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ACCELERATED COMMUNICATION

"Run-Down" of γ-Aminobutyric Acid$_A$ Receptor Function during Whole-Cell Recording: A Possible Role for Phosphorylation

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SUMMARY

When using whole-cell recording methods and a minimal intracellular medium containing only inorganic ions, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, and *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid, we have observed a time-dependent decrease in the responsiveness of cultured chick spinal cord neurons to γ-aminobutyric acid (GABA). The current evoked by 30 μM GABA progressively declined to approximately 30% of its initial value after five applications at 10-min intervals. This was accompanied by an equivalent decline in the GABA-evoked membrane conductance. "Run-down" of the response was reduced when Mg^{2+}-ATP was present in the pipet solution. Inclusion of ATP-γ-S, an analog that donates a thiophosphate group resistant to hydrolysis, also reduced run-down. The nonhydrolyzable analog β,γ-imidoadenosine-5′-triphosphate was without effect. These results suggest that an ATP-dependent process, possibly phosphorylation, is involved in the maintenance of GABA$_A$ receptor function.

GABA, a major inhibitory neurotransmitter in the vertebrate central nervous system, mediates fast synaptic inhibition via the activation of a GABA$_A$ receptor-linked chloride ionophore. The ability of numerous drugs and toxins to alter the activity of this receptor is well documented (for reviews see Refs. 1–3). In contrast, comparatively little is known of the role of intracellular factors in the control of GABA$_A$ receptor function.

The whole-cell configuration of the patch-clamp recording technique (4) provides an attractive approach by which to investigate this problem. Diffusional exchange between the cell and pipet contents (5, 6) not only allows manipulation of the intracellular ionic environment but may also result in the loss of cytoplasmic components necessary to maintain the current under investigation. By determining the intracellular conditions necessary for preservation of the current, it is possible to gain an insight into the mechanisms responsible for the control of receptor or channel activity (7, 8).

In the present report, we describe the progressive loss, or "run-down," under whole-cell recording conditions of GABA-evoked currents in cultured chick spinal cord neurons and provide evidence that an ATP-dependent process, most likely phosphorylation, is involved in the maintenance of GABA$_A$ receptor function.

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Materials and Methods

Tissue culture. Neuronal cultures were prepared from spinal cords of 7-day chick embryos as previously described (9). Briefly, dissociated cells were plated on collagen-coated 35-mm tissue culture dishes in Eagle's minimal essential medium supplemented with glutamine (2 mM), heat-inactivated horse serum (10%, v/v), chick embryo extract (5%, v/v), penicillin (50 μg/ml), and streptomycin (50 μg/ml). To control the proliferation of nonneuronal cells, cytosine arabinoside (1 μM) was added after 24 hr. This medium was removed 24 hr later and replaced with a similar one supplemented with glucose (20.5 mM) and KCl (18 mM), containing only 2.5% chick embryo extract. Fresh medium was added twice weekly. Cultures were kept in a humidified atmosphere of 5% CO_2/95% air at 37° and were used for recording 2–4 weeks after plating.

Electrophysiology. Experiments were carried out on the stage of an inverted microscope equipped with phase contrast optics. Conventional patch-clamp methods were used to record whole-cell currents (4). Electrodes were pulled from thin-wall borosilicate glass (Fisher Scientific, Pittsburgh, PA) and had resistances of 2–8 MΩ when filled with intracellular recording solution. This contained (in mM) NaCl 3, KCl 140, $MgCl_2$ 1, EGTA 11, and HEPES 10, adjusted to pH 7.2 with KOH. Cells were bathed in a solution containing (in mM) NaCl 150, KCl 4, $CaCl_2$ 1, $MgCl_2$ 1, and HEPES 10, adjusted to pH 7.2 with NaOH.

Recordings were made using a Yale Mk V amplifier or an Axopatch 1B patch-clamp (Axon Instruments, Burlingame, CA). Currents were filtered using an eight- or four-pole Bessel filter (1–5 kHz, −3 dB) and either recorded on a chart recorder (MFE 1200) or digitized (16–40

ABBREVIATIONS: GABA, γ-aminobutyric acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; ATP-γ-S, adenosine-5′-*O*-(3-thiotriphosphate); AMP-PNP, β,γ-imidoadenosine-5′-triphosphate.

msec/point) using an on-line data acquisition system (pClamp; Axon Instruments). After forming a tight seal (typically 1–5 GΩ) the patch of membrane within the pipet was disrupted by gentle suction to obtain the whole-cell configuration. Cells had resting potentials of −55 to −70 mV and input resistances in excess of 200 MΩ. All recordings were made with the cell membrane potential clamped at its resting value.

GABA (3 or 30 μM in bath solution) was applied by pressure ejection (15 psi) from blunt-tipped pipets positioned approximately 50 μm from the neuronal soma (10). Responses to GABA applications of 15-, 20-, or 25-sec duration were obtained at fixed times (2, 10, 20, 30, 40, 50, and 60 min) after the whole-cell recording was established.

In a number of cases, the conductance change associated with the GABA response (g_{GABA}) was determined. Hyperpolarizing voltage steps (10 or 20 mV, 350 msec; 1 Hz) were applied before and during GABA application and g_{GABA} was calculated from the magnitude of the resulting current responses (11). Throughout, results are expressed as mean ± standard error of the mean; statistical comparison of groups was carried out using Student's *t* test.

Results and Discussion

Virtually all cells tested were responsive to GABA, which at negative membrane potentials produced an inward current (I_{GABA}). The GABA response was blocked by bicuculline, potentiated by chlordiazepoxide, and reversed at or near 0 mV in symmetrical Cl^-, consistent with it being a $GABA_A$ receptor-mediated Cl^- current.

Responses to 3 μM GABA (180 ± 26 pA, 14 experiments) developed slowly, were maintained for the duration of GABA application, and could be repeated at low frequency with little change in their peak amplitude (Fig. 1). In contrast, currents evoked by 30 μM GABA rapidly reached a peak (2.1 ± 0.3 nA, 13 experiments), slowly 'desensitized' during the time course of GABA application, and progressively declined in amplitude with repetition. As shown in Fig. 2A, after five applications of 30 μM GABA (40 min of recording) less than 30% of the initial peak I_{GABA} could be elicited. The time after patch rupture at which peak I_{GABA} declined to 50% of that obtained in the initial response ($T_{½}$) was 21.6 ± 2.7 min (13 experiments). The different stability of the currents evoked by 3 μM and 30 μM GABA was also apparent when these two concentrations were applied in sequence to the same cell (Fig. 2B).

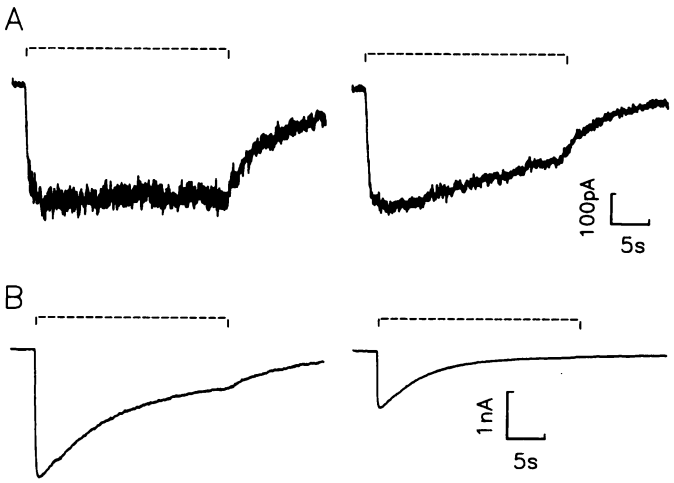

Fig. 1. Representative responses (I_{GABA}) to 3 μM GABA (A) and 30 μM GABA (B) obtained from two different neurons. In A and B the *left trace* shows the inward current resulting from the first application of GABA, at 2 min, and the *right trace* shows the current resulting from the fourth application, at 30 min. The *bar* above each trace indicates the period of GABA application. The holding potential was −60 mV in both A and B.

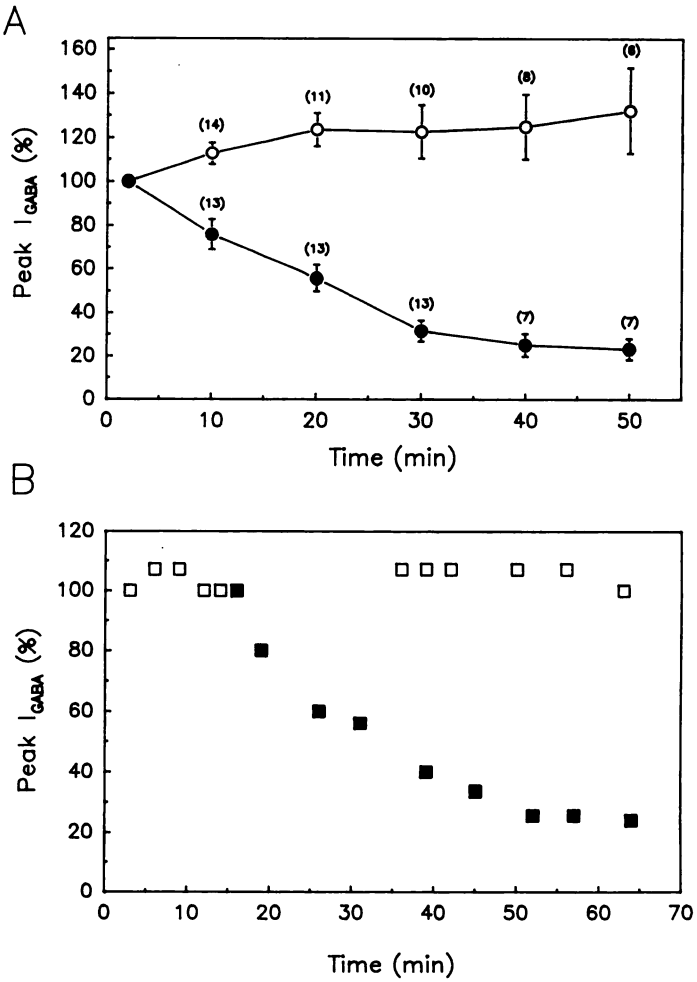

Fig. 2. Peak I_{GABA} produced by 30 μM but not 3 μM GABA declines during repeated transmitter application. A, Pooled results from cells tested with either 3 or 30 μM GABA. The normalized peak currents evoked by 3 μM (○) and 30 μM GABA (●) are shown as a function of time after establishing the whole-cell recording. *Vertical bars* represent standard error; the number of cells is shown in *brackets*. B, Plot showing the time course of changes in peak I_{GABA} evoked by 3 μM (□) and 30 μM GABA (■) applied in sequence to a single cell. The initial responses to 3 μM GABA and 30 μM GABA were 70 pA and 1.25 nA, respectively. Similar results were obtained with three other cells.

Conceivably, "run-down" of the response to 30 μM GABA could result either from the loss of activatable receptors (or altered channel-gating properties) or from a change in the driving force for Cl^- during the course of the experiment. A similar argument applies to the fading of I_{GABA} in the continued presence of agonist (11–14). If a decreased driving force for Cl^- were solely responsible for the decline in I_{GABA} then there should be no change in GABA-evoked conductance (g_{GABA}). The results shown in Fig. 3 demonstrate that this is not the case. For individual responses, the decline in I_{GABA} was accompanied by a parallel decline in g_{GABA} (Fig. 3A), suggesting that under our recording conditions this fading represents receptor desensitization. Likewise, the run-down of peak I_{GABA} with repeated GABA applications was associated with an equivalent loss of GABA-activatable conductance (Fig. 3B), thus ruling out possible Cl^- redistribution as its cause.

Run-down was accompanied by a consistent increase in the

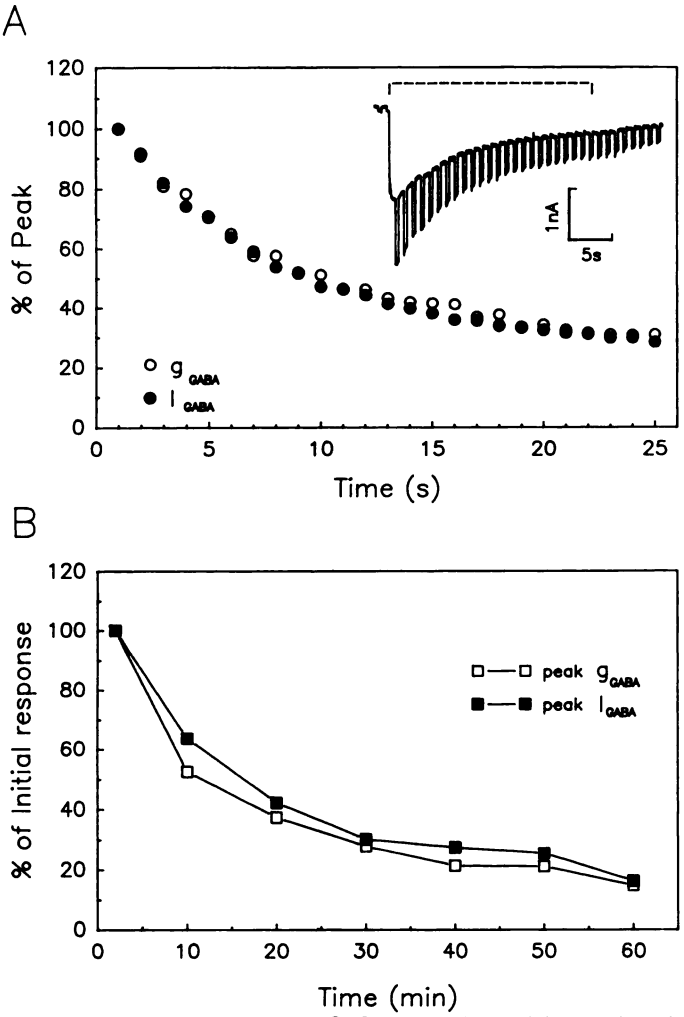

Fig. 3. The decline in I_{GABA} during GABA application and the run-down in peak I_{GABA} with repeated applications both reflect a loss of GABA-activatable conductance. A, Plot showing the decline of I_{GABA} and g_{GABA} during the course of a single GABA application. *Inset*, current record showing the effect of hyperpolarizing voltage steps (20 mV, 350 msec, 1 Hz) used to determine g_{GABA}. In this example, the holding potential was −60 mV, peak I_{GABA} was 1.8 nA and peak g_{GABA} was 56 nS. The *horizontal bar* above the trace indicates the period of GABA application. B, Plot showing the parallel decline of peak I_{GABA} and peak g_{GABA} during repeated GABA applications (same cell as A). Similar results were obtained in three other cells.

extent of desensitization (*%D*) produced by single GABA applications. This was calculated according to the formula;

$$\%D = [(\text{peak } I_{GABA} - \text{off } I_{GABA})/\text{peak } I_{GABA}] \cdot 100$$

where peak I_{GABA} is the current at the peak of the GABA response and off I_{GABA} is the current remaining immediately before the termination of the GABA pulse. Because a steady state I_{GABA} was generally not reached during the GABA application times employed, *%D* does not represent the maximum extent of desensitization attainable. Nevertheless, it provides a convenient way to compare responses. For example, *%D* at 30 min (81.6 ± 2.6%, 13 experiments) was significantly greater ($p < 0.01$, paired Student's t test) than that at 2 min (62.4 ± 4.4%). Although the rate of desensitization was not routinely quantified, an increase during run-down was apparent in many cells (Fig. 4), as would be predicted by the change in *%D*.

The acceleration of desensitization cannot be accounted for

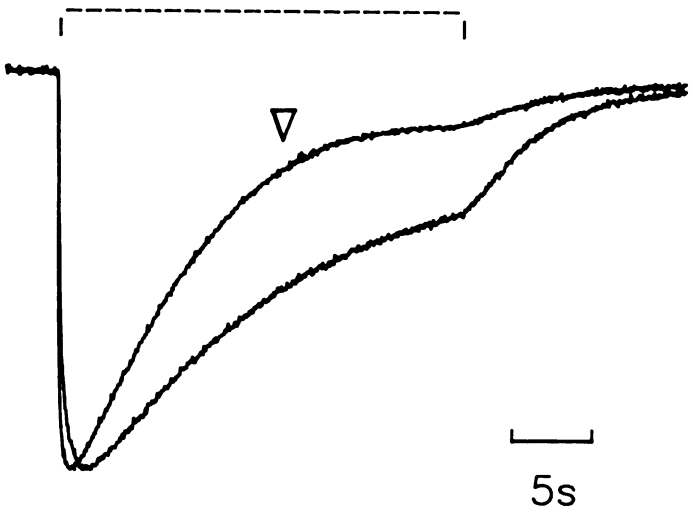

Fig. 4. The rate and extent of desensitization to 30 μM GABA increase during the recording period. Representative responses from a single cell showing the current obtained at 2 min (peak, 3.1 nA) and the current obtained at 20 min (peak, 2.0 nA) (∇). The *horizontal bar* above the traces indicates the period of GABA application. The traces have been scaled to the same peak. *%D* was 64.9% at 2 min and 87.8% at 20 min. In each case the decline of I_{GABA} was adequately described by a single exponential decay with a time constant of 14.5 sec at 2 min and 8.1 sec at 20 min.

by the *decrease* in peak I_{GABA}. Using conventional intracellular recording methods, we have previously shown that, under normal conditions, an *increase* in the amplitude of the GABA response results in an increased rate and extent of desensitization (15). Whether an acceleration of desensitization could itself account for the decline in peak I_{GABA} is less certain. Because the time required for 30 μM responses to peak was approximately 200–800 msec, rapid desensitization of the GABA receptor, such as that observed in studies of $^{36}Cl^-$ uptake ($t_{1/2}$ = 32 msec; Ref. 16), may have taken place during the rising phase of the response. An increase in the rate of such desensitization would be expected to attenuate peak I_{GABA}.

Importantly, although run-down was always associated with an increase in *%D*, the converse was not true. Responses to 3 μM GABA did not exhibit run-down and were always initially nondesensitizing, but in 6 of 14 cells desensitization developed progressively during the course of the recording. In the example shown in Fig. 1A, *%D* was less than 5% at 2 min but increased to 39.5% at 30 min. It is thus unclear whether the time-dependent changes in the pattern of desensitization to 30 μM GABA are related to run-down or whether they represent an independent phenomenon.

Given that the responsiveness of chick spinal cord neurons to GABA does not decline when recordings are made using conventional intracellular microelectrodes (e.g., Refs. 15 and 17), it is tempting to ascribe the run-down observed with whole-cell recording to the loss of a soluble cytoplasmic constituent necessary to maintain channel function. Rapid diffusional exchange between the cell and the recording pipet is well documented (5, 6) and the loss of high energy phosphates by this route has been shown to account, at least in part, for the "washout" of voltage-activated calcium currents (7, 8, 18). To investigate the possibility that a similar process may be involved in the run-down of GABA responses, we examined the effect of cell dialysis with ATP. Addition of 5 mM Mg^{2+}-ATP to the pipet solution decreased run-down such that in only one of five

cells did peak I_{GABA} decline to less than 50% of its initial value within the 50-min recording period. At 30 min, the current remaining was significantly greater than that in the absence of ATP (Fig. 5). A more striking protection against run-down was afforded by ATP-γ-S, a phosphorothioate analog of ATP (19). When added to the intracellular solution at a concentration of 28 mM (plus 8 mM Mg^{2+}, a combination previously shown to slow the rate of Ca^{2+} channel inactivation in dialyzed *Helix* neurons) (7) run-down was markedly reduced (Fig. 5). A similar result was produced by 5 mM ATP-γ-S (plus 5 mM Mg^{2+}). For none of the 13 cells tested did peak I_{GABA} fall below 50% of its initial value, and in some cases it actually increased slightly during the recording period.

ATP-γ-S can not sustain the activity of ATPases such as the Na^+-K^+ pump and the Ca^{2+} pump (20, 21) but it can act as a substrate for protein kinases (22, 23), with resultant protein thiophosphorylation. It therefore seems most likely that exogenous ATP reduces run-down by acting as a phosphate source for protein phosphorylation. The failure of AMP-PNP, a nonhydrolyzable analog of ATP, to prevent run-down (Fig. 5; $T_{½}$ = 18.8 ± 2.6 min, six experiments), argues against ATP stabilization of the GABA receptor through a noncovalent interaction.

Because the thiophosphate group donated by ATP-γ-S is removed from proteins only slowly by phosphatases (23, 24), one would expect a greater number of target proteins to be phorphorylated at any given time in the presence of ATP-γ-S than in the presence of ATP. The slightly greater protective effect of both 5 and 28 mM ATP-γ-S compared with that of 5 mM Mg-ATP (Fig. 5) may be explained in this manner. Interestingly, only 28 mM ATP-γ-S prevented the increase in *%D* seen during the course of recording (Table 1). This high concentration could conceivably have led to an increased *degree* of phosphorylation of a given protein, an increased *number* of phosphorylated molecules, or an increased *variety* of phosphorylated species. There was no significant difference between any of the recording conditions with regard to the extent of

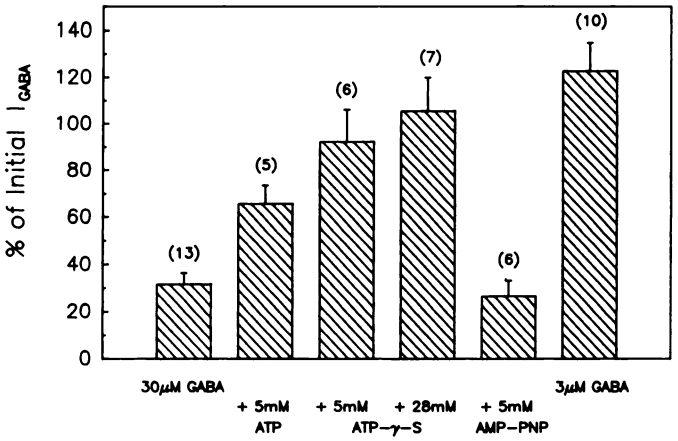

Fig. 5. The run-down of peak I_{GABA} is reduced by Mg^{2+}-ATP and ATP-γ-S but not by AMP-PNP. Pooled results showing the percentage of initial peak I_{GABA} remaining after 30 min under different recording conditions (ATP was added as the Mg^{2+} salt; 5 mM $MgCl_2$ was added with AMP-PNP and 5 mM ATP-γ-S; 8 mM $MgCl_2$ was added with 28 mM ATP-γ-S). The number of cells is shown in *brackets*; *vertical bars* represent standard error. The currents remaining at 30 min with Mg^{2+}-ATP and 5 mM and 28 mM ATP-γ-S were significantly greater than that in minimal recording medium ($p < 0.005$, unpaired Student's *t* test).

TABLE 1

Effect of ATP, ATP-γ-S, and AMP-PNP on the extent of desensitization produced by the first and fourth applications (2 and 30 min, respectively) of 30 μM GABA

Data are mean ± standard error.

Addition	Desensitization (%*D*)		*n*[a]
	2 min	30 min	
	%		
None	62.4 ± 4.4	81.6 ± 2.6[b]	13
5 mM AMP-PNP	68.9 ± 2.0	80.7 ± 4.1[b]	6
5 mM Mg^{2+}-ATP	62.2 ± 4.4	76.3 ± 2.0[b]	5
5 mM ATP-γ-S	52.6 ± 5.8	70.3 ± 5.2[b]	6
28 mM ATP-γ-S	56.5 ± 3.9	45.8 ± 5.5	7

[a] Number of experiments.
[b] $p < 0.05$ for difference between %*D* at 2 and 30 min (paired Student's *t* test).

desensitization observed for the initial GABA application (2 min; Table 1).

The primary structure of the bovine $GABA_A$ receptor contains a cAMP-dependent serine phosphorylation consensus sequence on a presumed intracellular portion of the GABA-binding β subunit (25). Although phosphorylation at this particular site has not been demonstrated, two proteins in a preparation of $GABA_A$ receptor from rat brain have been shown to be phosphorylated, possibly by a receptor-associated protein kinase (26). In light of this evidence, it seems not unreasonable to suggest that the GABA receptor itself may serve as a target for thiophosphorylation by ATP-γ-S. If one makes this assumption, then our results would indicate an effect of phosphorylation on the $GABA_A$ receptor that is clearly different from both the acceleration of nicotinic acetylcholine receptor desensitization seen in *Torpedo* (27) and the regulation of functional receptor number proposed for nicotinic receptors on chick ciliary ganglion neurons (28). This latter distinction follows from the concentration dependence of run-down. If run-down was simply due to a decrease in the number of functional GABA receptors, then responses to 3 μM and 30 μM GABA would be expected to decline in parallel. They did not. There are two possibilities. Either the concentration dependence of run-down results from the existence of a necessary phosphorylation step in the operation of the receptor-linked ionophore or, more parsimoniously, two receptor populations exist. The present results do not allow us to distinguish between these alternatives. However, previous electrophysiological and ion-flux studies have suggested the existence of more than one $GABA_A$ receptor subtype (16, 29, 30). Further studies are required to resolve this issue.

Run-down of GABA-evoked currents under whole cell recording conditions is not restricted to spinal cord neurons. A similar loss of responsiveness has been noted in cultured cortical neurons (31) and both cultured (32) and acutely dissociated (11, 32) hippocampal neurons. In the latter case, run-down has also been shown to be prevented by intracellular dialysis with ATP (32). On the basis of the Mg^{2+} sensitivity of this stabilizing effect, together with the ineffectiveness of β,γ-methyleneadenosine-5′-triphosphate, a nonhydrolyzable ATP analog, the authors suggest an involvement of protein phosphorylation. Our results with ATP-γ-S (and a different nonhydrolyzable ATP analog) strongly support this proposal. Protein phosphorylation is now recognized as an important regulatory mechanism governing the activity of several voltage- and ligand-gated membrane ion channels (33). These new findings

raise the possibility that $GABA_A$ receptors may also be subject to its controlling influence.

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