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$GABA_A$ RECEPTOR FUNCTION IS REGULATED BY PHOSPHORYLATION IN ACUTELY DISSOCIATED GUINEA-PIG HIPPOCAMPAL NEURONES

BY QIANG XIAO CHEN*, ARMIN STELZER, ALAN R. KAY† AND ROBERT K. S. WONG

From the Department of Neurology, College of Physicians and Surgeons, Columbia University, 630 West 168th Street, New York, NY 10032, USA

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SUMMARY

1. Current mediated by $GABA_A$ receptors was examined in pyramidal cells acutely dissociated from the hippocampus of mature guinea-pigs. Current responses were measured using whole-cell voltage-clamp recordings. An internal perfusion technique was used to change the intracellular contents during recording.

2. Application of GABA (100–300 μM) by short duration pressure pulses produced outward current responses at a holding potential of -10 mV. When recordings were made with intracellular solutions which did not contain Mg-ATP, GABA responses progressively decreased to less than 10% of their initial values after 10 min. This 'run-down' of the GABA response could not be accounted for by desensitization since the rate of run-down was not dependent upon agonist application.

3. The run-down of the $GABA_A$ response was reversed when Mg^{2+} (4 mM) and ATP (2 mM) were introduced into the intracellular perfusate. In addition to the presence of Mg-ATP, buffering of Ca^{2+} in the intracellular solution to low levels ($\sim 10^{-8}$ M) was also necessary to stabilize the $GABA_A$ response.

4. The role of a phosphorylation process in regulating the $GABA_A$ receptor was tested. After the GABA response stabilized, introduction of alkaline phosphatase (100 μg/ml) to the intracellular perfusate caused a complete run-down of the GABA response.

5. Stable GABA responses were obtained when ATP was replaced by ATP-γ-S (adenosine 5′-*O*-(thiotriphosphate), an analogue of ATP that donates a thiophosphate group resulting in a product that is more resistant to hydrolysis. Following such treatment GABA responses declined more slowly after the introduction of intracellular alkaline phosphatase.

6. Run-down of GABA responses accelerated when intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was elevated to about 5×10^{-4} M. The run-down caused by elevated $[Ca^{2+}]_i$ could be stopped and reversed by reducing $[Ca^{2+}]_i$ to about 10^{-8} M.

* To whom reprint requests should be sent.

† Present address: AT & T Bell Laboratories, 600 Mountain Avenue, Murray Hill, NJ 07974, USA.

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7. The introduction of ATP-γ-S to the intracellular medium retarded the run-down of GABA responses caused by elevation of $[Ca^{2+}]_i$.

8. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7), a calmodulin inhibitor, reduced the rate of run-down induced by elevated $[Ca^{2+}]_i$.

9. These results suggest that the function of the $GABA_A$ receptor is maintained by phosphorylation of the receptor or some closely associated regulatory molecule. Elevation of $[Ca^{2+}]_i$ destabilizes the function of the $GABA_A$ receptor, probably by activating a Ca^{2+}/calmodulin-dependent phosphatase.

INTRODUCTION

There is considerable evidence that γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian cortex (Krnjevic, 1974). In the hippocampus, intrinsic GABAergic neurones are driven by afferent fibres and local pyramidal cells to bring about feedforward and feedback inhibition, respectively (Andersen, Eccles & Loyning, 1963; Alger & Nicoll, 1982). Although the exact function of GABAergic inhibition in the integrative activity of the cortex remains unclear, its importance is underscored by studies showing that the pharmacological blockade of $GABA_A$ receptor lead to epileptiform activity (Schwartzkroin & Prince, 1980) and that deficiencies in the GABAergic inhibitory system may be involved in some forms of human epilepsy (Lloyds, Bossi, Morselli, Munari, Rougier & Loiseau, 1986). In addition, modification of inhibition may be involved in both short-term (Ben-Ari, Krnjevic & Reinhardt, 1979; Wong & Watkins, 1982; McCarren & Alger, 1985; Thompson & Gaehwiler, 1989) and long-term (Stelzer, Slater & ten Bruggencate, 1987; Miles & Wong, 1987) changes in neuronal activity following tetanic stimulation of afferent fibres to the hippocampus. Simulation studies (Traub, Miles & Wong, 1989), based on data obtained from the hippocampal slice preparation, showed that reverberating, synchronized activity appears in groups of hippocampal cells when inhibition is reduced. The frequency and amplitude of synchronized activity can be regulated by variations in the strength of inhibition.

There are at least two types of GABA receptors in the central nervous system, which are termed types A and B. Activation of both types of receptors leads to inhibition of hippocampal neurones, but through different mechanisms (Nicoll, 1988). Ligand binding to $GABA_B$ receptors leads to a transmembrane activation of G-proteins which in turn open K^+ channels. Binding of GABA to $GABA_A$ receptors opens up Cl^- channels, presumably via allosteric modification of the receptor–channel complex (see Olsen, 1982 for review). In addition to the GABA recognition site, the receptor can be allosterically modified by ligand binding to three extracellular sites on the macromolecule. The agonists for these sites are picrotoxin-like convulsants, benzodiazepines and barbiturates. In contrast to the detailed knowledge about ligand–receptor interactions of the $GABA_A$ receptor, it was, until recently, unclear whether $GABA_A$ receptors could be modulated intracellularly. Our initial studies showed that the activation of the $GABA_A$ current required the presence of intracellular Mg-ATP and the maintenance of low levels of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Stelzer & Wong, 1987; Stelzer, Kay & Wong, 1988). We hypothesized that the magnitude of $GABA_A$ conductance of hippocampal cells was determined by the

outcome of a competition between a phosphorylation process that maintains the conductance and a Ca^{2+}-dependent dephosphorylation process that reduces the conductance. In this paper we present results from additional experiments carried out to test this hypothesis. We have developed a method for perfusing the intracellular medium of neurones during whole-cell recording. In this way we were able to obtain more direct information about intracellular modulation sites for the $GABA_A$ receptor.

TABLE 1. Composition of intracellular solutions

Solution	ATP (mM)	$MgCl_2$ (mM)	$BaCl_2$ (mM)	ATP-γ-S (mM)	BAPTA (mM)	$CaCl_2$ (mM)	Alkaline phosphatase (μg/ml)
Mg-ATP	2	4	1	—	10	—	—
Mg-ATP-free	—	—	1	—	10	—	—
High Ca^{2+}	2	4	—	—	0·5	1	—
Phosphatase	2	4	1	—	10	—	100
ATP-γ-S	—	5	1	7	10	—	—

BAPTA, bis(σ-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

METHODS

Experiments were carried out using acutely isolated cells from mature guinea-pigs of both sexes weighing 200–400 g. The animals were killed by cervical dislocation. The isolation procedure has been published previously (Kay & Wong, 1986).

Recording pipettes were made from 1·5 mm outside diameter thin-walled borosilicate glass tubing (WPI). The solution perfusing the outside of the cell had the following composition (mM): NaCl, 140; CsCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; tetraethylammonium chloride, 15; 4-aminopyridine, 5; *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES), 10; D-glucose, 25; $CoCl_2$, 2; pH 7·4. The presence of K^+ channel blockers should prevent activation of K^+- currents via $GABA_B$ receptors. GABA responses elicited under these conditions were completely blocked by picrotoxin (10 μM), suggesting the selective activation of the $GABA_A$ receptor. The intracellular solution used in all experiments consisted of (mM); Tris-methanesulphonate, 130; HEPES, 10 and leupeptin (a Ca^{2+}-activated neutral protease inhibitor), 0·1. The latter was added to prevent irreversible loss of activity attributable to proteolysis. The pH of the solution was adjusted to 7·3. Table 1 lists the experimental solutions that were obtained by adding the listed components to the intracellular solution.

All chemicals, unless otherwise stated, were obtained from Sigma. The ATP-γ-S was purchased from Boehringer Mannheim Biochemicals.

The concentration of intracellular Ca^{2+} was calculated using a program developed in the laboratory of Dr P. Palade (for example, see Palade, 1987). In the Mg-ATP solution free Ca^{2+} was about 10^{-8} M and in high-Ca^{2+} solution the concentration was about 5×10^{-4} M.

Cells were voltage clamped at -10 mV. Gamma-aminobutyric acid (100–300 μM) dissolved in extracellular solution, was applied by pressure ejection from a glass micropipette. Preceding the GABA response a -10 mV, 10 ms pulse was applied to estimate the cell capacitance and input conductance. Changes in the capacitance may signal a loss of effective neuronal membrane, collapse of dendrites or resealing of the patch, all of which could affect the GABA response. Data analysed in this report were obtained from cells where no significant changes in the capacitance were noted.

Whole-cell voltage clamp was carried out as described by Hamill, Marty, Neher, Sakman & Sigworth (1981). Current activated by GABA was monitored with a List L/M-EPC7 patch-clamp amplifier and stored on an IBM PC XT using a Tekmar 12-bit analog-to-digital converter board and the data capture program 'pClamp' (Axon Instruments). Data were analysed using a program (developed by Dr W. Wadman) written in TurboPascal (Borland International).

A modification of the recording configuration depicted in Fig. 1 allowed us to perform intracellular perfusion. Various intracellular solutions (up to three) were placed in containers connected to a common, pressurized chamber. An outlet from the chamber was fed into a valve and a thin tube was connected to the output of the valve and inserted into the recording pipette extending close to the tip. Outflow was achieved through another tube which was connected.

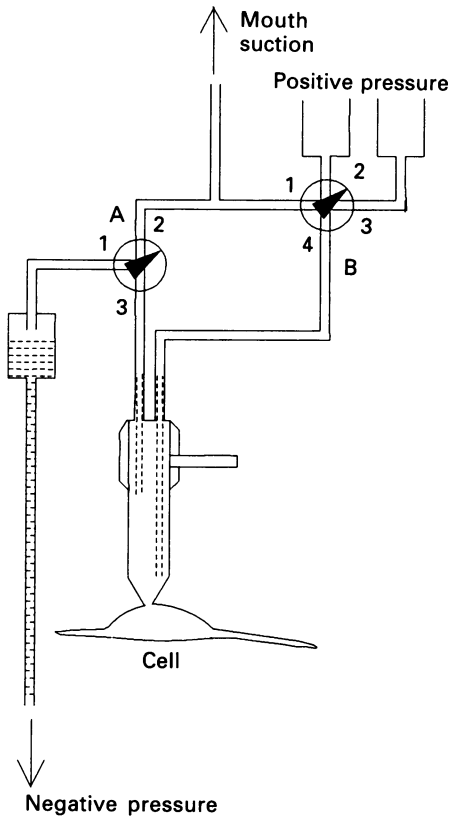


Fig. 1. Recording arrangement for switching intracellular perfusion solutions during whole-cell recording. Outlets 2 and 3 of valve A and 1 and 4 of valve B were connected during attempts to form high-resistance seals through suction applied by mouth. After cell penetration, outlets 1 and 3 of A and 2 (or 3) and 4 of B were connected. Positive pressure was applied at position 4 of B and negative pressure was applied at position 3 of A to facilitate the influx and efflux of solutions at the tip of the pipette.

through an air gap, to a column of water used to generate negative pressure. Solutions were perfused at 0·2–0·4 ml/min through the recording pipette. With such an arrangement, effects of different agents introduced into the cell became detectable after a latency of 3–5 min.

RESULTS

Stabilization of GABA-activated currents with Mg-ATP

Whole-cell voltage-clamp recordings were obtained from acutely isolated hippocampal pyramidal cells. To examine currents elicited by GABA (100–300 μM; 3–

50 ms pressure pulses), cells were held at −10 mV and outward currents were elicited. Maximum peak currents recorded from 104 cells ranged from 500 to 2000 pA.

When recordings were carried out with a Mg-ATP-free intracellular solution a decrease of the GABA response over time was observed (Fig. 2). This decrease could

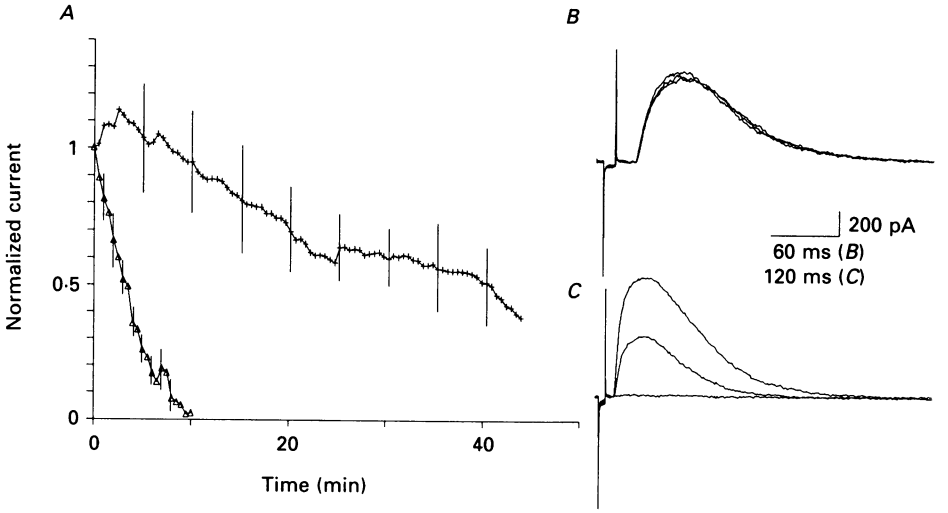


Fig. 2. Run-down of $GABA_A$ response. *A*, normalized peak current amplitudes (peak current amplitude of the first response = 1). In the absence of intracellular Mg-ATP, peak-current amplitudes were reduced to zero within 10 min (△; *n* (number of neurones) = 4, mean ± standard deviation (S.D.) are depicted in the diagram; S.D. was presented for every tenth averaged data point). When recordings were obtained in Mg-ATP solution (+; see Methods for content of solution), the fast run-down was prevented (mean peak-current amplitude after 10 min was 95% of the first response, *n* = 5;). *B*, outward current recordings elicited by GABA in Mg-ATP solution. Overlapping traces taken at 0, 5, and 10 min after penetration of the cell. *C*, recordings obtained with Mg-ATP-free intracellular solution. Short hyperpolarizing voltage pulses (−10 mV, 10 ms) were applied preceding each GABA pulse (10 ms) to monitor the leak conductance and capacitance of the cells. Holding potential in all cells was −10 mV.

not be attributed to desensitization since the interval between GABA applications was long enough (> 30 s) to avoid cumulative agonist-induced desensitization. Changes in the distribution of Cl^- ions could not account for run-down, as the reversal potential of the GABA-activated current remained constant throughout the time course of recording (Stelzer *et al.* 1988).

There are at least two possible causes for the time-dependent decrease of the GABA response (run-down). Firstly, the event could be caused by a wash-out of diffusible intracellular components essential for the maintenance of the GABA response. Secondly, GABA channels could be destroyed by proteolysis during whole-cell recording. Run-down of GABA responses was significantly reduced when 4 mM-Mg and 2 mM-ATP were included in the perfusion solution as shown in Fig. 2. With Mg-ATP-free intracellular solution, GABA-induced run-down was complete within 10 min of penetrating the cell, whereas with Mg-ATP perfusate the average response at this time was still 95·8 ± 7·6% (mean + S.D.) of the first response.

Figure 2*A* also illustrates that the GABA-activated current still slowly decreased with time in Mg-ATP solution. The peak-current amplitude was reduced to 56·2% following 40 min of recording. We have not been able to eliminate this slow run-down, but observed a significant retardation when low levels of Ba^{2+} were included in the pipette solution (see Table 1 for Ba^{2+} content). After 30 min of recording the responses were 62+11% ($n = 5$) of maximum when Ba^{2+} was present whereas responses were 29+15% ($n = 4$) in its absence.

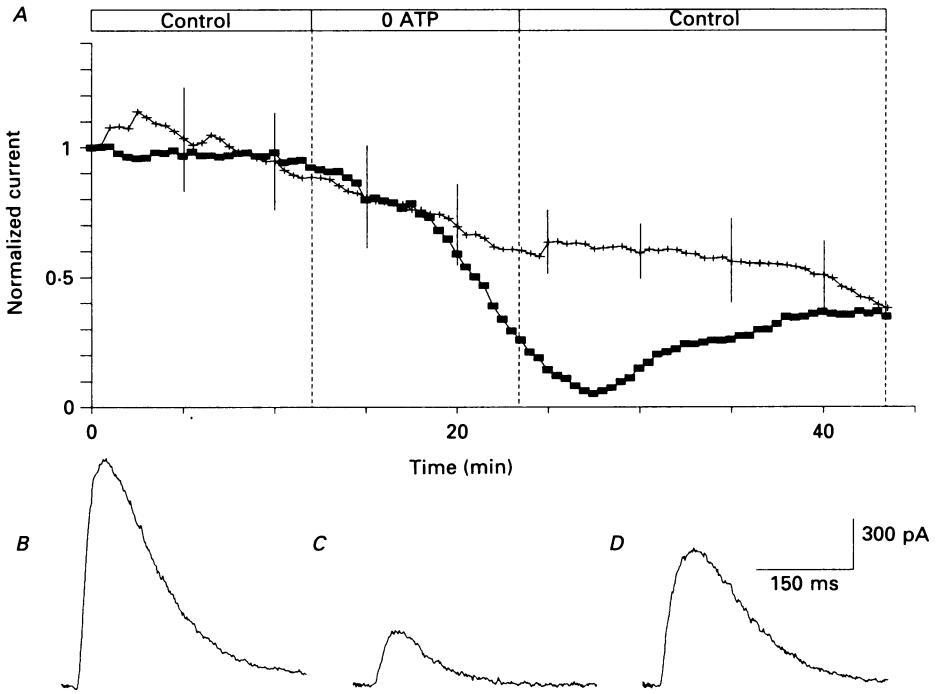


Fig. 3. Reversibility of $GABA_A$ response run-down. Symbols: +, average peak currents obtained with Mg-ATP solution ($n = 5$); solution without ATP (0 ATP) was introduced intracellularly at the time indicated in the box above. Following 12 min of ATP-free solution perfusion, Mg-ATP solution was reintroduced. *B* and *C*, experimental records taken just before the switches to 0 ATP and back to control solution. *D* shows the last GABA response plotted in *A* after 43 min of recording.

The results suggest that factors essential for the stability of the GABA response, *inter alia* Mg-ATP, are washed out during whole-cell recording with Mg-ATP-free intracellular solution. The requirement for Mg-ATP is suggestive of the involvement of an intracellular phosphorylation process in maintaining the GABA sensitivity of hippocampal cells. The persistence of the slow run-down in the presence of Mg-ATP may be caused by a wash-out of other diffusible molecules, such as endogenous protein kinases or other cofactors, that are also required for maintaining the functional state of the $GABA_A$ receptor.

Reversibility of run-down

Figure 3 shows GABA responses recorded during intracellular perfusion with Mg-ATP solution. The perfusion rate was set at 0·2–0·4 ml/min and GABA responses

were elicited once every 30 s. Recording was started, and 12 min later Mg-ATP-free perfusate was introduced; 5 min following the change of the perfusate, the run-down of the GABA response became noticeably faster, and the current was reduced to about 25% of the original response after 15 min. The response recovered 5 min after reintroduction of the Mg-ATP solution. For this cell the amplitude of GABA responses recovered to 35% of the original response. The slow run-down process observed in Mg-ATP solution probably prevented the full recovery of the GABA responses, since responses recorded in Mg^{2+}-ATP solution were reduced to a similar extent ($\sim$ 56%) after 40 min of recording.

The reversibility of the GABA run-down is inconsistent with it being the result of non-specific proteolysis of the GABA receptor. It is more likely that the $GABA_A$ receptor or a closely associated regulatory molecule needs to be phosphorylated for the receptor to respond to GABA. When phosphorylating factors, such as Mg-ATP, are absent, dephosphorylation dominates and run-down occurs. The following experiments provide additional tests for this hypothesis.

Alkaline phosphatase-induced GABA run-down

Alkaline phosphatases are non-specific enzymes that hydrolyse many compounds containing phosphorus, regardless of the chemical nature of the moiety to which the phosphorus is bound (McComb, Bowers & Posen, 1979). The inclusion in the Mg-ATP solution of alkaline phosphatase (100 μg/ml) triggered the onset of GABA-activated current run-down. The curve in Fig. 4*A* represents the average of four cells in which alkaline phosphatase solution was perfused intracellularly after stable GABA responses were established in Mg-ATP solution.

The involvement of phosphorylation in the regulation of the $GABA_A$ receptor was further explored using the ATP analogue adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S). This compound can serve as a substrate for protein kinases and the resulting thiophosphate is far more resistant to hydrolysis than is the corresponding phosphate group by phosphatases (Eckstein, 1985). If a phosphatase is involved in run-down it is expected that ATP-γ-S should retard the rate of run-down.

Figure 5 shows the time course of peak GABA responses recorded in ATP-γ-S solution. In six cells analysed, the GABA responses recorded after the establishment of whole-cell recording gradually decreased in amplitude. Within 10 min peak GABA responses decreased by about 50%. Subsequently the response stabilized and the peak amplitude remained relatively constant. The initial decline of the GABA responses may be due to a lower effectiveness of ATP-γ-S as a substrate in the phosphorylation reaction as compared to ATP. For example, cyclic nucleotide-dependent kinases are less effective in catalysing thiophosphorylation than phosphorylation (Palvimo, Linnala-Kankkunen & Maenpaa, 1985). Thus dephosphorylation of the GABA receptor regulatory site should proceed faster than phosphorylation when ATP-γ-S is the only available substrate. With time, thiophosphorylated products would accumulate and, since they are more resistant to hydrolysis by phosphatase, the GABA response should stabilize.

The stability of the thiphosphorylated protein product was further tested by application of intracellular alkaline phosphatase. After GABA responses had stabilized in ATP-γ-S solution, alkaline phosphatase solution was introduced intracellùlarly. As illustrated in Fig. 6, the decline in GABA current was significantly

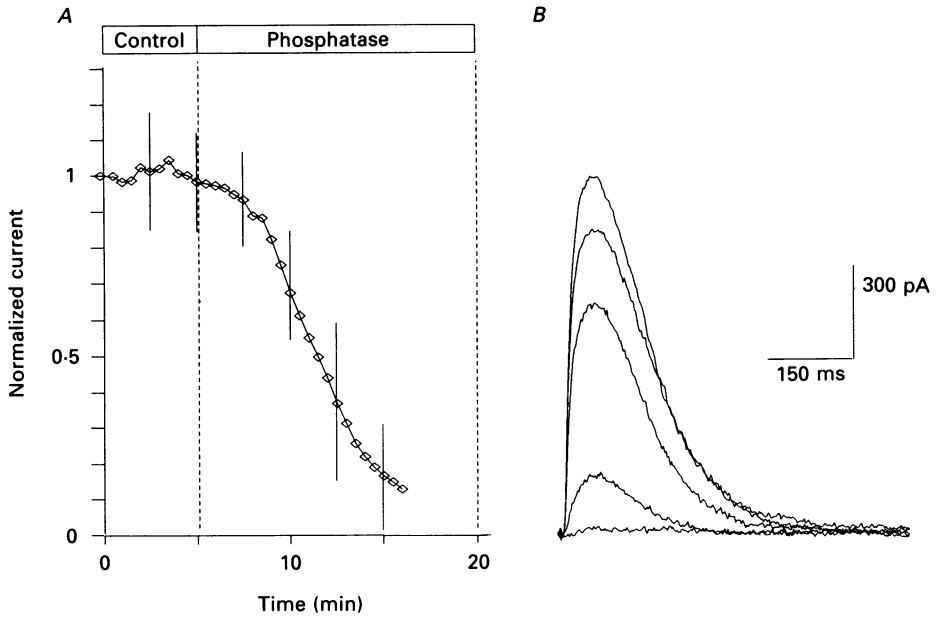


Fig. 4. Effect of intracellular alkaline phosphatase. *A*, plot of average peak-current amplitudes, first elicited with Mg-ATP solution. At the time indicated by the box above the curve, alkaline phosphatase was introduced intracellularly. The curve represents data obtained from four cells, the bars depict S.D. *B*, outward currents activated by GABA. Records were obtained just before the switch to phosphatase solution and 5, 7, 12 and 15 min after the switch. Corresponding records show a continuous reduction in peak-current amplitude.

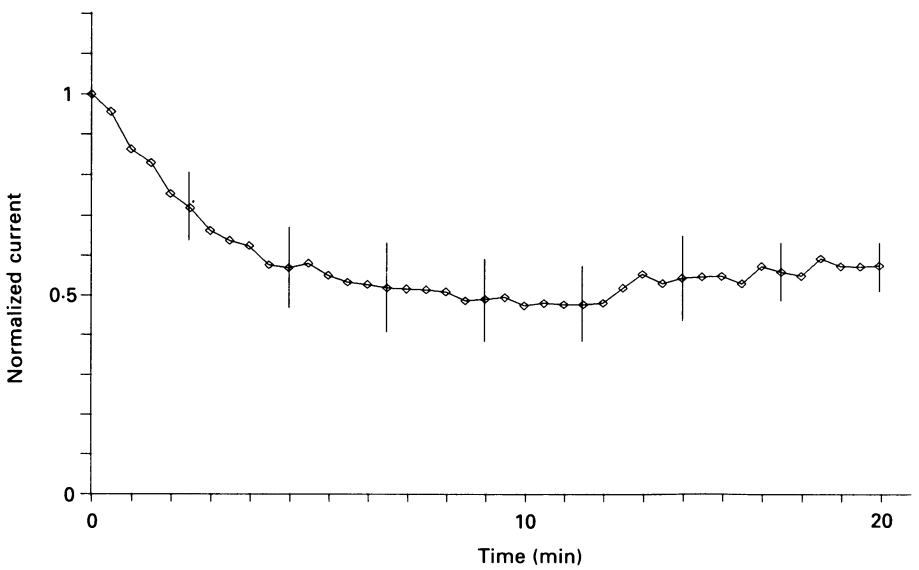


Fig. 5. Time course of peak $GABA_A$ responses recorded with a pipette containing ATP-γ-S solution. Data are averaged values from three cells. Time 0 represents the time when intracellular recording was first established.

slower in ATP-γ-S solution, being reduced to about 50% of the response (average of two cells) compared to 10% (average of four cells) in Mg-ATP solution, 10 min after introduction of alkaline phosphatase. These data indicate that ATP-γ-S reduced the effectiveness of alkaline phosphatase in destabilizing GABA responses.

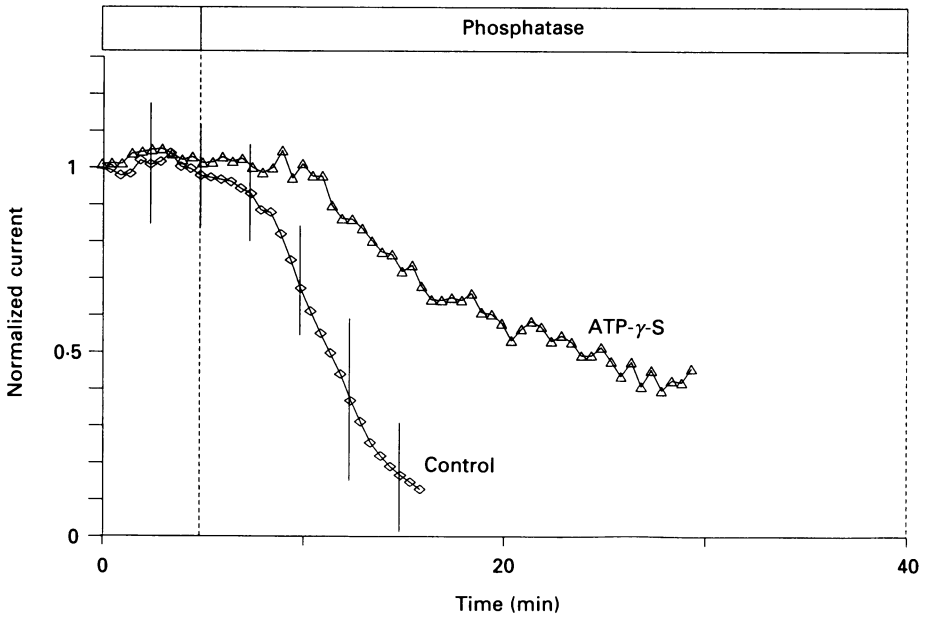


Fig. 6. Effect of alkaline phosphatase on GABA$_A$ responses stabilized in ATP-γ-S solution. After stable responses were obtained in ATP-γ-S ($\triangle$), phosphatase was introduced intracellularly at the time indicated on the top of the graph. Data represent peak currents averaged from two cells. Time 0 on the control trace represents 5 min after initiating recording, when the current had stabilized. Averaged data recorded in Mg-ATP solution ($n = 4$) ($\diamond$) are depicted for comparison.

Increased intracellular Ca^{2+} accelerates run-down

We observed that $[Ca^{2+}]_i$ had to be buffered to low levels in order to prevent the run-down of the GABA response. Stable responses could be obtained in Mg-ATP solution which contained low $[Ca^{2+}]_i$ (about 10^{-8} M). In the high $[Ca^{2+}]_i$ ($\sim 5 \times 10^{-6}$ M) solution, rapid run-down of the GABA response occurred despite the presence of Mg-ATP. The GABA-induced response fell to below 10% at 10 min following cell penetration (Fig. 7*A*).

Elevation of $[Ca^{2+}]_i$ may induce run-down through the proteolysis of GABA receptors by Ca^{2+}-dependent proteases. Alternatively intracellular Ca^{2+} may activate a Ca^{2+}-dependent phosphatase which catalyses dephosphorylation. Figure 7*B* shows that run-down of GABA responses in elevated $[Ca^{2+}]_i$ was reversible. Upon introduction of the Mg-ATP solution which has a low $[Ca^{2+}]_i$ ($< 1 \times 10^{-8}$ M), run-down ceased and recovery of the response was observed. In four other cells examined, intracellular perfusion with low $[Ca^{2+}]_i$ solution always stopped the run-down caused by high $[Ca^{2+}]_i$ solution. The reversibility of GABA-induced run-down triggered by an increase in $[Ca^{2+}]_i$ indicates that the run-down is probably caused by the

activation of a phosphatase rather than a protease. Furthermore, possible contributions of Ca^{2+}-dependent proteases were minimized by routinely including the neutral protease inhibitor leupeptin in the intracellular perfusion solutions (see Methods).

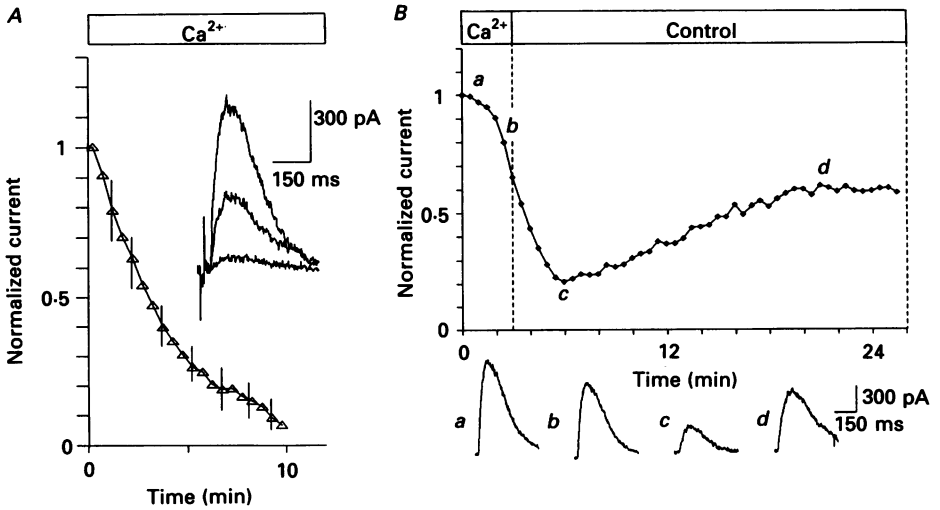


Fig. 7. Effect of elevated $[Ca^{2+}]_i$ on $GABA_A$-mediated currents. *A*, time course of peak GABA responses obtained in high $[Ca^{2+}]_i$ and optimal Mg-ATP. Records represent average of four cells. The inset shows outward currents obtained at 0, 5, 10 min following cell penetration in one cell. *B*, the reversal of run-down recorded in high $[Ca^{2+}]_i$ solution. Averaged values of peak currents recorded with high $[Ca^{2+}]_i$ solution, which contained Mg-ATP, are plotted below the box labelled Ca^{2+}. At the break between boxes, Mg-ATP solution (which has low $[Ca^{2+}]_i$) was introduced intracellularly. Records shown below the graph were obtained at the labelled points.

The possible involvement of a Ca^{2+}-dependent phosphatase was further tested by examining the effect of elevating $[Ca^{2+}]_i$ after GABA responses were stabilized by intracellular ATP-γ-S. Figure 8 shows that the run down caused by the high $[Ca^{2+}]_i$ solution was significantly retarded by intracellular ATP-γ-S, and GABA responses could still be recorded 35 min after the introduction of high Ca^{2+} solution (compare Fig. 7*A* with Fig. 8).

Possible involvement of a Ca^{2+}-dependent phosphatase

A probable explanation for the above observation is that elevation of $[Ca^{2+}]_i$ activates a phosphatase. A Ca^{2+}-dependent phosphatase calcineurin has been isolated from the mammalian brain (Klee, Crouch & Krinks, 1979). Activation of this enzyme requires the presence of Ca^{2+} and calmodulin (Cohen, 1985). We carried out a series of experiments to assess the contribution of calmodulin and calcineurin in the run-down of GABA response. Figure 9 shows that in the presence of W-7, a calmodulin inhibitor (Tanaka, Ohmura, Yamakado & Hidaka, 1982), the rate of run-down with elevated $[Ca^{2+}]_i$ was significantly reduced. In four cells, recorded with W-7 and elevated $[Ca^{2+}]_i$ 10 min after initiating the recordings the GABA currents were greater than 90% of the initial responses. On the other hand, GABA responses with

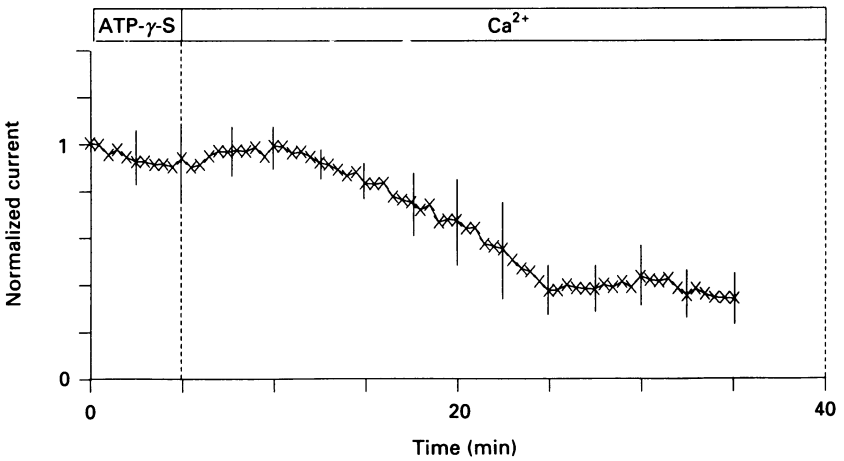


Fig. 8. Effect of ATP-γ-S and high $[Ca^{2+}]_i$ on GABA-induced currents. After GABA responses stabilized in ATP-γ-S solution, high $[Ca^{2+}]_i$ solution plus ATP-γ-S was introduced. Peak GABA responses were still about 20% of control ($n = 4$) 30 min after switch of the solution.

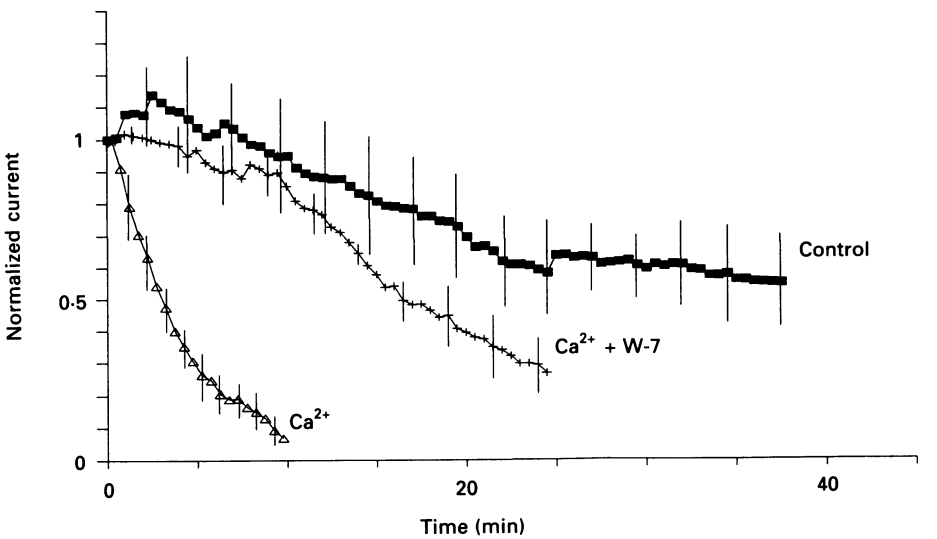


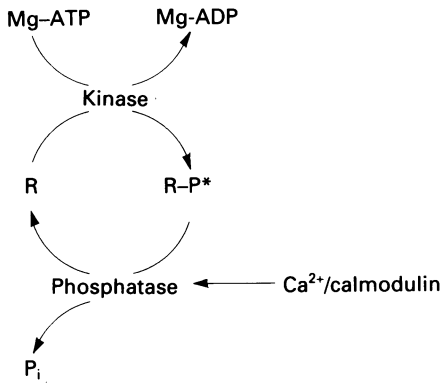
Fig. 9. W-7, a calmodulin inhibitor, retarded run-down of GABA activated currents induced by high $[Ca^{2+}]_i$ solution. Peak GABA currents obtained in Mg-ATP (■, $n = 5$) and high $[Ca^{2+}]_i$ (+, $n = 4$) solutions were plotted together with W-7 solution (in high $[Ca^{2+}]_i$; △, $n = 7$).

high $[Ca^{2+}]_i$ solution without W-7 fell to below 10% of the initial responses after 10 min.

DISCUSSION

In summary, the results presented here indicate that the $GABA_A$-mediated Cl^- current is regulated by a phosphorylation process that maintains the $GABA_A$ receptor function and a Ca^{2+}-dependent dephosphorylation that inactivates the GABA receptor.

Based on results presented in this paper we propose the following scheme for $GABA_A$ receptor function:



We propose that the $GABA_A$ receptor or a closely associated regulatory molecule can exist either in a phosphorylated (R–P*) or a dephosphorylated form (R). With the provision of Mg-ATP, a protein kinase phosphorylates the molecule and maintains the $GABA_A$ receptor in a functional form (R–P*). Phosphorylation is opposed by a dephosphorylation process which renders the $GABA_A$ receptor non-functional. The dephosphorylation process is catalysed by a phosphatase which is activated by elevated $[Ca^{2+}]_i$. A similar cycle has been suggested to play a role in the regulation of voltage-gated Ca^{2+} channels (Deroshenko, Kostyuk & Martynyuk, 1982; Chad & Ekert, 1986; Armstrong & Eckert, 1987).

Recent studies (Byerly & Yazejian, 1986) suggest that the effect of intracellular Mg-ATP might be to maintain a low Ca^{2+} concentration at the inner surface of the plasma membrane. Indeed, it has been shown that intracellular application of ATP helped to control the level of intracellular Ca^{2+} in perfused snail neurones. In contrast, our results suggest that intracellular Mg-ATP sustained the function of the $GABA_A$ receptor via a phosphorylation process. When $[Ca^{2+}]_i$ was maintained high (via intracellular perfusion) in the presence of intracellular ATP-γ-S, run-down of the GABA current was significantly retarded (see Fig. 8). If the sole effect of Mg-ATP was to remove Ca^{2+}, it is expected that ATP-γ-S would enhance the run-down, as it is a poor substitute for ATP in active transport processes.

We have been unable to identify the protein kinase involved in maintaining the functional state of the GABA receptor. As the $GABA_A$ receptor could be maintained in spite of intracellular perfusion, it seems likely that the protein kinase is bound to intracellular membrane sites. Sweetnam, Lloyd, Gallombardo, Malisan, Gallagher,

Tallman & Nestler (1988) recently demonstrated that the α-subunit of the $GABA_A$ receptor can be phosphorylated by a receptor-associated protein kinase, but they were unable to attribute any of the activity to known protein kinases. Their study showed that only about 10% of the receptors could be phosphorylated. A possible interpretation of this finding is that 90% of the receptors were already in a phosphorylated state. This interpretation of their data is consistent with our finding that maximal $GABA_A$ responses were obtained immediately following cell penetration, suggesting that in the intact cell most of the $GABA_A$ receptors exist in the phosphorylated form. A recent study on chick sensory neurones also shows that the maintenance of $GABA_A$ response required intracellular Mg^{2+} and ATP (Gyenes, Farrant & Farb, 1988). The results demonstrated that the rate of run-down was dependent on the concentration of GABA used to activate the cell reponse. In contrast, our studies show that run-down of the $GABA_A$ response in the hippocampus is independent of the concentration of agonist applied since the run-down still occurred without GABA application (Stelzer & Wong, 1987; Stelzer *et al.* 1988). The differences in the result could be due to variations in experimental conditions or the $GABA_A$ receptor structure in the two types of neurones.

The data presented here implicate a phosphatase activated by Ca^{2+} in the run-down of the GABA-induced response. Calcineurin, a phosphatase with this property has been identified in the mammalian CNS (Klee *et al.* 1979). Moreover, calcineurin is present in abundance in hippocampal neurones (Goto, Matsukado, Mihara, Inoue & Miyamoto 1986). The observation that W-7, a calmodulin inhibitor, retarded the run-down of $GABA_A$-activated current induced by the high Ca^{2+} solution, is consistent with the notion that calcineurin catalyses the run-down process. The stabilizing effects of Ba^{2+} on the GABA response provides additional confirmation of the involvement of calcineurin since Ba^{2+}, although it binds to calmodulin, only weakly activates it (Cheung, 1984).

A role for $[Ca^{2+}]_i$ in the regulation of GABA responses has been proposed in other systems. Increases in $[Ca^{2+}]_i$ reduced the open time of $GABA_A$ receptor channels in pituitary cells (Taleb, Trouslard, Demeneix, Feltz, Bossu, Dupont & Feltz, 1987). In bull-frog sensory neurones (Inoue, Oomura, Yakushiji & Akaike, 1986) elevation of $[Ca^{2+}]_i$ decreased the $GABA_A$ response by altering the binding affinity of GABA receptors. It is as yet unclear whether the run-down of the GABA response in CA1 pyramidal cells occurs as a result of a decrease in receptor affinity for GABA or because of an inactivation of the GABA receptor. Regardless of the exact mechanism of action, our results suggest a new intracellular site for regulating $GABA_A$-receptor mediated inhibition in the hippocampus. The physiological routes to this regulatory site remain to be demonstrated.

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