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$GABA_A$-Receptor Function in Hippocampal Cells Is Maintained by Phosphorylation Factors

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Gamma aminobutyric acid (GABA) mediates fast synaptic inhibition in the central nervous system by activating the chloride-permeable $GABA_A$ channel. The $GABA_A$ conductance progressively diminishes with time when the intracellular contents of hippocampal neurons are perfused with a minimal intracellular medium. This "run down" of the GABA-activated conductance can be prevented by the inclusion of magnesium adenosine triphosphate and calcium buffer in the intracellular medium. The amount of chloride conductance that can be activated by GABA is determined by competition between a calcium-dependent process that reduces the conductance and a phosphorylation process that maintains the conductance.

THE REDUCTION OF GABA-MEDIATED synaptic inhibition by pharmacological agents (*1*) or tetanic stimulation of afferent fibers (*2, 3*) causes convulsive discharges in the hippocampus. Inhibition may be reduced in the short term by intracellular accumulation of Cl^-, desensitization of the GABA receptor (*4, 5*), or elevation of intracellular calcium ($[Ca^{2+}]_i$) (*6*). There is little information on the long-term regulation of the GABA conductance.

A number of voltage-gated (*7*) and ligand-gated channels (*8*) are regulated by protein phosphorylation. The operation of such a mechanism may be signaled by the lability of the channel when recording from cells in the whole-cell mode of patch clamping (*9*); on penetrating the cell, small diffusible molecules and ions are rapidly lost (within 1 to 2 min). When the intracellular medium is supplemented with nucleotides and buffers to control the divalent ion concentration, it is sometimes possible to maintain channel activity (*10*). We used this approach to attempt to define the intracellular conditions necessary for the stability of the $GABA_A$ conductance in hippocampal neurons.

Acutely dissociated hippocampal neurons were prepared from 650-μm slices of the CA1 subfield of guinea pig hippocampus by trypsin digestion (*11*). Neurons were dissociated and used 3 to 10 hours after removal of the brain. Whole-cell voltage clamp was used to monitor the current response to GABA (50 to 200 μ*M*) applied by pressure ejection. The intracellular recording pipette contained 130 m*M* tris methanesulfonate; 10 m*M* Hepes; 10 m*M* BAPTA {[1,2-bis(2-aminophenoxy)ethane - *N,N,N′,N′′* - tetraacetic acid]}; 0.1 m*M* leupeptin (a Ca^{2+}-activated neutral protease inhibitor); *p*H, 7.3. We used the Ca^{2+} chelator BAPTA because it is *p*H-insensitive and binds Ca^{2+} faster than EGTA (*12*). The external solution contained 120 m*M* NaCl; 5 m*M* CsCl; 2 m*M* $CaCl_2$; 1 m*M* $MgCl_2$; 15 m*M* tetraethylammonium chloride; 5 m*M* 4-aminopyridine; 10 m*M* Hepes; 25 m*M* D-glucose; *p*H, 7.4. This solution was applied at a rate of 5 ml/min, close to the cell to prevent agonist-induced desensitization. All experiments were performed at 18° to 22°C.

Current responses to GABA were elicited in CA1 pyramidal neurons by pressure ejection (pulse duration 20 to 80 ms) at a low frequency (0.016 Hz) to avoid cumulative desensitization. The current was completely blocked by 10^{-6} μ*M* picrotoxin and reversed at the Cl^- equilibrium potential, which suggests it resulted from the activation of the $GABA_A$ receptor (*13*). The peak amplitude of the GABA-activated outward current (at a holding potential of −10 mV) progressively declined to less than 10% within 10 min of penetrating the cell (Fig. 1A), in spite of the presence of 10 m*M* intracellular BAPTA to prevent accumulation of $[Ca^{2+}]_i$ (*6, 11*). Short voltage pulses (−10 mV, 10 ms) were applied before eliciting GABA responses to monitor the leak conductance and input capacitance. A short hyperpolarizing voltage pulse (−10 mV, 10 ms) was applied at a fixed point during the current response to assess the conductance increase and the GABA rever-

Table 1. Effect of intracellular contents on GABA response in acutely dissociated and cultured hippocampal cells. GABA currents were elicited approximately 1 min after entry into the whole-cell recording mode and every 1 min thereafter. Duration of GABA pulses was 20 to 80 ms and was constant for a given experiment. The intracellular solution had the composition specified in text with concentration of Mg^{2+}, ATP, or BAPTA as indicated in the table. The rate of run down was quantified by measuring the percentage of current remaining 10 min after penetration of the cell.

ATP (m*M*)	Mg^{2+} (m*M*)	BAPTA (m*M*)	Percent GABA current ± SEM remaining after 10 min (*n*)
Acutely dissociated neurons			
			9.8 ± 3.9 (8)
0	0	10	33.0 ± 7.4 (10)
2	4	10	130.9 ± 14.7 (12)
	4	10	6.1 ± 1.2 (12)
2	0	10	16.5 ± 2.8 (10)
2	4		21.8 ± 5.9 (6)
*1 mM Ca^{2+}**			
2	4	1†	23.6 ± 7.0 (7)
2 mM AMP-PCP			
0	4	10	22.5 ± 6.0 (10)
Cultured rat hippocampal cells			
0	4	10	54.1 ± 11.3 (9)
2	4	10	116.7 ± 9.3 (4)

*$[Ca^{2+}]_i$ = 5 μ*M*. †EGTA.

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sal potential (*4*). The leak conductance and the capacitance of the neuron were unchanged during the "run down" of the GABA response (Fig. 1B). The decline of the GABA current was accompanied by a proportionate reduction of the GABA conductance, while the GABA reversal potential remained essentially unchanged. The run down of the GABA current thus results from the loss of GABA-activable conductance rather than from a redistribution of Cl^- ions (*14*). Similar results were obtained from analysis of 14 other cells. Buffering $[Ca^{2+}]_i$ to $<10^{-9}M$ was not sufficient to preserve the $GABA_A$-receptor function (Table 1). The run down was not the result of a general decline in the condition of the cell since the input resistance was maintained (Fig. 1B) and voltage-activated Na^+ current and glutamate response (Fig. 1C) were not affected.

The run down of the GABA conductance may be the result of desensitization of the GABA receptor (*4, 5*). Our results suggest that desensitization and run down are independent phenomena (Fig. 2). The GABA current elicited in a hippocampal neuron gradually declined during continuous GABA application (Fig. 2A). This decline, occurring during prolonged GABA application and recovering within 1 min, can be ascribed to receptor desensitization (*4, 5*). In contrast, run down of the GABA response was not contingent upon receptor activation. The independence of run down from desensitization is illustrated by the results shown in Fig. 2B where a GABA current elicited 10 min after the initial response, with no GABA applications in between, still showed a reduction of nore than 80%, suggesting that agonist activation is not required for the run down to occur.

Run down of the GABA response might arguably result from the enzymatic treatment used in the dissociation. Consequently, we examined the stability of the GABA response of cultured rat hippocampal neurons (*15*) with an intracellular medium that did not support stable responses in acutely dissociated cells [4 m*M* Mg, 10 m*M* BAPTA, 0 m*M* adenosine triphosphate (ATP)]. Our results show that the GABA response declined with time, but on average more slowly than that in the acutely dissociated neurons (Table 1). Moreover, the rate of decline was more variable in the cultured cells; some cells ran down as rapidly as the acutely dissociated cells, while others exhibited little decline. The variability may reflect cell to cell variation in the amount of endogenous "stabilizing" factors and the length of the dendritic processes on the cultured cells, which might retard the loss of these factors. The addition of stabilizing factors sustained the GABA current in cultured cells (Table 1). These results demonstrate that run down is not a unique feature of acutely dissociated neurons (*16*).

The inclusion of 2 m*M* ATP, 4 m*M* Mg^{2+}, and 10 m*M* BAPTA in the intracellular medium sustained GABA currents (Fig. 3) for up to 1 hour. Under these conditions the GABA current increased after penetrating the cell, reaching a value of 146 ± 26% (SEM) of the control after 19 min and then declined slowly to a value of 124 ± 28% of the control (n = 11) after 30 min (Fig. 3B). When ATP was omitted from the intracellular solution, the GABA current declined consistently to less than 10% after 10 min (Fig. 3B and Table 1).

To further explore the basis for the stability of the GABA response, we systematically omitted Mg^{2+}, ATP, or BAPTA from the intracellular medium (Table 1). Both Mg^{2+} and ATP were essential for the stability of the GABA conductance in the presence of 10 m*M* BAPTA (Table 1); ATP alone did not suffice. This points to the involvement of the Mg^{2+}-ATP complex and implicates an adenosine triphosphatase in the stabilizing reaction. The hydrolysis of ATP is confirmed by the fact that AMP-PCP [adenylyl(β,γ-methylene)diphosphonate (*17*)], a non-hydrolyzable analog of ATP, did not support the maintenance of the GABA conductance (Table 1 and Fig. 1C). This also militates against ATP acting as an intracellular modulator and stabilizing the GABA receptor through noncovalent interaction with the GABA receptor.

The presence of Mg^{2+}-ATP alone was insufficient to maintain the GABA sensitivity of hippocampal neurons; an intracellular medium that buffered $[Ca^{2+}]_i$ to less than $10^{-7}M$ was also needed to stabilize the GABA response (Table 1). This indicates that elevated $[Ca^{2+}]_i$ can destabilize the GABA receptor.

The minimal requirement for the maintenance of the GABA receptor under conditions of whole-cell recording was the provision of an intracellular solution with Mg^{2+}-ATP and low $[Ca^{2+}]_i$. Our data suggest that ATP needs to be hydrolyzed to confer stability on the receptor. This indicates that either the receptor or some entity associated with it is covalently modified, probably by phosphorylation.

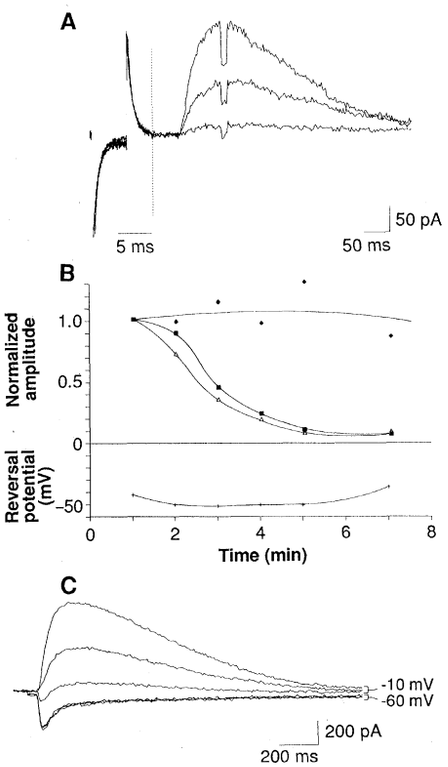

Fig. 1. Run down of GABA-activated current results from the loss of GABA conductance. (**A**) Whole-cell recording of outward GABA currents elicited by agonist application at 1, 3, and 5 min (in order of decreasing current amplitude) after cell penetration. The holding potential was −10 mV. GABA was applied by short-duration (20-ms) pressure pulses (20 psi) to a GABA-containing pipette (100 μ*M*). Voltage pulses of −10 mV, 5-ms duration, were applied before the onset of the GABA responses to monitor the leak conductance and cell capacitance (current response shown to the left of the dotted line; note the different time scales on either side of the dotted line). The average value for input capacitance was 12.4 ± 2.6 pF (mean ± SEM, n = 10). Voltage pulses of −10 mV and 20 ms were applied close to the peak of the GABA response to measure the instantaneous conductance elicited by GABA (the current response to the onset of the hyperpolarizing pulse was measured for the calculation). GABA conductance and reversal potential were calculated (*4*) on the assumption that the current-voltage (*I-V*) characteristic was linear. As the *I-V* relation rectified in the outward direction this method overestimates the GABA reversal potential; however, the form of the *I-V* relation did not change during run down. (**B**) The normalized GABA current (triangle, initial 211 pA), GABA conductance (square, peak 6.6 nS) and input resistance (diamond, initial 842 megohms), and GABA reversal potential (+). Similar results were obtained for all cells in this report. (**C**) Response of hippocampal neuron to pressure application of a solution containing GABA and glutamate (both 100 μ*M*). Neurotransmitters were applied every 30 s, alternating holding potential between −10 and −60 mV, to measure the GABA and glutamate response, respectively. GABA responses were recorded at 1, 5, and 10 min after cell penetration; glutamate responses were elicited 30 s after each GABA response. The reversal potentials for $GABA_A$ and glutamate responses were close to −65 and −10 mV, respectively. The early small inward current appearing at the final stage of GABA run down is induced by glutamate, which has a reversal potential slightly more depolarized than the holding potential of −10 mV. In this experiment the intracellular solution contained 10 m*M* BAPTA, 4 m*M* $MgCl_2$, and 2 m*M* AMP-PCP.

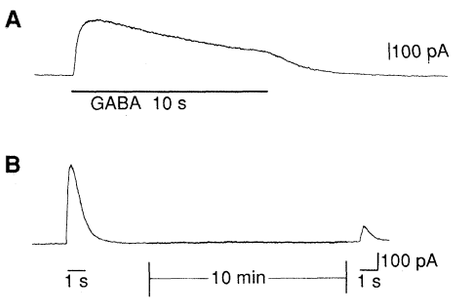

Fig. 2. Desensitization of GABA responses. (**A**) Time course of current during a 10-s application of GABA to cells in 4 m*M* Mg^{2+}, 2 m*M* ATP, and 10 m*M* EGTA. The record was obtained after 12 min of intracellular recording under stable conditions. (**B**) The first GABA current was elicited immediately after cell penetration. The second response was activated 10 min later. Intracellular solution in this case contained 10 m*M* BAPTA but no Mg^{2+}-ATP. Similar results were obtained in six out of six cells under various unstable conditions.

Until recently there was little information on the possible involvement of second messenger systems and protein phosphorylation in the regulation of the $GABA_A$ channel; however, two important findings have been made. First, the β-subunit of the $GABA_A$ channel contains a cyclic AMP (cAMP)–dependent kinase consensus sequence (*18*); but phosphorylation of this site has not been demonstrated. Second, the isolated $GABA_A$ receptor has been shown to be phosphorylated; however, the reaction could not be attributed to a known protein kinase (*19, 20*).

It has been postulated that the observed phosphorylation site may modulate GABA desensitization (*18*), as it does in the acetylcholine receptor (*21*). Our data suggest that phosphorylation may also serve to maintain the functional state of the $GABA_A$ receptor. A similar mechanism occurs in acetylcholine receptors of chick ciliary ganglion neurons (*22*).

The intracellular conditions for the preservation of the GABA conductance are similar to those described for the maintenance of noninactivating Ca^{2+} channels in neurons (*10, 23*) and the M current in frog ganglion neurons (*24*). In snail and GH_3 neurons evidence suggests that the Ca^{2+} channel is phosphorylated by cAMP-dependent protein kinase, and the phosphate group is removed by a Ca^{2+}-calmodulin–dependent phosphatase (*10, 25*). Only the phosphorylated form is proposed to be available for activation by membrane voltage. Our evidence suggests that a similar mechanism might be at work in $GABA_A$ channels.

We have shown that the GABA-mediated Cl^- conductance can be regulated by intracellular Mg^{2+}-ATP and $[Ca^{2+}]_i$. However, it remains to be seen how the GABA-activated Cl^- conductance is regulated in vivo. Earlier studies have shown that hippocampal neurons experience a reduction in GABA sensitivity after tetanic stimulation of afferent fibers (*3*). Our results raise the possibility that the modification of the $GABA_A$ conductance may result from a change in the state of phosphorylation of the receptor.

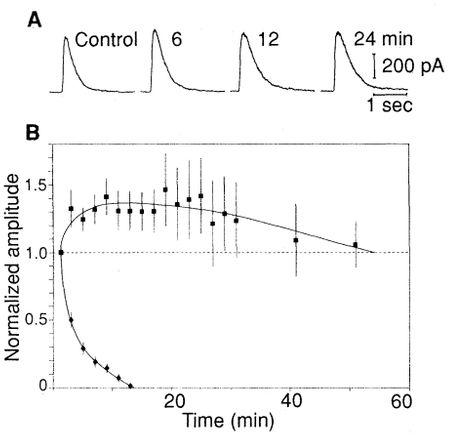

Fig. 3. Both Mg^{2+}-ATP and calcium buffers are necessary for the stability of GABA responses. (**A**) Outward GABA currents recorded by a pipette filled with a solution that included 4 m*M* $MgCl_2$ and 2 m*M* Na_2-ATP in addition to the constituents listed in Fig. 1. (**B**) Squares represent averaged GABA responses of 11 cells elicited under "stable" conditions; diamonds depict the average time course of responses elicited in 14 cells with 10 m*M* BAPTA, 4 m*M* $MgCl_2$, and 0 m*M* ATP in the intracellular solution. The bars mark the SEM.

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14. Huguenard and Alger (*4*) have noted a similar loss of GABA conductance in acutely dissociated hippocampal pyramidal cells.
15. We prepared cultured hippocampal neurons from neonatal rats by using a modified version of the method of M. Dichter [*Brain Res.* **149**, 279 (1978)]. Recordings were made from neurons maintained in culture for 8 to 24 days.
16. Run down of the GABA responses in cultured cells has been alluded to by some investigators. S. Vicini *et al.* [*Proc. Natl. Acad. Sci. U.S.A.* **83**, 9269 (1986)] reported a decline of inhibitory synaptic currents during whole-cell recording from cultured hippocampal neurons. O. Hamill (personal communication) has noted the run down of GABA channels in outside-out patches derived from cultured neurons. In cultured chick neurons, the GABA response declines when recordings are performed in the whole-cell configuration but not with conventional high-resistance electrodes (D. Farb, personal communication).
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20. We have attempted to identify the protein kinase involved in the maintenance of $GABA_A$-receptor function. Inclusion of cAMP-dependent protein kinase inhibitor (Peninsula PKI 5-24) (20 ng/ml) in the recording pipette resulted in a run down under stable conditions to 54.7 ± 11.31% (mean ± SEM, $n = 9$) as measured 10 min after penetration of the cells. However, extracellularly applied forskolin (50 μ*M*) and 8-bromo cAMP (10 μ*M*) produced inconsistent effects on GABA current. GABA currents measured 4 min after drug application were 118 ± 22% of the control in the presence of forskolin and 102 ± 17.4% in 8-bromo cAMP ($n = 7$ and 8, respectively). Phorbol 12,13-dibutyrate (200 n*M*) suppressed the GABA conductance to 69.5 ± 4.1% ($n = 10$) and to 45.1 ± 7.2% ($n = 5$) at 400 n*M*, whereas 4α-101 phorbol had no effect on GABA conductance. These data exclude protein kinase C as the enzyme of up regulating phosphorylation. Ca^{2+}-calmodulin–dependent kinase is unlikely to be operative, as Ca^{2+} destabilizes the GABA conductance. There could also be a unique kinase associated with the GABA receptor as for the β-adrenergic receptor (*26*).
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