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Effects of Glycine on the Mouse Neuromuscular Junction

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Glycine was applied at a range of different concentrations to test possible effects at the neuromuscular junction of the mouse. The presynaptic control of acetylcholine (ACh) release and the postsynaptic activation of the nicotinic receptor have been analysed by means of extracellular recording with an EPC7 Patch Clamp amplifier. The results indicated that glycine did not modify in a significant manner the release of ACh and the postsynaptic cholinergic receptor function. Nevertheless, a significant increase in the rate of the conformational change of the receptor–ion channel complex seemed to be noteworthy. Glycine at 30 and 300 μM increased in a dose dependent manner the decay time of the spontaneous miniature current. Concentrations of glycine exceeding 10mM completely blocked the activity of the end-plate in this preparation. In conclusion, we proved that glycine does not affect most of the parameters of neurotransmission in the mouse but, it increases the conformational change of the postsynaptic complex, perhaps inhibiting the acetylcholinesterase activity.

Keywords: Acetylcholine, Loose Patch Clamp, Neurorelease miniature end-plate current decay time.

INTRODUCTION

Glycine is the most abundant inhibitory amino acid present in the spinal cord and has been proved to be concentrated in the nervous terminals of spinal interneurons (Iversen, 1978; Gynther and Curtis, 1986). The pharmacology and physiology of this putative mediator is far from being completely outlined. Studies carried out on crayfish neuromuscular junctions, indicate that high concentrations of glycine elicited high rates of inhibitory postsynaptic currents (Finger, 1983). The present study concerns some of the actions on parameters underlying the function of the neuromuscular junction. Indeed, the physiological role of glycine in the release and in the action of ACh could be tested with this technique. The analysis of the glycine effect has been carried out using the Loose Patch Clamp technique at the mouse end-plate (Re *et al.*, 1989; Re 1993). With this technique we can measure either the evoked or the spontaneous release of ACh and monitor any interaction of the assayed drug with the postsynaptic receptor–ion channel complex. The amplitude of the spontaneous ACh release (mepc), of the evoked ACh release (epc),

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the quantal content (m) and the decay rate of the mepc (τ) have been analysed.

RESULTS

Table I reports the effects obtained with different concentrations of glycine on the analysed parameters. Glycine did not modify neither the epc's nor the mepc's amplitudes (See Figure 1). However, at these concentrations the drug exerted a significant increase of the rate of the conformational change of the receptor–ion channel complex, i.e. the mepc time constant τ. It is worth noting, this effect was well correlated with the dose of glycine suggesting a specific interaction of the drug.

Finger (1983) reported that doses of glycine exceeding 0.1 mol/l elicited high rates and 'giant' inhibitory postsynaptic currents in crayfish neuromuscular junctions. With the aim of testing the effects of glycine on the mouse neuromuscular junction, some experiments have been performed using glycine from 1 to 30 mM concentrations. In this preparation neither the evoked nor the spontaneous release were modified at doses of glycine below or equal to 10 mM. In all the experiments, excitability of the preparations was suppressed in an 'all or none' fashion between the two assayed concentrations. As can be seen, 10 mM glycine slightly reduced epc while the 30 mM dose abolished completely epc and mepc (Figure 2). It was noted that the presynaptic action potential disappeared, suggesting a concomitant presynaptic action of the drug. Furthermore, the recovery of mepc was not complete after the second wash out of the drug, while a long lasting reduction of the epc's amplitude was constantly seen. The membrane potential (RMP) of the postsynaptic muscle fibres was not modified by any concentrations of the drug. Its control value of 68.3 ± 3.8 mV from 8 fibres was modified to 72.5 ± 2.4 mV in the presence of 20 mM glycine. The pH of the solutions was not altered when measured in the presence of a higher concentration of glycine assayed.

DISCUSSION

The results of the present study demonstrate that in this preparation, physiologic concentrations of glycine do not modify, either presynaptically or postsynaptically, the function of the ACh release. However, the effect on the time course of the quantal decay obtained at μM ranges needs to be considered and discussed. Indeed, the signals obtained in this neuromuscular junction and the high sensitivity of the recording technique (Re *et al.*, 1993) enabled a careful estimation of this parameter. Taking into account the statistical significance and the dose correlated effect in the prolongation of the time course of spontaneous mepc's, we must explain the possible molecular event involved. The prolongation of the quantal conductance change could be due to some interactions with the molecular complex responsible for the gating action or to the inhibition of ACh hydrolysis (Katz and Miledi, 1973). The decay phase of the synaptic current normally arises from the rate constant of

TABLE I Effects of Glycine at the Mouse Neuromuscular Junction

	Control Values	30 μM	100 μM	300 μM
epc (nA)	0.60 (0.15; 7)	−13.8 (16.7; 5)*	−12.7 (15.3; 3)*	−1.7 (20.0; 3)*
mepc (nA)	0.18 (0.12; 8)	+16.6 (20.4; 5)*	+20.3 (24.8; 3)*	+8.0 (12.1; 3)*
m	4.20 (2.28; 8)	−13.2 (19.6; 4)*	+4.3 (13.6; 3)*	−4.3 (20.6; 3)*
τ (ms)	1.00 (0.19; 8)	+14.2 (8.4; 5)	+22.0 (7.5; 3)	+26.0 (6.1; 3)

Values show the effects of glycine on four parameters related to the function of the neuromuscular junction of the mouse. The amplitudes of the evoked (epc) and spontaneous (mepc) release, the quantal content of the epc (m) and the mepc's decay time constant (τ) have been analyzed. Data are expressed as the mean percentage variation from the respective control value. Standard deviations and number of experiments are indicated between brackets.

*Indicates means not significant. In all the other cases the p values were less than 0.05 with Student's *t* test.

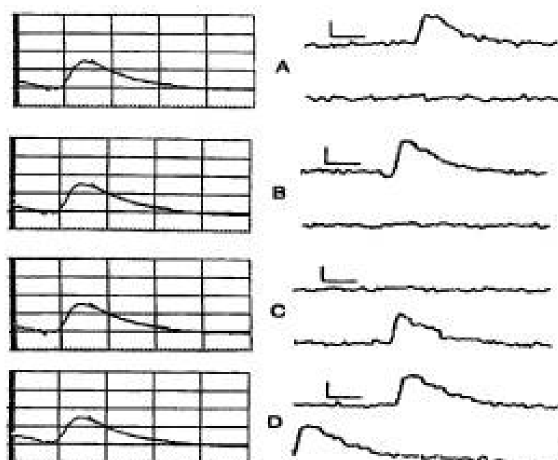

FIGURE 1 Averaged signal obtained by 100 evoked end-plate currents (left) and spontaneous miniature end-plate currents (right) at the mouse neuromuscular junction. The figure shows digitised raw data related to the Control (A), to the treatment with glycine 30 μM (B), 100 μM (C) and 300 μM (D) respectively. Left panel calibration; vertical bar = 0.5 nA, horizontal bar 2 ms. Right panel calibration; vertical bar = 0.2 nA, horizontal bar 1 ms.

the conformational change, reflecting the closing of the ACh-sensitive channels (Magleby and Stevens, 1972; Anderson and Stevens, 1973). These results do not clarify the real mechanism related to the effect induced by glycine but, as proposed by Katz and Miledi (1973), the prolongation of mepc decay time could be due to the possible inhibition of acetylcholinesterase activity.

Earlier studies showed that high concentrations of glycine in some neuromuscular junctions elicited high rates of spontaneous inhibitory postsynaptic currents (Finger, 1983). The Author discussed the hypotheses of an osmotic effect and of a possible block of presynaptic potassium channels. Here, we proved that high concentrations of glycine do not increase the frequency of excitatory miniature currents. Furthermore, the evidence that we can never observe 'giant' mepcs, indicates that the action mechanism of glycine at the presynaptic level could be due to different actions either in the membrane or in the cytosolic domains. Finally, the abrupt reduction of the excitability, both of the evoked and of the spontaneous activity, could be attributed to a common pathway in blocking the electric activity of the pre- and post-synaptic membranes.

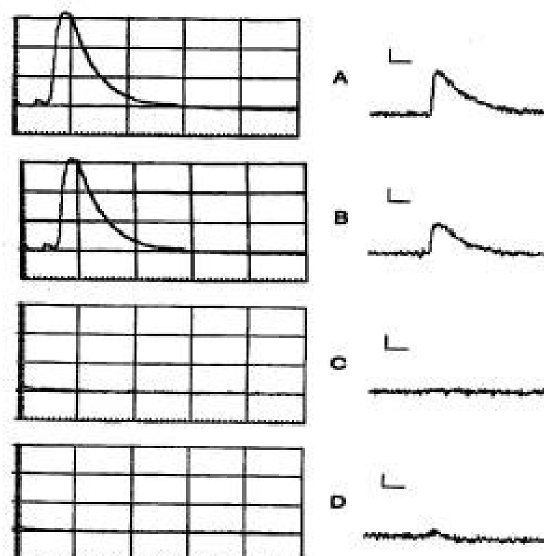

FIGURE 2 Averaged signal obtained by 100 evoked end-plate currents (left) and spontaneous miniature end-plate currents (right) at the mouse neuromuscular junction. The figure shows digitised raw data related to the Control (A), to the treatment with glycine 10 mM (B), glycine 30 mM (C) and the final wash out (D). Left panel calibration; vertical bar = 0.25 nA, horizontal bar 2 ms. Right panel calibration; vertical bar = 0.2 nA, horizontal bar 1 ms. Note the disappearance of the biphasic action potential which precedes the evoked signals in the C and D blocks.

In conclusion, our data fills the gap related to the possible role of glycine at the mammalian neuromuscular junction. The results obtained at this peculiar 'peripheral' synapse, i.e. the neuromuscular junction, are far from being completely outlined but, we think, could help in the definition of the complex pattern regulating neurorelease in 'central' synapses. The aim of stimulating this kind of research, must be intended as an attempt to also give a little scientific contribution to a better understanding of the most important CNS human pathologies.

MATERIALS AND METHODS

Preparation

Left hemidiaphragm of mouse was prepared as described previously (Re and Di Sarra, 1988; Re *et al.*, 1989). Briefly, Charles River mice of either sex,

30–40 days old, were killed and the left hemidiaphragm was dissected along with a short length of phrenic nerve. The preparation was bathed in Krebs' solution of the following composition (mM), NaCl (13)–KCl (4.7)–$MgCl_2$ (1.2)–$CaCl_2$ (7.2)–NaH_2PO_4 (1.3)–$NaHCO_3$ (16.3)–Glucose (7.8), pH 7.4, gassed with 95% O_2–5% CO_2 and maintained at room temperature (18–22°C). Concentrations of $MgCl_2$ (5 to 15 mM) and $CaCl_2$ (0.9 to 2 mM) were adjusted in order to abolish the twitch of the muscle fibre. The muscle was pinned on Sylgard resin, and placed on the stage of a Leitz inverted microscope. End-plates were visible by transillumination of the preparation with an optic fibre system. The preparation was equilibrated in saline for 30 minutes before starting the experiments.

End-plate Signals

Spontaneous and evoked end-plate currents were recorded with a focal extracellular pipette pressed against the edge of an end-plate (Schuetze, 1980). The pipettes from Drummond, 100 μl measuring pipettes of soft glass (1.4 mm) were pulled with a Kopf 700C Puller, fire polished with a Narishighe MF83 Microforge, and had a final tip diameter ranging from 3 to 15 μm. Following filling with physiological solution the electrode was connected to a List LM EPC7 current to voltage converter. Pipette resistances were of 100–300 KΩ and seal resistances, measured after pressing the pipette against the sarcolemma, ranged between 300–600 KΩ. The Loose Patch Clamp method (Stühmer *et al.*, 1983) enables a good control of the series resistance all over the experiment. The technique enables a good voltage control of the muscle fibre, virtually clamped at the resting value. Indirect stimulation of the muscle was achieved by means of a suction electrode. Supramaximal square wave pulses of 0.1 ms duration at 2 Hz were applied by a stimulator (model 300A, WPI) via a stimulus isolator unit (model 305–2R, WPI). The signals were visualized on a Tektronix 5113 dual-beam storage oscilloscope and fed to the input stage of the analog-to-digital converter of a computer system (HP486, Hewlett-Packard) which enables a fully automated analysis of the data. The decay phase of the mepc's were analyzed to calculate the decay time constant. The elaboration was carried out on the mepc decay part that fell within 10–90% of its peak amplitude. The function used was:

$$I_t = I_o e^{-t/\tau}$$

where I_t is the current at time t, I_o is the current at time zero (i.e. the peak current) and τ is the time constant of decay. Marquardt's least squares method was used for the fitting.

The parameters were first recorded after the flowing of each 10 ml of physiological solution at a constant rate of 1 ml/min and repeated until stable values were obtained. The acquisition started with the analysis of the decay time of spontaneous mepc's (12 to 20 signals) with the calculation of the mean τ value. The acquisition of each block proceeded with the capture of mepc's over a 60 s time period. The amplitudes of the mepc's captured in each 60 s block were averaged and the mean peak value (**mepc**) recorded. Finally, the preparation was stimulated and 200 evoked epc's were acquired and averaged to obtain a mean epc's peak value (**epc**). Subsequent similar blocks were performed either after the flowing of 10 ml of the tested drug at the desired concentration or after a final wash out. Quantal content (**m**) was calculated by the direct method (del Castillo and Katz, 1954).

The RMP was measured during the experiment by conventional intracellular microelectrodes (3M KCl) led to a P16 differential amplifier (GRASS).

Statistical Analysis

Given data are expressed as the means ± standard deviations of the means. The statistical significance was assessed by Student's *t* test. P values < 0.05 were considered as significant differences.

Materials

The drug used was glycine (Hydrochloride) Sigma, Lot 88F-5622. The drug was prepared daily and diluted before the experiment.

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