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Actions of picrotoxinin and related compounds on the frog spinal cord: the role of a hydroxyl-group at the 6-position in antagonizing the actions of amino acids and presynaptic inhibition

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1 The frog spinal cord has been used to test the effects of naturally occurring picrotoxane compounds (picrotoxinin, picrotin, tutin and coriamyrtin) and semisynthetic ones (6-acetylpicrotoxinin and anhydropicrotin) on the electrical activities of dorsal and ventral roots and on amino acid-induced depolarizations of primary afferent terminals.

2 Picrotoxinin, tutin and coriamyrtin (10^{-5}–3×10^{-5}M), which have a free hydroxyl group at the 6-position, caused a gradual depolarization in both ventral and dorsal roots. The depolarizations were accompanied by a reduction in the size of the dorsal root potential (DR-DRP), dorsal root reflex (DR-DRR) and ventral root potential (DR-VRP) and by an augmentation of the first spike potential and polysynaptic components in the ventral root reflex (DR-VRR).

3 6-Acetylpicrotoxinin (10^{-5}–3×10^{-5}M) caused a slight hyperpolarization in both roots, and this hyperpolarization was accompanied by the augmentation of DR-DRP, DR-VRP and DR-VRR. The DR-DRR was reduced or abolished by the compound.

4 Picrotoxinin, tutin and coriamyrtin reduced γ-aminobutyric acid (GABA)-, β-alanine- and taurine-induced depolarizations of primary afferent terminals. 6-Acetylpicrotoxinin showed almost the same degree of inhibition of β-alanine and taurine as did picrotoxinin, but the GABA-antagonizing action of the compound was significantly weaker than was that of picrotoxinin.

5 Picrotoxinin, tutin, coriamyrtin and 6-acetylpicrotoxinin all blocked presynaptic inhibition of the first spike potential caused by antidromic conditioning stimulation.

6 The present results suggest that the hydroxyl group at the 6-position of picrotoxane compounds are important for antagonism of the effects of GABA, but not of β-alanine and taurine and for the blocking action of the presynaptic inhibition in the frog spinal cord.

Introduction

Although picrotoxin, or its active component, picrotoxinin, has been described as having an action as a noncompetitive γ-aminobutyric acid (GABA) antagonist (Takeuchi & Takeuchi, 1969), its specific antagonizing action on the GABA effector has provided a pharmacological tool for identifying GABA-ergic pathways in vertebrate and invertebrate nervous systems as well as providing many important clues for elucidating the molecular mechanisms of the GABA receptor and effector interactions (Takeuchi, 1976; Olsen *et al.*, 1978; Ticku, *et al.*, 1978).

However, in the frog spinal cord, picrotoxin and picrotoxinin were reported to antagonize not only GABA-induced depolarizations of the primary afferent terminal but also those induced by β-alanine and taurine (Barker *et al.*, 1975; Kudo *et al.*, 1983). Since the receptor for GABA has been shown to be distinct from that for β-alanine and taurine (Barker *et al.*, 1975), those three amino acids may drive a common effector in the frog spinal cord. In our previous study we found that dendrobine, an alkaloid isolated from Orchidaceae plants and having the same picrotoxane skeleton as that of picrotoxinin, had no GABA antagonizing action, but had significant antagonizing actions on β-alanine and taurine-induced depolarizations on the primary afferent ter-

minal and on presynaptic inhibition in the frog spinal cord (Kudo *et al.*, 1983). These data suggested that the mechanisms of action of picrotoxane compounds in antagonizing the effects of GABA and β-alanine/taurine might differ and that the transmitter involved in presynaptic modulation in the frog spinal cord is not GABA but may be another amino acid, such as β-alanine or taurine. One of the differences in structure between dendrobine and picrotoxinin is that the former has no hydroxyl group at the 6-position in the picrotoxane skeleton. The importance of the hydroxl group for pharmacological activity has been shown in an earlier study, where the acetylation of the hydroxyl group caused the molecule to lose its activity as a convulsant in mice (Jarboe *et al.*, 1968). Recent studies using a receptor assay showed that 6-acetylated picrotoxinin had no ability to replace [^{3}H]-dihydropicrotoxinin from its binding sites in vertebrate and invertebrate nervous systems (Olsen *et al.*, 1978; Ticku *et al.*, 1978). In the present study, we tested the effects of picrotoxinin and its naturally occurring analogues, tutin and coriamyrtin, as well as the semi-synthetic analogues, 6-hydroxypicrotoxinin and anhydropicrotin, to elucidate the characteristics of the action of picrotoxane compounds on synaptic activity and on the effects of neutral amino acids, upon the primary afferent terminal.

Methods

The isolated intra-arterially perfused spinal cord of the bullfrog was used in these experiments. Recordings were made of the root potentials and root reflexes by a sucrose gap method.

Seventy-five bullfrogs (*Rana catesbeiana*) weighing 80–150 g were obtained from April to July 1981 and from October to December 1982. The technique for preparing the isolated, intra-arterially perfused spinal cord preparation was the same as that described by Kudo *et al.*, (1975). An arterial cannula was inserted into the ventral spinal artery and the isolated spinal cord was perfused with amphibian Ringer solution having the following composition (mM): NaCl 115, KCl 2.7 $CaCl_2$ 1.8, (in Ca-free media, $CaCl_2$ was omitted and replaced by $MgCl_2$ 9.0) and glucose 5.5, with the pH adjusted to 7.6 by addition of $NaHCO_3$. The perfusion rate was approx 0.3 ml min^{-1}. The experiments were performed at room temperature (20 ± 2°C).

The potential differences between the spinal cord and the peripheral root stumps (9th dorsal root and 10th ventral root) were assessed by means of a sucrose gap method (Kudo *et al.*, 1975; Kudo, 1978). The 10th dorsal root was stimulated to evoke the dorsal root potential (DR-DRP) and reflex (DR-DRR) and to evoke the ventral root potential (DR-VRP) and reflex (DR-VRR). In some experiments the ventral root (9th) was stimulated repetitively (50 Hz, 20 pulses) to evoke the VR-DRP in the 9th dorsal root. Presynaptic inhibition of the first spike potential in the DR-VRR induced by the test stimulation was produced by antidromic conditioning stimulation of the ventral root of the same segment (Holemans & Meij, 1968).

Drugs used were picrotoxinin (Sigma), tutin (iso-

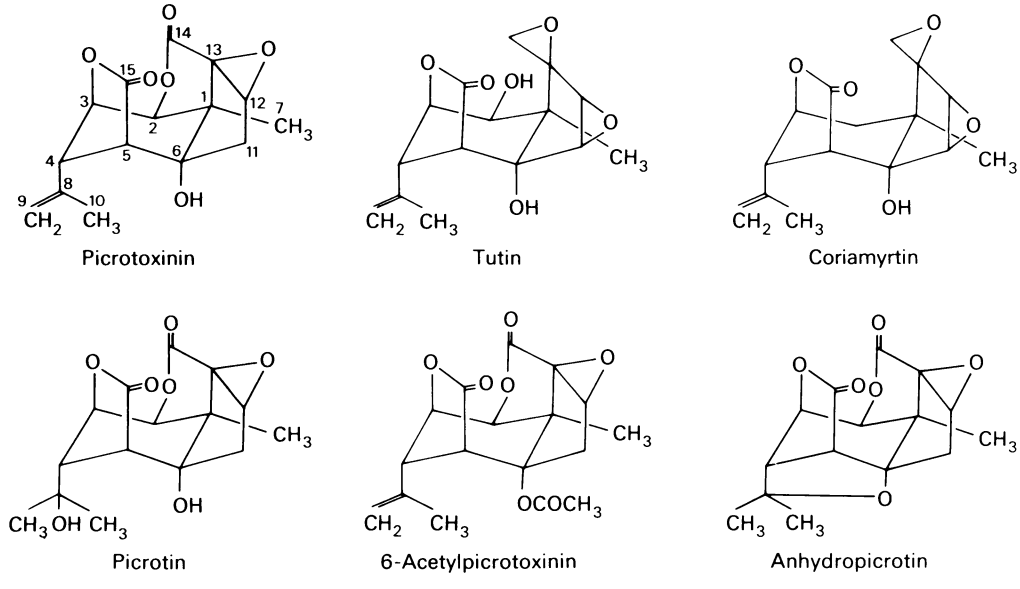

Figure 1 Chemical structures of picrotoxane compounds tested.

lated and purified by K.Y. and H.N., from *Coriaria japonica*, mol. wt. 294.3, m.p. 211–212°C), coriamyrtin (isolated and purified by K.Y. and H.N., from *Coriaria japonica*, mol. wt. 278.3, m.p. 228–231°C), 6-acetylpicrotoxinin (synthetized by Y.K. and H.N., mol. wt. 334.3, m.p. 256–258°C), anhydropicrotin (synthesized by K.Y. and H.N., mol. wt. 292.3, m.p. > 300°C (dec.)) and picrotin (Sigma) (Figure 1). Since 6-acetylpicrotoxinin and anhydropicrotin were insoluble in Ringer solution, these compounds were dissolved in 0.1 ml of acetic acid and diluted with Ringer solution (50 ml). The pH was adjusted to 7.6 by addition of 0.1 N $NaHCO_3$ and the drug was administered by exchanging the perfusion medium with a drug solution containing the substance through the anterior spinal artery. Amino acids used were γ-aminobutyric acid (GABA) (Wako Pure Chem), β-alanine (Wako Pure Chem.) and taurine (Wako Pure Chem.). All amino acids were dissolved in a Ca^{2+}-free, Mg^{2+}-containing Ringer solution. Three of these (3×10^{-3} M) were routinely perfused sequentially for 5 s at a rate of 0.01 mls^{-1},

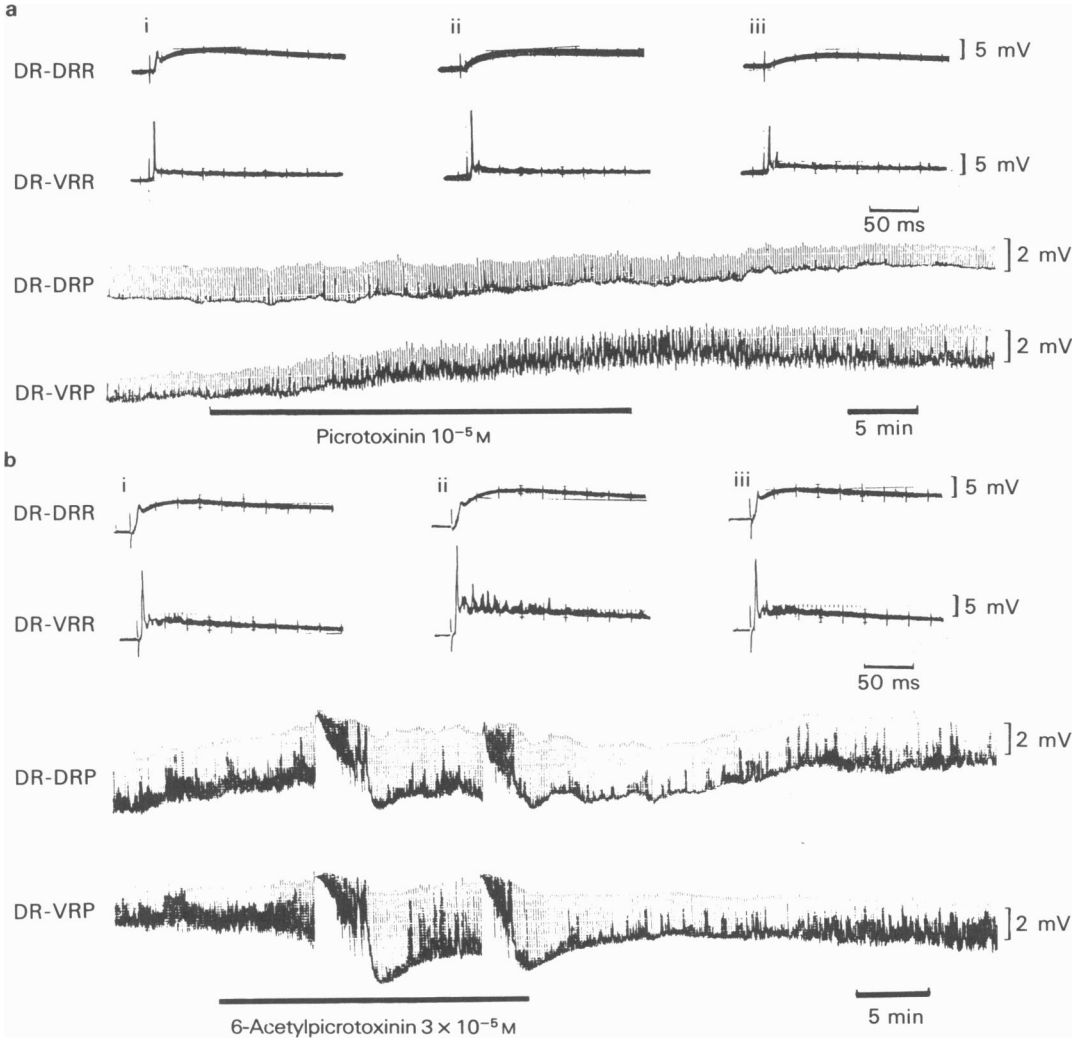

Figure 2 Effects of picrotoxinin and 6-acetylpicrotoxinin on root potentials and reflexes induced by stimulation of the dorsal root in the frog spinal cord. (a) Effects of picrotoxinin (10^{-5}M). Upper set of recordings indicate the oscilloscope displays of the DR-DRR and DR-VRR. The stimulation was applied to the 9th dorsal root. Lower set of recordings indicate the dorsal (10th) (upper) and ventral (9th) (lower) root potentials. (b) Effects of 6-acetypicrotoxinin (3×10^{-5}M) on root potentials and reflexes. Upper and lower sets of recordings were obtained as in (a). (i) Before the application of drugs; (ii) 20 min after the application; (iii) 30 min after washing.

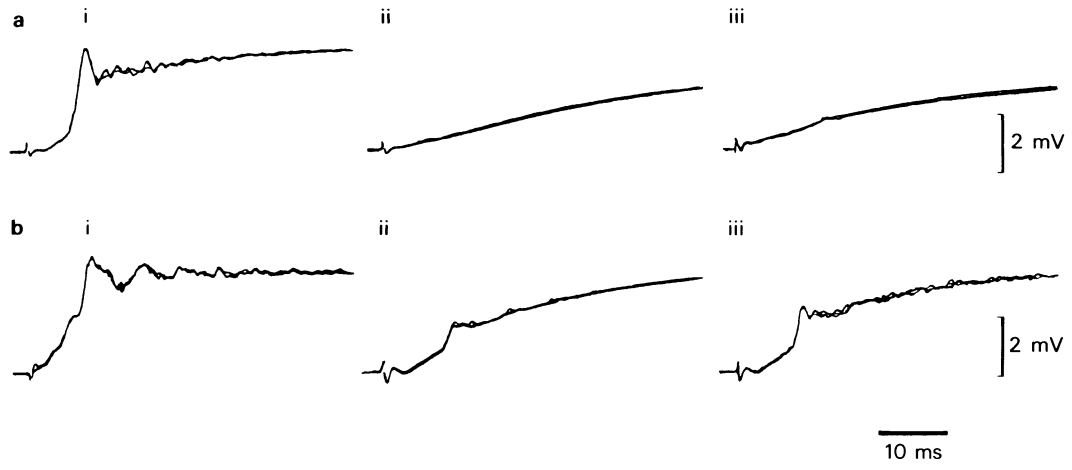

Figure 3 Effects of picrotoxinin and 6-acetylpicrotoxinin on DR-DRP and DR-DRR complex. DR-DRP and DR-DRR complex was rapidly swept, 5 ms cm^{-1}) and photographed (3 traces surperimposed). (a) Effect of picrotoxinin (10^{-5}M); (b) effect of 6-acetylpicrotoxinin (3×10^{-5}M). (i) Before the application of drugs; (ii) 20 min after the application; (iii) 30 min after washing.

once every 5 min by a multiperpex pump (LKB 2155) having three electric valves operated by a timer. The compounds flowed through three separate fine polyethylene tubes which were placed in a glass cannula, the latter having previously been inserted into the spinal artery.

Results

Effects on root potentials and reflexes

As shown in Figure 2a and Table 1, picrotoxinin, tutin and coriamyrtin (10^{-5} M) showed qualitatively similar effects on root potentials and reflexes. They caused gradually depolarizing shifts of the d.c. baseline in both ventral and dorsal roots. These shifts were accompanied by a reduction in the size of the DR-DRP and the DR-VRP. The first spike potential in the DR-VRR increased and corresponding polysynaptic components were augmented, while the DR-DRR was reduced or abolished (Figure 2a and 3a). These three compounds sometimes caused spontaneous oscillations in both roots (Table 1).

As has already been reported, picrotoxinin reduced the size of the DR-DRP and the VR-DRP evoked by repetitive stimulation (50 Hz, 20 pulses) of the dorsal and ventral roots, respectively (Kudo *et al.*, 1983). Such inhibitory actions on the DR-DRP and VR-DRP were also observed with tutin and coriamyrtin (Figure 4a, b). These effects were not completely reversed even after 30 min of washing with the drug-free medium.

By contrast however, 6-acetylpicrotoxinin (10^{-5}–3×10^{-5} M) caused a slight hyperpolarizing shift in the d.c. level of both ventral and dorsal root

Table 1 Effects of picrotoxane compounds on dorsal and ventral root potentials

Compounds	*Dose* (M)	n	*% amplitude of* DR-DRP	DR-VRP	*d.c. baseline*	*S.O.*
Picrotoxinin	10^{-5}	4	31.4±8.6	17.2±1.6	D	2/4
Tutin	10^{-5}	4	56.5±7.1	57.2±8.9	D	4/4
Coriamyrtin	10^{-5}	4	28.4±1.7	52.0±8.7	D	3/4
6-Acetylpicrotoxinin	3×10^{-5}	4	125.8±6.8	177.2±7.7	sH	4/4
Picrotin	5×10^{-5}	4	81.7±4.8	73.4±6.5	—	1/4
Anhydropicrotin	5×10^{-5}	2	103.8	111.8	—	2/2

D: depolarizing shift; sH: slight hyperpolarizing shift; S.O.: incidence of spontaneous oscillation of d.c. baseline during the drug application. Percent amplitudes of root potentials were calculated based upon the amplitudes of root potentials observed following 20 min of application of test compounds; responses before application taken as controls.

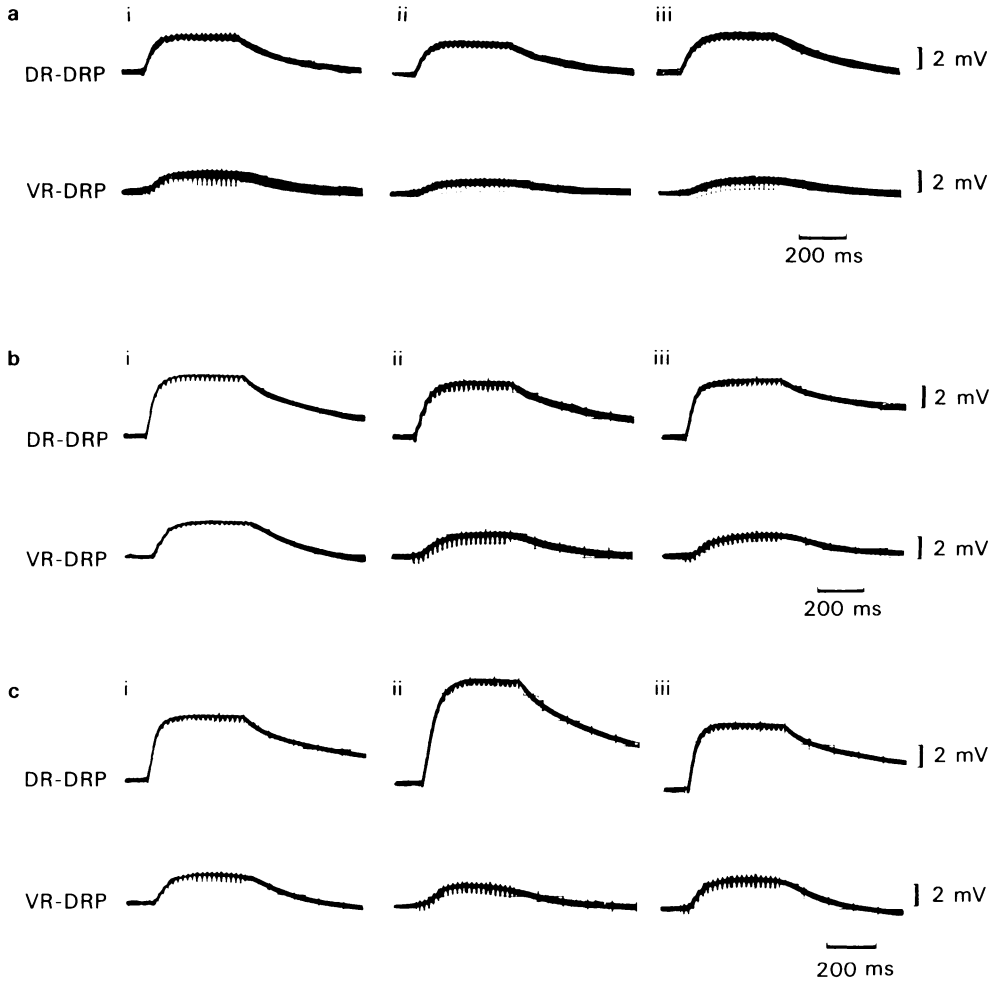

Figure 4 Effects of tutin, coriamyrtin and 6-acetylpicrotoxinin on the dorsal root potentials. Upper and lower tracings in each set of figures indicate the dorsal root potential induced by repetitive stimulation (50 Hz, 20 pulses) of an adjacent dorsal root (10th) and corresponding ventral root (9th), respectively. (a) Effect of tutin (10^{-5}M); (b) effect of coriamyrtin (10^{-5}M); (c) effect of 6-acetylpicrotoxinin (3×10^{-5}M). In each set of figures: (i) before the application of the drug; (ii) 20 min after its application; (iii) 30 min after washing.

potentials (Figure 2b and Table 1). These hyperpolarizations were accompanied by an increase in the size of the DR-VRP and of the first spike potential as well as an increase of the polysynaptic component of the DR-VRR. On the other hand, the early component of both the DR-DRP and the DR-DRR was reduced or abolished, while the maximum amplitude of the DR-DRP was augmented (Figure 2b and 3b). The DR-DRP induced by repetitive stimulation was augmented by 6-acetylpicrotoxinin, but the VR-DRP induced in the same manner was reduced, as it was with the above three compounds (Figure 4c). These effects were reversible within 30 min of washing with drug-free medium. The drug caused marked spontaneous oscillations in both roots in all preparations tested (Figure 2b and Table 1). The amplitudes of DR-DRP and DR-VRP were slightly but significantly reduced by picrotin (5×10^{-5} M) (Table 1). Anhydropicrotin (5×10^{-5} M) caused no detectable alteration in the size of the DR-DRP and DR-VRP, but caused marked spontaneous oscillations in both dorsal and ventral roots in two preparations tested (Table 1).

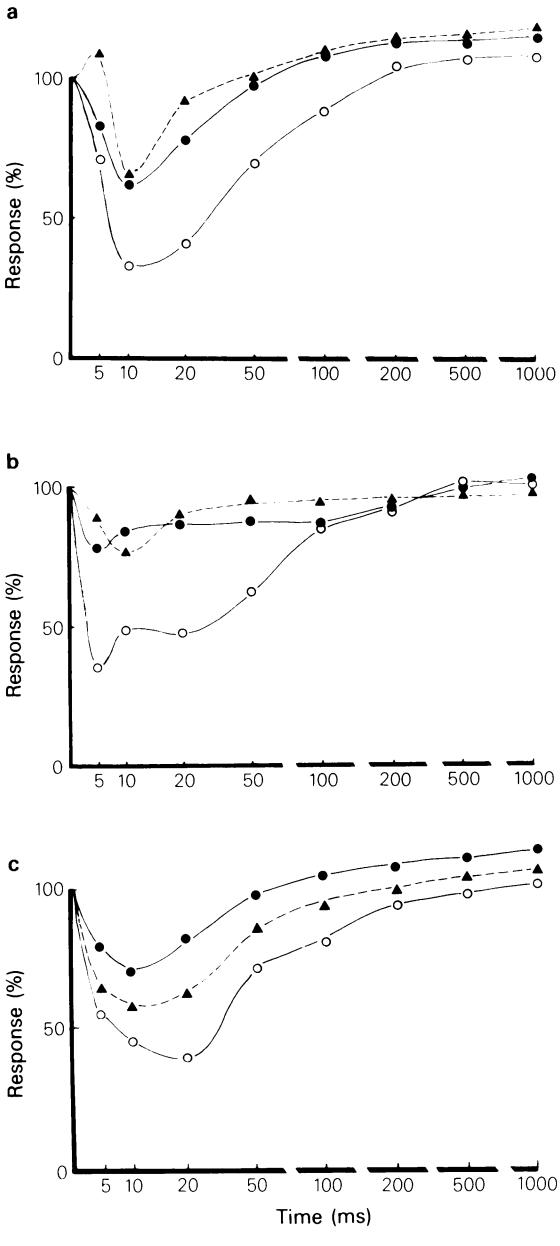

Figure 5 Alteration of presynaptic inhibition on the first spike potential in the DR-VRR. (a) Effects of tutin (10^{-5}M); (b) effect of coriamyrtin (10^{-5}M); (c) effect of 6-acetylpicrotoxinin (10^{-5}M). Each point represents the average of three separate experiments. Abscissa scale: interval between conditioning (9th ventral root) and test (10th dorsal root) stimulations. Ordinate scale: the percentage amplitude of the first spike potential in the 10th ventral root. (○) Control; (●) 20–30 min after the application of the drug; (▲) 20–30 min after washing.

Effects on presynaptic inhibition

6-Acetylpicrotoxinin caused similar inhibitory actions on both the DR-DRR and the VR-DRP. Since these effects have been regarded as indices of presynaptic modulation of synaptic transmission, the drug would be expected to produce an antagonism of presynaptic inhibition upon the first spike potential in the DR-VRR caused by antidromic conditioning stimulation of the ventral root of the same segment. Accordingly, the effects of 6-acetylpicrotoxinin on presynaptic inhibition were compared with those of picrotoxinin, tutin and coriamyrtin. As was shown in a previous paper (Kudo *et al.*, 1983), picrotoxinin (10^{-5}M) reduced presynaptic inhibition; this effect was maintained even after 20–30 min of washing with drug-free medium. Figure 5a and b shows that tutin (10^{-5}M) and coriamyrtin (10^{-5}M) produced similar blocking actions on presynaptic inhibition as did picrotoxinin. 6-Acetylpicrotoxinin (10^{-5}M) also caused a clear antagonism of presynaptic inhibition (Figure 5c). The effects of tutin and coriamyrtin are apparently irreversible within 20–30 min of washing, but the effect of 6-acetylpicrotoxinin was at least partly reversible.

Effects on amino acid-induced depolarizations of the primary afferent terminal

The characteristic actions of the picrotoxane compounds tested on root potentials, reflexes and presynaptic inhibition suggested that picrotoxinin and 6-acetylpicrotoxinin may cause different antagonizing actions on amino acid-induced depolarization of the primary afferent terminals, as has been demonstrated for dendrobine (Kudo *et al.*, 1983). The antagonizing action of picrotoxane compounds on amino acid-induced depolarizations was therefore tested in concentrations of 10^{-5}–5×10^{-5}M. The antagonizing effect of 6-acetylpicrotoxinin on GABA was significantly weaker than that of picrotoxinin, but the compound caused almost the same degree of antagonism of the effects β-alanine and taurine as did picrotoxinin (Table 2). As is shown in Table 3, tutin and coriamyrtin caused a significant antagonism of GABA, β-alanine and taurine at a concentration of 10^{-5}M. At a higher concentration (3×10^{-5}M) they caused a much more pronounced antagonism of the effects of the neutral amino acids. Picrotin and anhydropicrotin caused no significant antagonism of the amino acids in the dose range tested (Table 3).

Discussion

It has been demonstrated that tutin causes a picrotoxin-like antagonism of the action of GABA in

Table 2 Comparison of antagonism by picrotoxinin and 6-acetylpicrotoxinin of amino acid-induced depolarization of primary afferent terminals

Agent	*Dose* (M)	n	*% values of antagonism upon* GABA	*β-Alanine*	*Taurine*
Picrotoxinin	5×10^{-5}	4	63.5 ± 5.0	55.8 ± 7.8	57.0 ± 11.4
6-Acetylpicrotoxinin	5×10^{-5}	4	24.4 ± 9.0*	43.6 ± 10.1	52.9 ± 18.4

GABA: γ-aminobutyric acid. Each amino acid in a concentration of 3×10^{-3}M was infused for 5 s at a rate of 0.01 ml s^{-1} (1.5 × 10^{-5} mol) once every 5 min by a microtube pump and a timer-controlled micro-valve system. % values of antagonism were calculated based upon amino acid-induced responses observed following 15–25 min of application of test agents; the responses before application were taken as controls.
*Significantly different from picrotoxinin ($P < 0.01$, Student's t test).

the frog spinal cord (Nistri, *et al.*, 1974). The present study confirmed that in the frog spinal cord, pharmacological actions of tutin and of coriamyrtin, a molecule having the same picrotoxane skeleton and free hydroxyl group at the 6-position, are quite similar to those of picrotoxinin. These compounds caused: (1) a depolarizing shift in the d.c. level of the base-line, (2) reductions in the size of the DR-DRP, DR-DRR and VR-DRP, (3) antagonism of presynaptic inhibition and (4) antagonism of the effects of GABA, β-alanine and taurine. For the most part, these effects were observed to persist even after 30 min of washing. 6-Acetylpicrotoxinin was employed in the present study in order to examine the importance of the hydroxyl group at the 6-position. This substance also reduced the size of the DR-DRR and VR-DRP, antagonized presynaptic inhibition and blocked the effects of β-alanine and taurine. However, the compound caused (1) a hyperpolarizing shift of the d.c. level of the base-line, (2) augmentation of the size of the DR-DRP and (3) showed a significantly weaker action as an antagonist of GABA than did picrotoxinin. The effects of 6-acetylpicrotoxinin were at least partially reversed by replacement of the perfusing solution with drug-free medium for 30 min. These molecular properties are quite similar to those previously observed with dendrobine (Kudo *et al.*, 1983). The acetylation of the hydroxyl group at the 6-position of picrotoxinin caused the molecule to lose its convulsive, toxic action on mice (Jarboe *et al.*, 1968) as well as its property of replacing [^{3}H]-dihydropicrotoxinin from the binding site described for the substance in synaptic membranes (Olsen *et al.*, 1978; Ticku *et al.*, 1978). Thus, the drug has been thought to be pharmacologically inert. However, in the frog spinal cord, 6-acetylpicrotoxinin exhibited clear pharmacological actions which may reflect its activity as an antagonist of β-alanine and taurine. The characteristic presynaptic inhibition in frog spinal cord caused by antidromic stimulation of the ventral root is blocked by strychnine (Barker *et al.*, 1975a; Kudo *et al.*, 1983) and dendrobine (Kudo *et al.*, 1983), two compounds which are not antagonists of GABA but which do block the effects of β-alanine and taurine. Our previous study demonstrated that the DR-DRR and the early phase of the DR-DRP were reduced or abolished by dendrobine and strychnine (Kudo *et al.*, 1983). These observations suggest that in the frog spinal cord, the pathways responsible for the early phase of the DR-DRP which account for presynaptic inhibition may employ the compounds, β-alanine or taurine, as synaptic transmitter. Although GABA has been widely regarded as the most likely candidate

Table 3 Antagonistic effects of tutin, coriamyrtin, picrotin and anhydropicrotin upon amino acid-induced depolarization of primary afferent terminals

Compounds	*Dose* (M)	n	*% values of antagonism upon* GABA	*β-Alanine*	*Taurine*
Tutin	10^{-5}	4	14.4 ± 2.8	18.9 ± 7.9	25.0 ± 4.1
	3×10^{-5}	4	35.7 ± 2.9	41.1 ± 3.4	38.2 ± 5.0
Coriamyrtin	10^{-5}	4	19.5 ± 5.8	27.2 ± 9.2	31.4 ± 6.1
	3×10^{-5}	4	36.3 ± 3.2	52.8 ± 4.8	40.2 ± 15.4
Picrotin	10^{-4}	3	4.4 ± 4.3	0.8 ± 4.0	6.6 ± 9.5
Anhydropicrotin	5×10^{-5}	2	10.0	9.1	13.6

Experimental conditions and abbreviation are the same as those of Table 2.

for the transmitter responsible for presynaptic inhibition in the frog spinal cord, the present results and our previous studies suggest that in this species, β-alanine or taurine is the major synaptic transmitter for this inhibitory system.

The hydroxyl group at the 6-position may be important for high affinity anchoring of picrotoxane compounds on the GABA effector (Cl^--channel), but is not necessary for its actions in antagonizing the postsynaptic effects of β-alanine or taurine. It seems likely therefore, that the mode of antagonism of picrotoxane compounds on GABA and on the receptor for β-alanine and taurine may be different in nature. The long-lasting action of picrotoxinin depends upon the high affinity binding to the effector site through an interaction between the hydroxyl group at the 6-position and a corresponding site on the effector molecule. Anhydropicrotin was synthesized especially for the present study in anticipation that the lack of a hydroxyl group at both the 6- and 8-positions in the picrotin molecule might yield a compound having the same pharmacological properties as 6-acetylpicrotoxinin. Although anhydropicrotin caused spontaneous oscillations in both ventral and dorsal roots, as did 6-acetylpicrotoxinin, it had only a slight action on evoked root potentials and on the amino acid-induced depolarization of primary afferent terminals. Its parent compound, picrotin, which has a hydroxyl group at the 8-position, showed minor pharmacological actions on electrical activity and on amino acid-induced responses in the frog spinal cord; these actions are now generally accepted. Thus, the presence of the isopropyl bond or isopropenyl group at this position may be an important molecular requirement for access of picrotoxane compounds to their site of action. Although anhydropicrotin has no hydroxyl group at this position, it still has a bridge to the 6-position and this bridge may interfere with its binding to the receptor site.

From results obtained in the present study and from the data of our previous investigation (Kudo *et al.*, 1983), we have confirmed that in the frog spinal cord, the hydroxyl group at the 6-position of picrotoxane compounds is important for an action in antagonizing the effects of GABA but not of β-alanine or taurine on primary afferent terminals and for antagonism of presynaptic inhibition.

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