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THE ACTION OF ACETYLCHOLINE ANTAGONISTS ON AMINO ACID RESPONSES IN THE FROG SPINAL CORD *in vitro*

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1 The isolated hemisected frog spinal cord has been used to study the action of acetylcholine antagonists on amino acid responses by means of sucrose gap recording.

2 Primary afferents and motoneurones were shown to contain few, if any, cholinoceptors, since acetylcholine and carbachol responses were essentially abolished when synaptic transmission was blocked with magnesium ions or when action potentials were blocked by tetrodotoxin.

3 Curare antagonized the γ-aminobutyric acid (GABA) and β-alanine depolarizations of primary afferents and the hyperpolarizing action of these amino acids on motoneurones. Nicotine also antagonized β-alanine depolarizations and to a small extent GABA depolarizations of primary afferents. These actions are similar to but weaker than those obtained previously with picrotoxin.

4 Atropine selectively antagonized β-alanine depolarizations of primary afferents and blocked β-alanine and glycine hyperpolarizations of motoneurones. GABA responses were entirely resistant to the action of atropine. These actions are similar to but 50 times weaker than those obtained previously with strychnine.

5 Dihydro-β-erythroidine, tetraethylammonium, and gallamine were entirely ineffective in antagonizing amino acid responses. Since these agents are known to block the dorsal root potential elicited by ventral root stimulation but have no effect on the amino acid responses of primary afferents, it is evident that a cholinergic step is involved in this pathway.

Introduction

It is generally believed that the convulsant properties of such drugs as strychnine, picrotoxin, and bicuculline arise, in part, from their ability to antagonize amino acid mediated synaptic inhibition in the central nervous system. The fact that a number of agents which antagonize acetylcholine, including curare (Eccles, 1946; Chang, 1953; Feldberg, Malcolm & Darian Smith, 1957; Phillis & Tebecis, 1967; Bhargava & Meldrum, 1969; Banerjee, Feldberg & Georgiev, 1970; Feldberg & Lotti, 1970), gallamine (Salmoiraghi & Steiner, 1963; Curtis, Ryall & Watkins, 1966; Halpern & Black, 1967; Pixner, 1967; Galindo, Krnjević & Schwartz, 1968; Grinnell, 1970), tetraethylammonium (Furukawa, 1955, Pixner, 1967; Grinnell, 1970) and atropine (Bonnet & Bremer, 1952; Phillis & Tebecis, 1967; Bhargava & Meldrum, 1969), also have potent excitatory effects on central neurones, raises the possibility that these agents might also interfere with amino acid mediated transmission. Indeed, the observation that curare blocks both cortical inhibition and the action of γ-aminobutyric acid (GABA) (Hill, Simmonds & Straughan, 1972) supports this notion. Furthermore, the observation that picrotoxin, bicuculline (DeGroat, 1970; Bowery & Brown, 1974) and strychnine (Alving, 1961; Phillis & York, 1967; Kehoe, 1972; Faber & Klee, 1974; Bowery & Brown, 1974) can antagonize responses to acetylcholine, at least in high concentrations, indicates that acetylcholine and neutral amino acid responses have features in common. Thus, the present study was undertaken to examine the effects of a number of acetylcholine antagonists on amino acid responses in the frog isolated spinal cord.

Methods

The dissection and sucrose gap recording method used for recording drug responses from frog (*Rana*

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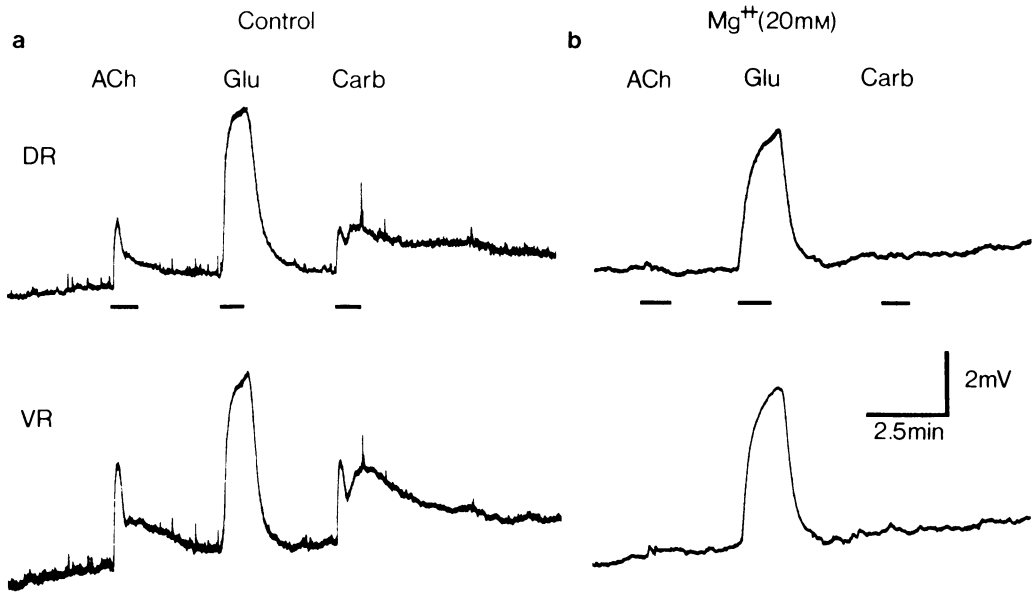

Figure 1 Effect of magnesium on cholinergic responses. (a) Upper record shows acetylcholine (ACh), carbachol (CCh) and glutamate (Glu) depolarizations on the primary afferents (DR) and lower record shows responses similarly recorded from motoneurones (VR). These responses were obtained in normal Ringer solution. Acetylcholine and carbachol are in a concentration of 10^{-2} M and glutamate 10^{-3} M. (b) Responses obtained after the addition of 20 mM magnesium sulphate to the Ringer solution. Note that the magnesium abolishes the responses to acetylcholine and carbachol.

pipiens) spinal roots has recently been described (Barker, Nicoll & Padjen, 1975a). In most preparations the responses from either two dorsal roots or two ventral roots were recorded simultaneously. On occasion, responses from one dorsal and one ventral root were recorded simultaneously (cf. Figure 1) (Barker *et al.*, 1975b). The drugs were made up in frog Ringer solution and the pH adjusted when necessary to 7.3 with HCl or NaOH. When the amino acids were tested during the application of an acetylcholine antagonist, the amino acids were prepared in the appropriate amount of antagonist, so that the antagonist would not be washed off during the amino acid application. To compare the sensitivity of various amino acid responses on the primary afferents to a particular antagonist, antagonist responses of equal size were usually used. For glutamate, GABA, and β-alanine the concentration range was from 2×10^{-4} M to 10^{-3} M. With this concentration range the responses for these amino acids were between 35 and 60% of their maximum response. Since the sensitivity of primary afferents to glycine is considerably less than to these other amino acids it was usually not possible to match this response without approaching the peak of the dose-response curve and using very high concentrations of glycine ($>5 \times 10^{-2}$ M). In such cases a compromise was reached whereby a glycine response of approximately 80% of the maximal response was used. However, to ensure that the resistance of the glycine response to a particular antagonist was authentic, responses ranging from 20 to 80% of the maximal response were also used. Since the size of the responses on the ventral root to the neutral amino acids was small and varied considerably in different preparations (Nicoll, Padjen & Barker, 1975), only a qualitative assessment was made as to whether a particular agent blocked the responses. Either magnesium sulphate (10 or 20 mM) or tetrodotoxin (50 μM) (Sigma) was added to the Ringer solution to block synaptic and interneuronal transmission, unless otherwise stated. The following drugs were used in the present study; β-alanine (Aldrich), γ-aminobutyric acid (Aldrich), atropine sulphate (Aldrich), dihydro-β-erythroidine (kindly donated by Merck, Sharp & Dohme), gallamine triethiodide (Davis & Geck), glutamate (K & K), glycine (Aldrich), nicotine (Sigma), and (+)-tubocurarine (Sigma).

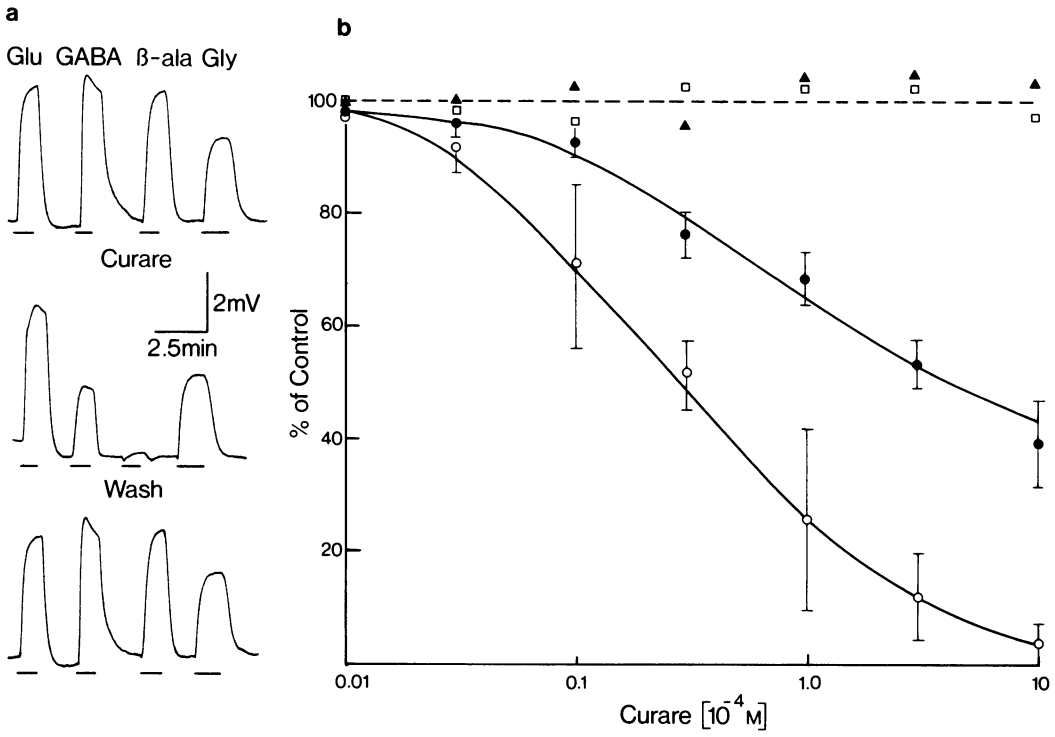

Figure 2 Curare antagonism of amino acid responses on primary afferents. (a) The effect of curare (10^{-3} M), applied for 95 min, on the depolarizing responses to glutamate (Glu, 3×10^{-4} M), γ-aminobutyric acid (GABA, 2×10^{-4} M), β-alanine (β-ala, 10^{-3} M) and glycine (Gly, 10^{-2} M). The bottom trace (Wash) was obtained 70 min after returning to a drug-free Ringer solution. In (b) the effects of increasing concentrations of curare on primary afferent depolarization produced by equi-effective concentrations of amino acids are plotted. The amplitude of the glycine response was 60-80% the size of the other amino acid responses (see methods section). The amplitude of the responses is expressed as a percentage of that obtained in the absence of curare. The responses at each concentration of curare were obtained after the responses had equilibrated (approximately 20 to 40 minutes). Each symbol represents the average of values obtained from at least three preparations and five dorsal roots. Responses to GABA (●); β-alanine (○); glutamate (▲) and glycine (□). The vertical bars give the standard deviation of the mean.

Results

Action of acetylcholine and carbachol on primary afferents and motoneurones

In studying the action of acetylcholine antagonists in the spinal cord it is important to know if the primary afferents and motoneurones have cholinoceptors, and if so, to characterize these pharmacologically. A depolarization of both the primary afferents and motoneurones was always produced when acetylcholine and carbachol (10^{-3}-10^{-2}M) were applied to the frog spinal cord with synaptic transmission intact. The acetylcholine and to a lesser extent the carbachol response faded quickly and often disappeared completely during the application (Figure 1a). Although a detailed analysis of the pharmacology of these responses has not been made, they were blocked entirely by atropine and were reduced by dihydro-β-erythroidine. These findings are in accord with previous investigations (Phillis & Tebecis, 1967; Koketsu, Karczmar & Kitamura, 1969). Since these responses were obtained in preparations exhibiting normal synaptic transmission, it is possible that they are mediated indirectly. Indeed, it was a consistent finding that the addition of magnesium or tetrodotoxin virtually abolished these responses, even when elicited with concentrations of 10^{-2} M (Figure 1b). In a few preparations magnesium ions or tetrodotoxin actually reversed the carbachol responses into small (<1 mV) hyperpolarizations (Nicoll, 1975). These results would suggest that the primary

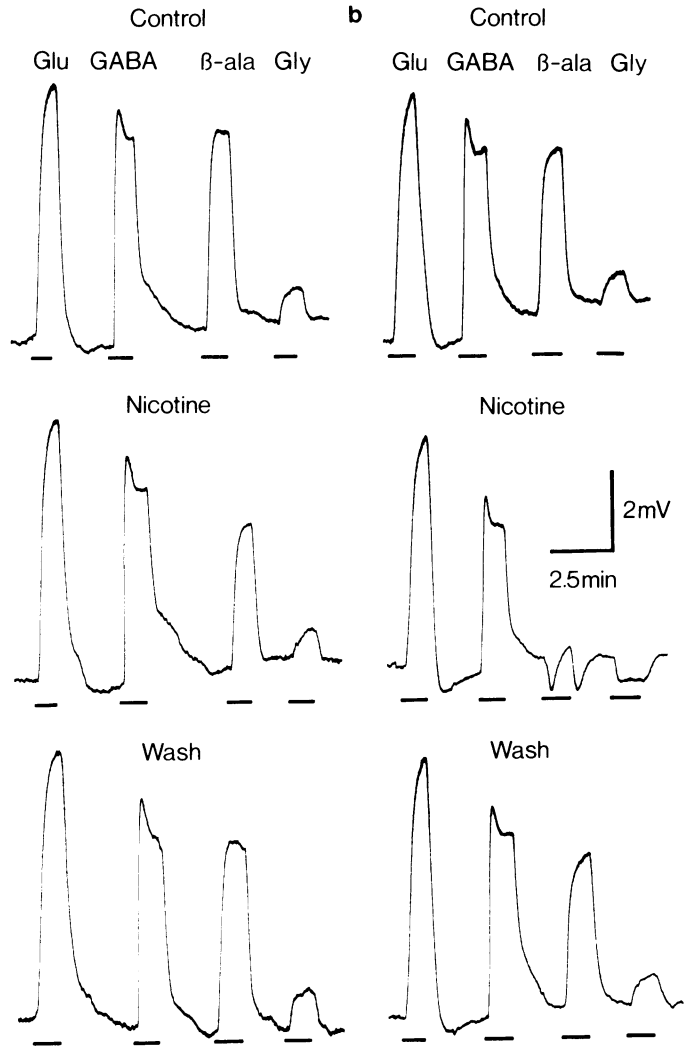

Figure 3 Nicotine antagonism of amino acid responses on primary afferents. In (a) 10^{-3} M nicotine, added 10 min prior to starting the middle record, reduced the β-alanine (β-ala) response but had little effect on the γ-aminobutyric acid (GABA) response. In (b) 4×10^{-3} M nicotine, added 13 min before starting the middle record, antagonized the GABA, β-alanine and glycine (Gly) depolarizations and revealed hyperpolarizing components to the β-alanine and glycine responses. The concentration of all the amino acids in (a) and (b) was 10^{-3} M except for glycine which was 2×10^{-3} M.

afferents and motoneurones have few, if any, receptors for acetylcholine.

Action of acetylcholine antagonists on amino acid depolarizations of primary afferent fibres

The acetylcholine antagonists tested did not have a common action on the amino acid responses. None of the agents had a direct effect on the membrane potential of primary afferents. Figure 2a shows the typical depolarization of the primary afferents by the amino acids, glutamate, γ-aminobutyric acid (GABA), β-alanine, and glycine. Curare was found to antagonize reversibly both the GABA and β-alanine response, but not the glutamate and glycine responses (Figure 2a). However, the β-alanine response appeared to be considerably more sensitive to curare than was the GABA

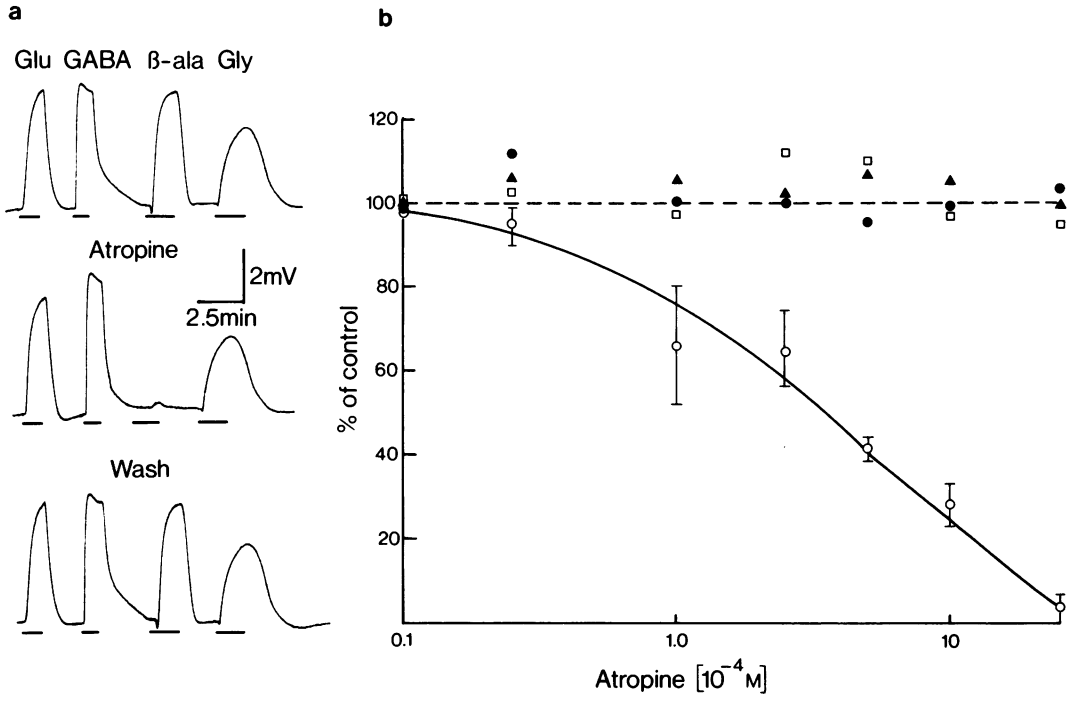

Figure 4 Atropine antagonism of amino acid responses on primary afferents. (a) The effect of atropine (2.5×10^{-3} M), applied for 75 min, on the depolarizing responses to glutamate (Glu, 4×10^{-4} M), γ-aminobutyric acid (GABA, 2×10^{-4} M), β-alanine (β-ala, 6.5×10^{-4} M), and glycine (Gly, 2×10^{-2} M). The bottom trace was obtained 90 min after returning to a drug-free Ringer solution. In (b) the effects of increasing concentrations of atropine on the primary afferent depolarization produced by equi-effective concentrations of amino acids are plotted. The amplitude of the glycine response was 60-80% the size of the other amino acid responses (see methods section). The amplitude of the responses is expressed as a percentage of that obtained in the absence of atropine. The response at each concentration of atropine was obtained after equilibration of the responses (approximately 15-30 minutes). Each symbol represents the average of values obtained in at least three preparations and four dorsal roots. Responses to GABA (●); β-alanine (○); glutamate (▲); and glycine (□). The vertical bars give the standard deviation of the mean.

response. To analyze the relative sensitivity of the amino acid responses to curare in more detail a wide range of antagonist concentrations were used in a number of preparations (Figure 2b). The threshold concentration for curare was approximately 3×10^{-6} M. At 10^{-3} M curare the β-alanine response was only 6.3% of control. The responses to both glutamate and glycine were unaltered by curare. Although not examined in detail, the response to taurine showed a similar sensitivity to curare as the β-alanine response. Thus the action of curare, although approximately 10 times weaker, is similar to that of picrotoxin (Nicoll & Barker, 1973; Barker *et al.*, 1975a).

Although nicotine (examined in six preparations) also antagonized amino acid responses, its action was very weak. With equimolar concentrations of amino acid and nicotine, nicotine reduced the β-alanine response, but had only a slight action against GABA (Figure 3a). With high concentrations (up to 4×10^{-3} M nicotine and 10^{-3} M amino acids) these effects became more pronounced (Figure 3b). However, these higher concentrations of nicotine also antagonized the glycine response and brought out a hyperpolarizing component to the glycine and β-alanine response (Figure 3b).

Unlike the action of curare and nicotine, the action of atropine was entirely selective against the β-alanine responses (Figure 4). GABA, glutamate and glycine responses were resistant to atropine, even in concentrations of 2.5×10^{-3} M. Figure 4b summarizes the effect of increasing concentrations of atropine on amino acid responses from a number of preparations. The antagonism of β-alanine response is detectable at approximately 2.5×10^{-5} M and becomes complete at approxi-

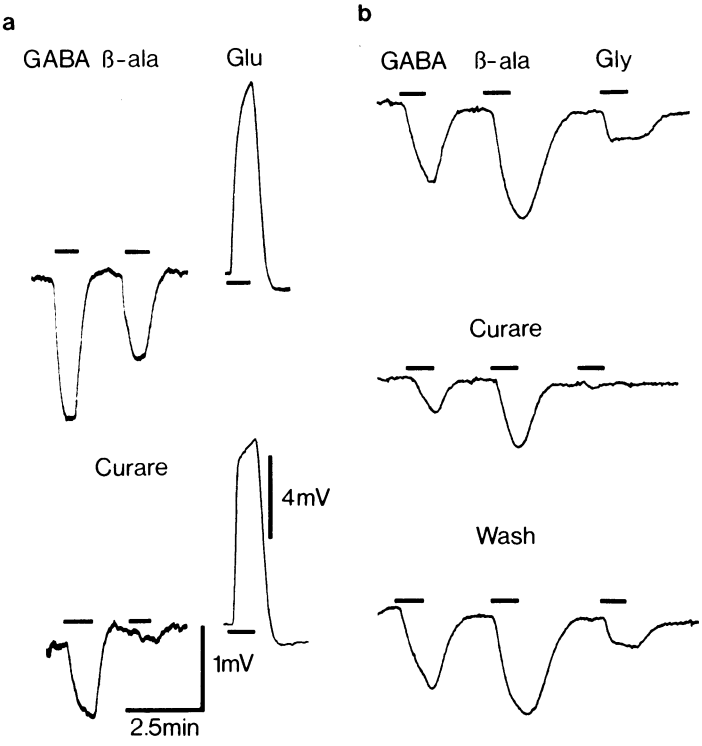

Figure 5 Curare antagonism of amino acid hyperpolarizing responses from motoneurones. (a) Antagonism of γ-aminobutyric acid (GABA) and β-alanine (β-ala) hyperpolarizations by curare, but not the glutamate depolarizing response. The responses in curare (5×10^{-4} M) were obtained 10 min after addition of curare. The concentration of all the amino acids was 5×10^{-4} M. In (b), from another preparation, curare (5×10^{-4} M), applied for 15 min antagonized all the amino acid hyperpolarizations, including glycine (Gly). The concentration of all amino acids was 2×10^{-4} M. The bottom trace (Wash) was obtained 10 min after returning to a drug-free Ringer solution. Calibration in (a) also applies to (b).

mately 2.5×10^{-3} M. Atropine also completely antagonized the response to taurine (not illustrated). This selective reduction of β-alanine (and taurine) responses is identical to the action of strychnine on responses of primary afferents to amino acids (Nicoll & Barker, 1973; Barker *et al.*, 1975a). However, the relative potency of atropine was considerably less than that of strychnine, being approximately 50 times weaker.

All of the other acetylcholine antagonists tested, including dihydro-β-erythroidine, tetraethylammonium, and gallamine did not antagonize amino acid responses. The latter two agents, especially when in high concentrations (5×10^{-4} M) actually increased all of the amino acid responses by about 10-20%. The presence of acetylcholine and carbachol in the Ringer in concentrations up to 5×10^{-3} M had no effect on the amino acid responses.

Action of curare and atropine on amino acid induced hyperpolarization of motoneurones

The experiments on the primary afferent fibres indicate that curare, and to some extent nicotine, have picrotoxin-like properties while atropine has strychnine-like properties. It is of interest to know if these similarities also exist for the hyperpolarizing responses of motoneurones. Since the hyperpolarizations of frog motoneurones by neutral amino acids are more variable than the primary afferent depolarizing responses, detailed quantitative studies have not been initiated. Curare, examined in five preparations, invariably reduced the GABA and β-alanine responses in concentrations greater than 10^{-4} M but did not reduce the glutamate response (Figure 5a) or affect the d.c. potential. Glycine had a hyperpolarizing action in only two of the

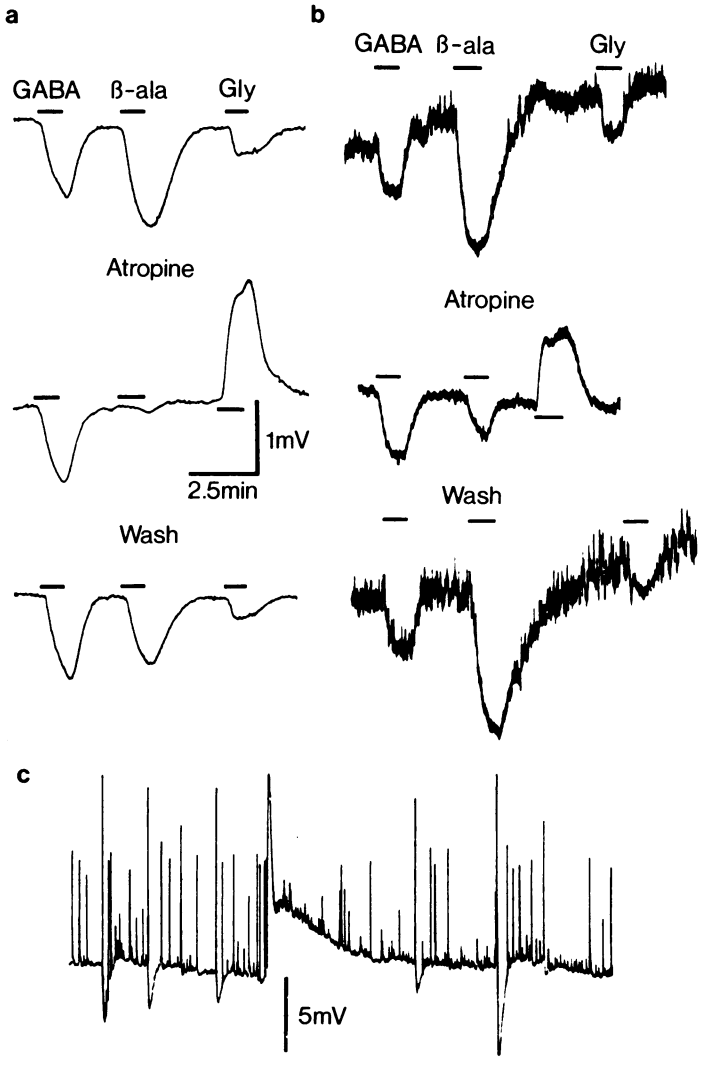

Figure 6 Atropine antagonism of amino acid hyperpolarizing responses from motoneurones. In (a) atropine (10^{-3} M), applied for 10 min, has no effect on the γ-aminobutyric acid (GABA) responses, but abolishes the β-alanine (β-ala) responses, and converts the glycine (Gly) hyperpolarization into a depolarization. The bottom trace (Wash) was obtained 20 min after washing in drug-free Ringer solution. (b) Is the same as in (a) but from another preparation in which synaptic transmission had not been blocked with magnesium ions. The concentration of atropine in this experiment was 3×10^{-3} M. The concentration of amino acids in (a) was 2×10^{-4} M and 5×10^{-4} M in (b). The calibration in (a) also applies to (b). (c) Shows the convulsant activity induced by 10^{-3} M atropine applied for 10 minutes. Time scale in (a) applies to (c).

preparations and in both cases curare antagonized the response (Figure 5b). Nicotine (4×10^{-3} M) had little effect on the hyperpolarizing amino acid responses in the two preparations tested.

The effect of atropine on amino acid hyperpolarizations was examined in four preparations. Atropine had no effect on GABA responses or d.c. potential but reduced and/or reversed the β-alanine and glycine hyperpolarizations. The action could be seen with concentrations above 10^{-4} M. The results were similar whether magnesium was (Figure 6a) or was not (Figure 6b) present to block synaptic transmission. Thus the action of atropine on the hyperpolarizing

responses is similar to that obtained with strychnine (Nicoll *et al.*, 1975). In concentrations of 10^{-4}-10^{-3} M, atropine induced convulsant activity in the spinal cord when magnesium was not present which was characterized by short lasting bursts of activity (Figure 5b). In higher concentrations, however, atropine actually reduced ongoing spontaneous activity as is shown by the background in Figure 5c before and after atropine (3×10^{-3} M).

Discussion

A number of investigations have shown that acetylcholine and related compounds depolarize the membrane of primary afferents and motoneurones of the amphibian spinal cord (Matsuura, 1961; Phillis & Tebecis, 1967; Koketsu *et al.*, 1969). In all of these studies, however, it was difficult to exclude indirect synaptic effects. In the present study similar effects were observed with acetylcholine and carbachol. However, when synaptic transmission was blocked with magnesium ions or when action potentials were blocked with tetrodotoxin, these effects were essentially abolished even with drug concentrations of 10^{-2} M. This would imply that the membranes of motoneurones and primary afferents possess few, if any, cholinoceptors. This finding is difficult to reconcile with the proposed cholinergic recurrent excitatory pathway to motoneurones (Matsurra, 1971), although it is conceivable that recording from the spinal roots might not have detected small responses generated in the motoneurone dendrites. The fact that unmyelinated nerve terminals of the primary afferents are unresponsive to acetylcholine and carbachol suggests that the acetylcholine sensitivity observed in some unmyelinated fibres and terminals (Armett & Ritchie, 1960; Hubbard, Schmidt & Yokota, 1965; Koketsu & Nishi, 1968) does not apply to all nerve fibres.

The present results are only partially in agreement with the idea proposed at the outset, i.e., that the central excitatory effects of acetylcholine antagonists might arise from their ability to block amino acid mediated synaptic inhibition. In agreement with results obtained in the cortex (Hill *et al.*, 1972) curare has the same action as picrotoxin and bicuculline on the amino acid responses evoked in primarly afferents and motoneurones. However, it is approximately 10 times weaker and somewhat less specific since it also antagonizes the glycine-induced hyperpolarization of motoneurones. Curare, like picrotoxin and bicuculline, antagonizes the dorsal root potential generated by stimulating an adjacent dorsal root (Kiraly & Phillis, 1961; Grinnell, 1966), which is thought to be mediated by GABA (Davidoff, 1972; Barker & Nicoll, 1972, 1973; Barker, Nicoll & Padjen, 1975b). Thus it seems possible that these three compounds share a common mode of action in their ability to generate convulsions, i.e., blockade of GABA-mediated synaptic potentials.

The effect of even high concentrations of nicotine ($>10^{-3}$ M) on the amino acid responses on primary afferents was weak but similar to picrotoxin, reducing the β-alanine and, to some extent, the GABA depolarizations. However, glycine depolarizations were also antagonized and hyperpolarizing components were observed with the β-alanine and glycine responses. These hyperpolarizing responses on primary afferents have not been observed with any other amino acid antagonist and raise the possibility that the responses to these amino acids in normal conditions may have a hyperpolarizing component which is masked by the more prominent depolarizing component. Indeed in a number of preparations (especially at a temperature below 18° C) the glycine response does have a hyperpolarizing component (unpublished observations; Barker & Nicoll, 1973; Barker *et al.*, 1975a). Nicotine had little effect on motoneurone hyperpolarizing responses elicited by the amino acids. Given the weak action of nicotine on the amino acid responses it would appear that the central stimulatory effects of nicotine are unrelated to amino acid-mediated synaptic inhibition. Presumably the cholinomimetic properties of nicotine play a predominant role in its central action.

Atropine behaved in a manner identical to strychnine (Nicoll & Barker, 1973; Barker *et al.*, 1975a; Nicoll *et al.*, 1975), specifically antagonizing the β-alanine responses on primary afferents and the hyperpolarizing motoneurone responses to glycine and β-alanine. This action of atropine was approximately 50 times weaker than that of strychnine. The unmasking of a depolarizing component to the glycine response on motoneurones supports the notion that there are two types of receptors for this amino acid on frog motoneurones (Nicoll *et al.*, 1975). In agreement with previous studies (Bonnet & Bremer, 1952; Phillis & Tebecis, 1967) the present results indicate that atropine can induce convulsant activity in the spinal cord, but in concentrations greater than 10^{-3} M, it tends to suppress ongoing activity. It is quite possible that in high concentrations the local anaesthetic action of atropine (Curtis & Phillis, 1961) tends to counteract its convulsant properties. The observation that atropine does not block strychnine-sensitive post-

synaptic inhibition in the cat spinal cord (Curtis & Duggan, 1967) presumably arises from the much lower concentration used.

The fact that dihydro-β-erythroidine does not antagonize amino acid responses and yet is a competitive antagonist of acetylcholine suggests that the amino acid antagonist properties of curare, and possibly atropine, may be unrelated to the properties they share in relation to acetylcholine receptors.

The failure of tetraethylammonium and gallamine to antagonize amino acid responses indicates that their well documented excitatory effects arise from some property other than that of blockade of amino acid-mediated synaptic inhibition. Both of the compounds are known to block the delayed increase in K^+-conductance during an action potential, which can prolong the action potential, leading to repetitive neuronal firing (Washizu, 1959; Grinnell, 1970) and enhanced transmitter release (Koketsu, 1958; Payton & Shand, 1966). This property could account for some of the observed central excitatory effects of these two compounds. Clearly the present results do not exclude the possibility that some of the excitatory actions of acetylcholine antagonists might, in fact, arise from blockade of cholinergic pathways although acetylcholine is generally considered to be an excitatory transmitter. Acetylcholine responses and the dorsal root potential generated by ventral root stimulation (VR-DRP), which is thought to contain a cholinergic synapse, appear to have a sensitivity to acetylcholine antagonists (Kiraly & Phillis, 1961; Grinnell, 1966; Koketsu *et al.*, 1969) similar to that of the amino acid responses in the present study.

The ability of curare, nicotine, and atropine to antagonize amino acid responses on primary afferents could contribute to the antagonism these compounds exert on the VR-DRP, which occurs at similar concentrations, since amino acids are thought to be involved in the final step in this pathway (Davidoff, 1972; Nicoll & Barker, 1973; Barker *et al.*, 1975b). However, the inability of tetraethylammonium gallamine, and dihydro-β-erythroidine to antagonize amino acid responses and yet block the VR-DRP (Kiraly & Phillis, 1961; Grinnell, 1966; Koketsu *et al.*, 1969) clearly indicates that there is a cholinergic step in this pathway.

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