

MULTIPLE MECHANISMS OF PICROTOXIN BLOCK OF GABA-INDUCED CURRENTS IN RAT HIPPOCAMPAL NEURONS

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SUMMARY

1. We have examined the effect of picrotoxin on GABA-induced currents in dissociated rat hippocampal neurons. In addition, we used the putative picrotoxin receptor antagonist, α -isopropyl- α -methyl- γ -butyrolactone (α IMGBL), and the picrotoxin agonist, β -ethyl- β -methyl- γ -butyrolactone (β EMGBL) to explore the mechanisms of picrotoxin's interaction with the GABA-Cl⁻ receptor-ionophore complex.

2. The picrotoxin block of GABA current was use dependent, suggesting that the site of picrotoxin block is exposed by the conformational change initiated by GABA binding to the receptor.

3. The alkyl-substituted butyrolactone antagonist, α IMGBL, selectively blocked the use-dependent mechanism of picrotoxin effect. After the apparent complete inhibition of the use-dependent effect, there was a residual picrotoxin effect that was independent of the time or concentration of GABA application. This indicates that the picrotoxin block of the GABA current is mediated by two different mechanisms. α IMGBL influences just one of these mechanisms.

4. The picrotoxin receptor agonist, β EMGBL, exclusively blocked the GABA current in a use-dependent manner. Consistent with a use-dependent mechanism, the rate of onset of block increased with GABA concentration. Surprisingly, the fraction of GABA current block decreased with increasing GABA concentration.

5. These results suggest that the relationship of picrotoxin and γ -butyrolactones with the GABA-Cl⁻ receptor-ionophore is quite complex. They are consistent with at least two possible models of agonist-antagonist interactions. Both cases require different antagonist affinities for the various kinetic states of the GABA-Cl⁻ receptor-ionophore. However, there is no need to require that either picrotoxin or β EMGBL acts as an open channel blocker.

INTRODUCTION

The activity of the GABA-Cl⁻ receptor-ionophore complex is modulated by different binding sites associated with its multiple subunits. For example, barbiturates and benzodiazepines clearly have specific binding sites on the complex. The latter compounds either enhance or diminish GABA-induced chloride currents, depending upon the specific ligand (Polc, 1988). In addition to these well-established modulatory sites, there are other specific binding sites within the complex. These include the neurosteroid receptor (Majewska, Demirgoren, Spivak & London, 1990) and picrotoxin receptor (Weissman, Burke, Rice & Skolnick, 1984; Levine, Ferrendelli & Covey, 1985).

This latter receptor is particularly interesting to us. Its known ligands, picrotoxin (PCTXN), t-butylbicyclopophosphoro-thionate (TBPS), pentylenetetrazol, and other picrotoxin-like alkaloids (Squires, Casida, Richardson & Saederup, 1983), are all convulsants. The majority of the ligand binding assays have been carried out with TBPS due to its high affinity. On the other hand, the majority of the electrophysiological experiments have been carried out with picrotoxin. One of the reasons for this is that the onset of activity of TBPS is slow (about 30 min to peak effect according to Van Renterghem *et al.* 1987) compared to PCTXN (milliseconds).

Both the onset and the recovery from GABA current block produced by activating the picrotoxin receptor in frog sensory neurons (Inoue & Akaike, 1988) and in oocytes injected with chick brain mRNA (Van Renterghem *et al.* 1987) depend on the presence of GABA or another agonist. Inoue & Akaike (1988), therefore, concluded that the mechanism of PCTXN block of the GABA-Cl⁻ ionophore required an open channel and that the PCTXN binding site was located within the channel. This hypothesis was supported by the report that PCTXN, applied intracellularly, was capable of blocking GABA-activated chloride current (Akaike, Hattori, Oomura & Carpenter, 1985).

We have now employed two alkyl-substituted γ -butyrolactones to examine the mechanism of PCTXN block of GABA-induced chloride currents in greater detail. One of these compounds, β -ethyl- β -methyl- γ -butyrolactone (β EMGBL) is a PCTXN agonist in that its actions resemble those of PCTXN at the PCTXN receptor (Holland, Ferrendelli, Covey & Rothman, 1990*a*). The other compound, α -isopropyl- α -methyl- γ -butyrolactone (α IMGBL), is an antagonist at this receptor, and reduces GABA-induced chloride current blockade produced by PCTXN and β EMGBL (Holland, Yoon, Ferrendelli, Covey & Rothman, 1990*c*). Our experiments indicate that PCTXN probably blocks the GABA-Cl⁻ receptor-ionophore complex by two separate mechanisms and points to an even greater level of complexity for this multimeric protein than had previously been anticipated.

METHODS

We used dissociated postnatal (day 1) rat hippocampal cell cultures for all experiments. The cells were plated on a layer of glia 10–18 days before the physiology experiments were performed. A detailed account of our culture methods has been described elsewhere (Yamada, Dubinsky & Rothman, 1989).

The tight-seal whole-cell voltage-clamp recording technique was used to measure the currents elicited in all experiments (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Prior to an

experiment, the culture medium was exchanged with extracellular fluid containing (mM): Tris-Cl, 120; CsCl, 3; NaHepes, 10; glucose, 5.5; MgCl₂, 5; at pH 7.3. This solution was used because it limited GABA desensitization seen in sodium-containing buffers. The electrodes were fabricated by pulling glass capillaries (1.2 mm o.d.) with a Brown Flaming-type puller and were filled with a solution containing (mM): CsCl, 130; TEA-Cl, 10; NaHepes, 10; EGTA, 1.1; glucose, 4; ATP-Mg₂, 2; at pH 7.2. The electrode resistance was usually between 5 and 10 M Ω . In the whole-cell configuration, the series resistance was in the range of 10–20 M Ω . With these solutions the estimated reversal potential of Cl⁻ at room temperature was -1 mV, which also limits desensitization. When we elicited GABA-induced chloride currents, the cells were usually held at -30 mV. When we constructed *I-V* plots, a cell was held at 0 mV (close to zero current potential), stepped to -100 mV and gradually depolarized to +60 mV over a 10 s period. In this experimental set-up, it is difficult to make an assessment of space-clamp problems. However, the phenomena described in this paper are so slow that small problems with the space clamp would not alter our general conclusions.

We applied GABA and additional drugs near a voltage-clamped neuron using the U-tube method and sometimes additional bath perfusion (Fenwick, Marty & Neher, 1982; Akaïke, Shirasaki & Yakushiji, 1989). The U-tube was fabricated from polyethylene tubing (1.5 mm o.d.) and an opening at the apex of the curve (about 1 mm diameter) was made using a hypodermic needle. The outflow of the U-tube was connected to a solenoid valve which was gated by a pulse generator. The inflow was switched between different solutions by a manual valve. With the solenoid closed, the usual flow rate into the dish was about 0.5 ml min⁻¹. When the solenoid was open, there was constant suction of the extracellular fluid through the opening at the tip leading to an outflow rate of 2–2.5 ml min⁻¹. To replenish the extracellular solution, control solution was infused through another inlet at 1.5–2 ml min⁻¹. The tip of the U-tube was manoeuvred to about 100 μ m from the cell body. With every closure of the valve there was an obvious distortion of the cell due to the sudden flow of solution out of the tube. However, no current was elicited when control solution was applied in this manner.

All drugs were mixed in the control extracellular solution on the day of the experiment. Both GABA and PCTXN were applied continuously through the U-tube usually for 7 s. The PCTXN receptor antagonist α IMGBL was applied both in the bath (2 min prior to U-tube application) and in the U-tube. After each drug application, the recording chamber (2 ml total volume) was bolus perfused with 10 ml of the desired solution to insure adequate wash. All drugs were applied at an interval of 3 min to insure complete wash-out of the bath and clearance of the U-tube. All chemicals were purchased from Sigma (St Louis, MO, USA) except for the lactones. The synthesis of these compounds is detailed in earlier publications (Klunk, Covey & Ferrendelli, 1982*a, b*; Canney, Holland, Levine, McKeon, Ferrendelli & Covey, 1991).

The data were digitized at 1 kHz, filtered at 2 kHz and stored on disk for later analysis. The currents were measured at the peak and after 6 s of continuous GABA application, with holding currents subtracted in all cases. The comparisons were expressed as the percentage of the control peak and as the percentage of the control current after 6 s of continuous application. We used commercial software (pClamp, Axon Instruments or Sigma plot, Jandel Scientific) for all data analysis and curve fitting. All numerical figures are expressed as means \pm s.e.m. and comparisons made by two tailed *t* test, paired *t* test, or *F* test statistics when appropriate.

RESULTS

With U-tube application of GABA (10 μ M), we were able to produce a Cl⁻ current with an on-rate in the range of 50–200 ms. Over a concentration range of 1–10 μ M, GABA currents remained constant over many seconds (Fig. 1). Desensitization was, therefore, not a big problem. At concentrations above 30 μ M, some desensitization was unavoidable. However, by extensively washing out GABA between applications and spacing applications at 3 min intervals, the recovery of the original control peak current was frequent.

The application of 40 μ M PCTXN along with GABA (10 μ M) caused a depression of peak current to $66.6 \pm 3.3\%$ of control peak ($n = 14$) followed by a time-dependent attenuation of the current with a time constant of about 2.2 s (Figs 1*B*, 2*A* and *B* and

4A). This time constant was usually obtained by fitting the current decay to a single exponential with a constant residual current. Occasionally, the current decay was better fitted by two exponentials especially at a higher concentration of GABA (Fig. 1C). The slower component of the decay was variable and was attributed to the effect

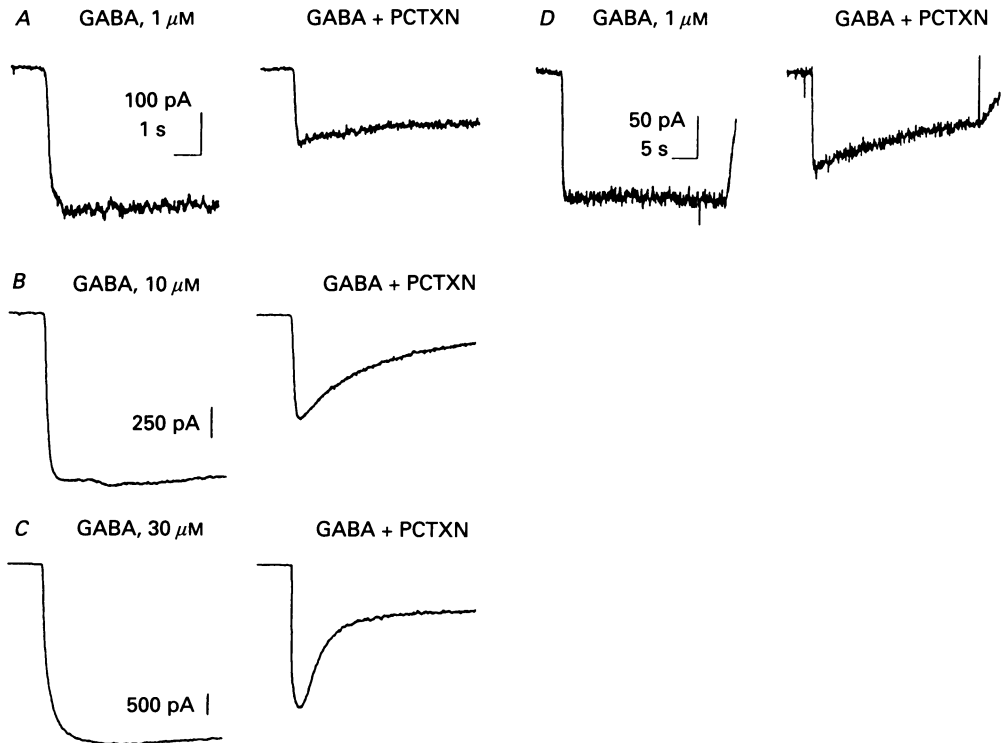


Fig. 1. *A–D*, the mode of 40 μM picrotoxin (PCTXN) block of GABA-induced Cl^- current at varying GABA concentrations. With increasing GABA concentration, the final extent of picrotoxin block is achieved at a faster rate. For 1 μM GABA the GABA + PCTXN application had to be extended to about 25 s to demonstrate the use dependence of the picrotoxin block. Note the difference in the time scale in *D* and that traces are all from different neurons.

of desensitization and was not included in the analysis. After 6 s of continuous application, the current was attenuated further to $24.5 \pm 2.2\%$ of the control. After the plateau PCTXN block of the current was established, the recovery of the GABA current required repeated (usually 2–4) GABA applications. The traces in Fig. 3*Aa–d* demonstrate the typical course of recovery which is facilitated by GABA application. This time-dependent block of the GABA-induced chloride current by PCTXN has been described by Inoue & Akaike (1988) as use dependence.

At a lower concentration of PCTXN (10 μM) (Fig. 2*A* and *B*) the peak 10 μM GABA-induced chloride current was depressed to $89.7 \pm 5.5\%$ ($n = 6$, $P < 0.001$ compared to 40 μM PCTXN) and the time constant for the use-dependent block increased from 2.2 ± 0.2 ($n = 14$) to 3.3 ± 0.2 s ($n = 6$) ($P < 0.001$, Student's *t* test). After 6 s of steady application, the GABA current was reduced to $54.5 \pm 4.5\%$ of

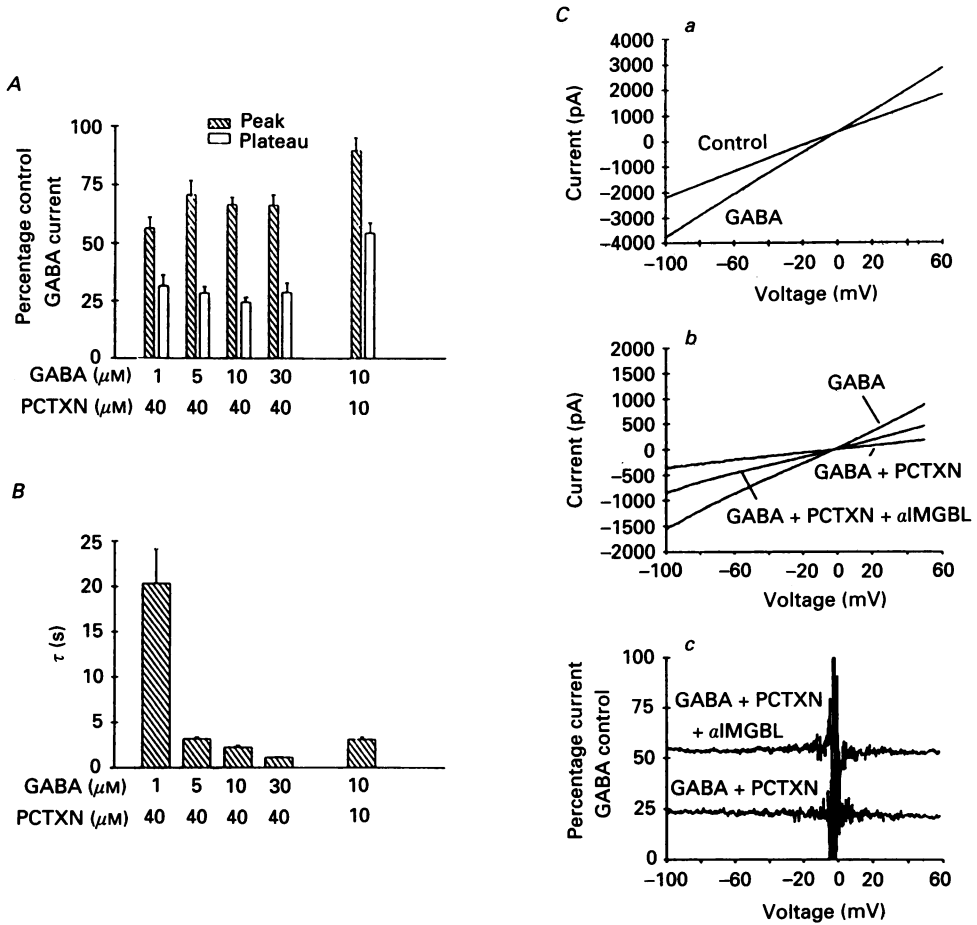


Fig. 2. *A*, the effect of picrotoxin on the GABA-induced current measured at the peak and after the plateau is established. The current was considered to be at a plateau at the end of the 6 s U-tube application of GABA and picrotoxin for GABA concentrations of 5–30 μM . For 1 μM GABA, the current was considered to have reached a plateau after a 30 s application. The amount of attenuation of the peak as well as the plateau showed no significant difference over the various GABA concentrations. However, both the peak and plateau attenuation was significantly dependent on the picrotoxin concentration (see text for detail). *B*, the time constant (τ) of the use-dependent block assuming that the decay is fitted by a single exponential curve with a constant residual current. The average time constant of block in 10 μM GABA + 40 μM PCTXN was significantly different from that in 10 μM GABA + 10 μM PCTXN (see text for detail). In both *A* and *B* n values (no. of experiments) for the GABA concentrations of 1, 5, 10 and 30 μM with 40 μM PCTXN are 7, 5, 14 and 8 respectively. The n value for 10 μM GABA + 10 μM PCTXN is 6. *C*, the effect of voltage on the 5 μM GABA current block by 10 μM PCTXN. *Ca*, the amount of membrane current elicited by applying a ramp voltage command from -100 to $+60$ mV over a 10 s period in the control and in solution containing GABA. *Cb*, resultant current after subtracting the current measurement in the control solution from the test solution. *Cc*, the amount of picrotoxin and picrotoxin + αMGBL (5 mM) block of GABA current expressed as percentage of control GABA current.

control, compared to the 24.5% of control seen with 40 μM PCTXN ($P < 0.001$) (Fig. 2*A* and *B*). Thus, the PCTXN block of GABA current as well as the rate of achieving the block both increase with PCTXN concentration.

PCTXN was more potent in blocking plateau than peak GABA currents over the GABA concentration range of 1–30 μM (Fig. 2*A*). While the antagonism of either

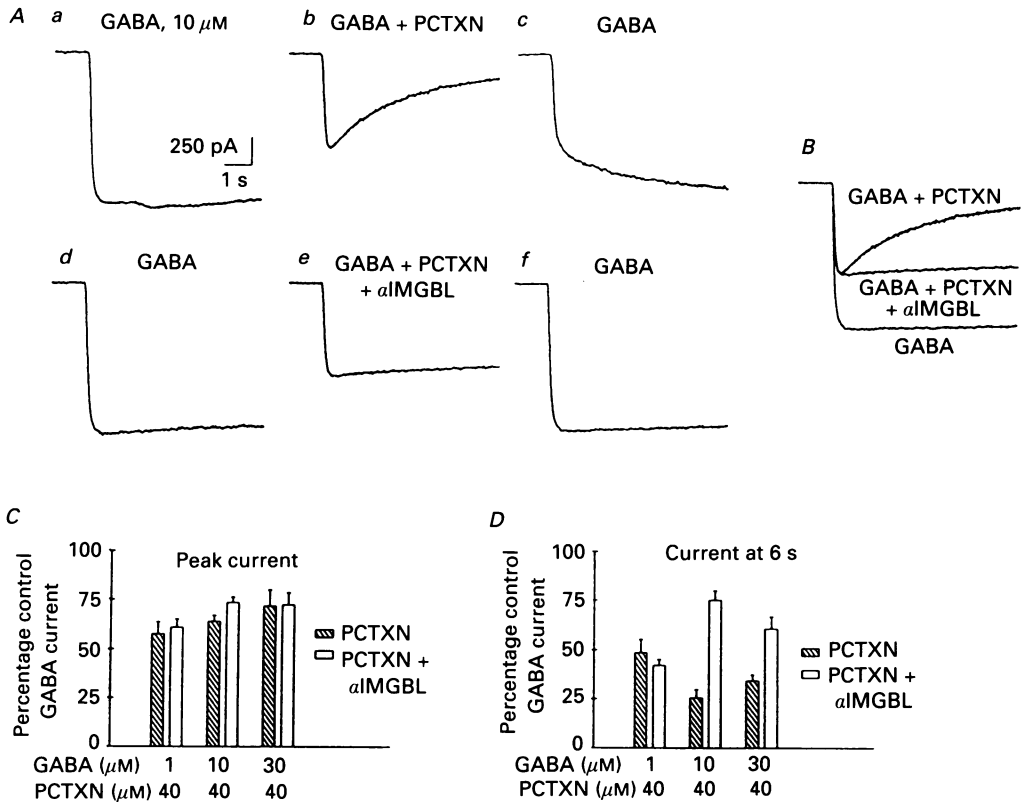


Fig. 3. The effect of 5 mM αIMGBL on microtoxin effect. *A*, traces *a*–*f* show current traces from the same cell with 10 μM GABA application and then GABA + 40 μM PCTXN. PCTXN remained in the bath between traces *b* and *c*. The recovery from the use-dependent block requires repeated GABA application (traces *c* and *d*). After a full recovery, αIMGBL selectively prevented the use-dependent portion of the block. At variance with the recovery from the use-dependent block, the recovery from the PCTXN + αIMGBL block is prompt (trace *f*). *B*, the traces *b*, *d*, and *e* are superimposed, demonstrating the selective block of use-dependent microtoxin effect. *C*, the effect of 5 mM αIMGBL on the peak current block of GABA current by PCTXN. The *n* values for the GABA concentrations of 1, 10 and 30 μM are 5, 7, and 4 respectively in both *C* and *D*. *D*, the effect of 5 mM αIMGBL on the microtoxin block of GABA current after 6 s of continuous application. Note that at 1 μM GABA, αIMGBL has no significant effect because the use-dependent microtoxin block has not developed (see Fig. 5).

current did not vary significantly over this concentration range ($P > 0.2$ for both, *F* test) the time constant for the use dependence was inversely related to the GABA concentration (Fig. 2*B*).

At 1 μM GABA, no apparent use dependence was seen with an application duration of 7 s even though the peak currents were attenuated to $56.5 \pm 4.7\%$ of control ($n = 7$) (Fig. 1A). To demonstrate use-dependent block at 1 μM GABA by 40 μM PCTXN, continuous application for a much longer period (25 s) was necessary (Fig. 1D). Once the PCTXN block was established at 1 μM GABA, recovery was rare because it required such prolonged GABA application. Occasionally, the recovery was facilitated by intervening application of the higher concentration of GABA (10–30 μM ; data not shown).

In some cases, PCTXN was added in the bath perfusion 2 min prior to the U-tube application. This pretreatment with PCTXN in the absence of GABA had no significant effect on the amount of PCTXN block or the development of the use-dependent block of the GABA-induced chloride current. In six cells with PCTXN application both through the U-tube and in the bath, 40 μM PCTXN attenuated the 10 μM GABA current to $62.6 \pm 8.9\%$ of control at the peak and $25.7 \pm 5.3\%$ at 6 s ($P > 0.2$ for both values compared to PCTXN effect without prior bath application).

Because use-dependent block suggests an obstruction to ion flow within the actual ionophore, we determined if the PCTXN block of GABA conductance was dependent on voltage or the direction of ionic flow (Fig. 2C). In these experiments, the drugs were applied by whole-bath perfusion instead of U-tube because it was awkward to maintain U-tube flow for the duration of voltage ramps. At a GABA concentration of 5 μM and PCTXN concentration of 10 μM , no voltage dependence of the PCTXN effect was seen using slow range depolarization from -100 to $+60$ mV. Although voltage dependence is not an absolute requirement for channel block, its absence provides no support for PCTXN acting through such a mechanism.

We were also interested in the interaction between the butyrolactone antagonist αIMGBL and PCTXN. Much to our surprise, 5 mM αIMGBL had no significant effect on the PCTXN block of the peak GABA current but selectively abolished the use-dependent portion of the PCTXN block (Fig. 3). In seven cells after control application of 10 μM GABA, subsequent treatment with 40 μM PCTXN diminished the peak current to $63.8 \pm 3.2\%$ of control. The combination of PCTXN and αIMGBL (in bath and U-tube) decreased the peak current to only $73.5 \pm 2.8\%$ of control ($P > 0.1$, paired t test, compared to the peak depression with PCTXN alone). However, after 6 s of continuous application, αIMGBL dramatically attenuated the PCTXN block and increased the GABA current from 25.6 ± 4.2 to $74.9 \pm 5.0\%$ of control ($P < 0.001$, paired t test).

Five millimolar αIMGBL alone had no significant effect on the GABA-induced chloride current. In three cells, the difference between the current induced by 10 μM GABA alone and GABA with αIMGBL was $-2.2 \pm 3.7\%$ at the peak and $0.3 \pm 1.0\%$ at 6 s.

When 40 μM PCTXN was repeatedly applied with 10 μM GABA in the constant presence of PCTXN in the bath ($n = 4$), the peak attenuation by PCTXN increased with each application, while the final extent of the PCTXN block with each 7 s application stayed the same (Fig. 4A). By the third application of GABA with PCTXN (Fig. 4Ad), the current elicited showed no evidence of the use-dependent decline. Five millimolar αIMGBL partially reversed the block. This suggests that by repetitively applying PCTXN with GABA, the use-dependent mechanism of the

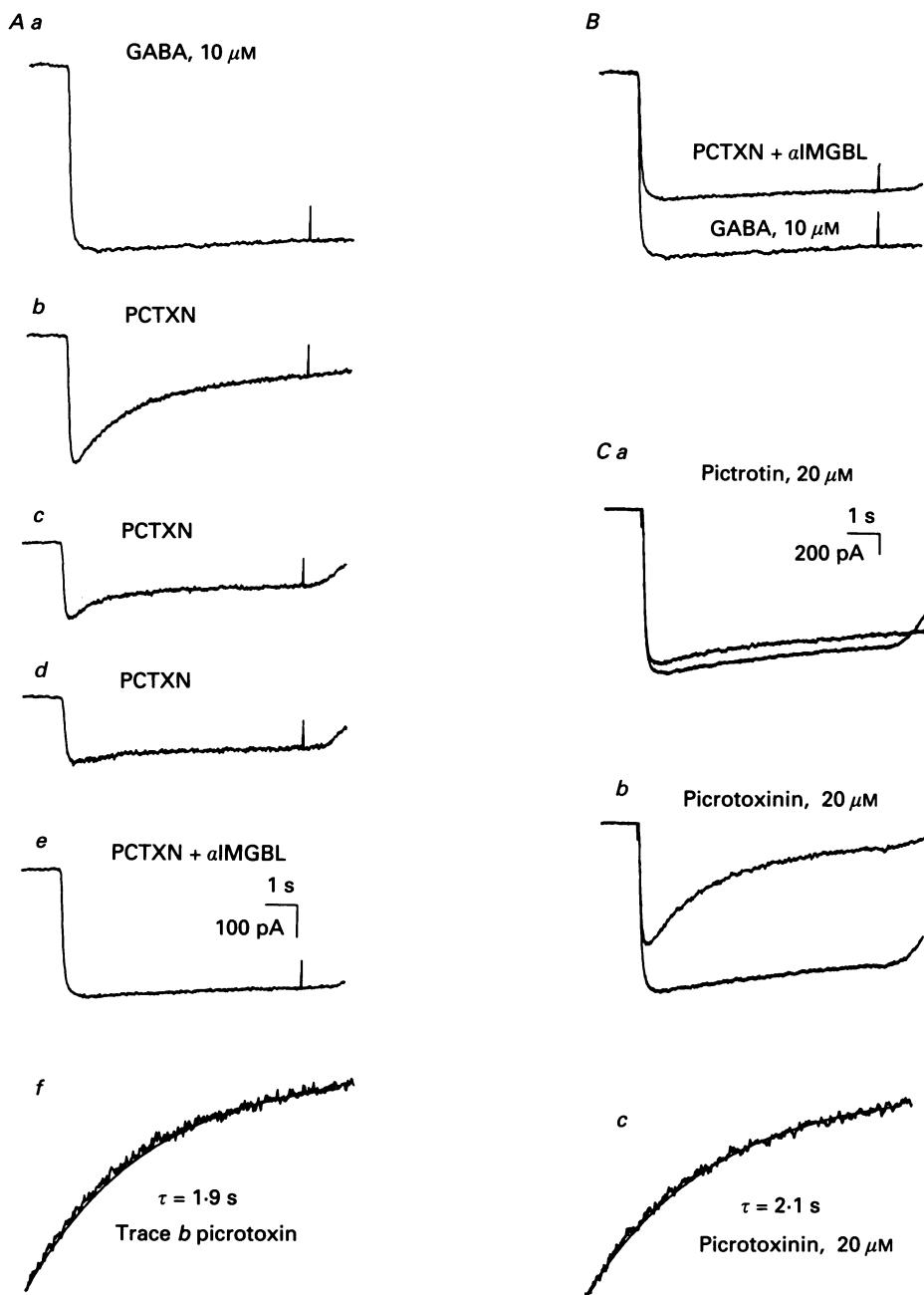


Fig. 4. *A*, the effect of repetitive application of 10 μM GABA in the presence of 40 μM picrotoxin (traces *b*, *c*, *d* and *f*) or picrotoxin + 5 μM αIMGBL (trace *e*) in the bath and U-tube. Note the progressive increase in the peak attenuation by picrotoxin. However, the final extent of the block with each GABA application is approximately the same with each application. By the third application (trace *d*), the time-dependent picrotoxin block is no longer evident. Five millimolar αIMGBL partially reverses this picrotoxin block. The declining portion of trace *b* is fitted by a single exponential curve with a constant residual in trace *f*. *B*, traces *a* and *e* from panel *A* are superimposed to demonstrate the portion of the PCTXN block that is not affected by αIMGBL . *C*, comparison of the effect of picrotin

block can be saturated and that this saturation can be selectively antagonized by α IMGBL (Fig. 4B).

Since picrotoxin is an equimolar mixture of picrotin and picrotoxinin these two different mechanisms of GABA-induced chloride current block could represent the different effects of these two compounds. We, therefore, compared the individual effects of picrotin and picrotoxinin, each at 20 μ M, on the 10 μ M GABA-induced chloride current (Fig. 4C). The composite effects of these concentrations of picrotin and picrotoxinin most probably simulate the effect of picrotoxin at 40 μ M. In four cells, picrotin had no significant effect on the GABA-induced chloride current. Picrotoxinin, on the other hand, mimicked the effect of picrotoxin. Picrotoxinin diminished both the peak ($65.0 \pm 2.9\%$ of control) and steady-state portion of the current ($19.2 \pm 2.8\%$ of control at 6 s) (Fig. 4C). In two cells, this use-dependent portion of the block was attenuated by α IMGBL ($53.6 \pm 1.2\%$ of control). This implies that at this concentration of picrotoxin, most of the effect is mediated by picrotoxinin.

The observations described above suggest that there may be two different PCTXN mechanisms for GABA current attenuation. The first effect has a rapid onset (at least as fast as the method of drug application) and is not blocked by α IMGBL. The second effect is use dependent, and is blocked by α IMGBL.

In an attempt to further isolate the fast (use-independent and α IMGBL-insensitive) component of the PCTXN effect, we studied the effect of α IMGBL on the PCTXN block at low concentrations of GABA (1 μ M) applied for a short period of time (7 s) (Figs 3C and D and 5). Since the time constant for use-dependent PCTXN block was about 20 s at this GABA concentration, the majority of the PCTXN block of GABA current should initially be mediated by the fast component of the PCTXN block. In these experiments, α IMGBL had no significant effect on the PCTXN block of the peak GABA current. In PCTXN alone, the average current was $57.5 \pm 6.0\%$ of control. In PCTXN and α IMGBL the average current was $60.8 \pm 4.2\%$ ($n = 5$, $P > 0.2$, paired t test). Even after 6 s of continuous application the currents were $48.1 \pm 6.8\%$ with PCTXN alone and $42.0 \pm 3.1\%$ with PCTXN + α IMGBL ($P > 0.2$) (Fig. 3C and D). This observation further substantiates the existence of a PCTXN effect that is insensitive to α IMGBL. The small decay of the 1 μ M GABA-induced current was inconsistently observed.

We also simultaneously applied PCTXN (10 μ M) and α IMGBL (5 mM) with GABA (5 μ M) during slow voltage ramps to look for voltage dependence of the PCTXN block of *peak* GABA current (Fig. 2C). This block was voltage independent too, failing to support a channel blocking mechanism for PCTXN.

Another alkyl-substituted butyrolactone, β EMGBL, displaces TBPS, causes seizures in rats, and attenuates GABA-induced Cl^- current (Klunk *et al.* 1982*a, b*; Levine *et al.* 1985; Holland *et al.* 1990*a, c*; Holland, McKeon, Covey & Ferrendelli, 1990*b*). It thus appears to be very similar to PCTXN. In the light of the observations described above, we were curious about its effect on the GABA- Cl^- receptor-ionophore complex.

and picrotoxinin (both at 20 μ M) on GABA (10 μ M)-induced chloride current. The scale on trace *a* is also valid for trace *b*. In trace *c*, the declining portion of the current in trace *b* is fitted with a single exponential curve.

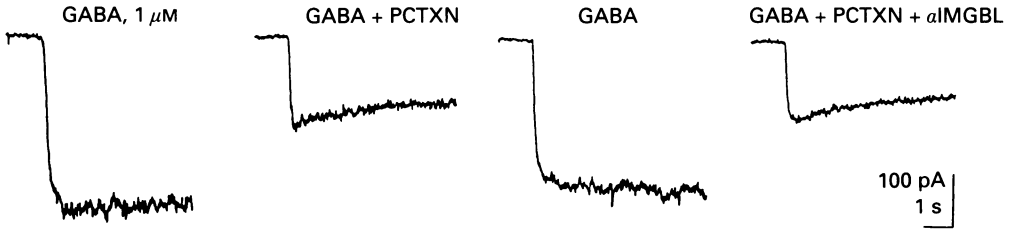


Fig. 5. The effect of 5 mM α IMGBL on the 40 μ M picrotoxin block of 1 μ M GABA-induced chloride current. Note that α IMGBL has no effect on the GABA + picrotoxin current at this low concentration of GABA because the use-dependent block has not developed with this short duration of picrotoxin application.

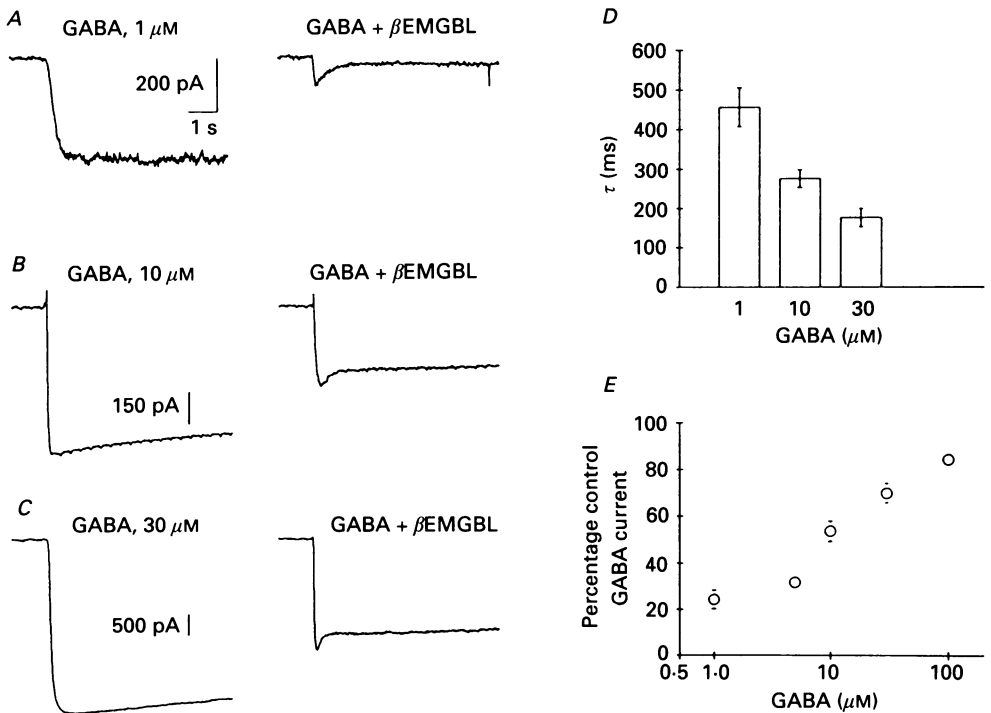


Fig. 6. The effect of β EMGBL on the GABA current. *A–C*, β EMGBL block with progressively higher GABA concentrations. Note that with increasing GABA concentration, the duration of the use-dependent portion of the block decreases and the final extent of the β EMGBL block also decreases. *D*, the time constant for decay of the GABA current transient at varying concentrations of GABA ($n = 11$, 10 and 11 for GABA at 1, 10 and 30 μ M, respectively). *E*, the amount of GABA current remaining after 6 s of continuous application by β EMGBL increases as GABA concentration increases ($n = 15$, 7, 13, 10 and 5 for GABA at 1, 5, 10, 30 and 100 μ M).

One millimolar β EMGBL attenuated the current produced by GABA (Fig. 6). At GABA concentrations of 30 μ M and below, there was a small transient component evident when GABA and β EMGBL were simultaneously applied. When the fall-off of this transient was fitted to a single exponential with a constant residual, the time

constant was shorter than observed with PCTXN but still dependent on the GABA concentration (compare Figs 6D and 2B). At 100 μM GABA, the use-dependent component of the βEMGBL block was no longer evident presumably because the rate of onset of the βEMGBL block was so fast at this high GABA concentration.

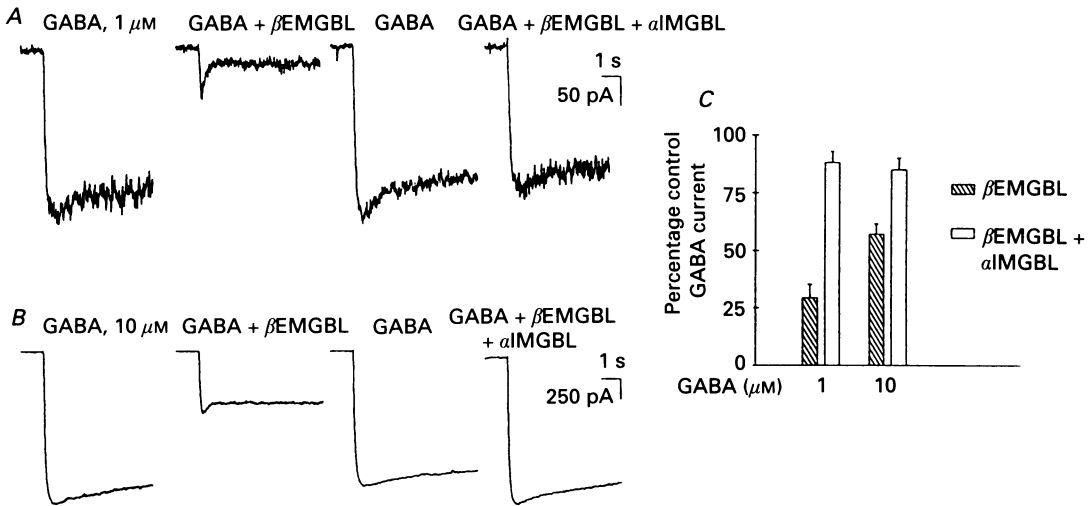


Fig. 7. The effect of 5 mM αIMGBL on the 1 mM βEMGBL block of GABA current at 1 μM (A) and 10 μM (B). Unlike picrotoxin, the recovery from the βEMGBL block does not require repeated GABA applications. C, the percentage of control, steady-state GABA current with βEMGBL and βEMGBL + αIMGBL .

The degree of GABA current attenuation by βEMGBL measured at the end of a 6 s application, however, was inversely related to the GABA concentration (Fig. 6E). In spite of the fact that the rate at which the block was achieved increased with higher GABA concentration, the final extent of blockade decreased with increasing GABA concentration.

In contrast to the lack of effect of αIMGBL on PCTXN block of 1 μM GABA current, the effect of 1 mM βEMGBL on 1 μM GABA was almost completely blocked by 5 mM αIMGBL (Fig. 7A and C). At 10 μM GABA, 1 mM βEMGBL attenuated the GABA current to $57.6 \pm 3.9\%$ at 6 s (Fig. 7B and C). This effect of βEMGBL was attenuated by 5 mM αIMGBL to $85.5 \pm 7.1\%$ at 6 s ($n = 5$, $P < 0.01$, paired t test). This suggests that the effect of βEMGBL on the GABA receptor is selectively mediated by a specific site that is antagonized by αIMGBL .

We pretreated a separate set of cells with βEMGBL for 2 min prior to the U-tube application of $\beta\text{-EMGBL}$ and GABA. Unlike PCTXN, the pretreatment with $\beta\text{-EMGBL}$ abolished the use-dependent aspect of the block (Fig. 8A and B). This loss of βEMGBL use dependence could be attributable to endogenous GABA causing spontaneous channel opening at low frequency or to the lipophilicity of βEMGBL , which may gain access to its binding site without the channel opening. As with PCTXN, the block of GABA current by βEMGBL was not voltage dependent (Fig. 8C).

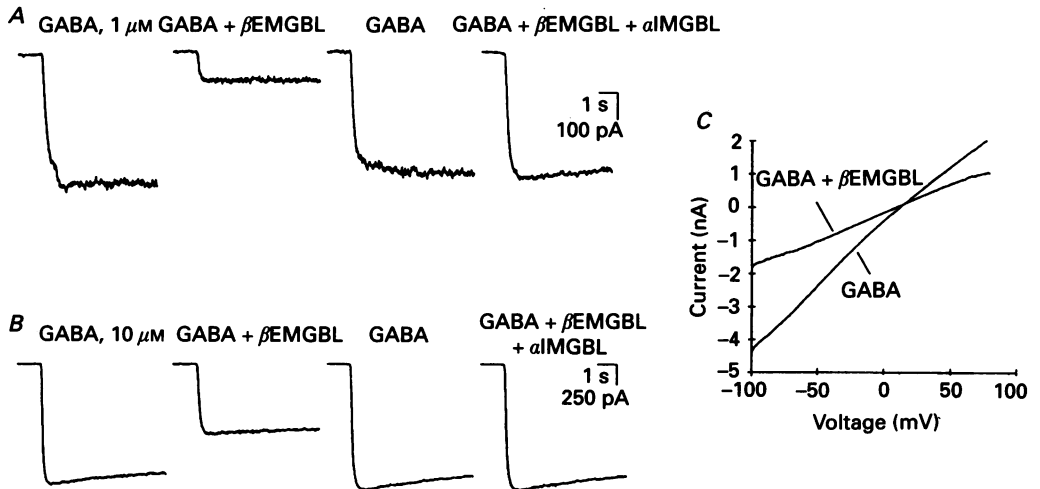


Fig. 8. Influence of pretreatment with βEMGBL 2 min prior to the U-tube application. Note that in both 1 μM (A) and 10 μM (B) GABA, the use dependence is no longer evident presumably due to the fact that βEMGBL is not dependent on the presence of GABA to reach the binding site. C, the currents induced by both applications of 10 μM GABA and GABA + 1 mM βEMGBL over a slow voltage ramp (see Fig. 2C). There is no apparent voltage dependence from -100 to +60 mV.

DISCUSSION

Our observations suggest that there are two separate mechanisms by which PCTXN affects the GABA- Cl^- receptor-ionophore complex in rat hippocampal neurons. The first mechanism is use dependent and is selectively antagonized by αIMGBL . The second mechanism is independent of GABA and is insensitive to αIMGBL .

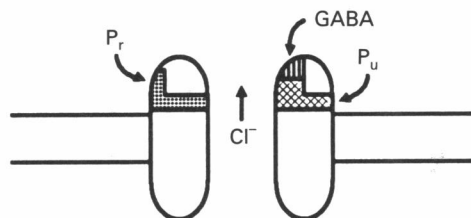
The majority of the binding studies examining the PCTXN (or convulsant) receptor thus far have demonstrated no evidence for two different sites for binding to TBPS (Levine *et al.* 1985; Holland *et al.* 1990b) or dihydropicrotoxinin (Ticku, Ban & Olsen, 1978). The effect of TBPS on chick GABA receptors reconstituted in oocytes is use dependent (Van Renterghem *et al.* 1987), so it is possible that TBPS binds solely to the use-dependent site we have identified. However, there is some evidence that the TBPS binding can be polyphasic (Squires *et al.* 1983; Trifiletti, Snowman & Snyder, 1985) suggesting that TBPS may bind to multiple sites.

Our results enable us to speculate on the location of the two sites of GABA blockade by PCTXN. Use dependence of non-competitive blocking agents is one of the characteristics of open channel blockade and is seen with local anaesthetic block of the nicotinic acetylcholine receptor (Steinbach, 1968; Skok, 1990) and with quaternary ammonium ion block of the delayed rectifier potassium channel (Hille, 1967; Armstrong, 1975; Stanfield, 1983). For different reasons both of these phenomena are voltage dependent.

Some of the observations described in this and other reports (Yasui, Ishizuka &

Akaike, 1985; Inoue & Akaike, 1988) are superficially consistent with the hypothesis that the PCTXN block of the GABA-Cl⁻ ionophore is an open channel blockade. It is use dependent and its efficiency increases with increasing agonist concentration. However, the voltage independence of the PCTXN effect and the reduction of

A Two PCTXN sites on each receptor



B Two receptors with separate PCTXN sites

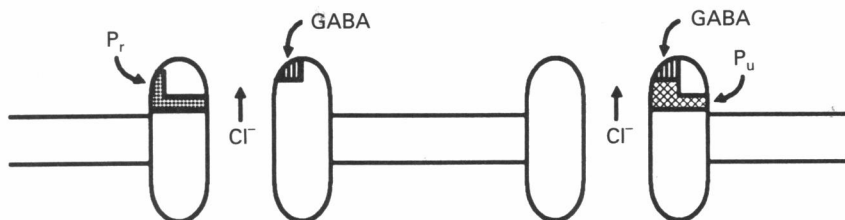


Fig. 9. Two possible models consistent with the PCTXN block of GABA-induced chloride current. *A*, in this model each GABA receptor-ionophore complex has both the P_r and P_u PCTXN binding sites. The P_u site is drawn next to the GABA site to indicate that it is influenced by the prior binding of GABA. *B*, in this alternate model, P_r and P_u sites are on different receptor-ionophore complexes.

β EMGBL antagonism with increasing GABA concentration make it less likely that either PCTXN or β EMGBL are channel blockers. Moreover, the existence of γ -butyrolactones that actually potentiate GABA-induced chloride currents makes it extremely unlikely that the binding site for this class of compounds is within an ion channel (Holland *et al.* 1990*a*). It, therefore, seems probable that the use-dependent site is outside the channel but in a position to be modified by GABA binding and channel opening. This site could actually be *within* the neuronal membrane, a conclusion completely compatible with recent nuclear magnetic resonance data on a fluorinated γ -butyrolactone (D. F. Covey, unpublished). The use-independent site may be distant from the actual GABA recognition site.

We have incorporated these two different PCTXN mechanisms into two simplified models which describe GABA current block (Fig. 9). The two PCTXN mechanisms (P) are differentiated by a subscript: 'r' for the rapid onset α IMGBL-independent effect and 'u' for the use-dependent, α IMGBL-sensitive effect. In this model the P_r mechanism can be described as *non-competitive* inhibition, while the P_u mechanism would be a form of *un-competitive* inhibition, requiring the prior binding of GABA to its site on the receptor-ionophore complex (Segel, 1976).

In the first model (Fig. 9A), the channels binding PCTXN at the P_r site are still capable of remaining partially open and binding at the P_u site as well. A second possibility is that PCTXN acts via the P_r mechanism at some receptors and the P_u mechanism at others, giving *average* behaviour consistent with our model. Our data are also consistent with a second model (Fig. 9B) in which the cultured hippocampal neurons have two different types of GABA_A receptors each capable of generating only P_r or P_u block but not both.

Based on this scheme, we would say that β EMGBL acts at the P_u site. This site has many of the properties that are characteristic of steady-state picrotoxin block of sympathetic neuron GABA currents (Newland & Cull-Candy, 1992). It is apparent that GABA activation of the receptor-ionophore complex does improve the ability of β EMGBL to access the picrotoxin receptor. However, unlike PCTXN, β EMGBL was capable of affecting the picrotoxin receptor if preapplied in the absence of GABA. The increased efficacy of β EMGBL compared to PCTXN could be attributed to the fact that β EMGBL is a non-polar molecule which might gain more rapid access to a site partially embedded in the neuronal membrane.

One intriguing aspect of the β EMGBL reduction of the GABA current was the fact that while the rate of onset of use-dependent block increased with higher GABA concentration, the final extent of GABA current blockade decreased (Fig. 6E). This was not seen with PCTXN (Fig. 2A). On casual inspection, this feature of the β EMGBL dose-response relationship resembles competitive GABA antagonism. However, there is no evidence for β EMGBL or any other alkyl-substituted γ -butyrolactone affecting GABA binding (Levine *et al.* 1985; Holland *et al.* 1990b). We believe that the complicated kinetics of GABA-Cl⁻ receptor-ionophore activation provides one possible explanation for this anomalous dose-response relationship. It is now well accepted that this receptor can be activated by binding one or two molecules of GABA (Mathers & Wang, 1988; Weiss & Magleby, 1989; Macdonald, Rogers & Twyman, 1989; Twyman, Rogers & Macdonald, 1990). We suggest that β EMGBL might produce a relatively selective and use-dependent block of the single-liganded receptor-ionophore complex. When the GABA concentration is low, a higher percentage of open channels is in the single-liganded state and vulnerable to β EMGBL block. However, as the GABA concentration increases, more receptors interact with two GABA molecules, thus decreasing the ability of β EMGBL to block the GABA current. Based on other data and mathematical simulations we have previously suggested that other alkyl substituted γ -butyrolactones, α -ethyl- α -methyl- γ -butyrolactone (Holland *et al.* 1990c) and 2,2-difluoro- α -ethyl- α -methyl- γ -butyrolactone (Yoon, Canney, Covey & Rothman, 1990), have higher affinities for single-liganded GABA channels.

A second possible explanation for the complicated effects of β EMGBL on GABA dose-response relationships is provided by a well-established model of the crustacean muscle GABA receptor proposed by Smart & Constanti (1986). They found a similar result with picrotoxinin, which they named 'mixed antagonism'. They attributed this to picrotoxinin having a different affinity for each of four possible states of the GABA-Cl⁻ receptor-ionophore complex. Higher affinity for closed states, which precede channel opening in their model, produces the apparent competition characteristic of 'mixed antagonism'. Newland & Cull-Candy (1992) have suggested

a similar mechanism for PCTXN block of sympathetic GABA responses. Of note, neither these earlier papers nor ours proposes that PCTXN and related compounds are open channel blockers.

An entirely different explanation for this dose-response anomaly is found in a paper by Van Renterghem and associates (1987), who obtained the same relationship for TBPS block of chick brain GABA receptors expressed in frog oocytes. This TBPS block has many features of the P_u mechanism discussed above. Based on other studies of [35 S]TBPS binding this latter group suggested that higher concentrations of GABA might actually displace TBPS. It is possible that something similar happens with β EMGBL. This, rather than the variations on state selectivity mentioned above, could account for the declining inhibition of GABA currents at higher concentrations. However, we suspect that this is not the case for two reasons. First, when using *intact* cells, other investigators have shown that the GABA agonist muscimol actually enhances [35 S]TBPS binding (Gallo, Wise, Vaccarino & Guidotti, 1985). Second, the ability of the related compound α -ethyl- α -methyl- γ -butyrolactone to act as either an antagonist or inverse agonist at the picrotoxin receptor is not consistent with these compounds being displaced at high GABA concentrations (Holland *et al.* 1990*c*).

We have to acknowledge that our present results are somewhat different from an earlier result with β EMGBL (Holland *et al.* 1990*a*). That paper reported that β EMGBL behaved like a non-competitive GABA antagonist. We suspect that the discrepancy is due to our applying drugs for only a brief period in the earlier set of experiments and, therefore, failing to obtain true steady-state currents.

Independent of the actual mechanistic explanation for our results, they are the first set of experiments which clearly identify two different PCTXN effects, one of which had not been anticipated (P_r). We are now looking for this mechanism in other GABA receptors in the central and peripheral nervous systems to see if it is common to other naturally occurring GABA-Cl⁻ receptor ionophores. We will also have to determine if artificially reconstituted GABA receptors in oocytes or cloned cell lines display it. Both the P_r and P_u effects should eventually give us important information about the underlying structure of this complex protein.

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