0026-895X/94/030542-08$03.00/0

MOLECULAR PHARMACOLOGY, 46:542-549, 1994.

Phosphorylation Factors Control Neurotransmitter and Neuromodulator Actions at the γ-Aminobutyric Acid Type A Receptor

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Received April 19, 1994; Accepted June 13, 1994

SUMMARY

Whole-cell and patch-voltage clamp experiments were carried out on cultured chick spinal cord neurons to investigate the dependence of γ-aminobutyric acid $(GABA)_A$ receptor function on intracellular phosphorylation factors. Without ATP in the intracellular solution, repeated application of 30 μM GABA results in a progressive decline (run-down) of the currents evoked by GABA in standard whole-cell recordings but not when the nystatin-perforated patch method is used. Run-down is also observed in outside-out excised patch recordings, indicating that any enzymatic factors required for run-down must be closely associated with the plasma membrane. Run-down is associated with decreases in both the maximum GABA-induced current and the GABA EC_{50}. Inclusion of magnesium adenosine-5′-*O*-(3-thio)triphosphate in the intracellular buffer prevents the decline in the maximum GABA response but the GABA EC_{50} still decreases, resulting in a "run-up" of the response at low (3 μM) GABA concentrations. Run-down is use dependent, requiring repeated activation of the $GABA_A$ receptor by high (30 μM) GABA concentrations. However, use-independent run-down can be induced by the inclusion of alkaline phosphatase in the intracellular buffer. The response to 3 μM GABA does not normally run down, but run-down is observed when the response to 3 μM GABA is potentiated with pentobarbital or allopregnanolone, suggesting that run-down is a consequence of GABA receptor activation and/or desensitization. Run-down of the potentiated GABA response can be prevented by addition of magnesium adenosine-5′-*O*-(3-thio)triphosphate to the intracellular solution. Strikingly, run-down results in a significant decrease in the potentiating effects of positive modulators, whereas the inhibitory effects of negative modulators such as pregnenolone sulfate and $ZnCl_2$ are unchanged. The results demonstrate that phosphorylation factors have the capacity to control $GABA_A$ receptor pharmacology, affecting the potency and efficacy of GABA, the kinetics of $GABA_A$ receptor desensitization, and the sensitivity of the receptor to modulators such as steroids, benzodiazepines, and barbiturates.

The inhibitory neurotransmitter GABA triggers an increase in the permeability of the nerve cell membrane to chloride ions by interacting with $GABA_A$Rs located in the plasma membrane. The $GABA_A$R has been the subject of intensive investigation because of its prevalence in the vertebrate central nervous system (1) and because of its emergence as a model system for the study of functional modulation of ligand-gated neurotransmitter receptors. Among the classes of compounds that positively modulate (potentiate) GABA responses are benzodiazepines (2, 3), barbiturates (4, 5), steroids (6, 7), and ethanol (8, 9). Conversely, β-carbolines (10, 11), picrotoxin (12), pregnenolone sulfate (13, 14), and several divalent cations (15, 16) negatively modulate GABA-induced responses.

There is considerable evidence to indicate that phosphorylation and dephosphorylation of receptors and/or associated ion channels can serve to control receptor-operated ion channel function (for review, see Ref. 17). Biochemical studies of the $GABA_A$R reveal possible phosphorylation of a protein kinase C site (18) and a cAMP-dependent protein kinase site (18, 19) in the β subunit and a tyrosine kinase site in the γ2 subunit (20); the α subunit of the $GABA_A$R is also phosphorylated by an unidentified kinase (21).

Phosphorylation of the $GABA_A$R/chloride channel complex may be required for the maintenance of its normal functioning (22–24). We have observed a progressive, use-dependent run-down of the GABA response, which is accompanied by an acceleration of desensitization. Both run-down and the acceleration of desensitization are reduced by the inclusion of Mg-ATP or, more effectively, Mg-ATPγS in the recording pipet,

Research support was provided by National Institutes of Health Grants NS23140 and NS22116.
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ABBREVIATIONS: GABA, γ-aminobutyric acid; $GABA_AR$, γ-aminobutyric acid type A receptor; $GABA_BR$, γ-aminobutyric acid type B receptor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; ATPγS, adenosine-5′-*O*-(3-thio)triphosphate.

consistent with the hypothesis that phosphorylation is required for the maintenance of $GABA_AR$ function.

We report here that $GABA_AR$ run-down also occurs in excised outside-out membrane patches, indicating that any phosphatases or other factors required for run-down must be closely associated with the plasma membrane. Run-down is absent during recording from whole cells by the nystatin-perforated patch method, supporting the view that run-down is due to the wash-out of soluble cytoplasmic factors. We have now investigated the influence of positive modulators of the GABA response on $GABA_AR$ run-down. We find that, although 3 μM GABA does not normally elicit run-down, run-down can be induced by applying 3 μM GABA in combination with a positive modulator of the GABA response, such as pentobarbital or allopregnanolone. Run-down results in a decrease in the maximum GABA response, a decrease in the EC_{50} for GABA, and decreased potentiation by benzodiazepine, barbiturate, and steroid modulators of the GABA response. Taken together, these results suggest that phosphorylation may play an important role in regulating the activity of the $GABA_AR$ and its sensitivity to modulators.

Materials and Methods

Tissue culture. Neuronal cultures were prepared from spinal cords of 7-day chick embryos as described previously (25). 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-activated 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 non-neuronal cells, cytosine arabinoside (1 μM) was added 24 hr after plating. 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 (26). Electrodes were pulled from thin-wall borosilicate glass (Fisher Scientific, Pittsburgh, PA) and had resistances of 4–5 MΩ when filled with minimal intracellular recording solution. This contained the following (in mM): NaCl, 3; KCl, 140; $MgCl_2$, 1; EGTA, 11; and HEPES, 10; adjusted to pH 7.2 with KOH. In the corresponding experiments, 5 mM Mg-ATP or Mg-ATPγS was added to the minimal intracellular solution. Cells were bathed in a solution containing the following (in mM): NaCl, 150; KCl, 4; $CaCl_2$, 1; $MgCl_2$, 1; and HEPES, 10; adjusted to pH 7.2 with NaOH.

GABA-evoked chloride currents were recorded using an Axopatch 1B patch-clamp amplifier (Axon Instruments, Burlingame, CA). Current records were digitized (16–40 msec/point) using an on-line data acquisition system (pClamp; Axon Instruments). 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 being held at −60 mV.

Voltage-clamp recording using the perforated patch technique (27) was carried out in the same manner as whole-cell recording, except that the neuronal membrane was not ruptured beneath the recording pipet. Instead, nystatin was added to the electrode buffer. It should be noted that we have found that the optimal nystatin concentration for perforated patch recording from chick spinal cord neurons tends to be somewhat higher than that reported for other cell types. We obtained optimal results with 250–400 μg/ml nystatin in the patch pipet; lower concentrations led to unacceptably high series resistances. In contrast to some reports, we have found no difficulty in obtaining good seals with nystatin in the patch pipet and have not found it necessary to back-fill the pipet tip with nystatin-free solution. A small capacitance current was observed immediately after establishment of the cell-attached configuration, due to the immediate pore-forming effect of nystatin. The capacitance current developed with time, and within 10 min a low access resistance was obtained. The data shown in this paper were obtained from cells with series resistances below 20 MΩ. Initial GABA responses were measured 3 min after stabilization of the capacitance current.

GABA was applied by pressure ejection (15 psi) from blunt-tipped pipets positioned approximately 50 μm from the neuronal soma. To elicit run-down, 30 μM GABA was applied for 20 sec at 2, 10, 20, and 30 min after the whole-cell configuration had been obtained.

For outside-out patch-clamp recordings, a whole-cell recording was first obtained and then the recording pipet was slowly elevated until the patch was separated from the cell body. GABA was applied by pressure ejection, as described above for whole-cell recordings.

The percentage desensitization ($\%D$) produced by a single GABA application was calculated according to the formula $\%D = 100 \times (I_{GABApeak} - I_{GABA5s})/I_{GABApeak}$, where $I_{GABApeak}$ is the current at the peak of the GABA response and I_{GABA5s} is the current remaining after 5 sec of continuous GABA application.

Dose-response curves were fit to the logistic equation by nonlinear regression. Results are means ± standard errors. All drugs were obtained from Sigma except pregnenolone sulfate (Steraloids) and Mg-ATPγS (Boehringer Mannheim Biochemicals).

Results

Prevention of run-down in excised membrane patches by ATPγS. As we have reported previously, repeated application of 30 μM GABA to intracellularly dialyzed neurons under whole-cell voltage-clamp conditions results in progressive run-down of I_{GABA}, which can be prevented by inclusion of Mg-ATP or Mg-ATPγS in the intracellular buffer. Fig. 1 shows that run-

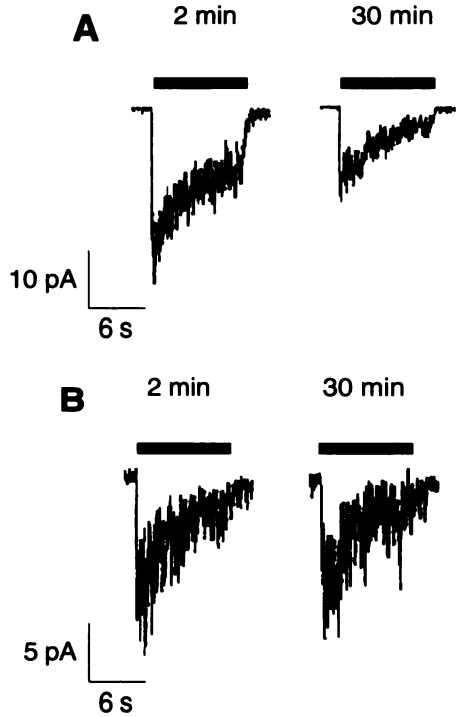

Fig. 1. Run-down of the GABA response in outside-out patches. GABA (30 μM) was applied before (*2 min*) and after (*30 min*) repeated application of 30 μM GABA to outside-out patches. *Bars*, periods of GABA application. A, Minimal electrode buffer; B, minimal buffer plus 5 mM Mg-ATPγS.

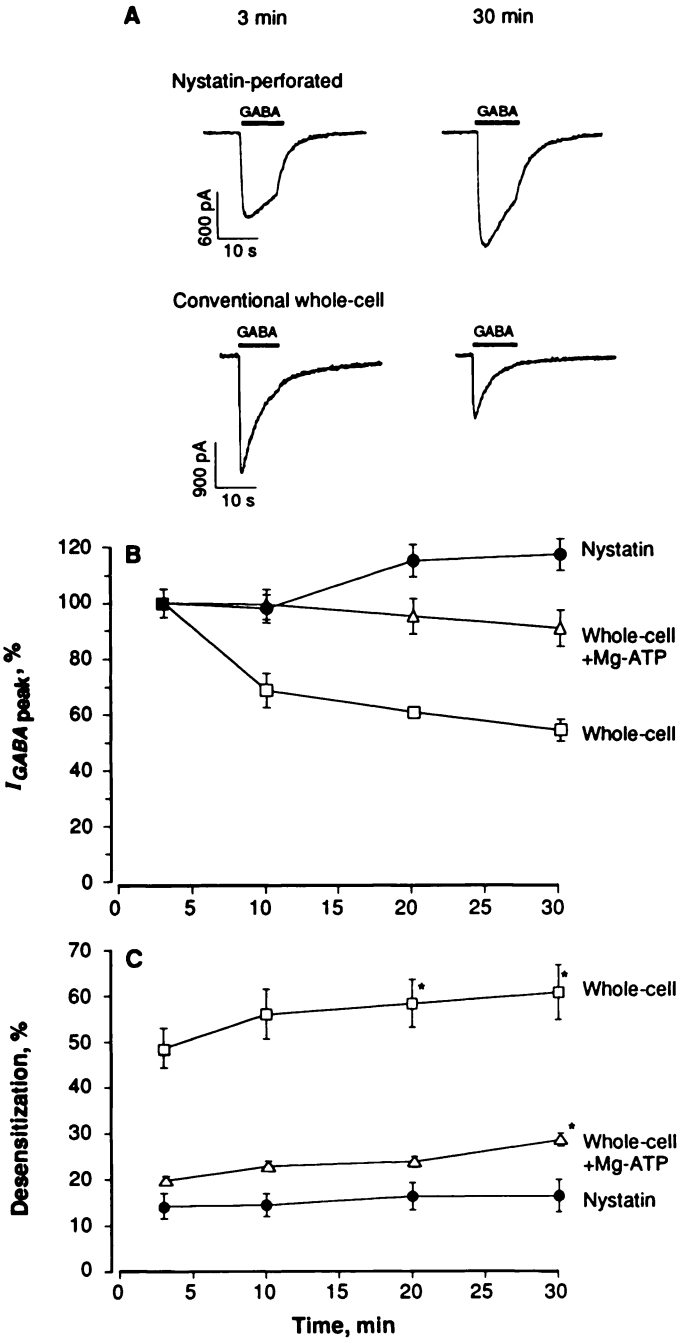

Fig. 2. Absence of run-down with the nystatin-perforated patch technique. A, Typical traces, recorded 3 min and 30 min after either a stable nystatin-perforated whole-cell clamp (*top*) or a standard whole-cell clamp (*bottom*) recording was obtained, are shown. GABA (30 μM) was applied for 10 sec at 3, 10, 20, and 30 min after a stable recording was obtained. Note both the change in peak response from 3 min to 30 min and the more rapid desensitization with the standard whole-cell clamp. B, Run-down of GABA-induced peak membrane currents occurred with conventional whole-cell recording without Mg-ATP (□) (n = 8) but not when the nystatin-perforated patch technique was used (●) (n = 8). Run-down was reduced when 5 mM Mg-ATP was included in the intracellular buffer (△) (n = 12). Patch electrodes contained minimal intracellular buffer with (△) or without (●, □) 5 mM Mg-ATP. For nystatin-perforated patch recording, 250–400 μg/ml nystatin was included in the pipette solution. *Data points*, percentage of initial I_{GABA} (30 μM, 10 sec). *Error bars*, standard errors. C, Desensitization after 5 sec of GABA application was less with the nystatin method (●) than during standard whole-cell voltage-clamp recording without ATP (□), at both early and late times after initiation of the recording. With the whole-cell method, a significant increase in desensitization was evident at the 20- and 30-min time points, compared with the initial (3-min) response. Desensitization was also reduced when 5 mM Mg-ATP was included in the whole-cell pipet (△), although some increase in desensitization was evident at 30 min. Desensitization after 5 sec of GABA application was calculated as a percentage of the peak response, as described in Materials and Methods. Desensitization increased with repeated application of GABA with the whole-cell method. *, Desensitization significantly greater than the first response ($p < 0.05$).

down is also observed when 30 μM GABA is repeatedly applied to excised, outside-out, membrane patches, indicating that any cytoplasmic factors required for run-down must be closely associated with the cell membrane. As with intact neurons, inclusion of 5 mM Mg-ATPγS in the recording pipet prevented run-down of the GABA response.

Absence of run-down with the nystatin technique. If run-down during whole-cell recording is due to depletion of intracellular ATP, then it follows that run-down should not occur during recording by the nystatin-perforated patch method, in which the cell membrane under the patch is not ruptured but rather "perforated" by the channel-forming antibiotic nystatin. Because nystatin channels allow passage of only small monovalent ions, this method should not result in depletion of cytoplasmic ATP. As shown in Fig. 2, there was no run-down during recording for up to 30 min using the perforated patch method, whereas substantial run-down was evident under standard whole-cell recording conditions. In other experiments, the interval between GABA applications was reduced from 10 min to 3 min, again with no evidence of run-down, using the nystatin method (data not shown). There was often a tendency for the peak I_{GABA} to increase somewhat at later times when the perforated patch method was used; this may reflect improvement of the access resistance due to insertion of additional nystatin channels.

During recording under whole-cell conditions without ATP in the recording pipet, desensitization of the GABA response tends to increase as run-down proceeds (Fig. 2C). As expected, this acceleration of desensitization did not occur with the nystatin method. Moreover, desensitization of I_{GABA} was consistently less under perforated patch conditions than under whole-cell conditions, even at the earliest time points, suggesting that a rapid change in the properties of the $GABA_AR$ occurs during the brief (~3-min) period between rupture of the patch and the first whole-cell response. Inclusion of 5 mM Mg-ATP in the whole-cell recording pipet also reduced desensitization at all time points, but there was still a tendency for desensitization to increase with repeated application of 30 μM GABA.

"Run-up" of the 3 μM GABA response with intracellular ATPγS. A striking feature of run-down in chick spinal cord neurons is that there is a pronounced decrease in the response to 30 μM GABA, whereas the response to 3 μM GABA does not decline and in fact tends to increase (22). When 5 mM Mg-ATPγS is present in the intracellular buffer, run-down of the 30 μM GABA response is prevented, whereas the 3 μM GABA response significantly increases or "runs up" with time (Fig. 3). To investigate the mechanism of run-up, the GABA (1–200 μM) dose-response curve was determined for the same neuron both early and late in a recording session. Fig. 4 shows that with 5 mM Mg-ATPγS present in the intracellular solution there was a significant decrease in the GABA EC_{50} but no significant change in the maximum response to GABA.

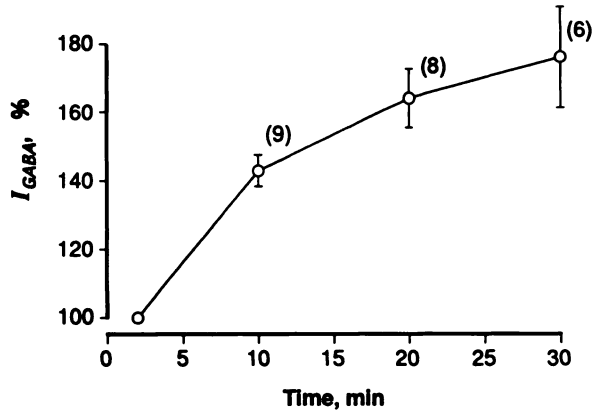

Fig. 3. Mg-ATPγS induction of run-up of the response to 3 μM GABA. I_{GABA} was determined at 2, 10, 20, and 30 min. Peak responses to 3 μM GABA are expressed as a percentage of the initial (2-min) peak I_{GABA}. Mg-ATPγS (5 mM) was included in the intracellular buffer. *Error bars*, standard errors. The number of neurons is given in parentheses.

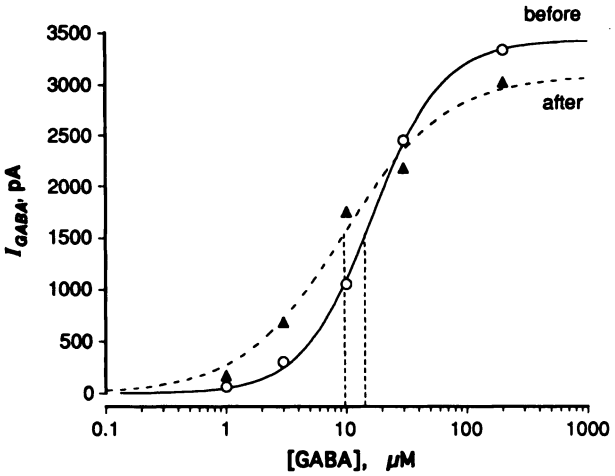

Fig. 4. Effect of ATPγS on the GABA dose-response curve. Typical single-neuron dose-response curves, determined for the same neuron before (○) and after (▲) the run-down protocol (four 20-sec applications of 30 μM GABA at 10-min intervals) with 5 mM Mg-ATPγS in the intracellular solution, are shown. The average EC_{50} values from eight such experiments were 17.0 ± 1.1 μM before and 12.5 ± 5.3 μM after the run-down protocol ($p = 0.012$, paired t test). There was no significant change in the maximum response to GABA (3182 ± 190 pA before and 2836 ± 358 pA after the run-down protocol; $p > 0.20$) or the Hill coefficient (1.77 ± 0.14 and 1.55 ± 0.14, respectively; $p > 0.24$).

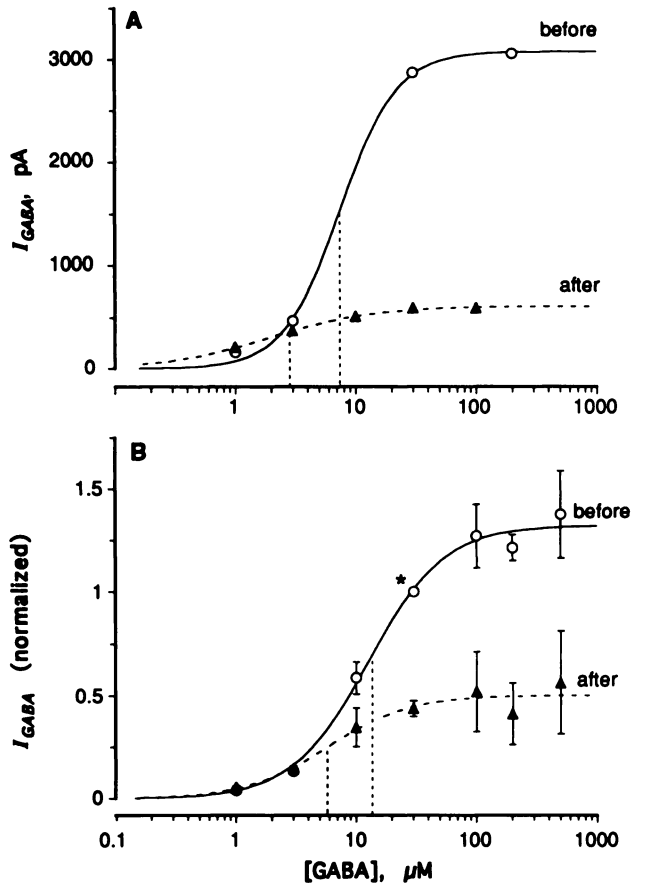

Fig. 5. Effect of run-down on the GABA dose-response curve. Run-down of the GABA response of chick spinal cord neurons under whole-cell voltage-clamp conditions is accompanied by a decrease of the maximal response to GABA and a leftward shift of the GABA EC_{50}. A, Typical GABA dose-response curves determined for a single neuron before (○) and after (▲) run-down was induced by four 20-sec applications of 30 μM GABA at 10-min intervals. Dose-response curves were determined using short GABA pulses to avoid contributing to run-down. The recording electrode contained minimal intracellular solution. In eight cells, the EC_{50} decreased from 16.7 ± 3.9 μM before run-down to 6.4 ± 1.3 μM after run-down. The change in the EC_{50} is statistically significant ($p = 0.015$, paired t test). B, Pooled dose-response data. To pool data, responses for each cell were normalized to the current induced by 30 μM GABA in the same cell before run-down (*). Each *point* is the mean of six to eight neurons. *Error bars*, standard errors. Results of logistic fits to pooled normalized data were in general agreement with means of fits to single-neuron dose-response curves (before rundown, $EC_{50} = 12.5$ μM and $n_H = 1.4$; after rundown, maximum response decreased by 62%, $EC_{50} = 5.8$ μM, and $n_H = 1.3$).

Effect of run-down on the GABA dose-response curve. The observation that run-down selectively decreases the response to high, but not low, concentrations of GABA implies that there must be changes in both the efficacy and potency of GABA. To examine this directly, the GABA (1–200 μM) dose-response curve was determined for a single neuron (using brief pulses of GABA to minimize run-down). Run-down was then induced by four 20-sec applications of 30 μM GABA, and the GABA dose-response curve was redetermined for the same neuron. Fig. 5 shows that run-down resulted in a 62% reduction of the maximum response to GABA, combined with a significant decrease in the GABA EC_{50}.

As reported previously, we do not observe run-down in response to repeated application of 3 μM GABA. To determine whether this is due to the small amplitude of the 3 μM GABA response, we applied 3 μM GABA in combination with either 500 nM allopregnanolone or 100 μM pentobarbital. In the presence of these positive modulators, 3 μM GABA elicits an enhanced I_{GABA} of about 2.5 nA, which is comparable to that elicited by 30 μM GABA alone. As shown in Fig. 6, the potentiated GABA response exhibited the same gradual decline with repeated application that we observe for the responses elicited by 30 μM GABA. As previously reported for run-down induced by 30 μM GABA, run-down was consistently associated with an acceleration of desensitization. The decrease in the peak I_{GABA} was effectively prevented by inclusion of 5 mM Mg-ATPγS in the intracellular solution.

As with run-down induced by 30 μM GABA, the decrease in the amplitude of the potentiated GABA response was use dependent rather than time dependent. Four 20-sec applications of 3 μM GABA plus 100 μM pentobarbital produced

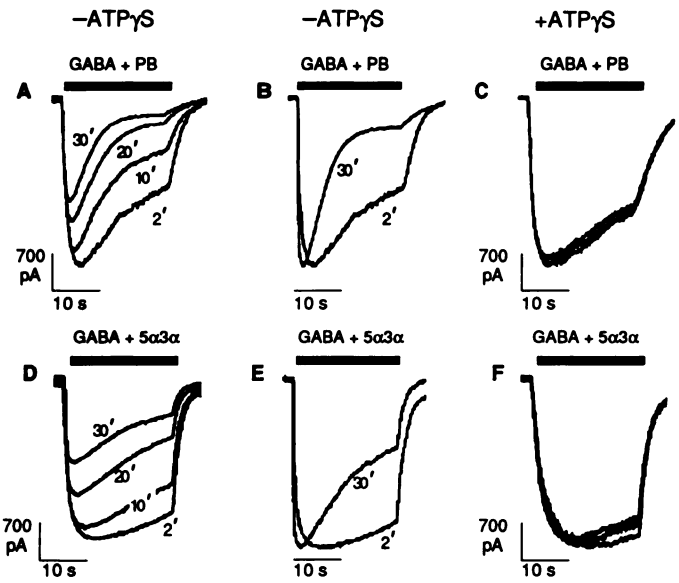

Fig. 6. Run-down of pentobarbital- and allopregnanolone-potentiated GABA responses. A, Response to 3 μM GABA plus 100 μM pentobarbital (*PB*) exhibits run-down during whole-cell recording. Recordings obtained 2, 10, 20, and 30 min after the whole-cell configuration was established, with minimal intracellular solution in the recording electrode, are shown. B, Traces from A, at 2 and 30 min, were rescaled to the same maximum to emphasize the change in desensitization kinetics. C, Responses are stable from 2 to 30 min when 5 mM Mg-ATPγS is present in the intracellular solution. D, Response to 3 μM GABA plus 500 nM allopregnanolone (*5α3α*) exhibits run-down during whole-cell recording. Recordings obtained 2, 10, 20, and 30 min after the whole-cell configuration was established, with minimal intracellular solution in the recording electrode, are shown. E, Traces from D, at 2 and 30 min, were rescaled to the same maximum to emphasize the change in desensitization kinetics. F, Responses are stable from 2 to 30 min when 5 mM Mg-ATPγS is present in the intracellular solution.

substantial run-down after 30 min, but there was no difference in the amplitudes of the responses elicited by two 4-sec applications at 2 and 30 min after patch formation (Fig. 7C; see also Figs. 6A and 7A for examples of traces). Inclusion of alkaline phosphatase in the intracellular solution eliminated the use dependence of run-down, such that prolonged repeated application of GABA was no longer required to elicit run-down (Fig. 7, B and C).

Effect of run-down on $GABA_AR$ modulation. The observation that the response to 3 μM GABA remains relatively stable, whereas the potentiated response declines, implies that run-down is associated with decreased potentiation. To determine the effect of run-down on the action of $GABA_AR$ modulators, we examined the ability of different modulators to potentiate or to inhibit the GABA response, before and after run-down. Table 1 shows that after run-down there was a significant decrease in potentiation of the GABA response by the positive modulators allopregnanolone, progesterone, pentobarbital, and chlordiazepoxide. In contrast, there was no change in inhibition of the GABA response by the negative modulators pregnenolone sulfate and $ZnCl_2$. As with run-down, the decrease in potentiation by positive modulators was prevented when ATPγS was included in the intracellular solution.

Discussion

As we have reported previously, application of 30 μM GABA to cells perfused with minimal intracellular solution (see Materials and Methods) evokes currents of about 2.5 nA, which progressively decline with repeated application of GABA (22). The decrease in the response evoked by 30 μM GABA is due to a change in conductance and not a change in the driving force for chloride (22). There is no change in the reversal potential

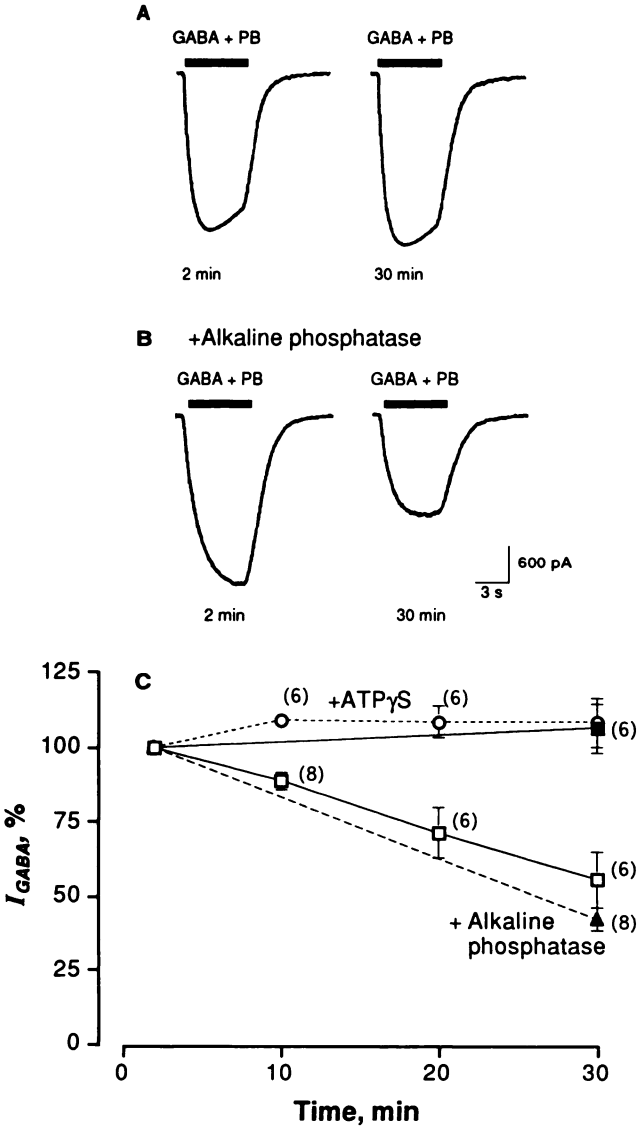

Fig. 7. Alkaline phosphatase conversion of use-dependent run-down into use-independent run-down. A, Response to single applications of 3 μM GABA plus 100 μM pentobarbital (*PB*) (*solid bars*) at 2 and 30 min, with minimal intracellular medium. No run-down is evident, due to the absence of intervening applications of GABA plus pentobarbital between 2 and 30 min. B, Responses to single applications of 3 μM GABA plus 100 μM pentobarbital at 2 and 30 min exhibiting run-down when 100 μg/ml alkaline phosphatase is included in the intracellular buffer. *Traces*, typical results from two different cells. C, Kinetics of run-down. Run-down of the pentobarbital-enhanced GABA response was use dependent. Although substantial run-down was evident with four prolonged (20-sec) applications of 3 μM GABA plus 100 μM pentobarbital at 10-min intervals (□), there was little if any run-down evident with two brief (2–5-sec) applications of 3 μM GABA plus 100 μM pentobarbital separated by 30 min (■). Neurons could be protected from run-down induced by frequent prolonged applications of GABA plus pentobarbital by inclusion of 5 mM Mg-ATPγS in the intracellular buffer (○). Conversely, when alkaline phosphatase was included in the intracellular buffer (▲), run-down occurred even with brief, widely separated, applications of GABA plus pentobarbital. *Error bars*, standard errors. The number of experiments at each time point is given in parentheses.

TABLE 1

Effects of different positive and negative modulators of the $GABA_AR$, tested before and after run-down

The percentage change of I_{GABA} was determined relative to the average of control GABA responses determined immediately before application and after washout of the modulator. Modulation of I_{GABA} was measured before and after run-down was induced by four 20-sec applications of either 30 μM GABA or 3 μM GABA plus 100 μM pentobarbital. Values in parentheses are the numbers of experiments.

GABA concentration	Modulator	I_{GABA}	
		Before run-down	After run-down
μM		% of control	
3	Allopregnanolone (0.5 μM)[a]	885 ± 145 (3)	350 ± 71 (3)[b]
3	Progesterone (30 μM)[c]	201 ± 21 (4)	106 ± 7 (4)[b]
3	Pentobarbital (100 μM)[a]	1509 ± 174 (6)	686 ± 89 (6)[b]
3	Pentobarbital (100 μM)[a,d]	1108 ± 107 (6)	1157 ± 51 (6)
3	Chlordiazepoxide (100 μM)[c]	288 ± 32 (5)	151 ± 19 (5)[e]
30	Pregnenolone sulfate (10 μM)[c]	63 ± 5 (8)	71 ± 4 (8)
3	$ZnCl_2$ (500 μM)[c]	36 ± 7 (6)	40 ± 4 (6)

[a] Run-down was initiated by 3 μM GABA plus 100 μM pentobarbital.
[b] $p < 0.03$, paired t test.
[c] Run-down was initiated by 30 μM GABA.
[d] Mg-ATPγS (5 mM) was added to the intracellular solution.
[e] $p = 0.002$.

for the GABA-induced currents after run-down, indicating that the decrease in I_{GABA} is not due to a change in the chloride concentration gradient (data not shown). The decline of the 30 μM GABA response may be decreased or prevented by the inclusion of Mg-ATP or Mg-ATPγS in the intracellular solution, strongly suggesting that run-down is a consequence of dephosphoryation of some target closely associated with the $GABA_AR$. Although the nature of this target has not been established, the simplest hypothesis is that run-down is related to a change in the phosphorylation state of the $GABA_AR$ itself, which contains numerous potential phosphorylation sites and has been shown to be a substrate for a variety of protein kinases *in vitro* (18–21).

Run-down is also observed with excised, outside-out patches, demonstrating that any intracellular factors required for run-down, such as phosphatases or kinases, must be closely associated with the plasma membrane. In this context, it is interesting to note that Sweetnam *et al.* (21) found that a protein kinase capable of phosphorylating the $GABA_AR$ was copurified with solubilized $GABA_AR$ on a benzodiazepine affinity column. The finding that run-down occurs in isolated patches rules out the possibility that run-down could be due to a loss of electrical access to receptors located on processes.

A surprising finding is that run-down changes the pharmacology of the $GABA_AR$, altering both the potency and the efficacy of GABA and decreasing the magnitude of potentiation of the GABA response by positive modulators. These changes are probably not due to unmasking of a subpopulation of run-down-resistant $GABA_ARs$, because this cannot account for the fact that the response to 3 μM GABA does not decrease and may even increase as run-down proceeds (22). Moreover, unmasking of a receptor subpopulation cannot explain the run-up of the 3 μM GABA response that is observed when Mg-ATPγS is included in the intracellular buffer. These results argue that run-down is associated with some modification of the $GABA_AR$ that increases the potency of GABA while decreasing its efficacy. These two effects may be mediated by different mechanisms, because the inclusion of Mg-ATPγS prevents the decrease in the maximum response but does not prevent the leftward shift of the GABA dose-response curve, resulting in the observed run-up of the 3 μM GABA response.

The decrease in potentiation by positive modulators follows from the changes in the GABA dose-response curve that are induced by run-down. Because positive modulators act by shifting the GABA dose-response curve to the left, the magnitude of the enhancement produced by a given modulator concentration is influenced by the concentration of GABA, decreasing as the concentration of GABA is increased. After run-down, there is a decrease in the maximum GABA response and a decrease in the GABA EC_{50}. Thus, although the actual magnitude of the response to 3 μM GABA does not change much, it represents a much larger fraction of the maximum GABA response after run-down than before. As a result, even if a modulator produced the same degree of leftward shift in the GABA dose-response curve, the degree of potentiation of the 3 μM GABA response would be expected to decrease after run-down.

It is unclear whether the decreased potentiation of I_{GABA} after run-down is related to the homologous or heterologous biochemical "uncoupling" that occurs after chronic (48-hr) exposure of neuronal cultures to GABA or muscimol (28) or to positive modulators such as benzodiazepines (29), steroids, or barbiturates (30) and that results in decreased enhancement of [^{3}H]flunitrazepam binding by agonists or modulators. The decrease in enhancement of I_{GABA} by positive modulators after run-down is predominantly due to a decrease in the GABA EC_{50}, whereas biochemical uncoupling reflects a decrease in the strength of the allosteric coupling among modulatory sites of the $GABA_AR$. It remains possible, however, that both effects may be consequences of changes in $GABA_AR$ phosphorylation.

As reported previously, run-down is associated with an acceleration of $GABA_AR$ desensitization, which is prevented if Mg-ATP or Mg-ATPγS is included in the whole-cell pipet (22). Similarly, no acceleration of desensitization was observed during recording by the nystatin method, which prevents the loss of intracellular ATP. Moreover, desensitization of the initial GABA response was greater under whole-cell recording conditions than with the nystatin method, implying that a rapid change in the properties of the $GABA_AR$ occurs during the brief interval between the establishment of the whole-cell configuration and the initial application of GABA. When Mg-ATP was included in the whole-cell pipet, desensitization of the initial response was reduced, resembling the nystatin case and confirming that phosphorylation is involved, although an increase in desensitization after repeated GABA application was

still observed. It therefore seems likely that there are at least two phosphorylation-mediated mechanisms involved in the regulation of $GABA_AR$ desensitization, i.e., a rapid GABA-independent process that accounts for the different rates of desensitization of the initial responses and a slower, use-dependent mechanism that mediates the progressive acceleration of desensitization that accompanies run-down.

Positive modulators, such as allopregnanolone and pentobarbital, increase $GABA_AR$-mediated chloride currents. Although steroids and barbiturates potentiate GABA responses by different mechanisms (31), both induce run-down of the 3 μM GABA response. As when run-down is induced by exposure to higher concentrations of GABA alone, run-down of the pentobarbital- or allopregnanolone-enhanced responses can be prevented by adding Mg-ATPγS to the intracellular solution, indicating that run-down of the potentiated GABA response is due to dephosphorylation.

The observation that run-down is accelerated by positive modulators of $GABA_AR$ function demonstrates that run-down is mediated by activation of $GABA_ARs$ rather than $GABA_BRs$ and indicates that the determining factor in the rate of run-down is the degree of activation of the $GABA_AR$, rather than the concentration of GABA. Run-down thus resembles desensitization in that it is induced by prolonged $GABA_AR$ activation but differs from classical $GABA_AR$ desensitization in that run-down develops more slowly. Moreover, whereas $GABA_AR$ desensitization is reversible on a time scale of a few minutes, $GABA_AR$ run-down is irreversible on the time scale of our experiments. The observation that run-down is associated with an acceleration of $GABA_AR$ desensitization (22) strongly suggests that there is a link between run-down and desensitization. It is noteworthy that run-down is observed only under conditions that result in a prolonged high level of $GABA_AR$ activation, conditions that produce substantial $GABA_AR$ desensitization, raising the possibility that desensitization leads to run-down. This might be the case if, for example, the desensitized $GABA_AR$ is dephosphorylated more rapidly than the receptor at rest.

It is also possible that the decrease in the peak GABA response could be a direct result of an increase in the rapidity of desensitization. The observed rate of desensitization, even after run-down, is not fast enough to account for the observed attenuation of the peak response; however, we cannot exclude the possibility that the decline in the peak I_{GABA} could be due to the appearance of a rapid subsecond phase of $GABA_AR$ desensitization, as described by Cash and Subbarao (32) and Celentano and Wong (33), which would not be resolved on the time scale of our experiments.

$GABA_AR$ run-down in the absence of intracellular phosphorylating factors has also been observed in acutely dissociated adult guinea pig hippocampal neurons but, in contrast to spinal cord neurons, run-down in this system is time dependent rather than use dependent (23). Why run-down is agonist dependent in spinal cord neurons but not in hippocampal neurons remains unclear. One possible explanation is that adult hippocampal neurons have a higher level of endogenous phosphatase activity. Consistent with this hypothesis, spinal cord neurons can be made to exhibit agonist-independent run-down by inclusion of alkaline phosphatase in the intracellular buffer.

In summary, the results suggest that receptor activation can lead to changes in $GABA_AR$ phosphorylation that result in profound changes in $GABA_AR$ pharmacology, affecting the potency and efficacy of GABA, the kinetics of $GABA_AR$ desensitization, and the sensitivity of the receptor to pharmacological modulators such as steroids, benzodiazepines, and barbiturates. Moreover, the results are consistent with the existence of a phosphorylation-dependent feedback mechanism that is capable of regulating GABA-mediated synaptic inhibition and the development of tolerance toward $GABA_AR$ modulators.

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