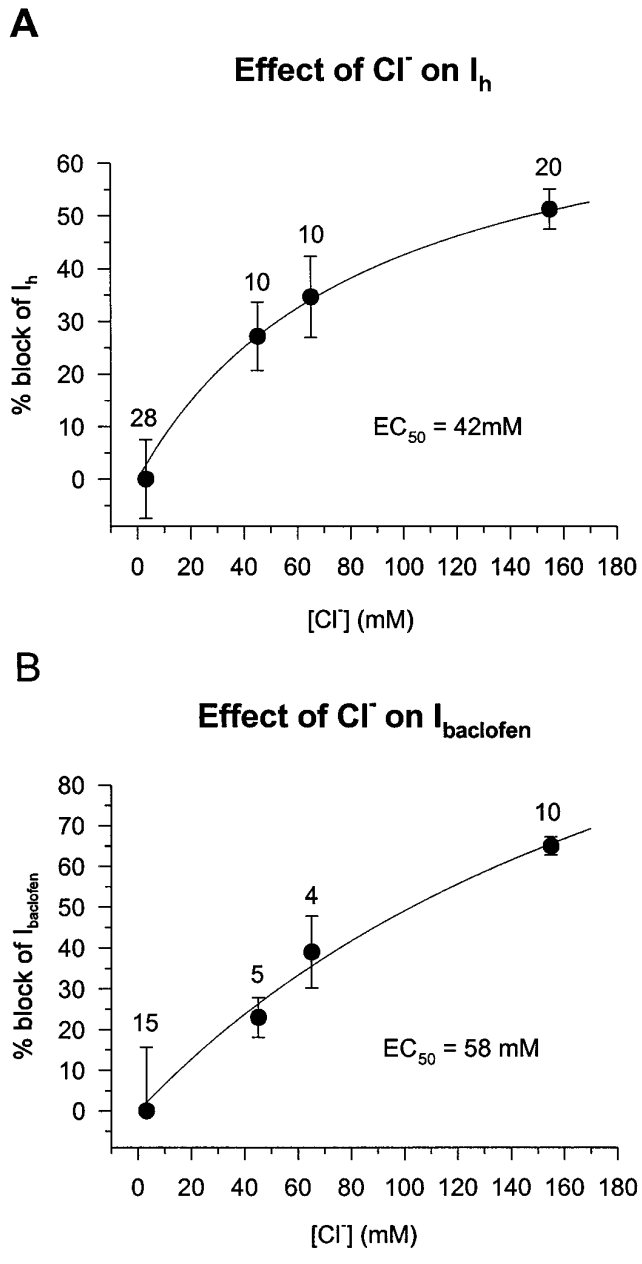

Figure 5. Intermediate concentrations of Cl_i^- reduce the baclofen-induced current and I_h in a dose-dependent manner. *A*, I_h was elicited by a 1 sec, 20 mV hyperpolarizing voltage step from a holding potential between −55 and −58 mV. I_h was measured from cells recorded with electrodes containing 3, 45, 65, and 155 mM Cl^-. The current obtained from cells filled with 3 mM Cl^- was designated 0% control, and the data were normalized to this. The dose–response curve was obtained by fitting the data with a computer-generated best-fit equation of the form: % block = % max. block · [KCl]/(EC_{50} + [KCl]). There was a 51% block of I_h at 155 mM Cl^- and an EC_{50} of 42 mM. The *numbers* of cells are indicated *above* the mean values. *B*, A 2 min bath application of 5 μM baclofen to cells filled with the same $[Cl^-]_i$ as in *A* produced a similar dose–response curve. The curve was obtained as in *A*. Cl^- (155 mM) produced a 65% block of the baclofen response with an EC_{50} of 58 mM.

It is difficult to determine which intracellular site(s) the Cl^- ions affect. Because the currents elicited by both baclofen and 5-HT were similarly reduced in KCl-filled cells, as compared with those in KCH_3SO_3-filled or K-gluconate-filled cells, it is unlikely that Cl^- ions interact directly with the $GABA_B$ receptor or a

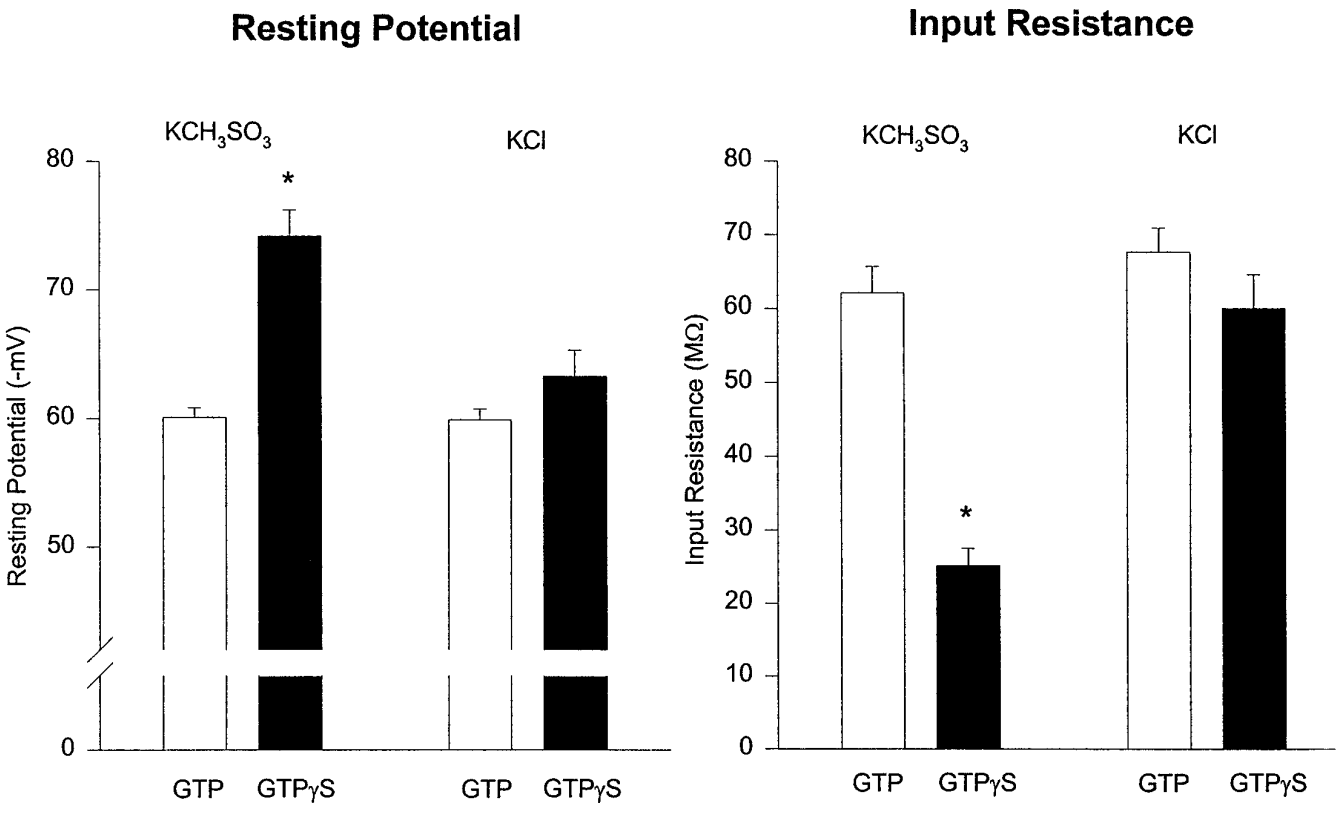

Figure 6. High $[Cl^-]_i$ blocks the effects of GTPγS on input resistance and resting membrane potential. Substituting 300 μM GTPγS for 300 μM Tris-GTP in the whole-cell recording electrode reduces input resistance in, and hyperpolarizes significantly, cells recorded with KCH_3SO_3 electrodes by activating a K^+ conductance ($p < 0.0001$). Contrariwise, in cells filled with KCl there was no significant difference in either input resistance or resting membrane potential when equimolar GTPγS was substituted for GTP. Additionally, there was no difference in input resistance or resting membrane potential between KCl-filled and KCH_3SO_3-filled cells recorded with 300 μM Tris-GTP ($p > 0.2$).

unique $GABA_B$-receptor-linked pathway. The observations that I_h was reduced in KCl-filled cells and that high $[Cl^-]_i$ blocked the effects of GTPγS on a K^+ conductance further argue against a unique interaction with the $GABA_B$ receptor. This is an important point, because the G-protein activated by the $GABA_B$ receptor is coupled very tightly (Andrade et al., 1986) to the inwardly rectifying K^+ channel that mediates the $GABA_B$ response (Gahwiler and Brown, 1985). The model is that these channels are gated directly by the activated G-protein. If indeed the effects of $[Cl^-]_i$ occur at a site downstream of the receptor, then it would seem that there are few possible sites of action. Two equally tenable, nonexclusive explanations are that high $[Cl^-]_i$ interferes with the normal functioning of either the G-proteins or the membrane channels themselves.

There is precedent for both of these possibilities. Anions affect G-proteins (Higashijima et al., 1987) and G-protein-mediated activation of K^+ channels (Nakajima et al., 1992), and in both cases Cl^- was the most potent anion tested. Another possibility is that Cl^- ions do not affect the G-protein but, rather, interact directly with monovalent cation channels. The possibility that high $[Cl^-]_i$ can modulate cation channels in the squid axon has been suggested. Adelman et al. (1966) found that sodium currents in squid axons progressively decline when the axon is perfused with high concentrations of KCl, and Cl^- ions suppress the amplitude and activation rate of delayed rectifier K^+ currents in these axons (Adams and Oxford, 1983). Our results would support an interaction with two separate channels: (1) the K^+ channel activated by GTPγS as well as by $GABA_B$ and 5-HT receptor activation, and (2) the nonselective cation channel mediating I_h. Velumian et al. (1996) reported that I_h was greatly reduced when internal $CH_3SO_4^-$ was replaced with Cl^- or gluconate$^-$. Our findings primarily agree with theirs, although we did not find that K-gluconate depressed I_h. This difference can be explained most easily as a difference in Ca^{2+} buffering, because Velumian et al. (1996) found that addition of 1–3 mM BAPTA to their internal recording solution could "rehabilitate" the attenuated I_h obtained from K-gluconate-filled cells. Zhang et al. (1994) reported that high $[Cl^-]_i$ appeared to inhibit the slow voltage-independent, Ca^{2+}-activated K^+ AHP in hippocampal cells, although they also suggested that Cl^- ions might act simply by disrupting Ca^{2+} homeostasis.

Our results cannot be explained by secondary effects on Ca^{2+}, because our buffering conditions always included 2 mM BAPTA, and the data constitute good evidence that indeed Cl^- can affect G-protein-linked conductances more directly. In view of the number of disparate channel types influenced by Cl^-, it is tempting to speculate that Cl^- affects some common intermediary, such as the G-protein itself.

$GABA_B$ receptor activation underlies many important physiological phenomena, such as synaptic inhibition and paired-pulse depression (Davies et al., 1990; Pitler and Alger, 1994), and it has been shown that $GABA_B$ receptor antagonists block LTP induction by certain stimulation protocols (Olpe and Karlsson, 1990; Davies et al., 1991). I_h and various I_h-like currents have been characterized widely in several mammalian nerve preparations (Mayer and Westbrook, 1983; Maccaferri et al., 1993) as well as

in cardiac atrial cells [referred to there as I_f (DiFrancesco et al., 1986)]. This current plays an integral role in the slow rhythmic burst-firing properties of thalamic relay neurons (McCormick and Pape, 1990), in pacemaking the action potential characteristics of O-A interneurons in the hippocampus (Maccaferri and McBain, 1996), and in the pacemaker current of sino–atrial myocytes. Therefore, disruption of these physiological properties by introduction of high $[Cl^-]_i$ may seriously alter the normal functioning of the cell and obscure correct interpretation of the electrophysiological recordings.

Indeed, there are many examples in which high $[Cl^-]_i$ is correlated with reduced or absent $GABA_B$ responses. Using perforated patch to study the developmental change in the $GABA_A$ receptor reversal potential in embryonic and early postnatal rat neocortical cells, Owens et al. (1996) reported that $[Cl^-]_i$ is high (27–37 mM) at young ages and decreases with development. Luhmann and Prince (1991) found that baclofen-induced responses essentially were absent from newborn rat cortical neurons. Interestingly, both somatic and dendritic $GABA_B$ responses matured during the second and third postnatal week, simultaneous with a shift of E_{GABA_A} to more hyperpolarized potentials because of decreasing $[Cl^-]_i$. Misgeld et al. (1984) found that baclofen produced only slight hyperpolarizations and small conductance increases in granule cells, whereas it elicited large hyperpolarizations accompanied by large conductance increases in CA3 cells. E_{GABA_A} was depolarized significantly more in the granule cells than in the CA3 cells, thus implying a higher $[Cl^-]_i$ in granule cells. It is also possible that the apparent difficulty in observing $GABA_B$-mediated miniature IPSCs (Alger and Nicoll, 1980; Otis and Mody, 1992) is related in part to the use of Cl^--based solutions in these experiments. Furthermore, it is interesting to note that investigators have had difficulty obtaining functional expression of $GABA_B$ receptors in *Xenopus* oocytes, which have high resting $[Cl^-]_i$ (~35 mM).

In light of our finding that $[Cl^-]_i$ ~40 mM can reduce $GABA_B$ and I_h currents significantly, it appears that there are many instances (such as during development) when Cl^- can reach concentrations that will interfere with these currents and potentially compromise normal cellular functioning. These results may be particularly relevant to the understanding of pathophysiological phenomena, such as spreading depression, thought to involve massive influx of Cl^- (Lux et al., 1986). Our results suggest that some of the K^+ conductances potentially available for repolarizing strongly depolarized cells and limiting the extent of pathological activity would, in fact, be compromised by high $[Cl^-]_i$. Reducing $GABA_B$ conductance in particular should contribute to more pronounced epileptiform activity (Traub et al., 1993). Our data support the growing recognition that internal anions may have important influences on cellular excitability in the CNS.

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